General introduction

I. History of antimicrobial drugs (Antibiotics)

The word antibiotic was used for the first time by Selman A. Waksman (1888-1973) in the medical sense in 1943 and they described "antibiotics" as a substance produced by a microorganism that is antagonistic to the growth of other microorganisms (Waksman, 1947). This definition has excludes synthetic antibacterial compounds (compounds that may kill bacteria or inhibits bacterial growth, but not produced by a microorganism) such as the sulfonamides (Scholar & Pratt, 2000; Davies & Davies, 2010). However, in the present time, the word "antibiotic" is used for any drug or compounds that kill bacteria or inhibit bacterial growth.

In 1928, Alexander Fleming (1881-1955) discovered penicillin and after 12 years of discovery, in 1942 three scientists, Ernst Chain, Howard Florey and Edward Abraham successfully purified penicillin which began the era of antibiotics (Tan & Tatsumura, 2015; Jones & Jones, 2014). However, the discovery of penicillin was not a former contribution to the use of antibiotics. There are some good evidences which suggest that antibiotics were used since the ancient period of times. In ancient civilizations, a variety of natural compounds were used as a treatment for infection, for example, herbs, honey, mouldy bread and animal faeces (Keyes *et. al.,* 2003). There are some references, which indicate that in ancient Egypt, China, Serbia, Greece and Rome, where the mouldy bread was used as a treatment for infection. John Parkinson (1567-1640) had also mentioned the benefits of mouldy bread in his book *Theatrum Botanicum,* which was published in 1640 (Gould, 2016). Even some modern antibiotics (devolved in the 20th century) may have been available in the ancient period. A notch of tetracyclines antibiotic has been detected in human skeletons dig out in Nubia and in ancient time Nubia was the part of roman occupied Egypt (Bassett *et. al*., 1980; Nelson *et. al*., 2010).

Most people have heard the story of how Alexander Fleming by coincidence contaminated his agar plates with mould and discovered penicillin back in 1929, but before the invention of penicillin, in 1909 Paul Ehrlich and Sahachiro Hata discovered a novel drug salvarsan, which is employed for treating the sexually transmitted disease *syphilis* that's is caused by the spirochete *Treponema palladium* (Ehrlich & Hata, 1910). Salvarsan was the foremost prescribed drugs until it was replaced by penicillin in the 1940s (Aminov,

2010). The systematic screening method used by Ehrlich and Hata in the invention of salvarsan became the path for identifying novel drugs and led to the discovery of the first sulfa drug (first systematically active antibacterial drug) in 1934, sulfonamidochrysoidine (KI-730, Prontosil), a precursor of the active compound sulfanilamide, which inhibits bacterial folic acid (vitamin B9) synthesis and *de novo* synthesis of nitrogen bases like purines and pyrimidines (Aminov, 2010; Achari *et. al*., 1997).

II. The golden era of antimicrobial drugs

We usually associate the beginning of the modern "antibiotic era" with the discovery of sulfa drug and release of penicillin. After the discovery of both drugs a period of 30 years known as the golden age of antibiotics (1940-1970), in which almost the entire antibiotic drug classes used in the clinic today was discovered (Figure 1) (Gould, 2016 and Clatworthy *et. al*., 2007).

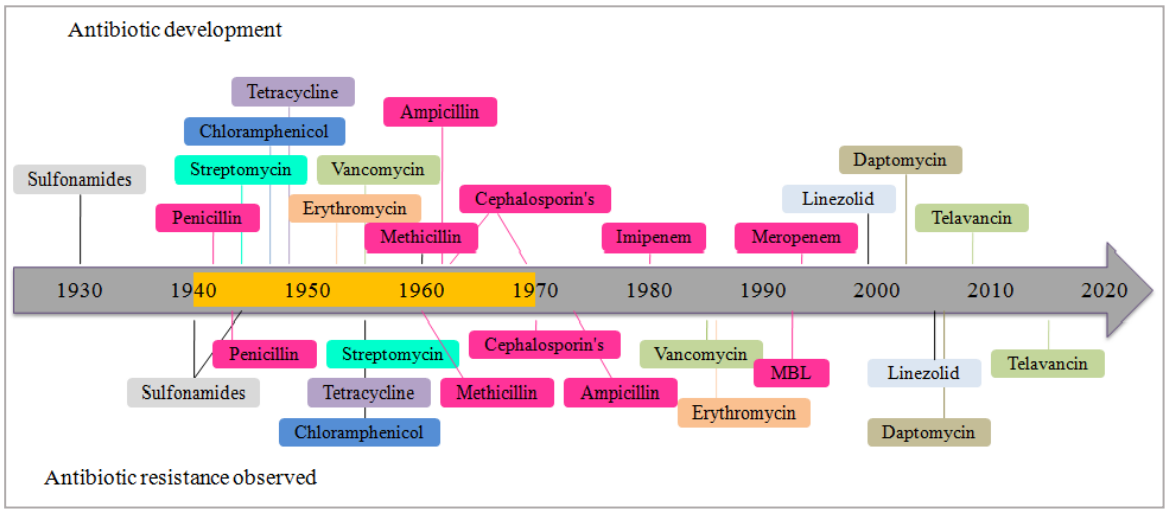

Figure 1: Timeline of new antibiotics developments and antibiotic resistance developments

The upper panel indicates the time at which antibiotics were discovered. The down panel indicates when resistance was observed. Modified from Clatworthy *et. al.*, 2007.

Most of the antimicrobial compounds discovered in the early days of the golden era of antibiotics were isolated from different naturally occurring microorganisms, and this type of research work began worldwide after the isolation of an antimicrobial compound "streptomycin". In 1944 streptomycin was isolated from soil growing bacteria *Streptomyces griseus* (Saga, 2009). Eli Lilly had given a good idea and requested Christian

missionaries to send him a sample from every exotic place that they had visited and through this way they collected the soil samples from around the world. Among them, one soil sample was from Borneo and vancomycin producing bacteria was isolated from these soil samples. In 1952 vancomycin producing *Streptomyces orientalis* was isolated from Borneo soil samples and this vancomycin antibacterial compound was released in 1958 for clinical use (Levine, 2006). Amidst all of this, Scientists were engaged in improving these existing agents so that they could overcome this obstacle of antibiotic resistance. As a result in 1959 first penicillinase-resistant β-lactam antibiotic "methicillin" was found. The penicillin's spectrums of activity pharmacodynamics/pharmacokinetics were improved by the introduction of ampicillin in 1961.

Cephalosporin was first introduced in 1960 and their evolution classified into three generations, according to their spectrum of activity. Third-generation cephalosporin ceftazidime appeared in the late 1970s (Russell, 1957).

The first β-lactamase inhibitors clavulanic acid was identified in 1976, which was isolated from gram-positive bacteria *Streptomyces clavuligerus* (Drawz & Bonomo, 2010). It was introduced for clinical use in combination with amoxicillin and thienamycin. First carbapenem antibiotic imipenem was evolved from Thienamycin in 1983 which was very effective *in vitro* condition; however, it had very short half-life in the human body. After addition of cilastatin in imipenem, the half-life ($t_{1/2}$) of imipenem had increased and the combination of imipenem + cilastatin was available for clinical use around the world. Second, most prescribed carbapenem antibiotics "meropenem" was introduced in 1995 and it had similar activity. It was associated with fewer adverse effects (Papp-Wallace *et. al.*, 2011).

There were plenty of antibiotic compounds discovered during the golden era, along with the start of antibiotics resistant bacteria. However, this was the beginning of the race between the evolution of antibiotic resistance and development and discovery of antibiotics "a race that currently seems to be led by the microorganism".

III. Classification of antibiotics

There are different classifications of antibiotics according to the mode of action, spectrum efficacy, the killing (bactericidal) or inhibitory (bacteriostatic) effects and their

Table 1: Classification of antibiotics, based on to their chemical structure and mechanism of action

Class of antibiotics	Examples	Mechanism of action
Aminoglycosides	Neomycin	Inhibit the bacterial protein synthesis by binding to 50S ribosomal subunit.
	Streptomycin	
Beta-lactam antibiotics		
Carbapenems	Imipenem	Inhibit the bacterial cell wall formation by inhibiting the cross-linking of stem peptides on peptidoglycan chain.
Cephalosporins	Cefepime	
Monobactam	Aztreonam	
Penicillin	Ampicillin	
Fluoroquinolones	Levofloxacin	Inhibit bacterial DNA synthesis by binding to DNA gyrase enzymes.
	Ciprofloxacin	
Folate pathway inhibitors	Trimethoprim Sulphamethaxazole	Folate pathway inhibitors.
Furanes	Nitrofurantoin	Damage bacterial DNA.
Imidazoles	Metronidazole	Inhibit bacterial DNA synthesis.
Lipopeptides	Polymyxins	Alters the bacterial membrane functions.
Lincosamides	Clindamycin	Interfering bacterial protein synthesis.
Macrolides	Erythromycin	Inhibition of bacterial protein synthesis.
	Azithromycin	
Peptides	Bacitracin	Prevent the bacterial cell wall synthesis.
Phenicols	Chloramphenicol	Inhibit the bacterial protein synthesis by binding to 50S subunits.
Phosphonic acids	Fosfomycin	Prevent the bacterial protein synthesis.
Polymyxins	Colistin	Destroying bacterial outer membrane integrity.
	Polymyxin B	
Tetracyclines	Tetracycline	Inhibit the bacterial protein synthesis by binding to 30S subunits.
	Doxycycline	

structural similarities. The classification of antibiotics according to their mode of action depends on the inhibition of a cellular structure or a metabolic channel that's present within the bacterial cell but not in the host cell. Several mechanisms have been reported

like the inhibition of bacterial cell wall synthesis, obstruction with cell membrane functions, inhibition of protein synthesis, and inhibition of nucleic acid synthesis (Levinson, 2008). However, the foremost common way of antibiotics classification is based on their chemical structure (Table 1), as antibiotics having closely related chemical structure tend to have the same antibacterial activity and same spectrum of activity against the host.

IV. Beta-lactam antibiotics

Beta-lactams are antibiotics that contain a β-lactam ring (2-Azeidinone) in their chemical structure (penicillins, cephalosporins, carbapenems and monobactams) (Figure 2). β-lactam antibiotics have a broad spectrum of antimicrobial activity, including gram-positive and gram-negative pathogens. Because of their favourable characteristics, β-lactams are the most broadly used antibiotics worldwide (Livermore *et. al.*, 2006). The β-lactam ring is essential for the antimicrobial activity and which mimics the D, D-alanyl-alanine structure of the pentapeptide chain of the bacterial cell wall. The D, D-alanyl-alanine structure is the site of catalysis for PBPs catalytic domain thus; the β-lactam rings of beta-lactam antibiotic binds irreversibly to the PBPs and inhibit the synthesis of the peptidoglycan layer in the bacterial cell wall (Konaklieva *et. al.*, 2014). All the β-lactam antibiotics are classified into four major groups based on their molecular structure.

(a) Penicillin: These antibiotics were among the first antibiotics prevalent in the treatment of infection caused by gram-positive bacteria. Drugs in the penicillin class work by indirectly bursting the bacterial cell walls. The basic structure of the penicillin group of antibiotics must contain a heterocyclic thiazolidine nucleus which is attached with a β-lactam ring and a side chain at the C6 position (Dalhoff *et. al.*, 2006). Based on the molecular composition of penicillin's side chain represent a different group of penicillin's: benzylpenicillin (Penicillin G), phenoxymethylpenicillin (Penicillin V), Penicillin O etc. However, due to the extensive use of natural penicillin, frequent resistance was observed. Also, the natural penicillins have a limited scope of use due to stability and extraction process.

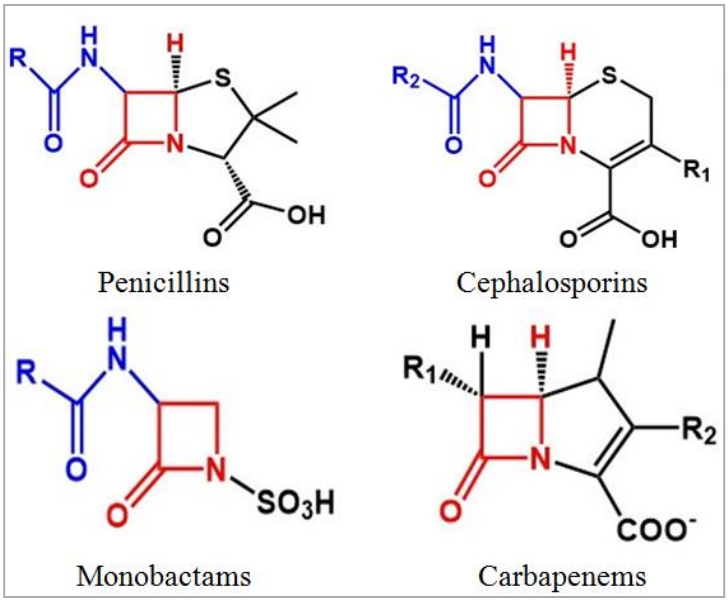

Figure 2: Beta-lactam antibiotics core structure. All β-lactam antibiotics contain the same core structure (highlighted in red)

(b) Cephalosporin: Groups of cephalosporin antibiotics are considered as the largest group of broad-spectrum antibiotics and cephalosporin antibiotic was discovered by the Italian pharmacologist Giusseppe Brotzu from the mould *Cephalosprium* (Torok *et. al*., 2016). They contain a nucleus (7- Aminocephalosporanic acid), which is fused with the β-lactam ring. Based on the antimicrobial activity, development period and modification of cephalosporin, the group has been subdivided into four generations (Table 2).

(c) Carbapenem: These antibiotics are the broad-spectrum β-lactam antibiotics used against all types of multidrug-resistant bacteria. All the carbapenem antibiotics are the most effective against both types of drug resistance microorganism (gram-positive and gram-negative bacteria) and therefore, often it is called the last resort of antibiotics. The widely used carbapenems are imipenem (developed in 1980 by Merck & Co.), meropenem (discovered by Dainippon Sumitomo Pharma).

(d) Monobactam: These antibiotics are the compounds which have a single β-lactam ring without any fused ring (Figure 2). It is narrow-spectrum β-lactam and they are active against only gram-negative bacteria. MBL (bla_{NDM}) positive strains are only sensitive against (Aztreonam) monobactam antibiotics (Aztreonam is the only monobactam antibiotics which is used presently in clinical practices).

Table 2: Generations of cephalosporin antibiotics

1st generation	2nd generation	3rd generation	4th generation	5th generation
Cefadroxil Cephalothin Cephalexin Cefapirin	Cefaclor Cefotetan Cefoxitin Cefprozil	Cefotaxime Cefixime Cefoperazone Ceftazidime	Cefepime Cefpirome Cefquinome	Ceftaroline Ceftobiprole Ceftolozane
Effective against gram positive bacteria and less effective against gram negative	Effective against gram negative bacteria and moderate against gram positive	Effective against gram negative bacteria and less effective against gram positive	Effective against gram negative bacteria and less effective against gram positive	Less susceptible to the development of resistance when combined with β-lactamase inhibitors.

V. Mechanism of resistance to β-lactams

Bacteria employ multiple mechanisms to resist β-lactam antibiotics. These resistance mechanisms have developed in bacteria either via mutation in the bacterial gene or by the acquisition of resistance genes from other bacteria. Bacteria can avoid the bactericidal effect of β-lactam antibiotics by several mechanisms; active efflux pumps promoting the efflux of the antibiotic out of the cell, a mutation in the penicillin-binding protein, lack/reduced the expression of porin channels, inactivation of β-lactam antibiotics by chromosomally encoded β-lactamases or through acquired specific β-lactamases gene (Figure 3) (Wanda, 2018). Often, multiple β-lactam resistance mechanisms could be also present in a single bacterial organism and some cases, they will work synergistically.

i. Reduced permeability: Porin is a channel, present in the outer membrane of a bacterial cell and it facilitates the transport of β-lactam antibiotic molecules across the cell membrane. Mutated porin channel can be reduced or lost to inhibit β-lactam invasion into the cell. *Pseudomonas aeruginosa* OprD porin is the substrate-specific porin and it facilitates the diffusion of imipenem. Modified/mutated OmpC and OmpF outer membrane Porins are a common in *E. coli* and *Enterobacter* spp. and increase the MIC value of antibiotics (Ochs *et. al*., 1999; Wozniak *et. al*., 2012; Novais *et. al*., 2012).

ii. **Efflux pumps:** Efflux pumps actively transport antibiotics and toxic substrates across the cell membrane to outside of the cell. Thus increase the expression of efflux pump genes

in bacterial cell increase the MIC values of antibiotics and decrease the level of antibiotics inside the cell. Total six different types of antibiotic resistance efflux pump have been reported and among them, RND efflux pumps are responsible for β-lactam antibiotic resistance in gram-negative bacteria. MexAB-OprM RND type efflux pump played an important role in carbapenem resistance in *Pseudomonas* spp. (Fernandez and Hancock, 2012).

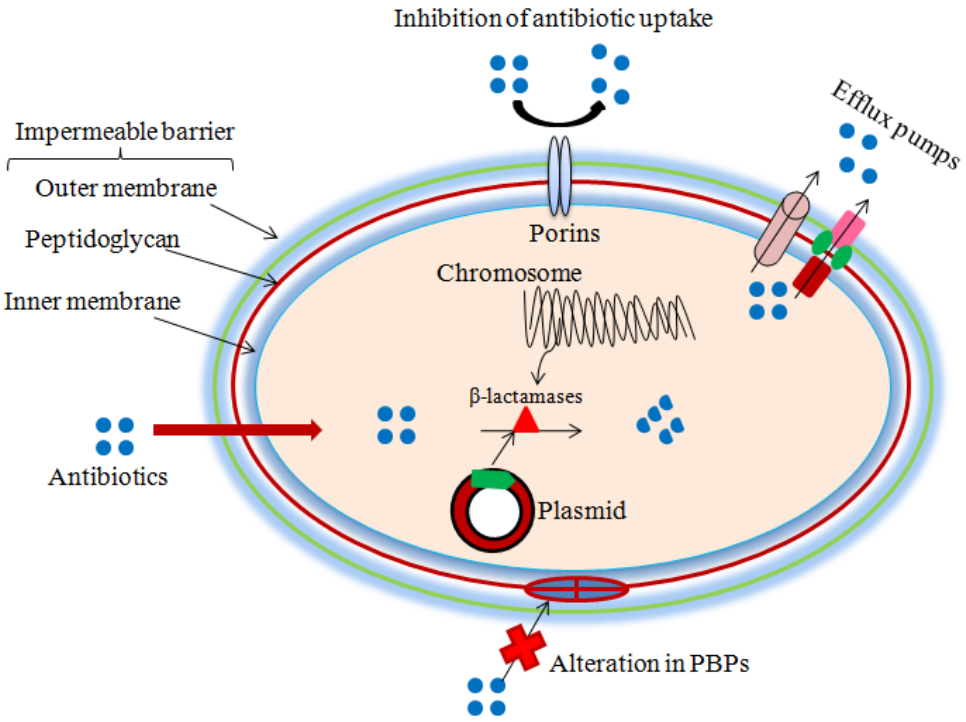

Figure 3: Mechanisms of resistance of β-lactam antibiotics in gram-negative bacteria

This diagram shows the mechanism used by gram-negative bacteria confer resistance to β-lactam antibiotics, which include enzymatic inactivation, activation of different types of efflux pumps, porins channel and alteration of β-lactam binding sites in the cell (Modified from Wanda, 2018).

iii. Penicillin-binding protein: Penicillin-binding protein is the first target of β-lactam antibiotics and due to mutation in PBP site the antibiotic molecule is unable to bind to them and this type of resistance mechanism observed in gram-negative and gram-positive bacteria (Zapun *et. al*., 2018). Due to this alteration or mutation *Staphylococcus aureus* show, methicillin resistance and *Pneumococci* show penicillin resistance (Grebe and Hakenbeck, 1996).

VI. Beta-lactamases

Enzymes are able to reduce the activation barrier of a reaction, thus accelerating reaction rates by many orders of magnitude. For this elemental ability, enzymes are both essential and ubiquitous in all the kingdoms of life (Wilcox, 1996 and Schenk, *et. al*., 2012). Enzymes can catalyze diverse reactions, being involved in a wide range of chemical pathways. For example, hydrolases are enzymes that can hydrolyze ester or amide bonds in specific substrates. Hydrolytic enzymes are involved in many physiological pathways and are necessary to recycle metabolites needed by the cells (Wilcox, 1996; Schenk, *et. al*., 2012 and Meyers, *et. al*., 1996). Among them, the enzyme β-lactamases are produced by both gram-positive and gram-negative bacteria and they hydrolyzed amide bond of the four-membered β-lactam ring of penicillin, cephalosporin, monobactam and cephalosporin group of antibiotics, destroying their antimicrobial activity (Wilcox, 1996; Schenk, *et. al*., 2012; Mitic *et. al*., 2006). In the case of gram-negative bacteria, they accumulate in the periplasmic space of cell wall, whereas in the case of gram-positive bacteria, they get excreted from the cell (Ghuysen, 1991).

Presently, more than 850 types of β-lactamases have been recognized, we speculate that the fast replication rate, recombination rates, and high mutation frequency allow bacteria to adapt to novel β-lactams by the evolution of these β-lactamases (Perez *et. al*., 2007).

VII. Classification of beta-lactamases

Beta-lactamases have been classified based on various criteria such as relative rates of hydrolysis and inhibition of β-lactamases by various compounds and their relation to molecular structure and functional properties. The molecular structure (sequenced based) classification of β-lactamases enzyme was proposed by Ambler (Ambler, 1980) and also called the ambler classification. It is based on amino acid sequence and the nature of the catalytic site. It distinguishes four different classes of the β-lactamases, where class A, C and D contain evolutionary distinct serine β-lactamases and class B contains Zinc metal ions (Table 3) (Ambler *et. al*., 1991). The second classification of β-lactamases was functional classification and it proposed by Bush, Jacoby-Medeiros (Bush and Jacoby 2010) and known as Bush Jacoby classification. It is more complex and based on substrate

affinity and inhibition properties. According to functional classification, the β-lactamases enzyme is divided into four major groups (group 1 to 4), with multiple subgroups. On the basis of functional classification metallo-β-lactamases placed in group 2f and 3 (Table 3).

Table 3: Classification of β-lactamase enzymes (Bush *et. al*., 2010)

Ambler class	Bush Jacoby-Medeiros group	Active site	Enzyme Type	Substrate
A	2b, 2be, 2br, 2c, 2e, 2f	Serine	ESBL (TEM, SHV, CTX-M)	Penicillins, 3 generation Cephalosporins
			Carbapenemase (KPC, GES, SME)	All beta-lactams
B	3	Zn binding thiol group	Carbapenemases (NDM, IMP, VIM)	All beta-lactams
C	1	Serine	AmpC, CMY, FOX	Cephamycins, 3rd Generation cephalosporins
	1e		GC1, CMY-37	Cephamycins, 3rd Generation cephalosporins
D	2d	Serine	ESBL (OXA)	Penicillins, 3rd generation cephalosporins
			Broad spectrum beta-lactamases (OXA)	Oxacillin, ampicillin, cephalothin
			Carbapenemases (OXA)	All beta-lactams

VII. Metallo-β-lactamases

Metallo-β-lactamases are a class of binuclear metallohydrolases and the first metallo-β lactamases (B1 types) was reported in gram-positive bacteria *Bacillus cereus* (Sabath *et. al*., 1966; Hussain *et. al*., 1985). Metallo-β-lactamases have a broad substrate spectrum β-lactamases and it can hydrolyze virtually all β-lactams antibiotics including imipenem. The metallo-β-lactamases enzymes reveal a various range of sequences homology with minimum 25% similarity between some enzymes (Garau *et. al*., 2004). X-ray crystallographic study of metallo-β-lactamases revealed that the group is structurally similar and it has a characteristic αβ/βα sandwich fold with the active site (Ullah *et. al*.,

1998; Concha *et. al*., 1996). The structural fold, a characteristic of MBLs was first identified in 1997 (Bebrone, 2007; Neuwald *et. al*., 1997). This three-dimensional fold structure supports up to six amino acids residues at the active site which manages either one or two zinc metal ions that are responsible for the catalytic mechanism. Enzymes belonging to the MBLs family of β-lactamases share a common three-dimensional structure as well as five conserved motifs in their sequences, i.e. Asp84, His116-X-His118-X-Asp120-His121, His196, Asp221 and His263 (Bebrone, 2007; Crowder *et. al*., 2006; Daiyasu *et. al*., 2001).

Based on the structural homology between two halves of the MBL protein, it has assumed that MBLs may have developed due to a gene-duplication. But no solid evidence has been established yet to substantiate such hypothesis. Rather, a solid authentication has been made that such beta-lactamases evolved from an ancient superfamily of metallohydrolases with distinct activities, called the MBL superfamily (Daiyasu *et. al.*, 2001). Moreover, the αβ/βα fold and a metal-binding motif in the active site are more conserved across the MBL superfamily, which is assumed to have evolved billions of years ago (Daiyasu *et. al.,* 2001). The metal ligands present in MBLs are distinctive to enzymes activity, substantiate that this metal-binding motif has been specific for β-lactam hydrolysis (Gonzalez *et. al.,* 2012).

Based on the sequence homology, substrate binding ability and Zinc ion requirements, the MBLs are divided into three subgroups (B1, B2 and B3) based on amino acid similarity (Bebrone, 2007; Garau *et. al*., 2004; Heinz *et. al*., 2004; Page *et. al*., 2008). Among all the three subclasses, B1 subclass is the most prevalent and structurally most extensively studied class of MBLs (Bebrone, 2007; Bellais, *et. al*., 2002; Carfi A., *et. al*., 1995). Among the class B β-lactamases the most common and biggest MBLs groups are B1 subgroups MBLs, which harbours some good studies MBLs such as Verona integron-encoded metallo-β-lactamases (VIM-1) from *P. aeruginosa*, New Delhi metallo-β-lactamases (NDM-1) *K. pneumoniae*, CcrA metallo-β-lactamases from *Bacteroides fragilis*, BcII metallo-β-lactamases from *Bacillus cereus*, and IMP-1 from *P. aeruginosa*. and Sao Paulo metallo-β-lactamases (SPM-1) from *P. aeruginosa* (Yong *et. al*., 2009; Poirel *et. al*., 2010; Rolain *et. al*., 2010; Zhang *et. al*., 2011; Walsh *et. al*., 2011; Li *et. al*., 2014). Such β-lactamases efficiently hydrolyse various β-lactam antibiotics groups, which

included penicillin's, cephalosporins, and carbapenems. Examples of subclass B2 is ImiS metallo-β-lactamases from *A. veronii* and CphA from *A hydrophila* (Segatore *et. al*., 1993; Crawford *et. al*., 2004) which have poor activity towards penicillins and cephalosporins groups of β-lactam antibiotics but easily hydrolyses the antibiotics of the carbapenems. Finally, subclass B3 is represented by monomeric FEZ-1 metallo-β-lactamases isolated from *F. gormanii*, and L1 metallo-β-lactamases isolated from *S. maltophilia*, both hydrolyse penicillins and cephalosporins groups of β-lactam antibiotics effectively (Costello *et. al*., 2006; Ullah *et. al*., 1998; Hu *et. al*., 2008; Garcia-Saez *et. al*., 2003).

The first NDM-1 was reported in 2009, a Swedish patient, who travelled to New Delhi and got infected with *Klebsiella pneumonia* (Yong *et. al*., 2009). NDM-1 belongs to the B1 subclass of Metallo-β-lactamase family containing two zinc ions and other divalent cations as cofactors. It catalyses almost all classes of β-lactam antibiotics including carbapenems. NDM-1 is more effective and broad in inactivating β-lactam antibiotics than known MBLs. The most common bacteria that make this enzyme are gram-negative bacteria such *Enterobacteriaceae* species, *Acinetobacter* sp., and *Pseudomonas aeruginosa* but the gene of NDM-1 can spread from one species/strain of bacteria to another by horizontal gene transfer (Zheng *et. al*., 2011).

VIII. Environmental perspective of abundance and spread of metallo-β-lactamases

The soil and water environment is a repository of antibiotics resistant bacteria and antibiotic-resistant genes, where these bacteria transmit antimicrobial resistance genes within the same or different genus and species. It is not yet clear whether an antibiotic-resistant bacterium originated from the clinical setting or environment, but it has been confirmed that clinical setting antibiotic-resistant bacteria are also found in the environment setting like soil and water. The antibiotic resistance gene of the clinical setting and soil share almost 100% similarity and these bacteria are a source of these antibiotics resistance genes for human infection (Forsberg *et. al*., 2012). However, now it was confirmed that antibiotic resistance genes are an essential element of bacteria and these types of bacteria come from different sources into the soil and water environment and where they are exchanged or transfer antibiotic resistance genes within the same species or other species. Due to this horizontal gene transformation method, different types of

antibiotic-resistant bacteria are evolving and spread in human and between the various environments (Figure 4).

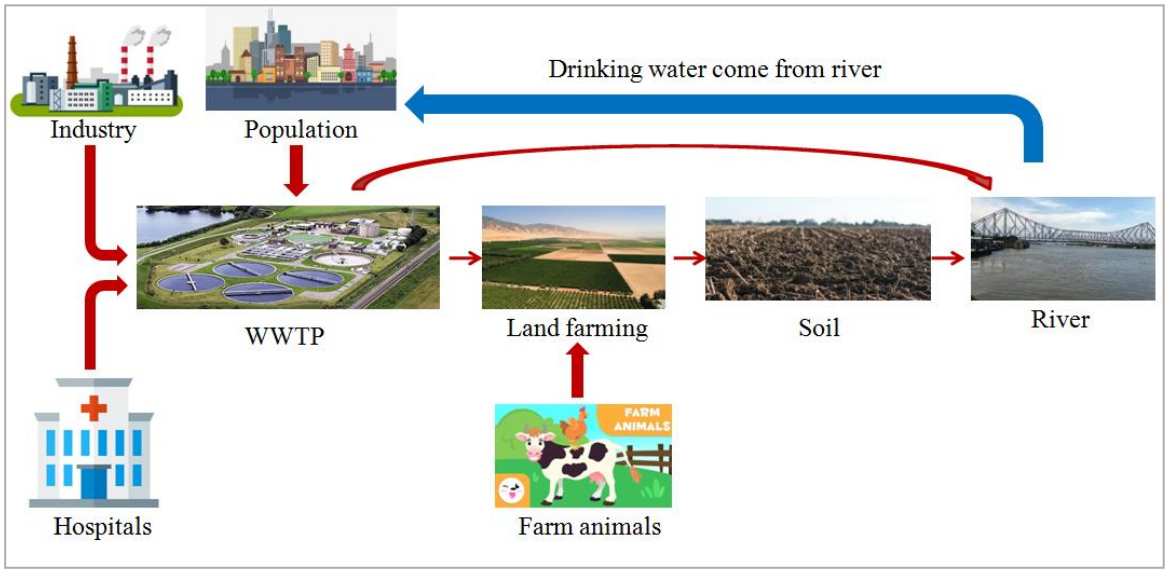

Figure 4: Various routes for antibiotic resistant bacteria and gene spread from human activity origins to the environment

The above figure shows the spread and dissemination routes of antibiotic resistant bacteria and gene between human and different environment, industry, farm animal and agriculture farm (WWTP= waste water treatment plants) (adapted and modified from Berendonk *et . al*., 2015).

Antibiotic-resistant bacteria are present in soil and represent the evolutionary reservoir of resistance for most bacteria. The soil environment is the largest and most divergent among other types of environments. Several studies have provided evidence for the transmission of ARGs between soil bacteria and clinical pathogens (D'Costa *et. al*., 2006).

Some of the most dangerous clinical infections are caused by the metallo-β-lactamase (MBL) producing bacteria (Van Duin & Doi, 2017). Some MBLs genes are plasmid-mediated and some are carried on smaller, independent chromosomes (Queenan & Bush, 2008). Plasmid-mediated MBLs producing genes include variants of New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and imipenem resistance metallo-β-lactamase (IMP). These MBLs producing genes are found in environmental samples like soil and water but they are typically found in conjunction with at least one of the other β-lactamase genes (Diene & Rolain, 2014; Meletis, 2016).

The study about MBLs type antibiotic resistance is often focused around clinical setting and it is an important source of MBLs and other types of ARGs or ARB and these MBLs producing bacteria release to the environment through the hospital sewage system to water and soil environment (Ray *et. al*., 2016; Satlin *et. al*., 2017). Two independent studies, one from Bangladesh and another one from Switzerland have reported increased MBLs-producing isolates downstream of hospitals (Islam *et. al*., 2017; Zurfluh *et. al*., 2017). Untreated hospital wastewater has the main source of MBLs producing bacteria in a developing country, which has been reported in several studies. NDM types MBLs have been isolated from hospital wastewater in Seoul, South Korea (Hwang and Kim, 2018). In Saudi Arabia, NDM was detected in municipal wastewater (Mantilla-Calderon *et. al*., 2016). In North China, NDM was detected in the final effluent of WWTP and dewatered sludge, which is often used in agriculture field. VIM was also identified in freshwater and sediment sludge samples of WWTP in Tianjin (Yang *et. al*., 2017). In India, NDM was not detected in WWTP samples, but it was identified in two treated tap water samples from around the Delhi city (Walsh *et. al*., 2011). Water samples from a first Nations Community in Canada also detected VIM types of MBLs in their drinking water, (Fernando *et. al*., 2016). NDM types of MBLs have been isolated from surface water in the Danube River, Brazil (Kittinger *et. al*., 2016). VIM types MBLs have been isolated from water in Canada, Spain and Austria. Sediment sludge is also a reservoir of AR bacteria and a higher concentration of AR bacteria are reported than waterways (Yang *et. al*., 2017). A study of sediment soil in Mula Mutha River, India detected MBLs producing genes prevalence in the upstream region of a city and within the city. Whereas only one type of MBLs VIM was detected in the upstream region of the city and two genes of interest NDM and VIM were detected within the city (Marathe *et. al*., 2017). NDM has been also detected in sediment soil samples from the Scioto River watershed in Ohio, USA (Lee *et. al*., 2019).

IX. Epidemiology

Metallo-β-lactamases (MBL) producing bacterial isolates mainly *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp. and other genera have a strong impact on clinical and therapeutic decisions. MBL-producing gram-negative bacteria have been reported continuously worldwide. Continues emergence and worldwide spreading of MBLs producing bacteria are becoming major threats to public health. The current

epidemiology of MBLs identification usually follows a pattern of increasing occurrences that are country-specific. Apparently, this depends upon multiple factors, including excessive use of antibiotics, dosing procedure, and clinical practices concerning the isolation of patients with multidrug-resistant pathogens.

IMP-4 was the first MBL found in China, which was detected in *Citrobacter youngae* (Hawkey *et. al*., 2001) and it was subsequently reported in four different unrelated strain of *Acinetobacter* spp. and *Klebsiella pneumoniae* (Chu *et. al*., 2001 and Mendes *et. al*., 2008). IMP-1 has been detected in *P. aeruginosa* and *Enterobacter cloacae* (Cheng *et. al*., 2008; Chen *et. al*., 2009). In 2006 in China the first VIM types MBL VIM-2 was detected in *P. aeruginosa* (Wang *et. al*., 2006). Recently NDM-1, NDM-3 and NDM-5 producing bacterial strain were detected and these MBLs producing strains was isolated from eight city hospital in China from January 2013 to December 2015. (Xiaofeng Hu *et. al*., 2017).

IMP-1 positive *P. aeruginosa* and *Serratia marcescens* which were isolated from Japanese patients were first MBL positive strain detected in Japan (Watanabe *et. al*., 1991; Osano *et. al*., 1994). However, now in Japan, IMP-1 has been found in *Enterobacteriaceae* and other non-fermenting bacterial strain. The VIM type MBLs were later identified in Japan with low prevalence than that of the IMP type MBLs.

A VIM-2 type MBL was common MBL in South Korea and it was detected in various gram-negative bacteria such as; *P. aeruginosa*, *Enterobacteriaceae* and *Acinetobacter* spp. (Walsh *et. al*., 2005; Yum *et. al*., 2002). Various type MBLs like SIM-1, IMP-1 and NDM-1 were reported from South Korea and first NDM-1 producing *Klebsiella pneumoniae* was detected in 2011.

In the Middle East region of Asia, the alleles of VIM and NDM-1 MBLs were identified. VIM-2 MBLs were detected in *P. aeruginosa*, which was isolated from Saudi Arabia and Iran and first NDM-1 was reported from Oman in 2011 (Al-Agamy *et. al*., 2009; Khosravi *et. al*., 2008).

NDM-1 and their alleles are widely spread in Indian subcontinents (Pakistan, Bangladesh, and Nepal). The NDM-12 and NDM-14 first appeared in Nepal. It has now widely identified in *P. aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp. and *Klebsiella pneumoniae* (Rolain *et. al*., 2010; Kumarasamy (a) *et. al*., 2010 and Kumarasamy (b) *et.*

al., 2010). Several VIM types MBLs like VIM-2, VIM-5, and VIM-11 are highly widespread in *Pseudomonas* spp. (Castanheira *et. al*., 2009). IMP types MBLs identified in *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Azim *et. al*., 2010). In Asian countries like China, Japan, Korea, India, Pakistan and Gulf countries the most common MBLs are IMP, VIM and NDM-1 among them VIM and NDM most predominant.

Among the European country, Italy was the first country where MBLs were reported, namely the IMP-2 and VIM-1 (Chu *et. al*., 2001 and Cornaglia *et. al*., 1999). Various type of IMP and VIM were reported from Italy, and among them VIM-1, VIM-2 and IMP-13 are the most common (Cornaglia *et. al*., 2007). The novel IMP-12, IMP-13 and VIM-14 was also reported from Italy (Docquier *et. al*., 2003; Pagani *et. al*., 2003 and Mazzariol *et. al*., 2010). NDM-1 positive *E. coli* have also been reported (Poirel *et. al*., 2010).

The first MBL found in France was VIM-2 and it was detected in *P. aeruginosa* (Poirel *et. al*., 2000). The novel MBL IMP-19 was also reported from France, which was detected in *Aeromonas cavial*. NDM-1 and VIM-4 have been detected in a *C. freundii* bacterial isolate in a hospital patient (Poirel *et. al*., 2010).

A. baumannii derived IMP MBL was detected first time in U.K. (Walsh *et. al*., 2005). VIM-2 producing *P. aeruginosa* was identified during a British Society for Antimicrobial Chemotherapy Surveillance Programme (Miriagou *et. al*., 2010). Two novels VIM types MBL VIM-9 and VIM-10 was also reported from UK (49). NDM-1 producing *A. baumannii* also reported (Kumarasamy *et. al*., 2010).

A novel MBL IMP-5 found in *A. baumannii* was the first MBLs which were first time reported in Portugal (Da Silva *et. al*., 2002). The multiple MBLs containing strain was also reported. VIM-2 MBL was also identified in several isolates of *P. aeruginosa* in 1995 (Walsh *et. al*., 2005).

In Germany VIM-1 producing *P. aeruginosa* and *Enterobacteriaceae* was reported (Valenza *et. al*., 2010; Weile *et. al*., 2007). GIM-1 was detected in five isolates of *P. aeruginosa* from different patients in Dusseldorf (Castanheira *et. al*., 2004) and it's highly widespread in *Pseudomonas* spp. and *Enterobacteriaceae* bacterial isolates.

In European countries, the most common MBLs are IMP, VIM, DIM, SIM and NDM and among them, VIM-2 and NDM are most common MBLs widely distributed in European subcontinents.

X. Aims and Objectives

1. To investigate the existence of imipenem resistance bacteria in river water of Karala and Mahananda of Jalpaiguri and Siliguri respectively, in the northern West Bengal, India.
2. To enumerate the incidence of metallo-β-lactamase resistant bacteria in both river water of Karala and Mahananda.
3. To ascertain molecular phylogeny of the metallo-β-lactamase genes carrying isolates.
4. To characterize metallo-β-lactamase genes using molecular techniques of PCR, cloning and sequencing.
5. To ascertain of metallo-β-lactamase genes with mobile DNA elements like integrons.
6. To sequence the entire genome of a selected metallo-β-lactamase-producing bacterial strain *Pseudomonas* sp. MR 02.
7. To elucidate additional role of $bla_{\text{NDM-1}}$ genes, if any, in ampicillin catabolism.