7. Summary

1. Introduction

*Helicobacter pylori* is a gram-negative bacillus, curved, microaerophilic and motile organism with multiple polar flagella (Mendall, 1995). *H. pylori* is a fragile bacteria that has found an ideal home in the protective mucous layer of the stomach. These bacteria have long threads protruding from them that attach to the underlying stomach cells. The mucous layer that protects the stomach cells from acid also protects from *H. pylori*. Infection-fighting white blood cells move into the area, and the body even develops *H. pylori* antibodies in the blood (Megraud, 1995). Ulceration is caused by an imbalance between damaging factors (gastric acid and peptic enzymes) and protective factors (gastric mucus secretion, local secretion of alkali). *H. pylori* appears to cause direct damage to the epithelial cells as well as produce enzymes that break down the surface mucus. This allows access of acid to mucosal tissues leading to the formation of an acute ulcer. If the process proceeds unchecked, then the ulcer can erode through the full thickness of the stomach or duodenal wall, leading to perforation and escape of gut contents into the peritoneal cavity. Generally, the destructive process is arrested by an acute inflammatory response. Tissue repair then begins with the formation of granulation tissue; repair may be effective if conditions are favorable, leaving a fibrous scar. However, if tissue destruction continues, the concurrent organization and repair result in chronic inflammation. A chronic peptic ulcer reflects an imbalance between tissue destruction and tissue repair (Logan and Hiscal, 1996). Colonization of *H. pylori* occurs by producing urease and gastric acid inhibitory protein. Metaplasia, which is present in more than 90% of patients of duodenal ulcer, occurs by replacing the columnar cells, normally covering the duodenal villi, by gastric type epithelium. Adhesion of *H. pylori* to the gastric epithelium occurs by tissue
specific proteins. Colonization of the duodenal bulb by *H. pylori* leads to mucosal inflammation which makes it vulnerable to attack by acid or pepsin or bile resulting into ulceration. However, factors leading to gastric metaplasia in the duodenal bulb are not known (Noach *et al*., 1993).

Treatment of *H. pylori* infections with a combination of proton pump inhibitor (PPI), pantoprazole plus an antibiotic has shown increased efficacy. Such combination therapy ensured increased bacterial killing and prevention of the emergence of drugs resistance (Dore *et al*., 1999; Louw *et al*., 1998). However, improper combination and selection of antibiotic for the treatment often results in failure of therapy. The best possible solution for eradication of *H. pylori* to use a Pantoprazole and Antibiotic with best efficacy. There does not exist a fixed dose combination of PPI and suitable antibiotic so far to offer best possible treatment of infection caused by *H. pylori*.

The present study is therefore, aimed at evaluating efficacy of potent fixed dose combination of Pantoprazole with various Antibiotics. The best antibiotic in combination with pantoprazole can be considered for clinical evaluation to establish efficacy.

2. Review of Literature

Gastrointestinal disturbances which include gastritis, duodenitis, gastroduodenal ulcers and other inflammatory conditions are a world wide problem among all age and sex groups. Several causative factors have been identified contributing to the development of these complications. These include age, gender, hyperacidity, smoking habit, alcohol consumption and use of nonsteroidal anti-inflammatory drug. Besides these factors there is strong evidence that bacterial agent *Helicobacter pylori* can cause gastritis, gastric ulcer, duodenal ulcer and non-ulcer dyspepsia. Although this spiral shaped organisms in the gastric mucosa were first seen and reported by Bizzozero in
1893 (Bizzozero, et al., 1893) in the histological sections of the biopsies taken from the patients with gastroduodenal disturbances, it is only with the isolation of this organism by Warren and Marshall (1983) that the relationship with gastroduodenal pathology was established (Marshall, 1983). The organism was previously named as *Campylobacter pylori*, but later it was placed in a new genera and now referred to as *Helicobaccter pylori* (H. pylori), (Goodwin et al., 1989). The association between the presence of this spiral organism in antral mucosa and histological gastritis as well as peptic ulceration has now been well established both in adults (Graham 1989; Buck, 1990) and children (Cadranel et al., 1986; Drumm et al., 1987). The discovery of this organism act as a etiological factor of peptic ulcer disease have resolved the enigma which has puzzled physicians for many years. It has been postulated that long term gastritis caused by *H. pylori* sets the scene for the initiation of gastric cancer, a disease that is more common in countries with a very high prevalence of *H. pylori* infection (Correa et al., 1990).

In the recent years it has been postulated that *H. pylori* infection may have some role in development of gastric cancer. This hypothesis is supported by the fact that prolonged presence of the organism in progression from the relatively benign chronic gastric with neutrophil activity to atrophic gastritis, a condition in which inflammatory infiltration and lymphoid follicle aggregate and disrupts the mucosa to such a degree that ablation of function occurs. This tissue destruction results in intestinalization of gastric mucosa and achlorhydria followed by overgrowth of intestinal bacteria in the stomach (Correa and Ruiz, 1989). If other conditions are present for example consumption of certain dietary factors (e.g., salts) or reduction of protective antioxidant such a vitamin C, then the scenic is set for induction of cancer (Reeavarren-Arec et al., 1991). *H pylori* probably does not itself induce gastric cancer.
but may promote either the production of carcinogens or mutagenic events, e.g., the atrophic gastritis which has been known for many years to be an essential prerequisite in certain forms of gastric cancer (Correa, 1988). Should this hypothesis prove to be true, and at present all the epidemiological study support (Forman et al., 1991; Nomura et al., 1991; Parsonnet et al., 1991), then intervention strategies resulting in prevention of H. pylori infection could eliminate a disease with high rate of major morbidity/mortality in certain developing countries.

3. Materials and Methods

Three biopsy specimens of Helicobacter pylori groups (HP-B₁, HP-B₂, HP-B₃) isolated from gastric biopsy of the specimen of patient with gastric and peptic ulcer obtained from Post Graduate Institute of Medical Education and Research (PGIMR), Chandigarh and one Helicobacter pylori ATCC 43504 (HP-B₄) strain obtained from American Type Culture Collection through LGC Promochem India Private Limited Bangalore (India) were used for these experiments. All the chemicals used in present study were purchased from Merck, Sigma, and Himedia (India). The culture medium Mueller-Hinton broth (MHB) and Brain Heart Infusion (BHI), supplemented with 5% defibrinated sheep blood, purchased from Himedia were used according to manufacturer’s instructions for susceptibility, MIC, Time Kill Curve and resistant developments experiments. Clarithromycin, Amoxicillin, Metronidazole, Tetracycline, Cefotaxime, Cefazidime, Ceftriaxone, Piperacillin, Tazobactum, Ciprofloxacin, Streptomycin, Erythromycin, Gentamycin, Tobramycin, Amikacin, Vancomycin, and Nitilmycin were used for in vitro studies. All antibiotics and Pantoprazole were obtained as gift sample from manufacture, Venus Remedies Limited India. All above Helicobacter pylori groups were tested for response of effective antibiotics combinations with PPI, pantoprazole in vitro. The best combination found in vitro was
selected for in vivo studies.

The bacteria were identified by Rapid Urease Test, Gram's staining, Campylobacter Like Organism (CLO) Test and Helicobacter pylori IgG ELISA. H. pylori were tested for response of antibiotic combinations with pantoprazole in vitro. The best three combination found in vitro were selected and infection was induced in experimental animal i.e. mice. These combinations of antibiotics were tested for the treatment of ulcer induced in experimental animals. This study may be helpful in developing best Fixed Dose Combination (FDC) therapy for the treatment of ulcers caused by infection of H. pylori.

Best three combinations of antibiotics on basis of in vitro microbiological analysis were selected for the in vivo studies on mice Mus musculus. 360 mice used for the study, were divided into three groups. Each group had subgroups with non infected control, infected control and infected with treatment animals. Mice were maintained at 25°C ± 2°C and 12 hours light / 12 hours dark period in animal house. These animals were fed with mice feed and tap water. Each group, marked as A (treatment with the best combination), B (treatment with the second best combination), and C (treatment with the third best combination) were evaluated for bacteriological eradication by combination based on microbiological and histological studies. Best possible combination was suggested for clinical evaluation. The mice Mus musculus were infected with standard reference culture of ATCC 43504 (HP-B4) in all three combination groups. The establishment of infection was confirmed by histologically.

4. Results

Helicobacter pylori groups HP-B₁, HP-B₂, HP-B₃, HP-B₄ were taken for Rapid Urease Test, Gram's staining, Campylobacter Like Organism (CLO) Test and
Helicobacter pylori IgG ELISA. These samples were examined for the conformation of H. pylori by CLO test. All tests showed the positive results in all specimens of H. pylori groups. Growth curve of all groups (HP-B₁, HP-B₂, HP-B₃ and HP-B₄) were found to have normal pattern of growth in culture media.

Susceptibility, MICs, time kill curve and resistance development were found against at least 11 antibiotics (Clarithromycin, Amoxicillin, Metronidazole, Tetracycline, Cefotaxime, Ceftazidine, Ceftriaxone, Ciprofloxacin, Erythromycin, Gentamycin, and Tobramycin) in the combination of pantoprazole. Best three combination having maximum killing effect as per susceptibility, MICs, time kill curve and resistance development studies were found to be of Pantoprazole with Clarithromycin, Amoxicillin and Tetracycline on the basis of in vitro analysis.

The susceptibility, MIC, and resistance developments data from our study demonstrate that Pantoprazole-Antibiotics (Piperacillin, Tazobactum, Streptomycin, Amikacin, Nitlimicin, and Vancomycin), a fixed dose combination and antibiotics alone were not having bacterial inhibition properties and bactericidal activity after in vitro analysis in groups HP-B₁, HP-B₂, HP-B₃ and HP-B₄.

Histological Evaluation

Stomach

Control stomach showed normal histological structure normal muscularis mucosae, submucosa and mucosa in all antibiotics combination groups. The degradation of tissue was found on infection site of stomach. There was necrosis reported in circular layer of muscularis, submucosa and muscularis mucosae. At higher magnification internal metaplasia of the tissue was clearly seen with H. pylori infection. After treatment for 15 days with combination of A, B and C, tissue degradation and H. pylori infection was evident. Necrotic tissue at Submucosa signs, muscularis mucosae
and neutrophils were also marked. After treatment for 30 days with A, B and C still
signs of *H. pylori* infection was seen in antibiotics groups A, but not in B and C. The
treatment of 45 and 60 days started showing recovery of the histological attributes with
regeneration of mucosa.

**Small Intestine**

Control histopathological structure showed normal muscularis mucosae, Lamina
propria and villi. After infection of *H. pylori* minor impact of infection was seen.
Treatment with a combination (A, B and C) for 15 days showed almost normal
histological structure was marked with little degradation and *H. pylori* infection at
Lamina propria. Normal structure of small intestine was seen at 30, 45, and 60 days of
treatment with A, B, and C.

**Rectum**

Control rectum showed normal structure of vein, muscularis mucosae, lamina
propria, submucosa and crypt of lieberkühn. After infection and treatments of 15, 30,
45 and 60 days with combination of A, B, and C, showed almost normal structure
lamina propria, crypt of lieberkühn, muscularis mucosae, vein, and submucosa were
marked as normal.

**5. Discussion**

It has been reported that Pantoprazole has superior complementary treatment to
diagnostic therapy in high-risk bleeding ulcers (Hsu *et al.*, 2004). Pantoprazole
appeared to be superior to the H₂ receptor antagonists in the treatment of ulcer (Huggins
*et al.*, 2003). Pantoprazole therapy combined with anti-infective agents like
clarithromycin, tetracycline, amoxicillin, Metronidazole etc. can effectively eradicate
*H. pylori* in gastric infection. The Pantoprazole also has low therapeutic potential and
bactericidal activity at neutral pH, there by requiring short incubation periods against
*H. pylori* (Diez Enciso, 1999).

Clarithromycin and Erythromycin are a macrolid antibiotic, acid stable, phagocytes and actively transported to the site of infection antibiotic. Clarithromycin and Erythromycin prevents bacteria from growing by interfering with their protein synthesis. It binds to the subunit 50S of the bacterial ribosome and thus inhibits the translation of peptides (Graham, 1993). These antibiotics are currently accepted as the most effective drug in *H. pylori* infection, with a 42% eradication rate (Graham, 1995). Moreover, the eradication rate increased when the antibiotics were used in conjunction with Pantoprazole (Daw *et al*., 1991; Graham, 1993; Isabel Garcia-Arata *et al*., 1999).

Amoxicillin is a penicillin group antibiotic used for treatment of gram negative infection (Dore *et al*., 1999). Treatment of *H. pylori* infection usually involves a combination of this antibiotic and acid suppressors proton pump inhibitor protection (Noophun *et al*., 2004). Very high resistance rates to amoxicillin have been reported in some prevalence studies (Godoy *et al*., 2003 and Wu, *et al*., 2000).

Tetracycline is acid stable, readily absorbed antibiotic which is actively transported to the site of infection. Tetracycline prevents bacteria growth by interfering with their protein synthesis. It binds to the subunit 30S of the bacterial ribosome and thus inhibits the translation of peptides (Piolett *et al*., 2001). These antibiotics are currently accepted as the most effective drug in *H. pylori* infection, with a 76% eradication rate (Triber *et al*., 2002). Moreover, the eradication rate increased when combined with other antibiotics (Kim *et al*., 2001; Wu *et al*., 2000), including combinations of Pantoprazole (Wu, *et al*., 2000).

Metronidazole is one of the most successful drugs used in combination to eradicate *H. pylori*. Metronidazole resistance is strongly believed to be caused by *H. pylori* genetic drift and adaptation to environment (Tolia *et al*., 2000). Increased prescription
of metronidazole for parasitic infection among children and infection with resistant strain *H. pylori* are the other factors leading to high metronidazole resistance rate (Kim *et al.*, 2001). Moreover, the eradication rate increased with the use of antibiotics metronidazole and combinations including PPIs pantoprazole.

Ceftriaxone, Cefotaxime and Ceftazidime are broad spectrum, third generation parenteral cephalosporin effective against many Gram negative organisms. Serious infections of the respiratory tract and gastrointestinal tract infections (Angehm *et al.*, 1980; Barza, 1982). MICs of Ceftazidime and Cefotaxime for some Gram-negative strains isolated from clinical cases have been determined (Cars and Qgren, 1985). Ceftazidime is a compound that has potent bacterial activities against gram negative organism *H. pylori*, particularly clinically relevant pathogen (NAEJA Pharmaceutical Inc. Canada). Ceftriaxone inhibits the synthesis in the bacterial cell wall. Like other cephalosporins of the 'third generation', it has a very broad anti-bacterial spectrum and it is stable against most β-lactamases. Ceftriaxone owes its popularity mainly to its relatively long plasma half-life (Craig, 1980).

Ciprofloxacin is one of a second generation of fluorinated quinolones structurally related to nalidixic acid. The primary mechanism of action of ciprofloxacin is inhibition of bacterial DNA gyrase. It is a broad spectrum antibacterial drug to which most Gram-negative bacteria are highly susceptible *in vitro* (Egerbacher *et al.*, 2000). Ciprofloxacin is a compound that has potent bacterial activities against gram negative organism *H. pylori*, particularly clinically relevant pathogen (Boyanova *et al.*, 2000; NAEJA Pharmaceutical Inc. Canada).

Gentamycin and Tobramycin are broad spectrum aminoglycoside antibiotic. Aminoglycosides work by binding to the bacterial 30S ribosomal subunit, causing misreading of t-RNA, leaving the bacterium unable to synthesize proteins vital to its
growth (Kaye et al., 1974; NCCLS, 1993). Aminoglycosides are useful primarily in infections involving anaerobic, Gram-negative bacteria. Aminoglycosides like Gentamycin "irreversibly" bind to specific 30S-subunit proteins and 16S rRNA. Specifically Gentamycin binds to four nucleotides of 16S rRNA and a single amino acid of protein S12 (Riff et al., 1971). Aminoglycosides are compound that have potent bacterial activities against gram negative organism *H. pylori*, particularly clinically relevant pathogen (NAEJA Pharmaceutical Inc. Canada).

The antibiotics used in common clinical practice to treat *H. pylori* infection, either alone or in combination are Clarithromycin, Amoxicillin, Tetracycline, Metronidazole, Cefotaxime, Ceftazidime, Ceftriaxone, Piperacillin, Tazobactum, Ciprofloxacin, Streptomycin, Erythromycin, Gentamycin, Tobramycin, Amikacin, Niltimicin, and Vancomycin. (Refer to the literature cited earlier.)

The susceptibility data from our study demonstrated that Pantoprazole-antibiotics, a fixed dose combination have higher zone diameter (significant reduction, P value is < 0.001 in all groups), and lower MIC value than individual antibiotics and Pantoprazole, suggesting higher bactericidal activity in Pantoprazole-antibiotics combination in isolated gastric biopsy and ATCC 43504 *H. pylori* groups (HP-B1, HP-B2, HP-B3, and HP-B4). This was confirmed by time-kill analysis, which demonstrated that Pantoprazole-antibiotic, a fixed dose combination has better bactericidal activity than Antibiotics and Pantoprazole alone, even at a concentration of 2 X the MIC after 12 hours. Indeed, in all organisms under study, Pantoprazole-antibiotic, demonstrated similar pattern of bactericidal activities when compared with alone. The resistance developments of our study demonstrated that the Pantoprazole-Tetracycline, Pantoprazole-Clarithromycin, and Pantoprazole-Amoxicillin have higher zone value than other combinations in well concentration 30μg, 20μg, and 10μg as well as higher
microbial efficacy as compared to other antibiotics alone or in combination in all \textit{H. pylori} groups, suggesting that chance of development of resistance is least in Pantoprazole-Tetracycline, Pantoprazole-Clarithromycin, and Pantoprazole-Amoxicillin.

Analysis of susceptibility of combination by cup plate method, MIC, time kill curve, and resistance developments against \textit{H. pylori in vitro} has not be regular feature in earlier studies (Goodwin et al., 1986). In the present study, the activity of most of the above mentioned antibiotics against \textit{H. pylori} exhibited a bimodal phenomenon, suggesting that clinical and ATCC groups of \textit{H. pylori} are either highly susceptible or moderately susceptible to the combination. Decrease in MIC value was maximum with Pantoprazole-Tetracycline combination. These might be because of synergistic affect of Pantoprazole and Tetracycline in combination proton pump inhibitor Pantoprazole with tetracycline given maximum bactericidal affect (Goodwin, \textit{et al.}, 1988; Mathai \textit{et al.}, 1990).

\textit{H. pylori} eradication regimen usually entails a proton pump inhibitor (Pantoprazole) in combination with one of the antimicrobial agents. However, various clinical trials and \textit{in vitro} studies have reported \textit{H. pylori} antimicrobial resistance all over the world that represents a serious public health challenge. \textit{H. pylori} antimicrobial resistance varies between different geographical regions. Therefore, health care awareness of the \textit{H. pylori} susceptibility to each of the commonly used antibiotics is necessary to be able to recommend the most effective therapy regimen. Patient noncompliance and the location of the bacterium which is beneath the gastric epithelium and out of reach of antibiotics are among the most common causes of treatment failure in existing therapy (Duck \textit{et al.}, 2004; Tolia \textit{et al.}, 2000). There are no reports of comparisons of different antibiotics including Cephalosporins, Penicillins,
microlid, Fluorinated quinolones, and Aminoglycosides with PPI pantoprazole for prospective therapy of *H. pylori* infection.

*In vitro* results demonstrate that the combination of Pantoprazole-Tetracycline (PT), Pantoprazole-Clarithromycin (PC), and Pantoprazole-Amoxicillin (PA) are more effective with high bactericidal activity against *H. pylori*. These combinations were taken for further *in vivo* studies.

There has been gradual recovery in treatment of infection induced in *Mus musculus*, with Pantoprazole-Tetracycline (A), Pantoprazole-Clarithromycin (B), Pantoprazole-Amoxicillin (C). After 60 days of treatment there was completely recovery in all groups. Best *in vivo* results was found in the case of combination of Pantoprazole-Tetracycline, as compared with Pantoprazole-Clarithromycin and Pantoprazole-Amoxicillin. The histological recovery in case of stomach ulcer was almost similar in all combination of antibiotics with Pantoprazole. In time kill curve analysis, there has been maximum reduction in the culture of *H. pylori* growth in the case of Pantoprazole-Tetracycline followed by Pantoprazole-Clarithromycin and Pantoprazole-Amoxicillin.

6. Concussion

The study clearly indicates that the combination of Pantoprazole-Tetracycline, Pantoprazole-Clarithromycin and Pantoprazole-Amoxicillin are effective in treatment of infections caused by *H. pylori* in laboratory condition. The *in vitro* results show that maximum efficacy of Pantoprazole-Tetracycline has been there for eradication of *H. pylori*. Pantoprazole mediated successful treatments of *H. pylori* infections were done in laboratory animals in combination with Tetracycline, Clarithromycin and Amoxicillin. The efficacy of Pantoprazole-Tetracycline combination was found to be more in comparison to other combinations. It may be concluded that any of these three
combinations can be used for the treatment of *H. pylori* infections in humans after successful clinical evaluation.