European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 05 , 2020

ISOLATION OF HELICOBACTER PYLORI FROM DIFFERENT GASTRODUODENAL DISEASES

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ABSTRACT

Helicobacter pylori infection is regarded as the causal factor of most of the diseases of the gastrointestinal tract in adults and children. Isolation of H. pylori by culture is highly specific and is considered as the gold standard technique. But often H. pylori are difficult to isolate in proper culture media. Helicobacter pylori infect about half of the world population causing chronic gastritis, peptic ulcer or 175 gastric cancers. This study investigated the prevalence of H. pylori and determined the antimicrobial susceptibility patterns of isolates; the molecular basis of the resistance pattern of isolates.

Keywords: H.pylori, Culture, Endoscopy, Rapid urease test.

INTRODUCTION

The human gastrointestinal tract is colonized by an abundance of bacteria, which are in constant interaction with the epithelial lining usually leading to an intricate balance between tolerance and immunological response. There is ample evidence that the abundant presence of bacteria thus play a role in the maintenance of human health, as well as in the induction of chronic inflammatory diseases of the gastrointestinal tract (Kuipers et al., 1995). Helicobacter pylorus (H.pylori) is recognized as a chronic colonizer of the human stomach. It is a small, curved or spiral, highly motile, gram-negative bacillus. It chronically infects billions of people worldwide, is one of the most genetically diverse of bacterial species, and has been implicated as the major cause of various diseases since the Nobel-winning discovery by Warren and Marshall in 1982 (Marshall and Warren, 1983)**.**

The basic morphology of H.pylori shows the bacterium as a spiral-shaped Gram negative bacterium (Rhead et al., 2007). It is a flagellated bacterium with 5 to 7 polar flagella. The spiral shapes along with the polar flagella were linked to its high bacterial motility. In addition to the spiral shape of the bacteria, H.pylori may form coccoid cells as it ages. Fresh cultures are mainly spiral bacteria; however, after 3 to 4 days of culture, coccoid cells dominate leading to a dramatic decrease in culturability (Benaissa et al., 1996).

The prevalence of H.pylori infection has also been reported to vary widely by geographic area, age, race, and socioeconomic status (Malcolm et al., 2004). Although there is geographical

and socio–demographic variation in the prevalence of human infection, prevalence does not parallel the incidence of morbidity caused by the infection
(Mukhopadhyay et al., 2000).

MATERIALS AND METHOD

Sample collection

One hundred and ninty-four consecutive patients referred for endoscopy at Kovai Medical College (KMC), Coimbatore, Tamil Nadu, India between January to August 2018 were evaluated.Patients were divided broadly into 4 categories between the age groups of 10 to 70 years of both sex with or without gastrointestinal endoscopic evidences were selected for the present study.

Group I – Patients with Acute gastritis (Both male and female)
Group II – Patients with chronic gastritis (Both male and female)
Group III - Patients with Gastric atrophy (Both male and female)
Group IV - Patients with Intestinal metaplasia (Both male and female)

The patients were taken for this study under belongs to both and middle groups of socioeconomic status, the patients have different dietary habits like vegetarians and non vegetarians, who have consume spicy food, pickles and chutneys with excess chillies. The life style of patients who are positive for H.pylori having a family history who are addicted to alcohol, smoking or beetle nut. And also the different blood groups of the patients both sex examined randomly.

Isolation of H.pylori from gastric biopsies

Two gastric biopsies were collected (one from the antrum and the other from the corpus) and the organism was isolated on agar base. Therefore, Helicobacter pylori can be diagnosed by its isolation, histology, smear microscopic examination, biochemical assays based upon its characteristic metabolic activities such as urease, catalase and oxidase tests and other serological tests. Taking different factors like sex, age, diet, life style, family history and blood groups, this study was undertaken to evaluate the prevalence of Helicobacter pylori in patients.

Antibiotics susceptibility testing

A sterile cotton swab dipped into the standardized bacteria suspension was used to evenly inoculate BHI agar plates and allowed to dry for 10-15 minutes. Thereafter, all the disks for this study were placed on the plates and pressed gently to ensure complete contact with agar. A distance of at least 15mm was maintained from the edges of the plates to prevent overlapping of inhibition zones (Njume et al., 2009). The plates were incubated at 37^{O}C for 2-5 days. They were then examined and the diameter of the zone of inhibition measured.

Determination of Minimum Inhibitory Concentration (MIC)

The range of antibiotic concentrations obtained for clarithromycin was (0.06-1.0µg/mL), while for amoxicillin, tetracycline, ciprofloxacin, gentamicin, erythromycin and metronidazole it was (0.625-10µg/mL). A volume of 13.5mL of the base medium (Brain Hearth Infusion Agar) enriched with 7% horse blood and selective supplement was prepared and 1.5mL of the serially

diluted antibiotic was added to the medium to obtain the desired concentration. This was poured into sterile petri dishes and then allowed to solidify. Fresh pure isolates were grown for 3 days and the inoculums prepared.The inocula were plated and incubated under microaerophilic condition for 3 to 5 days at 37^{o}C. After incubation, the MIC value was read as the lowest concentration of the antibiotic that inhibited bacteria growth (no visible growth). A plate free of antibiotic was included as a negative control in every MIC determination.

Results

H. pylori culture positive gastric biopsies

Out of the 194 patients tested, direct smear positive are 140, rapid urease positive are 54 and 121 are culture positive. Anaerobic jar failure resulted in the loss of 12 cultures. Another 17 cultures could not be recovered after storage at -80°C, which left a total of 121 primary cultures available for genotyping studies (Table-1). High prevalence of acute and chronic gastritis was observed (81% and 84% respectively) in participants, while atrophy and intestinal metaplasia were less frequently observed, in 21% and 9% of participants, respectively (Table-2).

Patients of both sexes are taken at random for the study and they are between the age group of 10 to 70 years. Between the age group of 41to 50 years, 44 patients are tested out of which 31 are positive with percentage positivity of 70.4 and between the age group 21 to 30 years, 28 cases are positive with percentage positivity of 71.7. Thirty nine patients tested out of 194 are between the age group of 31to 40 years. Out of which 19 are positive for H.pylori with percentage of positivity above 60 in the age group, 51to 60, the percentage positivity is about 51.6 and in the age group of 10 to 20 years the lowest percentage of about 77.7 is noted.

The patients under study belong to both and middle groups of socioeconomic status. Out of 194 patients studied, 121 patients below to low socio-economic status and 73 belong to middle socioeconomic status. Eighty six out of 121 belonging to low - socio- economic status are positive for H.pylori with percentage positivity of 73.9% .54 out of 73 belonging to middle socio economic status are positive for H.pylori with percentage positivity of 71%.

The patients under study have different dietary habits out of 194 patients, 25 are vegetarian and 169 are non vegetarians. 11 out of 25 vegetarians are positive for H.pylori with percentage positivity of 44, 148 out of 169 non vegetarians are positive for H.pylori with percentage positivity of 87.5. Both vegetarians and non vegetarians, who are positive for H.pylori consume spicy food, pickles and chutneys with chilies. Out of 140 positive cases 62 are positive for H.pylori who consumes spicy food with percentage positivity of 44.2. One hundred and thirteen patients consuming excess use of chilies are positive for H.pylori with percentage positivity of 80.7.

The life style of patients who are positive for H.pylori having a family history of gastroduodenal diseases and who are addicted to either alcohol, smoking or beetle nut. The patients having family history of gastroduodenal diseases disease are positive for H. pylori with percentage positivity of 80.9. Among the patients positivity for H. pylori, 23 patients are addicted to alcohol with percentage positivity of 36.5 and 48 are smokers with percentage

positivity of H. pylori is 76.2. Two patients out of 63 patients are positive for H. pylori, which are addicted to beetle nut. Eighty seven patients out of 140 positive patients for H.pylori belong to O^+ve blood group with percentage positivity of 42.8. The percentage positivity of B^+ve blood group is 12.7 and A^+ve blood group 9.5.

Culture

Colonies of H. pylori were small, convex and translucent on Brucella chocolate agar after 72 h of incubation. These colonies were confirmed by H.pylori specific biochemical tests and also by microscopically using modified Gram's staining (Fig-1). The standard graph was obtained by serial dilution of H. pylori culture.

Demographics of H. pylori Project participants

Cultures included in this study were isolated from 121 H. pylori participants. Seventy two (60%) females and 49 (41%) males were included in this study. The majority of participants (95%) were Aboriginal people. Participants' ages ranged from 10-70 years; 58 (48%) were 11 - 34 years old, 42 (35%) were 35 - 54 years old and 21 (17%) were older than 55 years. H.pylori was isolated from both the antrum and the corpus. The percentage positivity for antrum was 61.02% (310/198), and for corpus 55.51% (282/198).

Antimicrobial patterns

Of the 200 isolates subjected to antimicrobials, 100% susceptibility was recorded for ciprofloxacin and 97.5% for amoxicillin. Marked resistance were noted for metronidazole (95.5%). A total of 19 antibiotypes were noted. Of the 200 strains, 6 (3%) showed no resistance to all the antibiotics. The predominant resistant pattern METR was observed in 49 (26.06%) of isolates (Table-3). Thirty-two (17.02%) showed multidrug-resistance to metronidazole and erythromycin (METRERTR). The least resistance pattern were exhibited by ERTR (0.53%) and CLARTETRAMXRMETRGENRERTR (0.53%).

Antibiotic Resistance by Sex

Of the 200 strains tested for susceptibility, 49 were from males and 72 from females. The prevalence of metronidazole resistance in females and males was 65.44% and 34.55% while in gentamicin it was 69.09% and 30.90% respectively.In general there was a higher prevalence of resistant isolates in females when compared with male patients (Table-4).

MIC determination

Of the seven antibiotics, metronidazole showed no MIC within the susceptible breakpoint range. MIC values for the other antibiotics ranged from 0.125 -1.0μg/mL for clarithromycin; 1.25–2.0μg/mL for tetracycline, 2.5–5μg/mL for amoxicillin, gentamicin 5-8.0μg/mL, erythromycin 2.5–5.0μg/mL, and ciprofloxacin 0.0625–1.0μg/mL (Table-4).

DISCUSSION AND CONCLUSION

Helicobacter pylori is a major human pathogen which inhabits the mucous layer overlying the gastric epithelial cells in humans (Olivier et al., 2006). It is recognized by the World Health

Organization to be the most important causal factor of gastric carcinoma as well as chronic gastritis and gastro duodenal ulcer disease (Peek and Crabtree, 2006). Diagnosis of H. pylori infection by culture and histopathology are considered highly specific. We attempted to isolate this organism by culturing 194 biopsy specimens on blood based non selective media where 121 out of these 75 samples tested positive for H. pylori in Rapid Urease Test. Besides determining the presence of urea the rapid urease test was also done to determine the actual number of H. pylori positive specimens which could not be isolated by culture.

But the sensitivity and specificity of the culture techniques were greatly affected by the presence of bacteria other than H. pylori on the culture media. A single culture plate revealed a mixed bacterial population consisting of two, three or more different type of organisms. For this reason, suspected H. pylori colonies were masked by the growth of these bacteria. The rate of isolation of H. pylori by culture was relatively low as compared to the rapid urease test which detects the presence or absence of the organism by detecting the production of the enzyme urease indicated by colour change from yellow to pink (Aditya et al., 2009). For this reason we were able to isolate only 9 positive H. pylori culture isolates among the contaminants. We have not yet used selective media such as Skirrow's supplement in the culture media since it was important for us to evaluate the extent of contamination of the plates and the different types of contaminants on it.

The gastric mucosa of the human stomach harbours a wide range of normal microbial flora which may become opportunistic pathogens if the host defense mechanism fails (Cotton, M. C. (1996). This mostly comprises of organisms such as Streptococci, Micrococci, Enterobacteriaceae, yeasts and anaerobic Gram-positive cocci and rods. In Tonks et al(2003)demonstrated various microflora of gastric biopsies such as Streptococci, Micrococci, Staphylococci, Enterobacteriaceae and yeasts from patients with duodenal ulcer and gastric cancer (Bytzer, P. and O'Morain, C. (2005). According to their study, at least one type of organism with different colony morphology was present on the culture plate. These findings may be compared to the present study in which the plates consisted of two or more different type of organisms. In Dharmalingam et al. (2003) developed a novel approach of reproducing better yields of H. pylori through culture from contaminated specimens (Palmer et al., 2002). This study suggested that exposing and pre-treating the patients' gastric biopsy as well as saliva specimens with hydrochloric acid (HCl) and urea facilitated the growth and isolation of H. pylori. Although the above mentioned studies correlate with the, present study and also recommends ways of improving H. pylori isolation.

Based on these findings, this study conclude that improved environmental and socioeconomic conditions (either by way of education which will lead to employment and improved living standards) which have been associated with increased prevalence of H. pylori. This is of particular importance in this study population which is predominantly rural with deprived living conditions.

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Table-1: Positive by standard tests

TOTAL NO. OF CASES	DIRECT SMEAR	RAPID UREASE TEST	CULTURE
194	140	54	121

Table-2:Distribution of gastritis severity, atrophy and intestinal metaplasia among research participants

Histopathology	Distribution	
	n (total)	%
Acute gastritis	96 (119)	81
Absent	23	19
Mild	56	47
Moderate	32	27
Severe	8	7
Chronic gastritis	102 (121)	84
Absent	19	16
Mild	9	7
Moderate	44	36
Severe	49	40
Gastric atrophy	26 (121)	21
Absent	95	79
Mild	23	19
Moderate	2	2

Severe	1	1
Intestinal metaplasia Absent Mild Moderate Severe	11 (121) 110 7 3 1	9 91 6 2 1

Table-3: Antimicrobial resistance patterns of H. pylori

No.	Antibiotics (%)	Number of strains showing patter
A1	MET^R	49(25.93)
A2	ERT^R	1(0.53)
A3	MET^R GEN^R	12(6.35)
A4	MET^R ERT^R	32(16.93)
A5	CLA_RAMX^R	2(1.06)
A6	CLA^RMET^R	2(1.06)
A7	TET^RMET^R	11(5.85)
A8	$MET^RGEN^RERT^R$	13(6.91)
A9	$TET^RMET^RGEN^R$	10(5.3)
A10	$TET^RMET^RERT^R$	16(8.51)
A11	$CLA^RTET^RMET^R$	9(4.78)
A12	$CLA^RMET^RGEN^R$	5(2.65)
A13	$CLA^RMET^RERT^R$	6(3.19)
A14	$AMX^RMET^RGEN^RERT^R$	2(1.06)
A15	$CLA^RTET^RMET^RGEN^R$	2(1.06)

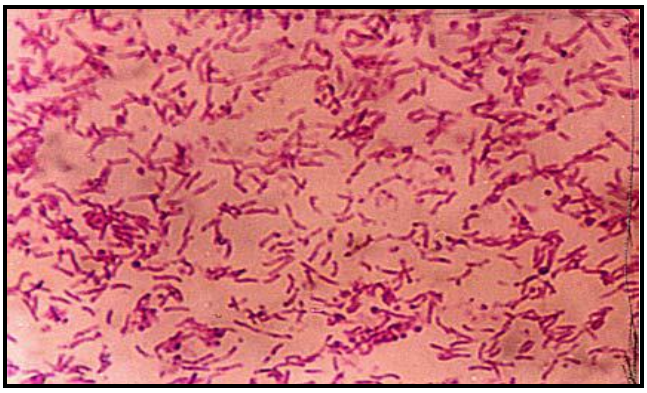
A16	$CLA^{R}TET^{R}MET^{R}ERT^{R}$	8(4.25)
A17	$TET^{R}MET^{R}GEN^{R}ERT^{R}$	5(2.65)
A18	$CLA^{R}TET^{R}MET^{R}GEN^{R}ERT^{R}$	3(1.59)
A19	$CLA^{R}TET^{R}AMX^{R}MET^{R}GEN^{R}ERT^{R}$	1(0.53)

CLA- clarithromycin; TET- tetracycline; AMX- amoxicillin, MET-metronidazole; GEN-gentamicin; ERT-erythromycin; CIP-ciprofloxacin

Table-4: Antibiotic sensitivity results of H. pylori strains isolated from gastric biopsy specimen

Antibiotics	No. sus (%)		No. res (%)		Overall	Overall	MIC
	Antrum	Corpus	Antrum	Corpus	No. sus (%)	No. res (%)	
Clarithromycin	87(82.07)	73(77.65)	19(17.92)	21(22.34)	160(80)	40(20)	0.125 - 1.0
Tetracycline	69(66.34)	66(68.75)	35(33.65)	30(31.25)	135(67.5)	65(32.5)	1.25-2.0
Amoxicillin	103(98.09)	92 (96.84)	2(1.90)	3(3.15)	195(97.5)	5(2.5)	2.5–5.0
Metronidazole	6 (5.94)	3 (3.03)	95(94.05)	96(96.96)	9(4.5)	191(95.5)	-
Gentamicin	84(75.67)	61(68.53)	27(24.32)	23(31.46)	145(72.5)	55(27.5)	5-8.0
Erythromycin	69(64.48)	42(45.16)	38(35.51)	51(54.83)	111(55.5)	89(44.5)	2.5-5.0
Ciprofloxacin	107(100)	93(100)	00(0)	00(0)	200(100)	00(0)	0.0625-1.0

Fig-1:H. pylori in culture smear



(modified Gram’s stain x 1000)