Investigating Host Dynamics Under Antibiotic Treatments for Resistant *Helicobacter pylori*

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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Investigating Host Dynamics Under Antibiotic Treatments for Resistant *Helicobacter pylori*

A THESIS

Submitted in partial fulfilment of the
requirements for the award of the degree

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Investigating Host Dynamics Under Antibiotic Treatments for Resistant *Helicobacter pylori* "** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2024 to May 2025 under the supervision of Dr. Hem Chandra Jha, Associate Professor, Indian Institute of Technology Indore. The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Sanjana. 22/05/25

Signature of the student with date

(Sanjana Kumari)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

22/5/25

Signature of MSc thesis supervisor

(DR. HEM CHANDRA JHA)

Sanjana Kumari has successfully given his M.Sc. Oral Examination held on **May 2025**

Signature(s) of Supervisor(s) of MSc thesis

Date: 22/5/25

P.V. Kodgire

Convener, DPGC

Date: 22/05/2025

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ABSTRACT

Helicobacter pylori (H. pylori) infection is a major global health issue linked to the onset of chronic gastritis, peptic ulcers, and gastric cancer. Although antimicrobial treatments are widely utilized, the rise of antibiotic-resistant (AMR) *H. pylori* strains has significantly undermined the effectiveness of these therapies. This study seeks to examine the response of host cells to the antimicrobial medications deployed for *H. pylori* treatment, especially in cases involving resistant bacterial strains. The research will emphasise two main areas: the cellular reactions of the host to these medications and the possible synergistic effects of combination therapies within the scope of AMR *H. pylori* infection. Evidence indicates that these drugs, in addition to their intended antibacterial effects, can influence mammalian cells directly, modifying pathways related to inflammation, immune responses, and cellular stress. This study will investigate how these medications affect host cell biology when targeting resistant *H. pylori* strains, evaluating whether they intensify inflammatory reactions, trigger cytotoxic effects, or alter signalling networks in host cells. Further, the study will assess the potential synergistic impacts of combination therapies on host cells. While these therapies seek to improve bacterial elimination, their unintended consequences for mammalian cells in the context of resistance are still not clearly understood. Through cellular assays, molecular pathway analysis, and synergy modelling, this research will shed light on how combination therapies engage with host cells during infections with resistant *H. pylori* strains. The results will aid in refining *H. pylori* treatment strategies by finding a balance between effective bacterial elimination and reducing negative effects on the host, addressing the important challenges that AMR presents in gastric infections.

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ACRONYMS

AGS	Adenocarcinoma gastric cell
BHI	Brain heart infusion
CagA	Cytotoxin associated gene
A	Amoxicillin
C	Clarithromycin
M	Metronidazole
DCFDA	2, 7- dichlorofluorescein diacetate
FBS	Foetal bovine serum
LC3A	Light chain 3 alpha
LC3B	Light chain 3beta
M+C	Metronidazole + Clarithromycin
C+A	Clarithromycin + Amoxicillin
A+M	Amoxicillin + Metronidazole
PBS	Phosphate buffer saline
AKA	Aurora Kinase A
PARP	Poly (ADP-ribose) Polymerase
BabA	Blood Group Antigen Binding Adhesin
qRT-PCR	Quantitative RT-PCR
RMSD	Root means square deviation
RMSF	Root means square fluctuation
MALT	Mucosa-associated lymphoid tissue
VacA	Vacuolating cytotoxin a
GC	Gastric Cancer
WHO	World health organisation
μM	Micro-molar

Chapter 1: Introduction

1.1 Overview of *Helicobacter pylori*

Helicobacter pylori, commonly known as *H. pylori*, is a Gram-negative bacteria shaped like a spiral, which resides in the gastric mucosa of more than half the global population. It thrives in the highly acidic stomach environment by locally neutralizing acidity and embedding itself within the protective mucus layer. These adaptations allow it to stick to the cells lining the stomach and evade attacks from the immune system, even though immune cells tend to accumulate around infection sites. Additionally, *H. pylori* interfere with local immune responses, which hampers the body's capacity to eliminate the bacteria (Kusters et al., 2006).

H. pylori infections are prevalent, especially in low- and middle-income nations, where factors such as inadequate sanitation, high population density, and poverty elevate the risk. Transmission generally occurs during childhood through faecal-oral, oral-oral, or gastric-oral routes. The prevalence of the bacterium varies by geographical and ethnic factors, with approximately two-thirds of the world's population infected

While many individuals with *H. pylori* infection remain symptom-free, chronic infections can lead to ongoing inflammation (non-atrophic gastritis) that may progress to atrophic gastritis. This progression heightens the risk of developing gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Acknowledging its link to stomach cancer, the World Health Organization designated *H. pylori* as a human carcinogen in 1994, and it was added to the National Toxicology Program's carcinogen list in 2021 (Wroblewski et al., 2010). There is also emerging evidence

suggesting a connection between *H. pylori* infection and colorectal cancer, although its relationship with pancreatic cancer is still not clearly established. Notably, *H. pylori* have been associated with a lower risk of oesophageal adenocarcinoma, which is linked to gastroesophageal reflux disease and Barrett's oesophagus. Besides cancer risks, *H. pylori* is a major contributor to peptic ulcers in the stomach and upper small intestine, significantly affecting gastrointestinal health worldwide. Effective management and enhanced sanitation are essential to decrease the prevalence and associated risks of this infection (Polyzos et al., 2017)**.**

1.2 *H. pylori* and gastric cancer

Gastric cancer (GC) is a complex disease closely associated with infection by *H. pylori*, which is identified as the primary risk factor for its onset. Additional contributors to GC risk include genetic factors in the host, the virulence attributes of the bacteria, and environmental elements like diet and socioeconomic status. The World Health Organization designated *H. pylori* as a class I carcinogen in 1994 due to its strong link to gastric adenocarcinoma, especially the intestinal type. This form of cancer develops through defined histological stages, progressing from chronic active gastritis to atrophic gastritis, followed by intestinal metaplasia, dysplasia, and finally, adenocarcinoma (Malfertheiner et al., 2023).

H. pylori utilize various virulence factors, such as cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), and outer inflammatory protein A (OipA), to evade the host's immune defenses and promote cancer development (Figure 1). These factors lead to chronic inflammation, enhance the production of reactive oxygen and nitrogen species, and result in cellular injury. Strains that test positive for CagA significantly heighten the risk of GC by altering pathways like

Src/ERK, JAK/STAT3, and the Hippo tumor suppressor pathway. Strains characterized as CagA + VacA + facilitate the differentiation of cancer-associated fibroblasts and initiate epithelial-mesenchymal transition (EMT), both of which increase cell invasiveness (Conteduca et al., 2013).

Recent research has underscored the interactions between *H. pylori* and host cell adhesion molecules, such as CEACAMs, which aid in the translocation of CagA through the T4SS. These interactions highlight the pathogen's ability to adapt while colonizing the gastric mucosa and fostering cancer development. Ongoing molecular characterization of *H. pylori* strains and their virulence factors, including differences in vacA genotypes, offers critical insights into their roles in gastritis and the progression of GC. These results stress the importance of developing targeted approaches to reduce the risk of gastric carcinogenesis linked to *H. pylori.*

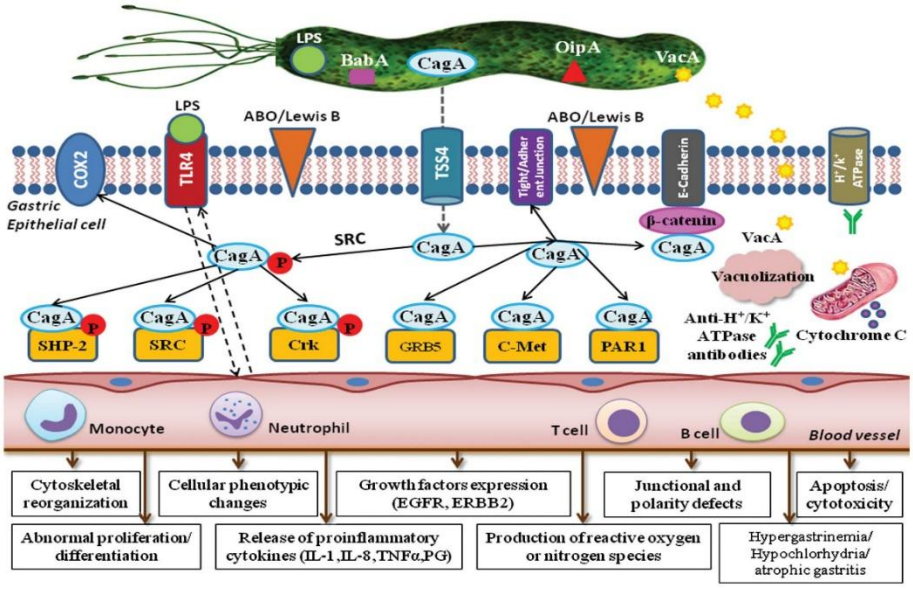


Figure 1. Pathogenesis of *H. pylori* infection

[Source: Conteducai et al. International Journal of Oncology, 42: 5-18, (2013)]

1.3 Antibiotic resistance in *H. pylori*

H. pylori have several mechanisms of antibiotic resistance which make treatment difficult and may lead to gastric complications. The resistance is mainly brought about by mutations in the chromosomal genes rather than by gene transfer or loss. These mutations are usually gene mutations that may lead to alteration of antibiotic targets or inhibition of drug activation within the bacterium. Missense, nonsense and frameshift mutations are the common types which cause the resistance to single drugs or MDR (Figure 2). The use of antibiotics at subtherapeutic doses also leads to the selection of the resistant populations with some regions having reported MDR strains in more than 40% of the infections.

Besides genetic mutations, some physiological mechanisms also play a role in the development of resistance. Some of these protein mechanisms include biofilm formation in which efflux all pump work expression, to decrease in the outer uptake and diffusion of the antibiotics. Biofilms are made of EPS which acts as a shield that reduces the effectiveness of the antimicrobials, enhances the gene transfer and supports the persistence of the resistant strains. Biofilm producing *H. pylori* has been found on gastric mucosa and these may act as a reservoir for infection and resistance development.

Also, *H. pylori* can exist in a coccoid state which is a non-dividing cellular state which is linked with reduced antibiotic susceptibility. These forms have the following structural and metabolic changes which may increase the antibiotic resistance; however, the current literature does not support the existence of coccoid forms or genetic their and viability. Phenotypic changes threaten the increasing effectiveness of the MDR current *H. pylori* treatment approaches. This therefore both shows the need to enhance the current understanding of the mechanisms of resistance and find new ways of dealing with it (Mannion et al., 2021).

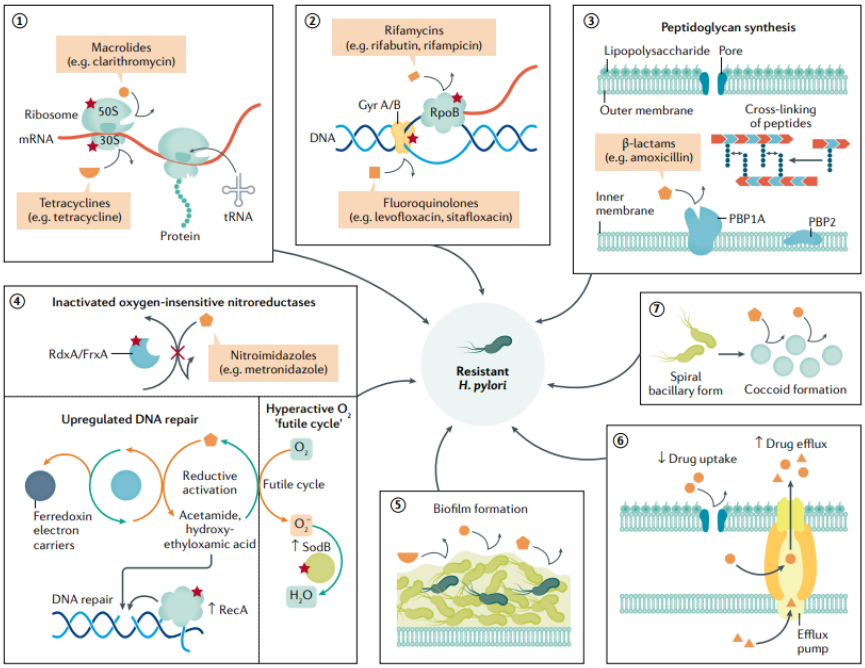


Figure 2. Mechanism of antibiotic resistance in *H. pylori*.

(Source: Kabamba et al. Nat Rev Gastroenterol Hepatol 18, 613–629 (2021).)

1.4 Effects of antibiotics on host cells

Antibiotics commonly used in *H. pylori* eradication regimens have been shown to exert various harmful effects on host cells, extending beyond their antimicrobial properties. Bactericidal antibiotics such as quinolones, aminoglycosides, and β-lactams—including amoxicillin—can induce mitochondrial dysfunction and excessive generation of reactive oxygen species (ROS) in mammalian cells. This oxidative stress results in damage to DNA, proteins, and membrane lipids, both in vitro and in vivo. In mice, such antibiotic exposure led to elevated oxidative stress biomarkers and tissue damage, alongside the upregulation of antioxidant defence genes—effects that were alleviated by antioxidant treatment with N-acetyl-L-cysteine or avoided by using bacteriostatic antibiotics instead (Kalghatgi et al., 2013). Amoxicillin, a β-lactam antibiotic, also exhibited genotoxic effects in human peripheral blood lymphocytes and gastric mucosa cells, with oxidative DNA damage linked to ROS formation. Although this damage was repairable within 60 minutes, it was significantly reduced by antioxidants such as vitamins C and E, melatonin, and PBN. Notably, *H. pylori*-infected gastric cells showed impaired DNA repair compared to non-infected cells, suggesting an increased susceptibility to oxidative stress–induced carcinogenesis (Zaharieva et al., 2012). Clarithromycin, another commonly used antibiotic, has been shown to impair autophagic flux and promote apoptosis in cancer cells such as colorectal carcinoma and multiple myeloma, primarily by disrupting PI3K/Akt signaling and inducing autophagosome accumulation (Spina et al., 2015; Nakamura et al., 2010). Additionally, both amoxicillin and clarithromycin directly induced apoptosis in a murine B cell lymphoma line via activation of the TNF signaling pathway, as evidenced by increased expression of TNFR1, Fas, and caspases-3, -8, and -9 (Inoue et al., 2004). Beyond these effects, amoxicillin was also found to reduce transcription and secretion of apolipoprotein A-I (ApoA-I) in HepG2 and Caco-2 cells,

likely through inhibition of PPAR transactivation and downregulation of KEAP1, CPT1, and CHOP gene expression (Plat et al., 2018). Collectively, these findings emphasize that while antibiotics are essential for *H. pylori* eradication, they may also disrupt host cell homeostasis through oxidative stress, genotoxicity, altered autophagy, apoptosis, and impaired lipid metabolism—underscoring the importance of understanding and mitigating these side effects during treatment.

1.5 Role of Autophagy in gastric cancer and *H. pylori* infection

Autophagy plays a complex and context-dependent role in both host defense and cancer biology. During *H. pylori* infection, autophagy is initially activated as a host defence mechanism to clear the pathogen. However, *H. pylori* subvert this process, particularly through virulence factors such as VacA, which not only induces autophagy but disrupts autophagosome maturation, ultimately promoting bacterial persistence (Yahiro et al., 2012; Tsugawa et al., 2012). Additionally, *H. pylori* modulate autophagy via reactive oxygen species (ROS)-mediated signaling, LRP1 receptor interaction, and microRNA regulation—for instance, MIR30B targets key autophagy genes such as ATG12 and BECN1, impairing the host's autophagic response (Tang et al., 2012).

Interestingly, recent studies suggest that antibiotics commonly used to eradicate *H. pylori*—amoxicillin, metronidazole, and clarithromycin—can also modulate autophagy in host cells. These antibiotics have been shown to either activate or suppress autophagy through mechanisms involving mitochondrial stress and cellular homeostasis (Kim et al., 2021; Cao et al., 2023). The overlapping effects of *H. pylori*-induced autophagy manipulation and antibiotic-driven autophagic modulation may synergistically disrupt autophagy pathways. This dual interference

could significantly affect treatment outcomes, influence bacterial persistence, and potentially enhance the risk of gastric carcinogenesis by destabilizing autophagic balance in gastric epithelial cells. Therefore, understanding how these antibiotics interact with host autophagy during *H. pylori* infection could help develop adjunctive therapeutic strategies aimed at restoring autophagic homeostasis for improved infection control and cancer prevention.

Chapter 2: Review of Past Work and Problem Formulation

2.1 Review of Past Work

H. pylori, a gram-negative and communicable pathogen, is responsible for infecting approximately 4.4 billion people worldwide, or roughly 70% of the population (J. K. Y. Hooi et al., 2017). Typically acquired in childhood, the infection persists throughout one's lifetime, leading to progressive chronic gastric inflammation that can result in clinical complications in 1-10% of those infected, such as peptic ulcer disease, gastric atrophy, gastric intestinal metaplasia, and eventually, gastric cancer or mucosa-associated lymphoid tissue (MALT) lymphoma (P. Sipponen et al., 2015). As there is no effective vaccine, managing chronic *H. pylori* infection has become the primary approach for controlling the spread of the bacterium in the population, resolving gastric lesions in infected individuals, and preventing the development of subsequent gastric cancer (J.-Y. Wu et al., 2019). Furthermore, a significant paradigm shift has occurred since the Kyoto *H. pylori* conference in 2015, which recommended that all *H. pylori* infections be eradicated, unless there are valid reasons to avoid doing so, such as comorbidities, high rates of reinfection in a particular region, or competing health priorities of society (K. Sugano et al.,15).

Consequently, despite regional variations in rates and profiles, increasing *H. pylori* antibiotic resistance has been reported globally over the past two decades in parallel with a continuous decrease in the success rates achieved with eradication therapies (I. Thung et al.,2016). In the past decade, with rare exceptions, all regimens recommended worldwide in treatment guidelines as first-line and rescue therapies continue to face failures in approximately 10–30% of patients. These treatment failures

led to a therapeutic dilemma in patients who are not cured by consecutive drug regimens for whom no logical empirical (third) treatment remains thereafter (J. P. Gisbert et al., 2017). Thus, since 2017, *H. pylori* have been listed by WHO (World Health Organization) among the 20 pathogens that pose the most serious threat to human health because of their drug resistance (Bahrain et al., 2019).

Due to overdose of antibiotics along with amoxicillin, clarithromycin (CAM), or metronidazole (MNZ), which can be used to treat *H. pylori* contamination good sized cell modifications occur, ordinarily in reaction to the oxidative pressure and DNA damage they induce. Amoxicillin, although powerful in opposition to bacterial infections, has been shown to cause DNA harm in human lymphocytes and gastric mucosa cells. This harm is linked to the production of reactive oxygen species (ROS), which can result in oxidative DNA damage, especially in cells inflamed with *H. pylori*. Appreciably, the potential of infected cells to restore this DNA harm is impaired, doubtlessly increasing the hazard of mutations and cancer promotion. Further, clarithromycin and different macrolides can induce autophagy and apoptosis in cancer cells, highlighting their antitumor effects (Eguchi et al., 2022). But these antibiotics additionally influence mobile signalling pathways like ERK and PI3K/Akt, that are critical for mobile proliferation and survival. Those results can also alter cellular responses to strain or growth alerts, in rapidly dividing most cancers or infected cells.

Despite these adjustments, while treating infections due to antimicrobial-resistant bacteria, the therapeutic efficacy of these antibiotics may be seriously constrained. Resistant bacteria, unaffected by means of the antibiotic’s action, stay viable and preserve to proliferate, rendering the meant antibacterial consequences useless. In such cases, while host cells may also go through DNA damage, oxidative stress, or altered mobile cycles because of the drug remedy, the bacteria continue to thrive, evading the drug's mechanism of action

(Kalghatgi et al., 2013).

For this reason, even as those antibiotics can also cause considerable organic responses in host cells, their loss of effectiveness against resistant pathogens poses a task, necessitating alternative treatments or combinatorial treatments to cope with both bacterial resistance and host cell safety (Tshibangu-Kabamba & Yamaoka, 2021).

2.2 Problem Formulation

The rise of antimicrobial-resistant *H. pylori* strains and their persistence despite conventional antibiotic therapy presents a critical public health challenge, with poorly understood implications for host-pathogen dynamics and gastric carcinogenesis. While drug-resistant *H. pylori* evade treatment, prolonged antibiotic exposure-even when ineffective at bacterial eradication-may exacerbate host cell stress responses, including DNA damage, oxidative stress, and dysregulation of cellular clearance mechanisms like autophagy. This dual burden of persistent infection and subtherapeutic drug activity could synergistically remodel the gastric microenvironment, fostering chronic inflammation, genomic instability, and survival of mutation-prone cells. However, the mechanistic links between treatment-resistant infections, drug-induced host stress, and progression to aggressive gastric cancer remain uncharacterized. There is an urgent need to evaluate how these combined factors alter host signalling networks, epigenetic landscapes, and tumor suppressor pathways to drive malignant transformation. Addressing this gap is essential to redefining therapeutic strategies for resistant *H. pylori* infections, which currently lack approaches to mitigate unintended host cell damage while combating bacterial persistence. Without such insights, standard therapies risk inadvertently fuelling the very oncogenic processes they aim to prevent.

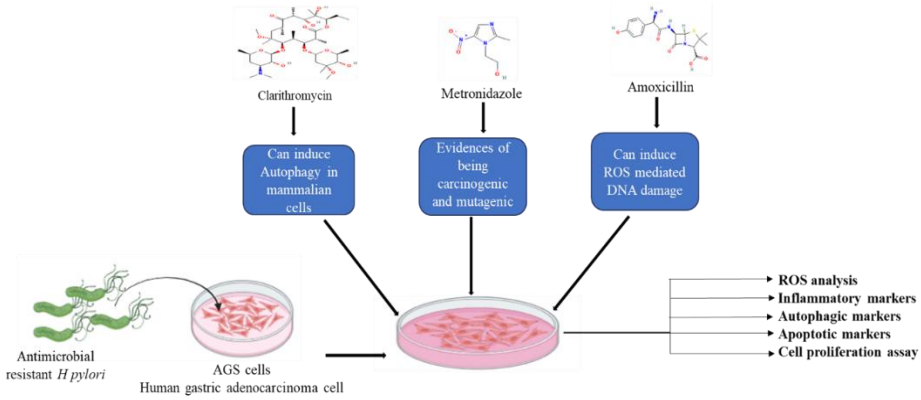


Figure 3. Deciphering the role of antibiotics in gastric cancer development when infected with antimicrobial resistant *H. pylori.*

Incubation with H. pylori in AGS cells for 24 hrs, and treatment with different drug Combination. In this experimental setup, AGS gastric epithelial cells were cultured under standard conditions and allowed to reach approximately 60% confluency, at which point they were infected with clinical strains of H. pylori—I10 and HB1. Following 24 hours of infection, the cells were treated with different combinations of standard antibiotics used in H. pylori eradication therapy, namely amoxicillin, clarithromycin, and metronidazole, for an additional 12 hours. This treatment strategy aimed to evaluate the synergistic or individual effects of the drugs on infected gastric epithelial cells.

Chapter 3: Aim and Objectives

3.1 Aim

This study aims to understand the modulatory changes that occur in AGS cells following *H. pylori* infection and drug treatment at transcript level and protein level. It seeks to understand how antibiotics, including amoxicillin, clarithromycin, and metronidazole, interact with host gastric epithelial cells in the presence of antibiotic-resistant *H. pylori*, probably inducing oxidative strain, DNA damage, and changes in key cellular signalling pathways. By combining these advanced techniques, we aim to unravel the molecular mechanisms underlying *H. pylori*-induced cellular processes and provide a comprehensive view of the interactions between *H. pylori* and host cells. Also, the study aims to investigate how apoptotic pathways in gastric epithelial cells are altered by reactive oxygen species (ROS) generated during *H. pylori* infection and antibiotic treatment. It addresses a key gap in understanding the potential pro-carcinogenic effects of failed antibiotic therapy, particularly against resistant *H. pylori* strains.

3.2 Objectives

There are majorly two objectives and various experiments under them.

Objective 1: To examine host responses to antibiotic drugs in the context of antibiotic-resistant *H. pylori* infection.

This objective focuses on understanding how the host gastric epithelial cells respond when exposed to antibiotics like amoxicillin, clarithromycin, and metronidazole. It considers the additional factor of bacterial resistance, which diminishes therapeutic efficacy and might

exacerbate cellular stress and damage. Experiments involved under this objective are:

1. To check the antibiotic susceptibility of different Helicobacter pylori strains. 2. To assess the cytotoxicity of amoxicillin, metronidazole, and clarithromycin on AGS cells.

3. To perform checkerboard analysis to determine the effective drug concentrations and synergy between them for treatment.

4. To investigate cellular responses, including ROS production, cancer markers, and autophagy.

Objective 2: The second objective of this study is to systematically investigate how apoptotic pathways in gastric epithelial cells are altered by the combined effects of reactive oxygen species (ROS) generated from both *H. pylori* infection and antibiotic drug treatment. This research objective addresses a critical knowledge gap regarding the potential pro-carcinogenic consequences of failed antibiotic therapy against resistant.

1. Characterize the distinct ROS signatures produced during resistant *H. pylori* infection versus reference strain infection, identifying strain-specific oxidative stress markers.
2. Quantify changes in intrinsic versus extrinsic apoptotic pathway components following exposure to both bacterial infection and antibiotic compounds.
3. Elucidate the molecular mechanisms through which ROS signalling influences apoptotic threshold and cell fate decisions.

These objectives represent a critical component of understanding how antimicrobial resistance affects not just treatment efficacy but also creates a potentially dangerous cellular environment host cell survival pathway.

Chapter 4: Materials and Methods

4.1 Materials

Biosafety level 2A cabinet (cell culture), Laminar airflow hood (for working on bacteria), Centrifuge, Refrigerator (-80ºC, -20ºC, 0ºC, 4ºC), Microaerophilic chamber, Heat block, pH meter, Vortex shaker, CO2 incubator, Liquid Nitrogen Container, Pipette with tips, Serological pipettes, Sterile disposable Culture dish (60mm and 100 mm), 14 ml round bottom snap-cap tubes, 15 ml screw-cap centrifuge tubes, 1.5 ml micro centrifuge tubes, 6/ 12/ 24 well plates, Glass slides and coverslip, Homogenizer, 0.45µm filter, Microplate reader, Trans illuminator, Ethanol, PBS, Phenol, Chloroform, Sodium Acetate, Loading dye, triton X100, Trypsin 0.05%, tris-HCl, Sodium dodecyl sulfate, BHI agar, DMSO, Isopropanol, 100 and 70% Ethanol, Ponceau, Glycerol, Tris free base, Iso-amyl alcohol, Phenol, $ChCL_3$.

Selective media for *H. pylori* - Brain heart infusion broth/agar**,** Fetal bovine serum (10.0% vol/vol)**,** Amphotericin (5.0 µg/ml)**,** Trimethoprim (5.0 µg/ml)**,** Cefsulodin (5.0 µg/ml)**,** Vancomycin (10.0 µg/ml).

4.2 Methods

4.2.1 Culture of *H. pylori* clinical isolates in liquid and solid growth medium

A single colony from the BHI agar plate of each strain was inoculated into brain heart infusion (BHI) (BD-DIFCO, USA), broth supplemented with 10% foetal bovine serum (FBS) (FBS; Hi-Media, Mumbai, India) and 1x *H. pylori* selective antibiotics, in a snap cap tube. concurrently,

the colony became streaked onto a BHI agar plate containing 10% FBS and 1x antibiotics. Both the broth and agar plates have been incubated at 37°C within a microaerophilic chamber optimized for *H. pylori* increase.

For the infection study, clinically isolated *H. pylori* lines, HB1 (human biopsy sample #1) and HB5 (human gastric biopsy sample #5), had been used. Each trace demonstrated triple resistance to antibiotics. The bacteria had been grown in selective BHI broth within 14 mL round-bottom snap cap tubes (BD, Cat. No. 352001). and incubated inside the microaerophilic chamber (Whitley DG 250) situations for 24 hours. After incubation, 150 µL of the bacterial subculture was transferred in duplicate into a 96-properly flat-backside plate, and optical density (OD) became measured at 600 nm. An OD 600 of 0.3 corresponds to approximately 500 million CFU/mL. The final OD values were normalized with normal media OD values. The bacterial cell count (CFU/mL) calculated based on the OD values, and the desired volume for infection was determined. For contamination experiments with the bacterial isolates, a multiplicity of contamination (MOI) of 100 become utilized.

4.2.2. Bacterial characterisation and antibiotic susceptibility test

(a) Gram staining: To identify the isolated strains, Gram staining was performed. A smear was prepared by diluting the bacterial colony in 100 µL of PBS, accompanied by using air-drying and heat-fixation over a flame. The smear was first flooded with crystal violet stain for 60 seconds, then rinsed with distilled water to do away with extra stain. Subsequently, Gram's iodine is applied to the smear for 60 seconds to repair the crystal violet, followed by a way of decolorization with 95% ethanol. The slides had been once more rinsed with distilled water, blot-

dried, and counterstained with safranin for 30 seconds. After a very last rinse with distilled water, the slides were dried and observed under a light microscope (Kashyap et al., 2020).

(b) Genomic DNA isolation: *H. pylori* cultures have been harvested in phosphate-buffered saline (PBS) as soon as the optical density (OD) at six hundred nm reached more than a few 0.2 - 0.6. The pelleted bacterial cells had been resuspended in an extraction solution containing 10 mM Tris (pH 8.0), 15 mM NaCl, 10 mM EDTA, and 0.5% SDS, and incubated at 55°C for 1 hour. Proteinase K (20 mg/mL) was added to achieve a final concentration of 1 mg/mL, and the aggregate turned into incubated in a single day at 37°C. Following this, RNase-A changed into delivered at a concentration of 0.1 mg/mL, and the solution was incubated for 1 hour at 37°C. DNA was then purified using the phenol-chloroform-isoamyl alcohol extraction method (Kashyap et al., 2020).

(c) PCR of universal and *H. pylori* specific 16s rRNA:

H. pylori DNA samples were amplified using PCR with a reaction volume of 10 μL for each strain. Specific primers targeting the 16S rRNA gene were employed: forward primer 5′-CTGGAGAGACTAAGCCCTCC-3′ and reverse primer 5′-ATTACTGACGCTGATTGCGC-3′, generating a product size of 169 bp. The amplification process began with an initial denaturation at 95°C for 7 minutes, followed by 40 cycles consisting of denaturation at 94°C for 2 minutes, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for 10 minutes.

The PCR products were analysed using gel electrophoresis on a 2.5% agarose gel stained with 0.5 μg/mL ethidium bromide. The expected product size was confirmed by comparing bands with a 50-bp DNA ladder. Gel images were captured using a gel documentation system (Kashyap et al., 2020)**.**

4.2.3 Antibiotic susceptibility test:

The antibiotic susceptibility of *H. pylori* isolates was evaluated using the agar dilution method. Conventional antibiotics which include amoxicillin, clarithromycin, and metronidazole were prepared, and serial -fold dilutions were included into Brain heart Infusion (BHI) agar supplemented with 10% foetal bovine serum (FBS). clean *H. pylori* colonies had been suspended in phosphate-buffered saline (PBS) to attain turbidity matching a 0.5 McFarland general, then diluted 1:10. A 1–2 μL aliquot of the bacterial suspension became inoculated onto agar plates containing unique antibiotic concentrations, alongside a control plate without antibiotics. The plates had been incubated at 37°C for 72 hours underneath a microaerophilic ecosystem (5% O_2, 10% CO_2, 85% N_2). Bacterial inhibition became visually assessed, and the minimal inhibitory awareness (MIC) became recognized as the lowest antibiotic awareness that completely inhibited bacterial increase. MIC values were interpreted as the use of installed scientific breakpoints to categorize the isolates as inclined, intermediate, or resistant, offering a detailed resistance profile (Singh et al., 2024).

4.2.4 Cell culture

Adenocarcinoma gastric (AGS) cell line was procured from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Kashyap et al., 2021).

4.2.5 Cell Cytotoxicity assay

The cytotoxicity of amoxicillin, metronidazole, and clarithromycin on AGS cells changed to determine the usage of the MTT assay. Equal

numbers of AGS cells (1×10^4) have been seeded into 96-well plates and incubated for a single day. Cells were then treated with 8 different concentrations of amoxicillin and metronidazole (250, 125, 62.5, 31.25, 15.625, 7.812, 3.9, and 1.95 µg/mL) for 24 hours, even as clarithromycin treatment commenced at a preliminary awareness of 50 µg/mL. After 24 hours, the culture medium becomes carefully removed, and 150 µL of MTT reagent is delivered to every well, followed with the aid of a 4hr incubation at room temperature. In the end, the MTT reagent was replaced with dimethyl sulfoxide (DMSO), and the plates had been incubated on an orbital shaker for 1.5 hours. Optical density turned into measured at 590 and 570 nm using a microplate reader, and the information was analysed and plotted using GraphPad Prism (Kashyap et al., 2024).

4.2.6 Checkerboard analysis for drug synergy estimation

The synergistic impact of clarithromycin, metronidazole and amoxicillin on AGS cells changed into evaluated using the checkerboard technique with the MTT assay. In this technique, varying concentrations of the 2 antibiotics were combined in a two-dimensional matrix format to analyze their interactive consequences. The concentrations of every drug were decided on based totally on their IC_{10} values (the concentration inhibiting 10% of bacterial viability) acquired from prior cytotoxicity assays. AGS cells have been seeded in 96-well plates at a density of 1×10^4 cells according to nicely and allowed to stick in a single day. Clarithromycin and metronidazole had been then introduced to the wells in special combinations of concentrations, creating a matrix wherein every properly contained a completely unique drug pair. Following a 24-hour incubation, the cell viability changed to assess the use of the MTT assay. The used medium was removed and with a 150 µL of MTT reagent and incubated for 4 hours. The reagent changed into then removed, dimethyl sulfoxide (DMSO) became added, and plates

have been incubated for 1.5hours on an orbital shaker. Optical densities have been recorded at 595 and 570 nm using a microplate reader. The results were then plotted to visualize drug synergy and optimize antibiotic combination for treatment.

4.2.7 Cellular ROS estimation

AGS cells were seeded and infected on a 6-well plate, and they will receive medication therapy for 12 hours according to the aforementioned plan. DCFDA dye was used to measure the amount of ROS produced by cells after the incubation period was over. To put it briefly, the live cells were stained with 10 μg/mL of the dye in PBS, incubated for 20 to 25 minutes, and then washed with PBS. The cells were then visualised at 10× objective magnification using an Olympus IX83 fluorescent microscope that was assisted by cell Sens imaging software. ImageJ software was used to quantify the amount of intracellular ROS, which was proportional to the intensity of DCF fluorescence. Fold increases over the control cells were used to represent relative changes in DCF fluorescence. (Kashyap et al., 2021).

The combinations for drug treatment after checkerboard analysis:

1. Control (AGS)

2. Control + *H. pylori* (I10)

3. Control + *H. pylori* (HB1)

4. Control + *H. pylori* (I10) + amoxicillin + clarithromycin

5. Control + *H. pylori* (I10) + clarithromycin + metronidazole

6. Control + *H. pylori* (I10) + amoxicillin + metronidazole

7.Control + *H. pylori* (HB1) + amoxicillin + clarithromycin

8. Control + *H. pylori* (HB1) + clarithromycin + metronidazole

9. Control + *H. pylori* (HB1) + amoxicillin + metronidazole

4.2.8 RNA isolation and qRT-PCR

Using the well-established Trizol technique, the total RNA content of cells treated to *H. pylori* and various antibiotic combinations for 24 hours will be determined. TRIzol reagent was used to extract total RNA, and a reverse transcription kit will be used to prepare cDNA in accordance with the manufacturer's instructions. qRT-PCR will be performed on cDNA using the SYBR green real-time master mix. Sequence-specific primers will be created for the analysis of the genes linked to autophagy (atg5, atg7, lc3a, lc3b, and Beclin 1), apoptosis (casp 3 and 8), antioxidants (sod2, cat, keap1, and hsf1), and Gankyrin (Sonkar et al., 2020).

4.2.9 Western blotting

Following treatment with the drugs obtained from the aforementioned groups, the infected cells will be extracted, rinsed with ice-cold PBS, and lysed in RIPA buffer, which contains protease and phosphatase inhibitors and contains 10 mM Tris (pH 7.5), 150 mM sodium chloride, 2 mM EDTA, and 1% NP-40. Bradford protein assay reagents will be used to quantify the proteins in the supernatant. SDS-PAGE will be used to separate equal amounts of protein from each group, which will then be deposited onto 0.45 μm nitrocellulose membranes. Following membrane blocking with 4% BSA, the membranes will be treated for 12 hours at 4 °C or with primary antibodies specific to anti-NF-κB, anti-PARP, autophagy-related genes (ATG7, LC3A, LC3B, and Beclin 1), and apoptotic genes (Caspase 3, 9 and 8). The membrane will be treated with a 1:3000 dilution of horseradish after incubation and washing

peroxidase-conjugated anti-rabbit antibodies for 1 h. The chemiluminescent detection will be based on the Pierce ECL Western blotting substrate Image analysis and quantification will be performed using Image J software (Sonkar et al., 2020).

4.3 Experimental model

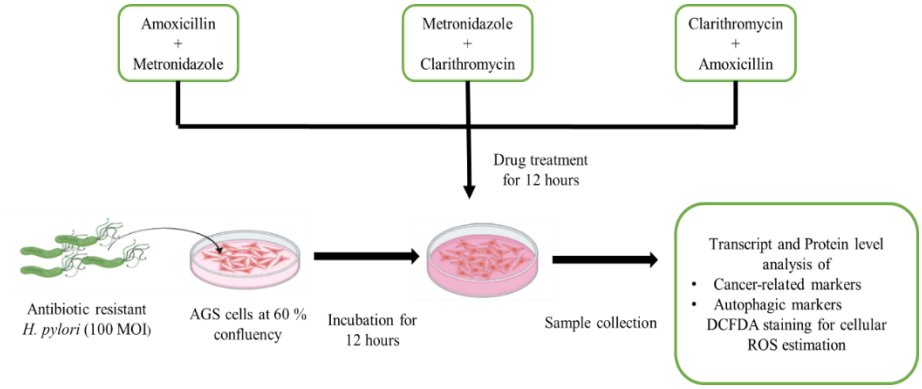


Figure 4. Experimental model to mimic infected and drug treated AGS cells to H. pylori. Incubation with *H. pylori* in AGS cells for 24 hrs, and treatment with different drug Combination.

In this experimental setup, AGS gastric epithelial cells were cultured under standard conditions and allowed to reach approximately 60% confluency, at which point they were infected with clinical strains of *H. pylori*—I10 and HB1. Following 24 hours of infection, the cells were treated with different combinations of standard antibiotics used in *H. pylori* eradication therapy, namely amoxicillin, clarithromycin, and metronidazole, for an additional 12 hours. This treatment strategy aimed to evaluate the synergistic or individual effects of the drugs on infected gastric epithelial cells. Post-treatment, transcript-level analysis using qRT-PCR and protein-level analysis via western blotting and/or immunofluorescence were performed to assess changes in gene and protein expression associated with inflammation, oxidative stress, and other *H. pylori*-related pathogenic mechanisms. This model provides insights into the host-pathogen interaction and the therapeutic efficacy of antibiotic combinations at both the molecular and cellular levels.

Chapter 5: Results and Discussion

5.1 Bacterial characterization

Bacterial characterization is a fundamental step in microbiological analysis, beginning with Gram staining, a differential staining technique that categorizes bacteria into Gram-positive or Gram-negative based on the structural differences in their cell walls. *H. pylori*, for example, appears as a Gram-negative, spiral-shaped bacillus under the microscope. Molecular identification is further refined using polymerase chain reaction (PCR) targeting the 16S rRNA gene, a highly conserved region across bacterial species, allowing universal detection and taxonomic classification. To confirm the identity of *H. pylori*, species-specific primers targeting unique sequences within the 16S rRNA gene are employed, ensuring precise identification. Following molecular confirmation, antibiotic susceptibility testing is conducted to guide effective treatment. This includes the minimum inhibitory concentration (MIC) broth dilution method, which determines the lowest concentration of an antibiotic that inhibits visible bacterial growth in liquid media. Additionally, the disc diffusion method (Kirby-Bauer test) is used to assess the inhibition zone around antibiotic-impregnated discs placed on agar plates, providing qualitative data on bacterial sensitivity or resistance. Together, these methods offer a comprehensive approach for the identification and antimicrobial profiling of *H. pylori* and other bacterial pathogens.

5.1.1 Gram staining

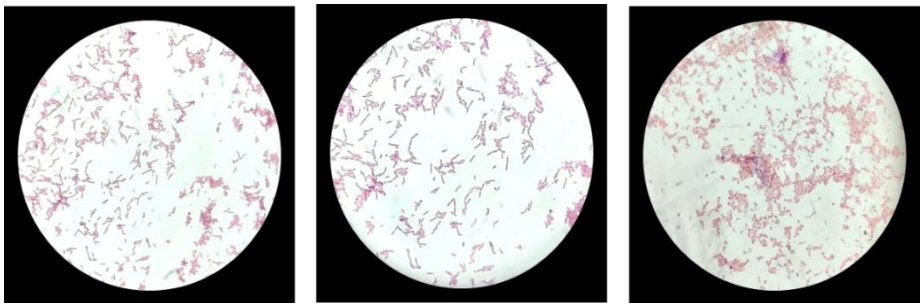


Figure 5. Gram staining of I10, HB1 and HB5

represented as (a), (b) and (c) respectively. Identification of bacteria through Gram staining. Gram staining of different clinical isolates of *H. pylori*, namely, I10 (a), HB1(b), and HB5(c) were showing typical gram-negative bacteria.

5.1.2 Genomic DNA isolation

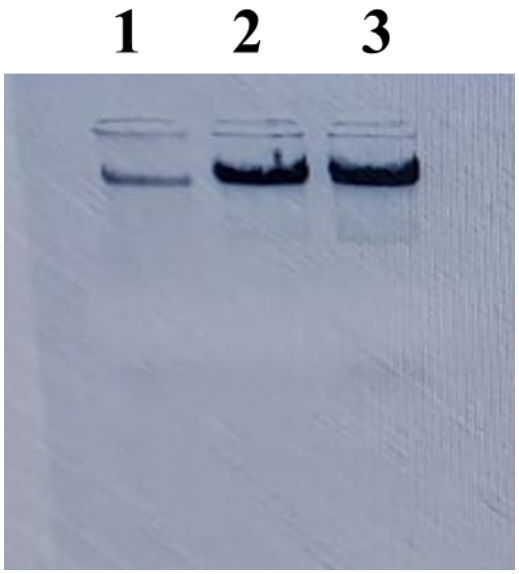


Figure 6.Gram staining of I10, HB1 and HB5

Similar bands of genomic DNA were observed in I1O (1) (laboratory strain) and

H. pylori extracted from human biopsy samples [HB1 (2) and HB5 (3)].

5.1.3 PCR of universal and *H. pylori* specific 16s rRNA

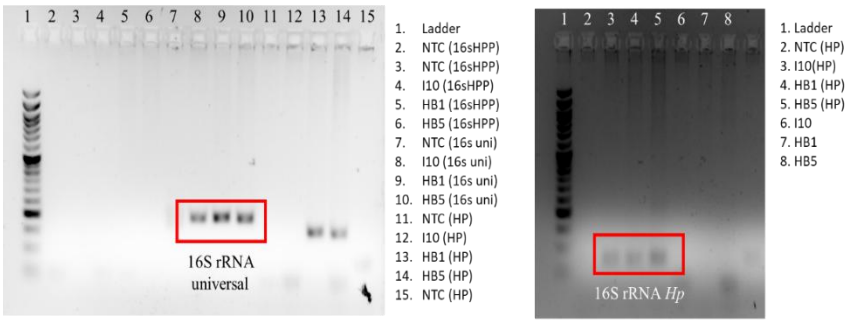


Figure 7. 2.5% Agarose gel for PCR product of universal 16s rRNA and *H. pylori* specific 16s rRNA

In this gel image the well 8, 9 and 10 shows amplified PCR product of universal 16s rRNA of the selected bacterial strains. Similar bands were observed in I1O (laboratory strain) and *H. pylori* extracted from human biopsy samples (HB1 and HB5). In the 2nd gel image well 3, 4, and 5 shows the amplified PCR product of *H. pylor*i specific 16s rRNA. Similar bands were observed in I1O (laboratory strain) and *H. pylori* extracted from human biopsy samples (HB1 and HB5).

The sequence of these primers and their product size are as follow:

1. Universal 16s rRNA

Forward - 5'-CCTACGGGAGGCAGCAG-3

Reverse - 5'-ATTACCGCGGCTGCTGG-3'

Product size – 169 bp

2. *H pylori* specific 16s rRNA

Forward - 5’- CGGACACACTGGAACTGAGA-3’

Reverse – 5’- CGGACACACTGGAACTGAGA-3’

Product size – 116 bp

5.1.4 Antibiotic susceptibility test:

Table 1: Antibiotic susceptibility of *H. pylori* isolates

H.pylori isolates	Clarithromycin	Metronidazole	Amoxicillin
HJ1	---	+++	+++
HJ9	---	---	---
HJ10	---	+++	+--
HB10	---	+++	+++
I10	---	+++	---
HB1	+++	+++	+++
HJ14	---	+++	+--
HB14	---	+++	+++
HJ17	+++	+++	+++
HB5	+++	+++	+++

Susceptibility determined using minimum inhibitory concentration (MIC) vis Disc diffusion and broth dilution method. HB denotes *H. pylori* obtained from biopsy samples infected patients and HJ denotes *H. pylori* obtained from gastric juice of infected patients. + denotes susceptibility and – denotes resistance.

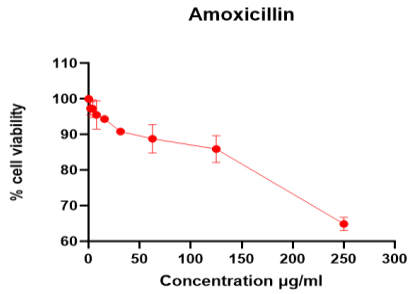
Table 2: Antibiotic drug concentrations recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) for antimicrobial susceptibility test.

Clarithromycin	0.25 μg/ml	0.5 μg/ml	1 μg/ml
Metronidazole	4 μg/ml	8 μg/ml	16 μg/ml
Amoxicillin	0.0625 μg/ml	0.125 μg/ml	0.25 μg/ml

We did the bacterial characterisation of the *H. pylori* strains in comparison to a reference lab strain. In this study, *H. pylori* strains were isolated from gastric biopsy and juice samples, including two triple antibiotic-resistant strains, HB1 and HB5, along with a reference strain, I10. Gram staining confirmed the isolates were Gram-negative.

Genomic DNA isolation showed similar banding patterns across all strains, indicating genomic similarity. PCR using *H. pylori*-specific 16S rRNA primers confirmed the identity of the isolates, with clear amplification bands observed on agarose gel electrophoresis. These results validate that HB1 and HB5 are *H. pylori*. Antibiotic susceptibility was done using MIC via Disc-diffusion and Broth-dilution method. The susceptibility was assessed using the following concentrations of antibiotics: Amoxicillin (0, 0.0625, 0.125, and 0.25 ug/ml), Metronidazole (0, 4, 8, and 16 ug/ml), and Clarithromycin (0, 0.25, 0.5, and 1 ug/ml). Resistance is indicated by +, and susceptibility by -. Susceptible for two higher concentrations, --- indicates susceptibility for all concentrations, and +++ indicates resistance for all three concentrations. The experiment was run in duplicate and twice.

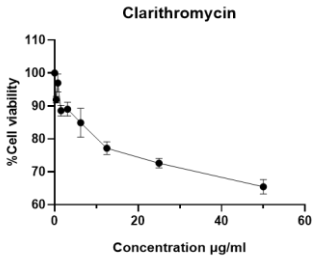
5.2 Cell Cytotoxicity assay of antibiotic Drugs on AGS cells



MTT of Amoxicillin

Control: Media
IC50: 512.8 µg/ml
IC10: 31.25 µg/ml

(a)



MTT of Clarithromycin

Control: DMSO
IC50: 66.73 µg/ml
IC10: 1.5 µg/ml

(b)

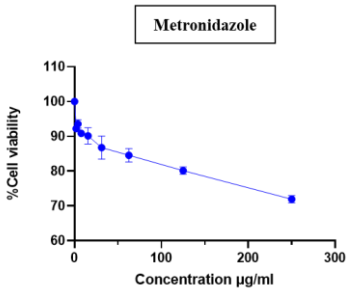


Fig. 10: MTT of Metronidazole

Control: Media
IC50: 377 µg/ml
IC10: 15.62 µg/ml

(c)

Figure 8. MTT assay to determine the toxicity of the antibiotic drugs on AGS cells.

(a), (b) and (c) respectively shows the % cell viability of AGS cells at different concentrations of the Amoxicillin, Clarithromycin and metronidazole.

To check the optimal dose of the drugs to be given for treatment after *H. pylori* infection MTT assay of these drugs was performed on AGS cells. IC10 values of these drugs were obtained as 31.24 µg/ml, 1.4 µg/ml and 15.62 µg/ml for Amoxicillin [Figure 8(a)], Clarithromycin [Figure 8(b)] and Metronidazole [Figure 8(c)] respectively. The IC20 values of these drugs were further used for checker-board analysis**.**

5.3 Checkerboard analysis

Checkerboard analysis is a widely used method to evaluate the interaction between two drugs, particularly to determine whether their combined effect is synergistic, additive, or antagonistic. In this assay, varying concentrations of two drugs are combined in a matrix (checkerboard) format across a microtiter plate, allowing for the assessment of their effects on cell viability or bacterial growth at different dose combinations. In this study, we are employing checkerboard analysis to evaluate the synergy between drug combinations conventionally used for *H. pylori* treatment. These combinations will be administered to AGS gastric epithelial cells following *H. pylori* infection. This approach will help in optimizing the effective dose range for these treatments, ensuring maximum efficacy while minimizing cytotoxicity to host cells.

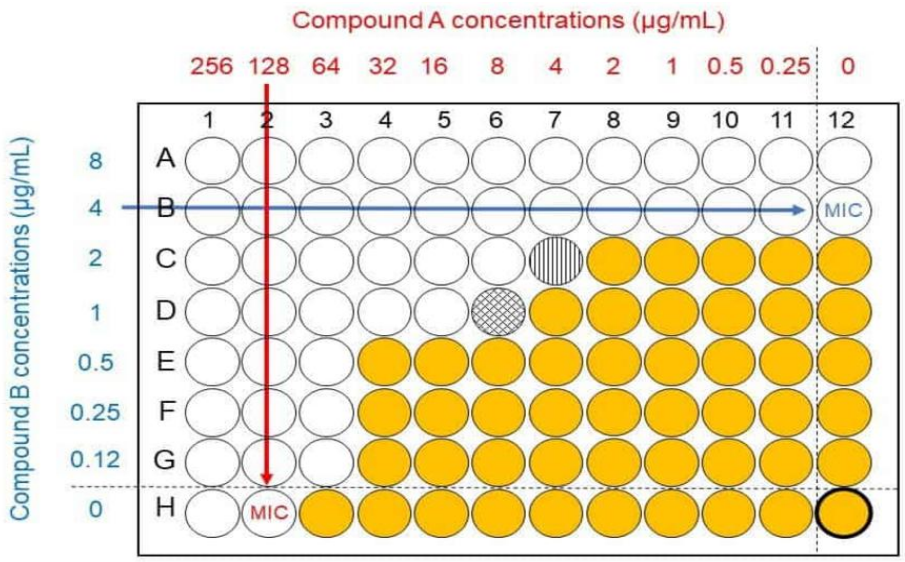
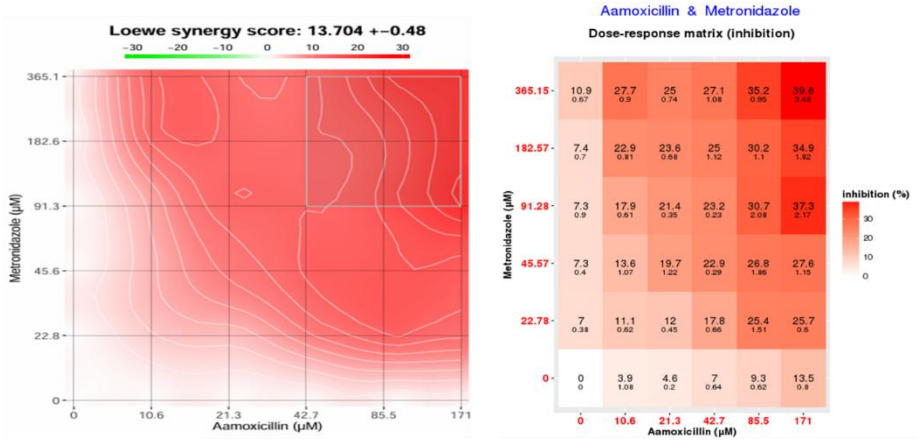


Figure 9. Checker board for synergy determination between drugs

The primary goal was to identify a combination dosage that maintains cellular viability while exerting therapeutic effects, particularly for use in host cell-based infection models. Starting with the IC20 values of two selected drugs, serial dilutions were performed along orthogonal axes in a 96-well format. This approach allowed for systematic evaluation of multiple concentration combinations. Cell viability at each drug combination was assessed using the MTT assay, which provided quantitative insights into cytotoxicity across the treatment matrix.

5.3.1 Checker board analysis for Metronidazole and Amoxicillin

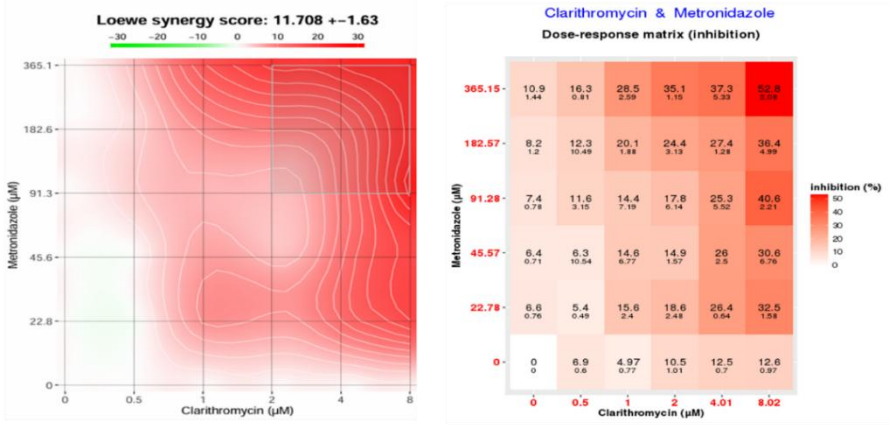


(a) Bliss-Loewe synergy heatmap for Amoxicillin and Metronidazole

(b) Dose response matrix for Amoxicillin and Metronidazole

Figure 10. Checker board analysis of Metronidazole and Amoxicillin. (a) Bliss-Loewe heat map and (b) dose response matrix showing synergy between Amoxicillin and Metronidazole at different concentrations.

5.3.2 Checker board analysis for Metronidazole and Clarithromycin

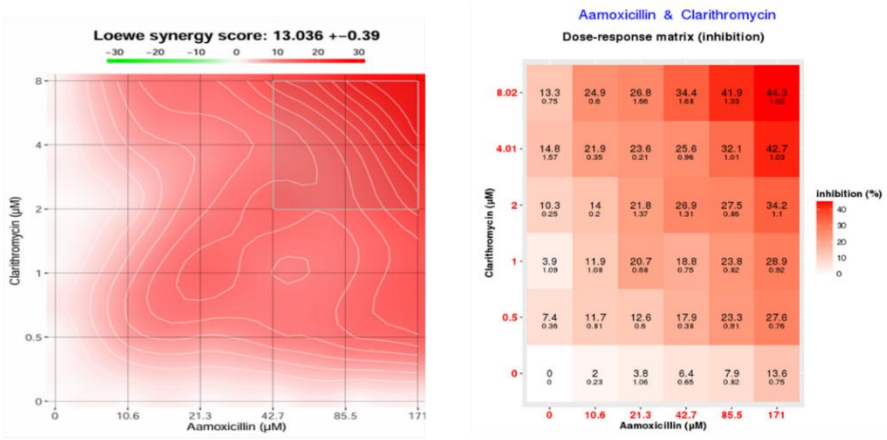


(a) Bliss-Loewe synergy heatmap for Clarithromycin and Metronidazole

(b) Dose response matrix for Clarithromycin and Metronidazole

Figure 11.Checker board analysis for Metronidazole and Clarithromycin. (a) Bliss-Loewe heat map and (b) dose response matrix showing synergy between Clarithromycin and Metronidazole at different concentrations.

5.3.3 Checker board analysis for Amoxicillin and Clarithromycin



(a) Bliss-Loewe synergy heatmap for Clarithromycin and Amoxicillin

(b) Dose response matrix for Clarithromycin and Amoxicillin

Figure 12. Checker board analysis for Amoxicillin and Clarithromycin. (a) Bliss-Loewe heat map and (b) dose response matrix showing synergy between Amoxicillin and Clarithromycin at different concentrations**.**

The obtained drug concentrations in combination for treatment after H. pylori infection were obtained as follow:

Table 3: The dose of the drug combinations obtained after checker board analysis

S. No.	Drug 1	Drug 2	Concentration of drug 1	Concentration of drug 2
1.	Metronidazole	Clarithromycin	33.62 µM	1.04 µM
2.	Clarithromycin	Amoxicillin	0.5 µM	10.6 µM
3.	Amoxicillin	Metronidazole	22.78 µM	10.6 µM

The dose-response matrix shows that higher inhibition percentages are achieved when both drugs are combined at elevated concentrations, suggesting potential synergy. Notably, the highest inhibition (50.1%) is observed at the combination of 8.02 µM clarithromycin and 365.15 µM

metronidazole (Figure 10). At lower concentrations, the inhibition values are significantly reduced, highlighting the concentration dependency of the observed effects. The data may indicate that the combined treatment of clarithromycin and metronidazole is more effective than either drug alone (Figure 11).

From the matrix, the drug combination that preserved at least 90% cell viability (i.e., approximating IC10 for each drug) was chosen for further use. This threshold was selected to ensure minimal host cell toxicity while still applying pharmacologically relevant drug pressure. The selected concentrations thus balance therapeutic relevance with biological tolerability, providing a robust foundation for downstream *in vitro* infection studies involving *H. pylori*. These findings underscore the utility of the checkerboard method in refining drug dosages for combinatorial therapies, especially in cell-based infection models where host cell health is critical.

5.4 ROS estimation in AGS cells due to *H. pylori* infection and antibiotic treatment

2′,7′-Dichlorofluorescin diacetate (DCFDA) staining is a widely used fluorescence-based assay to detect intracellular reactive oxygen species (ROS), which are key indicators of oxidative stress. The principle of the assay lies in the cell-permeable, non-fluorescent DCFDA molecule that, once inside the cell, is deacetylated by cellular esterases to form 2′,7′-dichlorodihydrofluorescein (DCFH). This compound is then oxidized by ROS to yield 2′,7′-dichlorofluorescein (DCF), a highly fluorescent molecule measurable by flow cytometry or fluorescence microscopy. In the context of *H. pylori* infection, DCFDA staining helps estimate the extent of ROS generation as a response to bacterial-induced host cellular stress. This assay can be further used to compare ROS levels in cells infected with *H. pylori* alone, treated with antibiotics (such as amoxicillin, metronidazole, or clarithromycin) alone, or exposed to a combination of infection and drug treatment. Such comparative analysis provides critical insights into whether drug treatment alleviates or exacerbates ROS-mediated damage. The significance of DCFDA staining lies in its ability to reveal early oxidative stress events, which are often precursors to inflammation, apoptosis, or carcinogenesis. Therefore, quantifying ROS levels using this method is instrumental in understanding host-pathogen interactions, evaluating drug efficacy, and potentially identifying combination therapies that modulate oxidative stress in favour of the host response.

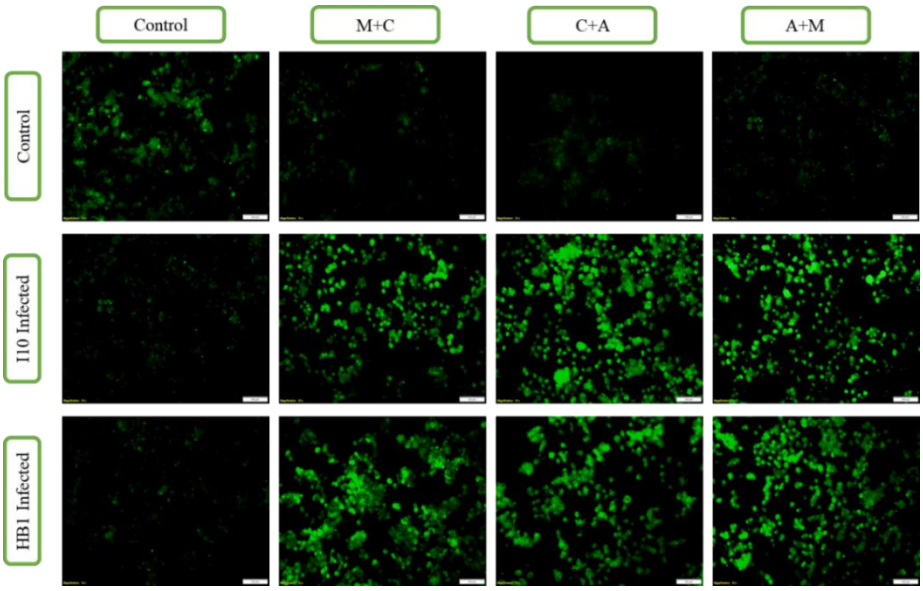


Figure 13. Cellular ROS production due to *H. pylori* infection and Drug treatment estimated via h2-DCFDA.

The above images (Figure13) show ROS induction in AGS cells in different conditions. Controls just having AGS cells, Drug controls consisting AGS cells treated with drug combinations, AGS cells infected with I10 and HB1, and AGS cells infected with I10 and HB1 treated with antibiotics in combination with Metronidazole and Clarithromycin (M +C), Clarithromycin and Amoxicillin (C+A) and Amoxicillin and Metronidazole (A+M).

It can be observed (Figure 13) that there is more ROS production in the AGS cells infected with *H. pylori* and then treated with drug combinations compared to the control (AGS), alone *H. pylori* infection or AGS cells treated with the drug combinations. This signifies that when the cells are infected with *H. pylori* and treated with the drug combination the ROS induction is significantly increased. Also, on comparison of AMR strain with reference strain it has been found that ROS induction was found to be more in AMR strain. This reflects the

oxidative stress burden imposed by both persistent infection and drug-induced stress.

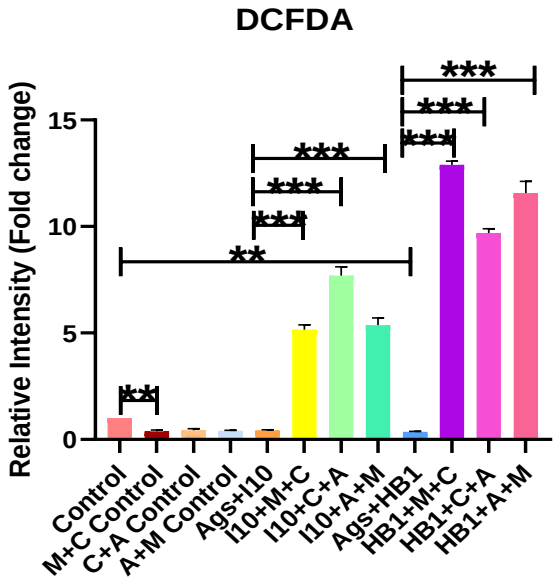


Figure 14. Quantitative analysis of relative fluorescence intensity.

The ROS estimation by DCFDA staining showed significantly increased ROS production in the treated group of both reference strains I10, HB1 and then in the drug control groups. In infected groups, the ROS is elevated more than in control, and in the treated group the ROS is more than in the infected group. Also, the resistant strain upon infection shows more ROS production compared to the reference strain (Figure 14).

5.5 Transcript level analysis of autophagy, antioxidant and cancer genes in AGS cells due to *H. pylori* infection and antibiotic treatment

Autophagy is a tightly regulated cellular process critical for maintaining homeostasis, particularly under stress conditions such as pathogen infection. Key genes involved in autophagy include *Beclin 1*, *SQSTM1/p62*, *ATG5*, *ATG7*, *LC3A*, and *LC3B*, each playing a distinct role in the initiation and progression of autophagosome formation and cargo degradation. *Beclin 1* is crucial for autophagosome nucleation, while *ATG5* and *ATG7* are essential for the elongation and maturation of the autophagosome. *LC3A* and *LC3B* are involved in autophagosome membrane formation and are often used as markers of autophagic activity. *SQSTM1/p62* acts as an autophagy adaptor protein, linking ubiquitinated cargo to the autophagic machinery. During *H. pylori* infection, these genes exhibit dynamic regulation, with evidence suggesting that *H. pylori* manipulate autophagy to favour its intracellular survival, often by impairing autophagic flux or altering LC3 processing. Antibiotic treatment following *H. pylori* infection may restore normal autophagic activity by clearing the pathogen, thereby reducing autophagy suppression or overactivation. Similarly, oxidative stress is another hallmark of *H. pylori* infection, often counteracted by host antioxidant defence involving genes such as *Catalase*, *SOD1*, and *SOD2*. These enzymes mitigate reactive oxygen species (ROS) accumulation, protecting cells from oxidative damage. Persistent infection can dysregulate these antioxidant pathways, compounding cellular stress and damage. Post-eradication antibiotic therapy may normalize the expression and activity of antioxidant genes, alleviating oxidative stress and restoring cellular redox balance. Together,

modulating autophagy and oxidative stress-related genes highlights a potential therapeutic window following *H. pylori* infection and underscores the importance of post-antibiotic monitoring for cellular homeostasis restoration.

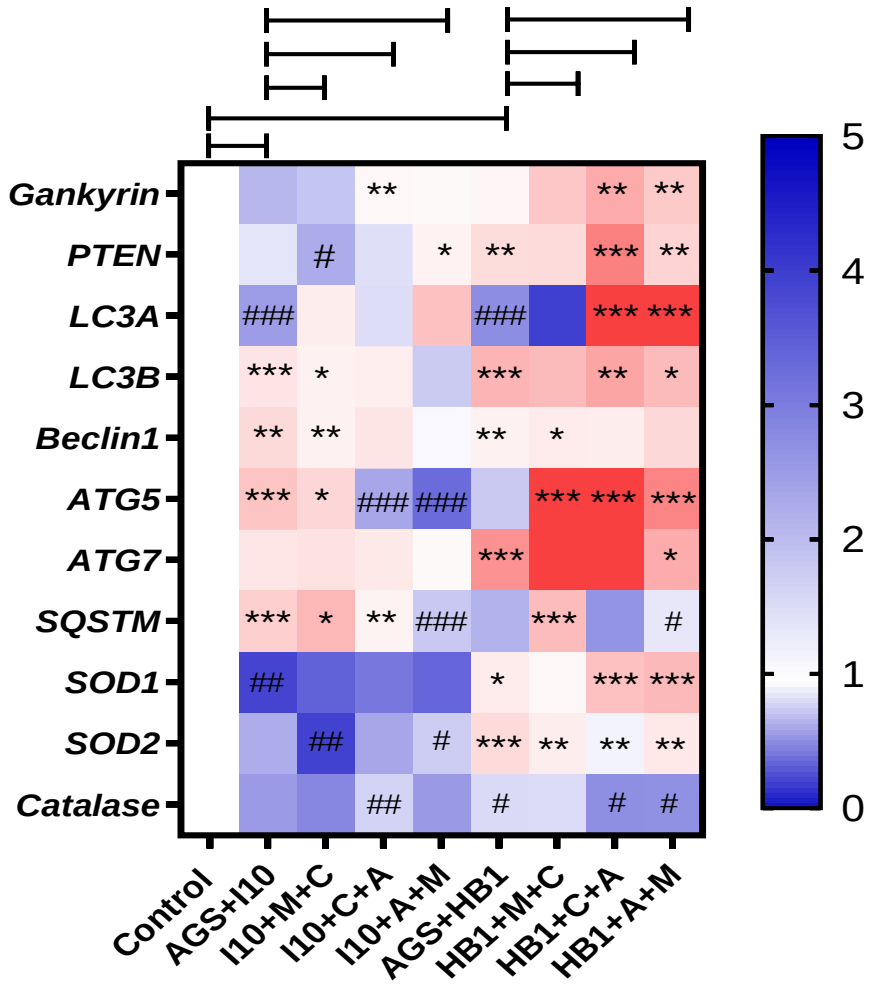


Figure 15. Relative transcript expression of post-treated model.

Genes are oncogene (Gankyrin and Aurora kinase) and oxidative stress (SOD1, SOD2 and Catalase), autophagic (LC3A, LC3B, Beclin 1, ATG5, ATG 7 and SQSTM).

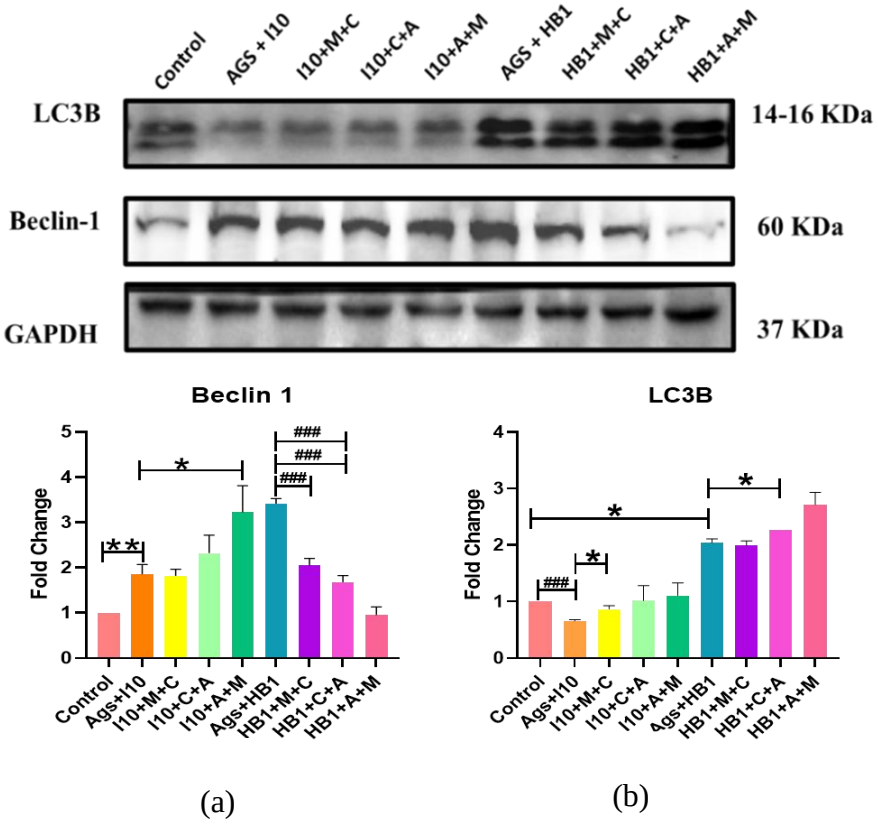
To monitor the effect of antibiotics along with infection of AMR *H. pylori* the AGS cells first infected with and then treated with different combinations of antibiotics were analysed at transcript level. Gankyrin,

PTEN, LC3A, ATG5, SOD1 and SOD2 were found to be significantly upregulated in HB1 treated group whereas the same except Gankyrin and PTEN were found to be significantly down regulated in I10 treated group (Figure 15). Catalase was found to be significantly down regulated in all both groups.

5.6 Protein level analysis of autophagy markers in AGS cells due to *H. pylori* infection and antibiotic treatment

Autophagy, apoptosis, and oxidative stress are closely interconnected cellular pathways that are significantly impacted during *Helicobacter pylori* infection. Core autophagy genes such as *Beclin 1*, *ATG5*, *ATG7*, *LC3A*, *LC3B*, and *SQSTM1/p62* are essential for the initiation, elongation, and maturation of autophagosomes, which help eliminate damaged organelles and intracellular pathogens. *H. pylori* can manipulate these genes to alter autophagic flux, often promoting its own survival by impairing effective autophagic degradation. Antibiotic treatment can potentially reverse this disruption, making the assessment of these genes crucial in infection models to understand the restoration of autophagy post-eradication. Simultaneously, *H. pylori* infection increases the production of reactive oxygen species (ROS), making antioxidant genes such as *catalase*, *SOD1*, and *SOD2* critical in mitigating oxidative stress. Dysregulation of these genes exacerbates oxidative damage and may trigger apoptotic signaling. Elevated ROS levels can activate both intrinsic and extrinsic apoptotic pathways, making it essential to analyse apoptosis-related genes like *Caspase 8*, *Caspase 9*, *Caspase 3*, *PARP*, and *Survivin*. *Caspase 8* and *Caspase 9* initiate apoptosis through death receptor and mitochondrial pathways, respectively, while *Caspase 3* executes the apoptotic process. *PARP* cleavage indicates DNA damage and active apoptosis, whereas *Survivin*

functions as an anti-apoptotic protein that may be upregulated in response to stress. Investigating the expression and activation of these genes in experimental models provides insight into how *H. pylori*-induced ROS influences the balance between cell survival and death, and how antibiotic therapy may modulate these pathways to restore cellular homeostasis.



(a) (b)

Figure 16. Protein level alterations in autophagic markers in antibiotic-treated *H. pylori* infected AGS cells.

(a) Western blot of Beclin 1 and LC3B, (B) and (c) relative quantification of western blots. *p*-values of < 0.05, < 0.01 and < 0.001 were represented with *, ** and *** respectively for significant upregulation and #, ## and ### for significant down regulation.

To check the alterations in autophagic markers at protein level due to AMR *H. pylori* infection and drug treatment, autophagic markers such as Beclin 1 and LC3B were analyzed via western blotting. In this Beclin

1 was found to be significantly downregulated in HB1 treated group compared to HB1 while upregulation was observed in I10 and I10 treated group [Figure 16 (a)]. LC3B was found to significantly upregulated in one of the HB1 treated group and in HB1 compared to the reference strain [Figure 16 (b)].

5.7 Protein level analysis of cancer markers in AGS cells due to *H. pylori* infection and antibiotic treatment

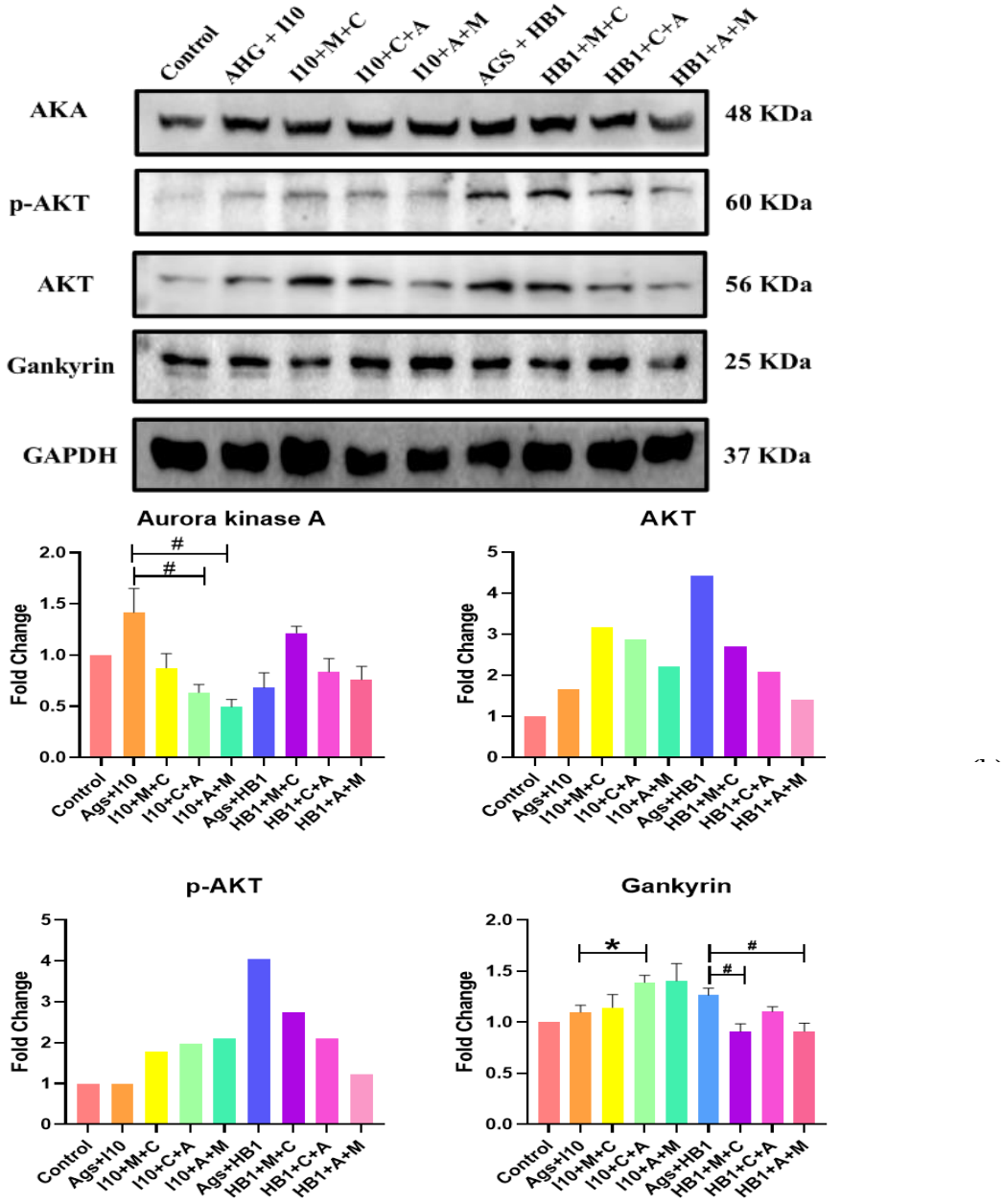


Figure 17. Protein level alterations in cancer markers in antibiotic-treated *H. pylori* infected AGS cells.

(a) Western blots for cancer genes and (b) relative quantification of western blots. *p*-values of < 0.05, < 0.01 and < 0.001 were represented

with *, ** and *** respectively for significant upregulation and #, ## and ### for significant down regulation.

To check the alterations in cancer markers at protein level due to AMR *H. pylori* infection and drug treatment, cancer markers such as Gankyrin, PTEN, AKT and p-AKT were analyzed via western blotting. In this Gankyrin was found to be significantly downregulated in HB1 treated group compared to HB1 while upregulation was observed in I10 and I10 treated group (Figure 17). Aurora kinase was found to significantly upregulated in I10 treated group.

5.8 Protein level analysis of apoptotic markers in AGS cells due to *H. pylori* infection and antibiotic treatment

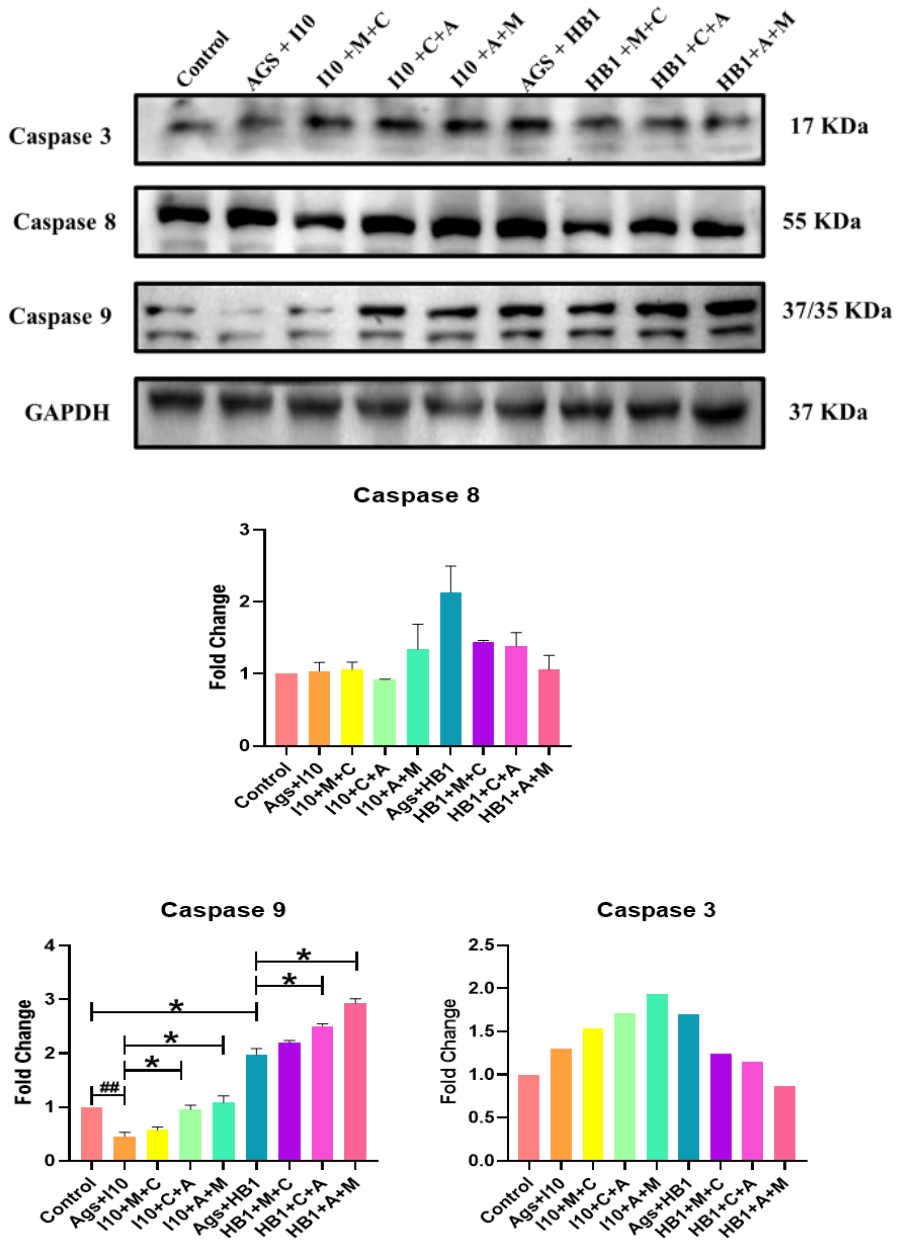


Figure 18. Protein level alterations in apoptotic markers in antibiotic-treated *H. pylori* infected AGS cells.

(a) Western blots for cancer genes and (b) relative quantification of western blots. *p*-values of < 0.05, < 0.01 and < 0.001 were represented with *, ** and *** respectively for significant upregulation and #, ## and ### for significant down regulation.

To check the alterations in apoptotic markers at protein level due to AMR *H. pylori* infection and drug treatment, apoptotic markers such as Caspase 8, 9 and 3 were analyzed via western blotting. In this Caspase 9 was found to be significantly upregulated in HB1 treated group compared to HB1 while upregulation was observed in I10 treated group and down regulated in I10 alone (Figure 18). Caspase 8 was found to downregulated in HB1 treated group compared to HB1.

5.9 Protein level analysis of apoptotic markers in AGS cells due to *H. pylori* infection and antibiotic treatment

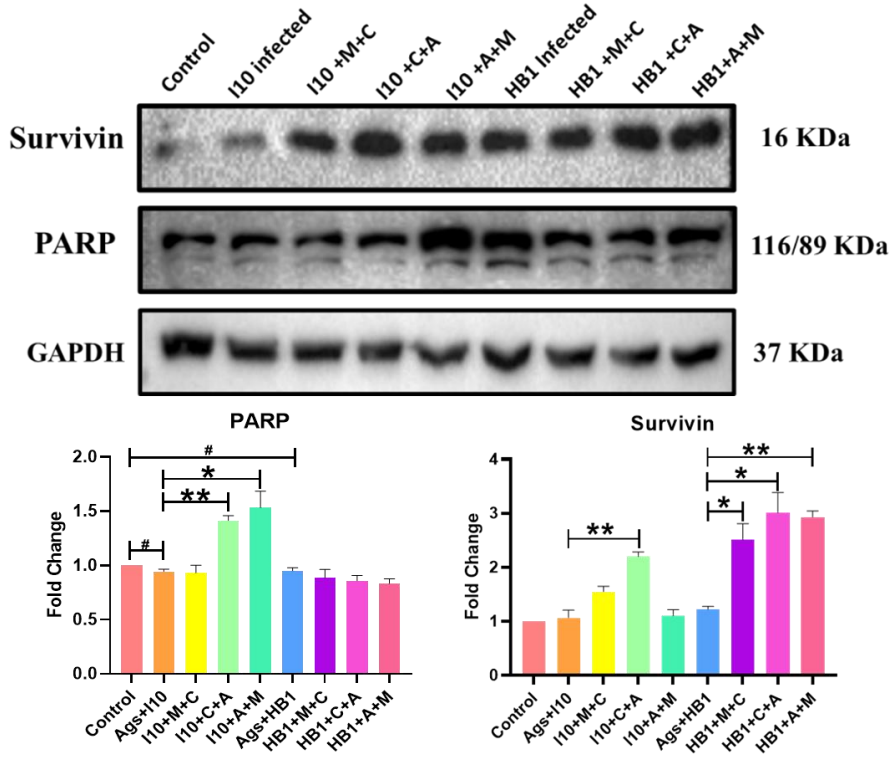


Figure 19. Protein level alterations in apoptotic markers in antibiotic-treated H. pylori infected AGS cells.

(a) Western blots for cancer genes and (b) relative quantification of western blots *p*-values of < 0.05, < 0.01 and < 0.001 were represented with *, ** and *** respectively for significant upregulation and #, ## and ### for significant down regulation.

To check the alterations in apoptotic markers at protein level due to AMR *H. pylori* infection and drug treatment, apoptotic markers such as PARP and Survivin were analyzed via western blotting. In this PARP was found to be significantly upregulated in I10 treated group compared to HB1 while down regulated in I10 alone. Survivin was found to

significantly upregulated in HB1 treated group compared to HB1 and same in I10 treated with clarithromycin and amoxicillin (Figure 19).

5.10 Discussion

This study provides novel insights into how host gastric epithelial cells respond to antibiotic therapy in the context of *H. pylori* infection, particularly when challenged with a triple drug-resistant strain. The work is significant in addressing the dual challenge of treatment failure and potential pro-carcinogenic consequences resulting from antibiotic-resistant *H. pylori* infections. The objectives focused on deciphering host cellular responses to antibiotics under resistant strain infection and exploring how ROS-induced signalling influences apoptosis and autophagy.

Initial bacterial characterization established HB1 as a clinically relevant triple-drug resistant strain, in contrast to the reference I10 strain. Antibiotic susceptibility profiling and MTT-based cytotoxicity assays enabled careful dose optimization, followed by checkerboard analysis that identified synergistic combinations mimicking conventional *H. pylori* eradication regimens. These combinations were further used in the in vitro infection model using AGS cells to probe host cellular changes at both transcript and protein levels.

ROS estimation through DCFDA staining revealed that infection with *H. pylori*, especially the HB1 strain, substantially elevated intracellular ROS levels. Treatment with antibiotics further augmented ROS accumulation, more markedly in the HB1-infected group. This reflects the oxidative stress burden imposed by both persistent infection and drug-induced stress. SOD1 and SOD2 upregulation in HB1-treated cells highlights the cellular attempt to counteract elevated ROS levels, whereas catalase downregulation across groups suggests compromised

hydrogen peroxide detoxification, exacerbating oxidative damage.

Transcriptional profiling revealed differential regulation of autophagy and apoptotic markers between the HB1 and I10 infection models. Notably, LC3A, LC3B, and ATG5 were upregulated in HB1-infected and treated cells (Figure 15), indicating enhanced autophagosome formation. However, downregulation of Beclin1—critical for canonical autophagy initiation—suggests a shift towards non-canonical autophagic pathways or incomplete autophagy. This dysregulation may facilitate pathogen survival and persistence, while fostering a pro-tumorigenic environment due to accumulation of damaged organelles and macromolecules.

Western blot analysis confirmed these transcriptional trends. LC3B protein levels were elevated in HB1-infected cells, while Beclin1 was suppressed, supporting the notion of dysfunctional or hijacked autophagy. Gankyrin and PTEN, which regulate cell cycle and tumor suppression, displayed differential regulation: Gankyrin was notably downregulated in HB1-treated groups, potentially relieving inhibition of p53 but also pointing to disrupted oncogenic signaling pathways. Upregulation of PTEN in these groups could be a compensatory tumor-suppressive response.

Apoptotic markers provided further clarity on the host cell fate. The HB1-treated group showed increased Caspase 9 (intrinsic apoptosis) and reduced Caspase 8 (extrinsic apoptosis), indicating a selective shift towards mitochondrial apoptosis. PARP, a DNA repair enzyme cleaved during apoptosis, was downregulated in HB1 groups, suggesting impaired DNA repair and heightened genomic instability. Conversely, Survivin—a known inhibitor of apoptosis—was upregulated, especially in HB1-treated cells, implying survival signalling despite accumulated cellular damage.

These findings collectively indicate that in the presence of drug-resistant

H. pylori, conventional antibiotic treatments not only fail to eradicate infection effectively but may also induce cellular environments conducive to cancer progression. The increased ROS burden, coupled with incomplete autophagy and altered apoptotic balance, favours survival of DNA-damaged, apoptosis-resistant clones. This aligns with growing literature suggesting that chronic infection and unresolved oxidative stress are drivers of gastric carcinogenesis.

In contrast, cells infected with the reference strain I10 and treated with the same antibiotics demonstrated a more regulated stress response, with reduced ROS levels, relatively preserved Beclin1 expression, and activation of both apoptosis pathways. These differences underscore the impact of antimicrobial resistance not just on therapeutic failure but also on the trajectory of host-pathogen interaction and host cell fate.

Chapter 6: Conclusion and scope for future work

6.1 Conclusion

This study highlights the critical impact of antibiotic resistance in *Helicobacter pylori* on host cell responses during infection and treatment. Using a comparative approach between a reference strain (I10) and a clinically isolated triple-drug resistant strain (HB1), we demonstrate that resistant *H. pylori* strains not only evade antibiotic action but also provoke heightened oxidative stress and disrupt normal autophagic and apoptotic regulation in gastric epithelial cells. The observed increase in ROS, along with dysregulated expression of autophagy markers (LC3A/B, ATG5, Beclin1) and apoptosis-related proteins (Caspases, PARP, Survivin), suggests a maladaptive host response favouring cell survival under stress, potentially contributing to genomic instability and carcinogenesis. These findings emphasize that treatment failure in resistant infections may extend beyond microbial persistence, posing a risk for long-term host cell damage. The results advocate for integrating host-targeted strategies—such as antioxidant supplementation or autophagy modulators—into current therapeutic regimens to mitigate adverse host outcomes. Ultimately, this work provides mechanistic insight into how antibiotic-resistant *H. pylori* strains influence host cell fate and highlights the need for more comprehensive treatment approaches in the era of rising antimicrobial resistance.

6.2 Scope for future work

The current findings open several promising avenues for future research on multidrug-resistant *H. pylori* (HB1) and its impact on host gastric epithelial cell dynamics. One key direction involves dissecting the autophagy-apoptosis crosstalk, particularly the suppression of Beclin1 alongside upregulation of LC3B and ATG5, which may indicate HB1's exploitation of non-canonical autophagy for intracellular persistence. Further, the imbalance between SOD1/SOD2 and Catalase highlights a pro-oxidative environment that may promote genomic instability and resistance to apoptosis. Investigating the caspase-9 versus caspase-8 response could clarify how HB1 manipulates intrinsic apoptosis to evade immune surveillance. Therapeutically, combining antibiotics with autophagy inhibitors (e.g., chloroquine) or ROS scavengers (e.g., NAC) offers a potential strategy to counteract HB1-induced host damage. Clinically, validating markers like LC3B/Beclin1 ratios and Caspase-9 activation may help identify patients at risk of progression to gastric cancer. Additionally, incorporating genomic resistance profiling and efflux pump inhibitors may improve treatment efficacy. Future studies using in vivo models, epigenetic mapping, and microbiome analysis will further elucidate HB1's oncogenic potential. On a broader scale, global health priorities should include the development of novel antimicrobials, phage therapy, and vaccines targeting AMR *H. pylori*-specific virulence factors to curb the rising threat of drug-resistant *H. pylori* infections.

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