

Drug resistance pattern and clonality in *H. pylori* strains

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Abstract

Background: This aim of this work was to determine the *in vitro* activity of clarithromycin, amoxicillin, metronidazole and tetracycline against *Helicobacter pylori* and clonality among resistant and sensitive strains isolated from North India.

Methodology: A total of 68 *H. pylori* isolates from peptic ulcer disease and non ulcer dyspepsia patients were examined. These strains were subjected for determination of minimum inhibitory concentration of clarithromycin, amoxicillin, metronidazole and tetracycline. For molecular characterization of resistant and sensitive strains, enterobacterial repetitive intergenic consensus sequences (ERIC) and random amplified polymorphic DNA-PCR (RAPD-PCR) methods were used.

Results: All the tested isolates were found resistant to metronidazole, while 65% were resistant to amoxicillin and 4.7% were resistant to clarithromycin. However, none of the isolates were found to be resistant to tetracycline. Molecular fingerprinting and cluster analysis of resistant and sensitive strains did not give clues for clonal spread of resistant strains.

Conclusions: Various chromosomal mutations were seen in the putative resistance genes of resistant strains, possibly indicating selection pressure as the major cause of high resistance.

Keywords: Metronidazole, amoxicillin, clarithromycin, tetracycline, β -lactamase, clonality, *H. pylori*

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Introduction

Helicobacter pylori is a gram-negative bacterium that colonizes the gastric mucosa of more than half of the world's population [1]. The association between *H. pylori* infection and subsequent development of chronic active gastritis, peptic ulcer disease, gastric cell carcinoma and B cell MALT lymphoma has been well established [2,3].

H.pylori is known for its panmictic population structure [4,5], *i.e.*, genetic recombination is so frequent that it randomizes the DNA sequences and generates linkage equilibrium [4]. However, mutation is considered as the key for phenotypic variation as well as ability of cellular adaptations to stress. Microorganisms can resist environmental challenges by changing their genetic material by either mutation or horizontal gene transfer and/or by genetic rearrangement. A recent report has indicated that genomic regions encoding phenotypic features required in drug resistance and virulence mechanisms tend to evolve faster than other regions due to diversifying selection pressure exerted by the host immune response [6]. The development of antibiotic

resistance can be considered one of the significant examples of this strategy.

There is general agreement that increasing antimicrobial resistance is related to the selection pressure exerted by the use of antibiotics. A significant variation in the resistance to antibiotics in *H. pylori*, especially to metronidazole, amoxicillin and clarithromycin, has been reported across the globe. Metronidazole resistance in *H. pylori*, is primarily associated with mutational inactivation of the *rdxA* gene, and amoxicillin resistance was suggested to result from alterations in penicillin binding proteins (*pbp1A*) [7].

The prevalence of *H. pylori* infection in India has been reported to be very high, ranging from 70-90% in patients with duodenal and peptic ulcer and 50-80% in patients with non-ulcer dyspepsia (NUD) as well as healthy asymptomatic adults [8]. The majority of the countries, including India, lack a regional surveillance program for antimicrobial susceptibility against *H. pylori*. The therapeutic recommendations are mostly based on either

insufficient data or those obtained from other geographically unrelated regions [9,10].

Since there is no report on drug resistance patterns of this bacterium from the thickly populated Gangetic belt of North India, the present study, was conducted to determine antibiotic susceptibility of *H. pylori* strains isolated from this zone.

Whole genome fingerprinting was done, based on ERIC sequences and RAPD-PCR, to determine the clonal relationship, if any, among resistant and sensitive isolates of *H. pylori*.

Material and Methods

Patients and Strains

Consecutive patients in aged 18 to 75 years attending the Department of Gastroenterology at University Hospital of Banaras Hindu University, Varanasi, for upper gastrointestinal endoscopy with signs and symptoms of duodenal or gastric ulcer/gastritis/gastric adenocarcinoma/non ulcer dyspepsia based on endoscopic findings were included in this study. This hospital provides tertiary level health services to about 100 million people spread over the eastern part of Uttar Pradesh, the western part of Bihar and Jharkhand, and the adjoining part of Chattisgarh and Madhya Pradesh. However, patients having history of previous gastric surgery, active upper gastrointestinal bleeding, chronic alcoholism, prior intake of antibiotic and proton pump inhibitors during the previous 4 weeks, or those on non-steroidal anti-inflammatory drugs were excluded from the study. Individuals younger than 18 years of age, pregnant or lactating women, or those with cirrhosis and ischemic heart diseases were also excluded. The study was conducted during the period from January 2005 to December 2006.

Informed consent was taken from each subject included in the study. The study was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University.

Three biopsy pieces from the gastric antrum of each patient were collected during upper gastrointestinal endoscopy and were transported on ice in brain heart infusion (BHI) broth containing 20% glycerol to the microbiology laboratory. *H. pylori* were isolated by streaking homogenized biopsies on brain heart infusion agar (BHI) (Becton Dickinson, Sparks, MD, USA) plates supplemented with 7% defibrinated sheep blood; 0.4% Iso Vitale X (Becton Dickinson, Sparks, MD, USA) and Skirrow selective supplement (vancomycin, 10µg/ml; polymixin B sulfate 2.5 IU/ml; trimethoprim lactate

5µg/ml) (Becton Dickinson, Sparks, MD, USA). These plates were incubated at 37°C in microaerophilic atmosphere (5% O₂; 10% CO₂; 85% N₂) for three to six days.

H. pylori isolates were identified by typical colony morphology, characteristic Gram negative spiral morphology on microscopy, and positive tests for production of catalase, urease and oxidase enzymes. These isolates were further confirmed by PCR-based amplification for two conserved *ureC* and *hsp60* genes of *H. pylori* as reported earlier [11,12]. A total of 63 *H. pylori* isolates thus confirmed by PCR were included in this study. Suspensions of *H. pylori* were preserved at -80°C in BHI broth containing 20% glycerol until used.

Histopathological Examination

Biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Very thin (4 µm) sections were cut and stained with hematoxylin-eosin to observe the presence of curved rod-shaped bacteria on the mucosal surface and also abnormal histopathological changes.

Minimum Inhibitory concentrations

The minimum inhibitory concentrations (MICs) of *H. pylori* isolates for clarithromycin (CLA), amoxicillin (AMX), metronidazole (MTZ) and tetracycline (TET) (Sigma, St. Louis MO, USA) were determined by agar dilution method per NCCLS recommendations [13].

Twofold serial dilutions of antibiotics were used: CLA, 0.125-16mg/L; AMX, 0.125- 64 mg/L; MTZ, 0.5-256 mg/L; TET, 0.125-8 mg/L.

Frozen stock cultures were revived on BHI agar containing 7% defibrinated sheep blood and incubated for three days under microaerophilic conditions. Isolates were subcultured on fresh BHI agar plates and incubated for 24 hours. Exponentially growing *H. pylori* cells were suspended in sterile 0.85% NaCl and adjusted to an optical density of 0.1 at 600 nm. Ten microliters of the adjusted inoculum was delivered to Muller Hinton agar (MHA) (Hi media, India) plates containing twofold serial dilutions of antibiotics. Antibiotic-free control plates were inoculated at the beginning and end of each series of plates. The plates were incubated under microaerophilic conditions at 37°C for five days. If *H. pylori* growth appeared on the plates, the isolate was considered as resistant to the corresponding drug concentration. Following are the recommended break

points for various drugs: MTZ \geq 8 mg/L, AMX \geq 8 mg/L, CLA \geq 2 mg/L and TET \geq 2 mg/L.

The MIC was determined twice by agar dilution method and once by Epsilon meter test (E test; AB BIODISK, Solna, Sweden) and appropriate control strains were used during each set of experiments.

Determination of β -lactamase production

Lactamase production was determined by means of acidometry using β -lactamase detection paper. A penicillinase producing *Staphylococcus aureus* strain was used as a positive control.

Preparation of genomic DNA for PCR assay

Extraction of genomic DNA from bacterial isolates was done by using a standard proteinase K and phenol-chloroform method [14].

Primers used for amplification of *rdxA* and *pbp1* genes

To investigate the molecular mechanisms for resistance, we amplified and sequenced putative resistance genes from five randomly selected *H. pylori* MTZ- and AMX-resistant strains using the following primers. Amplification cycle and conditions were the same as those described in a previous study [7]: 5'*rdxA*1 ATGGGTTGCTGATTGTGGTTTATGG and 3'*rdxA*2 GCTTGAAAACACCCCTAAAAGAGCG and 5'*pbp*1-1 AATCAAGCGGTGAGTATCCTTGTGG, 3'*pbp*1-2 CTACGGTTTCTAAACCCCTTTTACG.

ERIC and RAPD PCR

Fingerprinting of resistant and sensitive strains was performed based on ERIC and RAPD PCR methods by using following primers: ERIC 5' ATGTAAGCTCCTGGGGATTAC 3', 5' AAGTAAGTGACTGGGGTGAGCG 3' [15] and RAPD 5'-CCGAGCCAA-3' [16].

PCR was carried out in 25 μ l volume using 10 ng of genomic DNA, 1U of Taq polymerase (Bangalore Genie), and 10 pmol of each primer (Bangalore Genie), 0.25mM (each) deoxynucleotide triphosphate (MBI, Fermentas) and 2-3 mM MgCl₂ in standard PCR buffer. Amplification reactions were carried out in a thermal cycler (Biometra, Goettingen, Germany).

Cloning of amplified products

DNA manipulations were performed according to standard protocols [17]. Oligonucleotides (Bangalore Genie), PCR-core system I (Promega, Madison, WI,

USA) and pGEM-T Easy vector (Promega) were used according to the manufacturer's recommendations. Plasmid DNA was isolated with Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Recombinant plasmids were outsourced for sequencing to Bangalore Genie, India. .

Gel analysis and strain relatedness:

All the gel images were analyzed under ultraviolet light in a gel documentation system (Alpha Innotech, USA). Cluster analysis of all the isolates was constructed on the basis of the fingerprint generated. Based on the presence or absence of different DNA fragments in the fingerprints of the *H. pylori* strains, a binary data matrix was created. Overall similarity between the pair of strains was calculated from the binary data matrix using the simple matching-dice coefficient. The resulting similarity matrix was used as the input data for cluster analysis by the unweighted-pair-group method to generate a dendrogram [18].

Results

Incidence of *H. pylori*

Of the 108 patients, 68 (62%) were males and 40 (37%) were females. Seventytwo (66.7%) of the patients were diagnosed with PUD and 36 (33.3%) had NUD. Only 62% (68/108) of the subjects were positive for *H. pylori* by culture. All 68 isolates were Gram negative rods and oxidase, urease and catalase enzyme producers. Furthermore, 63 isolates were found positive for expected size of amplicon for the two conserved genes of *H. pylori* (*ureC*, *hsp60*). Of them, 76% (48/63) were isolated from peptic ulcer patients (PUD) and 23% (15/63) from non-ulcer dyspepsia (NUD) patients.

Histopathologically, the diagnosis made on endoscopy was confirmed; however, no correlation was seen between drug resistance and stage of the disease.

MIC

All 63 isolates (100%) were found resistant to MTZ, but with different MIC values, *i.e.*, 32, 64 and 256 mg/L for 12 (19.1%), 10 (15.8%) and 41 (65.1%) strains respectively. Resistance rate against AMX was observed to be 65% (41/63) with MIC values of 16, 32 and 64 mg/L for 26 (41.2%), 12 (19.1%), and 3 (4.7%) isolates respectively. All the MIC values remained stable after storage at -80°C and after repetitive sub culturing on AMX-free agar plates.

Only 4.7% (3/63) of the isolates were resistant to CLA (MIC values of 8mg/L for two isolates and 16 mg/L for one isolate). Furthermore, all the isolates from this part of India were highly sensitive to TET (100%) with MICs of 0.5 mg/L for 52 isolates and one mg/L for 11 isolates followed by CLA (96%) with MIC values of 0.125 mg/L for 37 and 0.25 mg/L for 23 strains. All the MIC values were the same by agar dilution method as well as by E-test method.

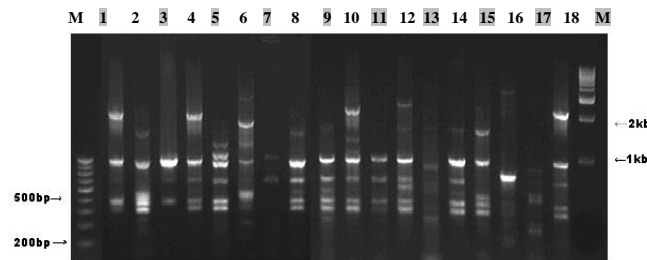
β -lactamase activity

No β -lactamase activity was demonstrable in any of the *H. pylori* isolates when tested by using the chromogenic cephalosporin method.

Sequencing of *rdxA* and *pbp1* genes

The *rdxA* and *pbp1* genes of each Mtz^r and Amx^r strains had at least one mutation compared to the copy of the wild-type sensitive strain (data not shown).

Figure 1. ERIC profiles generated from representative *H. pylori* resistant and sensitive isolates.



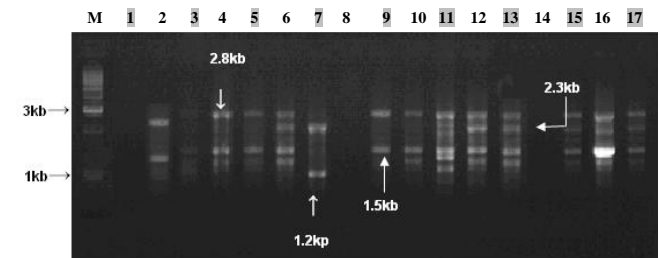
Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 correspond to profiles from isolates resistant to MTZ and AMX. Lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 are profiles from sensitive isolates. M, molecular mass markers with 100bp (left) and 1kb (right) ladders.

Amplification of *H. pylori* genomic DNA with EIRC and RAPD primers

All the tested strains yielded significant PCR products with ERIC and RAPD primers. Depending upon the abundance and availability of amplified fragments, fingerprints of resistant and sensitive strains were compared (Figures 1 and 2).

Cluster analysis based on ERIC-PCR showed that no two isolates generated identical profiles. Whereas RAPD-PCR based method showed that some isolates were identical. However, analysis of these clusters showed that no independent cluster was obtained containing exclusively either resistant or sensitive strains (Figures 3 and 4).

Figure 2. RAPD profiles generated from representative *H. pylori* resistant and sensitive isolates.



Lanes 3,5,7,9,11,13,15 and 17 correspond to profiles from isolates resistant to MTZ and AMX. Lanes 2,4,6,10,12 and 16 are profiles generated from sensitive isolates. M, molecular mass marker with 1kb (left) ladders.

Discussion

The present study shows that the prevalence of MTZ- and AMX-resistant *H. pylori* is quite high in this zone. A multicentric study has reported quite high prevalence of MTZ-resistant *H. pylori* from South India (Hyderabad, 100%, Chennai, 88.2%) and from Northern India (Lucknow, 68%) [9]. In contrast to these observations, two centers from the western part of Northern India have reported relatively lower prevalence of resistant strains (Delhi, 37.5% and Chandigarh, 38.2%) [9]. However, a report from Eastern India has shown high MTZ resistance (85%) in *H. pylori* [10]. Geographical variation in the prevalence of MTZ-resistant *H. pylori* may be attributed to (i) indiscriminate use of drugs in different areas and (ii) lack of uniformity in identification of the *H. pylori* strains as well as drug susceptibility testing in different laboratories.

Identification based on phenotypic characters of the isolates may be misleading and likely to include other urease and oxidase producing bacteria present in the stomach apart from *H. pylori* [19]. However, in the present study, the strains have been identified by amplifying at least two *H. pylori* specific conserved genes (*ureC* and *Hsp60*). Such high level of MTZ resistance may also be explained on the basis of the null mutation in *rdxA* gene which encodes for nicotinamide adenine dinucleotide phosphate nitroreductase, which in turn is responsible for activation of prodrug [20]. Higher prevalence of MTZ resistant *H. pylori* has also been reported from other developing countries (China, 77.8%, Bangladesh, 77.8% and Mexico, 76.3%) [21,22,23]. Resistance to AMX was observed in the present study to be 65%. Although reports from Central India (Mumbai, 73%) and Southern India (Hyderabad, 80%) shows quite similar levels of resistance against AMX, the majority of the reports

Figure 3. Dendrogram based on ERIC-PCR profiles of representative *H. pylori* isolates resistant and sensitive to MTZ and AMX. Isolates 1, 3,5,7,9,11,13,15 and 17 are resistant to MTZ and AMX. and 2,4,6,8,10,12,14,16,and 18 are sensitive isolates.

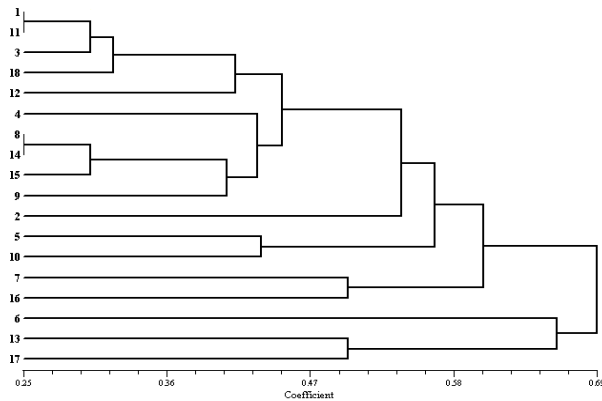
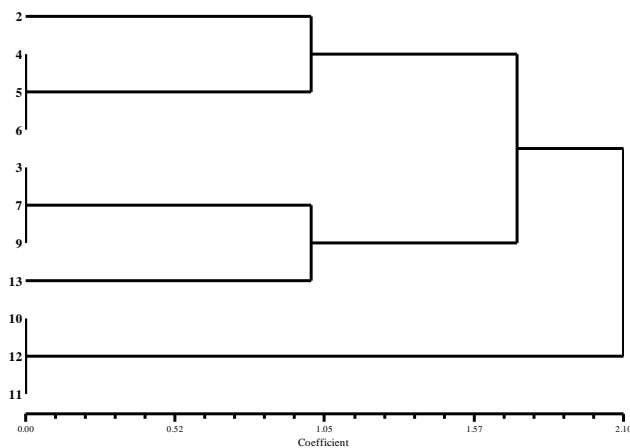


Figure 4. Dendrogram based on RAPD-PCR profiles of representative *H. pylori* isolates resistant and sensitive to MTZ and AMX. Isolates 3, 5, 7, 9, 11 and 13, are resistant to MTZ and AMX. and 2, 4, 6, 10, and 12 are sensitive isolates.



from Northern India (Lucknow, Delhi, Chandigarh), Eastern India (Kolkata), and also one city in Southern India (Chennai) showed no AMX-resistant isolates [9,10]. China, Bangladesh, Mexico, and Italy have also reported quite variable prevalence rates for AMX resistance, *i.e.*, 41.2%, 6.6%, 19.4%, and 45% respectively [21,22,23, 4]. Similarly, it is again difficult to explain such variations of AMX-resistant strains of *H. pylori* in different parts of the country as well as throughout the world.

It is important to mention here that although the strains were resistant to AMX, none of them produced β -lactamase enzyme. Moreover, there is no report across the globe showing production of β -lactamase by *H. pylori*. It is again difficult to explain

the CLA resistance prevalence rate of 96% and 91% in Hyderabad and Mumbai respectively in *H. pylori*, but only 4% resistance in our study. However, our observation of quite low resistance rates against CLA is in accordance with those of many other Indian cities (Lucknow, Delhi, Chandigarh and Chennai) [9,10]. Detection of higher CLA-resistant strains may be due to the fact that our study has been conducted very recently compared to the above studies. Reports from Bangladesh, China, and Mexico have shown a wide range of resistance prevalence (10-24%) for CLA. Many of the industrialized countries have reported still higher CLA resistance rates [25]. Higher frequency of CLA usage in developed countries to treat upper respiratory tract and dental infections may be one of the explanations. However, emergence of CLA resistance in *H. pylori* strains isolated in the present study seems to be alarming since it is the key drug used in the first-line therapy.

Similar to the *H. pylori* strains found in most of the cities in India, none of the *H. pylori* strains isolated in the present study was resistant to TET. However, Kolkata and Hyderabad were exceptional with resistance rates for TET at 7.5% and 4% respectively [9,10]. Frequent use of TET in cholera-endemic zones may be the possible explanation for this resistance, which can very well be explained by the isolation rate of 15% TET-resistant *H. pylori* strains from Bangladesh.

Genetic relationships among *H. pylori* strains were examined through cluster analysis of the ERIC and RAPD-PCR based generated patterns from representative isolates and were presented in the form of dendrogram (Figures 3 and 4). Clustering, however, was independent of the isolate's drug resistant/sensitive status. Hence it seems that there is no clonal spread of resistant genes. However, chromosomal mutations in the putative resistant genes under antibiotic selection pressure seems to be the most likely cause of high resistance in *H. pylori* strains isolated from this zone, as demonstrated by our sequencing results.

With the exception of TET, all three drugs (MTZ, AMX, CLA) tested in the present study have been found to have unique patterns of antibiotic resistance in different geographical regions, even in India.

Since very few laboratories in India are conducting susceptibility testing for *H. pylori* and the majority of them lack consistency in methodology, and conflicting results are not unexpected. Therefore, refinement of protocols and participation in a quality control scheme will improve reproducibility of the

tests and allow national and international surveillance of antibiotic resistance, both to monitor the prevalence of resistant/sensitive strains and to guide empirical treatment protocols on the basis of local resistance patterns against *H. pylori*.

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