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ORIGINAL ARTICLE

Emerging levofloxacin and multidrug resistance in clinical isolates of helicobacter *pylori* in Peshawar Pakistan

Momena Ali[1], Muhammad Waqas[2*], Saman Hussain[3], Adeeba Zahid[4] and Hafiz Danyal Khan[4]

[1] Department of Pathology, Khyber Medical College Peshawar Pakistan, [2] Department of Pathology, Khyber College of Dentistry Peshawar, Pakistan, [3] Department of Pathology, North West School of Medicine Peshawar, Pakistan, [4] Khyber Medical College, Peshawar Pakistan

ABSTRACT

Background: *Helicobacter pylor*i infection persists as a significant public health concern in developing countries and is a principal cause of gastritis, peptic ulcer disease, and gastric carcinoma. Rising resistance to clarithromycin and metronidazole jeopardizes empirical triple therapy and necessitates local molecular surveillance.

The objective of the study is to do molecular characterization of *H. pylori* isolated from dyspeptic patients and to perform antimicrobial susceptibility testing to guide the physicians for treatment strategies with symptoms of dyspepsia.

Methods: Gastric biopsies from symptomatic patients were cultured for *H. pylori*. DNA was extracted from 27 confirmed isolates and quantified by NanoDrop. Sample size was calculated using a standard formula based on a 92% expected prevalence, 95% confidence interval, and 5% margin of error. Molecular confirmation targeted the 16S rRNA gene using established primers. Antibiotic susceptibility testing was carried out by Kirby-Bauer disk diffusion method. Data were analysed descriptively and associations between prior antibiotic exposure and resistance were assessed.

Results: Molecular confirmation succeeded for the majority of cultured isolates. Markedly high resistance levels were observed to metronidazole and clarithromycin while amoxicillin resistance remained uncommon. Prior macrolide exposure was associated with increased likelihood of clarithromycin resistance.

Conclusion: *H. pylori* isolate from this tertiary care setting demonstrate clinically significant resistance to commonly used antibiotics, undermining empirical triple therapy. Routine susceptibility testing, molecular-guided therapy and antimicrobial stewardship are recommended to improve eradication rates and limit spread of resistance. Larger, regional multicentre surveillance and incorporation of molecular resistance testing into diagnostic workflows are urgently warranted.

Keywords: Antibiotic Stewardship, Drug Resistance, Helicobacter Pylori

This article may be cited as: Ali M, Waqas M, Hussain S, Tariq S, Zahid A, Khan HD. Emerging levofloxacin and multidrug resistance in clinical isolates of helicobacter pylori in Peshawar Pakistan. Int J Pathol 23(4):371-8. https://doi.org/10.59736/IJP.23.04.1031

CORRESPONDING AUTHOR

Dr. Muhammad Waqas

Department of Pathology

Khyber College of Dentistry Peshawar, Pakistan

Email: drmuhammadwaqas4@gmail.com

Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium exhibiting a spiral or rod-like morphology and multiple flagella (1). It predominantly colonizes within the

gastric mucosal layer in close proximity to the surface of gastric epithelial cells (1). Since its initial identification as the etiological agent of peptic ulcer disease, *H. pylori* has been consistently linked to a wide range of gastrointestinal as well as extra-gastrointestinal disorders including chronic gastritis, duodenal ulceration, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma, along with a variety of various extra-gastrointestinal pathologies comprising neurological, ocular, hematologic, cardiovascular, and dermatological manifestations, collectively affecting millions of individuals across the globe (2).

Global prevalence of *H. pylori* is reported to be approximately 50%(3). The World Health Organization (WHO) has classified *H. pylori* as a Group 1 carcinogen, indicating definitive evidence of its established carcinogenicity in humans (3). The incidence of *H. pylori* infection is considerably high in Pakistan, with reported values encompassing 85% to 95%(4). Diagnosis of *H. pylori* infection can be performed via non-invasive techniques, such as urea-breath test (UBT), stool-antigen assays, and serological IgG antibody detection or via invasive modalities using endoscopic gastric biopsy specimen with subsequent histological assessment or PCR testing (5). The microscopic tissue analysis of gastric mucosal specimens, adopting hematoxylin–eosin or Giemsa staining procedures, is gold standard for the definitive diagnosis of *H. pylori* infection. This modality not only assists in precise identification of the organism but simultaneously allows comprehensive evaluation of the extent and nature of gastric mucosal inflammation (6).

Antibiotic eradication regimen of *H. pylori* is substantial to prevent recurrence of peptic and duodenal ulcers and to lower the risk of gastric adenocarcinoma and MALT lymphoma (7). A major contemporary challenge in the treatment of *peptic ulcer disease* is the surging global rates of antimicrobial resistance, particularly to major therapeutic agents, which distinctly compromises the efficacy of standard eradication regimens (8). Revised Maastricht VI/Florence Consensus Report (2022) has recommended bismuth-containing quadruple therapy (BQT) as the preferred first-line regimen (9). In the context of antibiotic-resistant *H. pylori isolates*, non-bismuth concomitant quadruple therapy has exhibited greater therapeutic efficacy compared to successive eradication protocols, with reported eradication rates of approximately 92% and 62%, respectively(10). Nevertheless, growing evidence indicates that antibiotic resistance in *H. pylori* is a multifactorial phenomenon (11).

Given the exceptionally high prevalence of *H. pylori* infection in Pakistan, therapeutic success rates remain unsatisfactory despite continued efforts to optimize therapeutic regimens (12). Due to irrational use of antibiotics, antibiotic-resistance rates of Helicobacter pylori vary substantially by sociodemographic and clinical determinants, region-specific regional antibiotic resistance surveillance is essential to guide the selection of optimal H. pylori eradication regimens (13). This study aimed to determine the prevalence of levofloxacin and multidrug resistance in *H. pylori* isolates from gastric biopsies of patients undergoing endoscopy in tertiary care centers of Peshawar, Pakistan, and to evaluate their antimicrobial susceptibility patterns to guide effective local treatment strategies, thereby contributing local evidence for improved therapeutic and antimicrobial-stewardship strategies.

Methods

A cross-sectional study was conducted over a period of one year, from August 2018 to August 2019, at the Department of Pathology, Khyber Medical College (KMC) and the Institute of Basic Medical Sciences (IBMS), Khyber Medical University (KMU), Peshawar. A non-probability, purposive sampling technique was employed, whereby patients with suspected peptic ulcer disease undergoing endoscopy were included. Sample size was calculated using standard statistical formula based on the expected prevalence of 92% from a previous study(14), keeping confidence interval of 95% and 5% margin of error. A total of 115 participants were recruited for this study based on clearly defined inclusion and exclusion criteria to ensure the reliability and validity of the findings. We included adult patients of both genders, aged 18 years and above presenting with symptoms of dyspepsia or suspected peptic ulcer disease undergoing esophagogastroduodenoscopy (EGD). Only participants who exhibited endoscopic findings suggestive of gastritis, gastric or duodenal ulcer, or mucosal erosions were enrolled in the study. Eligible individuals were required to have no prior history of *H. pylori* eradication therapy and had not received antibiotics, proton pump inhibitors (PPIs), Bismuth containing compounds, or H_2- receptor Antagonist in the preceding four weeks as could interfere with microbial isolation and PCR testing. Only those patients who gave written consent and agreed to the use of their biopsy specimens for research purpose were included in the study. Exclusion criteria encompassed individuals with a history of partial or total gastrectomy, gastric carcinoma or previous gastric surgery, given the potential alterations in gastric physiology, that could influence bacterial colonization and persistence. Additionally, patients with severe systemic conditions such as hepatic cirrhosis, chronic renal failure, malignancy, or any immunosuppressive condition were excluded to avoid confounding effects on culture and antibiotic susceptibility testing. Pregnant and breastfeeding women were excluded due to ethical considerations and effects of hormonal variations on study outcomes. Additionally, patients with endoscopic findings inconsistent with *H. pylori* associated disease such as esophageal varices, malignancy, or NSAID-induced gastritis—differentiated based on a documented history of chronic NSAID use and those who declined consent or provided inadequate biopsy material, were excluded from the study.

Ethical approval for the study was obtained from the Institutional Review Board (IRB) of Khyber Medical College (KMC), Peshawar (Reference No. 377/ADR/KMC). All participants provided written informed consent prior to enrollment.

Three gastric biopsy samples were obtained from the antrum and corpus during endoscopy using sterile forceps. One specimen was fixed in 10% formalin for histopathology, while the remaining samples were transported in thioglycolate broth for microbiological evaluation. Biopsies were cultured on Columbia blood agar with 10% sheep blood and DENT selective antibiotics under microaerophilic conditions at 37°C for 48-72 hours. Detection of *H. pylori* was done using Gram staining and standard biochemical assays (catalase, oxidase, urease). Antibiotic sensitivity was assessed by the Kirby-Bauer disk diffusion method on Mueller-Hinton

agar incorporated with 5% sheep blood and interpreted according to CLSI 2019 guidelines, using amoxicillin, clarithromycin, levofloxacin, metronidazole, and tetracycline. DNA from confirmed isolates were extracted and evaluated qualitatively and quantitatively via Nanodrop spectrophotometry.

The collected data were analyzed using SPSS software version 23.0. For categorical variables, including antibiotic resistance profiles and gender distribution, frequencies and percentages were calculated to provide a clear representation of proportional distribution within the study population. For continuous variables, such as patient age, central tendency and dispersion were assessed using mean values and standard deviation (SD). 95% confidence intervals were computed for the key estimates where applicable. No inferential statistical tests were applied.

Results

A total of 115 endoscopic biopsy specimens were obtained, were collected from selected participants with symptoms suggestive of peptic ulcer disease or gastro-intestinal discomfort. The gastric tissue samples were evaluated and interpreted at pathology department, KMC (Khyber medical college) and IBMS, KMU (institute of basic medical sciences, Khyber medical university).

Among the 115 patients who underwent endoscopy, 64 were male (55.7%) and 51 were female (44.3%), with a mean age of 41.7 years for males and 43.7 years for females. *Helicobacter pylori* was successfully isolated from 27 patients (23.47%), including 15 males (55.6%) and 12 females (44.4%) as shown in table 1. The age range of male patients was 18 to 80 years (mean 42 years), whereas the female patients varied from 21 to 70 years (mean 49 years).

Table 1. Demographic distribution and Helicobacter pylori culture positivity among study participants (n = 115)

Parameter		Male	Female
Total Patients	115	64 (55.7%)	51 (44.3%)
Mean Age (Years)		41.7	43.7
Age Range (Years)		18 - 80	21 - 70
Total *H. pylori* Isolated (+ve culture)	27 (23.5%)	15 (55.6%)	12 (44.4%)
H. pylori Group Mean Age		42	49

All 27 culture-positive isolates displayed the classical microbiological features of *H. pylori*. Colonies were small and translucent on Columbia blood agar and microscopy revealed Gram-negative curved bacilli. Biochemical testing was uniformly positive: every isolate produced rapid urease activity and tested positive for catalase and oxidase. These phenotypic results were corroborated by histopathology (modified Giemsa stain) as shown in figure 1, which showed organisms and chronic gastritis changes in the corresponding biopsy material. All culture-positive isolates exhibited positive outcomes in these biochemical assays, thereby validating their identification as *H. pylori*.

A

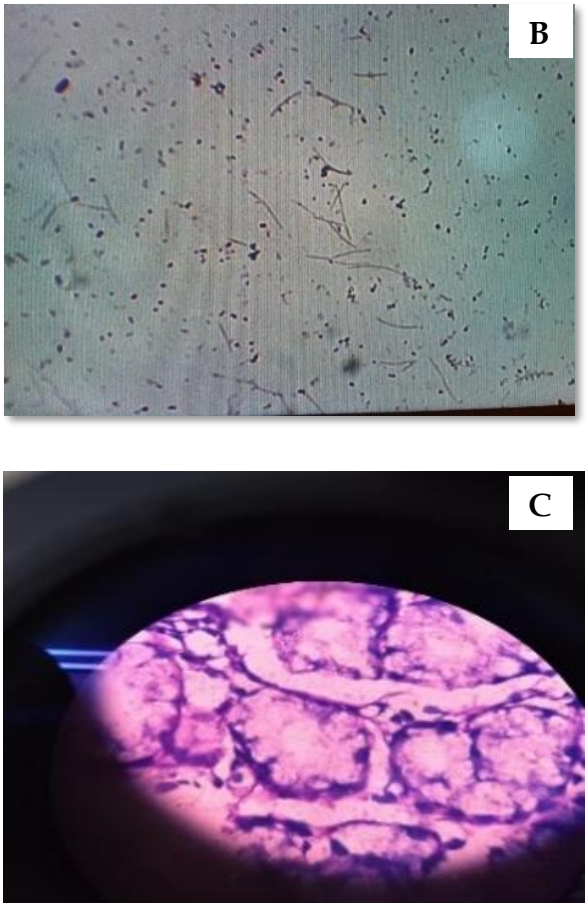

Figure 1. Identification of *H. pylori* by Culture, Gram Stain, and Histology.

(A) Small, translucent colonies of H. pylori grown on Columbia blood agar. Pseudomonas aeruginosa served as a negative control, and Campylobacter jejuni as a positive control.
(B) Gram-stained smear showing small, curved, Gram-negative, spiral-shaped *H. pylori* bacilli observed under oil immersion (100×).
(C) Giemsa-stained gastric mucosal tissue displaying *H. pylori* (arrows) within the lumen of gastric glands, confirming histological presence of the organism.

All culture-positive isolates exhibited positive outcomes in these biochemical assays, thereby validating their identification as *H. pylori*. For molecular testing of *H. pylori*, PCR technique was employed. Genomic DNA was extracted for testing from 27 confirmed isolates, and its quantitative assessment was done using a Nano Drop spectrophotometer. The extracted DNA was subsequently amplified to detect presence of the *H. pylori* 16S rRNA gene using the distinct primers: 5´-GCGACCTGCTGGAACATT-3´ and 5´-CGTTAGCTGCATTACTGGAG-3´. The amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 minutes, followed by cycles of denaturation at 94–95°C for 30 seconds, annealing at 55°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR products were analyzed by agarose gel electrophoresis. PCR amplification yielded a discrete band of 139 bp, thus confirming the presence of *H. pylori* specific gene as shown in figure 2.

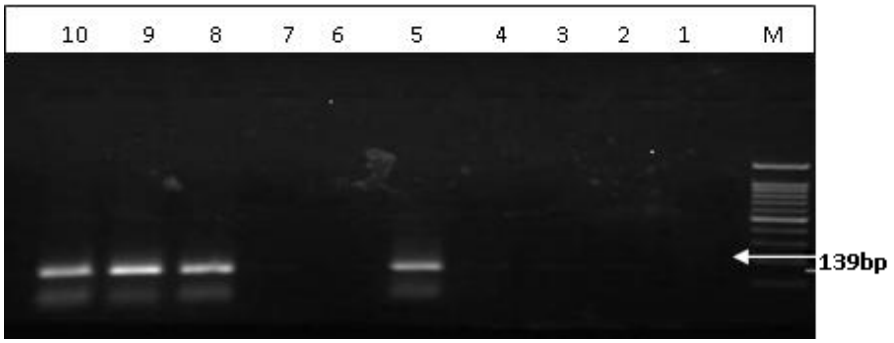

Figure 2. Molecular detection of H. pylori using species specific 16s rRNA gene (139 bp): 16s rRNA genes were amplified as follow: Lanes 1,2,3,4, 6, 7: negative, Lane 5,8,9 Biopsy samples, Lane10: positive control: M:100 bp.

Gastroesophageal duodenoscopy findings of the 115 enrolled participants demonstrated that gastritis involving gastric antrum was the most common lesion, identified in 90 patients (78.26%). Mild gastritis was observed in 15 patients (13%), whereas gastric mucosal erosions were observed in 6 patients (5.2%). More advanced pathological findings were relatively uncommon, with gastric carcinoma detected in 3 patients (2.6%) and adenocarcinoma in 1 patient (0.86%). Collectively, these findings indicate that gastritis—particularly involving the antrum—was the most common endoscopic finding in this study population.

Antibiotic susceptibility profiling using the Kirby–Bauer disc diffusion method identified significantly high resistance of *H. pylori* to several standard first-line antibiotics. Resistance towards metronidazole was universal (27/27, 100%), while amoxicillin and clarithromycin depicted markedly high resistance rates (25/27, 92.6% each), leaving only 2 isolates susceptible to either of these drugs. Levofloxacin resistance was reported in 14/27 isolates (51.8%), with the remaining 13/27 (48.2%) demonstrating phenotypic sensitivity. Tetracycline showed the highest activity among all, with 20/27 isolates (74.1%) susceptible, 2 (7.4%) exhibiting intermediate susceptibity, while 5 (18.5%) isolates were resistant. Overall, multi-drug resistance—defined as resistance to two or more antibiotic agents—was recognized in 9/27 isolates (33.3%), underscoring a significantly high prevalence of *H. pylori* resistance in this cohort.

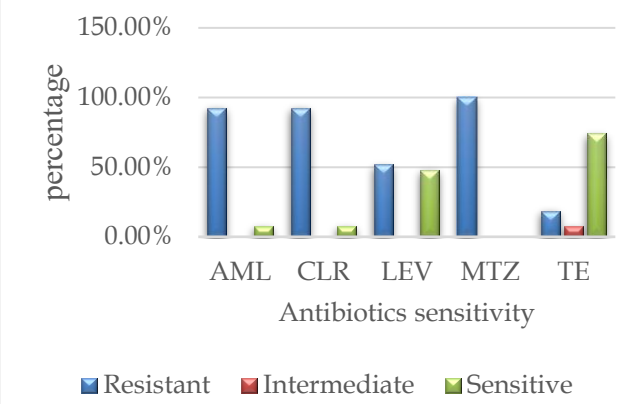

Figure 3: Antibiotic Susceptibility and resistance pattern in *H. Pylori*.

AML: Amoxicillin, AML(10µg), **CLR:** Clarithromycin(15µg), **LEV:** Levofloxacin(5µg), **MTZ:** Metronidazole(5µg), **TE:** Tetracycline(30µg)

Discussion

In this study we investigated 115 gastric biopsy specimens from patients with suspected peptic ulcer disease, *H. pylori* positive cultures were obtained in 27 cases (23.5%). This culture-positivity is comparable to recent reports. A study reported that 346 of 1540 adult biopsies (22.4%) were positive for *H. pylori* (15). Another study reported 20 of the 134 biopsies (14.9%) as positive(16). In contrast, some studies have found considerably higher culture positivity, such as 61.2% (17), 81.7%(18), 84.8% (19), 89.5% (20) and 65.7%(21). The culture detection rate was significantly lower than many local surveillance reports illustrating variability in culture methodology, prior antibiotic exposure, or other demographic/clinical factors. The relatively low rate of culture positivity here emphasizes the known challenge of *H. pylori* culture. The study population comprised 55.7% males and 44.3% females, with mean ages of 41.7 and 43.7 years, respectively. This corresponds to previous studies in which adults with

dyspepsia showed a major proportion of males (69.6%) and an age range of 18–70 years(22). The cohort characteristics, 55.7 % male, 44.3 % female with mean ages ~41.7 and ~43.7 years for males and females respectively, is consistent with earlier reports showing, 74.1 % were from males and 25.9 % from females, with age ranges of 18–69 years and 17–66 years, respectively (23). This aligns with our findings, showing that adult patients with dyspepsia present with *H. pylori infection* in these age groups. On the contrary, a study showed slightly higher proportion of females with positive culture for H. pylori, with 52 males (44.8%) and 64 females (55.2%)(17). Endoscopic findings indicated that antral gastritis was the predominant lesion, identified in 78.3% of the patients. This finding confirms the documented tendency of *H. pylori* for gastric antral colonization, primarily in symptomatic patients. Previous studies have demonstrated similar findings. A study reported positive *H. pylori* cultures from antral tissue biopsies in 80.1% of patients with peptic ulcer disease(18). Another study showed that 46.1% of patients had gastric antral biopsy samples positive for gastritis(22). These observations highlight the clinical significance of deriving biopsy samples from gastric antrum for *H. pylori* culture and genetic analysis in patients presenting with dyspeptic symptoms.

All 27 positive bacterial isolates manifested the distinct phenotypic attributes of *H. pylori*, including small, translucent colonies on blood-enriched agar, curved Gram-negative bacilli, and were positive for urease, catalase and oxidase reactions. The confirmation via PCR targeting the 16S rRNA gene reinforced the accuracy of culture-based identification. Our findings did not show any variation in sensitivity of detection of *H. pylori* by culture and PCR however, a study showed 37.9% positive and 50% negative cases by both the modalities. A slight variation was noted in 0.32% of cases, which were culture-positive but PCR-negative. Alternatively, 11.6% of tissue samples were PCR-positive and culture-negative (24). Another study reported significantly higher sensitivity and specificity of PCR than culture (25). The high sensitivity reported with PCR signifies its enhanced analytical precision, primarily in biological samples with low microbial load or minimal viability(24). These findings underscore the significance of using molecular confirmation techniques, as reliance on bacterial culture or phenotypic analysis alone can lead to suboptimal detection of atypical *H. pylori* strains.

A major finding of this study was significantly high antimicrobial resistance observed among the *H. pylori* isolates. Metronidazole resistance was found in 100% (27/27) isolates, while amoxicillin and clarithromycin each exhibited markedly high resistance rates (92.6%). levofloxacin was resistant in 51.8% (14/27) of isolates, whereas tetracycline demonstrated the highest susceptibility, with sensitivity of 74.1%. Multidrug resistance (MDR), defined as resistance to two or more antibiotic classes, was observed in 33.3% (9/27) of isolates. Consistent outcomes were documented by Metwally *et al.* with resistance rates of 100% for metronidazole, 95% for amoxicillin, 40% for clarithromycin, and 20% for levofloxacin(16). These findings are parallel to our results with distinct variation in resistance of clarithromycin and levofloxacin. Conversely, another study observed fraction of resistant isolates as; 27.5%, 50%, 67.5%, 35%, and 5% for amoxicillin, clarithromycin, metronidazole, levofloxacin, and tetracycline, respectively (26). A recent study reported

antibiotic resistance highest for levofloxacin (69.3%), followed by metronidazole (61.4%) and clarithromycin (19.3%) (27). In another study Metronidazole showed the highest resistance pattern (83.2%), followed by Amoxicillin (67.6%) (28). These studies show difference in antibiotic resistance patterns, nevertheless, tetracycline proved to be the most effective antibiotic across studies which aligns with our results. The proportion of resistant isolates observed in our study—particularly for levofloxacin—is higher than those observed in previous resistance reports, highlighting clinically significant burden of fluoroquinolone resistance in the tertiary-care population in Peshawar. This observation suggests irrational use of antibiotics, empirical antibiotic prescription, and long-term selective pressure from prior fluoroquinolone therapy. Extensive metronidazole resistance and high amoxicillin and clarithromycin resistance align with global resistance patterns, emphasizing the alarming rise in antimicrobial resistance. The strikingly high antimicrobial resistance in adult gastric biopsy specimens emphasize the regional issue for adequate and effective empirical therapy. The MDR rate in this study (33.3%) corresponds to rates reported in previous studies, such as clarithromycin–metronidazole–amoxicillin (30%), metronidazole–amoxicillin–levofloxacin (15%), and dual resistance to clarithromycin–metronidazole (40%) (16). A study reported 4.5% pooled prevalence of MDR isolates (29). Recent studies have reported 22.5%(26) and 21.8%(30) MDR isolates, which is lower than our findings, however high MDR of 53.5% has been observed (17). Given these resistance patterns, standard first-line triple therapy regimens are unlikely to attain satisfactory eradication of *H. pylori* infection in local population. This observation stresses the need for regional evidence-based empirical treatment protocols or, adopting routine culture and antimicrobial susceptibility testing (AST) of *H. pylori* isolates to direct precise therapy and lower the incidence of treatment failures.

Relatively high susceptibility of *H. pylori* isolates to tetracycline in our study population indicates that tetracycline-based regimens preserve therapeutic efficacy in the regional context. In contrast, 18.5% tetracycline resistance reflects that definitive efficacy cannot be guaranteed without susceptibility testing. Significantly high resistance of levofloxacin limits the use of fluoroquinolone-based antibiotic regimens without prior susceptibility testing. The variation in antibiotic resistance patterns of *H. pylori* between studies worldwide can be attributed to various factors, including regional variation, difference in antibiotic prescription practices, and antibiotic susceptibility assessment methods. Geographical differences in antimicrobial resistance often reflect local prescription trends, over-the-counter antibiotic availability, and self-medication, which influence the resistance patterns of *H. pylori* strains. The higher reported resistance to metronidazole and amoxicillin is contributed by the irrational and empirical use of these drugs. On the contrary, studies reporting less antimicrobial resistance often involve treatment-naïve study populations or regions with strict antimicrobial management. However, the concordant observation that tetracycline remains the most potent antimicrobial agent highlights its clinical significance, even with rising multidrug resistance.

Our study has several limitations. Firstly, the study was carried out at a single tertiary care

center with a comparatively small sample size of 115 patients, resulting in only 27 culture-confirmed *H. pylori* isolates. This constrains the statistical power of the study and limits its generalizability. Additionally, the study was dependent exclusively on phenotypic methods for assessing antibiotic susceptibility, while this approach provided reliable resistance patterns, however, were unable determine the genetic mechanisms of resistance. Moreover, due to inadequate of clinical data, such as patients' detailed prior antibiotic exposure records, or success/failure of antibiotic regimens, we could not establish a direct causal relationship between the observed resistance profiles and treatment outcomes. Future investigations should involve large, multicentric surveillance studies across Pakistan emphasizing molecular characterization of resistant isolates to establish a comprehensive regional map of *H. pylori* resistance patterns.

Conclusion

H. pylori isolate from this tertiary care setting demonstrate clinically significant resistance to commonly used antibiotics, undermining empirical triple therapy. Routine susceptibility testing, molecular-guided therapy and antimicrobial stewardship are recommended to improve eradication rates and limit spread of resistance. Larger, regional multicentre surveillance and incorporation of molecular resistance testing into diagnostic workflows are urgently warranted.

Source of funding: None
Acknowledgement: None
Conflict of Interest: None

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HISTORY	
Date received:	12-11-2025
Date sent for review:	18-11-2025
Date received reviewers' comments:	27-11-2025
Date received revised manuscript:	23-12-2025
Date accepted:	30-12-2025

CONTRIBUTION OF AUTHORS	
AUTHOR	**CONTRIBUTION**
Conception/Design	MA, MW, SH
Data acquisition, analysis and interpretation	MA, AZ, HDK
Manuscript writing and approval	MW, SH, AZ, HDK
All the authors agree to take responsibility for every facet of the work, making sure that any concerns about its integrity or veracity are thoroughly examined and addressed.	