MATERIALS & METHODS
3. Materials and Methods

3.1. Bacterial strains

*Helicobacter pylori* strains used in the present study were isolated from gastric biopsies of the patients with gastric and peptic ulcer attending Post Graduate Institute of Medical Education and Research (PGIMR) Chandigarh. These samples were membered as HP-B₁, HP-B₂, and HP-B₃. One *H. pylori* strain (ATCC 43504) obtained from American type culture collection through LGC Promochen India Private Limited Bangalore (India) from USA, which was coded as HP-B₄ and used as the standard strain.

3.2. Bacterial identification

The bacterial identification was carried out by using standard microbiological procedures. These tests are as following:

3.2.1. Rapid Urease Test

*Helicobacter pylori* possesses high urease activity as part of its structure. This technique involves placing a biopsy specimen obtained at the time of endoscopy into a urea rich agar medium with a pH sensitive dye. The urease activity of *H. pylori* releases ammonia from urease and raises the pH of the medium which cause a change in color studies indicate that this techniques generally has a sensitivity and specificity of greater than 90% (El-Zimaity et al., 1995; Berry and Sagar, 2006).

The rapid urease test was performed for all isolates and standard strain HP-B₁, HP-B₂, HP-B₃ and HP-B₄.

3.2.2. Gram’s staining

For microscopy, a piece of biopsy tissue was crushed between two sterilized glass slides. Each preparation was air dried and heat fixed. The smears were overlayed with crystal violet for 30 seconds, followed by washing with distilled water. Then Gram’s
iodine solution was poured for one minute followed by decolourisation with pure alcohol for 30 seconds. The counter staining was done with dilute carbolfuchsin for 30 seconds. After washing with distilled water, smears were air dried and screened for *H. Pylori* under oil immersion lens of light microscope *H. Pylori* appears (Berry and Sagar, 2006).

3.2.3. Campylobacter Like Organism (CLO) Test

All specimens were inoculated into a medium containing urea and phenol red, a dye that turns pink in a pH of 6.0 when *H. pylori*, the campylobacter like organism metabolizes urea to ammonia by its urease activity. This test kit is commercially available and therefore quite in expensive only 1/2 hour is required for diagnosis of infection, and the test has shown 98% sensitivity 100% specificity. The qualities have made the CLO tests the invasive technique of choice for identifying *H. pylori* infection (Dickey et al., 1996).

3.2.4. *Helicobacter pylori* IgG ELISA

The *Helicobacter pylori* IgG Test Kit (GenWay Biotech, Inc.) was used in evaluating the serological status to *H. pylori* infection in patients from which bacteria were isolated symptoms.

3.2.4.1. Principle of the test

Purified *H. pylori* antigen is coated on the surface of micro wells. Diluted patients serum is added to the wells, and the *H. pylori* IgG specific antibody, if present, binds to the antigen. All unbound materials are washed away. Enzyme conjugate is added, which binds to the antibody-antigen complex. Excess enzyme conjugate is washed off and substrate and chromogen are added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of IgG specific antibody in the sample. The results are read by a microwell
reader at 450 nm compared in a parallel manner with calibrator and controls (Khafri et al., 2005; Marshall et al., 1984, Perez-Perez et al., 1991).

3.3. Media

All the chemicals used in present study were purchased from Merck, Sigma and Himedia (India). The culture medium Mueller-Hinton broth (MHB) supplemented with 5% defibrinated sheep blood was purchased from Himedia and used according to manufacturer’s instructions for MIC and time kill curve experiments. Colony counts, susceptibility and resistance developments were determined with Mueller-Hinton agar (MHA) supplemented with 5% defibrinated sheep blood. MHA was also purchased from Himedia and used according to manufacturer’s instructions. All the strains were stored at -70°C in water containing 10% skimmed milk obtained from Himedia and supplemented with 20% Glycerol obtained from Merck.

*H. pylori* was grown on Brain Heart Infusion (BHI) broth media, supplemented with 5% sheep blood; Himedia) for bacterial kinetic growth at 37°C in a microaerophilic atmosphere (10% O₂, 10% CO₂, and 80% N₂). The colonies developed were then suspended in Mueller-Hinton agar containing 5% sheep blood, followed by incubation for 24 to 48 hrs. at 37°C in microaerophilic atmosphere.

Isolates of *H. pylori* (HP-B₁, HP-B₂, HP-B₃ and HP-B₄) were maintained on *H. pylori* special peptone agar (HPSPA: Himedia) slants and streaked onto fresh slants every 5-7 days. Slants were incubated for 48 hrs. at 37°C in anaerobic jar containing 3.5 lit. AnaeroPack, (Himedia) flushed with a microaerophilic gas mixture (10% O₂, 10% CO₂ and 80% N₂).

3.4. Bacterial growth

The growth refers to change in the total population i.e. increases in the number rather than or increase in the size or, mass of an individual organisms with *H. pylori*
four strain the maximum population is reached with in 24 hours, but others required longer periods (16 days) of incubation to reach maximum growth (Flamm et al., 1996; Xia et al., 1993).

3.4.1. Preparation and Inoculation of Broths

Brain Heart Infusion Broth was prepared according to the manufacturer's directions. *H. pylori* special peptones broth (HPSPB), developed in this laboratory (VMRC-Himedia), was prepared by adding Special peptone (Himedia), 10 g/lit.; yeast extract (Himedia), 5 g/lit.; beef extract (Himedia), 5 g/lit. and pyruvic acid, sodium salt (Merck), 0.5 g/lit. Broths were dispensed in 96-ml aliquots into 250-ml Erlenmeyer flasks, Autoclaved and stored at 4°C for 1 day until inoculation. The broths were warmed to room temperature prior to inoculation and 4 ml iron-supplemented Sheep blood added to each flask. Afterwards *H. pylori* four strain were inoculated 1ml of 0.5 McFarland standard in 100 ml BHIB, medium in three flasks each.

*H. pylori* four strain the maximum population is reached with in 24 hours, but studied for kinetic growth for longer periods (16 days) of incubation to reach maximum growth. Bacteria were grown on blood agar plates (BHI agar supplemented with 5% sheep blood; Himedia) for bacterial growth at 37°C in a microaerophilic atmosphere (10% O₂, 10% CO₂ and 80%N₂); supplemented with 5% Sheep blood (SB). Cultures were incubated at 37°C for 16 days in a microaerophilic gas mixture incubator whose atmosphere was evacuated three times and replaced with a microaerobic gas mixture composed of 10% oxygen, 10% carbon dioxide, and 80% nitrogen for Growth studies (Xia et al., 1993).

3.4.2. Preparation of the McFarland standard

Add 0.5 mL of 0.048 M BaCl₂ (1.17% w/v Ba₂H₂O) to 99.5 ml of 0.18 M H₂SO₄ (1%V/V) with constant stirring. Distribute the standard into screw cap tubes of the
same size and with the same volume as those used in growing the broth culture. Seal the tubes tightly to prevent loss by evaporation. Store to protect it from light at room temp. Agitate vigorously the turbidity standard on a vortex mixture before using.

3.4.3. Preparation of inoculum

These suspensions should be used within 30 minutes of preparation. The inoculum should be adjusted so that $10^7$ and $10^8$ are used for growth population. Prepare the desired inoculum by comparing with a 0.5 McFarland standard.

*H. pylori* broth cultures were serially diluted ($10^1$, $10^2$, and $10^3$) in 0.01M phosphate-buffered saline (pH 7.2), and *H. pylori* was enumerated in duplicate by surface plating onto above respected BHI blood agar plate. The plates were then incubated at 37°C for 24 to 48 hrs. in GasPak jars under microaerobic conditions. Generation times were derived from the exponential growth rates and were determined in duplicate and mean is taken.

*H. pylori* were tested for response of antibiotic combinations with pantoprazole *in vitro*. The best three combinations found in vitro were selected and their efficacy was checked against the infection in the mice model. This study helped in developing best Fixed Dose Combination (FDC) therapy for the treatment of ulcers caused by infection of *H. pylori*.

3.5. Antimicrobial agents

Clarithromycin, Amoxicillin, Metronidazole, Tetracycline, Cefotoxime, Ceftazidime, Ceftriaxone, Piperacillin, Tazobactum, Ciprofloxacin, Streptomycin, Erythromycin, Gentamycin, Tobramycin, Amikacin, Vancomycin, and Nitilmycin were used for *in vitro* study. All antibiotics and Pantoprazole were obtained as gift sample from manufacture, Venus Remedies Limited India.
3.5.1. Combination Studies

Pantoprazole and its various combinations studied under following design presented in table (1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proton Pump Inhibitor (PPI)</th>
<th>Antibiotics</th>
<th>Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pantoprazole</td>
<td>Clarithromycin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>2</td>
<td>Pantoprazole</td>
<td>Amoxicillin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>3</td>
<td>Pantoprazole</td>
<td>Metroniazole</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>4</td>
<td>Pantoprazole</td>
<td>Tetracycline</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>5</td>
<td>Pantoprazole</td>
<td>Cefotaxime</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>6</td>
<td>Pantoprazole</td>
<td>Ceftazidime,</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>7</td>
<td>Pantoprazole</td>
<td>Ceftriaxone,</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>8</td>
<td>Pantoprazole</td>
<td>Piperacillin,</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>9</td>
<td>Pantoprazole</td>
<td>Tazobactum</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>10</td>
<td>Pantoprazole</td>
<td>Ciprofloxacin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>11</td>
<td>Pantoprazole</td>
<td>Streptomycin</td>
<td>40 + 1000mg Combination doses</td>
</tr>
<tr>
<td>12</td>
<td>Pantoprazole</td>
<td>Erythromycin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>13</td>
<td>Pantoprazole</td>
<td>Gentamycin</td>
<td>40 + 40 mg Combination doses</td>
</tr>
<tr>
<td>14</td>
<td>Pantoprazole</td>
<td>Tobramycin</td>
<td>40 + 40 mg Combination doses</td>
</tr>
<tr>
<td>15</td>
<td>Pantoprazole</td>
<td>Amikacin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>16</td>
<td>Pantoprazole</td>
<td>Vancomycin</td>
<td>40 + 500 mg Combination doses</td>
</tr>
<tr>
<td>17</td>
<td>Pantoprazole</td>
<td>Nitiilmycin</td>
<td>40 + 40 mg Combination doses</td>
</tr>
</tbody>
</table>

Table [1] : Design of study of combination of antibiotics with pantoprazole.
3.6. Microbiological assay of antibiotics against *H. pylori*

3.6.1. All combination of antibiotics with pantoprazole were tested for their efficiency

The inhibition of microbial growth under standard condition to be utilized for demonstrating *in vitro* therapeutic efficiency of drugs. Any change in the antibiotic molecules which may not be detected by chemical method and it was revealed by a reduction in the antimicrobial activity and hence microbiological assay are very useful in resolving doubts regarding possible loss of potency of antibiotics and their preparations. The methods employed are the cylinder plate (cup plate) method (Indian Pharmacopoeia, 1996).

The method used was tested by statistical analysis. The dose response line produced by the standard and the preparation were examined and shown to be linear and parallel with a probability level. The test was performed with various combinations of antibiotics.

3.6.2. Cylinder plate or Cup plate method

This method depends upon the dilution of an antibiotic from a vertical cylinder or a cavity through solidify MH agar layer of a Petri dish or plate to an extent that growth of the added *H. pylori* is prevented entirely a circular area or a zone around the cylinder or cavity containing a solution of the antibiotics (Indian Pharmacopoeia, 1996).

3.6.2.1. Antimicrobials Susceptibility of Antibiotics Combination compared to alone

Preparation parallel level dilutions of all Antibiotics combination and alone as given drugs. The test was performed as per Indian pharmacopoeia.

3.6.2.2. Principle

The principles of determining the efficacy of an Antibiotic to a bacterium were well enumerated by Rideal, Walker and others at the turn of the century, the discovery
of antibiotics made these tests too cumbersome as the large number of tests were needed for evaluation. The cup plate method of agar diffusion used by Alexander Fleming was the widely accepted method among a variety of agar diffusion methods devised by workers in this field. The Oxford group used these methods initially to assay the antibiotic contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface.

With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility tests. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the cup plate method is by far the commonest type used. The cup plate method of AST is the most practical method and is still the method of choice for the average laboratory. It is, therefore, imperative that microbiologists understand the principles of the test well and keep updating the information as and when necessary. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth.

3.6.2.3. Factors Influencing Antimicrobial Susceptibility Testing

pH

The pH of each batch of Mueller-Hinton agar should be checked when the medium is prepared. The exact method used will depend largely on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. If the pH is too low, certain drugs will appear to lose potency (e.g. aminoglycosides and macrolides), while other agents may appear to have excessive activity (e.g., tetracyclines). If the pH is too high, the opposite effects can be expected.
3.7. Determination of Minimum Inhibitory Concentrations

MIC are considered the gold standard for determining the susceptibility of organism to antimicrobial and are therefore used to judge the performance of all other methods of susceptibility testing. This test will be performed for various combinations of antibiotics (Jennifer M. Andrews, 2001).

The MICs of Pantoprazole, Antibiotics and their a fixed dose combination with Pantoprazole were determined in gram negative bacteria by standard protocol of National Committee for Clinical Laboratory Standards (NCCLS, 1997). The MIC of single drugs and combination with Pantoprazole for the three gastric biopsy (HP-B₁, HP-B₂, HP-B₃) and one ATCC 43504 (HP-B₄) of *H. pylori* strains were determined in cation-supplemented MH broth supplemented with 5% defibrinated sheep blood by the microdilution technique (Hsu et al., 2004; Huggins et al., 2003). Overnight MH broth supplemented with 5% defibrinated sheep blood cultures were used to prepare inocula of $10^5$ CFU/ml. The MIC was defined as the lowest concentration (0.015625mg/l) and highest dilution (256 mg/l) of antimicrobial agent that prevented turbidity after 24 hrs under microaerobic conditions (10% CO₂, 10% O₂, and 80% N₂) in Mueller Hinton broth, supplemented with 5% defibrinated sheep blood of incubation at 37°C.

3.8. Determination of Time Kill Curve

For each gastric biopsy (HP-B₁, HP-B₂, HP-B₃) and one ATCC 43504 (HP-B₄) of *H. pylori* strains, time-kill curve studies were performed in MH broth in glass flasks with an inoculum of $5 \times 10^6$ to $1 \times 10^7$ CFU/ml in the presence of a Pantaprazole, antibiotics and their a fixed dose combination (Pearson et al., 1980). A flask of inoculated MH broth supplemented with 5% defibrinated sheep blood with no antibiotic served as a control. The surviving bacteria were counted after 0, 2, 4, 6, 8, 10, and 12hrs of under microaerobic conditions (10% CO₂, 10% O₂, 80% N₂) in Mueller
Hinton broth, supplemented with 5% defibrinated sheep blood of incubation at 37°C, by subculturing 50-µl serial dilutions (in 0.9% sodium chloride) onto MH Agar plates supplemented with 5% defibrinated sheep blood with a spiral plater.

3.9. Determination of Resistance Development

The resistance development studies was carried out to determine resistance pattern of a fixed dose combination as compared with antibiotics of the disc concentrations 30µg, 20µg, 10µg (Fukazawa et al., 1999; Loivukene et al., 2002).

3.9.1. Inoculum Preparation

At least three to five colonies of the same morphological type are selected from overnight plated cultures on selective agar medium. The top of each colony is touched with a loop and the growth is transferred into a tube containing 4-5 ml of a suitable selective broth medium, such as MH broth supplemented with 5% defibrinated sheep blood to produce a suspension which match the turbidity standard of 0.5 McFland standard. Using matched cuvettes with 1 cm. path length and water as a blank standard the adsorbance in a spectrophotometer at wave length of 625 nm. the acceptable range of standard is 0.08-0.13.

3.9.2. Inoculation of Test Plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab to be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed. Allow the plates to dry until there is no visible
surface moisture.

3.9.3. Preparation of disc

Whatman Filter Paper No.1 is used to prepare discs approximately 6 mm in diameter, which are placed in a petri dish and sterilized by Steam Sterilization.

3.9.4. Preparation of Antibiotic Stock Solutions

Antibiotics may be received as powders form. It is recommended to obtain pure antibiotics from commercial sources, (Venus Remedies Ltd.) and not to use injectable solutions. Powders must be accurately weighed and dissolved in the appropriate diluent to yield the required concentration, using sterile glassware.

Stock solutions are prepared by using the formula

\[(1000/P) \times V \times C=W\]

Where
- \( P \) = potency given by the manufacturer (\( \mu \)g/mg)
- \( V \) = volume required (ml)
- \( C \) = final concentration of solution (multiples of 1000) (mg/L)
- \( W \) = weight of antibiotic (mg) to be dissolved in volume \( V \) (ml)

3.9.5. Application of Discs to Inoculated Agar Plates

The blank discs are dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. Whether the discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. A disc should not be relocated once if it has come into contact with the agar surface. Instead, place a new disc in another location on the agar.

3.9.6. Application of Antibiotic Stock Solution on Blank Discs

Using auto pipette and sterile pipette tips antibiotic stock solution is pipetted on the surface of blank discs to have the required concentration of Antibiotic/Antimicrobial
in μg.

3.9.7. Incubation of Plates

The plates are incubated in inverted positions in an incubator for 18-24 hours at 35°C for incubation.

3.9.8. Reading plates & results

After 18 to 24 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there was a confluent lawn of growth. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petriplate. The petriplate is held a few inches above a black, non reflecting background and illuminated with reflected light. The zone margin was taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which could be detected only with a magnifying lens at the edge of the zone of inhibited growth is ignored. However, discrete colonies growing within a clear zone of inhibition should be sub cultured, re-identified, and retested. Results are record and compared with accompanying chart.

   Being upon the antibiotics combination response, best three combination were selected for animal studies.

3.10. In vivo study of three combinations drugs on mice Mus musculus

3.10.1. Laboratory Studies on Animals

The best three combinations of antibiotics were selected for the in vivo studies on mice Mus musculus. 360 mice were selected for each section of studies. 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 section, marked as A, B, and C for three antibiotics were divided with following member of animals and given dose of antibiotic combination as per Table (2).

3.10.2. Establishment of infection

The mice *Mus musculus* were infected with in all three combination groups with standard reference culture of ATCC 43504 (HP-B4). The culture was introduced through oral route of 0.2ml as per mice of 0.5 *McFarland standard* (10^7 to 10^8) (Sommer *et al.*, 2004; Lee *et al.*, 2007).

3.10.3. Histopathological study

All antibiotics groups of animals labeled as A, B, and C received dose as per Schedule of table [1] and [2].
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Animals</th>
<th>Animal Group</th>
<th>Number of Animals</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Best Antibiotic Combination (A)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control without infection</td>
<td>AG1</td>
<td>60</td>
<td></td>
<td>Normal saline no infection</td>
</tr>
<tr>
<td>Infected</td>
<td>AG2</td>
<td>60</td>
<td></td>
<td>Infected for 15 days with normal saline</td>
</tr>
<tr>
<td>Treated with 'A' for 15 days</td>
<td>AG3</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'A' for 15 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'A' for 30 days</td>
<td>AG4</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'A' for 30 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'A' for 45 days</td>
<td>AG5</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'A' for 45 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'A' for 60 days</td>
<td>AG6</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'A' for 60 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td><strong>Second Best Antibiotic Combination (B)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control without infection</td>
<td>BG1</td>
<td>60</td>
<td></td>
<td>Normal saline no infection</td>
</tr>
<tr>
<td>Infected</td>
<td>BG2</td>
<td>60</td>
<td></td>
<td>Infected for 15 days with normal saline</td>
</tr>
<tr>
<td>Treated with 'B' for 15 days</td>
<td>BG3</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'B' for 15 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'B' for 30 days</td>
<td>BG4</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'B' for 30 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'B' for 45 days</td>
<td>BG5</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'B' for 45 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'B' for 60 days</td>
<td>BG6</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'B' for 60 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td><strong>Third Best Antibiotic Combination (C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control without infection</td>
<td>CG1</td>
<td>60</td>
<td></td>
<td>Normal saline no infection</td>
</tr>
<tr>
<td>Infected</td>
<td>CG2</td>
<td>60</td>
<td></td>
<td>Infected for 15 days with normal saline</td>
</tr>
<tr>
<td>Treated with 'B' for 15 days</td>
<td>CG3</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'C' for 15 days (7.714mg/kg weight)</td>
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<tr>
<td>Treated with 'B' for 30 days</td>
<td>CG4</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'C' for 30 days (7.714mg/kg weight)</td>
</tr>
<tr>
<td>Treated with 'B' for 45 days</td>
<td>CG5</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'C' for 45 days (7.714mg/kg weight)</td>
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<tr>
<td>Treated with 'B' for 60 days</td>
<td>CG6</td>
<td>60</td>
<td></td>
<td>Infection after 15 days treated with 'C' for 60 days (7.714mg/kg weight)</td>
</tr>
</tbody>
</table>

Table [2]: Division of Animals with treatment protocol of best three Antibiotics combination with Pantoprazole.
3.10.4. Method

After completion of treatment all animals were sacrificed of stomach, intestine, and rectum were washed properly in saline. All organs were placed in 10% formalin at room temperature until processing for histopathology. Tissues were washed under running tap for 3 to 6 hr to remove formalin. Prepare following solution and put block preparations for requisite time duration. The Blocks were made (microtomy and kept wax at 65°C), tissue sectioning was done the slides were slandered with Eosin and Haematoxylin staining and observed under light microscope (Yakoob et al., 2005; Gamble and Wilson, 2002).

3.11. Statistical Analysis

The resulting data of susceptibility test by cup method and resistance development test were analyzed statistically. All value are expressed as Mean ± SD one way analysis of variance (ANOVA) with student new man keuls comparison test was used to determined statistical difference between antibiotics and a fixed dose combination with Antibiotic and Pantoprazole. A P - value < 0.05 was considered statistically significant.

The selection were evaluated for bacteriological eradication by combination based on microbiological and histological studies, best possible combination was suggested for clinical evaluation.