Original Article

Macrolide and Fluoroquinolone Resistance in Helicobacter pylori isolates: an experience at a tertiary care centre in Pakistan

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Abstract

Objective: To assess fluoroquinolone and clarithromycin susceptibility pattern along with the types of genomic mutations involved in the resistance of Helicobacter pylori isolates.

Methods: The cross-sectional study was conducted at the Department of Pathology and Microbiology, Aga Khan University Hospital, Karachi, from June 2009 to July 2010, and comprised 162 gastric biopsy samples which were tested with GenoType® HelicoDR (Hain Lifescience GmbH, Germany), a reverse hybridisation multiplex polymerase chain reaction (PCR) line probe assay (LiPA). Also, 23S rRNA (ribosomal ribonucleic acid) gene was analysed with three-point mutations at A2146G, A2146C and A2147G for clarithromycin, and gyrA gene was analyzed at two codon positions 87 and 91 for fluoroquinolone susceptibility testing. SPSS 19 was used for statistical analyses.

Results: Clarithromycin resistance was seen in 60 (37.0%) of the isolates mainly involving mutation at A2147G (85%) followed by A2146G (n=35; 21.6%) and A2146C (n=19; 11.6%). Fluoroquinolone resistance was noted in 101(62.3%) isolates, while gyrA mutations at codon 87 was seen in 64 (39.6%) and at codon 91 in 66 (40.6%). Isolates showing combined resistance to both antibiotics were 44 (26.9%).

Conclusion: High rate of resistance to fluoroquinolones was seen despite the fact that the drug was not part of the first-line anti-helicobacter therapy. There was moderate increase of clarithromycin resistance beyond the cutoff rates where empirical use of this antibiotic is abandoned. The findings warrant the need for pre-treatment susceptibility testing in Helicobacter pylori infections, especially in Pakistan where burden of disease is high and very limited data is available, to improve patient care by providing targeted therapy.

Keywords: H. pylori, Fluoroquinolone, Clarithromycin, A2147G, GenoType® HelicoDR. (JPMA 62: 1140; 2012)

Introduction

Eradication of Helicobacter pylori is a concern for developing countries where prevalence of H. pylori is more than 90% and acquisition of infection is seen at an early age.[1] Moreover, the spectrum of diseases caused by H. pylori in the developing countries is broader than chronic gastritis and peptic ulcer and includes conditions like chronic diarrhea, malnutrition, and predisposition to other enteric pathogens like typhoid and cholera.[1] Recently, acute decline in eradication rates of H. pylori has been noted and reported from various countries primarily due to emerging antibiotic-resistant isolates to clarithromycin and metronidazole.[2-4]

The Maastricht III Consensus report recommends triple therapy using a proton pump inhibitor (PPI) combined with clarithromycin and amoxicillin or metronidazole as first-line agents for the eradication of H. pylori.[5] Clarithromycin resistance can drop the eradication rate up to 58% and is frequently associated with point mutations within the peptidyltransferase-encoding region in domain V of the 23S rRNA (ribosomal ribonucleic acid) gene.[6] The fluoroquinolone group of antibiotics is considered an alternative with high prevalence of clarithromycin-resistance and is often recommended following failure with first-line therapy. But recently resistance among this class of antibiotic has also been reported with decline in eradication rates, warranting the need for susceptibility testing.[7]

Fastidious nature and need for the sophisticated culture techniques precludes routine susceptibility testing for H. pylori in diagnostic laboratories in Pakistan, thus susceptibility data is lacking from this part of the world. The study was conducted to analyse the fluoroquinolone and clarithromycin susceptibility pattern along with types of genomic mutations involved in the resistance of H. pylori isolates from Pakistan.

To the best of our knowledge, this is the first study from Pakistan reporting the frequencies of resistance and types of genetic mutations of clarithromycin and

fluoroquinolone using the line probe assay (LiPA) technique.

Materials and Methods

The cross-sectional study was conducted at the Department of Pathology and Microbiology, Aga Khan University Hospital, Karachi (AKUH), from June 2009 to July 2010, and comprised 162 biopsies positive for H. pylori diagnosed on haematoxylin and eosin (H&E) staining by an expert histopathologist. The biopsies were randomly selected using consecutive sampling technique, and subjected to process of DNA extraction, polymerase chain reaction (PCR) and reverse hybridisation. Patients' clinical information, endoscopic and histopathological findings were also reviewed. Ethical approval for the study was taken from the Ethics Review Committee of the AKUH.

GenoType® HelicoDR (Hain Lifescience GmbH, Germany), a multiplex-PCR based reverse hybridisation line probe assay (LiPA) was used in the study. The method employs simultaneous detection of resistances to clarithromycin and fluoroquinolone using the most common mutations at 23SrRNA and gyrA genes for clarithromycin and fluoroquinolone resistances respectively. This method has been previously evaluated with reported sensitivity and specificity of 87% and 98% respectively.[8]

DNA was extracted from paraffin embedded tissue biopsy specimens using QIAamp DNA FFPE Tissue kit (QIAGEN GmbH, Germany). A 10µm thick section of biopsy specimen was treated with xylene with aggressive agitation to remove paraffin from the sample and then absolute ethanol was added to remove residual xylene. The sample was digested using proteinase K at 56°C for 1 hour. For partial removal of formalin, cross-linking of released DNA (deoxyribonucleic acid) sample was then incubated at 90°C for 1 hour with Buffer ATL (animal tissue lysis). Subsequently, the entire lysate along with ethanol was transferred to a QIAamp MinElute Column and centrifuged at 8,000rpm for 1min. The columns were then washed multiple times using the commercial buffers supplied by the manufacturers (Buffers AW1 and AW2). The DNA was finally eluted using 55µl of ATE buffer and centrifuged at 14,000rpm to obtain purified DNA.

The protocol of amplification and hybridization was performed in line with methods available in literature.[9] Briefly, 5µl of extracted DNA was mixed with 35µl of 5'-biotinylated primers and nucleotide mix, 5µl of 10X PCR buffer, 2µl of 25mM MgCl 2, 3µl of molecular grade water to make the master mix. 1U of HotStarTaq® DNA polymerase (Qiagen GmbH, Germany) was then added to each reaction tube. The thermal cycling profile consisted of an initial denaturation cycle at 95°C for 15min, followed by 10 cycles at 95°C for 30sec and at 58°C for 2min. Then 25 cycles were composed of denaturation, annealing and extension at 95°C for 25sec, 53°C for 40sec and at 70°C for 40sec respectively, followed by a final extension step at 70°C for 8min. Negative controls were run with each batch of testing specimens.

Hybridisation was performed using reagents provided with Genotype® HelicoDR kit (Hain Lifescience GmbH, Germany). For the purpose, 20µl of the PCR product was mixed with denaturation solution and incubated for 5min at room temperature. After placing the DNA strips, 1ml of pre-warmed Hybridisation Buffer was added and incubated at 45°C for 30min in TwinCubator® (Hain Life science GmbH, Germany). After decanting the Hybridisation Buffer completely, DNA strips were immersed in 1ml of pre-warmed Stringent Wash Solution and incubated at 45°C for 15min. This was followed by the washing of DNA strips with 1ml of Rinse Solution, 1ml of Diluted Conjugate Solution was added and DNA Strips were incubated at room temperature for 30min. After removal of the conjugate solution and rinsing of DNA strips twice with the rinse solution and once with the molecular grade water, 1ml of Diluted Substrate solution was added and incubated at room temperature till the bands appeared on the strips. Reaction was stopped using molecular grade water and then DNA strips were allowed to air-dry and were pasted on the Evaluation Sheet. Results were interpreted using the given Evaluation Card and recorded as per the manufacturer's recommendations.

Four control probes were available on each GenoType® HelicoDR DNA-Strip: Conjugate control (CC) for the efficiency of conjugate binding and substrate reaction; amplification control (AC) for the correct performance of the test; HP for the presence of H. pylori in the sample; and locus controls for gyrA and 23S RNA to detect respective gene regions.

Isolate was considered sensitive if probes for Wild Type tested positive and there was no detectable mutation within the examined region, whereas the absence of the Wild Type band regardless of the presence of mutations in any of the examined region was reported as resistant strain. The mutation probes present on the DNA strips were only used for epidemiological studies.

The sample size was calculated on the basis of previous literature. The incidence of primary macrolide (clarithromycin) resistance is around 4-12%,[5] while fluoroquinolone resistance is reported as 2-22%.[7] To estimate the frequency of the resistance of H. pylori to clarithromycin and fluoroquinolone, with 95% confidence interval, level of significance (α) of 5%, an anticipated frequency of resistance (p= 8%), and bond on the error of

estimation of 4%, a sample size of 170 was required.

The data was analysed through SPSS version 19.0. Frequencies and percentages were generated by cross-tabulation for categorical variables like gender and clarithromycin and fluoroquinolone resistance.

Results

A total of 170 paraffin embedded tissue biopsy samples positive for H. pylori by histopathological diagnosis on routine H&E stain were randomly selected for the study. Of these, 8 (4.7%) samples could not be analysed due to insufficient amount of DNA extracted. As such, 162 samples were subjected to final analysis. There were 84 (51.8%) males and 78 (48.2%) females, with age ranging between 9 and 75 years. There was no statistically significant difference in endoscopic and histopathological features between clarithromycin and fluoroquinolone sensitive and resistant groups.

Of the 162 samples tested, 60 (37.0%) were found to be clarithromycin-resistant, while 101 (62.3%) were found to be fluoroquinolone-resistant, and 44 (26.9%) showed resistance to both the antibiotics.

A total of nine gyrA gene probes were present on each DNA strip; four corresponding to susceptible phenotypes (Wild Types) at codon 87 (N87-AAC, N87-AAT, T87-ACC and T87-ACT), one at codon 91 (D91-GAT), and four corresponding to resistant phenotypes (mutations) at positions 87 and 91 (N87K-AAA, D91N-AAT, D91G-GGT and D91Y-TAT).

Fluoroquinolone susceptibility was assessed at two codon positions 87 and 91. At codon 87, among the 4 Wild Type probes WT 1 and 2 were common in the strains. WT1 (N87) was the commonest of all wild types, 30.2% (n=49), followed by WT2 (N87) with 7.4% (n=12). None of them had WT3 and WT4 (T87). Mutation at codon 87 was observed in 40 (39.6%) isolates. At codon 91, MUT1 (D91N) was the most common mutation observed, 31 (30.6%) followed by MUT2 (D91G) 7 (6.9%) and MUT3 (D91Y) 3 (2.9%). Isolates not showing any signals for the WT or mutation either at codon 87 or 91 were 20 (19.8%) and were thus reported as fluoroquinolone-resistant strains as per the manufacturer's recommendations (Table-1).

Four, 23S rRNA gene probes were present on each DNA strip; one corresponding to susceptible phenotype (Wild Type) (2146 and 2147-AA), and three corresponding to resistant phenotypes (mutations) as A2146G, A2146C and A2147G.

WT (2146-2147 AA) was found in 102 susceptible strains. Among 60 resistant isolates, MUT3 (A2147G) was the commonest 51 (85%) followed by MUT1 (A2146G) 13 (21.6%) and MUT2 (A2146C) in 7 (11.6%). The A2146C mutation was observed only in association with other mutations.

Table 1: Details of resistant genotypes.

Genotype	n=101 (gyrA) n=60 (rrl)	%	Codon / Mutation Nucleotides*
gyrA gene			
Codon 87			
WT1 + MUT	21	20.7	N87 + N87K
WT2 + MUT	4	3.9	N87 + N87K
WT1 + WT2 + MUT	1	0.9	N87 + N87 + N87K
MUT	14	13.8	N87K
Codon 91			
WT + MUT1	16	15.8	D91 + D91N
WT + MUT2	2	1.9	D91 + D91G
WT + MUT3	2	1.9	D91 + D91Y
MUT1	15	14.8	D91N
MUT2	5	4.9	D91G
MUT3	1	0.9	D91Y
No WT or MUT band	20	19.8	
rrl gene			
23S rRNA			
WT + MUT1	4	6.6	2146-2147 + A2146G
WT + MUT 2	1	1.6	2146-2147 + A2146C
WT + MUT 3	24	40	2146-2147 + A2147G
WT + MUT1 + MUT3	5	8.3	2146-2147 + A2146G + A2147G
WT + MUT2 + MUT3	4	6.6	2146-2147 + A2146C + A2147G
MUT1	2	3.3	A2146G
MUT3	14	23.3	A2146C
MUT1 + MUT3	2	3.3	A2146G + A2147G
MUT2 + MUT3	2	3.3	A2146C + A2147G
No WT or MUT band	2	3.3	

*(Numbering system used in H. pylori J99 and 26695 (GenBank accession no. NC000921 and NC000915).

Table-2: Predominant mutations responsible for clarithromycin resistance in different regions.

Region	A2143G (%)	A2142G (%)	A2142C (%)	Year	Reference
Japan	22.2	66.6	0	2000	Umegaki[16]
Taiwan	83.3	8.3	0	2001	Yang[17]
Poland	69.5	17.3	8.6	2001	Dzierzanowska-fangrat[18]
Korea	66.7	0	0	2002	Kim[19]
Iran	73.6	5.2	21.5	2005	Mohammadi[20]
France	76	20	3	2009	Cambau[8]
Brazil	71.4	28.6	0	2010	Lins[9]
Pakistan	9	27	3	2010	Yaqoob[13]
Pakistan	85	21.6	11.6	2011	Current Study

Two samples did not show hybridisation bands either on Wild Type or mutation profile, suggesting unknown mutation.

A comparative analysis to assess the type of mutations detected in H. pylori isolates from Pakistan with those reported earlier from various parts of the world was also done (Table-2).

Discussion

Reported clarithromycin resistance from different parts of the world are concordant with our results - 52% in Brazil,[10] 27.7% in Japan.[11] In children alone, the reported resistance is 34.4% in Austria, 36.7% in France, 39.4% in Portugal and 49.2% in Spain.[12] Study on the peri-urban community of Karachi revealed 67% infant colonisation even at the age of 9 months[13] but interestingly in our study we had only one sample from paediatric population, reflecting either children being under-diagnosed or misdiagnosed or tested with non-invasive detection methods. However, children with upper gastrointestinal symptoms require multiple gastric biopsies for the accurate diagnosis of organic pathology[12] and pre-treatment susceptibility testing is specially warranted in children with areas of high prevalence. This population needs attention in future studies from our part of the world.

The commonest mutation related to clarithromycin resistance in our study was A2147G. Mutations with A2147G is associated with high level of clarithromycin resistance and lower cure rates.[14] On the other hand, mutation with A2146G have higher reported minimal inhibitory concentrations (MICs) but clinical relevance is minimal.[15]

Clarithromycin resistance is moderately high in our study, and the type of mutation responsible for its resistance in our population is the one with more chances of treatment failure (A2147G). Although reports from other studies indicate different results,[8,9,13,16-20] but variation in rates of prevalence among mutations within one country has been previously reported.[10] The prevalence of mutations appears to be different among strains of H. pylori obtained before and after treatment with clarithromycin.[15] We could not assess the primary and secondary resistance in our isolates due to lack of information about prior antibiotic therapy. The findings of our study strongly recommend pre-treatment susceptibility testing of clarithromycin as we were beyond the cutoff rates (more than 15-20%) where clarithromycin susceptibility testing is highly recommended.[5]

Fluoroquinolones are considered safe oral drugs with good anti-helicobacter activity. Our study showed high percentage of H. pylori isolates resistant to fluoroquinolone although it is not a part of first-line anti-helicobacter therapy. The results were in agreement with data published from China reporting 55% flouroquinolone resistance in H. pylori[21] and Japan, 47.9%.[22] Fluoroquinolone resistance is linked to past use of quinolones within 10-year period, number of courses prescribed, use in poultry industry and hospital setting.[21,23] One study from Germany has reported an alarming increase in fluoroquinolone resistance in H. pylori isolates and has linked it with increase prescription of fluoroquinolones in the out-patient department.[24] High resistance to fluoroquinolone in our population could also be related to increased fluoroquinolone use for concurrent infections leading to the emergence of resistance in H. pylori. Such consumption is likely since infections requiring fluoroquinolone usage, such as enteric fever and tuberculosis, are common in our region.[25] Isolates that had absence of both Wild Type and mutation band require further investigation for other mechanisms responsible for fluoroquinolone resistance.

As there are limited numbers of drugs currently in the pipeline for H. pylori, with such high prevalence, we have almost lost this drug for H. pylori. For third-line therapy, there are no current recommendations except targeted drugs that should be checked for susceptibility before the start of treatment like tetracyclines, rifabutin and furazolidone.

The limitation of our study was that we could not correlate our findings with patients' prior use of antibiotics or previous treatment failures.

The variable resistance rates and increasing trend towards resistance show a different geographical distribution

and call for more region-based studies to monitor emerging resistance.

Currently, Genotype® HelicoDR is the only available commercial assay that checks susceptibility of both macrolide and fluoroquinolone simultaneously. The main advantage of this test is it can be used retrospectively on paraffin embedded tissue samples in patients where diagnosis on histopathology is doubtful or patient has failed empirical therapy. This study is the first of its kind in terms of using Genotype® HelicoDR on the paraffin embedded biopsies. As pre-treatment susceptibility testing is cost-saving with high chances of treatment success, this test, therefore, can be a useful tool for both developing and developed countries.

Conclusion

The findings warrant the need for pre-treatment susceptibility testing in H. pylori infections, especially in Pakistan where burden of disease is high and very limited susceptibility data is available. Although fluoroquinolones are not part of first-line anti-helicobacter therapy, high resistance to this group and increasing clarithromycin resistance is alarming and warrants implementation of good antibiotic stewardship to prevent further increase in resistance.

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Conflict of Interest:

Syed Muhammed Zaheer Alam is the Chief Executive Officer at Official Sales Agency Pakistan of Hain Lifescience, Karachi, Pakistan and assisted in initial optimization of the assay.

Disclosure: A part of this study was presented as a poster in ECCMID, 2010, Vienna, Austria.

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