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Helicobacter Pylori Infection and Frequency of Clarithromycin Resistance by qPCR

Samia Perwaiz Khan, Rubina Ghani, Safia Izhar, Ajeet Kumar, Ambreen Irshad, Shaista Emad, Aemen Moeen, Ayesha Abbasi, Maham Sattar, Syed Sohaib Hasan

ABSTRACT.

Objective: Determine the frequency of Helicobacter pylori (H. pylori) infection in our population, the response to triple-quadruple regimen and resistance to clarithromycin.

Study Design and Setting: Study design is case-series, Medicare Cardiac & General Hospital, Karachi- 2020-2021. All patients (N=110) were selected from outpatient department (OPD) of the Jinnah Medical College Hospital (JMCH) and Medicare Cardiac and General Hospital.

Methodology: Patients with nausea, abdominal pain, diarrhea and IgG positive were included, and ELISA was done for detection of H. pylori infection.. IgG negative for H. pylori and having other gastrointestinal infections were excluded from this study. Patients positive with infection were prescribed the initial triple /quadruple regimen (triple regimen therapy including Proton pump inhibitor (PPI) 20 mg, Metronidazole 400mg, Amoxicillin 250 mg or Quadruple therapy by adding Bismuth subsalicylate). In ten cases of relapse Sequential / Rescue therapy were continued after a gap of 6 weeks included PPI 20 mg, metronidazole 400mg ciprofloxacin 200mg BD or Levofloxacin 400 mg OD. The qPCR was performed for the detecting resistant to clarithromycin in patients with H. pylori IgG positive after therapy.

Result: During the follow-up, 60 (54%) cases were recovered from initial triple regimen, whereas 40(36%) cases recovered quadruple therapy and remaining 10 (7%) had clarithromycin resistance and were prescribed sequential therapy replacing clarithromycin by fluoroquinolones.

Conclusion: The study showed that majority of H. pylori infected patient in our population recovered from initial triple/quadruple regimen. The alternate option with clarithromycin resistant was sequential and rescue therapy with high eradication rate.

Keywords: *Helicobacter pylori*, triple therapy, quadruple therapy, qPCR–qualitative polymerase chain reaction, sequential therapy, ELISA (Enzyme-Linked Immunosorbent Assays)

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INTRODUCTION:

Helicobacter pylori (H. pylori) is a frequent cause of gastrointestinal infection in humans. It is a gram-negative bacterium causing serious health issues such as chronic gastritis, peptic ulcer disease and gastric cancer. Spread of

infection affects nearly half of the global population. Appropriate management of *H. pylori* with combination of antibiotics is the best preventive measure against peptic ulcers and gastritis[1]. For effective healing after initial infection, it is important to select an appropriate antibiotic to treat the infection. Appropriate treatment is most important to avoid all the serious complications and antibiotic resistance[2] which is becoming serious problem in effective eradication of this infection. In the 90s, the standard triple therapy was considered as the gold standard for the treatment of H. pylor[3].,but with emergence of antibiotic resistance, sequential and rescue therapy are also in clinical use now[4]. The prevalence of *H. pylori* infection is high, in Pakistan [4, 5, 6]. A Study carried out in Lahore, three hundred patients were infected by *H. pylori*.4 Study participants, were divided into two equal groups, one group was on clarithromycin, amoxicillin and omeprazole. The other group was prescribed triple regimen with levofloxacin replacing clarithromycin. Outcomes were better in second group with less serious adverse-effects. Conditions which increase the risk of infection include poor sanitation conditions and fast urbanization. In 2015, it was reported that global burden of *H. pylori* infection, as an attributable fraction (AF) for chronic infection and cause of non-cardia gastric carcinoma.[7]. It was reported the mode of *H. pylori* transmission is unknown, but it is thought to be mainly through the fecal-oral route. Other modes of transmission of *H. pylori* are oral-oral, water-borne transmissions, or poorly disinfected endoscopes. *H. pylori* invades the luminal side of epithelial cells of the mucosal layer of stomach[8-10]. The aim of the study is to change *H. pylori* -treatment with its management for the implementation and targeting educational management to assess the relative effectiveness. *Helicobacter pylori* infection is most carcinogenic and produces gastric adenocarcinoma. Due to the alarmingly high antibiotic resistance in *H. pylori*, gastroenterologists should change the empiric *H. pylori* treatment according to an antimicrobial susceptibility testing-guided appropriate treatment. Antimicrobial susceptibility patterns for *H. pylori* should be conducted to monitor the antibiotic resistance pattern. Antimicrobial susceptibility testing may be laborious and time-consuming, although *H. pylori* can be cultured in almost every microbiology laboratory after training is provided to the microbiologists, so that they can provide the susceptibility testing to guide the treatment of *H. pylori*. Culture of *H. pylori* and subsequent susceptibility testing take 1–2 weeks, and although it is time-consuming; however, there is to initiate the treatment without knowing the susceptibility testing results because most of the patients have had the infection for decades sometimes *H. pylori* is usually acquired in childhood.[4] Thus getting culture and sensitivity done is highly recommended offers an opportunity for "treating it right the first time". This is very important as the cure rate is highest with initial therapy if the right antibiotics are chosen, whereas after the failure of initial therapy, the bacterium will mostly likely develop drug resistance and it will become more difficult to treat. That is why it is important to develop advancements to the initial treatment to be able to treat it the first time and eradicate it permanently. Therapeutic options include clarithromycin, metronidazole, amoxicillin, tetracyclines, colloid bismuth sub citrate, ranitidine, proton pump inhibitors (omeprazole) and other antimicrobial agents' duration is from one week, ten days to fourteen days.[5,6]

The main outcome of this study was to assess the efficacy of bismuth-based regimens[5],carried out on the principles of second-line therapy rather than the specific agents used. The Quadruple and 14-day regimens were generally more successful than shorter triple therapy. A study found quinolone-based therapies of more than 10 days duration to be the most effective second-line therapy. While in recent years the efficacy of bismuth-based quadruple therapy as a second-line therapy has been clearly established, there is now substantial evidence that it is the best performing first-line therapy. Antibiotic resistance was studied and a clear and dramatic increase in resistance is noted for clarithromycin and levofloxacin; most notably, it may not be possible to support these therapies in most regions of the world much longer without testing.

METHODOLOGY:

All hundred and ten participants were selected from the OPD of Jinnah Medical College Hospital (JMCH) and Medicare Cardiac and General Hospital, after taking the history of the patients on the bases of symptoms related dyspepsia, epigastric pain, and nausea. All the patients gave written informed consent to participate in the study.

Inclusion Criteria: These patients had IgG positive on ELISA (Enzyme-Linked immunosorbent Assays) for detection of H. pylori infection.

Exclusion Criteria: Patients with other gastrointestinal symptoms were excluded from this study.Patients with positive infection were prescribed the appropriate antimicrobial regimen. In this study the therapeutic options include clarithromycin, metronidazole, amoxicillin, tetracycline, colloid bismuth sub citrate, ranitidine, proton pump inhibitors and other antimicrobial agents' duration is going to be monitored for fourteen days to check management outcome. Hundred and ten blood samples collected from Medicare cardiac & JMH Korangi campus, by serological test, Eliza and PCR. Antibiotic regimen was prescribed consisting of triple regimen therapy including Proton pump inhibitor (PPI) 20 mg, Metronidazole 400mg, Amoxicillin 250 mg. In case of resistance quadruple therapy was prescribed included PPI 20 mg, metronidazole 400mg BD, Amoxicillin 250 mg BD, Bismuth subsalicylate. In ten cases of reoccurrence Sequential and Rescue therapy were continued after a gap of 6 weeks included PPI 20 mg,

metronidazole 400mg ciprofloxacin 200mg BD or Rescue therapy includes PPI 20 mg BD, Metronidazole 400 mg BD, ciprofloxacin 200mg BD or Levofloxacin 400 mg OD.

The qPCR was performed on the patients to confirm the H. pylori positive cases. The DNA extraction with performed by using a zymogen extraction kit (cat# D 3205) following the kit protocol. The DNA samples were amplified with qPCR (SLAN Instrument) amplification of the H. pylori gene fragment with slightly modification the qPCR was performed with the primer sequence 5'-AGATGGGAGCTGTCTCAACCAG-3' as forward primer and the reverse primer 5'-TCCTGCGCATGATATTCCC-3' (Integrated DNA Technologies, Inc., Coralville, Iowa) The total volume of master mix was 25ìl for qPCR. The master mix including 10 µl, (ABM One Step Bright Green q PCR kit G891), 2.5ìl set of primer, 5ìl extracted DNA and nuclease-free water to make the volume 25 ìl. The thermal cycle was programmed was pre-denaturation at 95°C for 10min, following with 40 cycles, denaturation at 95°C for 15 second, annealing at 60°C for 1 minute.

The ethical consideration was approval from ethnic research committee (ERC) of Jinnah Medical and Dental College (Protocol #. 00043/20).

RESULTS:

The patients included in study with general characteristics having the symptoms of dyspepsia and epigastric pain. In this study 50 % were the male and 60 % female with the age range between 20-70 years, with low to middle socioeconomic status as shown in table 1. Antibiotic regimen prescribed to the patients with *H. pylori* positive. The triple regimen therapy included Proton pump inhibitor (PPI) 20 mg x bid, amoxicillin 500 mg x tds, clarithromycin 500 mg x bid for fourteen days and were asked to come for follow-up. In 50 patients, prescribed quadruple therapy having PPI 20 mg, metronidazole 400mg bid, amoxicillin 250 mg bid, Bismuth subsalicylate showed complete recovery on follow-up. The n=10 with relapse, were prescribed sequential therapy. During time period 10 patients showed no response with clarithromycin and again IgG was performed and it was still positive and on performing the qPCR, *H. pylori* strain was observed the resistant pattern as shown in figure 2(a, b, c, d). The rescue therapy was prescribed that includes PPI 20 mg, metronidazole 400 mg, ciprofloxacin 500mg or levofloxacin 400 mg twice daily. On follow-up again the *H. pylori* antibodies were performed and it was noted that IgG – negative.

The qPCR was performed on the patients to confirm the H. pylori positive cases following the above-mentioned protocol. After the analysis curve and cycle threshold (Ct) value is noted as shown in figure 1and table 2. The lower Ct value is reported as positive and the Ct value is reported negative for H. pylori Figure 1 shows the positive cases which were identified on their low Ct (Cycle Threshold) value, and negative cases are identified on their high Ct (Cycle Threshold) value of *H. pylori* with qPCR. *This figure is s generated when qPCR gives the result and Ct value Table 2: shows the Cycle threshold (Ct) value of the positive patients with *H. pylori* infection. The highlighted with red shows positive cases.

After diagnosis with qPCR, the positive cases were given treatment. The first lineantibiotic regimen prescribed consisting of triple regimen therapy including proton pump inhibitor (PPI) 20 mg x bid, clarithromycin 500 mg x bid, amoxicillin 500 mg x tds. The second line quadruple therapy by including bismuth subsalicylate included after two weeks in these [n=40 (36%)] patients with symptoms. All the (N=110) cases were monitored, only [n=10(7%)] patients were found to be resistance to initial therapy were prescribed sequential therapy after a gap of 6 weeks included PPI 20 mg x bid, metronidazole 400 mg x tds`ciprofloxacin 500mg x bid.The medication was stopped for some time and again the samples were collected from these patients and this time RNA was isolated by using zymogen RNA extraction kit (cat# R1055) following the kit protocol. The qPCR was performed to identify the reason for the relapse by using type specific primer sequence of Clarithromycin resistant strain of H. pylori, 23S rRNA gene shown in table 3. The qPCR was performed on blood sample of the patients having relapse of infection due to antibiotic resistance. The qPCR protocol with some modification was performed by making total volume of reaction 30 ìl including qPCR master mix 10 µl, enzyme mix 1ìl (ABM One Step Bright Green q PCR kit G891), all the upstream primers 2 ìl, each, common downstream primer 2 ìl, extracted RNA 5ìl and volume was adjusted with nuclease-free water. The thermal cycle was programmed for cDNA synthesis at 42°C for 15min, pre-denaturation at 95°C for 10min, denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute for 45 cycles. The result was observed as shown in figure 2 with cycle threshold (Ct) value

Table 1: General characteristics of *H. pylori* infected patients

Variables	*H. pylori* **infected patients N= 110**	**Percentage**
Gender M:F	50:60	
Age	20-70 years	
Low /Middle class	110	100%
Dyspepsia	100	90%
Epigastric Pain	110	90%
Nausea	55	50%
Diarrhea	45	40%
Hematemesis & Malena	0	0

Figure1: Shows the positive cases which were identified on their low Ct (Cycle Threshold) value, and negative cases are identified on their high Ct (Cycle Threshold) value of *H. pylori* with qPCR

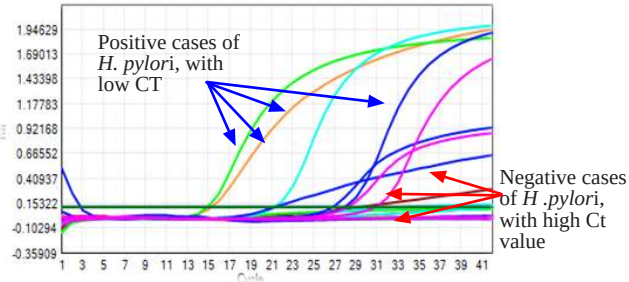


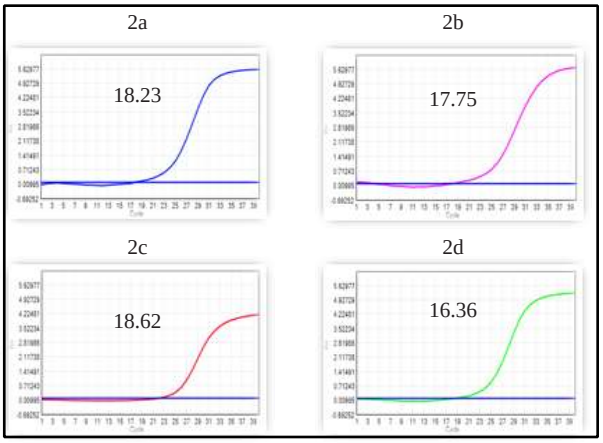
Table 2: shows the Cycle threshold (Ct) value of the positive patients with *H. pylori* infection. the highlighted with red shows positive case

Well	Project	Sample Name	Tube Name	Channel	Target	Ct Value
A1	*H. pylori*	S1	g1	1	sample	30.87
B1	*H. pylori*	S2	m1	1	sample	27.92
C1	*H. pylori*	S3	m2	1	sample	21.25
D1	*H. pylori*	S4	m3	1	sample	14.88
E1	*H. pylori*	S5	m4	1	sample	15.26
F1	*H. pylori*	S6	m5	1	sample	15.5
G1	*H. pylori*	S7	m6	1	sample	29.35
H1	*H. pylori*	S8	m7	1	sample	29.58
A2	*H. pylori*	S9	g2	1	sample	13.21
B2	*H. pylori*	S10	m2	1	sample	21.35
C2	*H. pylori*	S11	C5	1	sample	22.45
D2	*H. pylori*	S12	C6	1	NC	No Ct

Table 3: Primer sequence of information for the *H. pylori* 23S rRNA gene used for resistant with Clarithromycin

Primers	Sequence
	5'-CTACCCGCGGCAAGACTGA-3'
G upstream primer	5'-CTACCCGCGGCAAGACTGG-3'
C upstream primer	5'-CTACCCGCGGCAAGACTGC-3'
G upstream primer	5'-CTACCCGCGGCAAGACGTAG-3'
Common downstream primer	5'-ATAGGTGGGAGGCTTTGAAGTA-3'

Figure 2: a b c and d show the individual graph spikes of the resistant strain of the *H. pylori* with Clarithromycin



DISCUSSION:

Helicobacter pylori has been the causative organism for chronic gastritis, gastric /duodenal ulcers and the most serious outcome of this infection is gastric adenocarcinoma. After contact with *H. pylori* patient exhibits IgA, IgG and IgM antibodies. IgG antibody is detected (*Emproimmun Seekamp.31.23560*) in few weeks and remains in the serum for long time. *H. pylori* infection has serious complications such as duodenal ulcer and gastric cancer[7]. Thus, appropriate treatment is most essential to avoid such serious complications. The cause of *H. pylori* has been found as above fifty percent in undeveloped countries compared with nearly thirty five percent in developed countries and globally noted that 43% in females and 46% in males. The prevalence of infection in adults (=18 years) was significantly higher than in children[11], In our study females effected were 60 as compared to males 50. The high incidence of *H. pylori* is large in cities like Karachi, antibiotic resistance to clarithromycin was detected in 10 (7%) patients.

The importance of various approaches for the diagnosis of *H. pylori* and antibiotic therapy-based management is effective. The rate of recovery is greatest with initial therapy when right antibiotics have been prescribed, and the failure of initial therapy will mostly likely be due to the development of antibiotic resistance by the bacteria. Ierardi E et al 12, 2017 reported the molecular diagnosis has been used for the detection of *H. pylori* with evaluation of the virulence factors and antibiotic sensitivity. In our study out of total one hundred and ten patients having tested IgG positive for *H. pylori* were initially prescribed the triple regimen, 60 (54%) cases recovered completely, resistance or recurrence of infection 40 (36%) cases were prescribed quadruple regimen, sequential regimen prescribed 10 (7%).

H. pylori infection frequency in Pakistan is high 4,5,13 maybe due to large number of family members living together. The modification for using sequential regimen [14,15,16 17,18] showed beneficial therapeutic strategy for the management of H. pylori infection in clinical practice. The initial therapy includes triple regimen including proton pump inhibitors, clarithromycin, amoxicillin. The outcome of clarithromycin resistance has been reported worldwide[16]. Antibiotic resistance is a major problem in effective treatment to *H. pylori* infection, thus the initial triple regimen consisting of clarithromycin, amoxicillin, proton pump inhibitors are not that effective [18,19 20]. In this study resistance to initial therapy was high and recovery rate was 54% only and recovery from quadruple therapy was increase by 36%. The sequential therapy was prescribed to increase recovery rate. The triple therapy, quadruple therapy, sequential therapy and regimens containing fluroquinolones such as levofloxacin are routinely used [21, 22,23]. The continuous rise of *H. pylori* on secondary antimicrobial resistance, in particular to clarithromycin. Some patients on sequential therapy was carried out for either ten days or two weeks on esomeprazole

40 mg and amoxicillin 1 g for 7 days followed by esomeprazole 40 mg, clarithromycin 500 mg and tinidazole 500 mg for 7 days, all given twice daily. The efficacy of lansoprazole, bismuth, levofloxacin, and amoxicillin therapy compared to bismuth metronidazole tetracycline (BMT) quadruple therapy for second-line treatment of *H. pylori*. Studies found that sequential and hybrid therapies have found to be better eradication rate [24 , 25] in their studies showed triple and quadruple regimens patients with no compliance [2,25,] In study done in Korea[18], antibiotic resistance to clarithromycin has been reported 15%. Resistance against both clarithromycin and metronidazole reported was 8.6%. In our study 75% of first-line treatment is successful and whereas the participate achieved successful eradication with second-line treatment. The multidrug resistance is increasing, and standard triple therapy (STT) is no longer acceptable as first-line option eradication for *H. pylori* [14, 19] Similarly, our study has reported effectiveness consistent therapy of clarithromycin and metronidazole up to 89% and recurrence in 11% patients who were prescribed sequential and rescue therapy.

CONCLUSION:

The study was conducted to diagnosis and management of regimen to eradicate antibiotic resistant for *H. pylori.* The main challenge in treatment of *H. pylori* is resistance, which reduces the eradication by prescribed antibiotic regimens. Combination antibiotic therapy has been highly beneficial regimen for eradicating of antibiotic resistant strains of *H. pylori*. It is also concluded the importance of diagnosis of *H. pylori* by using different diagnostic tools before the treatment and on follow-up comparing with consistent guideline and new recommendations as well as the sensitivity pattern of the drug clarithromycin should be monitored in patient's detection with clarithromycin resistant *H. pylori* infection.

Authors Contribution:
Samia Perwaiz Khan: Constructing an idea or hypothesis for research and/or manuscript, Taking responsibility in logical interpretation and presentation of the results
Rubina Ghani: Planning methodology to reach the conclusion, Taking responsibility in the construction of the whole or body of the manuscript
Safia Izhar: Organising and supervising the course of the project or the article and taking the responsibility
Ajeet Kumar: Biological materials, reagents and referred patients
Ambreen Irshad: Taking responsibility in this necessary function
Shaista Emad: Reviewing the article before submission not only for spelling and grammar but also for its intellectual content.
Aemen Moeen: Taking responsibility in execution of the experiments, patient follow-up, data management and reporting
Ayesha Abbasi: Taking responsibility in execution of the experiments, patient follow-up, data management and reporting
Maham Sattar: Taking responsibility in execution of the experiments, patient follow-up, data management and reporting
Syed Sohaib Hasan: Taking responsibility in execution of the experiments, patient follow-up, data management and reporting

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