“Synergistic effect of combination antibiotics against *Salmonella typhi* after co-culture with resistant *Shigella flexneri*”

A dissertation submitted to the Department of Pharmacy, East West University, as the partial fulfillment of the requirements for the Degree of Master of Pharmacy

Submitted by:

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Declaration by Research Candidate

I, Azima Sultana Julie, hereby declare that this dissertation, entitled "Synergistic effect of combination antibiotics against *Salmonella typhi* after co-cultured with resistant *Shigella flexneri"* submitted to the Department of Pharmacy, East West University, in partial fulfillment for the requirement of the degree of Master of Pharmacy. This is a genuine & authentic research work carried out by me under the guidance of Dr. Sufia Islam, Professor, Department of Pharmacy, East West University, Dhaka, Bangladesh. The contents of dissertation has been carried out by me and has not been previously submitted to any other University/college/Organization any academic qualification/ certificate/ diploma or degree.

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Certificate by the Supervisor

This is certify that the dissertation, entitled "Synergistic effect of combination antibiotics against *Salmonella typhi* after co-cultured with resistant *Shigella flexneri"* is a bona-fide research work done by Azima Sultana Julie (ID: 2016-1-79-002), is a partial fulfillment for the requirement of the degree of Master of Pharmacy.

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Certificate by the Chairperson

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Dedication

This thesis paper

Is dedicated

To my parents &

Honorable Research Supervisor

Abstract

Some bacteria have gone evolutionary change and become resistant to several antibiotics. For that reason, the selection of effective antibiotics has become more difficult. In prospect of Bangladesh no standard combination antibiotics are available against resistant pathogens. So, we tried to find out the combination of antibiotics against resistant pathogen. In our present study, the anti-microbial activity with combination antibiotics was determined after co-culture of resistant pathogen with the sensitive strain. Disc diffusion technique was used for this study. This method is essentially a qualitative or semi-quantitative test indicating the sensitivity or resistance of microorganisms. Co-culture was performed with sensitive strain *Salmonella typhi* and registance strain *Shigella flexneri*. In this study, *Salmonella typhi* showed sensitivity against ciprofloxacin, vancomycin and imipenem with different concentration (20, 40, 60 and 80 μg/ml). This sensitive strain showed zone of inhibition of 20-27, 19-23 and 21-27 mm against ciprofloxacin, vancomycin and imipenem respectively. But *Shigella flexneri* did not give any zone of inhibition against these antibiotics at same concentration. After treatment with antibiotics ciprofloxacin, vancomycin and imipenem co-cultured newly resistant *Salmonella typhi* did not give optimum zone of inhibition. The resistance *Salmonella typhi* exhibited optimum zone of inhibition against combination antibiotics. Combination of ciprofloxacin and imipenem was exhibited optimum ZOI. Vancomycin and imipenem combined antibiotics was also exhibited optimum ZOI. The values of ZOI obtained individually were 0-4, 0-5, 4-9 mm for vancomycin, ciprofloxacin and imipenem against resistant *S.typhi*. The higher values of ZOI for the combination of vancomycin and imipenem were obtained which was 18-29 mm. For the combination of ciprofloxacin and imipenem 19-32 mm ZOI was obtained. The result of the study showed that co-cultured resistant *Salmonella typhi* was 10% more inhibited by combination antibiotics in comparison to single antibiotic. The present study indicates the synergistic effects of the antibiotics combination. These combinations therapy can be used as beneficial treatment approach in multi-drug resistant *Salmonella typhi* infections.

Key Words: *Shigella flexneri*; drug resistant; *Salmonella typhi*; co-culture; zone of inhibition; multi-drug resistant; synergistic effects.

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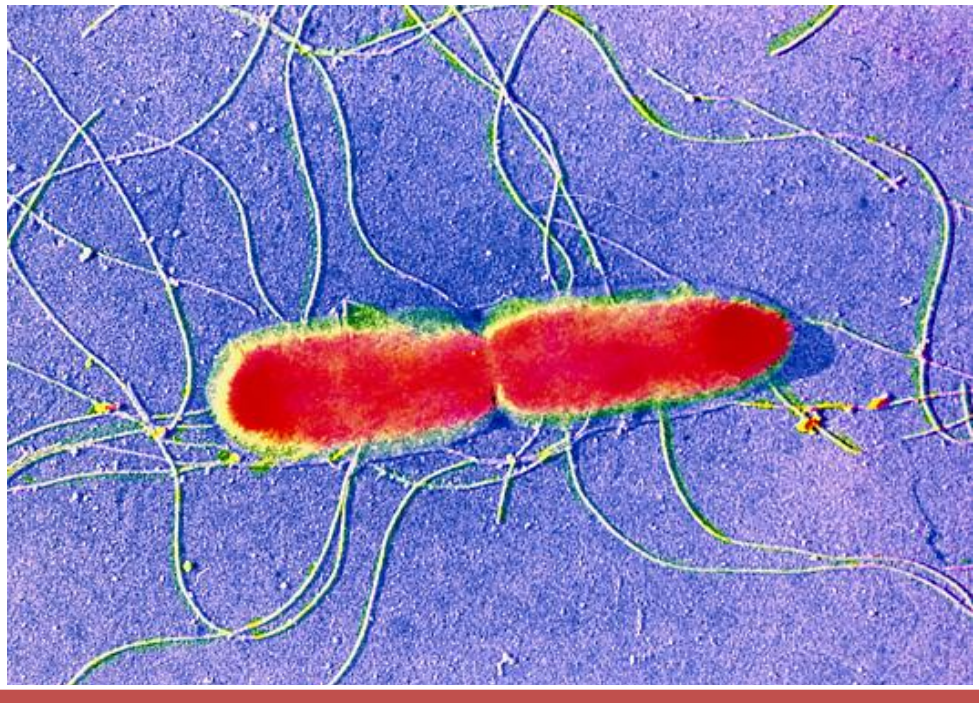


1.1 Introduction

Most commonly, *Salmonella* infections are self-limited, causing acute gastrointestinal illness in humans. Antimicrobial agents are commonly prescribed to those seeking medical attention. Severe infections which spread to the bloodstream, meningeal linings of the brain or other deep tissue can also occur. It is critical for the treatment of invasive infections. The selection of effective antibiotics has become more difficult as antibiotic-resistance has increased.

"Bacterium" or "pathogen" has many harmful effects. In a fascinating guide it is possible to observe bacteria with all the senses. Many groups of bacteria can be easily identified in the field with or without a microscope. (Dyer, *et al.* 2003)

'Pharmaceutical' combination therapy defined as to prescribe separate drugs that contain more than one active. In a recent study about solid cancers, clinical cases combination therapies will be needed to avoid the evolution of resistance to targeted drugs (Bozic, et al. 2013). Various systems biology methods have been utilized to investigate combination therapies to overcome drug resistance. (Korkut, *et al*. 2015)

A particular strain of MDR Salmonella, called as Salmonella Typhimurium DT104 (DT104), emerged in the U.S. This strain is typically resistant to five drugs: ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline.

Nalidixic acid-resistant *S. typhi* (NARST) was first isolated in 1993 from analysis of the quinolone resistance region. Three patterns mutation nucleotides were isolated. Treatment with ofloxacin for uncomplicated typhoid patients (78%) were infected with MDR *S. typhi*. (Wain, et al. 1997). The National Antimicrobial Resistance Monitoring System (NARMS) has identified increasing numbers of Salmonella isolates resistant. Many antimicrobial agents tested and showed resistant activity such as, amoxicillin/clavulanate, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. These isolates also have further decreased the susceptibility or resistance to ceftriaxone. Salmonella isolates with this resistance gene that produces AmpC-type enzymes that cause much of the drug-resistant pathogen.

A combination of rifazimin and cipro was effective in patients with active chronic, resistant pouchitis. The patients for the treatment using antibiotic agents need more specific and antibacterial spectrum of activity. (Gionchetti, *et al.* 1999)

MDR (gram-negative) bacteria, such as carbapenemase producer strains that are resistant to all current therapeutic options. We have reviewed the current available options for the treatment of MDR infections, including combination regimens are not optimum for patients. Only few new molecules have an adequate activity against MDR pathogens. Ceftozolane/tazobactam has been recently approved for clinical use. Some compounds, such as avibactam combinations, plazomicin and eravacycline have shown optimistic activity in phase 2 and 3 clinical trials. (Bassetti, 2015)

Accurate antimicrobial susceptibility testing is vital for patient care and emerging antimicrobial resistance. The National Committee for Clinical Laboratory Standards (NCCLS) outlines generally agreed upon guidelines for reliable and reproducible results in the susceptibility testing. (Julia, *et al.* 2000)

1.2 Outbreaks

Multi drug resistant bacteria are caused by the accumulation of gene, each coding for a resistance single drug. (Nikaido, H, 2009). Several outbreaks of multidrug-resistant Salmonella infections have been documented in the United States, including an outbreak associated. Outbreaks like these can result in multiple hospitalizations and death among individuals with the most severe infections. The multidrug-resistant nature of these organisms makes treatment failure more likely.

The growing number of bacterial pathogens that are resistant to numerous. There have been no new broad-spectrum antibiotics developed in the last 40 years, and the drugs we have currently are quickly becoming ineffective. The therapeutic strategies should be developed in conjunction with antibiotics and may help to prolong the life span of these life-saving drugs. (Gill, *et al.* 2014)

Treatment of infections is compromised worldwide by the emergence of bacteria that are resistant to multiple antibiotics. Although classically attributed to chromosomal mutations,

resistance is most commonly associated with extrachromosomal elements acquired from other bacteria in the environment. These include different types of mobile DNA segments, such as plasmids, transposons, and integrons. However, intrinsic mechanisms not commonly specified by mobile elements—such as efflux pumps that expel multiple kinds of antibiotics—are now recognized as major contributors to multidrug resistance in bacteria. Once established, multidrug-resistant organisms persist and spread worldwide, causing clinical failures in the treatment of infections and public health crises. (Alekshun, MN, *et al*. 2007)

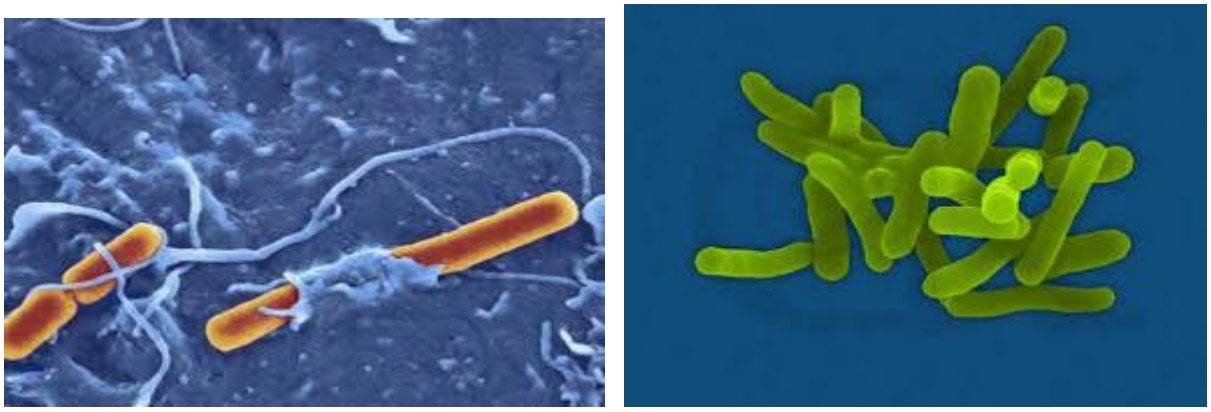


Figure 1.1: *Shigella flexneri*

Figure 1.2: *Salmonella typhi*

1.3 *Salmonella typhi*

The genome for *Salmonella typhi* has been completely classified. A majority of these genes have been inactivated, shows that the genes were recently modified for evolutionary changes. There are two commonly used strains of *Salmonella typhi*(CT18 and Ty2). *Salmonella typhi* CT18 has a large circular chromosome consisting of 4.8 Mb and two plasmids (pHCM1 and pHCM2). The plasmid pHCM1, has multiple drug resistance. *Salmonella typhi* Ty2 has one large chromosome. It does not have plasmids and can be affected by antibiotics. The current vaccine was developed using *S. typhi* Ty2. Out of the 204 pseudogenes in Salmonella, 195 genes are the same in both strains (CT18 and Ty2) making them 98% identical. (Parkhill, *et al*. 2001)

1.3.1 Description and significance:

There are over 2,000 various groupings (serovars), making *Salmonella typhi* . *Salmonella typhi* is a gram negative bacterium that causes systemic infections and typhoid fever in humans. This rod-shaped, flagellated organism's sole reservoir is humans. It has caused many deaths in developing countries where sanitation is poor and is spread through contamination of water and undercooked food. Eradication seems highly unlikely due to recent emergence of multi drug resistance strains. *Salmonella Typhi* strain Ct18 was originally isolated from a patient in a hospital in Vietnam. The chromosome sequence is 4,809,037 bp in length with a G+C content of 52.09%. The chromosome was sequenced though the method of shotgun sequencing with 97,000 shotgun reads. Since then, *Salmonella typhi* has undergone evolutionary change and has become resistant to antibiotics. (Den, W, *et al*. 2003)

1.3.2 Ecology:

Salmonella typhi is a food born pathogen and increasingly difficult to control. Salmonella's ability to change its phenotype and genotype with environmental changes make it almost impossible to eradicate from the food chain. When a culture of Salmonella was transferred to higher temperatures (60°C), it took 60 minutes to maximize heat resistance. When the pH was lowered, acid resistance increased to adjust their reliable pH. Salmonella cells experience gradual changes which is why Salmonella thrives in undercooked meat. It is able to adapt to survive the cooking process and also has the ability to cross the gastric acid barrier (this is how they enter

the human intestine). A high-fat matrix is protects *Salmonella* against various stressful environments. (Humphrey, 2004)

1.3.3 Pathology:

Salmonella typhi has killed over 600,000 people annually all over the world. It is a deadly bacterial disease that causes typhoid fever and is transmitted through food and water. It has become an epidemic in South Asian countries where sanitation is lacking. *S. typhi* usually invades the surface of the intestine in humans, but have developed and adapted to grow into the deeper tissues of the spleen, liver, and the bone marrow. Symptoms most characterized by this disease often include a sudden onset of a high fever, a headache, and nausea. Other common symptoms include loss of appetite, diarrhea, and enlargement of the spleen (depending on where it is located).

Salmonella typhi involves colonization of the Reticuloendothelial system. Some individuals who are infected with *S. typhi* become life-long carriers that serve as the reservoir for these pathogens. S typhi has an endotoxin (which is typical of Gram negative organisms), as well as the Vi antigen, which increases virulence. It also produces a protein called invasin that allows non-phagocytic cells to take up the bacterium and allows it to live intracellularly. Salmonella typhi is a strong pathogen for humans due to its resistance to the innate immune response system. (Falkow, *et al*. 2004)

Recently, strains of MDR (multi-drug resistant) Salmonella have been identified and grouped together in a single haplotype named H58. It has been found that these strains are now resistant to nalidixic acid and have reduced susceptibility to fluoroquinolones. This strain has been recently found in Morocco, which shows that the MDR strain has reached as far as Africa. (Parkhill, J, *et al*. 2001)

1.4 *Shigella flexneri*

Shigella flexneri is a rod-shaped bacterium that is physiologically similar to *Shigella dysenteriae*, *Shigella boydii*. It is important because it causes shigellosis, an acute bloody diarrhea. *Shigella flexneri* is the most common cause of the endemic form of shigellosis. *Shigella flexneri* (*S. flexneri* 2a) is a major public health concern in developing countries. *Shigella* was recognized as the cause of bacillary dysentery in the 1890s by Shiga. (Nato, *et al.* 2007). *Shigella flexneri* 2a strain 301 was isolated and sequenced by Jin et al. It was isolated the bacterium from a shigellosis patient in China in 1984. (Jin, *et al.* 2002)

1.4.1 Pathophysiology:

Shigella infection is a major public health problem in developing countries where sanitation is poor. Humans are the natural reservoir, although other primates may be infected. No natural food products harbor endogenous *Shigella* species, but a wide variety of foods may be contaminated.

Shigellosis is spread by means of fecal-oral transmission. Other modes of transmission include ingestion of contaminated food or water (untreated wading pools, interactive water fountain), contact with a contaminated inanimate object, and certain mode of sexual contact. Vectors like the housefly can spread the disease by physically transporting infected feces.

One possible explanation is that virulent *Shigellae* can withstand the low pH of gastric juice. Most isolates of *Shigella* survive acidic treatment at pH 2.5 for at least 2 h.

The incubation period varies from 12 hours to 7 days but is typically 2-4 days; the incubation period is inversely proportional to the load of ingested bacteria. The disease is communicable as long as an infected person excretes the organism in the stool, which can extend as long as 4 weeks from the onset of illness. Bacterial shedding usually ceases within 4 weeks of the onset of illness; rarely, it can persist for months. Appropriate antimicrobial treatment can reduce the duration of carriage to a few days.

1.4.2 Virulence

Virulence in *Shigella* species involves both chromosomal-coded and plasmid-coded genes. Virulent *Shigella* strains produce disease after invading the intestinal mucosa; the organism only rarely penetrates beyond the mucosa. (Edwards, BH, 1999)

The characteristic virulence trait is encoded on a large (220 kb) plasmid responsible for synthesis of polypeptides that cause cytotoxicity. Shigellae that lose the virulence plasmid are no longer pathogenic. *Escheria coli* (*E coli* O157:H7) that harbor this plasmid clinically behave as *Shigella* bacteria.

Regarding chromosomally encoded enterotoxin, many pathogenic features of *Shigella* infection are due to the production of potent cytotoxins known as Stx, a potent protein synthesis–inhibiting exotoxin. *Shigella* strains produce distinct enterotoxins. These are a family of cytotoxins that contain 2 major immunologically non–cross-reactive groups called Stx1 and Stx2. The homology sequences between Stx1 and Stx2 are 55% and 57% in subunits A and B, respectively. (Richardson, *et al.* 1992)

Stx1 and Stx2 are both encoded by a bacteriophage inserted into the chromosome. Stx1 increases inflammatory cytokine production by human macrophages, which, in turn, leads to a burst of interleukin (IL)-8. This could be relevant in recruiting neutrophils to the lamina propria of the intestine in hemorrhagic colitis and accounts for elevated levels of IL-8 in serum of patients with diarrhea-associated HUS. (Richardson, *et al.* 1992)

In summary, events that occur on exposure to *Shigella* toxin are as follows:

- The B subunit of holotoxin binds to the Gb3 receptor on the cell surface of brush-border cells of the intestines.
- The receptor-holotoxin complex is endocytosed.
- The complex moves to Golgi apparatus and then to the endoplasmic reticulum.

These genes help in invasion, multiplication, and resistance to phagocytosis by tissue macrophages.

1.4.3 Pathology

The host response to primary infection is characterized by the induction of an acute inflammation, which is accompanied by poly morpho nuclear cell (PMN) infiltration, resulting in massive destruction of the colonic mucosa. Apoptotic destruction of macrophages in subepithelial tissue allows survival of the invading shigellae and inflammation facilitates further bacterial entry.

Gross pathology consists of mucosal edema, erythema, friability, superficial ulceration, and focal mucosal hemorrhage involving the rectosigmoid junction primarily.

Shigella bacteria invade the intestinal epithelium through M cells and proceed to spread from cell to cell, causing death and sloughing of contiguously invaded epithelial cells and inducing a potent inflammatory response resulting in the characteristic dysentery syndrome. In addition to this series of pathogenic events, only *S dysenteriae* type 1 has the ability to elaborate the potent Shiga toxin that inhibits protein synthesis in eukaryotic cells and that may lead to extraintestinal complications, including hemolytic-uremic syndrome and death. Invasion of M cells, the specialized cells that cover the lymphoid follicles of the mucosa, overlying Peyer patches, may be the earliest event. (Phalipon, A, 2007)

In contrast, epithelial cells infected with *Shigella* undergo a stress response but do not die. Therefore, the objective of this study was to determine if *Shigella* has the ability to inhibit apoptosis in epithelial cells. A modified gentamicin protection assay was used to investigate if HeLa cells infected with *S. flexneri* are able to resist the induction of apoptosis following treatment with 4 μM of staurosporine. Nuclear staining and immunofluorescence revealed that infected cells remained healthy while uninfected cells appeared apoptotic. Only uninfected cells had detectable levels of activated caspase 3 upon immunofluorescence, and this was verified by Western blot analysis. Despite interfering with caspase 3 activation, *Shigella*-infected cells treated with staurosporine did have cytochrome *c* release and caspase 9 activation, indicating that *Shigella* protects epithelial cells from apoptosis by inhibiting caspase 3 activation. Analysis of *S. flexneri* mutants showed that invasion and a functional type III secretion system were required to block apoptosis. In addition, a mutant with a deletion in *mxiE*, which encodes a transcriptional activator for genes induced intracellularly, failed to inhibit apoptosis. Therefore, protection of epithelial cells from apoptosis by *S. flexneri* is regulated by one or more of the bacterial genes under the control of *mxiE*. We believe that *S. flexneri*, like other pathogens, inhibits apoptosis in epithelial cells but causes apoptosis in macrophages to ensure survival inside the host.

In contrast, epithelial cells infected with *Shigella* undergo a stress response but do not die. *Shigella* has the ability to inhibit apoptosis in epithelial cells. A modified gentamicin protection,

HeLa cells infected with *S. flexneri* are able to resist the induction of apoptosis. Despite interfering with caspase 3 activation, *Shigella*-infected cells treated with staurosporine. The infected cells have cytochrome *c* release and caspase 9 activation which indicating that *Shigella* protects epithelial cells by inhibiting caspase 3 activation. Analysis of *S. flexneri* mutants invasion and a functional type III secretion system were required to block apoptosis. A mutant with a deletion in *mxiE*, which encodes a transcriptional activator for genes induced intracellularly that failed to inhibit apoptosis. (Clark, *et al.* 2007)

1.5 Antibiotic:

Antibiotics are a type of antimicrobial drug used in the treatment and prevention of bacterial infections. They may either kill or inhibit the growth of bacteria.

A limited number of antibiotics also possess antiprotozoal activity. Sometimes the term antibiotic (which means "opposing life") is used to refer to any substance used against microorganisms. Some sources distinguish between antibacterial and antibiotic; antibacterials are used in soaps and disinfectants, while antibiotics are used as medicine.

Antibiotics revolutionized medicine in the 20th century. Together with vaccination, antibiotics have led to the near eradication of diseases such as tuberculosis in the developed world. However, their effectiveness and easy access have also led to their overuse, prompting bacteria to develop resistance. The World Health Organization to classify antimicrobial resistance as a

"serious threat is no longer a prediction for the future. It is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country.

The discovery of antibiotics more than 70 years ago initiated a period of drug innovation and implementation in human. This history has been interpreted to mean that antibiotic resistance in pathogenic bacteria is a modern phenomenon. The genes is encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics. A study was found on the complete vancomycin resistance element VanA confirmed its similarity to modern variants. (D'Costa, *et al*. 2011)

Antibiotic resistance is ancient and multi-drug resistance is common in bacteria isolated from Lechuguilla Cave. Here we use whole-genome sequencing, functional genomics and biochemical assays to reveal the intrinsic resistome of *Paenibacillus* sp. LC231, a cave bacterial isolate that is resistant to most clinically used antibiotics. (Pawlowski, *et al*. 2016)

1.5.1 Classification:

Antibiotics are commonly classified based on their mechanism of action, chemical structure, or spectrum of activity. Most target bacterial functions or growth processes. Those that target the bacterial cell wall (penicillins and cephalosporins) or the cell membrane (polymyxins), or interfere with essential bacterial enzymes (rifamycins, lipiarmycins, quinolones, and sulfonamides) have bactericidal activities. Those that target protein synthesis (macrolides, lincosamides and tetracyclines) are usually bacteriostatic (Finberg, *et al.* 2004)

Further categorization is based on their target specificity. "Narrow-spectrum" antibiotics target specific types of bacteria, such as gram-negative or gram-positive, whereas broad-spectrum antibiotics affect a wide range of bacteria. Following a 40-year break in discovering new classes of antibacterial compounds, four new classes of antibiotics have been brought into clinical use in the late 2000s and early 2010s.

1.5.2 Medical uses:

Antibiotics are used to treat or prevent bacterial infections, and sometimes protozoan infections. (Metronidazole is used for parasitic diseases). When an infection is suspected of being

responsible for an illness but the responsible pathogen has not been properly identified, an empiric therapy is adopted.This involves the administration of a broad-spectrum antibiotic based. (Leekha, *et al.* 2011)

When the responsible pathogenic microorganism is already known or has been identified, definitive therapycan be started. This will usually involve the use of a narrow-spectrum antibiotic. The choice of antibiotic given will also be based on its cost. Identification is critically important as it can reduce the cost and toxicity of the antibiotic therapy and also reduce the possibility of the emergence of antimicrobial resistance.

Antibiotics may be given as a preventive measure and this is usually limited to at-risk populations such as those with a weakened immune system, those taking immunosuppressive drugs, cancer patients and those having surgery. They have an important role in dental antibiotic prophylaxis where their use may prevent bacteremia and consequent infective endocarditis. Antibiotics are also used to prevent infection in cases of neutropenia particularly cancer-related.

1.5.3 Side effects

Antibiotics are screened for any negative effects before their approval for clinical use, and are usually considered safe and well tolerated. However, some antibiotics have been associated with a wide extent of adverse side effects. The ranging from mild to very severe depending on the type of antibiotic used, the microbes targeted, and the individual patient.Side effects may reflect the pharmacological or toxicological properties of the antibiotic or may involve hypersensitivity or allergic reactions. Safety profiles of newer drugs are often not as well established as for those that have a long history of use.

Common side-effects include diarrhea, resulting from disruption of the species composition in the intestinal flora, resulting, for example, in overgrowth of pathogenic bacteria. Antibacterials can also affect the vaginal flora. Additional side-effects may be result from interaction with other drugs, for example: a quinolone antibiotic with a systemic corticosteroid. (Lewis, et al. 2014)

1.5.4 Combination therapy:

In critical, severe infectious diseases, including tuberculosis, combination therapy (i.e., the concurrent application of two or more antibiotics) has been used. This therapy has play useful role to delay or prevent the emergence of resistance. In acute bacterial infections, antibiotics are part of combination therapy. These combination therapy are prescribed for their synergistic effects to improve treatment outcome as the combined effect of both antibiotics is better than their individual effect. *Staphylococcus aureus* infections may be treated with a combination therapy of fusidic acid and rifampicin. (Ocampo, *et al*. 2014) Antibiotics used in combination may also be antagonistic. The combined effects of the two antibiotics may be less than the individual antibiotic.

The combinations of imipenem plus ciprofloxacin and imipenem plus amikacin gave synergistic effects against *Pseudomonas aeruginosa.* For imipenem-susceptible *P. aeruginosa,* synergy of imipenem plus ciprofloxacin and imipenem plus amikacin was 36 and 45% of the strains. The synergistic effect against imipenem-resistant isolates of *P. aeruginosa* was 10% for both combinations. (Bustamante, *et al.* 1987)

The combination of ceftriaxone and sulbatam is used in clinical practice for getting better therapeutic value. This combination has showed MIC analysis. (Shrivatsava, *et al.* 2009) Accelerated biodegradation of antibiotics agricultural, wastewater, or pharmaceutical manufacturing effluents would attenuate environmental exposure to antibiotics. The merits are investigated to the context of assessing potential risks of antibiotic resistance development. (Topp, *et al*. 2013)

1.6 Antibiotic Resistance:

Even before the extensive use of penicillin, it could destroy it by enzymatic degradation. “Syphilis has now been treated with arsenicals for about 40 years without any indications of an increased incidence of arsenic-resistant infections, an increasing incidence of infections resistant to penicillin” Surprisingly, this is still true for *T. palladium*. But not for pathogenic bacteria the Enterobacteriaceae, which have become resistant.

There are a lot number of reviews elsewhere describing a variety of antibiotic resistance mechanisms and, within the frames of the bullet-target concept, these mechanisms can be classified as a target or bullet-related. Targets can be: (i) protected by modification (mutations resistant to rifampin, (ii) modified by an enzyme, (iii) replaced and (iv) protected at cellular or population levels etc.

Recent works in the area of antimicrobials and interactions of bacteria with antibiotics can be explained within the frames of the classical bullet-target concept. A recent work has showed on novel antibiotic resistance mechanism used the "kin selection" concept. (Lee, *et al.*, 2010). Moreover, in complex biofilm consortia, the protection against antibiotics is offered to all community members, which requires a conceptual framework operating at the system level. Thus the conceptual base of microbe–antibiotic interaction has been broadening beyond the bullet-target model. (Davies, *et al.*, 2006, Aminov, 2009, Lee, *et al.*, 2010)

Recent studies have demonstrated that antibiotic resistance, generally confers a reduction in growth, virulence or transmission. These findings imply that resistance might be reversible, provided antibiotic use is reduced. (Anderson, 2006)

There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. The types and levels of metal contamination and specific patterns of antibiotic resistance, several mechanisms underlie this co-selection process. These co-selection mechanisms include co-resistance and cross-resistance. (Baker-Austin, *et al*. 2006)

The gene sequence (vanH, vanA, vanX) is common to all glycopeptide producers tested. The glycopeptide-producing organisms may have been the source of resistance genes in vancomycin-resistant enterococci. (Marshall, *et al.*, 1998)

Newly acquired high-throughput data on virulent microbial agents need potential new drug targets. Many approaches have been used to evaluate proteins from infectious pathogens. From a biological perspective, most essential proteins or highly conserved proteins are act as potential drug targets. Ribosomal proteins comprise such an example. Many of them are well-known drug targets in bacteria. Antibiotics are mainly originating from natural products of microorganisms targeting other microorganisms. The good drug targets are evolutionary constrained and are

subject of evolutionary selection. This means that mutations in such proteins are deleterious and removed by selection in development of resistance. (Gladki, et al. 2013)

Antibacterial-resistant strains and species, sometimes referred to as "superbugs", now contribute to the emergence of diseases that were for a while well controlled. For example, emergent bacterial strains causing tuberculosis that resistant to previously effective antibacterial treatment pose many therapeutic challenges. Every year, nearly half a million new cases of multidrug-resistant tuberculosis (MDR-TB) are estimated to occur worldwide. For example, NDM-1 is a newly identified enzyme conveying bacterial resistance to a broad range of beta-lactam antibacterials.

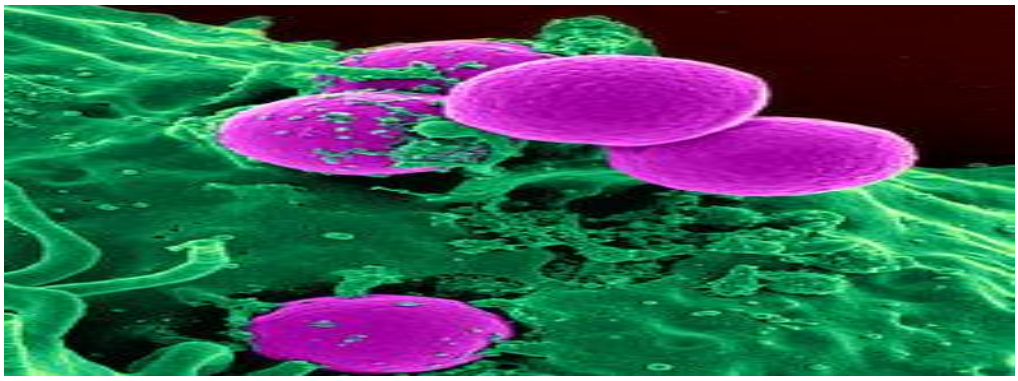


Figure 1.3: Resistant pathogen

1.7 Development of new antibiotics:

In April 2013, the Infectious Disease Society of America (IDSA) reported that the weak antibiotic pipeline does not match bacteria's increasing ability to develop resistance. Since 2009, only two new antibiotics were approved in the United States. The report has been identified that seven antibiotics against the Gram-negative *bacilli* (GNB) currently in phase 2 or phase 3 clinical trials. (Boucher, *et al*. 2013)

However, these drugs do not address the entire spectrum of resistance of GNB. Some of these antibiotics are combination of existent treatments:

- Ceftolozane/tazobactam (CXA-201;CXA 101/tazobactam):

- Antipseudomonal cephalosporin/β-lactamase inhibitor combination (cell wall synthesis inhibitor). FDA approved on 12/19/2014.
- Ceftazidime/avibactam (ceftazidime/NXL104): Antipseudomonal cephalosporin/β-lactamase inhibitor combination (cell wall synthesis inhibitor). In phase 3.
- Ceftaroline/avibactam (CPT-avibactam; ceftaroline/NXL104): Anti-MRSA cephalosporin/ β-lactamase inhibitor combination (cell wall synthesis inhibitor)
- Imipenem/MK-7655: Carbapenem/ β-lactamase inhibitor combination (cell wall synthesis inhibitor). In phase 2.
- Plazomicin (ACHN-490): Aminoglycoside (protein synthesis inhibitor). In phase 2.
- Eravacycline (TP-434): Synthetic tetracycline derivative / protein synthesis inhibitor targeting the ribosome. Development by Tetraphase, Phase 2 trials complete.
- Brilacidin (PMX-30063): Peptide defense protein mimetic (cell membrane disruption). In phase 2.

Prophylactic anti-bacterial vaccines have developed to drastic reduction in global bacterial diseases. Older vaccines have been largely replaced by less reactogenic a cellular vaccines. The vaccines made with purified components, including capsular polysaccharides and their conjugates to protein carriers. The current development of new vaccines to prevent diseases caused by *N. meningitidis* serogroup B, *S. aureus* and *C. difficle* is increased. Future progress will likely bring to the clinic passive immune therapies for use in vaccines against intracellular pathogens. (Donald, *et al.* 2011)

1.8 Synergistic drug combinations improve therapeutic selectivity:

Drug discovery approaches focus on compounds with molecular selectivity, inhibiting disease-relevant targets. However *in vivo*, many such agents are not therapeutically selective, either because of undesirable activity. In theory, drug combinations should permit increased control of such complex biology and therapeutic synergy will generally be mirrored by synergistic side-effects. Here we provide evidence, from 94,110 multi-dose combination experiments representing diverse disease areas, inhibited bacterial metabolism. The multi-target synergies are more specific than single agent activities to particular sites. Using an anti-inflammatory combination multi-target synergy can achieve therapeutic selectivity. Synergistic combinations

can increase the number of selective therapies using the current pharmacopeia and offer opportunities for more precise control of biological systems.

Synergistic combinations of two or more agents can overcome toxicity associated with high doses of single drugs. Multi-target effects can be identified using experimental and theoretical techniques. Combinations therapies are the most promising avenues towards treating multi-factorial diseases.

Drug combinations from thirteen phenotypic screens relevant to six disease areas and flux balance analysis simulations of chemically inhibited metabolism in *E. coli* bacteria. It shows that synergies do indeed operate in more narrow biological contexts than single drugs. Therapeutic selectivity can arise from the multi-target co-operativity underlying most phenotypic synergy. (Lehar, J, *et al.* 2009)

1.9 Literature review:

Parkhill, *et al.* 2001, "Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18". This article state that the 4,809,037-base pair (bp) genome of a *S. typhi* (CT18) is resistant to multiple drugs.The genome sequence was identified that are known to contribute to virulence in *Salmonella typhimurium*. This genetic degradation may contribute to the human-restricted host range for *S. typhi*.

Shanahan, *et al.* 1998, "Molecular Analysis of and Identification of Antibiotic Resistance Genes in Clinical Isolates of *Salmonella typhi* from India". This article suggests that *Salmonella typhi* strains isolated from cultures of blood from patients. The strains were tested for their susceptibilities to various antimicrobial agents. Eleven of the *S. typhi* strains possessed resistance to chloramphenicol (256 mg/liter), trimethoprim (64 mg/liter), and amoxicillin (>128 mg/liter). And four of the isolates were resistant to each of these agents except for amoxicillin. Six of the isolates were completely sensitive to all of the antimicrobial agents tested. All the *S. typhi* isolates were susceptible to cephalosporin agents, gentamicin, amoxicillin plus clavulanic acid and imipenem.

Sangeeta, J, *et al.* 2007, "Fluoroquinolone resistance in *Salmonella typhi* and *S. paratyphi* A in Bangalore, India". This work shows minimum inhibitory concentration (MICs) of ciprofloxacin, ofloxacin, levofloxacin and gatifloxacin against *S.typhi* and *S. paratyphi*. Nalidixic acid resistant to *S.typhi* and *S.paratyphi* was seen. The higher MICs were observed with fluoroquinolone by *S.typhi* and *S.paratyphi*.

Orsi R, *et al.* 2006 states that "Synergistic effect of propolis and antibiotics on the *Salmonella typhi".* The work wasdone to investigate a possible synergistic effect withethanolic extracts of propolis and some antibiotics (Amoxicillin, Ampicillin and Cefalexin) against *Salmonella typhi*. The propolis and anitibiotics were showed important antibacterial action. Synergistic effect against *Salmonella typhi* was also exhibited by propolis and antibiotics.

Nuding, S, *et al*. 2014, "Synergistic effects of antimicrobial peptides and antibiotics against *Clostridium difficile".* The article states that antimicrobial peptides and antibiotics were sensitive against *C. difficile*.The uptake of antibiotics and peptides increased antibacterial effect. This effect was enhanced by membrane perturbation in or pore formation on the bacterial cell wall.

Jiang, X, *et al.* 2014, "Studies of the drug resistance response of sensitive and drug-resistant strains in a microfluidic system". In this paper, a new microfluidic system to monitor the responses of sensitive and drug-resistant strains of *E. coli* in different β-lactam ceftriaxone concentrations. The drug-resistant strain can endure a much higher concentration of antibiotics than the sensitive strain. The antibiotic concentration ratio was investigated from the cell death state to the cell elongation state. The ratio is much larger than that of the sensitive strain.

Totsuka, K, *et al.* 1999, "The combined effects of vancomycin and imipenem against methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* and *in vivo.*" In that study, synergic and additive effects of the two drugs were observed. The clinical isolates of MRSA were determined by the chequer board method. The combined effects seemed to be synergistic. A single administration of either drug did not show anti-MRSA effects.

Rochon-Edouard, *et al*. 2000, *"In vitro* synergistic effects of double and triple combinations of β-lactams, vancomycin and netilmicin against methicillin-resistant *Staphylococcus aureuses* strains." The study was observed the effects of different combinations of a beta-lactam, vancomycin, and an aminoglycoside against 32 clinical MRSA strains. The effects of 26 different beta-lactam-vancomycin and 8 different aminoglycoside-vancomycin combinations were observed. The best effects of vancomycin were obtained with imipenem, cefazolin, or netilmicin.The combination of imipenem-vancomycin and cefazolin-vancomycin provided a synergistic bacteriostatic effect against 22 strains. The vancomycin-netilmicin combination provideddifferent effect against all of the 32 strains tested. The addition of netilmicin enhanced antibacterial activity of the combination of cefazolin or imipenem plus vancomycin.

Dong-M, et al. 2010, "*In vitro* efficacy of the combination of ciprofloxacin and cefotaxime against nalidixic acid-resistant *Salmonella enterica* serotype Typhi". The study was done to identify in vitro synergistic combinations of antibiotics against *S.* Typhi with ciprofloxacin, cefotaxime and azithromycin. The combination of ciprofloxacin (0.012–0.375 μg/mL) and cefotaxime (0.063–0.125 μg/mL) against all three NARST strains was significantly more effective.Combination therapy with ciprofloxacin and cefotaxime showed synergistic effects. The combination of a fluoroquinolone and a β-lactam, may improve efficacy compared with a

fluoroquinolone alone. And it may reduce the chance of fluoroquinolone-resistant mutants emerging in patients with severe typhoid fever.

Chapter-Two

Objectives

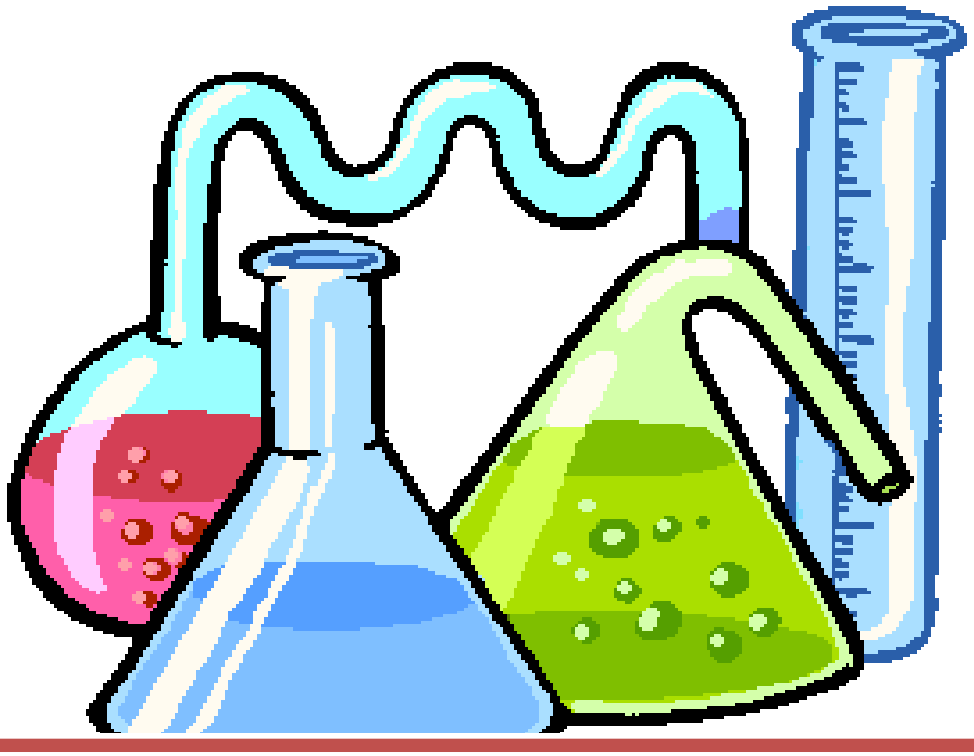
2.1 Research Objectives:

The objectives of the study are:

1. To develop antibacterial resistant by co-culturing of sensitive bacteria with resistance pathogenic strains
2. To determine the synergistic effect of combined antibiotics against microorganism after co-culture with resistant pathogenic bacteria.

Chapter-Three

Materials and Method



3.1 Disc diffusion method:

Disk diffusion technique is widely acceptable for the preliminary screening of antimicrobial activity. It is essentially a qualitative or semi-quantitative test indicating the sensitivity or resistance of microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be demonstrated by this method (Pelczar, *et al*. 1986)

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the in vitro fungal and bacterial growth. This ability may be estimated by any of the following three methods:

- ✓ Disc diffusion method
- ✓ Serial dilution method
- ✓ Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculums volume, culture medium composition (Bauer *et al*., 1966), pH, and incubation temperature can influence the results.

3.2 Principle of disc diffusion method

In this method-measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentration (µg/ml). Then sterile Matricel (BBL, Cocksville, USA) filter paper discs are impregnated with known amount of test substances using micropipette and dried. Standard antibiotic discs and discs on which the solvent used to dissolve the samples is adsorbed and dried are used as positive and negative control, respectively. These discs are then placed in Petri-dishes (120 mm in diameter) containing a suitable agar medium seeded with the test organisms using sterile transfer loop for antimicrobial screening. The plates are then kept at 40C for facilitating maximum diffusion. The test material diffuses from the discs to the

surrounding medium. The plates are then kept in an incubator (37˚C) for 12-18 hour to allow the growth of the microorganisms.

If the test material has any anti-microbial activity, it will inhibit the growth of microorganism giving a clear, distinct zone called "zone of inhibition. "The antibacterial activity of the test agent is determined by measuring the diameter of the zone of inhibition in term of millimeter. The experiments are carried out three times and the mean of the reading are recorded (Bayer et al., 1966).

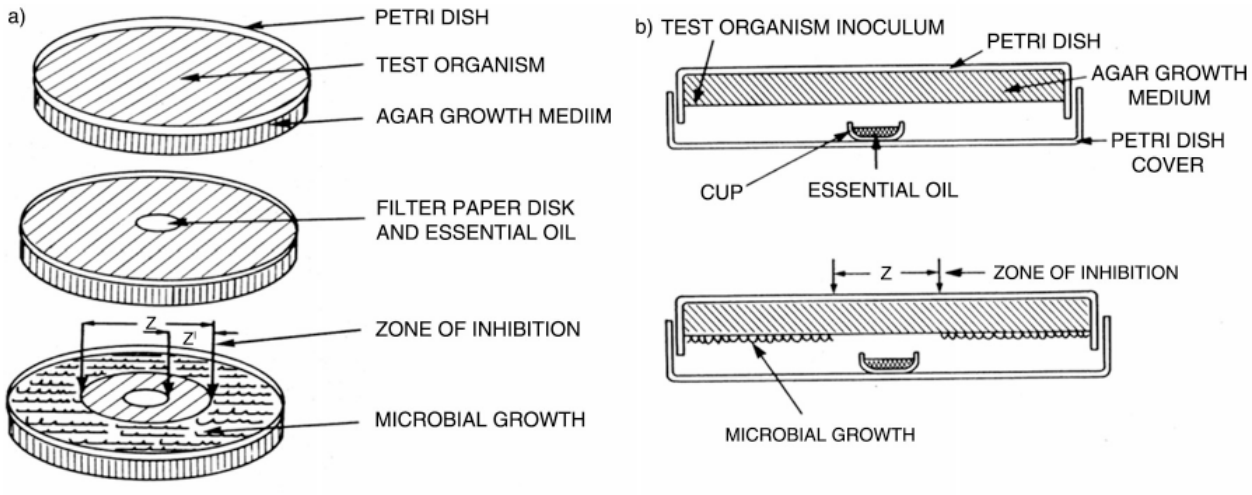


Figure: 3.1 Steps of disc diffusion method

3.3 Experimental

3.3.1 Apparatus and reagents

- Filter paper discs
- Nutrient Agar Medium
- Petridishes
- Sterile cotton
- Micropipette
- Inoculating loop
- Sterile forceps
- Screw cap test tubes
- Autoclave
- Laminar air flow hood
- Spirit burner
- Refrigerator
- Incubator
- Chloroform
- Ethanol
- Nose mask and Hand gloves

3.3.2 Test materials

In our presence study, the anti-microbial activity .

3.3.3 Test organisms

These organisms were collected from the Microbiology Lab. of Pharmacy Discipline, Dhaka University.

Table 3.1: List of bacteria used for screening of antimicrobial activity

Bacteria Sensitive	**Resistant Bacteria**
Salmonella typhi	*Shigella flexneri*

3.4 Composition of culture medium

The following media was used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

Table 3.2: Composition of nutrient agar medium

a) Nutrient Agar Medium	
Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
P^H	7.2 + 0.1

Table 3.3: Composition of macconkey agar medium

MacConkey Agar	
Ingredients	**Amount**
Proteose peptone or polypeptone	3 gm
Peptone or gelysate	17 gm
Lactose	10 gm
Bile salt	1.5 gm
NaCl	5 gm
Neutral red	0.03 gm
Crystal violet	0.001 gm
Agar	13.5 gm
P^H	7.1 + 0.2

Nutrient agar medium is the most frequently used and also used in the present study for testing the sensitivity. To prepare fresh cultures of organisms nutrient agar media was used.

3.5 Preparation of the medium

Nutrient agar media was prepared by adding water to a dehydrated product that contains all the ingredients. Practically all media are available commercially in powdered form (Pelczar, *et al.* 1986).

Media of the nutrient agar type was prepared by compounding the required individual ingredients or, more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form. The following steps were involved in the preparation of bacteriological media :

- Definite amounts of nutrient agar or macConkey agar were accurately weighed.
- It was taken in a volumetric flask containing distilled water (half of the required volume).

- A clear medium was obtained by thorough dissolving agar over a water bath with occasional shaking.
- Then the final volume was adjusted.
- The medium was then transferred in 16 ml and 5 ml volume respectively, to prepare plates and slants, in a number of test tubes.
- The test tubes were then plugged with cotton and sterilized in an autoclave at a temperature of 121°C and pressure of 15 lbs/sq inch for 15 minutes.

Figure 3.2: Media preparation

3.6 Sterilization procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121ºC and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized by UV light. (Pelczar, *et al*. 1986)

Figure 3.3: Laminar Hood

3.7 Preparation of sub-culture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures. With the help of loop the bacteria are transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test. (Pelczar, *et al*. 1986)

3.8 Preparation of test plates (Vineetha, *et al.* 2015)

- Each of the test organisms were transferred from the subculture to the test tube containing 16 ml autoclaved media with the help of the sterilized inoculating loop at 45°c in an aseptic area.
- The test tubes were shaken by rotation to get a uniform suspension of organism. The bacterial suspensions were immediately transferred to the sterile Petri dishes aseptically.
- The Petri-dishes were rotated several times, first clockwise and then anticlockwise, to assure homogeneous distribution of the test organisms.
- The medium was poured into Petri-dishes in such a way as to give a uniform layer, after the medium became cooled and stored in a refrigerator (4°c).

Figure 3.4: Preparation of test sample

3.9 Preparation of antibiotic disc (Vineetha, *et al*. 2015)

Three types of discs were used for antibacterial screening:

a) Sample discs

b) Standard discs and

c) Blank discs

Figure 3.5: **Preparation of disc**

3.9.1 Methods (Vineetha, *et al.* 2015)

- In this study the preparation of the filter paper discs involves the punching of holes of approximately 6mm diameter in whatman filter paper and was sterilized in an autoclave.
- Then the preparation of antibiotic stock solutions, where a known weight of the antibiotic powder is dissolved in the sterile distilled water. The stock solution was diluted to obtain the working solution.
- The following step was the impregnation of the discs where the antibiotic solutions are loaded on each a mechanical pipette.
- The discs were dried in an incubator and stored in small ampoules with a desiccant at minus 20 °C.

3.9.2 Preparation of filter paper discs (Vineetha, *et al.* 2015)

- For the purpose of production of antibiotics discs, whatman filter paper No.3 was used.
- To facilitate the identification of the discs, code name of the concentration of the drug in each disc.
- Using an ordinary office hole punching machine and precautions were taken to avoid overlapping of holes.
- The discs were then autoclaved at 15lbs pressure for 30minutes.

3.9.3 Preparation of Vancomycin antibiotic stock solution:

- Antibiotic powders were obtained from pharmaceuticals..
- Known weight of antibiotic powder was dissolved in sterile distilled water to obtain the stock solution.
- The stock solution was diluted at the time of disc preparation to obtain the working solution.
- A paper disc of 6mm diameter can absorb 0.02ml or 20µl of solutions. The concentrations of antibiotic solutions were expressed in µg/µl.

3.9.4 Preparation of Ciprofloxacin antibiotic stock solution:

- Known weight of antibiotic powder was dissolved in sterile distilled water to obtain the stock solution.
- The stock solution was diluted at the time of disc preparation to obtain the working solution.
- A paper disc of 6mm diameter can absorb 0.02ml or 20µl of solutions. The concentrations of antibiotic solutions were expressed in µg/µl.

3.9.5 Preparation of Imipenem antibiotic stock solution:

- Antibiotic powders were dissolved in saline water (0.9% NaCl solution).
- Before using the saline water, this was sterilized by autoclaving at a temperature of 121ºC and a pressure of 15-lbs/sq. inch for 20 minutes.
- The stock solution was diluted at the time of disc preparation to obtain the working solution.
- A paper disc of 6mm diameter can absorb 0.02ml or 20µl of solutions. The concentrations of antibiotic solutions were expressed in µg/µl.

3.10 Application of discs

Sample antibiotic discs (ciprofloxacin discs) and negative control discs (blank discs) were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were no closer than 15mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. The plates were then inverted and kept in refrigeration for about 4 hours at 4°c. This was sufficient time for the material to diffuse into a considerable area of the medium. Finally the plates were incubated upside down at 37°c for 12-18 hours.

3.11 Diffusion and incubation

The sample discs, the antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a

refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours. (Pelczar, *et al*. 1986)

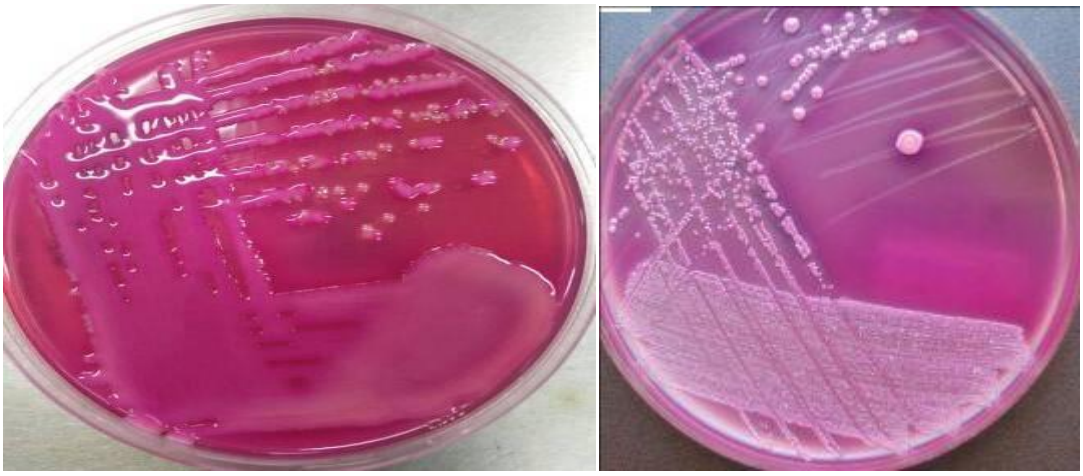


Figure 3.6: Test plates preparation

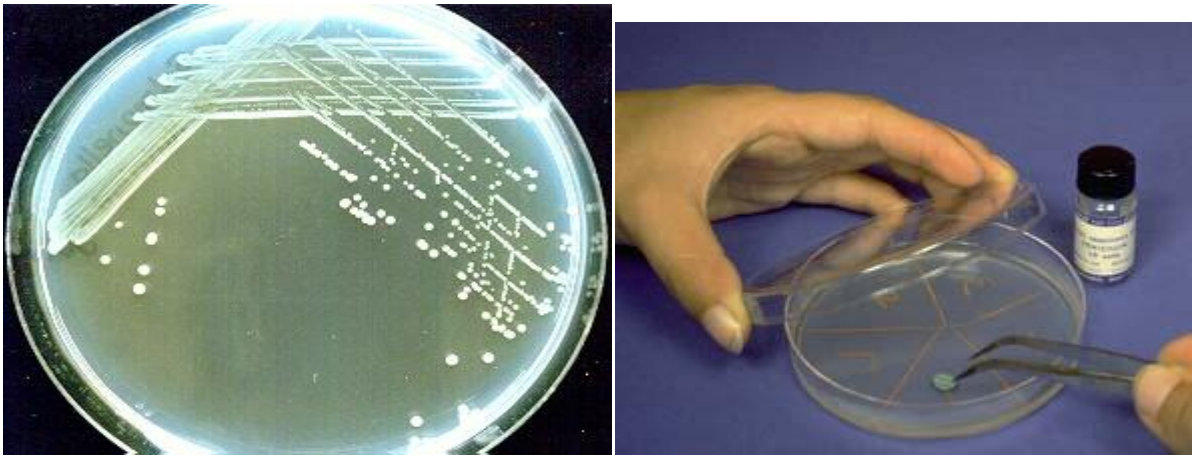


Figure 3.7: Applying antibiotics on test plates

Chapter-Four

Results

4.1 Zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

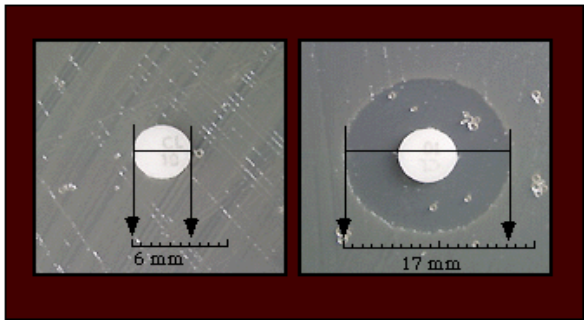


Figure 4.1: Determination of clear zone of inhibition

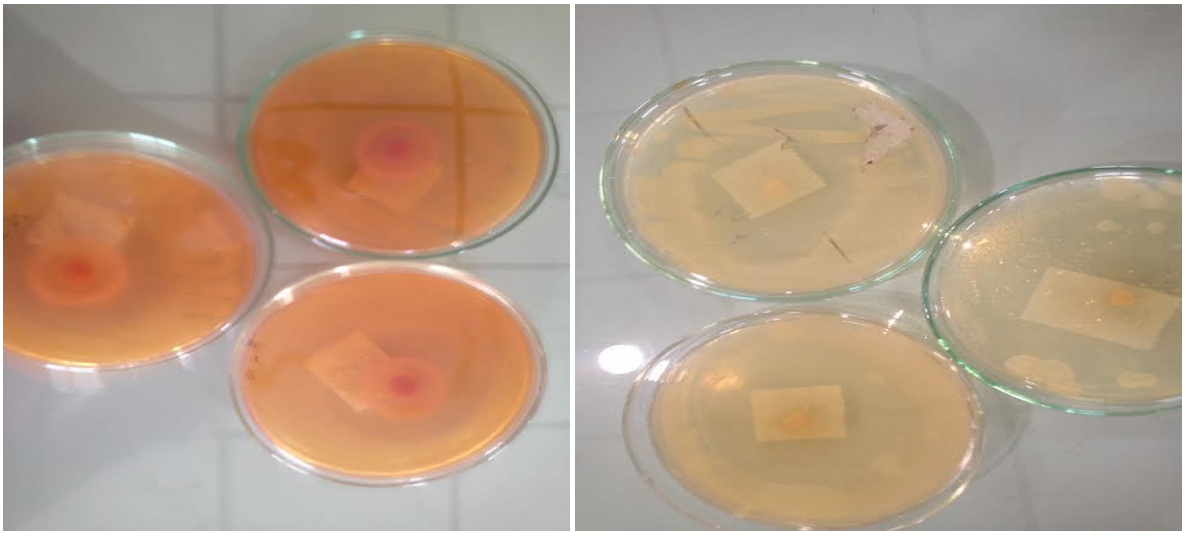


Figure 4.2: Clear zone of inhibition

4.2 Results

Table 4.1: Zone of inhibition of *Shigella flexneri* and *Salmonella typhi* with different concentration of ciprofloxacin, vancomycin and imipenem.

Media	Antibiotic	Concentration (μg/ml)	Bacteria	ZOI (mm)	Bacteria	ZOI (mm)
Nutrient Agar	Ciprofloxacin	20	*Shigella flexneri*	0	*Salmonella typhi*	20
		40		0		24
		60		0		25
		80		0		27
Nutrient Agar	Vancomycin	20	*Shigella flexneri*	0	*Salmonella typhi*	19
		40		0		20
		60		0		21
		80		0		23
Nutrient Agar	Imipenem (with cilastatin)	20	*Shigella flexneri*	0	*Salmonella typhi*	21
		40		0		23
		60		0		26
		80		0		27

According to table 4.1 *Salmonella typhi* showed sensitivity against ciprofloxacin. The zone of inhibition (ZOI) was 20 mm when the concentration of ciprofloxacin was 20μg/ml. Zone of inhibition was increased 24 , 25 and 27 mm when different concentration (40, 60 and 80μg/ml) respectively of ciprofloxacin. These concentration were applied to the culture plates of *Salmonella typhi.* On the other hand, *Shigella flexneri* did not show any ZOI when treated with ciprofloxacin.

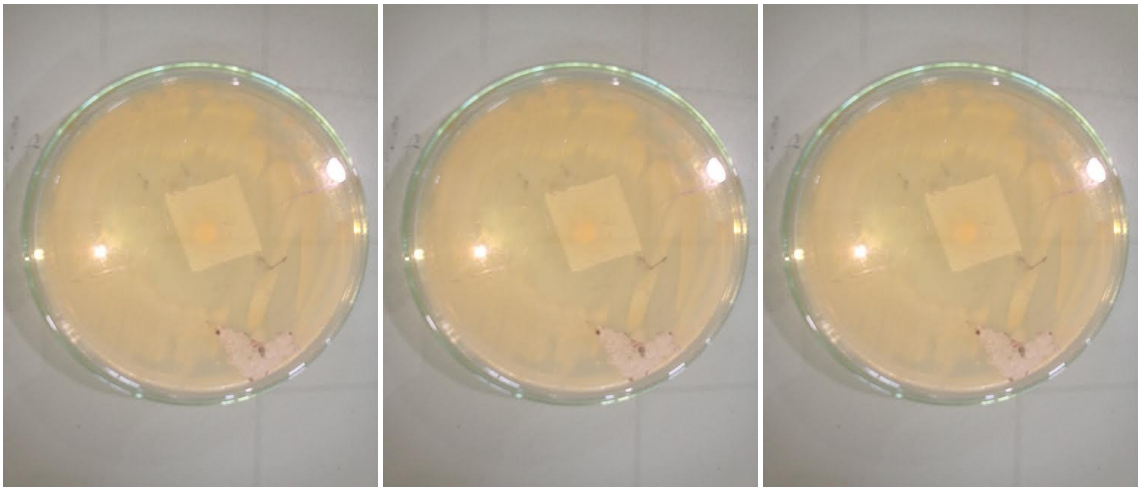


Figure 4.3 ZOI of *Shigella flexneri* and *Salmonella typhi* with different concentration of ciprofloxacin.

When the antibiotic vancomycin was applied *Salmonella typhi* showed sensitivity against vancomycin. Zone of inhibition was increased with different concentration. The ZOI was 19mm at the concentration 20 μg/ml of vancomycin. After the different concentration (40, 60 and 80 μg/ml) were applied to the culture plates different ZOI were obtained. The zone diameter 20-23 mm was exhibited by the *S.typhi* against antibiotic vancomyci. *Shigella flexneri* did not show any ZOI when treated with different concentration of vancomycin.

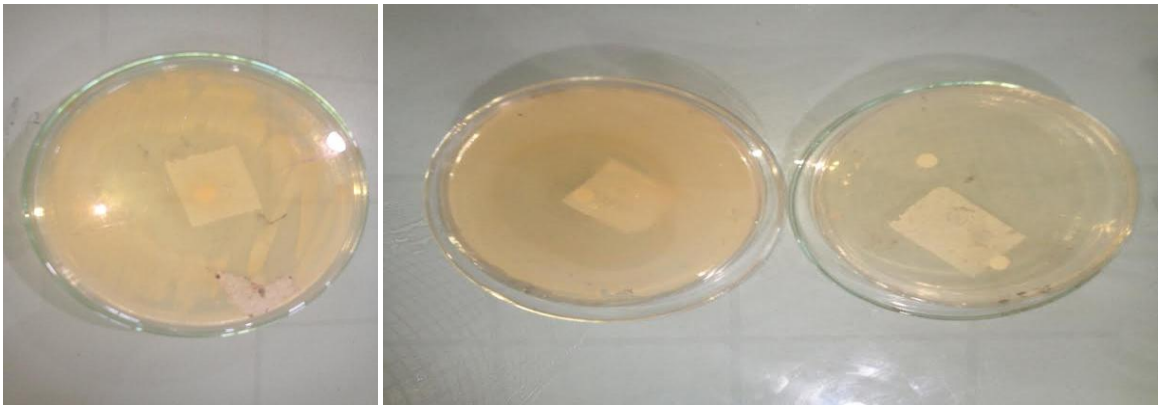


Figure 4.4 ZOI of *Shigella flexneri* and *Salmonella typhi* with different concentration of vancomycin.

Further study, *Shigella flexneri* did not show any zone when treated with different concentration 20, 40, 60 and 80 µg/ml of imipenem.

Salmonella typhi showed sensitivity against the antibiotic imipenem. The ZOI was 21 mm when the concentration of imipenem was 20 µg/ml. ZOI was increased respectively with the increasing different concentration. The *S.typhi* exhibited ZOI against imipenem were 23, 26 and 27 mm.

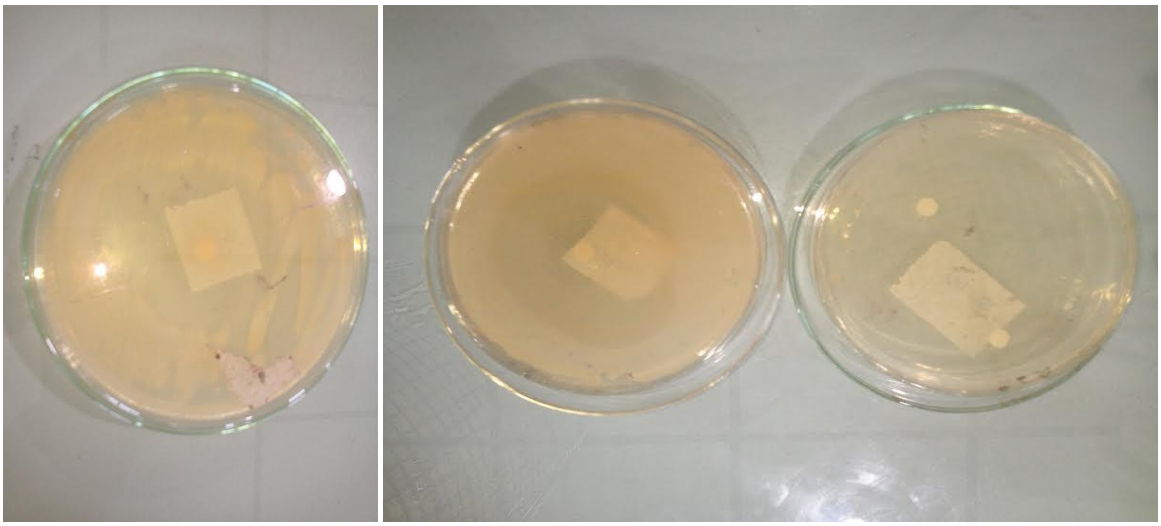


Figure 4.5 ZOI of *Shigella flexneri* and *Salmonella typhi* with different concentration of imipenem.

Table 4.2: ZOI after co-cultured *Salmonella typhi* with *Shigella flexneri* with different concentration of ciprofloxacin, vancomycin and imipenem.

Media	Antibiotic	Concentration (µg/ml)	Bacteria	ZOI (mm)
Nutrient Agar	Ciprofloxacin	20	Resistant*Salmonella typhi*	0
		40		0
		60		3
		80		5
Nutrient Agar	Vancomycin	20	Resistant*Salmonella typhi*	0
		40		0
		60		0
		80		4
Nutrient Agar	Imipenem	20	Resistant*Salmonella typhi*	0
		40		0
		60		4
		80		9

According to table 4.2: After co-cultured *S. typhi* with resistant *S. flex* treated with antibiotics ciprofloxacin, vancomycin and imipenem respectively. The co-cultured newly resistant *Salmonella typhi* did not give optimum ZOI. The different concentration was (20, 40, 60 and 80 µg/ml) respectively of antibiotics were applied to the co-culture plates. The values obtained individually were 0-4 mm, 0-5 mm, 4-9 mm for vancomycin, ciprofloxacin and imipenem.

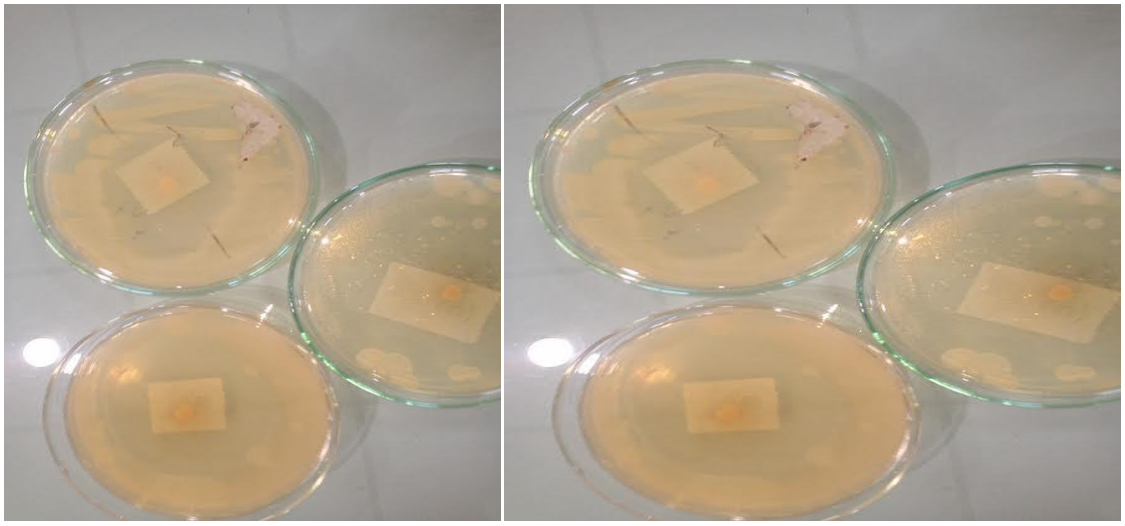
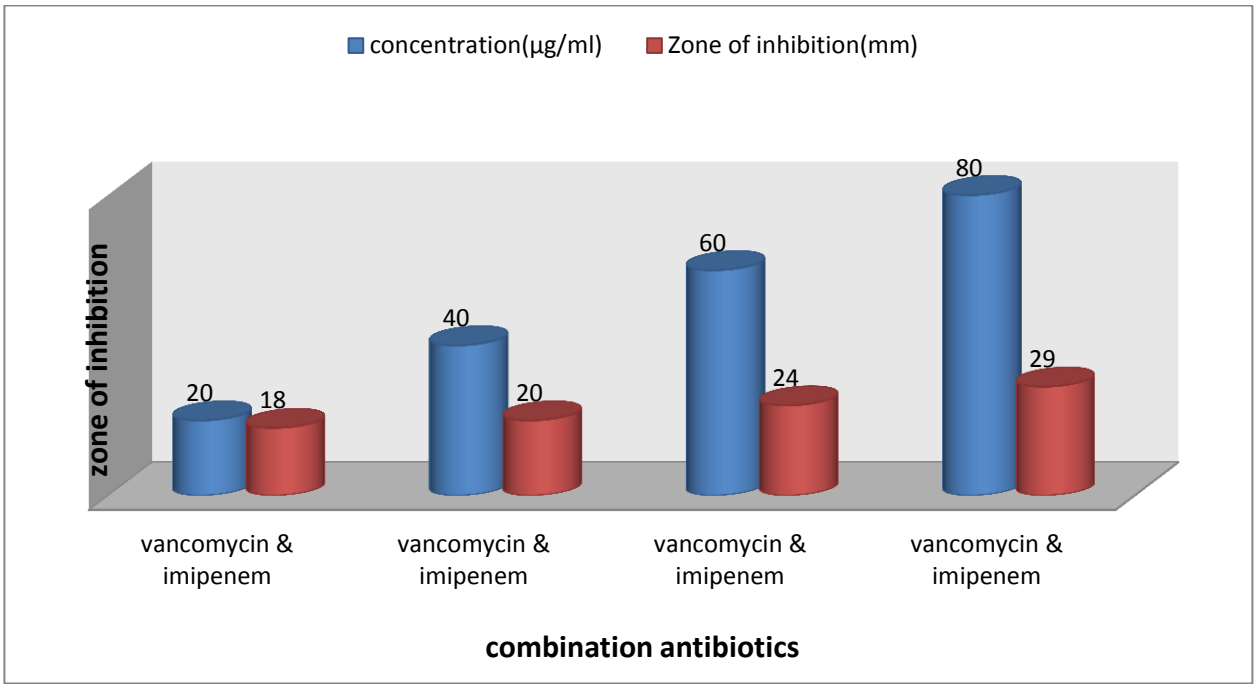


Figure 4.6 ZOI after co-cultured *Salmonella typhi* with *Shigella flexneri* with different concentration of ciprofloxacin, vancomycin and imipenem.

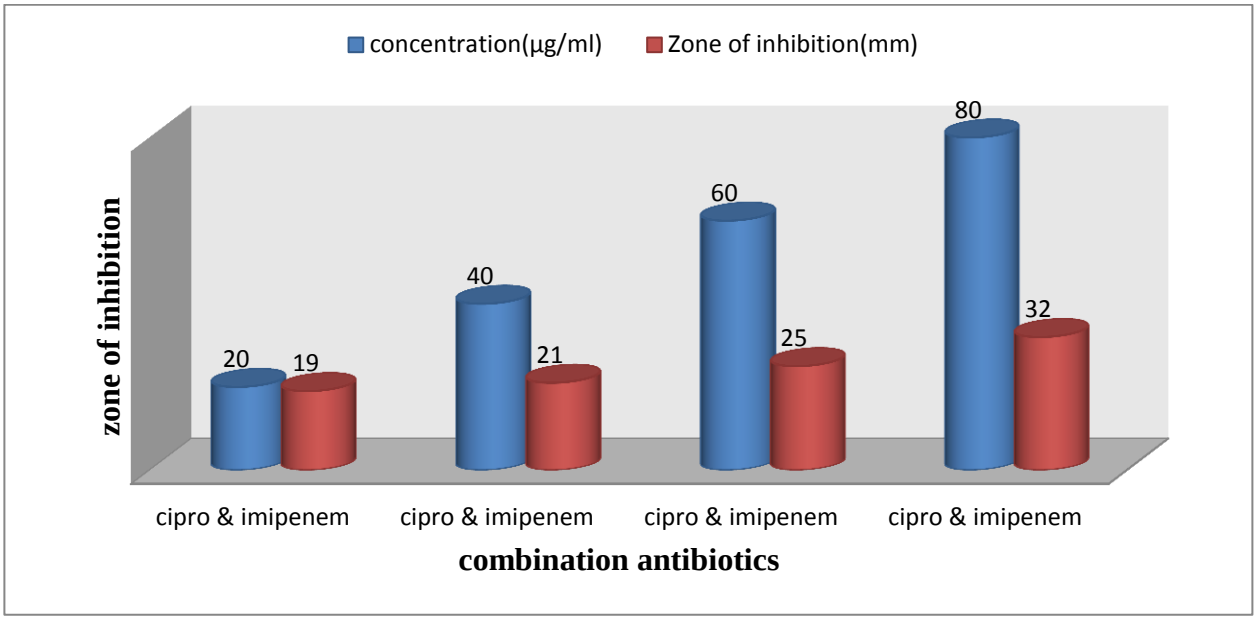
Figure 4.7: ZOI of resistant *Salmonella typhi* against combination vancomycin and imipenem with different concentrations.



This figure showed that the ZOI of resistant *Salmonella typhi* against the combination vancomycin and imipenem. The different concentration was 20- 80 µg/ml were applied to the test plates, 18, 20, 24 and 29 mm ZOI was observed respectively. The incidence of synergy against co-cultured strains was 10% more for both combinations.

The values obtained individually were 0-4 mm for vancomycin and 4-9 mm for imipenem, while higher values of 18-29 mm were obtained for the combination of vancomycin and imipenem. As co-cultured strains was 10% more inhibited by combinations which indicates the synergistic effects of the antibiotics combination.

Figure 4.8: ZOI of resistant *Salmonella typhi* against combination ciprofloxacin and imipenem with different concentration.

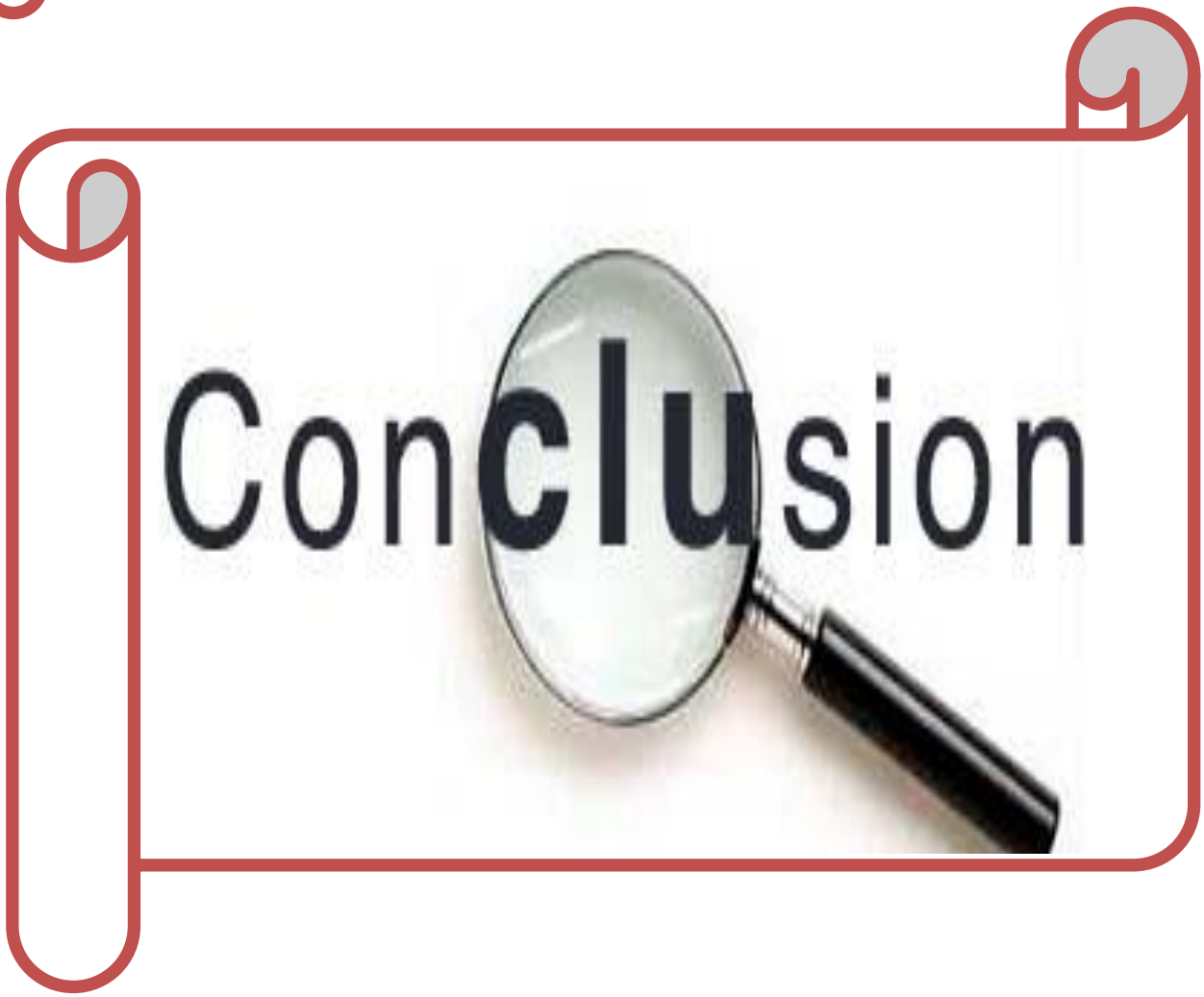


This showed the ZOI of combination ciprofloxacin and imipenem against resistant *Salmonella typhi*. The different concentration were applied to the co-culture plates. The ZOI were observed 19, 21, 25 and 32 mm respectively.

As the values obtained individually was 0-5 mm for ciprofloxacin, while higher values of 19-32 mm were obtained for the combination of ciprofloxacin and imipenem. This indicates the synergistic effects of the antibiotics combination.

Chapter-Five

Discussion & Conclusion



Discussion and Conclusion:

In our present study, resistant strain of *S.typhi* was developed after co-culture with *S. flexineri*. The fresh cultures were used for the sensitivity test by disc diffusion method. The study was done to find out effective combination antibiotics against microorganism after co-culture with resistant pathogenic bacteria. In recent year, some *S.typhi* has gone evolutionary change and become resistant to several drugs. Moreover, various antibiotics combinations are used to treat against multi-drug resistant *S.typhi.*

It has been showen from a study that eleven of the *S. typhi* strains were resistant to chloramphenicol and trimethoprim. But the strains were susceptible to cephalosporin, gentamicin, amoxicillin plus clavulanic acid and imipenem. (Shanban, *et al.,* 1998)

Our present study also showed that *S. typhi* strain was sensitive against ciprofloxacin, vancomycin and imipenem. The zone of inhibition (ZOI) was 20 mm when the concentration of ciprofloxacin was 20μg/ml. The ZOI were increased 24, 25 and 27 mm when different concentration (40, 60 and 80 μg/ml) respectively.
The zone diameter 20-23 mm and 23-27 mm were exhibited by the *S.typhi* against antibiotic vancomycin and imipenem respectively. However, *Shigella flexneri* did not show any zone after treatment with ciprofloxacin, vancomycin and imipenem.

The previous study shows minimum inhibitory concentration (MICs) of ciprofloxacin, ofloxacin, levofloxacin and gatifloxacin against *S. typhi* and *S. paratyphi*. Nalidixic acid resistant to *S.typhi* and *S.paratyphi* was seen. The higher MICs were also observed with fluoroquinolone by *S.typhi* and *S.paratyphi*. (Sangeeta, *et al.*, 2007)

In our present study we did not perform experiment on MICs against our resistant pathogen. Future studies are required to observe the MICs of antibiotics used in the study.

Our present study also showed resistant *Salmonella typhi* did not give optimum ZOI when treated with antibiotics ciprofloxacin, vancomycin and imipenem. The different concentrations (20, 40, 60 and 80 μg/ml) of antibiotics were applied to the culture plates.

Synergistic and additive effects of the two drugs vancomycin and imipenem against methicillin-resistant *S. aureus* were observed in previous study Totsuka, K *et al.* in 1999. The combined effects seemed to be synergistic. A single administration of either drug did not show anti-MRSA effects.

Here, we used two combination antibiotics to find out effective combination therapy against resistant *Salmonella typhi*. The ZOI of resistant *S.typhi*was obtained (18-29 mm) against the combination vancomycin and imipenem.
The effects of 26 different beta-lactam-vancomycin and 8 different aminoglycoside-vancomycin combinations were observed against methicillin-resistant *S.aureuses* strains. The best effects of vancomycin were obtained with imipenem, cefazolin, or netilmicin. The combination of imipenem-vancomycin and cefazolin-vancomycin provided a synergistic bacteriostatic effect against 22 strains. (Rochon-Edouard, *et al*. 2000)

In our present study, resistant *Salmonella typhi* showed sensitivity againstthe combination ciprofloxacin and imipenem. When the value of ZOI is exhibited more than the normal value it seemed to be synergistic. Usually two or more combined drugs give the synergistic effects. Our study showed synergistic effect which corroborates with the findings of Totsuka, K *et al.*,1999 and Rochon-Edouard, *et al*. 2000.

In conclusion, *Salmonella typhi* showed sensitivity when treated with different antibiotics. The different ZOI of *S. typhi* were observed against different antibiotics ciporofloxacin, vancomycin and imipenem. But *S.flex* did not give any ZOI against these antibiotics. The antibiotics ciprofloxacin, vancomycin and imipenem were applied against resistant *Salmonella typhi*. The newly resistant strain *S. typhi* did not give optimum ZOI.
Combination of vancomycin and imipenem was sensitive against *S.typhi* with zone diameter 18-29 mm. The different concentrations were applied to the test plates. The values obtained individually were 0-4 mm for vancomycin and 4-9 mm for imipenem. The higher values of ZOI

(19-32 mm) were obtained for the combination of ciprofloxacin and imipenem. Combination antibiotics were 10% more effective against *S. typhi*i n comparison to single antibiotic. This values are indicates the synergistic effects.

In Bangladesh prospect, the antibiotic combination should be tested in our lab against clinically isolates pathogen such as *K. pneumonia*. KP is responsible for nosocomial infection in hospitalized patients. Combination of two carbapenems was successfully evaluated and tested in 21 patients with carbapenem-resistant *K. pneumonia* isolates. About 81% clinical successes and 96% microbiological cure were observed. (Kontopidou, F, *et al.* 2014)

The antibiotics combination against various resistant can be used. In order to find out the efficacy combination therapy can be a potential source. As the antibiotic resistant is increasing day by day, combination antimicrobials with existing antibiotics may have more effective to inhibit multi-drug resistant pathogens.

Chapter-Six

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