Isolation and antimicrobial susceptibility testing of *Helicobacter pylori* strains from gastric biopsies from Pakistani patients

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Abstract: *Helicobacter pylori* is the etiological agent of gastritis and peptic ulcer. This importance had proposed antibiotics as a principle treatment of gastrointestinal pathologies. The focus of this research was to investigate the occurrence of *H. pylori* in patients having gastritis or gastric ulcer and also draw the susceptibility profile of isolates to several antibiotics. Blood and biopsy specimen from 96 acid peptic disease patients from both sexes were collected. Each sample was used for culture, gram staining, catalase, oxidase, urease and nitrate reduction test by conventional method. Serology using anti *Helicobacter pylori* IgG was done. The susceptibility profile to six common antibiotics was checked by E- test method. *H. pylori* was obtained from 40 patients (41.67%) with greater frequency in male (25%) than females (16.67%). With regards to age, *H. pylori* was recovered highest from the patients between 51-55 (75.86%) years of age. Tetracycline and rifampin were the most effective antibiotics *in vitro*, while metronidazole was less effective. Nine (22.5%) strains displayed resistance to at least one antimicrobial drug. Whereas, resistance to amoxicillin, ciprofloxacin, clarithromycin, metronidazole and combination of antibiotics like ciprofloxacin, clarithromycin and metronidazole, and ciprofloxin and metronidazole were 11.11, 55.56, 22.22, 33.33, 11.11 and 44.44% respectively. Lower susceptibility profile of *H. pylori* to antibiotics is because of frequent use of antibiotics to treat other infections.

Keywords: *Helicobacter pylori*, antibiotic susceptibility test, gastric ulcer, isolation

INTRODUCTION

Since isolation and characterization of *Helicobacter pylori*, formerly known as *Campylobacter pylori* and, is commonly associated with chronic bacterial infection to human (Hardin and Wright, 2002; Yvonne *et al*., 2001; Manyi-Loh *et al*., 2013). The condition caused by *H. pylori* infection affecting the stomach of approximately 50% of the human population worldwide (Otth and Wilson, 2011). The colonization can lead to chronic active gastritis and is directly related to the development of peptic ulcer. It is also designated as an important factor in initiation of gastric cancer and gastritis mucosa associated lymphoid tissue lymphoma (Suerbaum and Michetti, 2002; Hocker and Hohenberger, 2003).

The association between bacterium and gastroduodenal diseases demonstrates the requisite to identify the incidence of causative bacteria in dyspeptic patients. Conventionally, indicative techniques for *H. pylori* may be categorized as invasive, (which requires endoscopy to get biopsy samples form gastric tissue) and non-invasive (Ramis *et al*., 2012). The invasive techniques comprise histology, molecular methods and other biochemical tests like urease, oxidase etc, while the non-invasive techniques contain urea breathe test, serology, stool antigen and molecular tests (Hirschl and Makristathis, 2007). Although multiple diagnostic methods are available, yet there is no single method that can fulfil, on its own, the standards for adequate sensitivity and specificity in diagnosis of bacteria. Microscopy is considered standard method providing additional information of status of mucosa, but depends primarily on the number and localization of specimen taken. Culture and identification of *H. pylori* requires experience and dexterity, as identification and culturing is somewhat difficult (Abu-Sbeih *et al*., 2014). The rapid urease test is easy and delivers swift results, however this technique is influenced by the use of antimicrobial agents, proton pump inhibitor and bismuth containing compounds. Moreover the presence of other microorganism that produce urease can lead to false positive result (Mégraud and Lehours, 2007). Finally molecular method is extensively used for the analysis of infection. However, the greater genomic plasticity between strains obscures the target gene. On the other side serology test do not allow delination of active *H. pylori* infection. Urea breath test requires expensive equipment and reagent (Bazzoli *et al*., 1999).

The antimicrobial resistance properties are another important point in order to access the pathogenicity of bacterium. The combination of anti-acid and antimicrobial agents is known as standard treatment against gastric mucosal infections such as ampicillin,

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clarithromycin, tetracycline and metronidazole (Graham, 2000). The therapeutic choices have become somewhat narrow due to the occurrence of multi drug resistant bacterium strains. Like other bacteria, *H. pylori*, also exhibited variable response to antibiotics in diverse geographical areas, depending on the locally used drugs (Pajaras-García *et al*., 2007; Urrestarazu *et al*., 2003). Furthermore, to date, we could not find any single report on antibiotic resistance of *H. pylori* strains from gastric biopsy samples from Pakistan.

Since the data of *H. pylori* incidence in Pakistani patients is scarce and the present study was centered at investigating the rate of occurrence of *H. pylori* from gastritis or gastric ulcer patients and to also check the response of the strains to the commonly used antimicrobial drugs used for its treatment.

MATERIALS AND METHODS

Study design, participants and settings

A total number of four hundred and forty consecutive patients visited the different local hospitals of twin cities, Rawalpindi (33.62^{o}N and 73.07^{o}E) and Islamabad (33.66 ^{o}N and 73.22^{o}E) during December 2015 till December 2016. Males (age range from 35-50) and females (age range from 35-55) were asked to participate in the study. These patients were advised by the doctors to have clinical indications for an endoscopy in the gastrointestinal clinic of the hospital. The patients had undergone gastroendoscopy for dyspeptic symptoms namely epigastic pain, fullness and heartburn were asked to comply with the follow-up schedule. Among the tested group, pregnant and lactating women, patients who had received anti-secretory drugs, antibiotics, nonsteroidal anti-inflammatory drugs, bismuth or corticosteroids drugs were excluded and not considered in the study. After the initial screening, three hundred and forty four (n= 344) patients either did not meet the criteria or were not willingly to participate in the study. A total of 96 biopsy patients (48 males and 48 females) aged from 35 to 55 years were collected

Collection of biopsy sample

H. pylori was isolated according to procedure described by Mégraud and Lehours (2007) and Abu-Sbeih *et al*. (2014). Endoscopic analysis was done using Olympus video endoscopes (Olympus Tokyo, Japan). The biopsy tissues were collected from the corpus or the antrum of patients' stomach. Samples from the patients were collected in sterile normal saline at 4^{o}C. The collected isolates were instantly shifted (with in 1hr) to the laboratory for further analysis.

Culture of H. pylori from biopsy sample

For culture, biopsy samples was inoculated on to freshly prepared tryptone soy agar (TSA, Merck and Co., USA) and incubated at 37^{o}C for 3-5 days in Gas-Pak jars (BBL Microbiology System, MD, USA) with microaerophilic (Gas-Pak) generator envelop.

Colony morphology

Morphological studies of *H. pylori* colonies on TSA plates were done after incubating the culture at 37^{o}C for 3-5 days using magnifying glass.

Gram's staining

Gram's staining was done according to the procedure described by Berry and Sagar (2006). An individual colony was pulled out with sterilized loop and then mixed into water droplet on glass slides. Smear was fixed with heat through open flame. After 5 min the fixed smear was covered with Gram's iodine solution and rinsed with water. The ethyl alcohol was then poured until the color of crystal violet gone. Again, the smear was washed with distilled water and application of counter stain safranin was done for 30 sec and tailed by washing. Slides were dried with blotting paper. A drop of vegetable oil was immersed on smear ad examined under microscope.

Catalase test

Catalase activity was checked by drop method. One to three drops of 3% hydrogen peroxide were dropped on the smear and observed for immediate oxygen bubble.

Oxidase test

The oxidation of tetramethyl-*p*-phenylenediame dihydrochloride reagent from oxidase enzymes from organism was tested with the method described by Berg *et al*. (1997). One percent of N, N, N, N- tetramethyl-p-phenylenediame dihydrochloride was prepared and a loop containing the culure was added to it. The change in color was notice.

Urease test

The urease production ability of organism was determined using the standard method of Berry and Sagar (2006). The isolates were inoculated on the urea broth (10% at pH 6.8) with loop wire. The tubes were incubated at 37^{o}C for 1 hr and change in color from yellow to pink was recorded as positive result.

Nitrate reduction test

The following steps were done by using Pasteur pipette: A 50µl of the culture medium was added in solutions containing 3 dops of 1% sulphanilamide and 1 drop of 0.02% naphthylethylenediamine. The solution would change color to bright pink or purple in the presence of nitrate in medium. In the absence of any color, few grains of zinc powder were added. The appearance of purple color around the zinc particle indicates the presence of nitrate in the culture medium (Marietou *et al*., 2009).

Serum enzyme linked immunosorbent assay

Serum samples were used for anti *H. pylori* antibody IgG with NovaLisa^{TM} Enzye linked immunosorbant assay Kit

(Nova Tec Immundignosica GmbH) according to manufacturer direction. Value more than 30 NTU/ml were considered as positive.

Antibiotic susceptibility testing

Quantitative susceptibility testing was done to 40 strains, using the E-test Method (Farina *et al*., 2007; Piccolomini *et al*., 1997) with some modification. Briefly, numerous colonies of strain taken from a fresh culture on an agar plate. The strain was inoculated in 5ml of tryptone soy broth with turbidity of 3 Mac Farland standard. The suspensions were inoculated with sterile swab onto 150 mm diameter TSA plates and then surface of agar were allowed to dry. The strips were applied in plates. The plates were then placed at incubator at 37^{o}C for 3 to 5 days. Inhibitory concentrations were recorded at the point where the elliptical zone of inhibition interested the E-strip. Positive control was done using standard *H. pylori* ATCC 43504 strain. The used antimicrobial were amoxicillin (>2µg/ml), ciprofloxin (>4µg/ml), clarithromycin (2µg/ml), metronidazole (<8µg/ml), rifampin (5 µg/ml) and tetracycline (4µg/ml) respectively.

Ethical approval

The ethics committee approved the study vide letter No. PMAS-AAUR/IFNS/145 dated November 10th, 2015.

STATISTICAL ANALYSIS

The means were analyzed statistically for continuous data using statistical software 8.1. The frequency distribution were calculated by using Microsoft excel version 2010.

RESULTS

Gastric biopsies samples were collected from the patients with visible sign of *H. pylori* infection like abdominal pain, peptic ulcer, and heart burn. The patients included both male and female with age limit varying from 35 to 55 years. Samples were stored at 4^{o}C in sterilized normal saline and immediately transferred to laboratory within 1 hour and crushed with sterilized manual grinder. These samples were steak on TSA. The plates were microaerophilicaly cultured at 37^{o}C for 3 days. These colonies after incubation were morphologically and biochemically identified. The morphological characterization and confirmatory test was presented in table 1.

A total number of ninety six (96) biopsy samples were tested and forty (41.67%) were positive for *H. pylori*. Isolation frequencies were higher among males (n=24, 25%) then females (n=16, 16.67%) (fig. 1), which were statistically significant (p<0.05). The highest isolation frequencies were obtained in male from 51-55 years old (83.33%) followed by 46-50 years old (75%) and 41-45 years old (21.43%). The isolation frequencies for females showed same pattern as the highest isolates was obtained from 51-55 and 46-50 years old (63.63 and 58.30%). The data showed that the subject from 46-50 and 50-55 years old were highly infected or prone to disease (65.0 and 75.86%) (table 2).

Among the 40 strains tested, 9 (22.5%) were concurrently resistant to more than one antimicrobial drugs. The metronidazole showed the least activity as compare to other as MICs varied from <0.016 to >256µg/ml with 15% resistant (table 3). No resistance was found for rifampin and tetracycline whose CIMs ranged from <0.016 to 0.75 µg/ml respectively. CIMs for amoxicillin, ciprofloxacin, clarithromycin and metronidazole were ranges from <0.016 to >256, 0.0012 to >32, <0.016 to >64 and <0.016 to >256 µg/ml respectively.

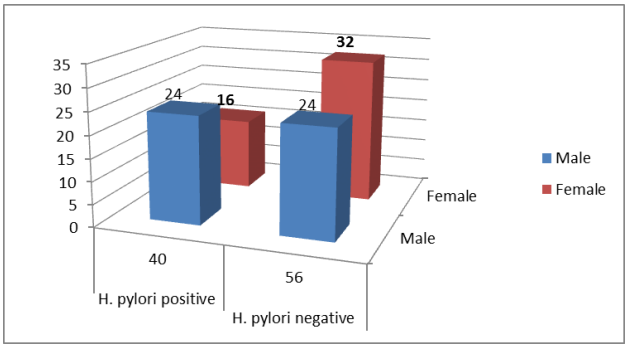

Fig. 1: Gastric biopsy samples for the screening of potential positive sign of infection

Nine *Helicobacter pylori* isolate showed the antibiotic resistance from the whole 40 biospy specimen samples which cultured on agar media in a microaerophilic jar for 3-5 days. The isolations were identified morphologically and biochemical examinations with oxidase catalase, urease and nitrate reduction activity were applied to all 9 bacterial colonies that are positive for *H. pylori* (table 4). The result showed that all of the 9 strains were oxidase positive, greyish and appeared curved, spiral rod shaped under the microscopic examination.

The nine *H. pylori* positive isolates (according to biochemical test and gram staining) were tested for susceptibility to different types of antibiotics (table 5). The frequent resistant patterns found were: 1 strain (11.11%) for amoxicillin, 5 strains (55.56%) for ciprofloxacin, 2 strains (22.22%) for clarithromycin, 3 strains for metronidazole (33.33%), 1 strain (11.11%) for combination of ciprofloxacin, clarithromycin and metronidazole and only 4 strains (44.44%) for combination of two antibiotic drugs (clarithromycin and metronidazole) respectively. On the other hand 7 strains (77.78%) were found sensitive to amoxicillin, 6 strains (66.66%) for clarithromycin while 4 strains showed sensitivity to combination of drugs.

Table 1: Morphological and confirmatory tests for *H. pylori*

Morphological characteristics		Confirmatory test		
Characteristics	*H. pylori* isolates	Test	Confirmatory observation	Results
Colony surface	Smooth, shiny	Oxidase	Blue color formation	Positive
Colony shape	Circular	Catalase	Bubble formation	Positive
Colony color	Greyish	Urease	Yellow to pink	Positive
Colony morphology	Curved, spiral rod	Nitrate reduction	No color or pink/purple color formation around zinc powder	Positive
Gram staining	Pink color	ELISA	>30 NTU/ml	Positive

Table 2: *Helicobacter pylori* isolation frequency related to age and sex

Age Range (Year)	Male	Female	Total positive culture	
	N°/n	N°/n	N°/n	%
35-40	0/8	0/15	0/23	0
41-45	3/14	2/10	5/24	20.83
46-50	6/8	7/12	13/20	65
51-55	15/18	7/11	22/29	75.86
Total	24/48	16/48	40/96	41.67

N°= number of positive culture, n= number of patient studied

Table 3: MIC ranges, MIC_{50}, MIC_{90} and resistant strain percentages of 40 isolates against antibiotic drugs

Antimicrobial drugs	MIC Ranges (mcg/ml)	MIC_{50}	MIC_{90}	Resistant strains N°/%
Amoxicillin	<0.016 - > 256	0.016	0.10	1/ 2.5
Ciprofloxacin	0.0012 - > 32	0.064	0.60	3/7.5
Clarithromycin	<0.016 - > 64	<0.016	0.10	4/10
Metronidazole	<0.016 - > 256	0.100	0.14	6/15
Rifampin	<0.016 - > 0.75	0.030	0.20	0/0
Tetracycline	<0.016 - > 0.75	0.025	0.15	0/0

DISCUSSION

Out of all the 96 samples only 40 samples were identified as *Helicobacter pylori* positive. The level of *H. pylori* infection in the present study is lower (41.67%) when linked to the testified occurrence level in previous investigation from sub-continent region (Memon and Ejaz, 2000; Ahmed *et al.,* 2008), that is due to the different method employed for detection. The other factors are age, ethinic group, area, social status, smoker, dietary habits, gender and so on. Different studies elsewhere revealed that risk of infection increases linearly with the age (Ertem *et al*., 2003). The present investigation is in the line with the study by Ahmed *et al*. (2008) and Alborzi *et al*. (2006) who concluded that chance of infection increase with age. The possible reasons are the increase in degree of contacts among people and weak defense system at old age (Rothenbacher *et al*., 2002; Rowland, 2000). The lower rate of isolation might be due to the delicate nature of organism as its survivability decrease with the delay in specimen transportation, transportation conditions and exposure to aerobic environment. The statement is further confirmed by Meunier *et al*. (1997). They demonstrated that *H. pylori* is a delicate in nature having lower isolation rate if biopsy samples are exposed to oxygen, room temperature or desiccation, during transportation.

The data suggest that males are highly prone to the exposure of inflammation caused by bacterium as compare to females (table 2). The data of our study indicates a significant difference between male and female infection outbreak, these differences are in line with the findings of Yang *et al*. (2003), however it negotiates with the study of Ahmed *et al*. (2008) who stated the non-significant difference between males and females. Kaltenthaler *et al*. (1995) concluded that it might be due to lack of hygiene in young male as compare to female or it may be due to the fact that males are more social as compare to female.

The phenotypic characterization of the colonies obtained on plates was done and grey, small, smooth and circular colonies appeared with no hemolytic act activity on the colony edges. However hemolysis was seen after 3 to 4 days of storage at 4°C. Similar results was also found by Megraud and Lehours (2007) after 3 to 5 days of incubation. Gram"s stained heat fixed smear when observed under microscope appeared to be gram negative and spiral shaped with round edges. This may be due to the reason that media favored the growth of bacteria. These findings are also in line with Owen (1998) who reported the cellular morphology of organism as negative, s-shaped and curved rod with 1 to 3 curves. The outcomes

Table 4: Patient data and test results (n=9 strains)

No	Sex	Age	Biochemical test						ELISA	Endoscopic examination
			Culture	Gram staining	Catalase	Oxidase	Urease	Nitrate reduction	IgG NTU. ml	
13	M	38	+	+	+	+	+	+	32	*H. pylori* gastritis
15	M	45	+	+	+	+	+	+	45	Gastric ulcer
23	F	43	+	+	+	+	+	+	35	*H. pylori* gastritis
24	M	55	+	+	+	+	+	+	31	*H. pylori* gastritis
29	F	55	+	+	+	+	+	+	30.3	Acute duodenal ulcer
31	F	50	+	+	+	+	+	+	35	*H. pylori* gastritis
32	M	48	+	+	+	+	+	+	38	*H. pylori* gastritis
38	F	40	+	+	+	+	+	+	104	Gastric ulcer
40	M	42	+	+	+	+	+	+	42	*H. pylori* gastritis

Table 5: Antibiotic susceptibility test of 9 *H. pylori* strains

Resistance profile	Resistant N^o (%)	Intermediate N^o (%)	Sensitive N^o (%)
Amoxicillin	1(11.11)	1 (11.11)	7 (77.78)
Ciprofloxacin	5 (55.56)	1 (11.11)	3 (33.33)
Clarithromycin	2 (22.22)	1 (11.11)	6 (66.67)
Metronidazole	3 (33.33)	4 (44.44)	2 (22.22)
Ciprofloxacin + Clarithromycin + Metronidazole	1 (11.11)	7 (77.78)	1 (11.11)
Clarithromycin + Metronidazole	4 (44.44)	1 (11.11)	4 (44.44)

are also in agreement with the Medouakh *et al*. (2010) who reported gram negative and curved rods of *H. pylori* under microscope.

Different confirmatory tests like catalase, urease, oxidase and nitrate reduction test were performed while a non-invasive technique namely enzyme linked immunosorbant assay was also used. The isolated strains produced bubbles in the presence of 30 percent hydrogen peroxide (H_2O_2) indicates the positive results for catalase. The production of blue color in the presence of N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (1%) indicated that isolates of *H. pylori* is oxidase producing. The change in color of urea broth from yellow to pink was noted for the positive urease test. The change in color to pink is due to activity of urease enzyme produced by the bacteria. A non-development of any color or formation of pink/ purple color around zinc dust was taken as positive and considered as a confirmation of particularly *H. pylori* strain. Similar activities for catalase, urease and nitrate reduction test were also observed by Marietou *et al*. (2009). They also observed the production of a powerful enzyme: urease, which changes the pH of urea broth resulting change in color from yellow to pink. It also does not possess any ability to use nitrates. The fallouts were also consistent with the result of Medouakh *et al*. (2010). They described catalase, urease and oxidase positive results while negative for nitrate reduction test for the identification of clinical isolates of bacterium. The data regarding the confirmatory test of *H. pylori* has been presented in table 1. The biopsy samples were also confirmed by using serum linked immunosorbant assay test. Due to interaction of antibodies, change in optical density was observed for the formed color (if they present in serum) with the substrate solution form yellow color. The change in color of yellow is also reported by Abu-Sbeih *et al*. (2014) and Enomoto *et al*. (2010).

In vitro sensitivity analysis of *H. pylori* is considered as a vital test as no management individually proved to be universally successful. Before the start of the treatment it is recommended to investigate the sensitivity profile of the bacterium. In this study, six frequently used antibiotics were investigated against 40 clinical isolates obtained from different groups. Our data showed a high resistance to metronidazole (15%) that could be because of frequent use of this drug for cure of diarrhea (Cederbrant *et al*., 1993). The consumption of metronidazole for tooth infection may also add to selection pressure. Additionally, repeated usage of antibiotics without prescription is also one of the reason (Aboderin *et al*., 2007). The usage in gynecological infections could elucidate the higher resistance detected in strains obtained from female. In addition the higher resistance in developing countries was well explained by the frequent use of drug against giardiasis and amebiasis (Vallejos *et al*., 2003). Otth and Wilson (2011) in Southern Chile reported 12.5% resistance while other authors reported resistance percentage in range of 26.2 to 67% (Farina *et al*., 2007; Solca *et al*., 2000; Urrestarazu *et al*., 2003; Vallejos *et al*., 2003).

Four strains were resistant to clarithromycin (10%). The resistance of *H. pylori* to this antibiotic is generally low and variable in different parts of the world. However the growing contextual rate of clarithromycin resistance shows at least a fractional explanation for the declining efficiency of conventional regime. It is vibrant that

resistance to this drug, which has been credited to numerous diverse point mutations in the peptidyltransferase region encoded in domain V of the 23S rRNA gene (142) is related with a high rate of treatment failure. Our results are lower than reported values of Vallejos *et al*. (2007) form Chile (20%). On the other hand many publications stated lower resistance rates ranging between 2 and 7% (Farina *et al*., 2007; Gonzalez *et al*., 2001; Lopez-Brea *et al*., 1997).

Three strains (7.5%) were resistant to ciprofloxacin. The findings are in line with the data reported by Otth and Wilson (2011) in Chili (5.7%), Toro *et al*. (2001) in Spain (7.9%) and Glocker *et al*. (2007) in Germany (9.5%) respectively. As shown in table 3, one strain showed a resistance (11.11%) against amoxicillin. Different studies elsewhere reported a low resistance to amoxicillin globally (Pajares- García *et al*., 2007; Vallejos *et al*., 2003). The present study was in line with Farina *et al*. (2007) and Otth and Wilson (2011) they reported a 2.2% and 2.3% resistance. On the other hand several studies reported absence of resistance of *H. pylori* strains to amoxicillin.

No strain was found resistant to tetracycline and rifampin. Similar results have already been reported by Lopez-Brea *et al*. (1997) in spain and Wolle *et al*. (2002) in Germany. However some scientists testified resistance percentage ranging from 7 to 26% for tetracycline, demonstrating that the resistance intensities are affected by the local use of antibiotics.

Resistance to several antibiotics was observed in 9 strains. Five were resistant to ciprofloxacin, four to combination of clarithromycin and metronidazole and three to metronidazole (table 5). On the other hand 7 stains were sensitive to amoxicillin and 6 to clarithromycin. As these antibiotics are often employed and suggested in *H. pylori* treatment. The present outcomes highlight a vital need to investigate the resistance profile of the bacterial isolates on regular basis to avoid treatment failures and further spread of the resistant strains.

CONCLUSION

The prevalence of *H. pylori* is high among the male patients with varying age between 51-55 years. Performance of invasive techniques enables determination of susceptibility profile antibiotic agent. Current rising antibiotics resistance for *H. pylori* is of concern. Resistance to clarithromycin, metronidazole and combination of clarithromycin + metronidazole isolated from adults of the twin cities in Pakistan is high. It is necessary to continuously monitor *H. pylori* resistance to drugs used in therapy.

REFERENCES

Aboderin OA, Abdu AR, Odetoyin B, Okeke IN, Lawal OO, Ndububa DA, Agbakwuru AE and Lamikanra A (2007). Antibiotic resistance of *Helicobacter pylori* from patients in Ile-Ife, South-West, Nigeria. *Africa Health Sci*., **7**(3): 147-148.

Abu-Sbeih RS, Hawari AD, Hassawi DS and Al-Daghistani HI (2014). Isolation and detection of *Helicobacter pylori* from patients suffering from peptic ulcer using biochemical tests and molecular techniques. *Am. J. Biochem. Biotechnol*., **10**(1): 58-68.

Ahmed T, Bilal R and Khanum A (2008). Prevalence of Helicobacter pylori infection in asymptomatic children of Islamabad suburbs (Pakistan). *Int. J. Agri. Biol*., **10**(6): 685-688.

Alborzi A, Soltani J, Pourabbas B, Oboodi B, Haghighat M, Hayati M and Rashidi M (2006). Prevalence of *Helicobacter pylori* infection in children (south of Iran). *Diagn. Microbiol. Infect. Dis*., **54** (4)**:**259-261.

Bazzoli F, Zagari M, Pozzato P, Varoli O, Fossi S, Ricciardiello L, Alampi G, Nicolini G, Sottili S, Simoni P, Roda A and Roda E (1999). Evaluation of short-term low-dose triple therapy for the eradication of Helicobacter pylori by factorial design in a randomized, double-blind, controlled study. *Aliment Pharmacol. Ther*., **12** (5): 439-445.

Berry V and Sagar V (2006). Rapid urease test to diagnose *Helicobacter pylori* infection. *Sci*., **8**(2): 86-88.

Berg D and Hoffman P *et al*. (1997). The *Helicobacter pylori* genome sequence: Genetic factor for long life in gastric mucosa. *Trends Microbiol*., **5**(12): 468-473.

Cederbrant G, Kahlmeter G and Ljungh A (1993). The E test for antimicrobial susceptibility testing of *Helicobacter pylori*. *J. Antimicrobial Chemother*., **31**(1): 65-71.

Enomoto S, Yanaoka K, Utsunomiya H, Niwa T, Inada K, Deguchi H, Ueda K, Mukoubayashi C, Inoue I, Maekita T, Nakazawa K, Iguchi M, Arii K, Tamai H, Yoshimura N, Fujishiro M, Oka M and Ichinose M (2010). Inhibitory effects of Japanese apricot (*Prunus mume* Siebold et Zucc. Ume) on *Helicobacter pylori* related chronic gastritis. *European J. Clin. Nutr. Adva*., **64**(7): 714-719.

Ertem D, Harmanci H and Pehlivanoglu E (2008). *Helicobacter pylori* infection in Turkish preschool and school children: Role of socioeconomic factors and breast feeding. *Turkish J. Pediatr*., **45**(2): 114-122.

Fariña N, Kasamatsu E, Samudio M, Morán M, Sanabria R and Laspina F (2007). Susceptibilidad a antibióticos de cepas paraguayas de *Helicobacter pylori* aisladas de pacientes con enfermedad gastroduodenal. *Rev. Med. Chile*., **135**(8): 1009-1014.

Glocker E, Stueger HP and Kist M (2007). Quinolone Resistance in *Helicobacter pylori* Isolates in Germany. *Antimicrob. Ag. Chemother*., **51**(1): 346-349.

Gonzalez C, Garcia A, Daroch F, Kawaguchi F, Solar H, Rivera N and Vega E (2001). Susceptibilidad *in vitro* de cepas de *Helicobacter pylori*: Aislamiento de cepas resistentes a claritromicina. *Rev. Med. Chile,* **129**(6): 643-646.

Graham DY (2000). Helicobacter pylori infection is the primary cause of gastric cancer. *J. Gastroenterol. Suppl.,* **35**(12): 90-97.

Hardin FJ and Wright RA (2002). *Helicobacter pylori* review and update. *Hosp. Phys.,* **1**: 23-31.

Hirschl AM and Makristathis A (2007). Methods to detect Helicobacter pylori: From culture to molecular biology. *Helicobac.,* **12**(2): 6-11.

Hocker M and Hohenberger P (2003). *Helicobacter pylori* virulence factors. One part of a big picture. *Lanc.,* **362**(9391): 1231-1233.

Kaltenthaler EC, Elsworth AM Schqeiger MS, Mara DD and Braunholtz DA (1995). Faecal contamination on children's hands and environmental surfaces in primary schools in Leeds. *Epidemiol. Infect.,* **115**(3): 527-534.

López-Brea M, Domingo D, Sanchez I and Alarcon T (1997). Evolution of resistance to metronidazole and clarithromycin in *Helicobacter pylori* clinical isolates from Spain. *J. Antimicrob. Chemother.,* **40**(2): 279-281.

Manyi-Loh CE, Clarke AM, Green E and Ndip RN (2013). Inhibitory and bactericidal activity of selected South African honeys and their solvent extracts against clinical isolates of *Helicobacter pylori*. *Pak. J. Pharma. Sci.,* **26**(5): 897-906.

Marietou A, Griffiths L and Cole J (2009). Preferential reduction of the thermodynamically less favorable electron acceptor, sulfate, by a nitrate reducing strain of the sulphate reducing bacterium *Desulfovibrio desulfuricans*. *J. Bacteriol.,* **191**(3): 882-889.

Medouakh L, Abdeslam A and Bensoltane A (2010). Antagonistic activity of *Lactobacillus* Sp. against *Helicobacter pylori*. *Intern J Microbiol Res.,* **1**: 80-86.

Mégraud F and Lehours P (2007). Helicobacter pylori detection and antimicrobial susceptibility testing. *Clin. Microbiol Rev.,* **20**(2): 280-322.

Memon IA and Ejaz MS (2000). *Helicobacter Pylori*: A correlation between symptomatology and endoscopy in children. *J. Coll. Physicians Surg. Pak.,* **10**: 87-89.

Meunier O, Walter P, Chamouard P, Piemont Y and Monteil H (1997). Isolation of *Helicobacter pylori*: Necessity of control of transport conditions. *Pathologie Biologie,* **45**(1): 82-85.

Otth L and Wilson M (2011). Isolation of *Helicobacter pylori* in gastric mucosa and susceptibility to five antimicrobial drugs in Southern Chile. *Braz J Microbiol.,* **42**(2): 442-447.

Owen RJ (1998). *Helicobacter* species classification and identification. *Br. Med. Bull.,* **54**(1): 17-30.

Pajares-c JM, Pajares-Villarroya R and Gisbert JP (2007). *Helicobacter pylori* infection: Antibiotic resistance. *Rev. Esp. Enferm. Dig.,* **99**(2): 63-70.

Piccolomini R, Bonaventura D, Catamo G, Carbone F and Neri M (1997). Comparative evaluation of the E test, agar dilution and broth microdilution for testing susceptibilities of *Helicobacter pylori* strains to 20 antimicrobials agents. *J. Clin. Microb.,* **35**(7): 1842-1846.

Ramis IB, Moraes EP, Fernandes MS, Sassi RM, Rodrigues O, Juliano CRV, Scaini CJ and Almeida da Silva PE (2012). Evaluation of diagnostic methods for the detection of Helicobacter pylori in gastric biopsy specimens of dyspeptic patients. *Braz. J. Microbiol.,* **43**(3): 903-908.

Rothenbacher D, Bode G and Brenner H (2002). Dynamics of *Helicobacter pylori* infection in early childhood in high risk group living in Germany: Loss of infection higher than acquisition. *Aliment. Pharmacol. Ther.,* **16**(9): 1663-1668.

Rowland M (2000). Transmission of *Helicobacter pylori*: Is it all child's play. *Lanc.,* **355**(9201): 332-333.

Solca NM, Bernasconi MV, Piffaretti JC (2000). Mechanism of metronidazole resistance in *Helicobacter pylori*: Comparison of the *rdxA* gene sequences in 30 strains. *Antimicrob. Ag. Chemother.,* **44**(8): 2207-2210.

Suerbaum S and Michetti P (2002). *Helicobacter pylori* infection. *New England J. Med.,* **347**(15): 1175-1186.

Toro C, Garcia-Samaniego J, Carbo J, Iniguaz A, Alarcon T, Lopez-Bera M and Baquero M (2001). Prevalencia de la resistencia primaria de *Helicobacter pylori* a ocho antimicrobianos en un hospital de Madrid. *Rev. Esp. Quimioter.,* **14**(2): 172-176.

Urrestarazu MI, Serrano N, Pinero R and Cavazza ME (2003). Susceptibilidad de *Helicobacter pylori* a los antimicrobianos. *Rev. Soc. Ven. Microbiol.,* **23**(1): 14-15.

Vallejos C, Cerda O, Valenzuela M and Toledo H (2003). Resistencia antimicrobiana en *Helicobacter pylori*: Aspectos clínicos y moleculares. *Rev. Méd. Chile,* **131**(11): 1313-1320.

Vallejos C, Garrido L, Caceres D, Madrid AM, Defilippi C, Defilippi C and Toledo H (2007). Prevalencia de la resistencia a metronidazole, clarithromycin y tetraciclina en *Helicobacter pylori* aislado de pacientes de la region metropolitana. *Rev. Med. Chile,* **135**(3): 287-293.

Wolle K, Leodolter A, Malfertheiner P and Konig W (2002). Antibiotic susceptibility of *Helicobacter pylori* in Germany: Stable primary resistance from 1995 to 2000. *J. Med. Microbiol.,* **51**(8): 705-709.

Yang Y J, Wang SM, Chen CT, Huang MC, Chang CJ and Liu CC (2003). Lack of evidence for fecal oral transmission of *Helicobacter pylori* infection in Taiwanese. *J. Formos Med. Assoc.,* **102**(6): 375-378.

Yvonne THP, Duynhoven V and De Jonge R (2001). Transmission of *Helicobacter pylori*: A role for food. *Bull. World Heal. Org.,* **79**(5): 455-460.