ANTIMICROBIAL SUSCEPTIBILITY STUDY OF DIFFERENT BRANDS OF AZITHROMYCIN

A

Dissertation submitted to the Department of Pharmacy,

East West University Dhaka in partial fulfillment of the requirement for

the degree of Bachelor of Pharmacy

Submitted by

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DEDICATION BY THE CANDIDATE

I hereby declare that this thesis entitled "**Antimicrobial Susceptibility Study of Different Brands of Azithromycin**" is a genuine research work carried out by me, Layla Azmayri Kabir, ID: 2009-3-70-050, under the guidance of Farhana Rizwan, Associatte Professor, Department of Pharmacy, East West University, Dhaka.

Date: **Signature by the candidate**

Place: Dhaka _____

CERTIFICATE

This is to certify that the Project entitled "**Antimicrobial Susceptibility Study of Different Brands of Azithromycin**" is submitted to the department of pharmacy, East West University in partial fulfillment of the requirements of the degree of Bachelor of Pharmacy which was carried out by **Layla Azmayri Kabir** (ID # 2009 – 3 – 70 – 050), a student of our Department under the guidance and supervision of **Farhana Rizwan**, Assistant Professor of our Department and that no part of the Project has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed of in this connection is duly acknowledged.

…….

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CERTIFICATE

This is to certify that the Project entitled "**Antimicrobial Susceptibility Study of Different Brands of Azithromycin**" is submitted to the department of pharmacy, East West University in partial fulfillment of the requirements of the degree of Bachelor of Pharmacy which was carried out by Layla Azmayri Kabir (ID # 2009 – 3 – 70 – 050) under my guidance and supervision and that no part of the Project has been submitted for any other degree. I further certify that all the sources of information and laboratory facilities availed for the conduction of this Project is properly accredited.

.....

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LIST OF ABBREVIATION

cm	Centimeter
UVC	Ultraviolet Light C
µl	Micro liter
MIC	Minimum Inhibitory Concentration
ml	Milliliter
HEPA	High Efficient Particulate Air
UV	Ultraviolet

ABSTRACT

Azithromycin is a macrolide antibiotic used in Bangladesh, India & other parts of the world as the treatment of bacterial infections, most often those causing middle ear infections, strep throat, pneumonia, typhoid, bronchitis, sinusitis etc. It is derived from erythromycin. It has a similar antimicrobial spectrum like erythromycin. It is more effective against certain gram-negative bacteria than erythromycin. The purpose of this research was to identify antibacterial activity of different brands of azithromycin.

To give a small dose & to inhibit the bacterial growth to know the MIC is very important to know. Azithromycin of the company Beximco, Orion, ACI, Navana & Opsonin were used in this research. We identified the antibacterial activity of these brands to know the MIC using disk diffusion method by measuring the zone of inhibition in cm range. According to this method the bacterial isolation was isolated at a standard concentration with 0.9% NaCl. This isolation was given on the agar plate. Nutrient agar is used as agar medium. Here ethanol was used as antiseptic to clean the spreader at the time of spreading the bacterial isolation. Commercially prepared disks were dispensed onto the agar surface on agar plate. Before dispensing each of the disks was impregnated with that standard concentration of a particular antibiotic. An overnight incubation was done to observe the bacterial growth around each disk. Using scale the zone of inhibition was measured in cm range to know the better antibacteial activity of brand of azithromycin of those five companies. The higher data represented the MIC of a specific brand comparing with the data of azithromycin as standard & proves the better antibacterial activity of that brand.

Keywords: Azithromycin, MIC, 0.9% NaCl, Nutrient agar, Ethanol, etc.

Chapter 1

Literature review

1.1 Azithromycin and the risk of cardiovascular death.

Azithromycin is considered as having minimal cardiotoxicity. The people who do not take antibiotics have less risk of cardiovascular death. Besides it patient taking azithromycin have an increased risk of cardiovascular death. They have also possibility of death from any cause. Azithromycin is specially related with an increased risk of cardiovascular death. They can face death from any cause. That means the risk of cardiovascular death is significantly greater with the use of azithromycin ciprofloxacin. (Ray WA, *et al*, 2012).

1.2 Meta-analysis of the adverse effects of long-term azithromycin use in patients with chronic lung diseases.

In this study the adverse effects of azithromycin is evaluated. For this purpose the patients with chronic lung diseases were chosen. Date of this study was compared with other data on microbiological study & articles. This meta-analysis shows higher risk of bacterial resistance in patients receiving long-term azithromycin. The patients receiving long-term azithromycin treatment have lower risk of bacterial colonization. They also have risk of increased impairment of hearing. This analysis shows the potentiality of being aware for the use of azithromycin for long-term use. (Li H, *et al*, 2013).

1.3 Antimalarial drug resistance in Bangladesh, 1996-2012.

Malaria causes by the attack of Plasmodium falciparum. In this study, data is about the summary antimalarial drug resistance from Bangladesh comparing to other articles frpm published journal. This study shows the variation of resistance to chloroquine, mefloquine & sulfadoxine-pyrimethamine. Artemisinin derivatives like artesunate are highly effective as antimalarial drug. Azithromycin-artesunate combination therapy shows high efficacy in the treatment of malaria in Bangladesh. (Haque U *et al*, 2012).

1.4 Effectiveness and safety of macrolides in bronchiectasis patients: A meta-analysis and systematic review.

In case of bronchiectasis patients macrolides are considered as effective therapeutic anti-inflammatory agent. In this study the efficacy & safety of macrolides in bronchiectasis is evaluated. This study shows that if patients having bronchiectasis take macrolides for long-term use they have opportunity of facing less incidence of pulmonary exacerbation. This analysis shows no evidence of increased adverse effects with the use of macrolides as such as azithromycin. (Shi ZL, *et al*, 2013).

1.5Azithromycin for the treatment of gastroparesis.

In this study the efficacy of azithromycin is evaluated for the treatment of gastroparesis. Azithromycin is used as prokinetic agent. Although initially the treatment of gastroparesis is done with erythromycin but it has some side effect. Erythromycin is initially used for the greatest effect on gastric emptying. This analysis shows the effectivenees of another microlide as such as azithromycin. It has fewer drug interactions & a longer half-life. It has less gastrointestinal adverse effects. The patients with gastric & small bowel dysmotility get higher benefit with the use of azithromycin. Thus this analysis proves the evidence of its use in gastroparesis. **(**Potter TG, Snider KR, 2013).

1.6 Local and systemic antimicrobial therapy in periodontics.

The aim of this analysis is to show the efficacy of antimicrobial therapy like with azithromycin in the treatment of periodontitis. Azithromycin is clinically effective in periodontitis. Amoxicillin, metronidazole is only effective in aggressive periodontitis but azithromycin is effective in chronic periodontitis. Besides its efficacy azithromycin is avoided because of the debate on its cost effectiveness & adequate indications. (Herrera D, *et al*, 2012).

1.7 Azithromycin: a new concept in adjuvant treatment of periodontitis.

Mostly patients with periodontitis are clinically treated by scaling & root planning. Azithromycin is effective against Gram-negative aerobic 7 anaerobic bacteria. It has a long half-life in periodontal tissues. The aim of this study is to show the efficacy of using azithromycin in the treatment of periodontitis. This analysis compares with different articles of published journal & indicates that azithromycin is used as an adjuvant treatment for chronic periodontitis. It significantly reduces probing depth & increases periodontal attachment. (Muniz FW, *et al*, 2013).

1.8 New life for macrolides.

The main target of this article was to anayze & discusses the role of azithromycin in non-eosinophilic severe asthma. It also discussed about antineutrophil activity, an effect on gastroesophageal reflux or antibacterial activity against an underlying chronic infection, such as Chlamydia pneumoniae. Macrolide antibiotics have a great role in the therapy of chronic inflammatory diseases. It is based on their additional anti-inflammatory and immunosuppressive properties. In this study it proved that the efficacy & safety of long-term treatment with azithromycin in severe non-eosinophilic asthma. It also showed no increased risk of pneumonia or other adverse effects on any cardiovascular events with.(Solidoro P, et al, 2013)

1.9 Prophylactic antibiotic therapy for chronic obstructive pulmonary disease (COPD).

This study was done with the macrolide antibiotics on coronary obstructive pulmonary disease. Here the continuous use of microlides like azithromycin gave benefited result. (Herath SC, et al, 2014).

Chapter 2

Introduction

2.1 Definition of Microbiology

The word microbiology has come from the greek word. According to that greek word it is the study of microscopic organisms. These oraganisms include bacteria, fungi, algae, protozoa & viruses. This part of science discuss about their form, structure, reproduction, metabolism & classification. (Pelczar, et al,1996).

These microscopic organisms can be unicellular, multicellular or acellular. Eukaryotic microorganisms are membrane bound cell organelles. They are the fungi & protists. On the other hand all of the prokaryotic organisms are microorganisms. They are conveniently considered as lacking membrane-bound organelles.Some of the microorganisms are beneficial & others are detrimental. (Vasanthakumari, 2007)

2.2 History of Microbiology

During the thirteenth century Roger bacon invented that there are some invisible living oraganisms in our world that produce disease. This same opinion was given again by the scientist Girolamo Fracastoro & Anton von Plenciz, although they had no proof to rely. Athanasius Kircher was the first person to find the importance of bacteria & other microbes in disease. In 1665 Robert Hooke gave description of cells in a piece of cork. (Pelczar, et al,1996).

In 1675 Antony van Leeuwenhoek is the first scientists to give accurate descriptions & drawings. He made more than 250 microscopes during his lifetine. He observed microorganisms by using these microscopes. His microscopes were capable to give clear view. So, it was easy for him to give clear description on his little animals in great detail. (Naveen, 2010)

His reports on the microbes helped the acientists 200 years later. Using this reports Loius Pasteur, Robert Koch, Theobald Smith discovered the relation of microbes with disease. Louis Pasteur & Robert Koch are considered as the father of microbiology. Pasteur established the theory of spontaneous generation. He is also famous for pasteurization. It is the method for food preservation. He invented vaccines against anthrax, fowl cholera & rabies. Koch established the

germ theory of disease & the guidelines which is known as Koch's postulates. This guideline helps to identify the causative agent of an infectious disease. He was the first scientist who isolated bacteria in pure culture that cause anthrax & tuberculosis. Pure cultures of bacteria were first obtained by Joseph Lister in 1878. He used serial dilutions in liquid media. (Pelczar, et al, 1996).

2.3Classification of microbiology

Microbiology is classified into pure & applied sciences. The major field of this microbiology is classified in different branches. (Pelczar, et al, 1996).

2.3.1Pure microbiology

According to the pure microbiology the different branches of microbiology are as follows:

- **Bacteriology**
  It is the study of bacteria. This term is also used as a synonym for microbiology. (Pelczar, et al, 1996).
- **Protozoology**
  It is the study of protozoa. In protozoology they are differentiated on the basis of morphological, nutritional, & physiological characteristics. (Pelczar, et al, 1996).
- **Parasitology**
  It is a special branch of protozoology. It is the study with parasitic or disease producing protozoa & also with other parasitic micro & macroorganisms. (Pelczar, et al, 1996).
- **Mycology**
  It deals with the study of fungi such as yeasts & molds. It discuss about its importance in the production of alcoholic beverages, responsibility in decomposition of many materials etc. (Pelczar, et al, 1996).
- **Phycology**
  It is the science that deals with algae. It discuss about the importance of it in the production of food in aquatic environments, in pharmaceutical preparations etc. (Pelczar, et al, 1996).

- **Virology**

  It is the study of viruses. It discuss about the involvement of it in various diseases in humans, other animals, & plants. (Pelczar, et al, 1996).

2.3.2 Applied microbiology

According to the applied microbiology the different branches of microbiology are as follows:

- **Medical microbiology**

  It is the science that deals with the study of causative agents of disease, diagnostic procedures for the identification of causative agents & preventive measures of disease. (Pelczar, et al, 1996).
- **Aquatic microbiology**

  It has role in water purification, microbiological examination, biological degredation of waste & waste. (Pelczar, et al, 1996).
- **Aeromicrobiology**

  This science is applied on the areas of contamination & spoilage & dissemination of diseases. (Pelczar, et al, 1996).
- **Food microbiology**

  It is applied on the area of food preservation & preparation, foodborne diseases & their prevention. (Pelczar, et al, 1996).
- **Agricultural microbiology**

  It is applied on the area of soil fertility, plant & animal diseases. (Pelczar, et al, 1996).
- **Industrial microbiology**

  It deals with the production of medicinal products such as antibiotics, & vaccines, fermented beverages, industrial chemicals, production of proteins & hormones by genetically engineered microorganisms. (Pelczar, et al, 1996).
- **Exomicrobiology**

  It has role on the exploration for life in outer space. (Pelczar, et al, 1996).
- **Geochemical microbiology**

  It deals with the study of coal, mineral& gas formation, prospecting for deposits of coal, oil & gas etc. (Pelczar, et al, 1996)

2.4 Bacteria

Bacteria are the simplest organisms living on earth. They are considered as the first organisms to evolve on earth. They are few micrometers in length. They were the first life forms to appear on earth. They are present in most habitats on the planet. They inhabit water, radioactive waste, acidic hot springs, soil. They also live in animals & plants. They have flourished in manned space vehicles. (Vasanthakumari, 2007)

In the world most of the bacteria have not been characterized. In the laboratory only about half of the phyla of bacteria have species that can be grown. In the human flora there are approximately ten times as many bacterial cells as there are human cells in the body. There are also large numbers of bacteria on the skin and as gut flora. In our body the vast majority of the bacteria are rendered harmless because of the protective effects of the immune system. Some of them are beneficial. (Vasanthakumari, 2007)

They perform important role in natural cycling of elements which contributes to soil fertility. They are also useful in industry for the manufacture of valuable compounds. Some of them spoil foods. They plays a great role in sewage treatment, the breakdown of oil spills, the production of cheese & in yogurt through fermentation. They help in the recovery of gold, palladium, copper and other metals in the mining sector. (Presscott, et al, 1993).

Bacteria can be used for the industrial production of amino acids. *Corynebacterium glutamicum* is one of the most important bacterial species with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine. (Presscott, et al, 1993).

Both Gram-positive and Gram-negative bacteria may have a membrane called an S-layer. In Gram-negative bacteria, the S-layer is attached directly to the outer membrane. In Gram-positive bacteria, the S-layer is attached to the peptidoglycan layer. Unique to Gram-positive bacteria is the presence of teichoic acids in the cell wall. Some particular teichoic acids, lipoteichoic acids, have a lipid component and can assist in anchoring peptidoglycan, as the lipid component is embedded in the membrane. (Presscott, et al, 1993).

Fig: 2.1 Different shapes of bacteria

In biotechnology they are greatly used. Bacteria also help in the manufacture of antibiotics & other chemicals.In developed countries, antibiotic resistance is becoming common. The main reason of it is the use of antibiotics to treat bacterial infections & also in farming. (Prescott, et al, 1993; Pelczar, et al, 1996).

2.5 Morphology of bacteria

Bacteria shows a wide variety in case of its size & shape. It is called morphology of bacteria. They are very small. They are approximately 0.5 to 1.0 m in diameter. Some of them are visible to the unaided eye. They have high surface area. It limits the size of bacteria to microscopic dimensions. (Prescott, et al, 1993; Ananthanarayan, et al, 2005)

Most species of bacteria are of two shapes. One of them is spherical shape, called cocci & rod-shape, called bacilli**.** Elongation of bacteria is associated with swimming. Some bacteria are of slightly carved rod shaped or comma-shaped like vibrio. Other bacteria are of spiral-shaped like spirilla or are of tightly coil shaped like spirochaetes. A very small number of species are of tetrahedral or cuboidal shapes. (Prescott, et al, 1993; Ananthanarayan, et al, 2005)

The wide variety of bacterial shapes is determined by the bacterial cell wall& cytoskeleton. This variety of shape is very important. Because it helps the bacteria to acquire nutrients, attach to surfaces, swim through liquids & escape from predators. (Ananthanarayan, et al, 2005)

2.6 Structure of bacteria

Bacterial structure contains various components. Some of these are external to the structure which is known as extracellular structure. Others are internal to the call wall which is known as intracellular structure. (Ananthanarayan, et al, 2005)

2.6.1 Intracellular structure

The bacterial cell is made of a lipid membrane. It is also known as a cell membrane or plasma membrane. This membrane encloses the contents of the cell. It acts as a barrier. It holds nutrients, proteins & other essential components of the cytoplasm within the cell. Bacteria do not usually have membrane-bound organelles. It contains a few large intracellular structures. Nucleus, mitochondria, chloroplasts & the other organelles are absence in their structure. (Naveen, 2010)

Micro-compartments such as carboxysomes give a further level of organization. These are the compartments within bacteria. They are surrounded by polyhedral protein shells instead of by lipid membranes. These polyhedral organelles localize &compartmentalize bacterial metabolism. It is one type of function which is performed by the membrane-bound organelles in eukaryotes. (Pelczar, et al, 1996)

They follow the whole characteristics of prokaryotic.it is important for them to protect themselves by their cell wall from the antibiotics. They follows many rules to adjust with the environment. Their structure is made in that way to protect themselves. They are specially identified by gram staining test. Some of them retain the crystal violet color. They are called gram-positive bacteria. Some of them do not retain crystal violet color. They are called gram-negative bacteria. They contains different characteristics. As a result they show different result in case of different antibiotics. They are capable to pass nutrients & other materials through their cell wall. (Pelczar, et al, 1996)

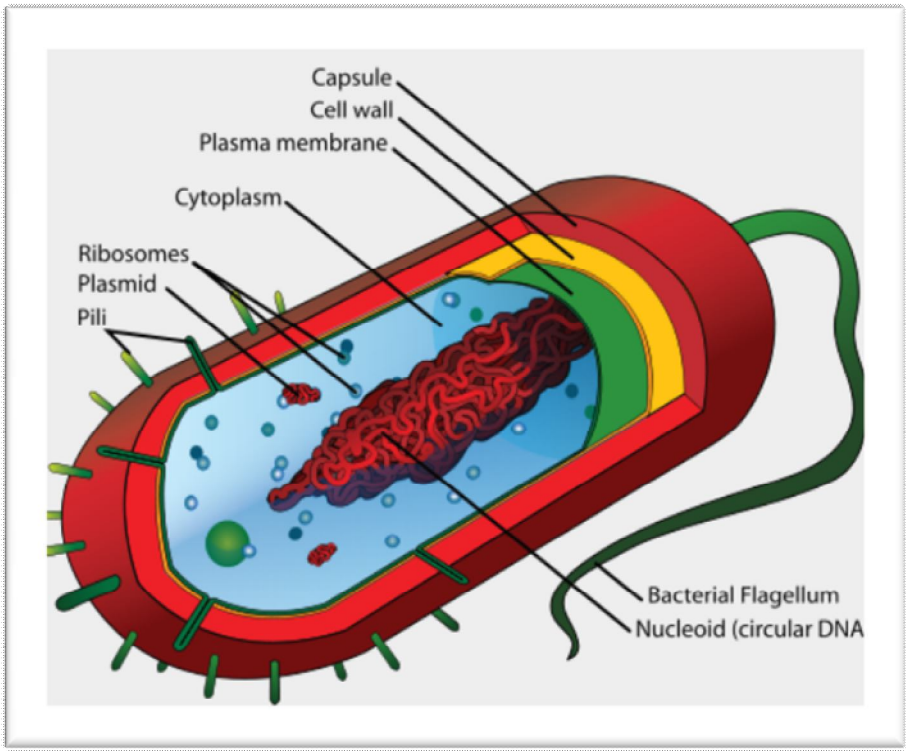

Fig: 2.2 Structure & the contents of a typical gram-positive bacteria cell

Many important biochemical reactions use the concentration gradients. These biochemical reactions are energy generation. This reactions use the concentfration gradients across membranes. Electron transports occur across the cell membrane between the cytoplasm & the periplasmic space. The plasma membrane is highly folded in many photosynthetic bacteria. This membrane fills most of the cell with layers of light-gathering membrane. These light-gathering complexes can form lipid-enclosed structures. This is called chlorosomes. It is formed in green sulfur bacteria. Other proteins import nutrients across the cell membrane. They also expel or remove undesired molecules from the cytoplasm. (Naveen, 2010).

In case gram-positive bacteria S layer is directly attached to the peptidoglycan layer. In case of gram-negative bacteria the S layer is directly attached to the cell wall. For that reason it is impossible for them to cope up with each other in case of characteristics. (Naveen, 2010).

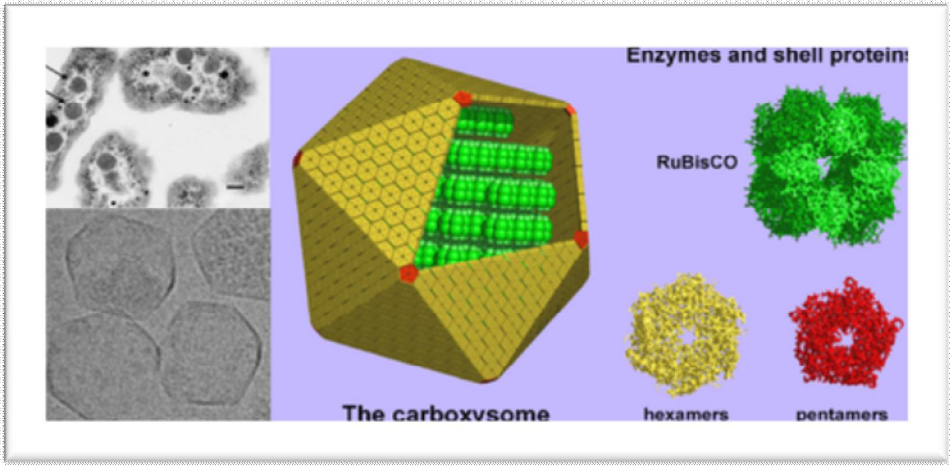

Fig:2.3 Microcompartments of the bacteria

Carboxysomes are organelles that are enclosed by protein. In the top left it is an image of carboxysomes of *Halothiobacillus neapolitanus* by electron microscope. Below this image it is an image of purified carboxysomes & on the right side of it this it is a model of their structure & its Scale bars are 100 nm. In most cases bacteria do not have a membrane-bound nucleus. Their genetic material is typically a single circular chromosome. It is located in the cytoplasm in an irregularly shaped body. This irregularly shaped body is called as nucleoid. It also contains associated proteins & RNA of chromosomes. (Pelczar, et al, 1996; Naveen, 2010)

The phylum Planctomycetes are an exception in bacteria. It remains in case of general absence of internal membranes in bacteria. They have a double membrane around their nucleoids. It contains other membrane-bound cellular structures. Bacteria contain ribosomes in their intracellular structure. They are often grouped in chains which are called polyribosomes. They remain in their body for the production of proteins. The structure of the ribosome of bacteria is totally different from that of eukaryotes & Archaea. Ribosome of bacteria contains a sedimentation rate of 70S. Their subunits have rates of 30S & 50S. Some of the antibiotics bind specifically to 70S ribosomes. This binding inhibits bacterial protein synthesis. These antibiotics kill bacteria without affecting the larger 80S ribosomes. They do not cause any harm to the host. (Ananthanarayan, et al, 2005)

Some of the bacteria have intracellular nutrient storage granules. They produce it for later use. They are glycogen, polyphosphate, sulfur or polyhydroxyalkanoates. Some species of bacteria like photosynthetic cyanobacteria can produce internal gas vesicles. They use it to regulate their buoyancy. This allow them to move up or down into water layers with different intensity of light & different levels of nutrient. Membranes of intracellular are called chromatophores. They are also found in the membranes of phototropic bacteria. They are used primarily for photosynthesis & contain bacterichlorophyll pigments & carotenoids. (Vasanthakumari, 2007)

At the time before it was considered that bacteria might contain membrane folds termed mesosomes. Now it has shown to are artifacts produced by the chemicals. This is used to prepare the cells for electron microscopy. Inclusions are considered to be nonliving components of the cell. They do not possess metabolic activity. They are not bounded by membranes. Glycogen, lipid droplets, crystals & pigments are the common inclusions. (Ananthanarayan, et al, 2005)

Volutin granules are the inclusions of cytoplasm. These granules are complexed inorganic polyphosphate. They are called as metachromatomatic granules at the time of showing their metachromatic effect. At the time of staining with the blue dyes methylene blue or toluidine blue, they appear as red or blue. Gas vacuoles are present in some species of Cyanobacteria. They are membrane-bound vesicles & are freely permeable to gas. By this vacuoles bacteria are allow to control their buoyancy. (Pelczar,et al, 1996)

Microcompartments are widespread membrane-bound organelles. They are made of a protein shell which surrounds & encloses various enzymes. Carboxysomes & megnetosomes are bacterial compartments. Carboxysomes contain the enzymes involved in carbon fixation. Magnetosomes are present in magnetotactic bacteria. These contain magnetic crystals. (Pelczar, 1993).

2.6.2 Extracellular structures

Most of the bacteria contain cell wall on the outside of their cytoplasmic membrane. Cell envelope is consisted of the plasma membrane & cell wall. Peptidoglycan is the common bacterial cell wall material. It is made from polysaccharide chains. This is cross-linked by peptides containing D-amino acids. Cell walls of bacteria are totally different from plants & fungi. Their cell walls are made of cellulose & chitin. Bacterial cell wall is also different from

Archaea because they do not contain peptidoglycan. The cell wall is very essential for the survival of many bacteria. Antibiotic like penicillin kill bacteria by inhibiting a step in the synthesis of peptidoglycan. (Ananthanarayan, et al, 2005)

There are two different types of cell wall in bacteria. They are called Gram-positive & Gram-negative. This name of bacteria has originated from the reactions of cells to the Gram stain. This test of Gram stain has been employed for the classification of bacterial species.
(Ananthanarayan, et al, 2005)

2.6.2.1Gram-negative bacteria

Gram-negative bacteria are the bacteria that show different views comparing to gram positive bacteria in Gram staining protocol. They do not retain crystal violet dye in the Gram staining protocol. In the test of gram stain a counterstain which is commonly known as safranin is added after the crystal violet which colors all gram-negative bacteria with a red or pink color. In this test the counterstain is used to visualize the colorless gram-negative bacteria. It's much thinner peptidoglycan layer does not retain crystal violet. The test is very useful in classifying two specific types of bacteria. This test is based on the structural differences of their bacterial cell walls. In case of gram-positive bacteria it retains the crystal violet dye after washing in a decolorizing solution. Between these two types of bacteria, gram-negative bacteria are more resistant against antibiotics than gram-positive bacteria. The main reason is that relatively impermeable lipid membrane of gram-negative bacteria. (Pelczar, et al, 1996)

Lipopolysaccharide layer is a particular component of gram-negative cell envelope. It is also known as LPS or endotoxin layer. This LPS is often associated with the pathogenic capability of gram negative-bacteria. In human body an innate immune response is triggered by LPS. This innate immune response is characterized by cytokine production & immune system activation. The common result of cytokine production is inflammation. It can also produce toxicity in host. Pathogenicity means the ability to cause disease. It is not synonymous with the innate immune response to LPS. Mainly the LPS trigger the innate immune response alone. (Naveen, 2010)

2.6.2.1.1 Characteristics of Gram-negative bacteria

- Cytoplasmic membrane
- Thin peptidoglycan layer which is much thicker in gram-positive bacteria
- In the outer leaflet of its outer membrane contains lipopolysaccharide & in the inner leaflet it contains phospholipids. LPS consists of lipid A, core polysaccharide, & O antigen.
- In the outer membrane porins exist. It acts like pores for particular molecules.
- Between the secondary cell membrane called the periplasmic space & the layers of peptidoglycan there is a space.
- The S-layer rather than the peptidoglycan is directly attached to the outer membrane.
- If present, instead of two flagella have four supporting rings
- They have no teichoic acids or lipoteichoic acids
- In the polysaccharide backbone lipoproteins are attached
- Most of the gram-negative bacteria have Braun's lipoprotein. It serves as a link between the peptidoglycan chain & the outer membrane by a covalent bond. (Ananthanarayan, et al, 2005)

2.6.2.1.2 Examples of Gram-negative Bacteria

Gram-negative bacteria are also known as proteobacteria. They are *Escherichia coli* or *E. coli, Salmonella, Shigella,* & *Pseudomonas, Moraxella, Stenotrophomonas, Bdellovibrio,* acetic acid bacteria, *Legionella* & numerous others. There are some other notable groups of gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur & green non-sulfur bacteria. (Ananthanarayan, et al, 2005)

The gram-negative cocci include the three organisms that causes a sexually transmitted desease *(Neisseria gonorrhoeae),* a meningitis *(Neisseria meningitidis),* & respiratory symptoms *(Moraxella catarrhalis).* Gram-negative bacilli include different types of species. Some of the species cause problems of respiratory systems. They are *Hemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa.* (Pelczar, et al, 1996)

2.6.2.2 Gram-positive bacteria

Gram-positive bacteria are stained dark blue or violet in the Gram stain test. They are capable to retain the crystal violet stain. It is because of their thick peptidoglycan layer. That is superficial to the cell membrane. (Ananthanarayan, et al, 2005)

PG layer, cell wall & the plasma membrane are three specific structures. As for example, plant cells contain rigid cell walls in addition to an outer plasma membrane. On the other side, animal cells contain only plasma membranes. For structural support & rigidity cell walls are very important. That is also needed for the survival of plant cells because they are not motile organisms. Their survival also depends on strong & rigid structures. Animal cells & gram-positive cells have some similarities. Both of them are amorphous & can easily change shape. (Pelczar, et al, 1996)

2.6.2.2.1 The characteristics of Gram-positive bacteria

- They contain cytoplasmic lipid membrane
- They have thick peptidoglycan layer. In this layer teichoic acids & lipoids are present. This forms lipoteichoic acids. It serves as chelating agents & also for certain types of adherence.
- Capsule polysaccharides which is only in some species
- Flagella which is only present in some species. If it is present, it contains two rings for support, because Gram-positive bacteria have only one layer of membrane.
- Pentaglycine chains helps in cross-linking of the individual peptidoglycan molecules. A DD-tranpeptidase enzyme also helps in this cross-linking. (Belland, 2012)

2.6.2.2.2 Examples of Gram-positive bacteria

Gram-positive bacteria are Staphylococcus, Streptococcus, Bacillus, Clostridium, Corynebacterium, Mycobacterium etc. (Byarugaba, 2010)

2.6.2.3 Difference of structure between Gram-negative & Gram-positive bacteria

Although Gram-positive bacteria & Gram-negative bacteria have a membrane called an S-layer, in case of Gram-negative bacteria it is attached directly to the outer membrane. In case of Gram-positive bacteria, S-layer is attached to the peptidoglycan layer. The basic difference between these two types of bacteria is the presence of teichoic acids in the cell wall. Some of the teichoic acids, lipoteichoic acids contain a lipid component. They help to assist in anchoring peptidoglycan. (Prescott, et al, 1993)

The differences of structure between these Gram-positive & Gram-negative bacteria can produce differences in antibiotic susceptibility. As for example, vancomycin is only effective against Gram-positive bacteria. These drugs are totally ineffective against Gram-negative pathogen like Haemophilus influenzae or Pseudomonas aeruginosa. After removing the cell wall of bacteria entirely, it is called a protoplast. When this cell wall is partially removed, then it is called as a spheroplast. (Naveen, 2010)

2.7 Antibiotic

The word antibiotic has come from two greek word, anti & bios. Here *anti* means against & *bios* means life. Antibiotics are the drugs that inhibit the growth of bacteria. These drugs also destroy the bacteria that cause infection. These drugs do not work against any type of viral diseases. As for example, the common cold or influenza is the viral diseases. Microbial infections are the infections that caused by microorganisms. (Ananthanarayan, et al, 2005)

2.7.1 History of antibiotics

Before 1930s, there were few effective ways of curing microbial infections. From 1930s, antibiotics have started to prevent a wide variety of infections in plants, animals & humans. It cannot prevent illnesses like pneumonia, tuberculosis, & typhoid fever. At that time minor infections cause death. (Ananthanarayan, et al, 2005)

The discovery & development of antimicrobial drugs has done in the year between 1928 & 1940. In 1928 a Scottish physician, named Sir Alexander Fleming has done a great job in the discovery

or the development of antimicrobial drugs. He tried to isolate the bacteria from infected wounds. Then he was working on ways to kill these bacteria. He found the mold growing in a laboratory culture. It was able to destroy that culture's bacteria. Then he identified the mold that produced the bacteria-killing substance. It was a species of *Penicillium*. He named this substance as penicillin. (Ananthanarayan, et al, 2005)

Till now it is unknown to us that when the first antibiotic was used. Various molds were used to fight infections for centuries. In 1935 the first class of antibacterial agents was discovered by Gerhard Domagk. He was a German chemist. The first class of antimicrobial agents is the sulfonamides. Sulfanilamide is the parent compound of the sulfonamides. It was originally part of a leather dye compound. This compound was being screened for its potential ability to kill bacteria. It was found as nontoxic. After the breakdown of the dye in the body, it was converted to the compound sulfanilamide. (Ananthanarayan, et al, 2005)

In 1941 penicillin was first tested in humans. It was being used to treat serious infections. This treatment was dramatic. The patients, who took this drug, got rapid & complete recoveries. In 1948, the naturally occurring antibiotics were discovered. These antibiotics are the bacitracin, chlortetracycline & streptomycin. In 1959 the chemical structure or the ring of penicillin was finally isolated by scientists of Britain & the United States. Then from 1948, a wide variety of substances or antibiotics have discovered. (Ananthanarayan, et al, 2005)

2.7.2 The process of making antibiotics

Majority of antibiotics are made from living organisms' such as bacteria. About 90% of antibiotics are isolated from bacteria fungi, and molds. Other antibiotics are discovered synthetically. It can be whole or in part. (Ananthanarayan, et al, 2005)

At first all antibiotics were produced from the living organisms. The process of producing the antibiotics from the living organisms is known as biosynthesis. It is still used in the manufacture of a number of antibiotics. In this method, only the organisms are used. They manufacture the antibiotic. The technician of laboratory gives favorable conditions to the organisms for multiplication. Then they extract the drug. As for example, mold oraganisms are placed in a medium. This medium is used for the growth of microorganisms. In this medium corn liquor

remains to which milk sugar has been added. This mixture forms a liquid. Then it is put into a tank. It is kept at a temperature of 25 degrees Centigrade or 77 degrees Fahrenheit. Then it is shaken for over 100 hours. In this warm liquid the mold organisms can multiply rapidly. As a result these organisms produce penicillin in the process. (Bearle, 2011).

All types of penicillin contain an identical ring. In each type of penicillin, the structure is different. As for example, the chemical chain attached to the ring is different. After modification of the molecules of the chain, scientists are able to create drugs with a wide range of effects on a variety of organisms. Most of these drugs are useful in treating infections. Pharmaceutical companies use to invent numerous structures. They use the computer-generated images of the rings. They experiment with a countless variety of possible chains. Scientists have developed an antibiotic with long half-lives. Because effectiveness depends on the long or shortness of half-lives. It proves that these drugs can be taken for every 24 hours instead of every few hours. The antibiotics that are invented recently are more effective against a wide range of bacteria. (Goodman, 2010)

2.7.3 How Antibiotics Work

Homeostasis is defined as the balance of body between health & illness. It mainly depends on the relationship of the body to the bacteria. As for example, on human skin we are confirm about the presence of Bacteria. After the injury of skin the bacteria become able to enter the body of the host. As a result it causes infection. If the bacteria can enter into the body, they are usually destroyed by blood cells called phagocytes. This destruction causes by various actions of the immune system. In the presence of too many bacteria, it becomes impossible to control or handle the situation. It causes illness & finally antibiotics are needed to help restore homeostasis. (Seth, 2007)

Antibiotics are of two types. They may be bacteriostatic or bactericidal. Bacteriostatic means that can easily prevent bacteria from multiplying & bactericidal means that kill bacteria. In most infections, these two types of antibiotics show effectiveness equally. A bactericidal antibiotic is usually more effective, when the immune system is impaired. Bactericidal antibiotic is also effective if the individual has a severe infection. Sometimes bactericidal drugs can be

bacteriostatic against some microorganisms. Bacteriostatic drugs can also be bactericidal against some microorganisms. (Katzung, 2010)

In most infections like in different types of pneumonia & urinary tract infections, the bacteriostatic drugs are more effective then bactericidal drugs. Bactericidal activity seems to be necessary in infections. In this activity host defense mechanisms are at least partially lacking locally or systemically. For example, endocarditis, meningitis, & last one is serious staphylococcal infections. Here endocarditis is the inflammation of the lining membrane of the heart. Then meningitis means inflammation of the membranes of the spinal cord or brain. (Molly, 2013)

All of the antibiotics kill or inhibit microorganisms following a unique way. Some of them irritate the structure of the bacterial cell wall. Others interfere with the production of essential proteins. They also interfere with the metabolism or transformation of nucleic acid of microorganisms. Antibiotics interfere in the surface of bacterial cells to make a great change in their ability to reproduce. In the laboratory an antibiotic is tested to see its action. Here the scientists observe that how much exposure to the drug is necessary to decrease reproduction or to kill the bacteria. (Pattrick, 2009)

2.7.4 Classification of Antibiotics

There are varieties of antibiotics produced by naturally, semi-synthetically or synthetically. They shows difference in primary target & variation of effectiveness on the species. From them most commonly used antibiotics are discussed here. (Vasanthakumari, 2007)

- **Fluoroquinolones**

  They are DNA synthesis inhibitor. Example of fluroquinolones are Nalidixic acid, ciprofloxacin, levofloxacin & gemifloxacin. They are Synthetic. their primary target is topoisomerase II & topoisomerase IV. (Rang, 2012)

- **Trimethoprim–sulfamethoxazole**

  They are DNA synthesis inhibitor such as Co-trimoxazole. It is combination of trimethoprim & sulfamethoxazole. They are synthetic. Their primary target is tetra hydrofolic acid synthesis inhibitors. (Vasanthakumari, 2007)

- **Rifamycins**

  They are RNA synthesis Inhibitor. Rifamycins, rifampin & rifapentine are the drug of this class. They are both Natural & semi-synthetic. Their primary target is DNA-dependent RNA polymerase. (Stephen, 2011)

- **β-lactams**

  They are Cell wall synthesis inhibitors. Penicillins like penicillin, ampicillin, oxacillin & cephalosporins like cefazolin, cefoxitin, ceftriaxone, cefepime & carbapenems like imipenem are the drug of this class. They are both natural & semi-synthetic. Their primary target is Penicillin-binding proteins. (Vasanthakumari, 2007)

- **Glycopeptides & glycolipopeptides**

  They are Cell wall synthesis inhibitors. Vancomycin is the drug of this class. They are both natural & semi-synthetic.their primary target is Peptidoglycan Units. (Pomares, 2011)

- **Lipopeptides**

  They are Cell wall synthesis inhibitors. Daptomycin & polymixin B are drugs of this class. They are both natural & semi-synthetic. Their primary target is cell membrane. (Vasanthakumari, 2007)

- **Aminoglycosides**

  They are protein synthesis inhibitors. Gentamicin, tobramycin, streptomycin & kanamycin are drug of this class. They are both natural & semi-synthetic. Their primary target is 30S ribosome. (Vasanthakumari, 2007)

- **Tetracyclines**

  They are protein synthesis inhibitors. Tetracycline & doxycycline is the drug of this class. They are both natural & semi-synthetic. Their primary target is 30S ribosome. (Powers, 2004)

- **Macrolides**

  They are protein synthesis inhibitors. Erythromycin & azithromycin is the drug of this class. They are both natural & semi-synthetic. Their primary target is 50S ribosome. (Vasanthakumari, 2007)

- **Streptogramins**

  They are protein synthesis inhibitors. Pristinamycin, dalfopristin & quinupristin are the drug of this class. They are both natural & semi-synthetic. Their primary target is 50S ribosome. (Saga, 2009)

- **Phenicols**

  They are protein synthesis inhibitors. Chloramphenicol is the drug of this class. They are both natural & semi-synthetic. Their primary target is 50S ribosome. (Vasanthakumari, 2007)

2.7.5 Choosing an Antibiotic

Nowadays physicians are usually able to determine the type of organism responsible for the infections or disease. They try to find the most effective antibiotic in combating it. To identify the invading microorganism, they examined a culture from the infection. This examination is done under a microscope. After the identification of the bacteria & knowing their sensitivity to antibiotics, it becomes difficult to make the choice of antibiotic. (Molly, 2013)

The treatment will be effective or not, it depends on a variety of factors. They are the absorbing capacity of the drug into the bloodstream, the presence of drug in various body fluids, & the rapidness of the elimination of the drug from the body. Drug selection depends on condition or seriousness of illness. It also depends on the side effects of drug, chance of being allergic reactions. Cost of the drug is also important to choose an antibiotic. (Molly, 2013)

2.7.6 Administering Antibiotics

An antibiotic can be applied externally or internally. Externally means to a cut on the skin's surface & internally means through the bloodstream. Antibiotics are manufactured in several forms. They are administered in a variety of ways like topically, orally & parenterally. Topically means the application of antibiotic on the skin or in the eyes. It can be the use of antibiotic on

mucous membrane. Topically used antibiotics are provided in the form of powders, ointments, drops, or creams. (Vasanthakumari,2007)

Antibiotics are given in the form of tablets, liquids & capsules. These forms of drug are easy to swallow. They are released in the small intestine. They are absorbed into the bloodstream. Troches dissolve in the mouth. In case of antibiotics, they are absorbed through the mucous membrane. (Molly, 2013)

Antibiotics are given parenterally. One form of it is an injection. This can be given subcutaneously, intramuscularly & intravenously. Antibiotics are given parenterally when physician think of the necessity of a strong, rapid concentration of these drug into the bloodstream. In case of severe bacterial infections, antibiotics are given as injection or intravenously. After controlling the severe condition they are given orally. These drugs are given until the infecting organisms are eliminated from the body. It may be days after the symptoms disappear. If the treatment is stopped as early as possible then chance of developing resistant bacteria increased. For that reason the antibiotics are given for several days. (Vasanthakumari, 2007)

2.7.7 Preventive Antibiotics

The aim of antibiotics is not only to treat infections. Its aim is also to prevent them like prophylactically. To avoid the development of resistance in bacteria, preventive antibiotic therapy must be used for only a short duration. We have to concern about the potency of the antibiotic against the particular type of bacteria involved. As for example, antibiotics are taken before or during travel in foreign countries to prevent diarrhea. Antibiotcs can be taken in case of people exposed to someone with meningitis. Here antibiotics are taken to avoid of the risk of infection. (Molly, 2013)

Antibiotics are also taken before, during, & after surgery. People with heart valve disorders routinely take antibiotics before surgery. People who have dental infections take antibiotics before dental surgery. These drugs are also taken even dental procedures such as cleaning. The people taking antibiotics before surgery have a risk of attacking endocarditis from bacteria. These bacteria are normally found in the mouth and other parts of the body. They enter the

bloodstream during the dental procedure. At last they travel to the damaged heart valve. Preventive antibiotics are taken by the people who have ineffective immune system, which is not worked properly. In case of leukemia, people receive chemotherapy for cancer. People with AIDS also receive chemotherapy. Healthy people before going to major orthopedic or intestinal surgery also take preventive antibiotics. They also have a high risk of infection. (Molly, 2013)

2.7.8 Therapeutic Use of Antibiotics

The use of antibiotics as therapeutically means to treat clinically ill animals. The importance of good management of medicine must not be underestimated. Antimicrobial therapy has superior power. It can easily address many diseases condition that is difficult to address by any other therapy. In case of animals the use of antibiotics as therapeutically is complicated. This therapeutic use of antibiotics is less complicated in case of human medicine. It gives the variations between species. (Vasanthakumari, 2007)

The main target of doing antimicrobial susceptibility test is to determine the various available options for therapy that will be suitable. We have to consider not only about the bacterial susceptibility at the time of selecting an antibiotic. There are some factors that have to be

Fig: 2.3 Therapeuctic uses of antibiotics.

considered during the selection of an antibiotic from a range of option. They are the drug's attributes like pharmacodynamics, pharmacokinetics, toxicity & tissue distribution. The second one is the host characteristics like age, species, & immune status. The accountability to the public & cost effectiveness of antibiotic should also be considered. Each of these issues is important to make a proper decision during the selection of an antibiotic. (Molly, 2013).

2.7.9 Non-Therapuetic use of antibiotics

Because of high density of population the demand of animal protein has also increased. So, the animals are raised to maximize the amount of utilizable product at the least cost. These rapid animal growths give facilities to the transmission of infectious agents. It also facilitates the susceptibility of the animals to infectious diseases. Antibiotics help in improving the production & prevent disease. That is why food animal producers utilize antibiotics for non-therapeutic purposes. Non-therapeutic applications of antibiotics are the use of antibiotics for growth promotion & the use of antibiotics in animals for metaphylaxis. (Molly, 2013)

2.7.10 Side Effects of Antibiotics

Allergic reactions are usually observed as skin rashes after taking antibiotics. Severe anemia, stomach disorders, & deafness are also observed. Many people outgrow their sensitivity. A large number of antibiotics are available. In most cases allergy-causing drugs can be avoided. (Vasanthakumari, 2007)

People who have taken antibiotics, they have faced side effects. Sometimes people take antibiotics with certain foods or medications. Then the effects of the antibiotic or the risk of side effects may increase. After taking an antibiotic, patient should report about any unusual symptoms to the physician. (Vasanthakumari, 2007)

2.7.11 Antibiotic Resistance

People take an antibiotic for a long period of time. As for example, in case of rheumatic fever, the targeted bacteria may develop their own defense against the drug. An enzyme is a complex protein. It is capable of inducing chemical changes. It remains unchanged. This can easily

destroy the drug. This enzyme may produced by the bacteria. Cell wall can become resistant to being broken by the action of the antibiotic. After being resistant to the antibiotic, an individual become fast against the drug. It means that antibiotic is no longer able to fight the infection. At that time another type of antibiotic must be administered. (Vasanthakumari, 2007)

Several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell, or modify target site so that it is not recognized by the antibiotic. (Vasanthakumari, 2007)

This figure shows different types of mechanisms involve in antibiotic resistance. The most common mode is enzymatic inactivation of the antibiotic. An existing cellular enzyme is modified to react with the antibiotic in such a way that it no longer affects the microorganism. An alternative strategy utilized by many bacteria is the alteration of the antibiotic target site. (Vasanthakumari, 2007)

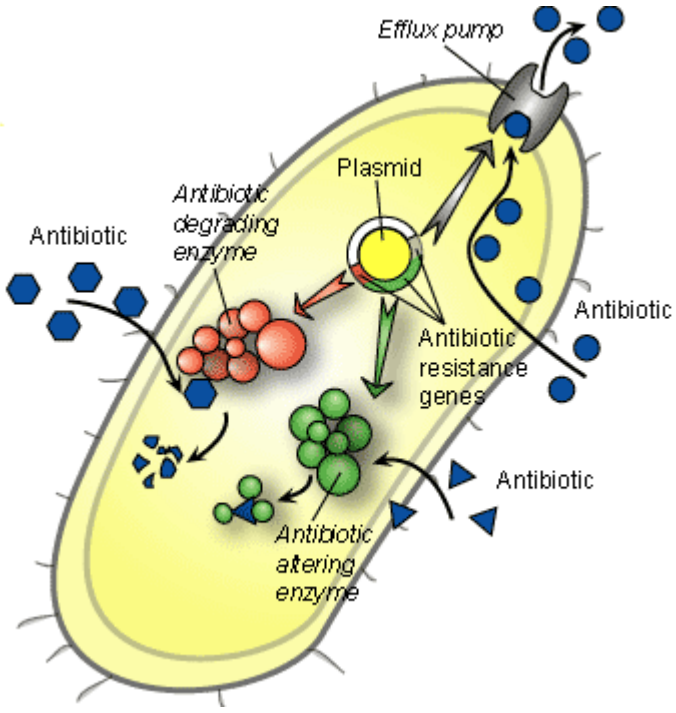

Fig: 2.4 Three types of mechanisms of antibiotic resistance.

2.7.12 Mode of Action Antibiotic

Different antibiotics have different modes of action. It depends on the nature of their structure & degree of affinity to certain target sites within bacterial cells. When the cells of humans & animals do not have cell walls, it becomes so tough for the survival of bacterial species. Drugs that target cell walls can easily kill or inhibit bacterial oraganisms. As for example these drugs are penicllins, cephalosporins, bacitracin & vancomycin. (Vasanthakumari, 2007).

Cell membranes are the important barriers. They play a great role to segregate & regulate the intra & extracellular flow of substances. Any disruption or damage to this structure can be happened. Then it could result in leakage of important solutes essential for the cell's survival. The main reason is that this this structure is found in both eukaryotic and prokaryotic cells. The actions of this class of antibiotics are often poorly selective. They can often be toxic for systemic use in the mammalian host. Example of these types of drugs is polymixin B and colistin. (Vasanthakumari, 2007)

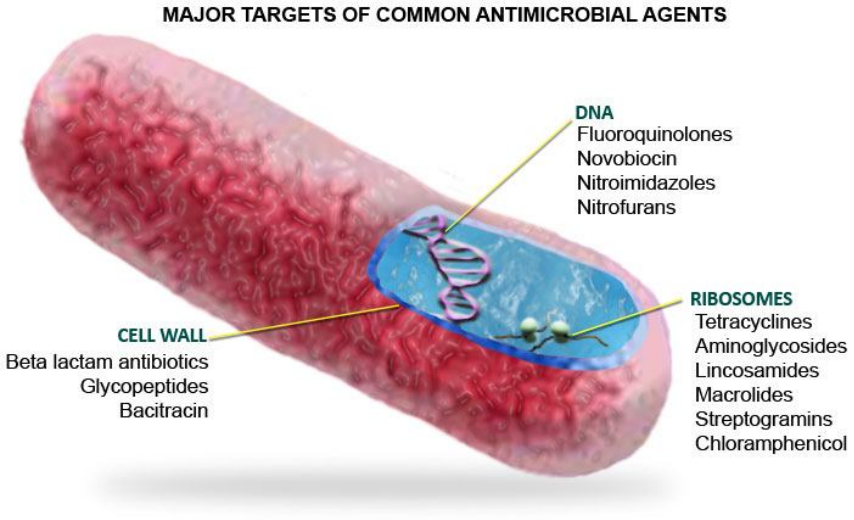

Fig: 2.5 Different modes of action of antibiotic.

Several types of antibacterial agents target bacterial protein synthesis. It can be happened by binding to either the 30S or 50S subunits of the intracellular ribosomes. Enzymes & cellular

structures are made of protein. Protein synthesis is an essential process especially for the multiplication & also for the survival of all bacterial cells. This activity then helps in the disruption of the normal cellular metabolism of the bacteria. It then consequently leads to the death or inhibition of growth of the organism & multiplication. Aminoglycosides, macrolides, lincosamides, streptogramins, chloramphenicol, tetracyclines show this type of action. (Vasanthakumari, 2007)

DNA and RNA play main role in the replication of all living forms. Some antibiotics perform by binding to components involved in the process of DNA or RNA synthesis. It causes interference of the normal cellular processes. It will ultimately compromise bacterial multiplication & survival. Quinolones, metronidazole, & rifampin are these types of antibiotics. Other antibiotics performed on selected cellular processes. These are essential for the survival of the bacterial pathogens. As for example, both sulfonamides & trimethoprim disrupt the folic acid pathway. It is a necessary step for bacteria to produce precursors important for DNA synthesis. Sulfonamides target & bind to dihydropteroate synthase. Trimethophrim inhibit dihydrofolate reductase. Folic acid is a vitamin synthesized by bacteria, but not humans. Both of these enzymes are essential for the production of folic acid. (Vasanthakumari, 2007)

2.8 Azithromycin

In our research work we have done the antimicrobial susceptibility test. In this test we have used different brands of Azithromycin. Azithromycin is an antibiotic. It is an azalide. It is a subclass of microlide antibiotics. It is specially derived from erythromycin. In its structure a methyl-substituted nitrogen atom incorporated into the lactone ring. This structure makes the lactone ring 15-membered. (Molly, 2013)

It is speciall made for the inhibition of the protein synthesis of different types of bacteria. Specially they are very active against gram-negative bacteria. They can easily inhibit the process of protein synthesis. That specially helps pathogen to be protected. It is more effective than erythromycin. So it is used more than the erythromycin. It has become popular for its mechanism. (Molly, 2013)

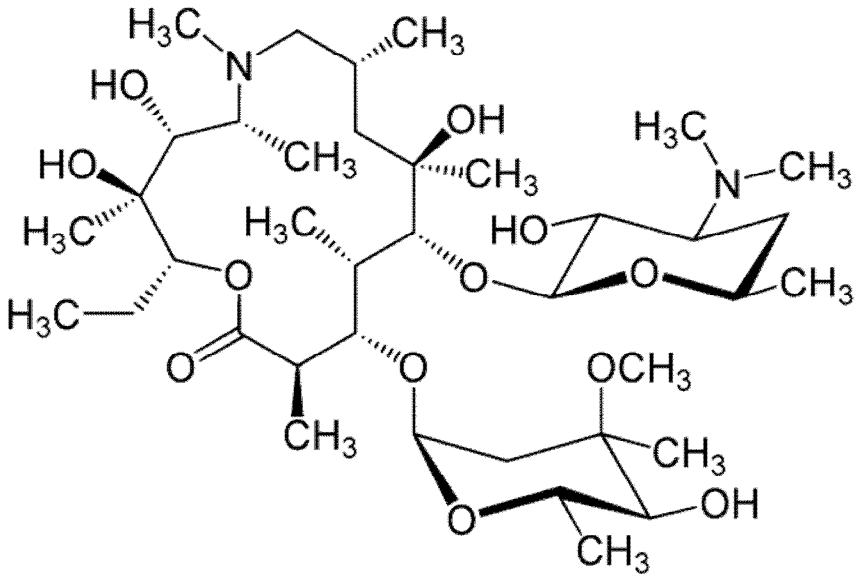

Fig: 1.6 Structure of Azithromycin

Azithromycin is an antibiotic which prevents bacteria from growing by interfering with their protein synthesis. It inhibits the translation of mRNA by binding to the 50S subunit of the bacterial ribosome. (Molly, 2013)

2.8.1 Uses of azithromycin

It is basically used to treat or prevent certain bacterial infections. In most cases these causing middle ear infections, strep throat, pneumonia, typhoid, bronchitis, & sinusitis. Recently this antibiotic has been used in infants to prevent bacterial infections. It has been also used for the patient with weaker immune systems. (Molly, 2013)

In case of sexually transmitted infections including those from unprotected sex or sexual assault, azithromycin plays very important role. As for example it is very effective against certain sexually transmitted infections like nongonococcal urethritis, chlamydia, & cervicitis.Nowadays researchers have showed that it is also effective against late-onset asthma. Although these type of findings are controversial. That is not widely used. (Molly, 2013)

Azithromycin has proved its activeness in different infections like acute otitis media, nonstreptococcal bacterial pharyngitis. It is used to treat gastrointestinal infections such as

traveler's diarrhea, in respiratory tract infections such as pneumonia, cellulitis, Bartonella infection, chancroid cholera, donovanosis, leptospirosis, lyme disease, malaria, Mycobacterium avium complex disease, Neisseria meningitis, pelvic inflammatory disease, pertusis, scrub typhus, toxoplasmosis and salmonellosis. It is used to prevent bacterial endocarditis. Azithromycin is also inactive against localized dental infections, uncomplicated skin & skin structure infections, urethritis & cervicitis & also genital ulcer disease. For the people who are allergic to penicillin, azithromycin is effectively used as a second line treatment for strep throat. (Molly, 2013)

2.9 Methods of antimicrobial sensitivity test

There are different types of antibiotic sensitivity testing methods. They are the dilution methods, disk diffusion methods, E-test, automated antimicrobial susceptibility testing systems, mechanism-specific tests, genotypic methods. (Schwalbe, et al, 2007)

2.9.1 Dilution Method

In dilution method broth & agar media both is used. In case of broth dilution the isolation of a series of concentrations of antimicrobial agents is done. It is performed in a broth environment. In the microdilution test, 0.05 to 0.1 ml total broth volume is used. It can be easily performed in a microtiter format. In macrodilution test, 1.0 ml broth volume is used in standard test tube. For both of these dilution methods, in microdilution & in macrodilution the lowest concentration is used. In this lowest concentration the isolate is completely inhibited. This lowest concentration is known as minimal inhibitory concentration or MIC. This inhibition is confirmed by the absence of visible bacterial growth. In this way MIC is the minimum concentration of the antibiotic. It will ultimately inhibit the particular isolate. The test is only considered as valid in two conditions. These two conditions are if the positive control shows bacterial growth & negative control shows no bacterial growth. (Schwalbe, et al, 2007)

In case of agar dilution same procedure of broth dilution is followed. In agar dilution method, it follows to establish the lowest concentration of the serially diluted antibiotic concentration. In this concentration bacterial growth is still inhibited. (Schwalbe, et al, 2007)

2.9.2 Disk Diffusion Method

Disk diffusion method is most probably widely used method. This method is widely used in the private veterinary clinics for determining antimicrobial resistance. In this process a growth medium is used which is known as Mueller-Hinton agar. At first this agar medium is first seeded throughout the plate. In this plate bacterial isolation is given after diluting this isolation at a standard concentration. Commercially prepared disks are used. Each of the disks is impregnated with a standard concentration of a particular antibiotic. All of these disks are then evenly dispensed & lightly pressed onto the agar surface. The antibiotic test is immediately begun to diffuse outward from the disks. At that time it starts to create a gradient of antibiotic concentration in the agar. As a result the highest concentration is found to close to the disk. The concentration decreases further away from the disk. An overnight incubation is done. Then the bacterial growth around each disk is observed. A clear area of no growth is observed around that particular disk. It means the test isolate is susceptible to a particular antibiotic. (Schwalbe, et al, 2007; Pelczar, et al, 1996)

Zone of inhibition is known as the zone around an antibiotic disk with no growth. This confirms that the minimum antibiotic concentration is sufficient to prevent the growth of the test isolate. After that the zone is then measured in mm. Then this measurement is compared to a standard Interpretation chart.This chart is used to categorize the isolate as susceptible & intermediately susceptible or resistant. By this qualitative testing method, MIC measurement cannot be determined. This test simply classifies the isolate as susceptible, intermediate or resistant. (Pelczar, et al, 1996)

2.9.3 E-Test

E-test is a commercially available test. In this test a plastic test strip is used. This strip is impregnated with a gradually decreasing concentration of a particular antibiotic. It also displays a numerical scale. This numerical scale is corresponded to the antibiotic concentration. This quantitative test is convenient for antibiotic resistance of a clinical isolate. In this test a separate strip is needed for each antibiotic. For this reason the cost of this method is high. (Schwalbe, et al, 2007)

2.9.4 Autamated Antimicrobial Susceptibility Testing Systems

Automated antimicrobial susceptibility testing system is used to reduce the technical errors & lengthy preparation times. Several commercial systems have been developed. So it provides conveniently prepared & formatted microdilution panels as well as instrumentation. These commercial systems also provide automated reading of plates. Most of these systems provide automated inoculation, reading & interpretation. These systems have benefit of being rapid because some results can be easily generated within hours. This method is also convenient. This method has one limitation in most laboratories. That is the cost entailed in initial purchase, operation, & maintenance of the machinery. There are some examples like Vitek System, Walk-Away System, Sensititre ARIS, Avantage Test System, Phoenix, Micronaut & many more.

In the mechanism-specific tests, the presence of a particular resistance mechanism is directly detected here. Thus resistance can be easily established through tests. As for example, different types of enzyme can be easily detected by these tests. Beta lactamase can be detected by using an assay like chromogenic cephalosporinase test. Chloramphenicol acetyltransferase, is known as chloramphenicol modifying enzyme is detected. It may utilize commercial colorimetric assays such as a CAT reagent kit. (Schwalbe, et al, 2007)

2.9.5 Genotypic Method

The last one is the genotypic method. Sometimes the test is done for the specific genes that confer antibiotic resistance. Nucleic acid-based detections systems are generally rapid & sensitive. In genotypic method there are various molecular techniques that are used for antimicrobial resistance detection. Some of the most common molecular techniques are Polymerase chain reaction, DNA hybridization, modifications of PCR & DNA hybridization. (Schwalbe, et al, 2007)

2.9.5.1Polymerase Chain Reaction

One of the most commonly used molecular techniques is Polymerase chain reaction. It is used for detecting certain DNA sequences of interest. Denaturation of sample DNA, annealing of specific primers to the target sequence is done in this reaction. The extension of this sequence is

facilitated by a thermostable polymerase. This leads to the replication of a duplicate DNA sequence in an exponential manner to a point. This will be visibly detectable by gel electrophoresis with the aid of a DNA-intercalating chemical. It fluoresces under UV light. (Schwalbe, et al, 2007)

2.9.5.2 DNA Hybridization

DNA hybridization is based on a specific fact. Pair up of DNA pyrimidines is done with purines specifically. From the test sample a known specific sequence can be paired up with opened or denatured DNA. If the hybridization occurs then the probe labels this. It labels this with a detectable radioactive isotope, antigenic substrate, enzyme or chemiluminescent compound. No attachment of probes will occur if no target sequence is present. The attachment will not happen if the isolate does not have the specific gene of interest. (Schwalbe, et al, 2007)

2.9.5.3 Modifications of PCR & DNA Hybridization

With the principle of modifications of PCR & DNA hybridization several modifications have been introduced. It further improve the sensitivity & specificity of these standard procedures as for example the use of 5'-fluorescence-labeled oligonucleotides, the development of DNA arrays & DNA chips etc. (Schwalbe, et al, 2007)

Chapter 3

Method & Materials

3.1 The reason of choosing the disk diffusion method

In our research, we have followed the disk diffusion methods for antimicrobial susceptibility test. Although there are various types of methods, but among all method we have used this because it is easy method than the other ones.

3.2 Collection of clinical isolation of bacteria

We have collected clinical isolation of different types of bacteria from Mirpur Sishu Hospital & LABAID.

Table 3.1: List of ATCC grade Microorganisms and Clinical Isolations Used in Antimicrobial Susceptibility Assay

Sl	Microorganism Type	Scientific Names
1	Gram – positive Bacteria	*Bacillus cereus*
2		*Bacillus megaterium*
3		*Bacillus subtilis*
4		*Sarcina lutea*
5		*Staphylococcus aureus*
6		*Staphylococcus pyrogenus*
7	Gram – negative Bacteria	*Escherichia coli*
8		*Pseudomonas aureus*
9		*Salmonella typhi*
10		*Salmonella paratyphi*
11		*Salmonella saprophyti*
12		*Shigella boydii*
13		*Shigella dysenteriae*
14		*Vibrio mimicus*
15		*Vibrio parahaemolyticus*
16	Fungus	*Aspergillus niger*
17		*Candida albicans*
18		*Saccharomyces cerevaceae*
19	Clinical Isolates	Pus
20		Blood – 1
21		Blood – 2

3.3List of chemicals & media

We have used various types of materials in the antimicrobial susceptibility test.

3.3.1 Nutrient agar

Nutrient agar is a microbiological growth medium. It is commonly used for the routine cultivation of non-fastidious bacteria. This agar is very useful. (Schwalbe, et al, 2007).

Fig: 3.1 Nutrient agars on agar plate

It remains solid even at relatively high temperatures. That is why, it is greatly used. If this nutrient agar is used bacteria can be easily grown in nutrient agar. This is clearly visible as small colonies. (Schwalbe, et al, 2007).

Table: 3.2 Composition of Nutrient agars

Name of the ingredients	Amounts of the ingridients
Peptone	0.5 %
Beef extract or yeast extract	0.3%
Agar	1.5%
NaCl	0.5%
Distilled water	q.s
Ph	Neutral like 6.8 at 25 °C.

(Ananthanarayan, 2005)

3.3.2 0.9 % Saline

It is used to make bacterial solution.

3.3.3 Ethanol

Ethanol is used to clean the spreader with cotton. It helps to sterile

3.3.4 Distilled water

It was used in case of autoclave. It was also used in case of making the bacterial solution & drug solution.

3.4 List of apparatus

List of apparatus that are used in the antimicrobial susceptibility test are discussed here with the application & figure.

3.4.1 Agar plate & agar bottle

This plate is used to contain the agar media for microbial growth. Agar bottle was used to contain the agar media.

Fig: 3.2 Agar bottle

3.4.2 Eppendorf tube

This tube is used for containing the bacterial solution.

3.4.3 Mortar & pestle

The mortar is a bowl. It is made of hard wood, ceramic or stone. The **pestle** is a heavy in weight. It is a club-shaped object. The end of pestle is used for crushing & grinding solid chemicals like tablet or capsule as drug.

Fig: 3.3 Mortar & Pestle

3.4.4 Pipette

This serological pipette is used to read in accurate volume by users. These pipettes are varied from 1ml, 2 ml, 5ml, and 10ml. they contain four types of color code on tips. It helps pipettes easily recognizable. (Schwalbe, et al, 2007)

Fig: 3.4 Pipette

3.4.5 Pumper

It is used to rise, compress & transfer fluids.

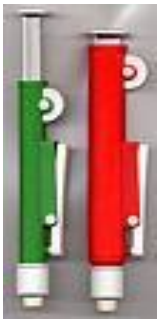

Fig: 3.5 Pumper

3.4.6 Micropipette

They are used for transferring small volumes of liquid. To get accurate result they were used to deliver the milliliter & microliter range. This equipment contains a plunger for drawing up & expelling liquids, a dial that is used for tunning the pipette to different volumes, the display & an ejector that helps to launch tips towards wastebaskets. The volume is set by turning the dial. (Schwalbe, et al, 2007)

Fig: 3.6 Micropippette

3.4.7 Micropipette tips

These tips are of different size. With them 2 to 1000 µL liquid can be taken easily.

Fig: 3.7 Micropipette tips

3.4.8 Inoculation loop

An inoculation loop is used to transfer bacteria for bacterial culture.

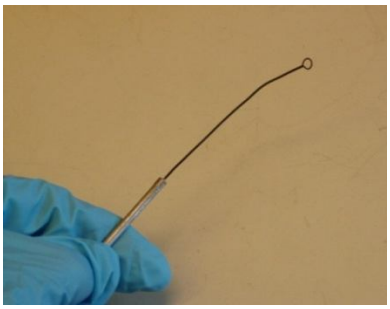

Fig: 3.8 Inoculation loop

3.4.9 Spreader

It is used to spread the bacterial solution on the agar plate containing agar media.

3.4.10 Disks

On these disks the solution of drugs is given. Then disks are kept on the agar plate containing agar media.

3.4.11 Forcef

It is used to hold the disks to set on the agar plate.

3.4.12Drug container

It is made of class. It is used to contain the drug solution.

3.4.13 Spatula

It is used to contain solid particle. We use this to carry agar & drug to weigh.

3.4.14 Foil paper

Foil paper is used to cover various apparatus made of class. It is specially used in case of using the autoclave & hot air oven.

Fig: 3.9 Foil paper

3.4.15 Cotton, gloves, towel, Scale, marker pen, burner, masking tape, cotton bud etc

Cotton is used for clean any apparatus if needed. In absence of spreader cotton bud is also used to spread the bacterial solution. In case of using hot air oven, autoclave device the use of gloves or towel is must to hold the apparatus.

Marker pen is used to write down the important info on the label. Here scale is used to measure the length of zone of inhibition. The burner is used to burn the microorganism remaining on the loop. The masking tape is used in case of labeling the name of microorganism & the name of the brand of azithromycin.

3.5List of machines

Here are some names of machines that are used in the antimicrobial susceptibility test.

3.5.1 Analytical balance

An analytical balance is used in our research work. It is designed to measure small mass in the sub-milligram range. The measuring pan of this balance is remained inside a transparent enclosure with doors. So, it is impossible for the entry of dust into this machine. Another advantage of using this balance is that no air currents in the room can affect the balance's operation. (Vasanthakumari, 2007)

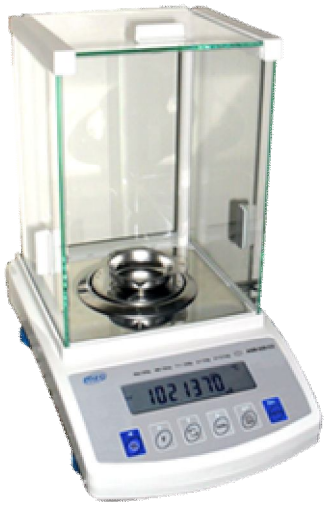

Fig: 3.10 Analytical balance

After crushing & grinding tablet in mortar with pestle, fixed amount of this drug is weighed using this machine. Here paper is kept on the pan of machine. Then this balance is tarred. After taring the balance that drug is kept on the paper to weigh. (Vasanthakumari, 2007).

3.5.2 Vortex mixer or shaker

A vortex mixer is also known as vortexer. It is a simple device that is used commonly in laboratories to mix small vials of liquid. It contains an electric motor with the drive shaft oriented vertically. This motor is attached to a cupped rubber piece mounted slightly off-center. The rubber piece oscillates rapidly at the time of motor running.This vortex machine is capable of fully mixing liquids in the test tube. It is small in size & also has large scope of application. Its mixing capacity is 5ml to 50 ml. (Vasanthakumari, 2007).

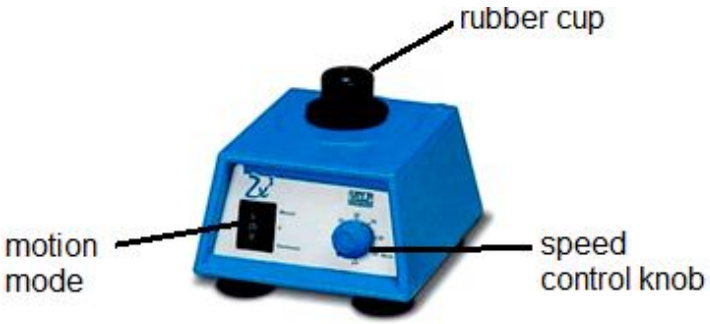

Fig: 3.11 Vortex mixer or shaker

In case of antimicrobial susceptibility testing appended tube & the container with drug solution are pressed into the rubber cup. At that time the motion is transmitted to the liquid inside. With the transmission of motion a vortex is created. Most of them are made in way that they will run only when downward pressure is applied to the rubber piece. (Vasanthakumari, 2007).

3.5.3 Autoclave

An autoclave is a device that is used to sterilize. This machine is widely used in microbiology & also in other sector of science. They vary in size. Their function depends on the size of the load of the contents. In this machine high pressure is provided with saturated steam at 121 °C. This machine is able to neutralize potentially infectious agents by utilizing pressurized steam & superheated water. (Vasanthakumari, 2007)

Before activation of this machine all of the trapped air is removed from the autoclave. It is done because hot air is a very poor medium for achieving sterility. in this machine downward displacement is maintained. It is maintained because steam entering the chamber fills the upper areas first as it is less dense than air. In case of steam pulsing air is diluted by using a series of steam pulses. In this device air or steam mixtures is sucked by the vacuum pump. (Vasanthakumari, 2007).

Fig: 3.12 Autoclave

Superatmospheric cycles are achieved with a vacuum pump. These cycles starts with a vacuum followed by a steam pulse. Subatmospheric cycles are similar to the superatmospheric cycles. Although in case of subatmospheric cycles chamber pressure never exceeds atmospheric pressure until they pressurize up to the sterilizing temperature. (Vasanthakumari, 2007)

3.5.4Hot air oven

Hot air ovens are electrical devices that are greatly used in sterilization. These machines are priginally invented by the scientist Pasture. This ovens use dry heat. This heat helps to sterilize articles. 50 to 300 °C are maintained in this machine. This oven contains thermostat to control the temperature & this oven is digitally controlled to maintain the temperature. (Vasanthakumari, 2007).

They contains double wall as insulator to keep the heat in & conserves the energy. The inner layer of this wall is a poor conductor & the outer layer is metallic. They also contain an air filled space in between to aid insulation. (Vasanthakumari, 2007)

Fig: 3.13 Hot air oven

3.5.5 Laminar flow cabinet

A laminar flow cabinet is also known as laminar flow closet or tissue culture hood. This device is carefully enclosed bench. This device is designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive device. In this device air is drawn through a HEPA. Here the air is blown in a very smoothly & laminar flow towards the user. The device is usually made of stainless steel. (Vasanthakumari, 2007)

Fig: 3.14 Laminar flow cabinet

This steel contains no gaps or joints where spores might collect. In this device hoods exist in both horizontal & vertical configurations. This device has a UV-C germicidal lamp. This lamp helps to sterilize the shells & contents when not in use. At the time of using this device it is important to switch this light off. Otherwise this light will quickly give any exposed skin sunburn & may also cause cataracts. (Vasanthakumari, 2007)

3.5.6 Incubator

This device is used to grow & maintain microbiological cultures. It maintains the optimal temperature, humidity & other conditions. In other conditions carbon dioxide & oxygen content of the atmosphere are included. (Vasanthakumari, 2007)

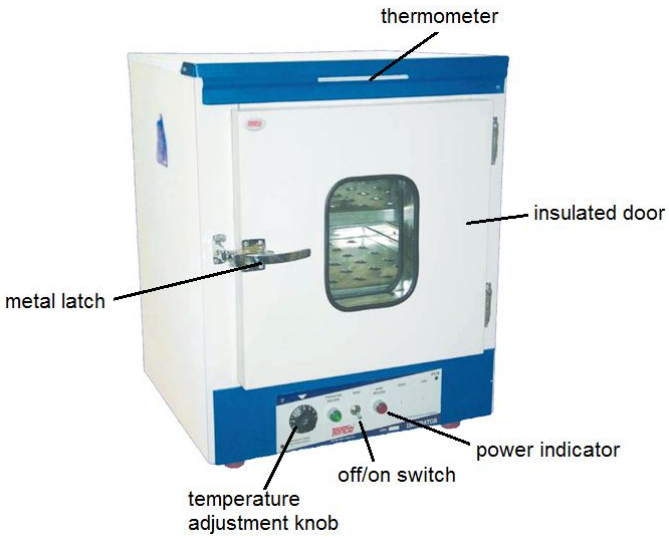

Fig: 3.15 Incubators

In this device the temperature is maintained at 28 to 30°C for bacteria. In case of mesophillic bacteria the temperature is maintained at 35-37°C. For the extremely thermophilic organisms the temperature is maintained as high as A temperature as 100°C. (Vasanthakumari, 2007)

3.6 Name of brands of azithromycin

Table: 3.3 Name of the brands of azithromycin

Generic name	Name of company	Brand name
Azithromycin 500mg/tablet	Beximco Pharmaceuticals Ltd.	AZITHROCIN
	Opsonin Pharma Limited	Azicin 500
	ACI Limited.	ODAZYTH
	Orion Pharma Limited	AZALID
	General Pharmaceuticals Ltd.	AZOMAC

3.7 Methods for Antimicrobial Susceptibility Assay

3.7.1 Preparation of nutrient agar plates

In order to prepare nutrient agar plates, the agar solution is prepared by dissolving 28 gm in 1000 ml. Then, the solution in agar bottle is autoclaved. Preparation of agar plates involves pouring of the agar solution onto the sterile petri-dish in such as way that the thickness of the agar plate is 4 mm. It needs some time to being semisolid. (Karlmose,2010).

3.7.2 Preparation of subculture

To prepare the inoculums, the agar plates containing the test organism are needs to examine visually. If culture appears mixed, a fresh sub-culture will be prepared with a loop to touch the top of at least 4 to 5 times on isolated colonies. It is because picking cells from more than one colony ensures the selection of sufficient bacterial numbers. It also minimizes the risk of selecting bacteria that have lost their resistance. The growth is then transferred to the saline in eppendorf tube and then the inoculum on the inside is emulsified to avoid clumping of the cells. This is very important because, confluent growth will not be obtained if the suspension is too heavy, zone sizes will be artificially small.(Karlsmose, 2010).

The agar plates should be free of visible contamination. It must be of a uniform depth of approximately 4 mm, not be excessively wet, and should not be cracked or dry. Within 15 minutes of preparing the adjusted inoculum, a sterile cotton swab is dipped into the inoculum. The swab is rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab is streak over the entire surface of the agar plate and then the plate is rotated approximately 60^{o} then repeat streaking motion and then again rotated 60^{o} and streaking is repeated for the 3^{rd} time. Inoculation is completed by running the swab around the rim of the agar. (Karlsmose, 2010).

3.7.3Preparation of 0.9% saline

To make bacterial dilution saline was prepared. For 100 ml water 0.9 gm NaCl is taken in a volumetric flask. (Karlsmose, 2010).

3.7.4. Inoculation of the Agar plate

Agar plate is prepared following the same way as the subculture. In this case homogeneous plating is important to yield reliable results. It is allowed to any excess moisture on the agar surface to be absorbed prior to applying the antimicrobial disks. For that reason the lid of the plate may be left for 3-5 minutes not more than 15 minutes to allow any excess moisture to be absorbed beore applying disks. (Karlsmose, 2010).

3.7.5 Despensing Antimicrobial Disks

Dispense disks to the agar surface with a disk dispenser or sterile forceps. Here forceps can be sterilized by flaming with alcohol. Do not relocate any disks after contact with the agar. After application of disks it is needed to insure that the disk has made complete contact with the agar surface by touching the top of the disk with forceps. (Karlsmose, 2010).

The disks cannot be moved after being placed onto the agar surface. Diffusion of the drug begins immediately when the disk contacts the agar. Moving the disks after contact with the agar will produce distorted zones & result in unreliable results. Selection of disks should be guided by plate size and the intended use of the results. Ordinarily, no more than 5 disks should be placed on a 10 cm agar plate and no more than 12 disks should be placed on a 15 cm agar plate. After

setting the disks on the agar plate it is kept into the incubators for overnight incubation. (Karlsmose, 2010).

Fig: 3.16 Agar plate after overnight incubation.

After this overnight incubation next day these agar plates is carefully observed to see & measure the zone of inhibition. The zone of inhibition is measured by scale in cm range.(Karlsmose, 2010).

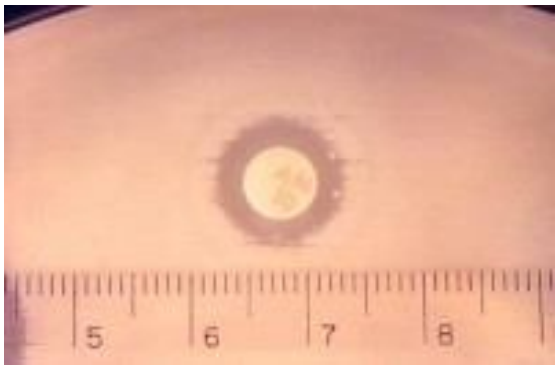

Fig: 3.17 Zone of inhibition measuring with scale.

Chapter 4

RESULTS

4.1: Disc Diffusion Assay of ATCC Grade Gram Positive Bacterial Strains

Table 4.1: Average Diameter of Zone of Inhibition of Azithromycins against ATCC grade Gram positive Bacterial Strains (Avg = Average of the sample; SD = Standard Deviation; M/O = Microorganisms; RS = Reference Standard)

M/O	Orion (Avg ± SD)	ACI (Avg ± SD)	General (Avg ± SD)	Opsonin (Avg ± SD)	Beximco (Avg ± SD)	RS (Avg ± SD)
Bacillus cereus	2.2 ± 0.4	2.3 ± 0.1	2.4 ± 0.6	2.2 ± 0.1	2.7 ± 0.9	2.7 ± 0.1
Bacillus megaterium	1.3 ± 0.2	2.1 ± 0.1	2.5 ± 0.4	2.4 ± 0.1	2.5 ±0.1	2.7 ± 0.2
Bacillus subtilis	2.5 ± 0.3	2.2 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
Sarcina lutea	1.9 ± 0.3	2.8 ± 0.5	2.5 ± 0.5	2.6 ± 0.1	2.8 ± 0.2	2.6 ± 0.2
Staphylococcus aureus	2.2 ± 0.1	2.3 ± 0.0	2.3 ± 0.3	2.4 ± 0.1	2.0 ± 0.1	2.5 ± 0.0
Staphylococcus pyrogenus	2.1 ± 0.2	2.2 ± 0.1	2.4 ± 0.2	2.3 ± 0.1	2.3 ± 0.2	2.3 ± 0.1

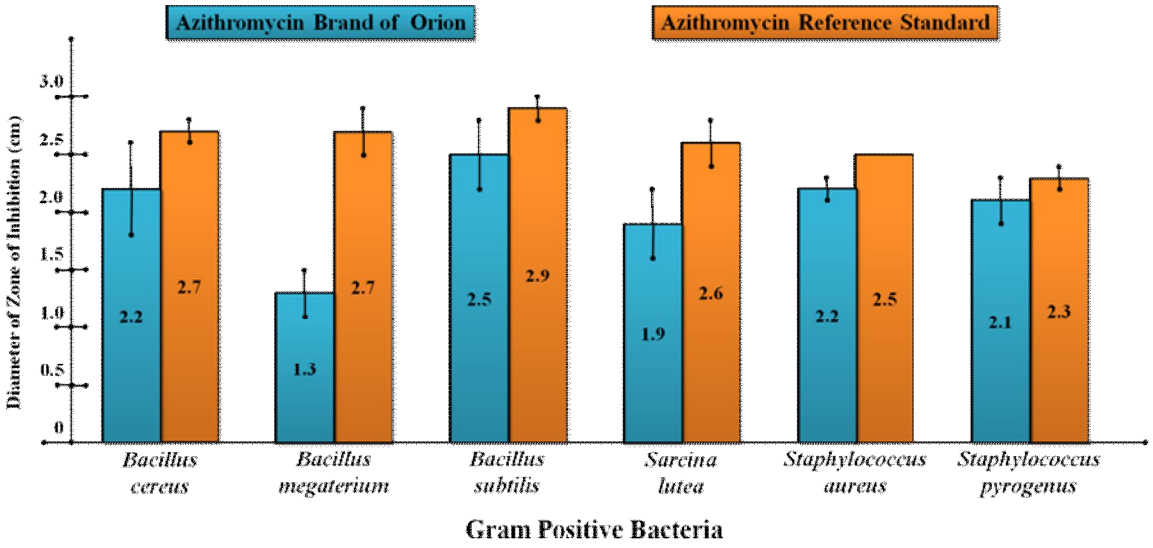

Figure 4.1 Average Diameter of Zone of Inhibition of Azithromycin of Drug Orion vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

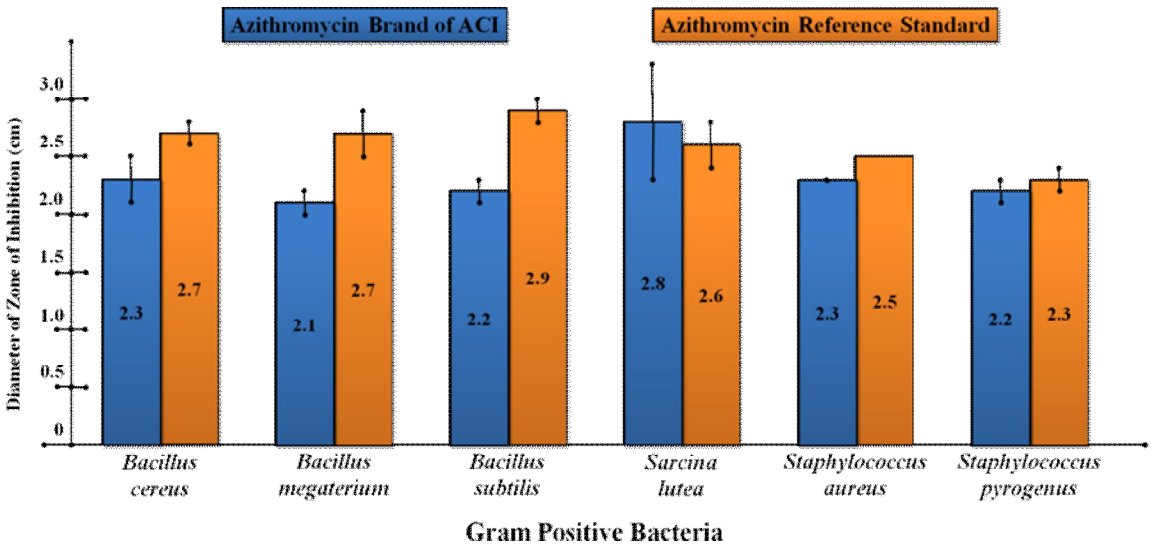

Figure 4.2: Average Diameter of Zone of Inhibition of Azithromycin of ACI vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

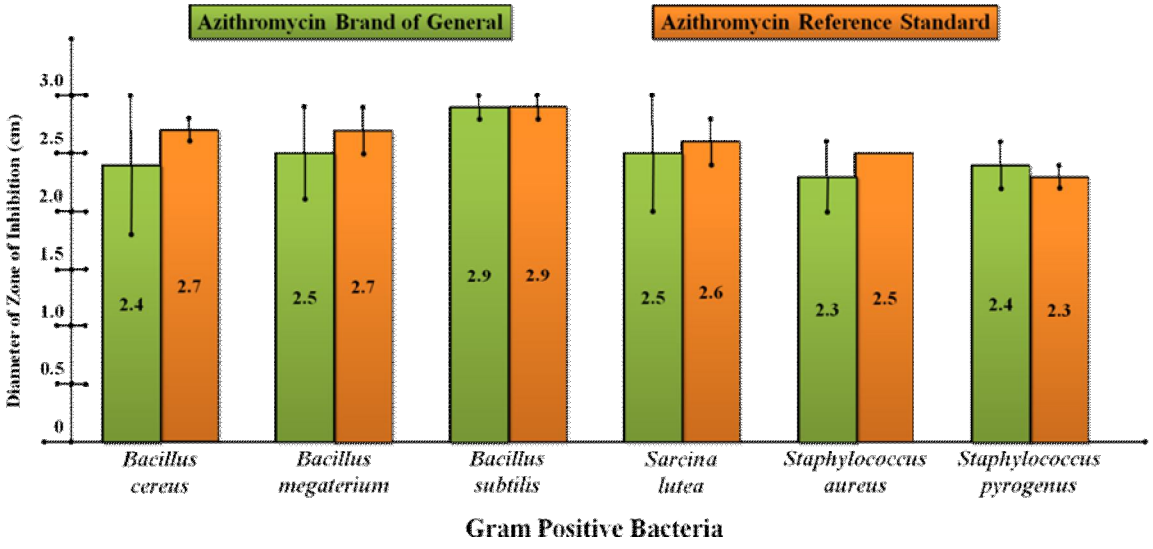

Figure 4.3: Average Diameter of Zone of Inhibition of Azithromycin of General vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

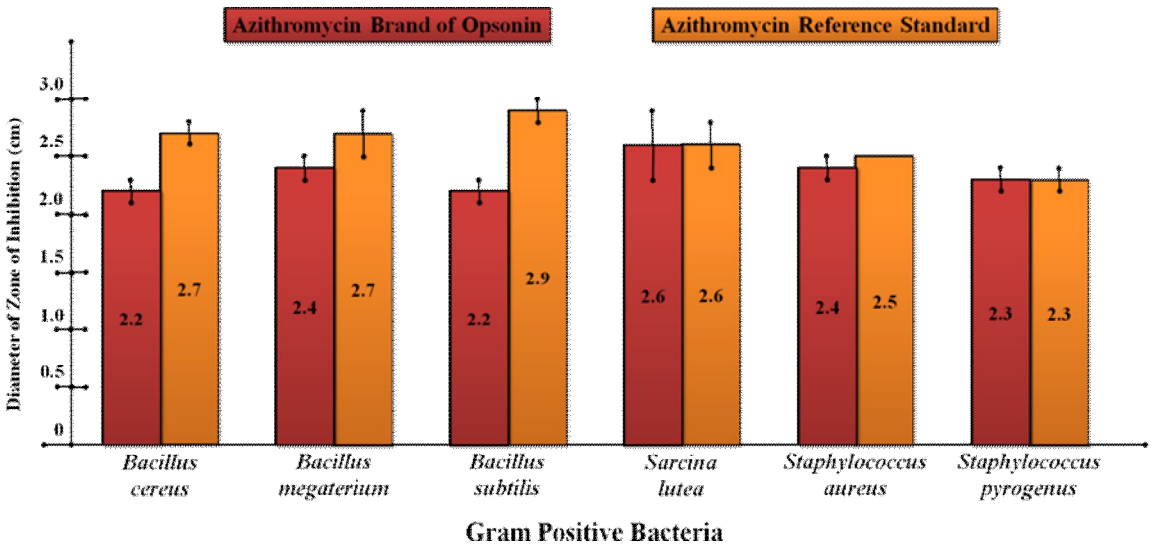

Figure 4.4: Average Diameter of Zone of Inhibition of Azithromycin of Opsonin vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

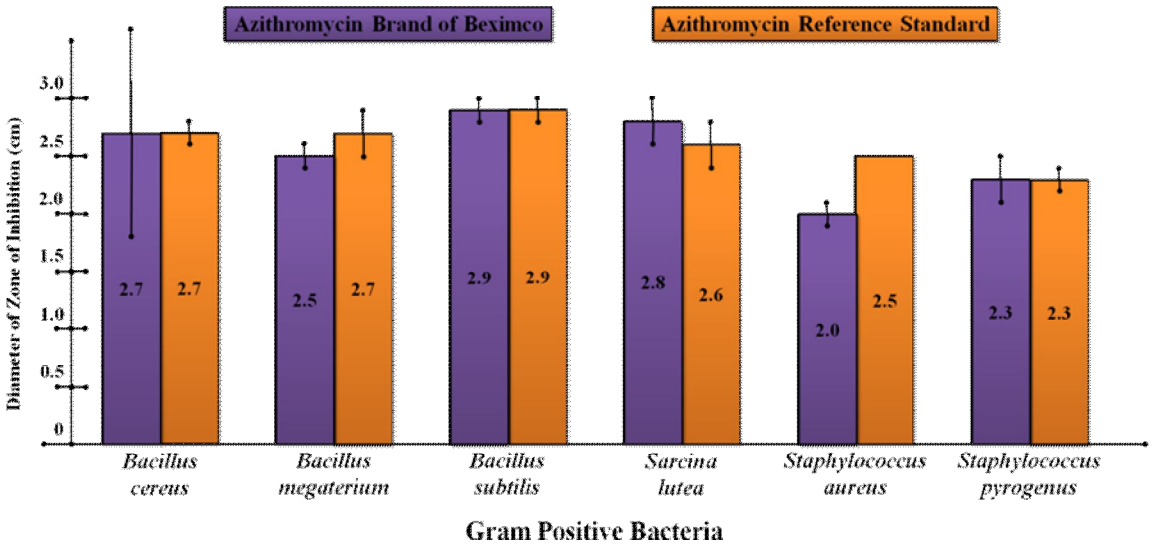

Figure 4.5: Average Diameter of Zone of Inhibition of Azithromycin of Beximco vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

4.2: Disc Diffusion of ATCC Grade Gram Negative Bacterial Strains

Table 4.2 Average Diameter of Zone of Inhibition of Azithromycins against ATCC grade Gram negative Bacterial Strains (Avg = Average of the sample; SD = Standard Deviation; M/O = Microorganisms; RS = Reference Standard)

M/O	Orion (Avg ± SD)	ACI (Avg ± SD)	General (Avg ± SD)	Opsonin (Avg ± SD)	Beximco (Avg ± SD)	RS (Avg ± SD)
Escherichia coli	2.2 ± 0.2	2.4 ± 0.1	3.5 ± 0.2	2.5 ± 0.1	3.4 ± 0.1	3.5 ± 0.0
Pseudomonas aureus	2.1 ± 0.1	2.2 ± 0.3	2.2 ± 0.2	2.6 ± 0.2	2.4 ± 0.3	2.8 ± 0.2
Salmonella typhi	2.5 ± 0.3	2.5 ± 0.1	2.6 ± 0.2	2.2 ± 0.2	2.7 ± 0.1	2.9 ± 0.1
Salmonella paratyphi	2.7 ± 0.2	2.0 ± 0.2	2.9 ± 0.4	2.4 ± 0.1	2.8 ± 0.5	3.3 ± 0.2
Salmonella saprophyti	2.7 ± 0.2	2.0 ± 0.7	2.9 ± 0.4	2.7 ± 0.3	2.5 ± 0.4	2.9 ± 0.1
Shigella boydi	2.4 ± 0.1	2.5 ± 0.2	2.6 ± 0.3	2.6 ± 0.2	2.8 ± 0.3	2.5 ± 0.1
Shigella dysenteriae	1.6 ± 0.5	2.5 ± 0.2	2.6 ± 0.3	2.4 ± 0.4	2.6 ± 0.1	2.7 ± 0.2
Vibrio mimicus	1.8 ± 0.7	2.6 ± 0.1	2.4 ± 0.2	2.6 ± 0.4	2.4 ± 0.2	3.0 ± 0.2
Vibrio parahaemolyticus	1.2 ± 0.2	2.9 ± 0.2	2.5 ± 0.1	2.6 ± 0.3	2.8 ± 0.2	2.6 ± 0.2

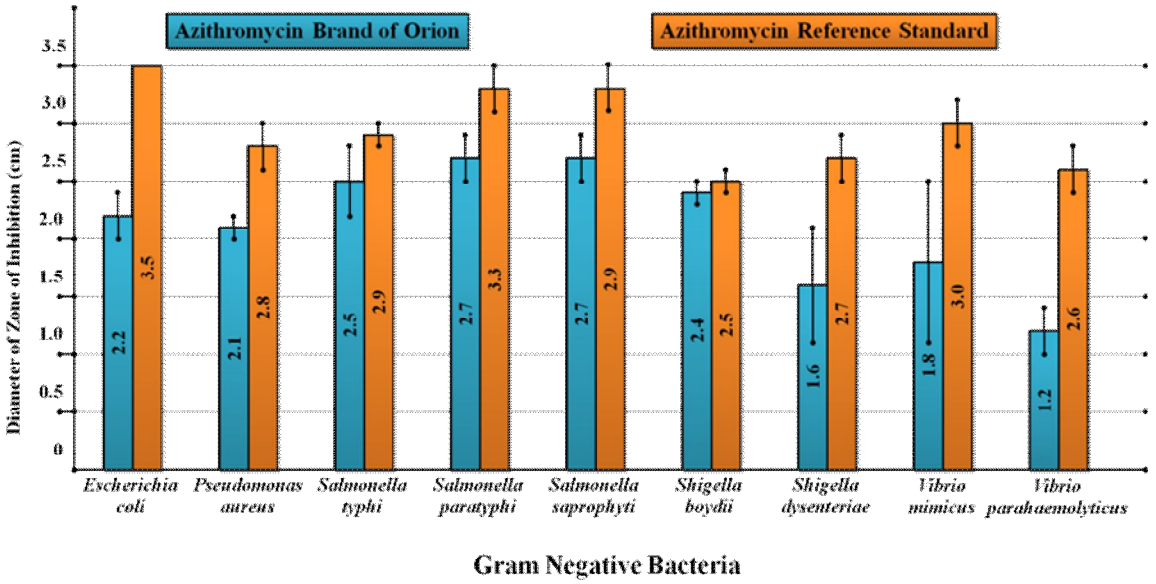

Figure 4.6: Average Diameter of Zone of Inhibition of Azithromycin of Orion vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

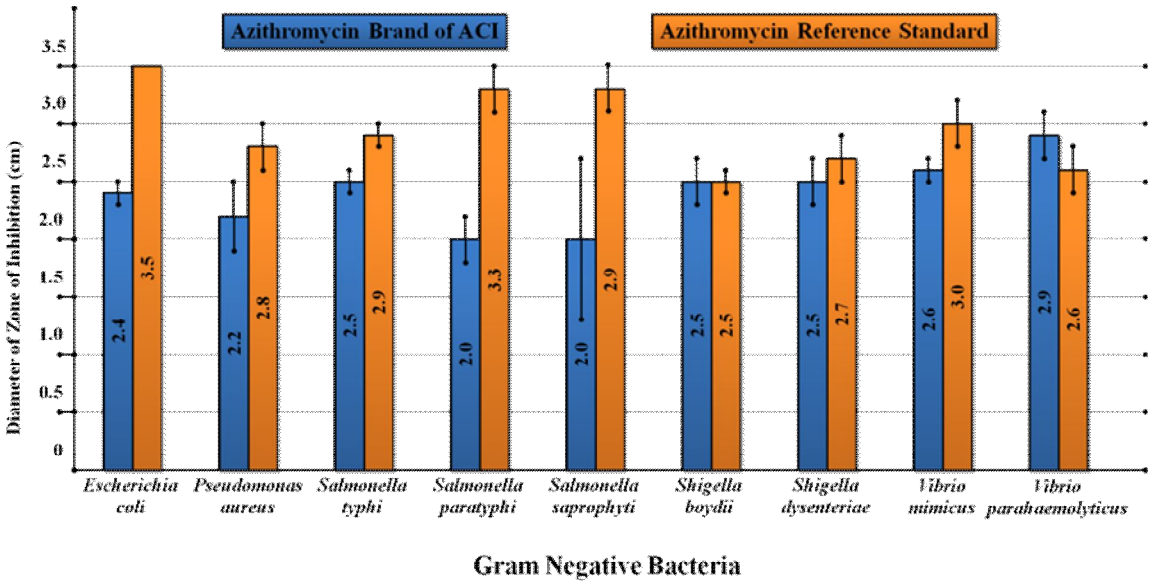

Figure 4.7: Average Diameter of Zone of Inhibition of Azithromycin of ACI vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

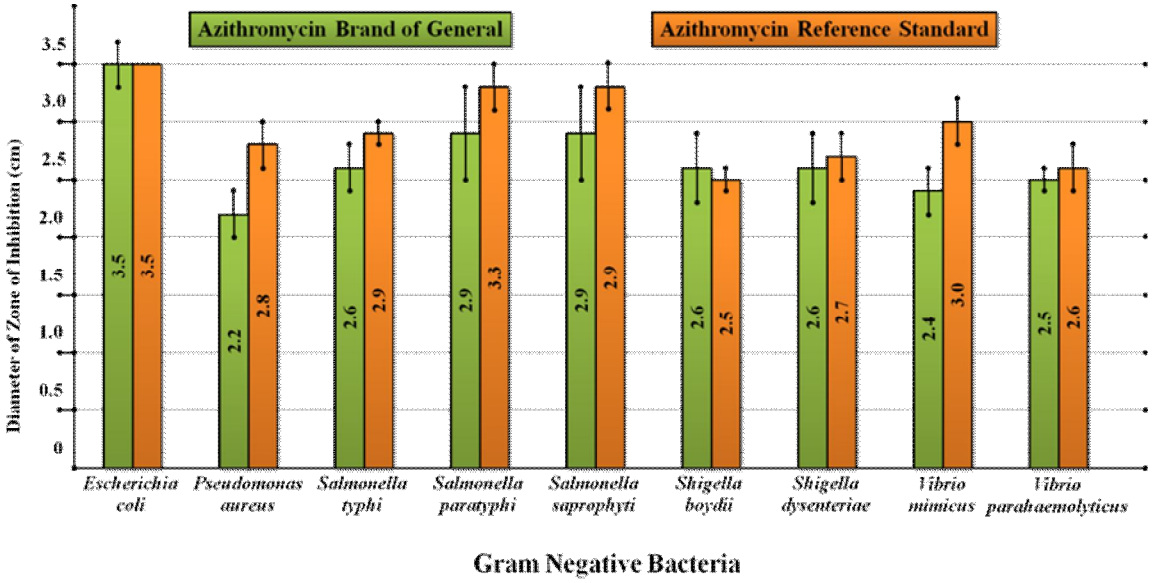

Figure 4.8: Average Diameter of Zone of Inhibition of Azithromycin of General vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

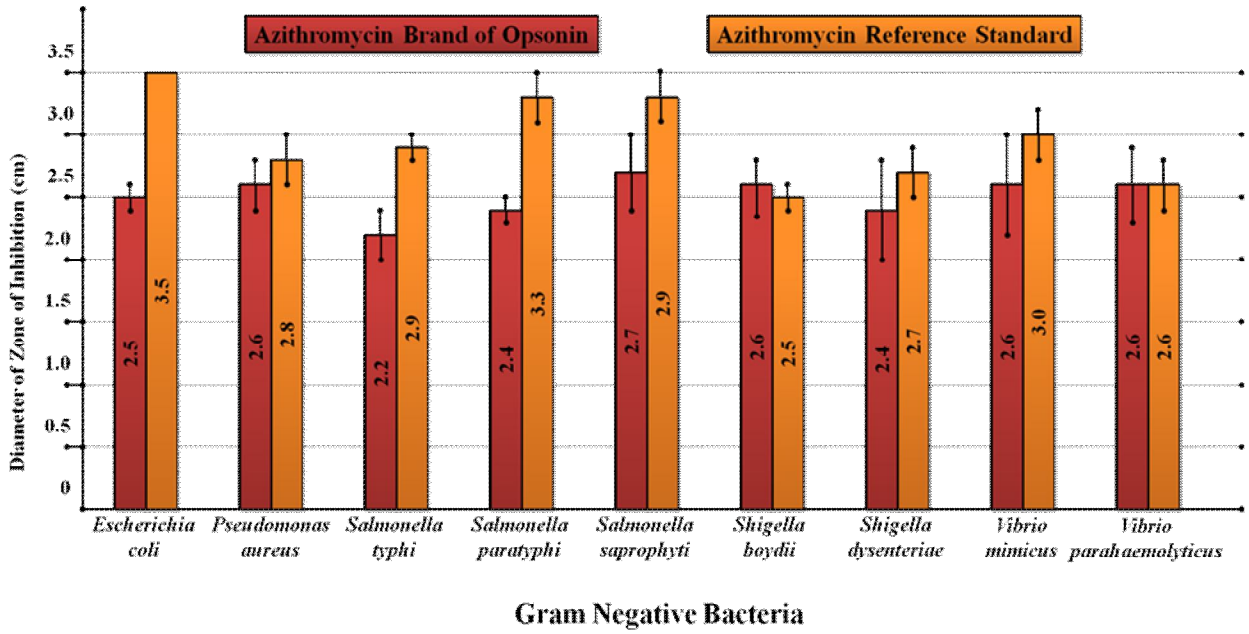

Figure 4.9: Average Diameter of Zone of Inhibition of Azithromycin of Opsonin vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

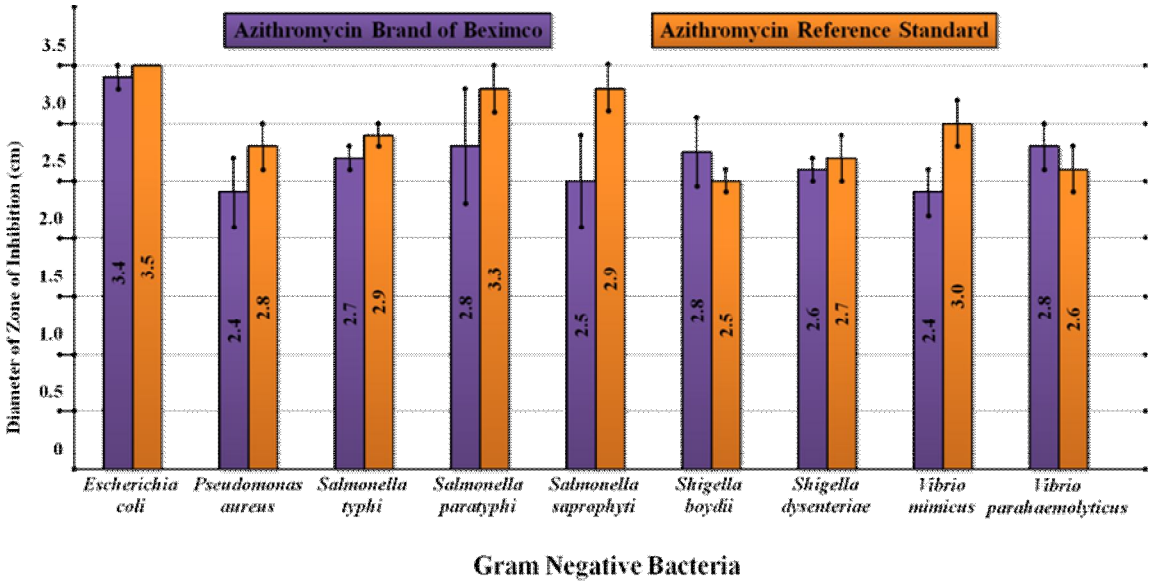

Figure 4.10: Average Diameter of Zone of Inhibition of Azithromycin of Beximco vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade gram positive bacterial strains

4.3: Disc Diffusion Assay of ATCC Grade Fungal Strains

Table 4.3: Average Diameter of Zone of Inhibition of Azithromycins against ATCC grade Fungal Strains (Avg = Average of the sample; SD = Standard Deviation; M/O = Microorganisms; RS = Reference Standard)

M/O	Orion (Avg ± SD)	ACI (Avg ± SD)	General (Avg ± SD)	Opsonin (Avg ± SD)	Beximco (Avg ± SD)	RS (Avg ± SD)
Aspergillus niger	2.4 ± 0.2	2.5 ± 0.2	2.5 ± 0.1	2.3 ± 0.2	2.8 ± 0.4	3.4 ± 0.2
Candida albicans	2.5 ± 0.2	2.4 ± 0.2	2.4 ± 0.2	2.4 ± 0.1	2.5 ± 0.2	2.8 ± 0.2
Saccharomyces cerevaceae	2.4 ± 0.1	2.2 ± 0.5	2.6 ± 0.4	2.5 ± 0.2	2.6 ± 0.2	3.2 ± 0.2

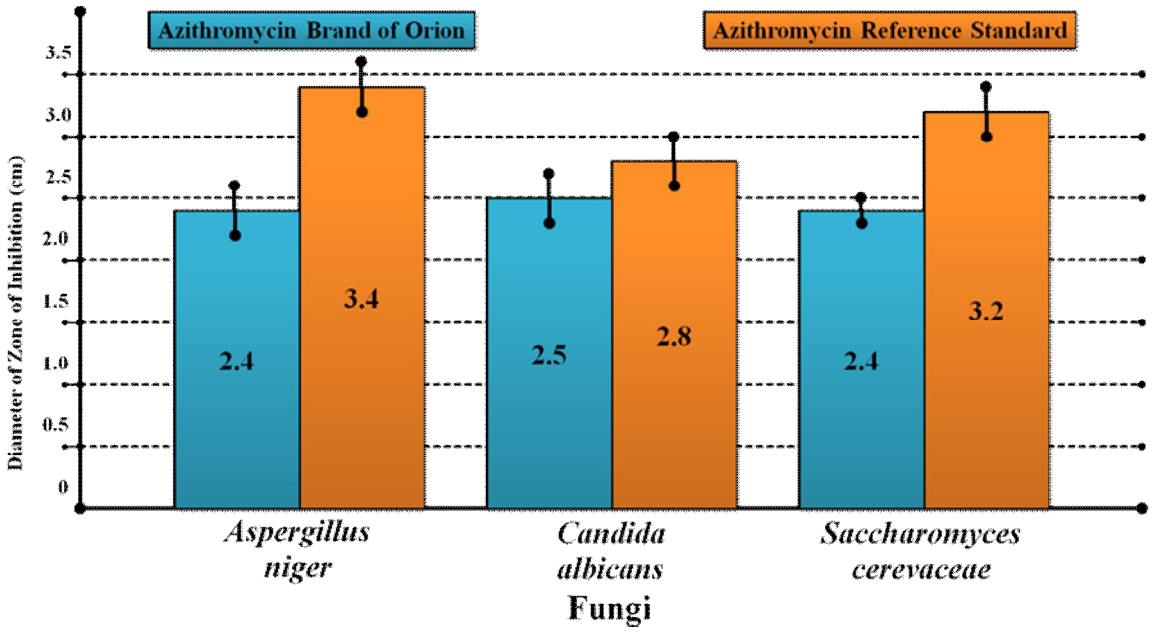

Figure 4.11: Average Diameter of Zone of Inhibition of Azithromycin of Orion vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade Fungal strains

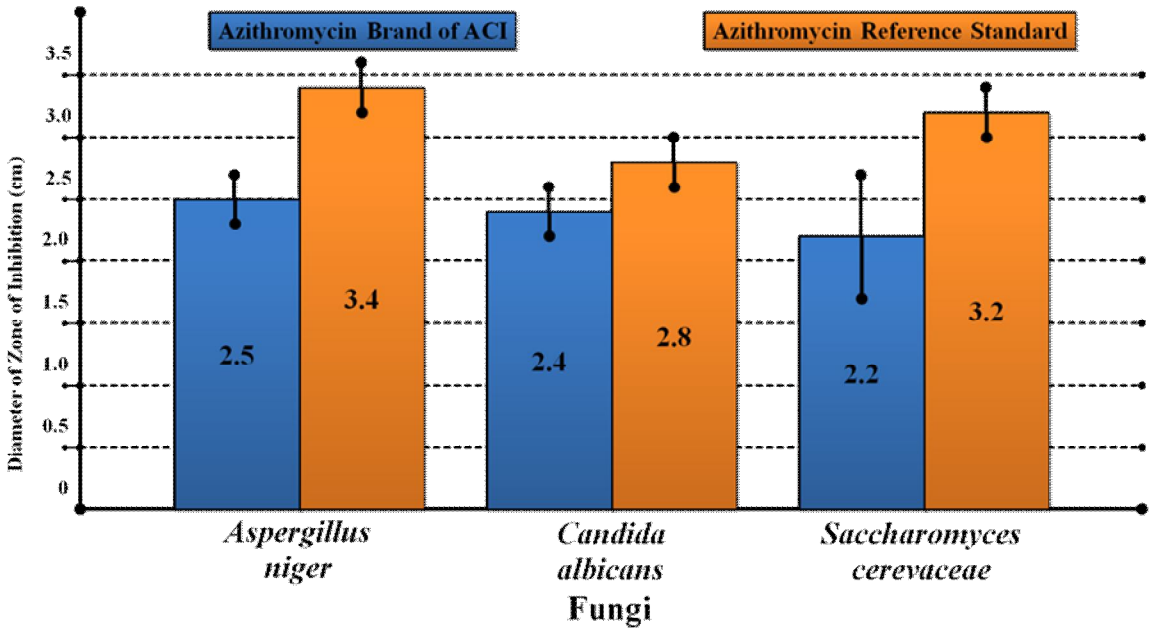

Figure 4.12: Average Diameter of Zone of Inhibition of Azithromycin of ACI vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade Fungal strains

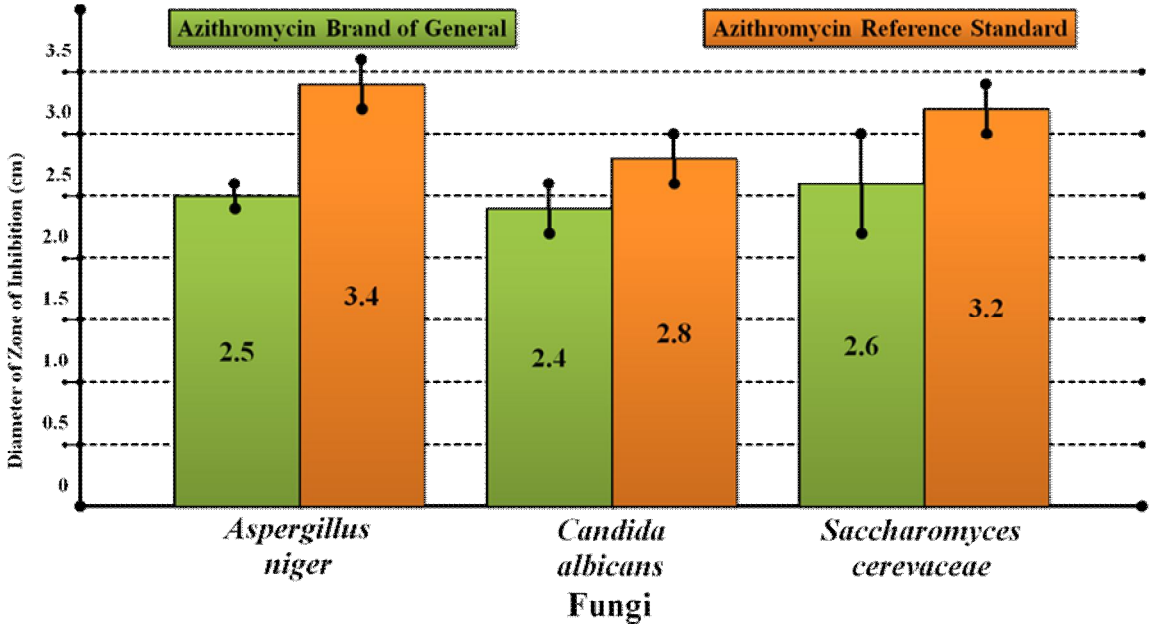

Figure 4.13: Average Diameter of Zone of Inhibition of Azithromycin of General vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade Fungal strains

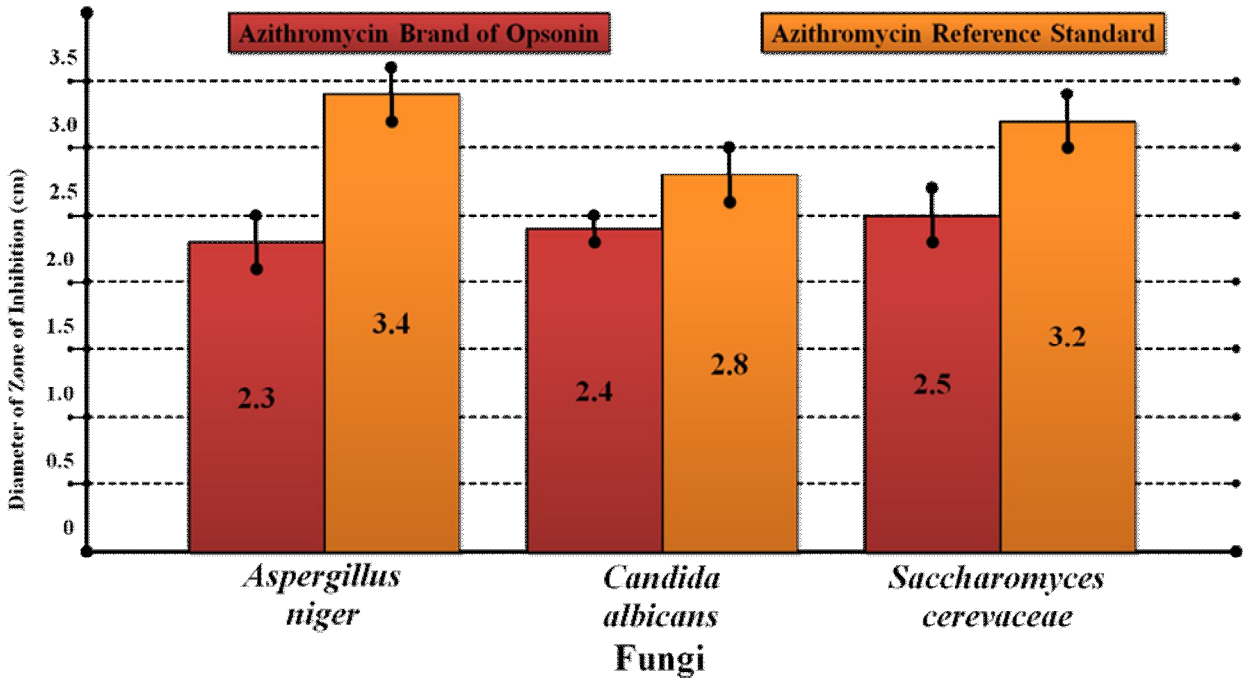

Figure 4.14: Average Diameter of Zone of Inhibition of Azithromycin of Opsonin vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade Fungal strains

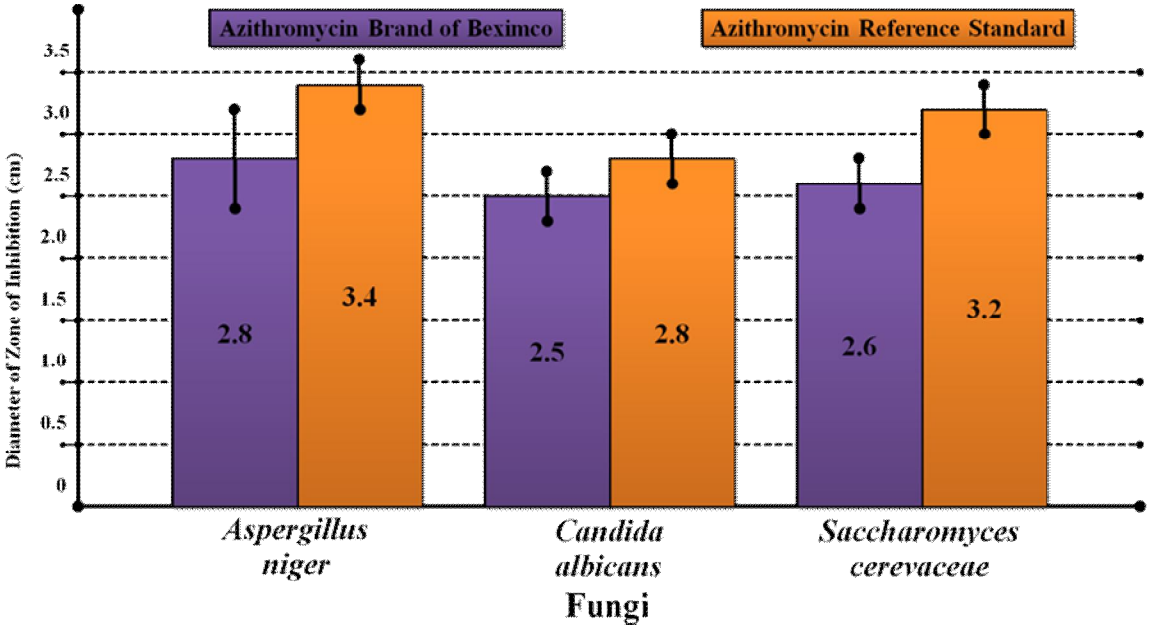

Figure 4.15: Average Diameter of Zone of Inhibition of Azithromycin of Beximco vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against ATCC grade Fungal strains

4.4 Disc Diffusion Assay of Clinical Isolates

Table 4.4: Average Diameter of Zone of Inhibition of Azithromycins against Clinical Isolates (Avg = Average of the sample; SD = Standard Deviation; C/I = Clinical Isolates; RS = Reference Standard)

C/I	Orion (Avg ± SD)	ACI (Avg ± SD)	General (Avg ± SD)	Opsonin (Avg ± SD)	Beximco (Avg ± SD)	RS (Avg ± SD)
Pus	2.4 ± 0.2	2.8 ± 0.3	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.9 ± 0.1
Blood – 1	2.2 ± 0.1	2.3 ± 0.6	2.2 ± 0.2	2.5 ± 0.2	2.6 ± 0.1	2.8 ± 0.2
Blood – 2	2.5 ± 0.1	2.5 ± 0.1	2.1 ± 0.1	2.3 ± 0.3	2.5 ± 0.2	2.7 ± 0.1

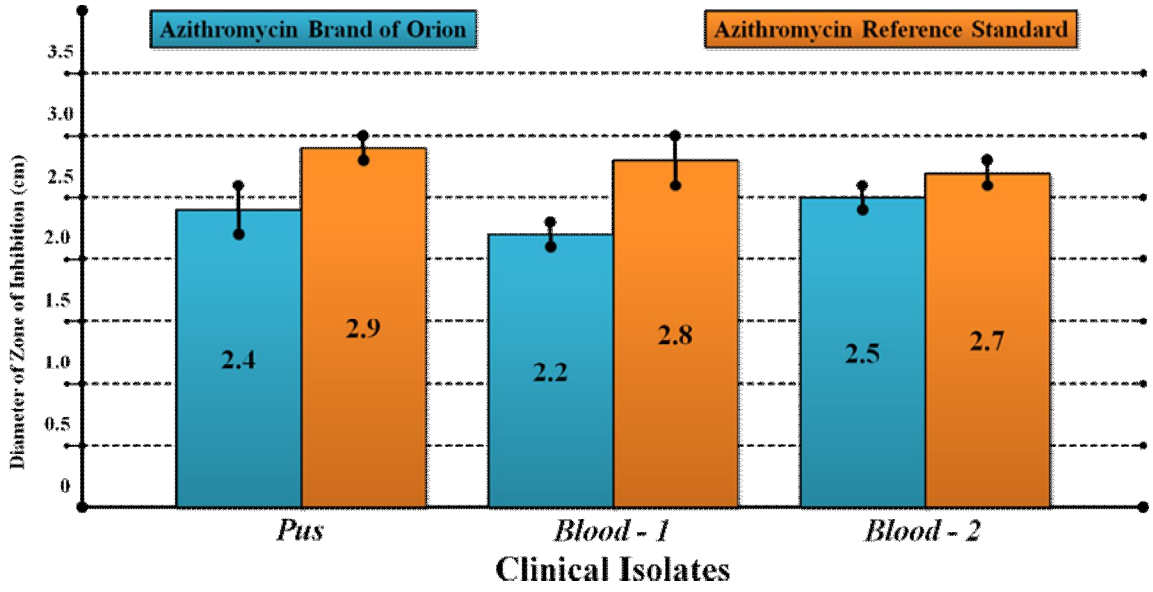

Figure 4.16: Average Diameter of Zone of Inhibition of Azithromycin of Orion vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against Clinical Isolates

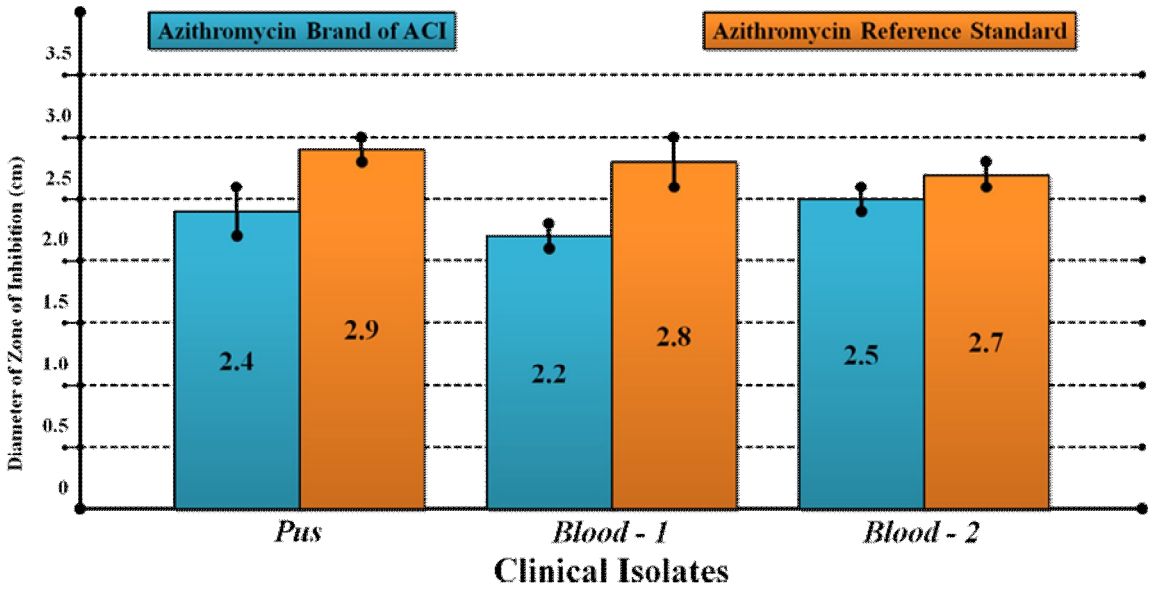

Figure17: Average Diameter of Zone of Inhibition of Azithromycin of ACI vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against Clinical Isolates

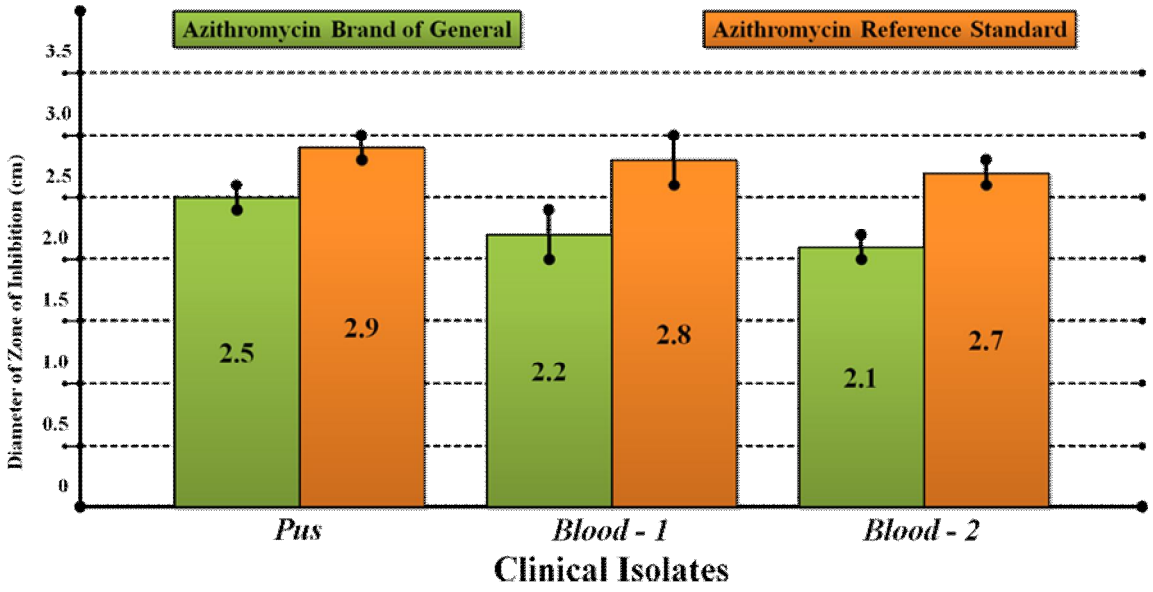

Figure 2: Average Diameter of Zone of Inhibition of Azithromycin of General vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against Clinical Isolates

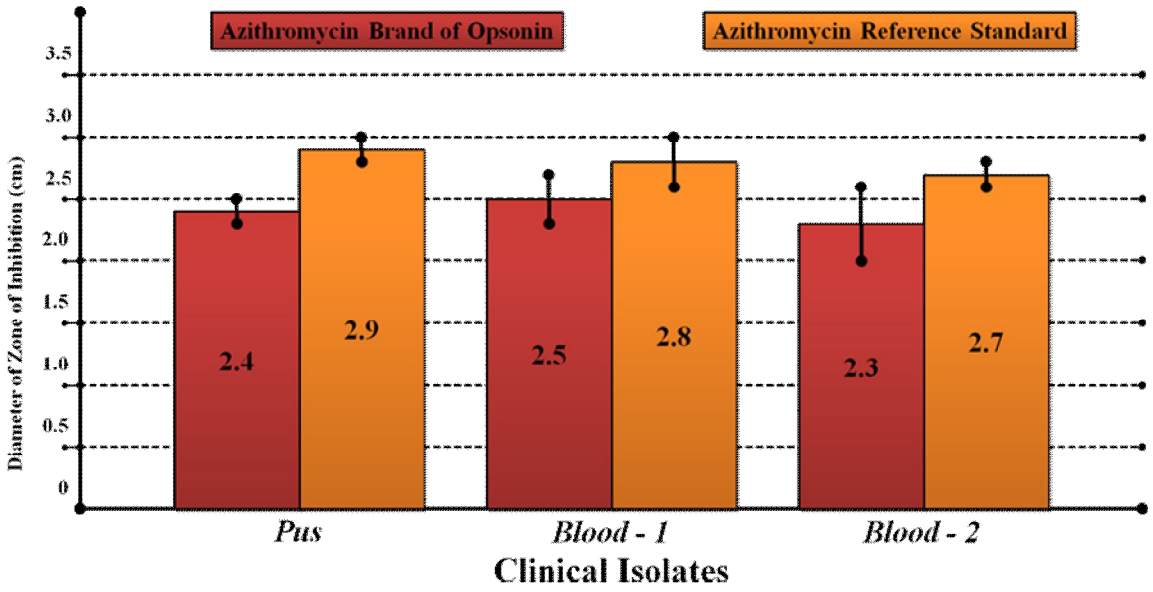

Figure 4.19: Average Diameter of Zone of Inhibition of Azithromycin of Opsonin vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against Clinical Isolates

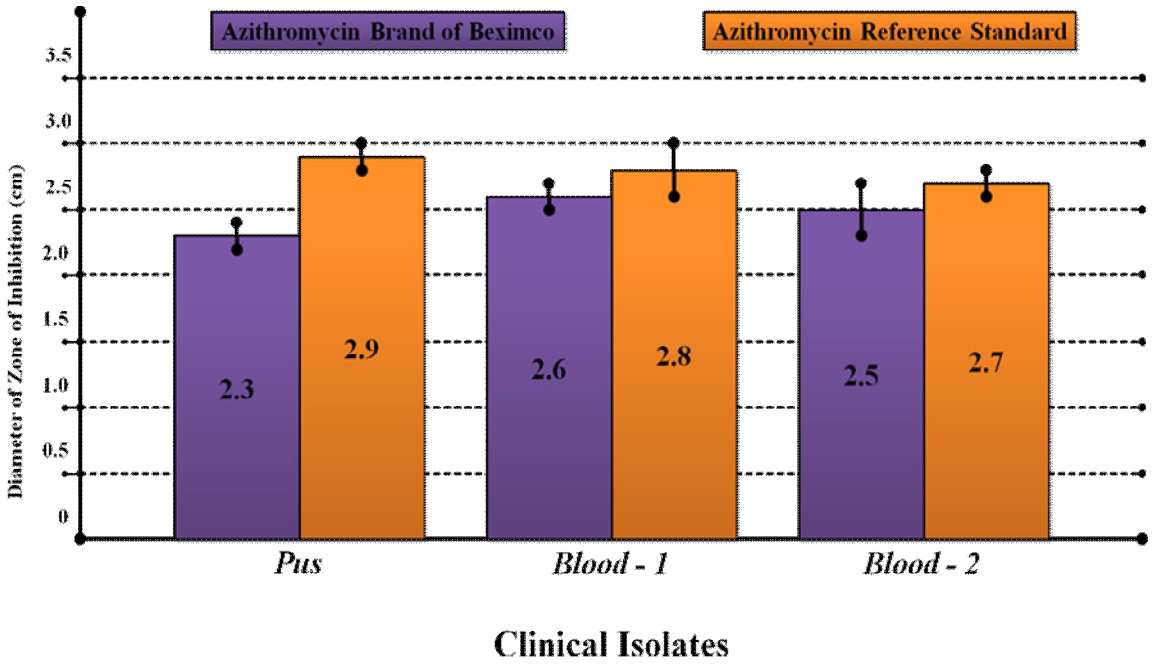

Figure 4.20: Average Diameter of Zone of Inhibition of Azithromycin of Beximco vs Azithromycin Reference Standard (Black Bold Lines between the bars indicates the standard deviations) against Clinical Isolates

4.5 Maximum & Minimum Activity Against ATCC Grade Gram Positive Bacterial Strains

Table 4.5: Maximum Activity against ATCC grade Gram Positive Bacterial Strains (SD = Standard Deviation; UL = Upper Limit; LL = Lower Limit)

Activity	Company	Diameter of Zone of Inhibition (cm)	SD	UL	LL	Species
Maximum	General	2.9	0.1	3.0	2.8	*Bacillus subtilis*
	Beximco	2.9	0.1	3.0	2.8	
Minimum	Orion	1.3	0.2	1.5	1.1	*Bacillus megaterium*

4.6 Maximum & Minimum Activity Against ATCC Grade Gram Positive Bacterial Strains

Table 4.6: Maximum Activity against ATCC grade Gram Negative Bacterial Strains (SD = Standard Deviation; UL = Upper Limit; LL = Lower Limit)

Activity	Company	Diameter of Zone of Inhibition (cm)	SD	UL	LL	Species
Maximum	General	3.5	0.2	3.7	3.3	*Escherichia coli*
Minimum	Orion	1.2	0.2	1.4	1.0	*Vibrio parahaemolyticus*

4.7 Maximum & Minimum Activity Against ATCC Grade Gram Fungal Strains

Table 4.7: Maximum Activity against ATCC grade Fungal Strains (SD = Standard Deviation; UL = Upper Limit; LL = Lower Limit)

Activity	Company	Diameter of Zone of Inhibition (cm)	SD	UL	LL	Species
Maximum	Beximco	2.8	0.4	3.2	2.4	*Aspergillus niger*
	Beximco	2.6	0.2	2.8	2.4	*Saccharomyces cerevaceae*
Minimum	Beximco	2.2	0.5	1.7	2.7	*Saccharomyces cerevaceae*

4.7 Maximum & Minimum Activity Against Clinical Isolates

Table 1: Maximum Activity against Clinical Isolates (SD = Standard Deviation; UL = Upper Limit; LL = Lower Limit)

Activity	Company	Diameter of Zone of Inhibition (cm)	SD	UL	LL	Clinical Isolates
Maximum	ACI	2.8	0.3	3.1	2.5	*Pus*
Minimum	General	2.1	0.1	2.0	2.2	*Blood – 2*

Chapter 5

DISCUSSION & CONCLUSION

5.1Discussion

5.1.1 Disc Diffusion Assay of ATCC Grade Gram Positive Bacterial Strains

- Disc Diffusion assay on six ATCC grade gram positive bacterial strains were performed by using five different Azithromycin brands of Orion, ACI, General, Opsonin, & Beximco Pharmaceuticals.
- Maximum activity of **Orion** was observed against *Bacillus subtilis* (2.5 ± 0.3) and Minimum activity was against *Bacillus megaterium* (1.3 ± 0.2).
- Maximum activity of **ACI** was observed against *Sarcina lutea* (2.8 ± 0.5) and Minimum activity was against *Bacillus megaterium* (2.1 ± 0.5).
- Maximum activity of **General** was observed against *Bacillus subtilis* (2.9 ± 0.5) and Minimum activity was against *Staphylococcus aureus* (2.3 ± 0.3).
- Maximum activity of **Opsonin** was observed against *Sarcina lutea* (2.6 ± 0.1) and Minimum activities were against *Bacillus cereus* & *Bacillus subtilis* (2.2 ± 0.1).
- Maximum activity of **Beximco** was observed against *Bacillus subtilis* (2.9 ± 0.1) and Minimum activity was against *Staphylococcus aureus* (2.0 ± 0.1).

5.1.2 Disc Diffusion Assay of ATCC Grade Gram Negative Bacterial Strains

- Disc Diffusion assay on 9 ATCC grade gram negative bacterial strains were performed by using 5 different Azithromycin brands of Orion, ACI, General, Opsonin, & Beximco Pharmaceuticals.
- Maximum activities of **Orion** were observed against *Salmonella paratyphi & Salmonella saprophyti* (2.7 ± 0.2) and Minimum activity was against *Vibrio parahaemolyticus* (1.2 ± 0.2).
- Maximum activity of **ACI** was observed against *Vibrio parahaemolyticus* (2.9 ± 0.2) and Minimum activities were against *Salmonella saprophyti* (2.0 ± 0.7) & *Salmonella paratyphi* (2.0 ± 0.2).
- Maximum activities of **General** was observed against *Escherichia coli* (3.5 ± 0.2) and also against *Salmonella paratyphi* and *Salmonella saprophyti* (2.9 ± 0.4) and Minimum activity was against *Pseudomonas aureus* (2.2 ± 0.2).

- Maximum activities of **Opsonin** were observed against *Salmonella saprophyti* (2.7 ± 0.3) and also against *Pseudomonas aureus* & *Shigella boydii* (2.6 ± 0.2) and Minimum activity were against *Salmonella typhi* (2.2 ± 0.2).
- Maximum activities of **Beximco** were observed against *Bacillus cereus* (3.4 ± 0.1) and *Vibrio parahaemolyticus* (2.8 ± 0.2) and Minimum activities were against *Pseudomonas aureus* (2.4 ± 0.3) and *Vibrio mimicus* (2.4 ± 0.2).

5.1.3 Disc Diffusion Assay of ATCC Grade Fungal Strains

- Disc Diffusion assay on 3 ATCC grade fungal strains were performed by using 5 different Azithromycin brands of Drug International, Orion, ACI, General, Opsonin, & Beximco Pharmaceuticals.
- Maximum activity of **Orion** was observed against *Candida albicans* (2.5 ± 0.2) and Minimum activity was against *Aspergillus niger* (2.4 ± 0.2).
- Maximum activity of **ACI** was observed against *Aspergillus niger* (2.5 ± 0.2) and Minimum activity was against *Saccharomyces cerevaceae* (2.2 ± 0.5).
- Maximum activity of **General** was observed against *Saccharomyces cerevaceae* (2.6 ± 0.4) and *Aspergillus niger* (2.5 ± 0.1) and Minimum activity was against *Candida albicans* (2.4 ± 0.2).
- Maximum activity of **Opsonin** was observed against *Saccharomyces cerevaceae* (2.5 ± 0.2) and Minimum activities were against *Aspergillus niger* (2.3 ± 0.2).
- Maximum activity of **Beximco** was observed against *Aspergillus niger* (2.4 ± 0.4) and Minimum activity was against *Candida albicans* (2.5 ± 0.2).

5.1.4 Disc Diffusion Assay of Clinical Isolates

- Disc Diffusion assay on 3 Clinical Isolates were performed by using 5 different Azithromycin brands of Orion, ACI, General, Opsonin, & Beximco Pharmaceuticals.
- Maximum activity of **Orion** was observed against *Strains Clinically Isolated from Blood – 2* (2.5 ± 0.1) and Minimum activity was against *Strains Clinically Isolated from Blood – 1* (2.2 ± 0.1).

- Maximum activity of **ACI** was observed against *Strains Clinically Isolated from Pus* (2.8 ± 0.3) and Minimum activity was against *Strains Clinically Isolated from Blood – 1* (2.3 ± 0.6).
- Maximum activity of **General** was observed against *Strains Clinically Isolated from Pus* (2.5 ± 0.1) and Minimum activity was against *Strains Clinically Isolated from Blood – 2* (2.1 ± 0.1).
- Maximum activity of **Opsonin** was observed against *Strains Clinically Isolated from Blood – 1* (2.5 ± 0.2) and Minimum activities were against *Strains Clinically Isolated from Blood – 2* (2.3 ± 0.3).
- Maximum activity of **Beximco** was observed against Strains Clinically Isolated from Blood – 1 (2.6 ± 0.1) and Minimum activity was against Strains Clinically Isolated from Pus (2.3 ± 0.1).

5.1.5 Maximum & Minimum Activity against Gram Positive Bacterial Strains

- Six ATCC grade gram positive bacterial strains were subjected to Kirby – Bauer Disc Diffusion Assay against 5 different Azithromycin brands manufactured by Orion, ACI, General, Opsonin, & Beximco pharmaceuticals.
- Maximum average diameter of zone of inhibition were observed for Azithromycin from General and Beximco Pharmaceuticals against *Bacillus subtilis* with 2.9 ± 0.1 and minimum was for Orion with 1.3 ± 0.2 against *Bacillus megaterium*.

5.1.6 Maximum & Minimum Activity against Gram Negative Bacterial Strains

- Nine ATCC grade gram negative bacterial strains were subjected to Kirby – Bauer Disc Diffusion Assay against 5 different Azithromycin brands manufactured by Orion, ACI, General, Opsonin, & Beximco pharmaceuticals.
- Maximum average diameter of zone of inhibition were observed for Azithromycin from General against *Escherichia coli* with 3.5 ± 0.2 and minimum was for Orion with 1.2 ± 0.2 against *Vibrio parahaemolyticus*.

5.1.7 Maximum & Minimum Activity against ATCC grade Fungal Strains

- Three ATCC grade fungal strains were subjected to Kirby – Bauer Disc Diffusion Assay against 5 different Azithromycin brands manufactured by Orion, ACI, General, Opsonin, & Beximco pharmaceuticals.
- Maximum average diameter of zone of inhibitions were observed for Azithromycin from Beximco against *Aspergillus niger* with 2.8 ± 0.4 and *Saccharomyces cerevaceae* with 2.6 ± 0.2 and minimum was for ACI with 2.2 ± 0.5 against *Saccharomyces cerevaceae*.

5.1.8 Maximum & Minimum Activity against Clinical Isolates

- Three strains clinically isolated Pus, and 2 different blood samples (Blood – 1 and Blood – 2) from were subjected to Kirby – Bauer Disc Diffusion Assay against 5 different Azithromycin brands manufactured by Orion, ACI, General, Opsonin, & Beximco pharmaceuticals.
- Maximum average diameter of zone of inhibitions were observed for Azithromycin from Beximco against Strains Clinically Isolated from Pus with 2.8 ± 0.3 and Strains Clinically Isolated from Blood – 2 with 2.1 ± 0.1.

5.2 Conclusion

Azithromycin is a subclass of macrolide antibiotics. It inhibits the translation of mRNA by binding to the 50S subunit of the bacterial ribosome. A large dose of an antibiotic can be taken to kill the bacteria at one time but these large doses cause an illness. This dose can easily create severe side effects. For that reason, antibiotics are given in small doses. So to know the MIC is very important to kill the bacteria. (Molly, 2013).

Taking too little dose of antibiotic has some disadvantages. At that time bacteria can develop methods to protect themselves & become resistant to those antibiotics. So the next time the antibiotic will be ineffective against these bacteria because of bacterial resistance. (Molly, 2013).

Azithromycin –IR tablets are given as 500 mg daily for 3 days. This single dose is also achieved higher exposure in white blood cells & at the infection site. As for example they are lung tissue & sinus fluid. (Muto, et al, 2011).

In this research work 5 different brands of Azithromycin are subjected to 6 ATCC gram positive & 9 ATCC gram negative bacteria, 3 ATCC fungi and 3 strains of microorganisms isolated from 1 clinical samples of pus, and 2 blood samples by utilizing Kirby – Bauer Disc Diffusion method. The findings of the study shows satisfactory results against the microorganisms for all brands of Azithromycins since according to USP, Azithromycin monograph, (2010) the diameter of zone of inhibition between 1.3 to 2.8 cm or more is considered susceptible towards the microorganisms. Thus, further studies are needed on more in vitro clinical samples to find out the effect of these Azithromycin brands on the mutated samples of microorganisms and bioequivalent studies are required to establish the range of doses required to treat the infected patients of Bangladesh.

Chapter 6

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