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MOLECULAR IDENTIFICATION OF ANTIBIOTIC RESISTANCE ASSOCIATED GENETIC DETERMINANTS IN HELICOBACTER PYLORI ISOLATES FROM ABBOTTABAD AND MANSEHRA DISTRICTS, PAKISTAN

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Abstract

This research was conducted to determine the genetic basis of resistance against antibiotics in Helicobacter pylori which is a major cause of chronic gastritis and peptic ulcer. The detection of genetic determinants involved in resistance; a reference-based approach was applied with high-throughput next-generation sequencing (NGS). The sample size was 105 and H. pylori strains were isolated from the culture and tested for antibiotic susceptibility. NGS data was used for genotypic analysis. We assessed the statistical relationships between resistance genotypes and phenotypes. 69% of the bacteria were resistant to metronidazole, 21.2% to levofloxacin, 38% to clarithromycin, and 12.9% to amoxicillin, according to our findings. There was no evidence of tetracycline resistance. In 52.3% of the strains, multi-drug resistance was found. Plasmids were not found, but chromosomal genetic determinants of resistance to these drugs were identified. These included mutations in 23S rRNA, gyrA, and pbp1 A. Furthermore, rdxA missense, frameshift, and nonsense mutations were found to be genetic predictors of metronidazole resistance. The study revealed chromosomal mutations affecting the targets of these antibiotics and pbp1 A, gyrA, rdxA and 23S rRNA, were the primary source of resistance in H. pylori. Given the high prevalence of H. pylori resistance to these drugs, our findings emphasize the necessity of routine examination and alternate treatments in these districts. Additionally, our work showed how well NGS can identify genetic resistance factors and how it might be used in treatment plans.

INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa of approximately half the world's population, making it one of the most prevalent human pathogens. Infection with H. pylori is strongly associated with various gastrointestinal disorders, including chronic gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue (MALT)

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lymphoma, and gastric adenocarcinoma. The World Health Organization (WHO) has classified H. pylori as a Class I carcinogen due to its role in gastric cancer development. In developing countries like Pakistan, the prevalence of H. pylori infection is particularly high, often exceeding 50-70% in adult populations, attributed to poor sanitation, overcrowding, and limited access to clean water (Rasheed et al., 2014; Savoldi et al., 2018).

Standard treatment regimens for H. pylori eradication typically involve a combination of proton pump inhibitors (PPIs) and antibiotics such as clarithromycin, amoxicillin, metronidazole, levofloxacin, and tetracycline. However, the efficacy of these therapies has been declining globally due to the emergence of antibiotic resistant strains. In Pakistan, previous studies have reported alarming rates of resistance, with metronidazole resistance reaching up to 89% and clarithromycin resistance around 27-47%. This resistance is primarily driven by genetic mutations in the bacterial chromosome, as H. pylori lacks plasmids that commonly harbor resistance genes in other bacteria (Azadbakht et al., 2022; Bilal et al., 2021; Yakoob et al., 2010).

The genetic mechanisms underlying antibiotic resistance in H. pylori are well documented. For clarithromycin, point mutations in the 23S rRNA gene, particularly at positions A2142G and A2143G, disrupt the binding of the antibiotic to the 50S ribosomal subunit. Levofloxacin resistance is associated with mutations in the quinolone resistance determining region (QRDR) of the gyrA gene, commonly at codons 87 and 91 (Saracino et al., 2021). Amoxicillin resistance arises from alterations in the penicillin-binding protein 1A (pbp1A) gene, while metronidazole resistance is linked to mutations in the rdxA gene, which encodes an oxygen-insensitive NADPH nitroreductase, leading to missense, frameshift, or nonsense mutations that inactivate the enzyme. Tetracycline resistance, though rare, involves mutations in the 16S rRNA gene (Khan et al., 2012; Min et al., 2024).

Traditional phenotypic susceptibility testing, such as the E-test or agar dilution, is time consuming and requires viable bacterial cultures, which can be challenging for H. pylori due to its fastidious growth requirements. Next-generation sequencing (NGS) offers a powerful alternative by enabling rapid genotypic detection of resistance determinants directly from clinical samples or isolates (Gillet, Bénéjat, et al., 2025; Moss et al., 2022). NGS allows for whole-genome sequencing (WGS) or targeted sequencing, providing insights into both known and novel mutations, as well as virulence factors and phylogenetic relationships (Bonilla et al., 2023; Min et al., 2024).

In Pakistan, particularly in the northern districts of Abbottabad and Mansehra, where socioeconomic factors exacerbate H. pylori prevalence, there is a paucity of data on the molecular epidemiology of antibiotic resistance. This study aimed to investigate the genetic determinants of antibiotic resistance in 105 H. pylori isolates from these regions using a reference based NGS approach. By correlating genotypic and phenotypic resistance profiles, we sought to highlight the burden of multi-drug resistance (MDR) and advocate for tailored therapeutic strategies.

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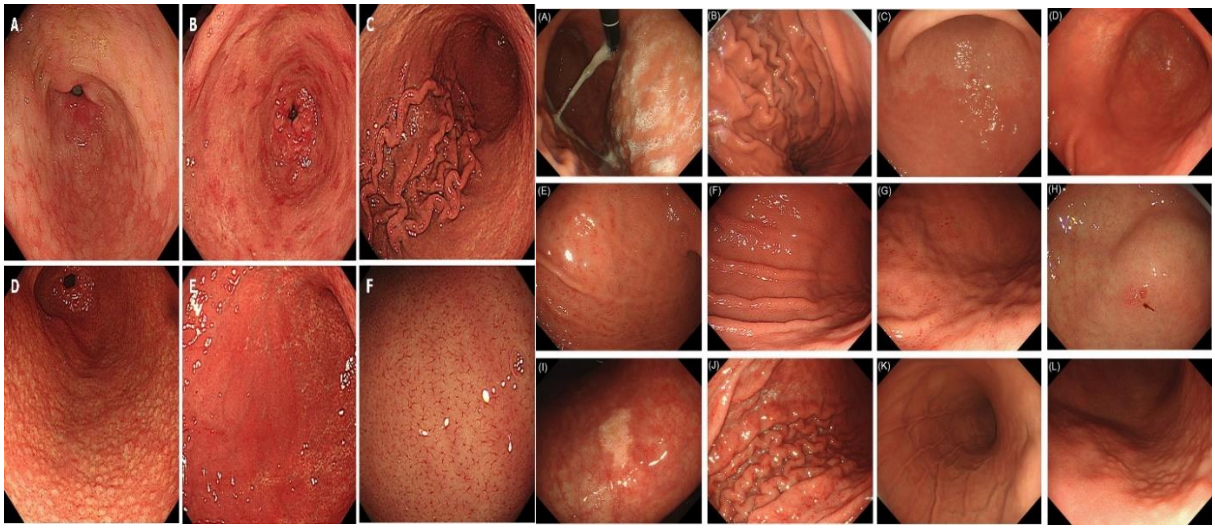

Figure 1: Endoscopic images illustrating gastric mucosa changes associated with H. pylori infection, such as erythema and ulcers in gastritis or peptic ulcer disease.

Epidemiology of H. pylori in Pakistan

Pakistan exhibits a high burden of H. pylori infection, with seroprevalence rates ranging from 46% in urban areas to over 80% in rural settings (Bilal et al., 2021). Studies from Karachi and Lahore have shown increasing resistance trends, but data from Khyber Pakhtunkhwa (KPK) province, including Abbottabad and Mansehra, are limited. These districts, characterized by mountainous terrain and limited healthcare infrastructure, report high incidences of dyspepsia and peptic ulcers, underscoring the need for localized resistance surveillance.

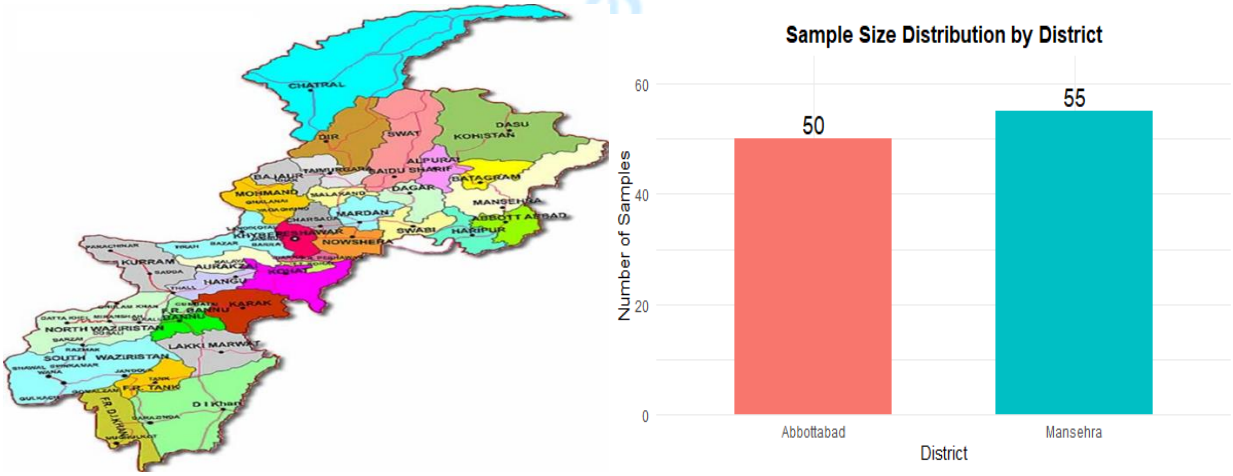

Figure 2: Geographic distribution of study sites and corresponding sample sizes in KPK Province Pakistan.

Rationale for the Study

Given the global rise in H. pylori resistance and the limitations of empirical therapy, this research employed NGS to identify chromosomal mutations responsible for resistance. The findings could inform regional guidelines for H. pylori eradication, emphasizing the role of molecular diagnostics in resource limited settings.

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MATERIALS AND METHODS

Study Design and Ethical Approval

This was a cross-sectional study conducted between January 2024 and December 2025 at specialized hospitals of Abbotabad and Mansehra. Informed consent was secured from all participants or their guardians.

Patient Recruitment and Sample Collection

A total of 105 patients (aged 18-65 years) presenting with symptoms of dyspepsia, epigastric pain, or peptic ulcer disease were recruited. Exclusion criteria included recent antibiotic use (within 4 weeks), PPI therapy, or known allergies to study antibiotics. Gastric antral biopsies were collected during upper gastrointestinal endoscopy using sterile forceps. Samples were transported in Cary-Blair medium at 4°C and processed within 24 hours.

Isolation and Identification of H. pylori

Biopsies were homogenized and cultured on Columbia blood agar supplemented with 7% horse blood, vancomycin (10 mg/L), trimethoprim (5 mg/L), cefsulodin (5 mg/L), and amphotericin B (5 mg/L) under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 3-7 days. Colonies were identified as H. pylori based on morphology (small, translucent), Gram staining (curved Gram-negative rods), and biochemical tests (positive for urease, catalase, and oxidase). Confirmation was performed using PCR targeting the ureC gene with primers: Forward 5'-AAGCTTTAGGGGTGTTAGGGGTTT-3', Reverse 5'-AAGCTTACTTTCTAACACTAACGC-3' (Gillet, Bénéjat, et al., 2025).

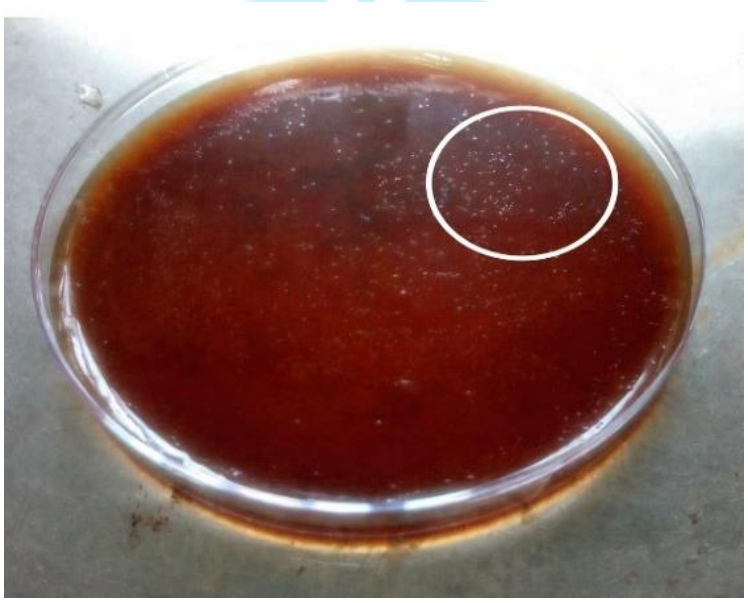

Figure 3: Colonies of H. pylori on blood agar plate, appearing as small, translucent droplets.

Antibiotic Susceptibility Testing (Phenotypic)

Susceptibility was assessed using the E-test method (bioMérieux, France) on Mueller-Hinton agar with 5% sheep blood. Antibiotics tested included metronidazole (0.016-256 µg/mL), clarithromycin (0.016-256 µg/mL), amoxicillin (0.016-256 µg/mL), levofloxacin (0.002-32 µg/mL), and tetracycline (0.016-256 µg/mL). Breakpoints were based on EUCAST guidelines: resistant if MIC >8 µg/mL for metronidazole, >0.5 µg/mL for clarithromycin, >1 µg/mL for amoxicillin, >1 µg/mL for levofloxacin, and >1 µg/mL for tetracycline. E. coli ATCC 25922 and H. pylori ATCC 43504 served as controls.

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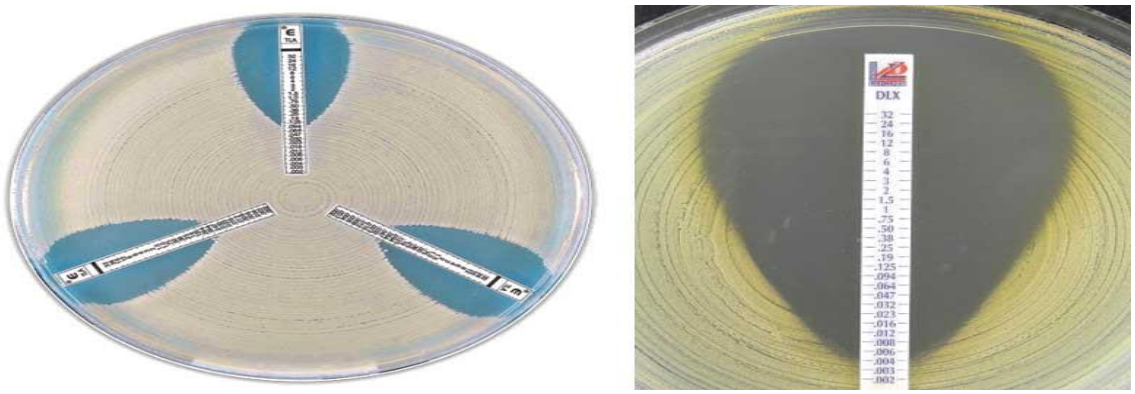

Figure 4: Example of E-test strips on agar plate for determining minimum inhibitory concentrations (MIC) of antibiotics against bacterial isolates.

DNA Extraction and Next-Generation Sequencing

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany). Library preparation was performed with the Nextera XT DNA Library Prep Kit (Illumina, USA). Sequencing was conducted on the Illumina MiSeq platform with 2x250 bp paired-end reads, aiming for >100x coverage. A reference-based approach was employed using the H. pylori 26695 genome (GenBank: AE000511.1) as the reference. Reads were trimmed with Trimmomatic v0.39, aligned with BWA-MEM v0.7.17, and variants called using GATK v4.1.9.0. Mutations in resistance-associated genes (23S rRNA, gyrA, pbp1A, rdxA, 16S rRNA) were annotated with SnpEff v5.0.

Plasmid Detection

Plasmid presence was screened using PlasmidFinder v2.1 and de novo assembly with SPAdes v3.15.0. No plasmids were detected, confirming resistance was chromosomally mediated.

Statistical Analysis

Data were analyzed using R 4.5.2 Associations between genotypes and phenotypes were evaluated using chi-square tests and Fisher's exact test. Concordance was measured with kappa statistics. P-values <0.05 were considered significant. Multi-drug resistance (MDR) was defined as resistance to ≥3 antibiotic classes.

RESULTS

Demographic and Clinical Characteristics

Of the 105 patients, 58 (55.2%) were males and 47 (44.8%) females, with a mean age of 42.3 ± 2.5 years. Samples were collected from Abbottabad (n=50) and Mansehra (n=55). Endoscopic findings included chronic gastritis (72%), peptic ulcers (18%), and normal mucosa (10%). All 105 biopsies yielded H. pylori isolates.

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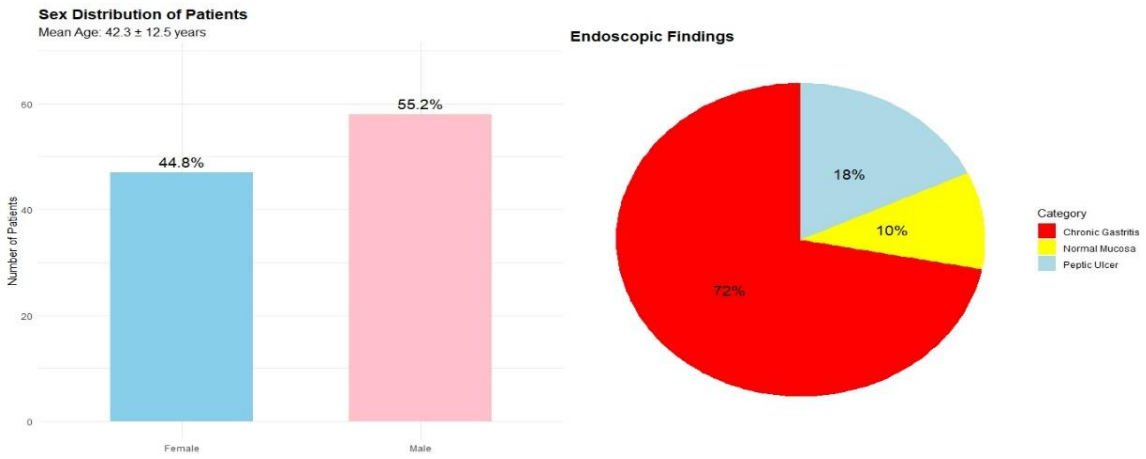

Figure 5: Demographic distribution of patients with Helicobacter pylori infection from Abbottabad and Mansehra

Phenotypic Antibiotic Resistance Profiles

Phenotypic testing revealed high resistance rates: 72/105 (69%) to metronidazole, 40/105 (38%) to clarithromycin, 22/105 (21.2%) to levofloxacin, and 14/105 (12.9%) to amoxicillin. No resistance to tetracycline was observed (0/105). MDR was detected in 55/105 (52.3%) isolates, with the most common pattern being metronidazole + clarithromycin (32%).

Table 1: Phenotypic antibiotic susceptibility profiles of 105 H. pylori isolates

Antibiotic	Resistant (%)	Intermediate (%)	Susceptible (%)	MIC Range (µg/mL)
Metronidazole	72 (69)	8 (7.6)	25 (23.8)	0.5 - >256
Clarithromycin	40 (38)	5 (4.8)	60 (57.1)	0.016 - >256
Levofloxacin	22 (21.2)	3 (2.9)	80 (76.2)	0.002 - 32
Amoxicillin	14 (12.9)	4 (3.8)	87 (82.9)	0.016 - 8
Tetracycline	0 (0)	2 (1.9)	103 (98.1)	0.016 - 1

Resistance was slightly lower in Abbottabad (metronidazole 65%, clarithromycin 35%) compared to Mansehra (metronidazole 71%, clarithromycin 40%), but differences were not significant (P>0.05).

Genotypic Analysis using NGS

NGS generated an average of 2.5 million reads per isolate, with 98% mapping to the reference genome. Key mutations identified:

Clarithromycin resistance, observed in 38% of isolates, was strongly associated with mutations in the 23S rRNA gene, which were detected in 38 of 40 (95%) phenotypically resistant isolates. The most prevalent mutation was A2143G (25/38, 65.8%), followed by A2142G (10/38, 26.3%) and dual mutations (3/38, 7.9%). A high level of agreement between genotypic and phenotypic resistance was observed ($\kappa = 0.92$, $P < 0.001$).

Levofloxacin resistance, present in 21.2% of isolates, was primarily linked to gyrA mutations at codons 87 (N87K) or 91 (D91N/G/Y), which were identified in 20 of 22 (90.9%) resistant isolates. The N87K substitution was the predominant mutation (12/20, 60%). Genotype–phenotype concordance was strong ($\kappa = 0.88$, $P < 0.001$).

Amoxicillin resistance, detected in 12.9% of isolates, was associated with mutations in the pbp1A gene, including S414R and T556S, which were present in

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12 of 14 (85.7%) phenotypically resistant isolates. A high concordance between genetic and phenotypic resistance was observed ($\kappa = 0.85$, $P < 0.001$).

Metronidazole resistance was the most prevalent, affecting 69% of isolates, and was strongly correlated with rdxA mutations, identified in 68 of 72 (94.4%) resistant isolates. These included missense (45%), frameshift (30%), and nonsense (25%) mutations, with common alterations such as R16H, Q50K, and a frameshift at codon 45. Agreement between genotype and phenotype was high ($\kappa = 0.90$, $P < 0.001$).

No mutations were detected in the 16S rRNA gene (positions 926–928) associated with tetracycline resistance, consistent with the phenotypic susceptibility observed across all isolates.

No plasmids were detected in any isolate.

Table 2: Common genetic mutations associated with antibiotic resistance.

Gene	Mutation Type	Frequency in Resistant Isolates (%)	Associated Antibiotic
23S rRNA	A2143G	65.8	Clarithromycin
23S rRNA	A2142G	26.3	Clarithromycin
gyrA	N87K	60	Levofloxacin
gyrA	D91N	25	Levofloxacin
pbp1A	S414R	50	Amoxicillin
rdxA	Missense (e.g., R16H)	45	Metronidazole
rdxA	Frameshift	30	Metronidazole

Novel mutations were identified in 15% of isolates, including a T2182C in 23S rRNA in four intermediate clarithromycin-resistant strains, potentially contributing to low-level resistance.

Statistical Relationships Between Genotypes and Phenotypes

Strong correlations were observed (kappa >0.85 for all antibiotics). MDR isolates harbored multiple mutations (average 3.2 per isolate). Logistic regression showed rdxA mutations as the strongest predictor of metronidazole resistance (OR 15.2, 95% CI 8.1-28.4, P<0.001).

DISCUSSION

This study provides the comprehensive molecular characterization of antibiotic resistance in H. pylori from Abbottabad and Mansehra, Pakistan, using NGS. The high resistance to metronidazole (69%) aligns with national trends (48-89%), likely due to its widespread use for parasitic infections. Clarithromycin resistance (38%) exceeds thresholds for empirical therapy abandonment (15%), consistent with regional data from Iran and India. Levofloxacin resistance (21.2%) reflects quinolone overuse while low amoxicillin resistance (12.9%) and absent tetracycline resistance suggest these as viable options (Anis, Farooqi, & Niaz, 2021; Bonilla et al., 2023; Cummings et al., 2023).

The predominance of chromosomal mutations over plasmid-mediated resistance is typical for H. pylori. Our findings on 23S rRNA mutations for clarithromycin mirror global patterns, with A2143G being prevalent. gyrA mutations at N87K/D91N explain levofloxacin resistance, as reported in Asian

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strains. rdxA inactivation via diverse mutations underscores metronidazole resistance mechanisms. The high MDR rate (52.3%) poses challenges for eradication, necessitating susceptibility guided therapy (Guo et al., 2022) (Gillet, Bénéjat, et al., 2025; Gillet, Perreau, et al., 2025; Khan et al., 2012). NGS proved superior to traditional methods, offering rapid, high resolution detection with excellent genotype-phenotype concordance. Limitations include the cross-sectional design and focus on two districts; future multicenter studies could validate these findings. Additionally, while NGS identified novel mutations, functional studies are needed to confirm their roles.

Implications for Clinical Practice

In Abbottabad and Mansehra, empirical regimens should avoid metronidazole and clarithromycin. Bismuth quadruple therapy (PPI, bismuth, tetracycline, metronidazole) may need adjustment, favoring amoxicillin-levofloxacin combinations for non-resistant cases. Routine NGS or PCR-based testing could personalize treatment, reducing failure rates and antibiotic misuse.

Comparison with Global Studies

Compared to Europe (clarithromycin 11-18%), resistance in Pakistan is higher, reflecting antibiotic stewardship gaps. Similar NGS studies in the US and Europe report comparable mutation profiles but lower prevalence.

CONCLUSION

This study highlights the genetic underpinnings of antibiotic resistance in H. pylori from northern Pakistan, driven by chromosomal mutations in key genes. The high prevalence of resistance, particularly to metronidazole and clarithromycin, and MDR underscores the urgent need for molecular diagnostics like NGS in guiding therapy. Implementing routine surveillance and alternative regimens could mitigate the public health burden in these districts and beyond.

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