Chapter 1
Review of Literature
Introduction:

1.1: History of Antimicrobial agents and the antibiotic era:

Antimicrobials are perhaps one of the most triumphant forms of chemotherapy in the history of therapeutics. Many lives have been saved due to antimicrobial agents and they have significantly contributed to the control of infectious diseases that were the leading causes of human morbidity and mortality. Paul Ehrlich, the father of chemotherapy, started the quest for the treatment of bacterial infections with chemicals. He believed in the concept of ‘silver bullet’ i.e., a compound that specifically acts on the disease causing agent but does not act on the host cells. In 1909, Paul Ehrlich identified a compound that cured syphilis-infected rabbits and showed significant promise for the treatment of patients with this venereal disease (Ehrlich and Hata, 1910). The compound was marketed under the name Salvarsan and proved to be a great success as an anti-syphilitic drug. This was the first antimicrobial to be marketed. Following his footsteps Gerhard Domagk, Josef Klarer and Fritz Mietzsch discovered antibacterial drug Prontosil (first of the sulfa drug family) in 1930’s (Domagk, 1935). This started the trend of using chemicals for the treatment of infections.

In 1928, Fleming discovered penicillin. He discovered that the growth of *Staphylococcus aureus* was inhibited in a zone surrounding a colony of fungus belonging to the genus *Penicillium*. This finding led to the understanding that a microorganism can produce substances that could inhibit the growth of other microorganisms (Fleming, 1929). Penicillin came into clinical use in 1940s and saved lives of many wounded soldiers during the Second World War. During the same time Waksman and Schatz discovered streptomycin, a compound active against both Gram-positive and Gram-negative bacteria (Schatz, et al., 1944; Schatz and Waksman, 1945). Waksman used the term ‘*antibiotic*’, for the first time, to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Waksman, 1947). The commercial production of penicillin and streptomycin started the golden antibiotic era. Many new antibiotic classes such as chloramphenicol, tetracycline, macrolides, quinolones etc., were discovered in subsequent years, thus improving the human health. The
period between the 1950s and 1970s was indeed the golden era of discovery of novel antibiotics classes, which improved the therapy against the infectious diseases. But the success of antibiotics was restricted by the development of resistance amongst pathogens. The decline in the rate of the discovery of new antimicrobials and the rise of antibiotic resistance among pathogens is a concern for the treatment of infectious disease today.

1.2: The rise of antibiotic resistance in pathogens:

The resistance to penicillin was detected even before the extensive use of penicillin started. Abraham and Chain showed that *E. coli* cell extract could destroy antimicrobial activity of penicillin by an enzymatic action (Abraham and Chain, 1940). Sir Alexander Fleming, also, had warned about the development of antibiotic resistance due to the overuse of antibiotic, in as early as 1945, stating:

“I would like to sound one note of warning. It is not difficult to make microbes resistant to penicillin in the laboratory, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantitites of the drug make them resistant.” Sir Alexander Fleming: Nobel Lecture, December 11, 1945.

His warning has, unfortunately, turned out to be true. With extensive use of antibiotics, pathogens, which were earlier sensitive to antibiotics, now started developing resistance to many classes of antibiotics. The timeline for the development of antibiotics and antibiotic resistance is represented in Figure 1.1. Emergence of penicillin resistant *Streptococcus pneumoniae* (PRSP), methicillin resistant *Staphylococcus aureus* (MRSA), multidrug resistant *Pseudomonas aeruginosa*, vancomycin resistant *Enterococcus* (VRE) and multidrug resistant *Mycobacterium tuberculosis* has led to difficulties in the treatment of these infections (Dulon, et al., 2011; Harbarth, et al., 2002; Lin and Martinez, 2006; Normark and Normark, 2002; Ormerod, 2005; Tang et al, 2002). Now days, high mortality rates are observed due to multidrug-resistant bacterial infections. Each year, around 25,000 people in the EU die from an infection with multidrug-resistant
bacteria (ECDC/EMEA Joint Working Group, 2009). This causes heavy economic losses each year.

**Figure 1.1**: Trend of development of antimicrobial agents and emergence of drug-resistant bacteria. Figure credited to (Saga & Yamaguchi, 2009).

Pathogens are becoming resistant by the mutations of pre-existing DNA or by the acquisition of DNA containing antibiotic resistance genes. These resistance genes confer varieties of different antibiotic resistance mechanisms to the pathogens. The modes of action of different classes of antibiotics and the mechanism of resistance are represented in Table 1.1. The mechanisms of resistance include:

1) **Modification of the antibiotic**: The resistance can be obtained by modifying the drug molecule so that it is no more effective. This kind of resistance is
observed in the case of β-lactams, macrolides and chloramphenicol. The β-lactam antibiotics are a group of antibiotics characterized by possession of a β-lactam ring. Intact β-lactam ring is necessary for action of penicillins. Bacteria produce a heterogenous group of enzymes called β-lactamases, which cleave the β-lactam ring and inactivate the drug, thus, conferring resistance (Jayaraman, 2009). Chloramphenicol resistance is generally due to inactivation of the antibiotic by chloramphenicol acetyl-transferase (Trieu-Cuot, et al., 1993), while resistance to aminoglycosides is widespread, with more than 50 aminoglycoside-modifying enzymes being discovered (Schmitz and Fluit, 1999).

2) **Resistance by influx–efflux systems:** Bacteria have different systems for transport of small molecules across the cell membrane. If the antibiotic does not stay in the cell, then it would not have any adverse effect. The antibiotics are flushed out of the cells by efflux pumps thus, conferring resistance. Resistance to fluoroquinolones and tetracyclines is commonly observed by efflux mechanism (Jayaraman, 2009; Byarugaba, 2010).

3) **Modification of the target site:** If the target site is modified, it means that antibiotic cannot have an effect. The modifications in the antibiotic target sites can be carried out by mutation e.g., mutation in DNA gyrase for resistance against quinolones, or enzyme modification of the target site e.g., methylation of an adenine residue in 23S rRNA making it insensitive to macrolides or by replacing targets e.g., ribosomal protection proteins conferring resistance to tetracyclines (Jayaraman, 2009; Byarugaba, 2010; Aminov, 2010).

There are other mechanisms of resistance such as overproduction of the target gene (dihydrofolate reductase overproduction for sulfonamide resistance), target protection (qnr genes for quinolone resistance), or production of other proteins which bind to the drug and prevents the original target (penicillin binding proteins), but the above discussed mechanisms are the major basic mechanisms of resistance widely encountered in pathogens (Jayaraman, 2009; Byarugaba, 2010; Aminov, 2010).
Table 1.1: Representative antibiotics, their modes of action and the mechanisms of resistance.

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Representative antibiotics</th>
<th>Mode of action</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>β–Lactams</td>
<td>Penicillins, Cephalosporins, Cefotaximes, Carbapenems</td>
<td>Inhibition of cell-wall synthesis</td>
<td>Enzymatic degradation of the drug. Drug binding proteins.</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin, Gentamycin, Tobramycin, Amikacin</td>
<td>Inhibition of protein synthesis</td>
<td>Enzymatic modification, efflux, ribosomal mutations, 16S rRNA methylation.</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin, Ofloxacin, Norfloxacin</td>
<td>Inhibition of DNA replication</td>
<td>Efflux, modification of target by mutations, protection of target.</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin</td>
<td>Inhibition of cell-wall synthesis</td>
<td>Altered cell walls, drug modification.</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td>Inhibition of translation</td>
<td>Efflux, modification of ribosomal proteins.</td>
</tr>
<tr>
<td>Rifamycins</td>
<td>Rifampin (Rifampicin)</td>
<td>Inhibition of transcription</td>
<td>Altered β -subunit of RNA polymerase.</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Azithromycin, Erythromycin</td>
<td>Inhibition of protein synthesis</td>
<td>Enzymatic modification, efflux, ribosomal mutations, 16S rRNA methylation.</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Sulfamethoxazole, Trimethoprim, Co-Trimoxazole</td>
<td>Inhibition of Folic acid synthesis</td>
<td>Modification of target.</td>
</tr>
</tbody>
</table>
1.3: Horizontal Gene Transfer (HGT) in bacteria:

Horizontal gene transfer is ‘the non-genealogical transmission of genetic material from one organism to another’ (Goldenfeld and Woese, 2007). The ability of bacteria to exchange genes across species boundaries is an important factor in the spread and persistence of antibiotic resistance genes. This often is facilitated by mobile genetic elements such as plasmids, transposons, integrons and genomic islands that harbor antibiotic resistance genes (Bennett, 2008).

Many plasmids carrying resistance genes are transferred by the process of conjugation. Conjugation is a replicative process in which both donor and recipient cells have a copy of the plasmid after the process (Wilkins, 1995). Conjugative plasmids exhibit broad or narrow host range. In narrow range, the transfer is restricted generally to and between a small number of similar bacterial species. Broad range resistance plasmids are known to be associated with pathogens, a resistance plasmid from *Pseudomonas aeruginosa* can be transferred to a wide variety of Gram-negative organisms (Bennett, 2008). These mobile plasmids work as one of the means of acquiring resistance genes for pathogens in the environment.

Integrons, especially class 1 integrons, are genetic elements that can capture and express any open reading frame (ORF). Integrons are usually associated with other mobile elements such as plasmids, transposons and genomic islands and hence, are mobile (Gillings, et al., 2008; Mazel, 2006; Stokes and Hall, 1989). Integrons are widespread in pathogens and form an important mechanism for pathogens to gain and exchange resistance genes (Mazel, 2006).

Genomic islands (GEIs) are relatively large segments of DNA, compared to plasmids and integrons. GEIs are detected by sequence comparison between closely related species and usually are between 10 and 200 kb in size. Genomic islands from pathogens usually carry antibiotic resistance and virulence genes. Such genomic islands have been well studied in pathogens such as MRSA, *Salmonella typhi* and *E. coli* (Janka, et al., 2005; Katayama, et al., 2000; Levings, et al., 2005).

All these different genetic exchange mechanisms help in the spread and exchange of antibiotic resistance between different bacterial species. Hence, controlling the
spread of resistance genes is difficult, especially if there is selective pressure in the environment due to the presence of antibiotics.

1.4: Environmental bacteria as a source of resistance genes for pathogens:

With the rise of antibiotic resistance in pathogens, people started questioning the origin of these antibiotic resistance genes. One school of thoughts suggested that antibiotic producing organisms are the source/origin of these resistance genes. Courvalin et al. (1977) showed that aminoglycoside-modifying enzyme from *Bacillus circulans* (aminoglycoside producer) confers resistance in *E. coli*. Similarly, *Streptomyces rimosus* (producer of tetracycline) has been reported to carry tetracycline resistance genes similar to those found in pathogens (Reynes, et al. 1988). Although, these studies supported the above hypothesis, the extent of drug resistance encountered in pathogens was not confined to the genes detected in antibiotic producing organisms. This indicated other sources of antibiotic resistance genes than the antibiotic producers.

A study by Riesenfeld et al. (2004) showed that uncultured soil bacteria are a source of novel antibiotic resistance genes. A recent study by Forsberg et al. (2012) showed that the antibiotic resistance genes of soil bacteria and the human pathogens share almost 100% similarity and soil bacteria are a source of these resistance genes for pathogens. Many other studies support this finding that the resistance genes carried by pathogens have their origin in environments other than the clinical world. The normal bacterial flora of different environments act as reservoirs of these resistance genes (Martínez, 2008; Allen, et al., 2008; Wellington, et al., 2013; Gaze, et al., 2013; Finley, et al., 2013). Hence, increased drug resistance in environmental bacteria essentially means more opportunity for pathogens to acquire resistance genes.

1.5: Antibiotic resistance in the environment:

Antibiotic resistance genes were present in the environment long time before the clinical use of antibiotics started (D’Costa, et al., 2011; Bhullar, et al., 2012). These resistance genes are reported to increase radically in abundance within the bacterial populations exposed to sufficient selection pressure from exposure to antibiotics (Dethlefsen, et al., 2008; Barbosa and Levy, 2000; Kristiansson, et al., 2011;
Jernberg, et al., 2010), thus, creating a pool of resistance genes for pathogens to acquire. The use, misuse and overuse of antibiotics have caused selection pressures in the environment by contaminating the pristine environments with antibiotics. There are several ways in which antibiotics enter the environment such as, hospital waste, sewage, agricultural waste, animal husbandry and industrial waste, represented in Figure 1.2 (Segura, et al., 2009; Kümmerer, 2009a).

**Figure 1.2:** The sources of pharmaceutical (antibiotics) pollution in the environment (Picture derived from PILLS project: http://www.pills-project.eu).

A significant proportion of antibiotics consumed by humans and animals are excreted by the body. This creates selection pressure due to presence of antibiotics in sewage treatment plants (Kümmerer, 2004; Kümmerer, 2009a). Hospitals have been considered as a major source of environmental pollution by antibiotics and antibiotic resistant bacteria, because of the extensive antibiotic use in the hospitals. Studies have shown that hospital effluent is a source of antibiotics which enter the sewage treatment plants (STP) (Kümmerer, 2004; Kümmerer, 2009b Segura, et al.,
Hospital effluents have also been reported to contain antibiotic resistant bacteria along with the presence of antibiotics (Diwan, et al., 2010; Segura, et al., 2009). Although, hospitals have been extensively studied as a source of environmental antibiotic pollution other sources such as animal husbandry, farming and aquaculture have been reported to cause environmental antibiotic pollution.

Antibiotics are used in agriculture as a growth promoter or as prophylactic agents, antibiotics are also sprayed on the infected plant parts (Khachatourians, 1998; Kümmerer, 2009a). Antibiotic applied in the farm, leaches out and contaminates the receiving waters and farm. Antibiotic resistant plant pathogens have been isolated from farms applied with antibiotic and the receiving waters; this suggests that antibiotic resistance is selected for due to the application of antibiotics in agriculture (Khachatourians, 1998; Kümmerer, 2009a; Martinez, 2009).

Aquaculture is farming of aquatic organisms such as fish, molluscs, crustaceans, etc. Several antibiotics are used for aquaculture, primarily for therapeutic purpose and sometimes as prophylactic agents (Kümmerer, 2009a; Smith, 2008). Oxytetracycline, florfenicol, sarafloxacin, erythromycin, trimethoprim and ormethoprim are antibiotics authorized for use in aquaculture (Serrano, 2005). The effect of this use of antibiotics was seen as an increase in antibiotic resistance in bacteria from fish and aquatic sediments. The residues of antibiotics used in aquaculture are often found in sediments, these antibiotics exert selection pressure on normal flora of the sediments, thus promoting selection of antibiotic resistant bacteria (Khachatourians, 1998; Kümmerer, 2009a; Smith, 2008; Cabello, 2006).

Antibiotics are used in animal farming to promote growth of animals, and also used in low doses in animal feeds to improve the quality of the product (Gaskins, et al., 2002; Cromwell, 2002). Significant proportion of the antibiotics administered to the animals is excreted unaltered in feces. Thus, antimicrobial use in livestock is an important source of antibiotics release into the environment (Dolliver, et al., 2007). Studies have shown that use of antibiotics for animal farming has led to the rise in antibiotic resistance in animal farms as well as receiving waters and surrounding environments. Tetracycline resistance genes have been reported to be prevalent in lagoons and ground water underlying swine production facilities (Chee-Sandford, et al., 2001), while multi-drug resistant bacteria have been reported from poultry
environments (Hayes, et al., 2004). A study by Pruden et al. showed a co-relation between animal farming and the prevalence of antibiotic resistance genes in the river sediments downstream of animal farms (Pruden et al., 2012). This suggests that the use of antibiotics in animal farming is exerting selection pressure in the environment and selecting out resistant bacteria.

Sewage treatment plants (STP) and waste water treatment plants (WWTP) serving urban waste and sewage play an important role in spread of antibiotic resistance (Kümmerer, 2009b; Segura, et al., 2009; Szczepanowski, et al., 2009; Da Silva, et al., 2005). Residual antibiotics from domestic sewage, hospital waste, animal farming waste, along with antibiotic resistant bacteria enter the WWTP. Antibiotic resistant bacteria have been isolated from WWTP processing urban waste (Da Silva, et al., 2005). The WWTP also serves as reservoirs of clinically important antibiotic resistance genes, which enter the natural environments such as rivers or estuaries (Szczepanowski, et al., 2009; Pruden et al., 2012). STP and WWTP thus, serve as an important source for polluting environments with antibiotics and antibiotic resistance genes.

1.6: Drug manufacturing industries as a source of antibiotic pollution:

Drug manufacturing industries are somewhat less studied as a source of antibiotic pollution compared to hospitals, although the waste generated contains high amount of antibiotics (Segura, et al., 2009). Drug manufacturing industries have been reported to be a source of antibiotic pollution in many countries like India, China, Croatia, Korea etc. The concentration of oxytetracycline in wastewaters from oxytetracycline production facilities from China was as high as 920 mg/L (Li, et al., 2008). This concentration is several times higher than MIC values for many pathogenic bacteria (Li, et al., 2008). Lincomycin has been reported in concentrations up to 44 mg/L in the effluent from a Korean factory (Sim, et al., 2011), while sulphonamides at concentrations up to milligrams per liter have been reported from Croatia (Babić, et al., 2007).

Li, et al. (2010) also showed the presence of multidrug resistant bacteria and tetracycline resistance genes in receiving river sediments downstream of oxytetracycline production WWTP. Similarly, presence of multidrug resistant
bacteria and extended spectrum beta-lactamases (ESBLs) has been reported in receiving river sediments downstream of penicillin production WWTP from China (Li, et al., 2009).

1.7: Antibiotic pollution and antibiotic resistance in India:

Recently, India has been in the news for the rise in antibiotic resistance in the clinics, especially with the emergence of NDM-1 carrying superbug (Kumarsamy, et al., 2010). The environmental antibiotic resistance is also increasing in India. India and China are the leading producers of active ingredients of pharmaceuticals in the world (World Health Organization 2004). This also means high chances for environmental contamination in these countries.

The studies on a waste water treatment plant (WWTP) in Patancheru, near Hyderabad, India operated by Patancheru Environment Technology Limited (PETL), showed the presence of high levels of antibiotics in treated effluent (Larsson, et al., 2007). The WWTP receives effluent from approximately 90 regional bulk drug manufacturers. The treated effluent contains a range of drugs at very high concentrations, including fluoroquinolone antibiotics, such as enrofloxacin, ofloxacin, norfloxacin, lomefloxacin and ciprofloxacin. The ciprofloxacin concentrations was as high as 30 mg/L (Larsson, et al., 2007). The levels of ciprofloxacin are even higher than those attained in the blood of patients taking this broad-spectrum antibiotic. Accordingly, concerns have been raised that antibiotic resistance will be promoted in such places (Larsson, et al., 2007; Pruden, et al., 2013).

A recent study, applying next-generation sequencing of microbial community DNA, demonstrated the promotion of resistance genes to several classes of antibiotics in the river sediment contaminated by the effluent from PETL (Kristiansson, et al., 2011). Additionally, elements associated with genetic mobility were detected in significantly higher frequencies down-stream from the treatment plant (Kristiansson, et al., 2011).

The PETL treatment plant applies an activated sludge technology. This technology results in an active selection for resistant bacteria, as the activated sludge bacteria are exposed to a strong antibiotic selection repeatedly during the recycling of the
sludge. Additionally, approximately 20% of raw human feces is added to the plant to maintain the biological activity. This inevitably adds pathogens to the treatment process (Larsson, et al., 2007). Furthermore, the treatment plant frequently operates at temperatures above 30°C. Such a close and extended contact among pathogens, resistant bacteria and antibiotics provide an environment with apparent opportunities for transfer of resistance to classical pathogens (Larsson, et al., 2007).

So much antibiotics are let out in the environment from PETL, as well as illegal dumping is carried out, that the ground and surface waters in the Patancheru area are heavily contaminated with antibiotics from the industrial releases (Fick, et al., 2009). Thus, not only is there selective pressure inside PETL, but also in the surrounding environment. Concerns have been expressed that antibiotic pollution in Patancheru area will promote the development of antibiotic resistance in normal bacterial flora of the environment (Larsson, et al., 2007; Pruden, et al., 2013).

With this background, the aim of the present study was to investigate the effect of antibiotic pollution in PETL and surrounding areas, on the prevalence of antibiotic resistance genes and the development of multidrug resistance among the bacteria living under such selection pressure.

1.8: Aims and Objectives:

1) To isolate, identify and study the antibiotic sensitivity profiles of the bacterial strains isolated from WWTP (PETL).

2) To study the prevalence of resistance genes upstream and downstream of PETL WWTP, using qPCR and functional metagenome analysis.

3) To study the impact of antibiotic pollution on human gut and the environment with reference to antibiotic resistance genes, by molecular methods.