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Analysis of Clinical Isolates of *Helicobacter pylori* in Pakistan Reveals High Degrees of Pathogenicity and High Frequencies of Antibiotic Resistance

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Abstract

Background—Antibiotic resistance in *Helicobacter pylori* contributes to failure in eradicating the infection and is most often due to point and missense mutations in a few key genes.

Methods—The antibiotic susceptibility profiles of *H. pylori* isolates from 46 Pakistani patients were determined by Etest. Resistance and pathogenicity genes were amplified, and sequences were analyzed to determine the presence of mutations.

Results—A high percentage of isolates (73.9%) were resistant to metronidazole (MTZ), with considerable resistance to clarithromycin (CLR; 47.8%) and amoxicillin (AML; 54.3%) also observed. Relatively few isolates were resistant to tetracycline (TET; 4.3%) or to ciprofloxacin

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 PCR primer pairs and amplification conditions for *H. pylori* pathogenicity genes.

Table S2 PCR primers pairs and amplification conditions for *H. pylori* genes associated with antibiotic resistance.

Table S3 Association of pathogenicity genes of *H. pylori* with key clinical findings.

Table S4 Frequency and association of pathogenicity genes with histopathological grade.

Table S5 Frequency and association of *vacA* genotypes with histopathological grades.

(CIP; 13%). However, most isolates (n = 43) exhibited resistance to one or more antibiotics. MTZ-resistant isolates contained missense mutations in oxygen-independent NADPH nitroreductase (RdxA; 8 mutations found) and NADH flavin oxidoreductase (FrxA; 4 mutations found). In the 23S *rRNA* gene, responsible for CLR resistance, a new point mutation (A2181G) and 4 previously reported mutations were identified. Pathogenicity genes *cagA*, *dupA*, and *vacA* s1a/m1 were detected frequently in isolates which were also found to be resistant to MTZ, CLR, and AML. A high percentage of CagA and VacA seropositivity was also observed in these patients. Phylogenetic analysis of partial sequences showed uniform distribution of the 3′ region of *cagA* throughout the tree.

Conclusions—We have identified *H. pylori* isolates in Pakistan which harbor pathogenicity genes and worrying antibiotic resistance profiles as a result of having acquired multiple point and missense mutations. *H. pylori* eradication regimens should therefore be reevaluated in this setting.

Keywords

23S rRNA gene; antibiotic resistance; cagA; clarithromycin resistance; *Helicobacter pylori* infection; metronidazole resistance

Helicobacter pylori is a common pathogen infecting approximately 50% of the world's population and is the causative infectious agent in the development of diseases including gastritis, peptic ulcer, and gastric cancer [1]. Emerging resistance of *H. pylori* strains to several classes of commonly used, widely available antibiotics is the major factor contributing toward the failure of eradication therapy. Wide variations in antibiotic resistance patterns have been described according to differing geographic regions [2,3]. Antibiotic resistance has been attributed to key mutations in a relatively small number of nucleotide and amino acid sequences. Amino acid substitutions in the sequences of oxygen-independent NADPH nitroreductase (RdxA) and NADH flavin oxidoreductase (FrxA) have been reported to be associated with metronidazole (MTZ) resistance [4,5]. Point mutations in 23S ribosomal RNA (23S *rRNA*) and amino acid changes in penicillin binding protein 1 (Pbp1) have been shown to cause clarithromycin (CLR) and amoxicillin (AML) resistance, respectively [6–9].

Several virulence factors are also known to influence the pathogenicity of *H. pylori.* These include the presence of the cytotoxin-associated gene pathogenicity island (*cag* PAI) [10,11], the induced by contact with epithelium gene (*iceA*) [12], the blood group antigen-binding adhesin (*babA*) [13], the duodenal ulcer-promoting gene A (*dupA*) [14], and vacuolating cytotoxin (*vacA*) [12]. Geographic variations in the distributions of these pathogenicity genes have also been described. This is also likely to affect the outcome of infection, particularly whether the more serious consequences such as gastric cancer result [15].

Polymorphism in CagA occurs as a result of the presence of variable number of repeat regions at the 3′ region of the *cagA* gene [16–18]. These repeat regions represent combinations of the EPIYA motif (Glu-Pro-Ile-Tyr-Ala). EPIYA-A and EPIYA-B motifs occur widely in all CagA proteins, whereas EPIYA-C and EPIYA-D motifs allow classification of strains as "Western" or "East Asian" types [19]. The "East Asian" variant is

regarded as being more harmful than the "Western" type [20]. Sequence analysis of the 3′ region of the *cagA* gene from *H. pylori* strains which have been cultured from patients with gastroduodenal diseases has been performed in many countries, but no such sequence analysis has previously been reported from Pakistan, although one key study has shown that clinical strains of *H. pylori* from Pakistan positive for the *cagA* promoter region to be significantly associated with gastric inflammation, ulceration, and carcinoma [21].

In Pakistan, *H. pylori* infection is highly prevalent and there is also indiscriminate consumption of commonly used antibiotics, which can be purchased over the counter without prescription from a healthcare professional. Therefore, we designed a study to investigate in this population; [1] the resistance patterns to commonly used antibiotics of *H. pylori* cultured from patients undergoing diagnostic endoscopy for investigation of upper gastrointestinal symptoms, [2] the gene mutations associated with this antibiotic resistance, and [3] the frequency and associations of *H. pylori* pathogenicity genes in the same cohort.

Materials and Methods

Patients

A total of 93 adult patients (with symptoms of acid reflux, abdominal pain, dyspepsia, heartburn, vomiting, or bloating) attending for endoscopy at the Gastrointestinal Endoscopy Department, Military Hospital, Rawalpindi, were enrolled in the study from July 2011 to March 2012. Seventy-one patients were male (mean age 45.8 ± 16.4; range 20–80 years) and 22/93 were female (mean age 49.1 ± 15.1; range 19–78 years). Informed written consent was obtained from each patient, and the study was approved by the Board of Advance Studies and Research, Quaid-i-Azam University, Islamabad. Patients were confirmed to have not taken any antibiotics or gastric acid inhibitors for at least 4 weeks prior to the time of their enrollment into the study. However, in view of the widespread over the counter use of antimicrobial agents in Pakistan, it was not possible to determine accurately to what extent patients had previously taken antibiotics to treat infections with bacteria including *H. pylori*.

Assessment of Helicobacter pylori Infection Status

Endoscopic biopsy tissue specimens were taken from the gastric antrum within 3 cm of the pylorus. The presence of *H. pylori* within these gastric antral mucosal biopsies was evaluated by routine histopathology. Briefly, each tissue specimen was formalin-fixed, paraffin-embedded, sectioned and stained with hematoxylin/eosin and Giemsa. *Helicobacter pylori* load, the degree of neutrophil and mononuclear cell infiltration, and the presence of atypia, atrophy, and intestinal metaplasia were each scored on the basis of the updated Sydney System (0: none, 1: mild, 2: moderate, 3: marked) [22], by an experienced pathologist who was "blinded" to the specimens and their *H. pylori* infection status. *Helicobacter pylori* infection was further confirmed by rapid urease test using Christensen urea agar slope (CM0071; Oxoid, Basingstoke, UK) and/or bacterial culture. *Helicobacter pylori* status was considered positive when histopathology showed positive results, and this was also confirmed in the majority of cases by rapid urease test and/or culture.

Isolation and Culture of Helicobacter pylori

Gastric antral biopsies were placed in 20% glucose solution [23], then chopped, and inoculated on Columbia blood agar (CM0331B; Oxoid) supplemented with Dent's *H. pylori* selective medium (SR0147E; Oxoid) and 7% laked horse blood (SR0048; Oxoid). Plates were incubated at 37 °C for up to 7 days in a moist microaerophilic atmosphere of 10% CO_2, 5% O_2, and 85% N_2 established using a *Campylobacter* gas generating kit (BR0056A, Oxoid). After 3 days of initial incubation, plates were evaluated for growth on a daily basis. Where no growth of *H. pylori* was observed after incubation for 7 days, plates were recorded as negative for *H. pylori* culture. Following positive growth, individual small rounded, translucent colony-forming units (CFU) were selected and subcultured twice to ensure pure cultures. Isolates were identified as *H. pylori* on the basis of morphology following Gram stain, positive findings of oxidase, catalase and urease tests as per [24], and by PCR for 16S *rRNA* (see Table 1).

In Vitro Antibiotic Susceptibility Tests

Susceptibility testing to the antibiotics MTZ, CLR, AML, TET, and CIP was performed using commercial Epsilometer test (Etest®) for minimum inhibitory concentration (MIC) (bioM_erieux SA, Marcy-ÍEtoile, France). Briefly, Columbia blood agar supplemented with 7% vol/vol laked horse blood (SR0048, Oxoid) was inoculated with a bacterial cell suspension calibrated at 3.0 McFarland and allowed to surface dry before application of Etest® strips as per the manufacturer's instructions. Following 3-day incubation at 37 °C under microaerophilic conditions, Etest® strips were read and resistance to an antibiotic determined using breakpoint concentrations as follows: MTZ (>8 μg/mL), CLR (>1 μg/mL), AML (>1 μg/mL), TET (>1 μg/mL), and CIP (>1 μg/mL). Breakpoint concentrations were defined on the basis of previously approved clinical laboratory standards [6,25–27]. *Helicobacter pylori* strain 26695 was included as an antibiotic susceptibility testing quality control.

Genomic DNA Extraction

Total genomic DNA was extracted from all *H. pylori* isolates by ethanol precipitation and proteinase K treatment, using a modification of a method previously described [28]. Briefly, a single bacterial colony was selected, resuspended in 20 μL of 1% sodium dodecyl sulfate, 40 μL proteinase K (100 μg/mL), 80 μL of proteinase K buffer (4M NaCl, 0.5M EDTA; pH 7.5), and incubated at 55 °C for 1 hour. Subsequently, 100 μL 6M NaCl was added, vortexed for 1 minute, and centrifuged at 16,863 × ***g*** for 1 minute, at 4 °C. Nucleic acids were then precipitated by adding absolute ethanol and harvested by centrifugation (16,863 × ***g*** at 4 °C for 1 minute). Resultant DNA pellets were each washed with 70% vol/vol ethanol and then resuspended in 100 μL 10 mM Tris, 1 mM EDTA buffer; pH 7.5. Samples were stored at −20 °C prior to PCR and DNA sequence analysis.

PCR Amplification of *Helicobacter pylori* Virulence Determinant Genes

PCR was performed on purified genomic DNA from all *H. pylori* to examine for the presence of 16S *rRNA* and markers of pathogenicity including, the *cag* PAI (consisting of *cagA, cagE, cagM*), the 3′ region of *cagA, iceA1, iceA2, babA, dupA* (jhp0917 and jhp0918),

and allelic variants of *vacA* (s1a, s1b, s1c, s2, m1, m2). Oligonucleotide primers for 16S *rRNA* were synthesized by Alpha DNA (Montreal, QC, Canada), primers for the 3′ region of *cagA* were supplied by Eurogentec (Hampshire, UK), and primers for all other pathogenicity genes were from Gene Link (Hawthorne, NY, USA). Primer sequences, PCR cycling conditions, and expected amplicon sizes are shown in Supporting Information File S1. Each PCR consisted of 5× FIREPol master mix (Solis BioDyne, Tartu, Estonia) and 5 μL of genomic DNA in a final reaction volume of 25 μL. Thermal cycling was performed using either a T1 Thermalcycler (Biometra GmbH, Goettingen, Germany) or a Multi-Gene OptiMax (Labnet, Edison, NJ, USA). Aliquots (10 μL) of PCR were subjected to electrophoresis on 1% wt/vol agarose gels in Tris-acetate buffer, with ethidium bromide staining for detection of amplicons visualized on a Transilluminator T12 (Biometra GmbH). Presence of *dupA* was defined as positive with PCR amplification for both jhp0917 and jhp0918.

PCR Amplification and Sequencing of *Helicobacter pylori* Genes Associated with Antibiotic Resistance

PCR was performed on purified genomic DNA from all *H. pylori* (resistant and sensitive) strains to obtain amplicons of genes *rdxA*, *frxA*, 23S *rRNA*, and *pbp1* to support DNA and amino acid sequence identification of point and missense mutations conferring resistance to MTZ (RdxA, FrxA), CLR (23S *rRNA*), and AML (Pbp1). Oligonucleotide primers for *rdxA* and 23S *rRNA* were synthesized by Gene Link, and the others were supplied by Eurogentec. Primer sequences, PCR cycling conditions, and expected amplicon sizes are shown in Supporting Information File S1. Each PCR mixture for the 3′ region of *cagA* and for the antibiotic resistance genes consisted of 2× DreamTaq Green PCR master mix (Thermo Scientific, Madison, WI, USA), 5 μL of genomic DNA. Aliquots (10 μL) from the PCR were subjected to electrophoresis on 1% wt/vol agarose gels in Tris-acetate buffer, with GelRed staining for detection of amplicons, visualized on a GelDoc™ XR System (Bio-Rad Laboratories, Hertfordshire, UK).

Prior to DNA sequencing, PCR-generated amplicons of the 3′ region of *cagA* and genes associated with antibiotic resistance (*rdxA*, *frxA*, 23S *rRNA*, and *pbp1*) were each purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). DNA was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific) followed by sequencing by two commercial services, Eurofins MWG Operon (London, UK) and GATC Biotech (Köln, Germany). Point and missense mutations were identified in resistant and sensitive *H. pylori* isolates and compared to available sequences of *H. pylori* reference strain 26695. Sequence comparisons were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA software v5.1. Nucleotide sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using BankIt.

CagA EPIYA Motifs and Phylogenetic Analysis

CagA EPIYA segments were characterized according to motif pattern as described previously [19]. Partial sequences of the 3′ region of the *cagA* gene of *H. pylori* reference strains 26695, J99, G27, SS1, and other deposited sequences retrieved from the National

Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/) were compared with our sequencing data.

CagA and VacA Seropositivity

Venous blood (5 mL) was collected from each patient to evaluate for the presence of specific serum antibodies against CagA and VacA. CagA and VacA seropositivity was determined using a Helico Blot 2.1 Western blot assay kit (MP Biomedicals SAS, Cedex, France) containing *H. pylori* CagA and VacA antigens with molecular weights of 116 kDa and 89 kDa, respectively. Test strips were incubated with sera for 1 hour at 25 °C and then incubated with an alkaline phosphatase-conjugated goat anti-human immunoglobulin G antibody for a further 1 hour. Strips were then developed with 5-bromo-4-chloro-2-indolylphosphate and nitroblue tetrazolium for 15 minutes. Helico Blot kit reactive and nonreactive control sera were assayed along with patients' samples for each test run performed. Results were interpreted according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using SPSS v20.0 software (SPSS Inc, Chicago, IL, USA). Chi-square (χ^2) was used to assess the relationships between pathogenicity genes and clinical findings, histopathologic grades, and antibiotic resistance. Differences were considered significant when $p \leq .05$.

Results

Demographics of Helicobacter pylori-Colonized Patients

The frequency of *H. pylori* infection observed in this patient cohort was 57/93 (61.3%) as assessed by gastric histopathology. For males, 41/71 were positive for *H. pylori* [57.7%], and in females, 16/22 [72.7%] were found to be positive. From the 57 *H. pylori*-positive patients, a total of 46 *H. pylori* isolates (49.5%) were cultured from gastric biopsy tissue. In all cases, positive culture was confirmed by 16S *rRNA* PCR. In those patients in whom *H. pylori* was identified by histopathology, 26/46 (56.5%) patients aged between 19 and 44 years were infected, and 31/47 (66.0%) patients aged above 45 years were infected. Frequent symptoms in patients with *H. pylori* colonization were abdominal pain (45/57; 78.9%), heartburn (43/57; 75.4%), acid reflux (31/57; 54.4%), vomiting (29/57; 50.9%), and bloating (29/57; 50.9%). Regarding clinical disease states, 45/75 (60.0%) of patients with gastritis, 8/12 (66.7%) of those with peptic ulcer disease (PUD), and 4/6 (66.7%) of those with gastric cancer (GC) were found to be *H. pylori* positive. The histopathologic findings in *H. pylori*-colonized patients are described in Table 1. To note, we were not able to culture *H. pylori* organisms from all patients in whom this bacterium was detected by histopathology. A variety of reasons including density and localization of infection, strain type, and technical difficulties in a developing world setting may have contributed to this, as previously described [29,30].

Antibiotic Susceptibilities of *Helicobacter pylori* Clinical Isolates

Of the 46 *H. pylori* isolates cultured, 34/46 (73.9%) displayed resistance to MTZ, with MICs ranging from 8 to 96 μg/mL, while only 12/46 were seen to be sensitive to this

nitroimidazole antibiotic (MIC range 1–6 μg/mL). Over half the *H. pylori* isolates (25/46; 54.3%) were also found to be resistant to AML (MIC 1–12 μg/mL), with the rest being sensitive to this β-lactam antibiotic (MIC 0.19–0.5 μg/mL). Similarly, clinical isolates also displayed resistance to the macrolide antibiotic CLR in 22/46 (47.8%) cases, with MICs ranging from 1 to 32 μg/mL, while the remaining 24 isolates were all sensitive to CLR (MIC 0.125–0.50 μg/mL). The majority of isolates (44/46; 95.7%) were found to highly sensitive to TET (MIC 0.125–0.5 μg/mL), with only 2 isolates showing resistance (MIC 2–4 μg/mL). Likewise, isolates were also sensitive to CIP (40/46; 87.0%) with MICs ranging from 0.125 to 0.5 μg/mL. Resistance to CIP was observed in only 13.0% (6/46) of *H. pylori* isolates, with MIC ranging from 1 to 4 μg/mL (see Fig. 1A).

Resistance against a single antibiotic was only found for a small number of *H. pylori* clinical isolates; MTZ (n = 6) and AML (n = 5). More frequently the isolates were resistant to more than one antibiotic, with dual antibiotic resistance being observed for MTZ & CLR (n = 10), MTZ & AML (n = 9), CLR & AML (n = 2), and MTZ & TET (n = 1). Multidrug resistance including triple resistance was seen to MTZ, CLR, & AML (n = 4), MTZ, CLR, & CIP (n = 2), and CLR, AML, & CIP (n = 2), and quadruple resistance to MTZ, CLR, AML, & CIP was found in one *H. pylori* isolate. Only one *H. pylori* isolate showed resistance against all five antibiotics tested and 3 clinical isolates were found to be sensitive to all five antibiotics studied (see Fig. 1B).

Nucleotide (and Amino Acid) Substitutions Identified in Antibiotic Resistant *Helicobacter pylori*

PCR-amplified DNA sequences of both resistant and sensitive clinical isolates were examined for nucleotide substitutions as compared to *H. pylori* reference strain 26695. We were able to sequence amplicons for *rdx* and *frxA* from 16 MTZ-resistant *H. pylori* isolates (with MICs of 12–96 μg/mL) and also amplicons from isolates resistant to CLR (n = 10; MICs of 4–32 μg/mL) and AML (n = 6; MICs of 2–12 μg/mL). For all sensitive *H. pylori* isolates (sensitive to MTZ, CLR, and AML), no nucleotide substitutions were found, with DNA amplicon sequences obtained all being identical to the reference strain.

MTZ resistance—Nucleotide sequence information for *rdxA* (Accession no. KC733771-90) and *frxA* (KC831769-70) was submitted to GenBank. Amino acid substitutions were identified in RdxA in the 16 *H. pylori* isolates that were resistant to MTZ (MICs 12–96 μg/mL) at positions R16C/H (n = 4/16 isolates), C19Y (n = 1), T31E (n = 8), R90K (n = 5), H97T (n = 8), S108A (n = 2), A118T/S (n = 11), and G189C (n = 1).

Likewise, four known amino acid substitutions were identified in FrxA in two *H. pylori* isolates which were resistant to MTZ (for isolate *SUK55*, at F72S, and G73S; for isolate SA18, F72S, G73S, N111H, and A153V), with MICs of 12 and 24 μg/mL, respectively. Interestingly, both of these isolates also possessed amino acid substitutions in RdxA (isolate SUK55, T31E, and H97T; isolate SA15, T31E, and A118T). All other MTZ-resistant *H. pylori* isolates which possessed amino acid substitutions in RdxA had no identified substitutions in FrxA. Amino acid substitutions such as R16C, C19Y, S108A, and G189C in

RdxA and N111H and A153V in FrxA were connected with high MICs (from 24 to 96 μg/mL) in MTZ-resistant *H. pylori* isolates.

CLR resistance—A previously unreported adenine to guanine (A>G) nucleotide substitution was identified at position 2181A>G in the 23S *rRNA* gene sequence of two *H. pylori* clinical isolates found to be resistant to CLR following antibiotic susceptibility testing (with MICs 4 and 16 μg/mL). In addition, four previously reported nucleotide substitutions were identified in 9 other isolates with MICs ranging from 4 to 32 μg/mL, 2116A>G (n = 3/10 CLR-resistant isolates), 2142A>G (n = 1), 2143A>G (n = 3), and 2182T>C (n = 2) [6,7,31]. Variation at position 2116A>G was associated high MICs to CLR (16 and 32 μg/mL) as compared to other observed nucleotide substitutions in the 23S *rRNA* gene. Nucleotide sequence information for 23S *rRNA* was submitted to GenBank (KC556778 and KC733766-70).

AML resistance—*Helicobacter pylori* isolates (n = 6, with MICs ranging from 2 to 12 μg/mL) were found to have previously reported amino acid substitutions in Pbp1 at positions D535N (n = 5 of 6 isolates), S543R (n = 3), and T556S (n = 1) [8,32,33]. Nucleotide sequence information for *pbp1* was submitted to GenBank (KC763636-41).

Frequency of Pathogenicity Genes in Clinical Isolates

Of the 46 *H. pylori* clinical isolates, 37 (80.4%) were positive for *cagA*, with other genes within the *cag* PAI, namely *cagE* and *cagM*, being detected with frequencies of 34.8% (16/46) and 54.3% (25/46), respectively. The frequency of *iceA1* among the isolates was 41.3% (19/46), whereas *iceA2* was not found in any isolate. Thirtythree of 46 isolates (71.7%) possessed *dupA*, and 18/46 (39.1%) *babA*. Notably, all *H. pylori* clinical isolates expressed *vacA*, with 42/46 possessing the allelic variant s1a (91.3%) and 27/46 the variant m1 (58.7%). Other *vacA* allelic variants found in clinical isolates included s2 (4/46, 8.7%) and m2 (19/46, 41.3%). Variants *vacA* s1b and s1c were not observed in any isolates tested. The three main *vacA* genotypes were therefore categorized as *vacA* s1a/m1 (27/46 isolates, 58.7%), *vacA* s1a/m2 (15/46, 32.6%), and *vacA* s2/m2 (4/46, 8.7%).

Presence of the *cagA* gene was predominant among *H. pylori* isolates that had *vacA* alleles: s1a (89.2%, 33/37) and m2 (51.4%, 19/37), whereas *cagA*-negative isolates harbored the *vacA* alleles s1a (100%, 9/9) and m1 (100%, 9/9). When *vacA* genotypes were compared with presence of the *cagA* gene, there was a significant association between presence of the *cagA* gene and *vacA* s1a/m1 48.7% (18/37), *vacA* s1a/m2 40.5% (15/37), and *vacA* s2/m2 10.8% (4/37) genotypes (all $p < .05$, χ^2 test).

Association of Pathogenicity Genes with Key Clinical Findings

The pathogenicity genes *cagA, cagM, dupA*, and *vacA* genotype s1a/m1 were detected more frequently in *H. pylori*-colonized patients who had gastritis. In patients with PUD, *H. pylori* isolates had higher frequencies of *cagA, cagM, iceA1, dupA*, and *vacA* genotype s1a/m1. *H. pylori* containing *cagA, babA, dupA*, and *vacA* genotype s1a/m1 were also more frequently observed in GC patients. However, none of these differences were statistically significant. For detailed information on these genotypes, see Supplementary Information File S2.

Single (*vacA* genotype s1a/m1)- and double (*vacA*, *cag* PAI)-positive genotypes were identified only in a few *H. pylori* isolates (n = 2 and n = 4, respectively, with these specific isolates all cultured from gastritis patients). Seven *H. pylori* isolates displayed a triple-positive genotype (*vacA*, *cag* PAI, and *dupA*), two of which were isolated from patients with PUD, one from patient with GC, and four from patients with gastritis. The most frequent quadruple-positive genotype was *vacA*, *cag* PAI, *dupA*, and *iceA1*, which was observed in six of *H. pylori* isolates. One of the isolates which had a quadruple-positive genotype was cultured from the stomach of a PUD patient, whereas all other isolates containing quadruple-positive genotype were isolated from gastritis patients. Commonly occurring quintuplepositive genotype (*vacA*, *cag* PAI, *dupA*, *iceA1*, and *babA*) was found in six isolates. Two *H. pylori* isolates which had quintuple-positive genotype were associated with PUD, and 4 *H. pylori* isolates were cultured from patients suffering from gastritis.

Association of Pathogenicity Genes with Histopathologic Grade

There were high frequencies of most of the *H. pylori* pathogenicity genes with the more marked histopathologic grades of *H. pylori* load, neutrophil infiltration, and mononuclear cell infiltration as compared to the scores of atypia, atrophy, and intestinal metaplasia. However, significant differences were only found between *babA*- and *dupA*-positive strains and scores of *H. pylori* load (Supplementary Information File S3). Among *H. pylori*-colonized patients with moderate mononuclear cell infiltration, *vacA* s1a/m1 was frequently observed (66.7%), while those with a marked score possessed *vacA* s1a/m2, and those with a mild score were *vacA* s2/m2 positive. The overall difference was found to be statistically significant ($p = .032$). However, no significant associations between *vacA* genotypes and *H. pylori* load, neutrophil infiltration, atypia, atrophy, and intestinal metaplasia were observed (Supplementary Information File S4).

Association of Antibiotic Resistance and Pathogenicity Genes in Clinical Isolates

High frequencies of *cagA*, *cagM*, and *dupA* genes were found in MTZ-resistant *H. pylori* isolates, and similar observations (with the exception of *cagM*) were made in cases of CLR and AML resistance. There were only two isolates that showed resistance against TET and both these isolates also had *dupA* and *iceA1*. The majority of CIP-resistant isolates harbored *dupA* and *iceA1*, whereas CIP-resistant isolates showed an even distribution of *H. pylori*-containing *vacA* genotypes s1a/m1 and s1a/m2. Most *H. pylori* isolates that showed resistance against all antibiotics had the *vacA* genotype s1a/m1 (Table 2). Differences between antibiotic resistance and pathogenicity genes were found to be nonsignificant.

CagA EPIYA Motifs and Phylogenetic Analysis

We attempted to sequence the 3′ end of *cagA* in all *H. pylori* isolates, but only obtained sequencing data in 10 cases. Several of these sequences contained multiple motifs, but only two types of motifs were found, namely EPIYA (22 occurrences in total) and EPIYT (seven occurrences in total). EPIYA-ABC was detected in most of the sequences, and this was classified as a Western type of *cagA*, while EPIYA-ABD was only found in the sequence of a single *H. pylori* isolate (QAU93). To study variations in sequences of *cagA* gene in *H. pylori* of different countries, un-rooted phylogenetic tree was drawn. Partial sequences of the

cagA gene were close to the sequences published from other developed and developing nations, that is, QAU93 was genetically similar to J7 (Japan) and K15 (Thailand), SAR30 and HF30 were similar to RIGLD-OC151 (Iran), and SKS43 and RZ99 were similar to I (Sweden). The sequences of *H. pylori* isolates SR28, AR31, BJC32, and PAEC83 were similar to CR61 (Costa Rica), 27s (China), and 368H (Mexico), whereas DMP63 was similar to 26695 and 101UK (United Kingdom); see Fig. 2 for un-rooted phylogenetic tree. DNA sequence information for the 3′ region of *cagA* (Accession no. KC626079-88) was submitted to GenBank.

CagA and VacA Seropositivity

All patients infected with a *cagA*-positive *H. pylori* strain were seropositive, suggesting that *cagA* was fully transcribed and translated into CagA protein. Antibodies to vacuolating cytotoxin VacA were found in 71.7% of *H. pylori*-infected patients. In patients infected with type s1a/m1 and s1a/m2 *H. pylori* strains, antibodies to vacuolating cytotoxin were observed in 70.4% and 80.0%, respectively. Only 50% of patients infected with type s2/m2 had antibodies against VacA. There were no significant associations between histopathologic findings and CagA or VacA seropositivity.

Discussion

This study was designed to determine the molecular basis of antibiotic resistance and pathogenicity in *H. pylori* bacteria isolated from gastric biopsies from symptomatic patients in Pakistan, a developing country where the frequency of *H. pylori* infection is high and emerging antibiotic resistance is a threatening crisis.

Classical triple therapy eradication regimes for *H. pylori* eradication have recently shown disappointing efficacy mainly due to the emergence of resistant strains of this bacterium to MTZ and CLR [34,35]. We also report high MTZ resistance, considerable resistance to CLR and AML but little resistance against TET and CIP in the Northern region of Pakistan. Three previous studies investigating *H. pylori* resistance have been published from the Southern part of Pakistan (approximately 1400 km from the Northern region). These showed wide variations in the frequencies of antibiotic resistance to MTZ (89.0% and 48% of *H. pylori* isolates with resistance to MTZ not being analyzed in the third study), CLR resistance in 36.0%, 64.0%, and 33.0% of isolates, and AML resistance in 37.0%, 98.0%, and 2.0% of isolates, respectively [36–38]. Geographic variations in the antibiotic resistance of *H. pylori* are related to the consumption of antibiotics in different communities [39]. In Pakistan, there is unregulated and extensive use of antibiotics specially MTZ, CLR, and AML to treat various infections including respiratory and intestinal disorders. This is likely to have contributed to the considerable resistance of *H. pylori* isolates toward these antibiotics. We therefore suggest that health authorities in Pakistan should pay serious attention to regulating the availability to these frequently consumed antibiotics.

The mechanism of antibiotic resistance in *H. pylori* is associated with point mutations and amino acid substitutions in a key resistance-specific gene (23S *rRNA*) and proteins (RdxA, FrxA, and PBP1). Resistance to MTZ in *H. pylori* isolates in the present cohort was high, and we detected previously reported amino acid substitutions at various sites (R16C/H,

C19Y, T31E, R90K, H97T, S108A, A118T, and G189C) in RdxA [9,25,40]. T31E, R90K, H97T, and A118T were the most common amino acid substitutions found in RdxA associated with MTZ resistance. However, it is very difficult when multiple mutations are present to determine which are significant particularly given that structural analysis of RdxA has shown that some of the amino acid changes noted may not in fact be responsible for the drug resistance [41]. Substitutions in both the MTZ resistance-associated proteins RdxA and FrxA were only identified in two of our MTZ-resistant *H. pylori* isolates. This suggests that most MTZ resistance in this setting resulted from inactivation of *rdxA*, which is in agreement with previous reports [4,42]. Jeong and colleagues concluded that development of MTZ resistance requires inactivation of *rdxA* alone or both *rdxA* and *frxA* depending on genotype. Only rarely does MTZ resistance occur as a result of inactivation of *frxA* alone, and in this case, it may be due to a difference in the regulation of nitroreductase gene expression [42]. High resistance against MTZ in *H. pylori* isolates supports a previous study which highlights that the extensive use of MTZ against other pathogens in persons positive with chronic *H. pylori* infection may stimulate the increased frequency of mutation in *H. pylori*, thus inducing the emergence of resistance against MTZ and other antibiotics [43].

Globally, the most frequently reported nucleotide substitutions within the 23S *rRNA* gene responsible for CLR resistance in *H. pylori* clinical isolates are 2142A>G and 2143A>G (along with 2116A>G and 2182T>C) [6,7,31]. Our findings of nucleotide substitutions in 23S *rRNA* for CLR resistance are in accordance with these previous studies. Of significant interest, we identified a new nucleotide substitution (2181A>G) in the 23S *rRNA* gene of two clinical *H. pylori* isolates that were identified as being resistant to CLR by antibiotic susceptibility analysis only. Given that nucleotide substitutions in other antibiotic resistance-associated genes, such as *rdxA* associated with MTZ resistance, may not in fact be responsible for the drug resistance, clearly further experiments (mutagenic analysis by transformation) are required to substantiate any direct association of this novel nucleotide variation in the 23S *rRNA* gene to CLR resistance. It is also worth noting that despite the high CLR resistance rates seen in *H. pylori* isolates from Pakistan, a recent study has reported a low incidence of recurrence of *H. pylori* infection in patients [38].

Amino acid substitutions were found at three positions (D535N, S543R, and T556S) in AML in *H. pylori* isolates which were resistant to this antibiotic. These substitutions have all been reported in previous studies [8,32,33]. In the current study, the most common amino acid substitution found in Pbp1 that is also responsible for AML resistance was D535N.

We also report a high frequency of the *cagA* gene (80.4%) in these Pakistani *H. pylori* isolates. This percentage was higher than in our recent previous report from the same geographic area. This reported 61.9% prevalence of the *cagA* gene in *H. pylori* detected in gastric biopsy specimens, but these were collected from children [28]. Other studies from the same region of Pakistan have shown 24.2% [44] and 56% [45] *cagA* prevalence in adult dyspeptic patients. The high frequency of *cagA*-positive *H. pylori* strains in our study is similar to reports from neighboring South and East Asian countries including India (78.4%) [46], China (82.3%) [47], Malaysia (94.0%) [48], and Japan (96.3%) [49]. Lower frequencies of *cagA*-positive strains have been reported in *H. pylori* isolates from Iran (67.0%) and Afghanistan (60.0%) [15].

The frequency of *cagE* in our *H. pylori* isolates was also similar to previous reports from South Asia (Iran, 44.0%) [15] and East Asia (Malaysia, 59.0%) [50]. *cagE* and *cagM* genes were highly prevalent with frequencies of 96.0% and 97.0%, respectively, in *H. pylori* isolates cultured in Taiwan (East Asia) [51], whereas we found comparatively lower frequencies of *cagE* and *cagM* genes in our Pakistani clinical *H. pylori* isolates.

In East Asia, *iceA1* is found commonly [52], whereas *iceA2* is more frequent in *H. pylori* strains from the USA [53]. In the present study, the considerable frequency of the more virulent allele *iceA1* in *H. pylori* isolates suggests that the predominant allelic form of *iceA* in Pakistan, like East Asia, is *iceA1*. The frequency of *babA*-positive *H. pylori* isolates in the current investigation was also similar to recent studies from South Asia (31.4%, India and 40.6%, Iran) [46,54], but lower than reports published from East Asia [55–57]. In the present study, the *dupA* gene was highly prevalent in clinical *H. pylori* isolates, and this frequency was higher than in other reports from South and East Asian countries [58–60].

The *vacA* s1a/m1 genotype was predominant among *H. pylori* isolates in this Pakistani cohort as compared to *vacA* s1a/m2, whereas the *vacA* s2/m2 genotype was found infrequently. This again contrasts with our previous study, in which the *vacA* s1a/m2 genotype was predominantly detected in *H. pylori* cultured from children from the same region [28]. In addition, the *vacA* s1b/m2 genotype has been reported among adult patients with dyspepsia in another study from the same region [44]. In previous studies from Karachi (Southern region of Pakistan), *vacA* s1a/m1 was found to be the predominant genotype [45,61]. It has been observed in this investigation and previous reports from Pakistan that different *vacA* genotypes were present among *H. pylori* strains. Frequently observed *vacA* genotypes in *H. pylori* from neighboring South and East Asian countries were s1/m2 (Iran), s1/m1 (Afghanistan), [15], s1a/m2 (India and China) [45,62], and s1c/m1b (Japan) [63].

These pathogenicity genes were therefore frequently found in these Pakistani *H. pylori* clinical isolates in accordance with previous studies from South and East Asia [46,54–57, 64]. However, the presence of these pathogenicity genes was not significantly associated with the severity of disease. *cagA*-positive *H. pylori* strains have previously been associated with more severe gastroduodenal diseases [12]. Similarly in this study, *cagA*-positive *H. pylori* strains were associated with more marked scores among histopathologic grades including *H. pylori* load, neutrophil infiltration, and mononuclear cell infiltration. Earlier reports suggested that *H. pylori* strains with *vacA* s1/m1 were likely to be more virulent than those with s1/m2 [65–67]. This is in agreement with our findings where the *vacA* s1a/m1 genotype was found more frequently in patients with high histopathologic scores as compared to other *vacA* genotypes.

In a recent report, MTZ resistance was found frequently in *cagA*-negative *H. pylori* strains, and it was suggested that absence of the *cagA* gene was the reason for acquisition of MTZ resistance [68]. However, our report of a relationship between MTZ resistance and *cagA* positivity is in agreement with another study in which *cagA* genotype (*cagA* 2a) was found more frequently in MTZ-resistant *H. pylori* strains [69]. In addition, high frequencies of the *cagA* and *dupA* genes as compared to other pathogenicity genes were found in MTZ-, CLR-, and AML-resistant *H. pylori* isolates. The most frequent *vacA* genotype in those *H. pylori*

isolates which were resistant to all antibiotics was the more toxin-producing genotype *vacA* s1a/m1. The high frequencies of these pathogenicity genes in resistant *H. pylori* strains highlight the potential threat and crisis in treatment of *H. pylori* infection.

In this study, the Western type of *cagA* was found more commonly in *H. pylori* isolates than the East Asian type of *cagA*, but our data are not sufficient to provide a conclusive prevalence. To analyze whether any evolutionary connection or mutational variation exists in *H. pylori* strains from Pakistan and other countries, an un-rooted phylogenetic analysis of partial gene sequences of Western and East Asian type of *cagA* was performed. A partial sequence of the *cagA* gene of *H. pylori* strain QAU93, which had East Asian type *cagA*, was genetically similar to *H. pylori* strains J7 (Japan) and K15 (Thailand), both of which also contained the East Asian type of *cagA*. Sequences of all other *H. pylori* isolates which possessed the Western type *cagA* were close to *H. pylori* strains isolated in East Asian, South Asian, and Western countries. This suggests that there is a uniform distribution of partial *cagA* gene sequences of Pakistani *H. pylori* isolates throughout the phylogenetic tree.

We observed that anti-CagA and anti-VacA antibodies were frequently detected in this cohort, in agreement with other studies [70,71]. This high frequency may be due to the different immunogenetic properties of vacuolating cytotoxin and CagA proteins. All the *cagA*-positive *H. pylori* strains in our population expressed CagA protein (100%) against which antibodies were produced in the sera of the corresponding *H. pylori*-colonized patients. Discrepancy has been reported between the results of Western blot and PCR assays by another group, in which they showed that Western blot analysis detected serum antibodies against CagA, while PCR did not reveal the presence of the *cagA* gene in some cases [72]. This is in contrast with our findings where we found that all patients infected with *cagA*-positive strains produced anti-CagA antibodies. This suggests that the *cagA* gene was translated into CagA protein and recognized by the host immune response.

The high frequency of anti-VacA antibodies (73.9%) in patients' sera also suggested that the *vacA* gene was highly expressed into VacA protein. *H. pylori* harboring *vacA* s2/m2 were isolated from patients (n = 2) who had antibodies against VacA protein in their sera. This shows the either mixed but patchy infection of *H. pylori* or viable but nonculturable *H. pylori* containing other *vacA* genotypes instead of *vacA* s2/m2 which could not be isolated from same patients. In the current analysis, our results of PCR and Western blot assay are in agreement with CagA and VacA status evaluation.

In conclusion, our findings show that the *H. pylori* strains which colonized Pakistani symptomatic patients had considerable resistance against commonly used antibiotics and that in some cases this was due to acquisition of novel gene variations. The isolates were also likely to be highly pathogenic and virulent due to the presence of several pathogenicity genes. This study therefore emphasizes the importance of reevaluation of treatment regimens for *H. pylori* in Pakistan, as the population of this country is currently at high risk of developing severe gastric pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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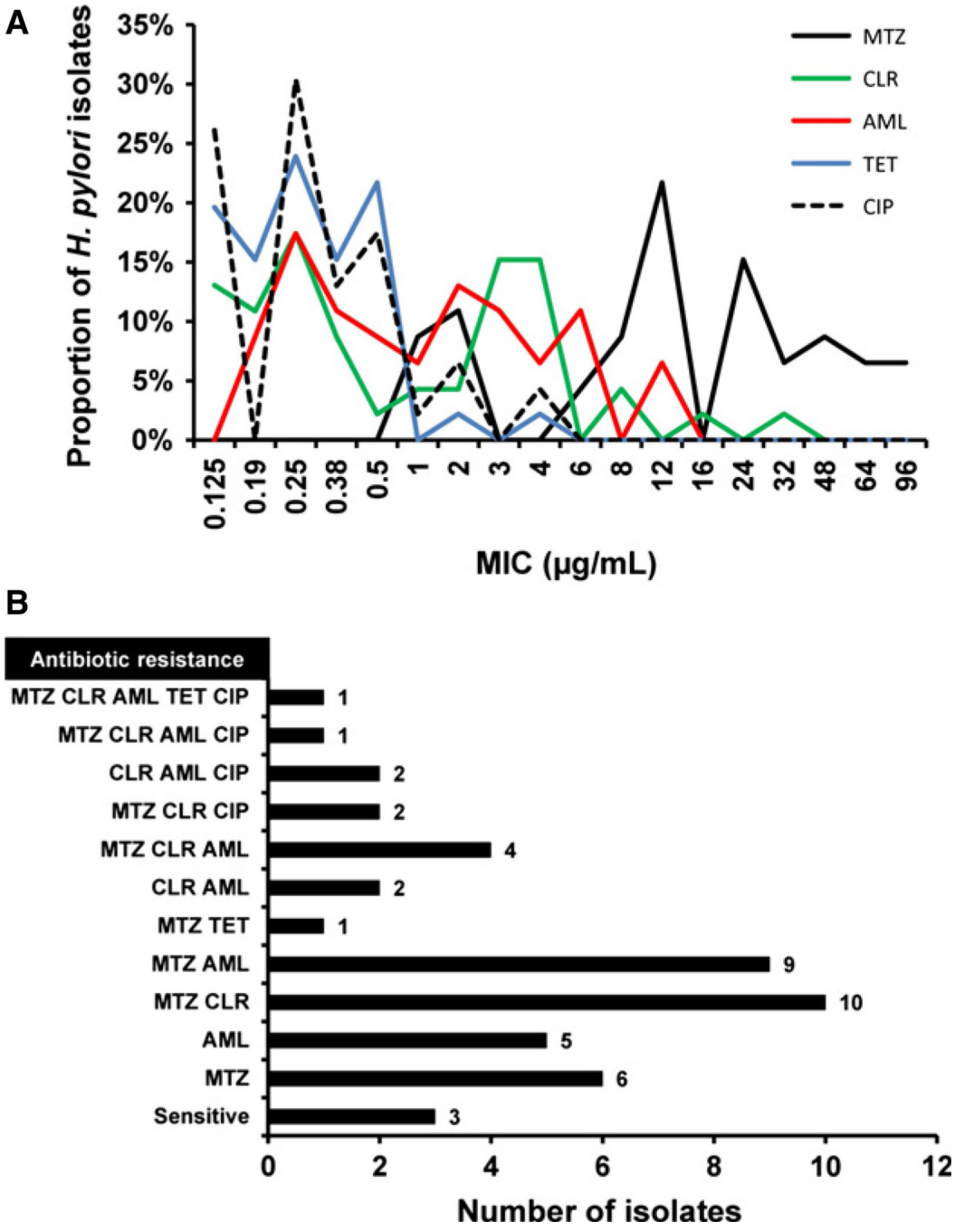

Figure 1.

Antibiotic susceptibilities of *Helicobacter pylori* clinical isolates. (*A*) Susceptibility testing to amoxicillin (AML), ciprofloxacin (CIP), clarithromycin (CLR), metronidazole (MTZ), and tetracycline (TET) among 46 *H. pylori* isolates cultured from gastric antral biopsy tissue. MICs were determined by Etest. (*B*) Distribution of multidrug resistant clinical isolates (i.e., resistance to two or more antibiotics), isolates showing resistance to MTZ or AML alone and those isolates sensitive to all five antibiotics. Resistance was determined

using breakpoint concentrations as follows: MTZ (>8 μg/mL), CLR (>1 μg/mL), AML (>1 μg/mL), TET (>1 μg/mL), and CIP (>1 μg/mL).

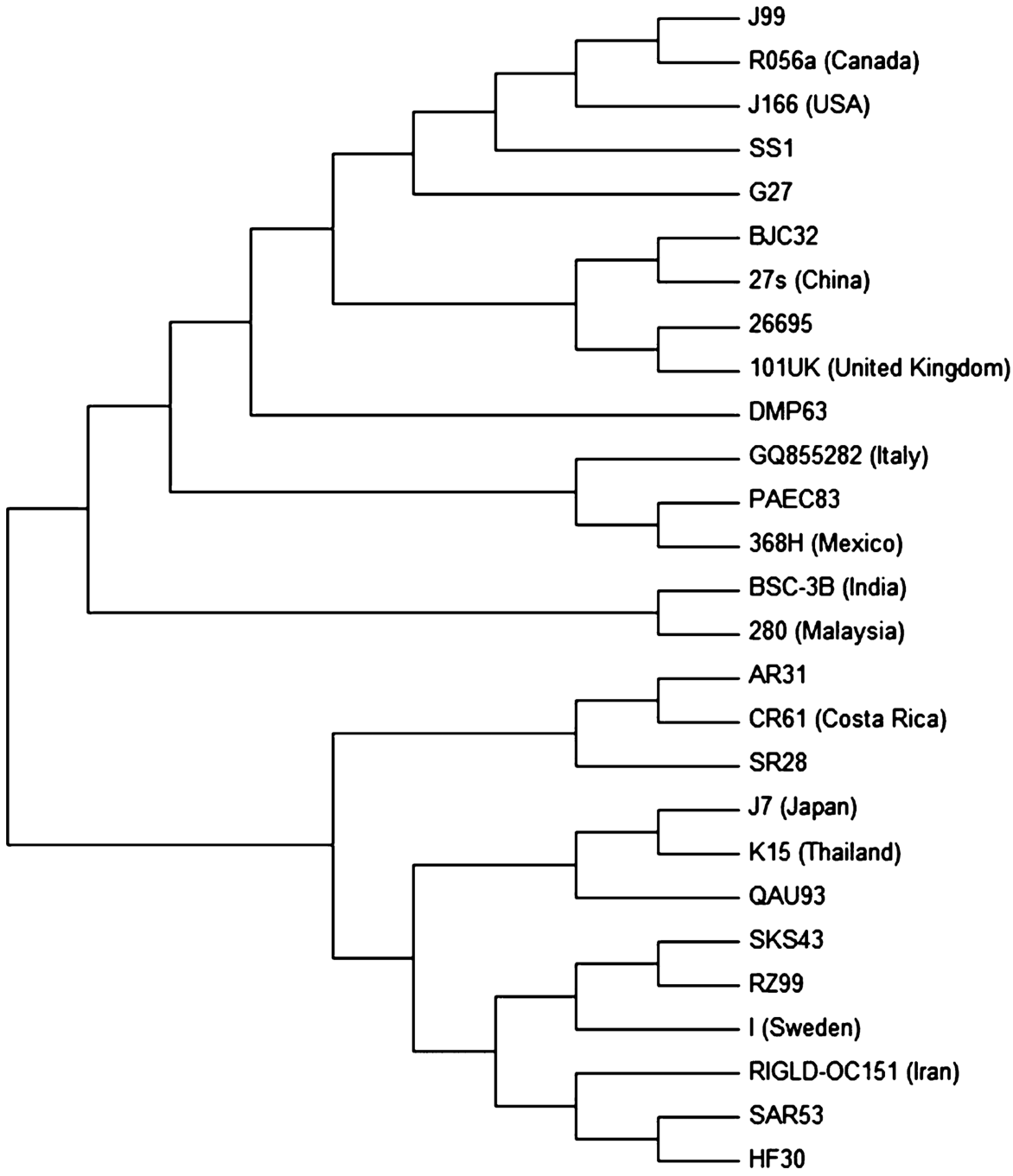

Figure 2.

Phylogenetic affiliation of 3′ region of *cagA* gene sequences of *Helicobacter pylori* isolates. An un-rooted neighbor-joining consensus tree was constructed with CagA EPIYA segments from *H. pylori* reference strains, sequences deposited for different *H. pylori* strains, and sequences of *H. pylori* isolates of the present study. Gene sequence of I (Sweden) was from gastric biopsy, while all other were sequences of *H. pylori* strains deposited by authors from developed and developing countries. CagA EPIYA sequences of this study were similar to

the sequences published from these countries. MEGA software version 5.1 was used to generate and view the phylogenetic tree by bootstrapping at 1000 bootstrap method.

Table 1

Scores of histopathologic grades in *Helicobacter pylori*-positive patients (n = 57)

	Grade			
Histopathology	**None (n)**	**Mild (n)**	**Moderate (n)**	**Marked (n)**
H. pylori load	0	20	25	12
Neutrophil infiltration	9	15	22	11
Mononuclear cell infiltration	1	7	39	10
Atrophy	16	27	9	5
Atypia	23	22	7	5
Intestinal metaplasia	48	7	2	0

n, number patients.

Table 2

Percentage distribution of key pathogenicity genes in *Helicobacter pylori* isolates demonstrating antibiotic resistance

Gene/allelic variant[a]	MTZ resistance (n = 34)	CLR resistance (n = 22)	AML resistance (n = 25)	TET resistance (n = 2)	CIP resistance (n = 6)
cagA+	82.4 (28)	77.3 (17)	76.0 (19)	0 (0)	50.0 (3)
cagE+	35.3 (12)	22.7 (5)	32.0 (8)	0 (0)	16.7 (1)
cagM+	58.8 (20)	45.5 (10)	48.0 (12)	0 (0)	33.3 (2)
babA+	44.1 (15)	45.5 (10)	40.0 (10)	50.0 (1)	33.3 (2)
dupA+	70.6 (24)	59.1 (13)	80.0 (20)	100 (2)	83.3 (5)
iceA1+	38.2 (13)	40.9 (9)	52.0 (13)	100 (2)	83.3 (5)
vacA s1a/m1	52.9 (18)	59.1 (13)	56.0 (14)	100 (2)	50.0 (3)
vacA s1a/m2	38.2 (13)	31.8 (7)	36.0 (9)	0 (0)	50.0 (3)
vacA s2/m2	8.8 (3)	9.1 (2)	8.0 (2)	0 (0)	0 (0)

AML, amoxicillin; CIP, ciprofloxacin; CLR, clarithromycin; MTZ, metronidazole; TET, tetracycline.

[a]Presence or absence of pathogenicity genes is based on PCR assay.