A DISSERTATION ON

Prevalence of Tetracycline Resistant Bacteria in the Gomti River Water

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES

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IN PARTIAL FULFILMENT

FOR THE

DEGREE OF MASTER OF SCIENCE

IN MICROBIOLOGY

BY

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M.Sc. Microbiology (IV semester)

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TO WHOM IT MAY CONCERN

This is to certify that Ronit Verma student of M.Sc. Microbiology (IV semester), Integral University has completed his four months dissertation work entitled "**Prevalence Of Tetracycline Resistant Bacteria In The Gomti River Water"** successfully. He has completed this work from March - June 2022 under the guidance of Dr. Mohd Ikram Ansari. The dissertation was a compulsory part of his M.Sc. degree.

I wish him good luck and a bright future.

Prof. (Dr.) Snober S.Mir

Head Department of Biosciences

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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ronit Verma** during March - June, 2022 reported in the present thesis was under my supervision and supervision. The results he reported are genuine and the candidate himself has written the thesis script. The thesis entitled "**Prevalence of Tetracycline Resistant Bacteria in the Gomti River Water"** is, therefore, being forwarded for the acceptance in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biosciences, Integral University, Lucknow (UP).

Dr. Mohd Ikram Ansari
Assistant Professor
Department Of Biosciences

DECLARATION

I hereby declare that the present work on "**Prevalence of Tetracycline resistant bacteria in the Gomti river water"** is a record of original work done by me under the guidance of **Dr. Mohd Ikram Ansari,** Assistant Professor, **Integral University**, during March -June 2022, at Integral University, Lucknow. All the data provided in this were through our original work.

I also declare that not any part of this thesis has previously been submitted to my University or any examining body for acquiring any diploma or degree.

Place: Integral University, Lucknow

Date:27-06-2022 Ronit Verma

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First of all, I bow in reverence to the Almighty for blessing me with solid will power, patience, and confidence, which helped me complete the present work. I want to express my special thanks to **Dr. Snober S. Mir (Head Department of Biosciences)** for allowing me to join the department laboratory and providing all the necessary facilities since I started my work.

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Ronit Verma

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List of Abbreviations

ARB : Antibiotic Resistant Bacteria 9

ARGs : Antibiotic Resistant Genes..... 9

CFU : Colony Forming Unit 27

E.coli. : Escherichia coli 20

kDa : Kilodaltons 18

otr : oxytetracycline resistance 20

RPPs : Ribosome Protection Proteins 9

rRNA : ribosomal Ribonucleic Acid..... 15

STDs : Sexual Transmitted Diseases..... 14

STPs : Sewage Treated Plant 21

tRNAs : transfer Ribonucliec Acid 15

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CHAPTER 1 - INTRODUCTION

Antibiotics play an important role in human and livestock health, but raising the resistance of microorganisms to antibiotics represents a severe cosmopolitan concern. An increase of ARB in the environment could be harmful to people's health. Wastewater is a significant source of ARB in the environment, and it serves as a pathway for ARB to enter natural systems from industrial effluents. Tetracyclines were the first primary class of antibiotics defined as "broad spectrum". These antibiotics have been widely used for over four decades as antimicrobial agents and growth promoters in agriculture. The pathogens showed no known resistance to tetracycline in the 1950s, but the recent discovery of tetracycline-resistant bacteria has resulted in limited use of these antibiotics. The emergence and distribution of tetracycline-resistant pathogenic bacteria have caused serious concerns. Tetracycline resistance involves various mechanisms, including efflux pumps, ribosome protection proteins (RPPs), and tetracycline modification (Aali and Hassanzadeh et al., 2014)

Human and animal sources of antibiotic-resistant microbes infiltrate aquatic ecosystems. These bacteria can transmit their genes to water-based microbes with resistance genes. Many antibiotics from industrial sources, on the other hand, circulate in aquatic habitats, potentially affecting microbial populations. Drug and resistant bacteria risk assessment techniques in water are being proposed based on improved technologies for antibiotic detection and antibiotic resistance microbiological source monitoring. Optimization of disinfection techniques and wastewater and manure management are methods for reducing resistant bacterial load in wastewaters and the amount of antimicrobial agents, which most often originate in hospitals and farms. A policy limiting the mixing of human- and animal-derived germs with environmental organisms appear sensible. (Baquero, Martınez,et-al;2008)

Antibiotics are frequently used as a food ingredient to protect human and animal health or to boost animal growth rate. Antibiotics are mainly excreted unchanged into the environment. As a result, in recent years, worries regarding the possible impact of antibiotic residues in the aquatic environment have grown. Antibiotics are challenging to detect in surface water, except in virgin sites in the mountains before rivers or streams pass through urban or agricultural regions. Antibiotics have been found in groundwater as deep as 10m.In addition to the chemical pollution generated by antibiotics, their usage may accelerate the development of antibiotic resistance genes (ARGs) in bacteria, causing health concerns to humans and animals. These bacteria could be passed from one person to the next by direct or indirect interaction with the environment.

Given the growing body of evidence linking environmental ARGs and bacteria to clinical resistance, it is evident that research efforts should be expanded to include nonpathogenic or environmental microorganisms. Although there have been many articles on the subject, there have been few articles on the occurrence of ARGs in various aquatic contexts. This study focuses on the antibiotic resistance development in the native bacteria of the Gomti river.

Pathogenic and potentially pathogenic microorganisms from humans and animals are regularly introduced into the aquatic environment through wastewater. Antibiotic resistance genes are found in many of these organisms, which are finally inserted into mobile genetic platforms (plasmids, transposons, and integrons) that can travel among water and soil bacterial communities. Water serves as a vector for spreading antibiotic-resistant organisms across human and animal populations and as a means of introducing resistance genes into natural bacterial ecosystems. Nonpathogenic bacteria could be a reservoir of resistance genes and platforms in such systems. Furthermore, antimicrobial agents, detergents, disinfectants, and residues from industrial pollutants, such as heavy metals, are introduced (and gradually accumulate) in the environment, contributing to the development and spread of such resistant organisms in the aquatic environment. On the other hand, environmental bacteria provide an endless

supply of genes that could be used as resistance genes in pathogenic species (Baquero, Martınez, et al. 2008). Considering all these points, the following objectives were designed for the study.

Objectives:

1. To evaluate the prevalence of tetracycline-resistant bacterial population in the Gomti river water of Lucknow.
2. To isolate the tetracycline-resistant bacteria from the Gomti river water of Lucknow.
3. To analyze the multiple antibiotic resistance in the bacteria isolated from the Gomti river water of Lucknow.

CHAPTER 2 - REVIEW OF LITERATURE

Tetracycline

Tetracyclines were developed in the 1940s and were effective against various microorganisms, including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. Shigella dysenteriae was the first tetracycline-resistant bacteria discovered in 1953. Tetracycline resistance is frequently caused by acquiring additional genes that code for a protein that shields bacterial ribosomes from tetracycline activity (Chopra and Roberts 2001). More than 70 % of tetracycline antibiotics are excreted after medication, and Via urine and feces from humans and animals, it is released in active form into the river. The occurrence of tetracycline antibiotics in the river inhibits the growth of some terrestrial and aquatic species because most wastewater treatment plants cannot remove tetracycline antibiotics (Daghrir and Patrick 2013).

Classification

Tetracyclines are grouped as follows:-

First Generation if they are obtained by biosynthesis For, e.g.,tetracycline, Chlortetecycline, Oxytetracycline, Demeclocycline.

Second Generation if they are derivatives of semi-synthesis such as: Doxycycline,

Lymecycline, Meclocycline, Methacycline, Minocycline, Rolitetracycline.

S. No.	Natural	S. No.	Semi-Synthetic
1.	Chlortetracycline	1.	Tetracycline
2.	Oxytetracycline	2.	Minocycline
3.	Demeclocyline	3.	Doxycycline
		4.	Methacycline
		5.	Lymecycline
		6.	Clomocycline
		7.	Rolicycline

Third Generation if they are obtained from total synthesis such as Tigecycline. However, some researchers consider Tigecycline to be distinguish from other tetracyclines drugs and are considered a new family of antibacterials called Glycylcyclines (Fuoco,2012).

TETRACYCLINE STRUCTURE:-

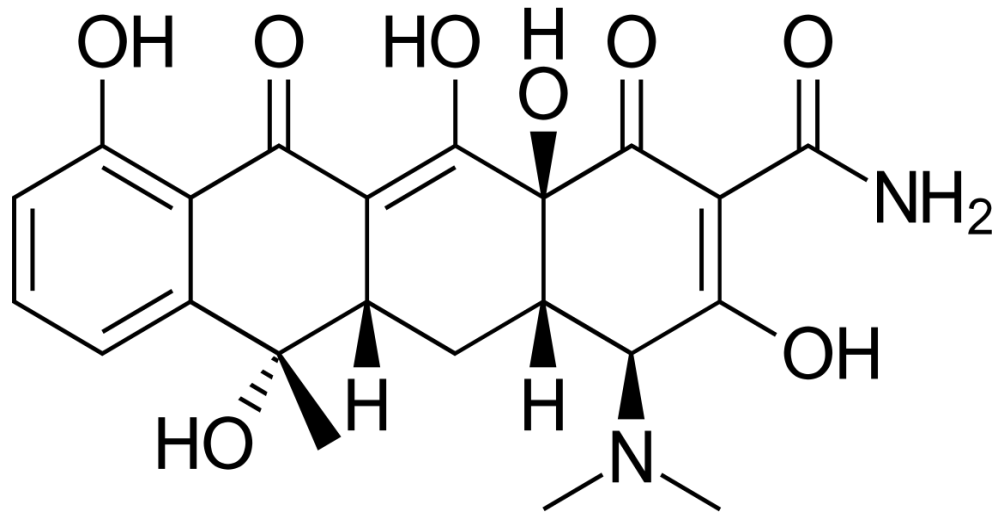


Figure 1:Tetracycline Structure

Ribosomal protection protein

The ribosomal protection proteins (RPP) are the most common type of tetracycline resistance determinant. RPPs in Gram-positive and Gram-negative bacteria come in 11 distinct types. Tet(O) and Tet(M) are the most common and well-studied RPP classes. Tet(M) is found in 24 different bacterial genera, whereas Tet(O) is found in 8 different bacterial genera. RPPs have a size of about 72.5 kDa. RPPs were previously thought to function as tetracycline-resistant elongation factors, allowing protein synthesis to occur in the presence of tetracycline, but substitution tests disproved this theory. Tetracycline resistance is achieved by decreasing the tetracycline-ribosome interaction, resulting in antibiotic release (Connell et al., 2003).

Uses of Tetracycline

It treats brucellosis, rickettsial infections, tularemia, early Lyme disease, and typhus (Standiford, H. C. 1990).Doxycycline is used to treat exotic diseases such as plague (Standiford, 1990). Dentists use tetracycline to treat periodontal disease (Slots et al., 1990). Furthermore, dermatologists treat acne. Tetracycline is an antibacterial drug that also has antiparasitic action against several protozoal parasites. Giardia lamblia, Trichomonas vaginalis, Entamoeba histolytica, Plasmodium falciparum, and Leishmania major are all inhibited by tetracycline derivatives (Katiyar,et-al, 1991.). The effectiveness of tetracycline derivatives in preventing the proliferation of these parasites varies. Because it enhances weight gain in some domestic animals. Oxytetracycline is commonly used as a livestock feed addition (Chopra and Richmond, 1981, Dupon and Steele, 1987). Calves, chickens, turkeys, sheep, and pigs have all been fed regularly (Dupon and Steele, 1987). Tetracycline is also used in commercial fisheries to increase the health and growth of fis

TETRACYCLINE AS A CLINICAL USES:-

In the United States, Tetracycline was one of the most widely used antibiotics during the 1950s and 1960s. It had a broad spectrum of activity against various bacteria and was effective against intracellular and extracellular pathogens(O'Brien et al. 1987).Tetracycline is helpful for outpatient therapy because it is cheap, taken orally, and has few side effects. It is bacteriostatic rather than bactericidal. It cannot be used to treat pregnant women or small children because it causes depression of skeletal growth in premature infants and discoloration of teeth in children(Standiford, 1990.)

Earlier tetracycline and its derivatives have limited use in treating clinical infections because Tet resistance is found in many groups of medically important bacteria (Levy, 1988.). For example, In the 1980s,tetracycline was used to treat STDs. With the appearance of resistant strains of Neisseria gonorrhoeae, tetracycline was discontinued as the first line of therapy. Nongonococcal urethritis and other chlamydial infections are still treated by oxy tetracycline and Tetracycline (Toomey et al. 1990). However, tetracycline is not frequently used as a drug of choice, but it is still used to treat brucellosis, rickettsial infections, and typhus. Plague which is a exotic disease is treated by Doxycycline (Standiford, H. C. 1990.). Sometimes, tetracycline is used by dentists to treat periodontal disease (Slots, J., and T. E. Rams. 1990.) and also by dermatologists to treat acne. Development of such drugs would

have to be based on a thorough understanding of the mechanisms of resistance. One goal of this review is to survey what is known about the mode of action of tetracycline, the various mechanisms of tetracycline resistance, and regulation of resistance genes. A second goal is to survey information about elements that transfer tetracycline resistance genes, including some novel types of gene transfer elements that are not plasmids. These elements have an unusually broad host range and carry genes encoding resistance to macrolides, lincos amides, and chloramphenicol. That is why tetracycline is become useless because of spreading of these elements.

Modes of Action

Tetracycline inhibits the bacteria's growth by entering the cell of bacteria, which binds to bacterial ribosomes and inhibits the synthesis of protein. After much research, it is still unclear exactly how tetracycline works. It is found that tetracycline binds to a single site on the 30S, and the 70S ribosomal protein appears to form part of the binding site (Goldmanetal. 1983.). A highly conserved region of 16S rRNA may also be part of the binding site, a feature that would explain the broad spectrum of tetracycline. There are many weaker tetracycline-binding sites on the ribosome, but their significance is unclear.

The direct effect of tetracycline binding to ribosomes is that aminoacyl-tRNAs do not bind to the A site on the ribosome (Epe et al. 1987). However, interference with binding aminoacyl-tRNAs to the A site could also induce a severe response and thus trigger numerous secondary effects. These secondary effects would include tRNA stability, rRNA synthesis, and amino acid metabolism (Cashel and Rudd, 1987). Although tetracycline and most of its derivatives have been shown to bind to ribosomes and inhibit protein synthesis, some of the derivatives may not act this way. Rasmussen et al (1991) have recently shown that although chelocardin and thiatetracycline have good antibacterial activity against Escherichia coli and Bacillus subtilis, they are very poor inhibitors of protein synthesis and appear to bind ribosomes ineffectively or not at all. Also, unlike other tetracyclines, they inhibit both DNA and RNA synthesis and protein synthesis. Rasmussen et al.

suggest that chelocardin and thiatetracycline may be exerting their effects on the cytoplasmic membrane of the bacteria.

Recently, Olivera and Chopra(1992) proposed that tetracyclines be divided into two types based on their modes of action: those that inhibit protein synthesis (e.g., tetracycline, chlortetracycline, minocycline) and those that interact with the cytoplasmic membrane (e.g., chelocardin, anhydro tetracycline, thiatetracycline).

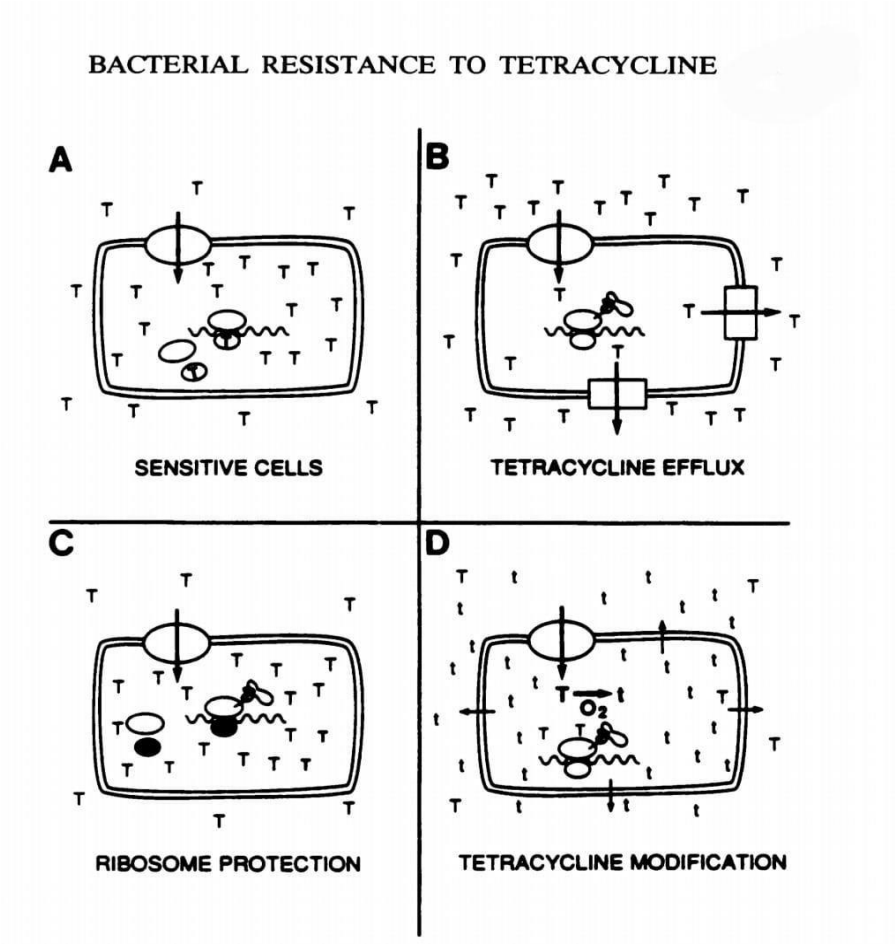


Figure 2: Different mechanisms of tetracycline resistance.

(A) Susceptible bacteria accumulate tetracycline to an internal concentration high enough to allow tetracycline to bind to ribosomes and stop protein synthesis.

(B) Bacteria carrying an efflux type of resistance gene produce a cytoplasmic-membrane protein (rectangular box), which pumps tetracycline out of the cell as fast as it is pumped in. This keeps the intracellular level low enough to allow protein synthesis to proceed.

(C) Bacteria carrying Ribosome- Protection type of resistance gene produce a 72-kDa cytoplasmic protein (not shown) that somehow interacts with the ribosomes and allows the ribosomes to proceed with protein synthesis even in the presence of high intracellular levels of the drug. Although the effect of the 72-kDa protein is indicated graphically by shading one of the ribosomal subunits, it is unknown whether the resistance protein binds to the ribosome. (D) Bacteria carrying a tetracycline modification resistance gene produce a 44-kDa enzyme that chemically modifies Tetracycline (T) to an inactive form (t), which diffuses freely out of the cell. The enzymatic reaction requires oxygen and NADPH. (Speer et al. 1990)

Limiting Tetracycline Access to Ribosomes

1. Reduced uptake

Tetracycline must enter the bacterial cell to inhibit protein synthesis and bind to the ribosome. The cytoplasmic membrane helps diffusion of the tetracycline in the protonated form. However, the accumulation of tetracycline in the cytoplasm of susceptible bacteria is not enough to explain simple diffusion. [McMurry et al.1980] showed that the tetracycline uptake was an energy-dependent phase in addition to diffusion. [Argast and Beck,1985] doubted the presence of energy-dependent uptake since they could not demonstrate saturation of tetracycline uptake, as expected if a tetracycline transporter existed. Recently, tetracycline can exist in a protonated form (TH2) and a magnesium-chelated form (THMg). Phospholipid bilayers help the diffusionofTH2, but they cannot diffuse THMg form. Tetracycline in THMg is kept inside the bacteria because the internal pH is higher than the exterior pH. Bacteria would be unable to develop resistance by inhibiting the flow of tetracycline across the cytoplasmic membrane because it has diffusion as its only mechanism of absorption. This form of resistance has not been observed, as expected. A suspected resistance mechanism in gram-negative bacteria is the modification of porin proteins (e.g., OmpF) to inhibit the diffusion of tetracycline into the periplasm.

2.Tetracycline efflux

A second method of inhibiting tetracycline entry into the ribosomes is to reduce intracellular tetracycline concentrations by pumping the antibiotic out of the cell at a rate equal to or greater than its uptake (Fig. 2).It is not known how this efflux protein pumps tetracycline out of the cell, nor is it clear how this pump protects the cells. The level of intracellular tetracycline is still relatively high compared with levels that inhibit protein synthesis. One possible explanation is based on the ability of tetracycline to exist in several ionic forms. One form may bind more readily to ribosomes than the other. Eight classes of tetracycline efflux genes have been identified. However, classification based on DNA-DNA hybridization is now the preferred method. Bacteria carrying resistance genes from classes A, B, E, and K are more resistant to minocycline than are other efflux classes. However, class B exhibits much higher resistance than classes A, E, and K. [Ives and Bott 1989] recently demonstrated that increasing the copy number of a region of the B. subtilis chromosome at the origin of replication can give resistance to tetracycline. The original strain that had this region in a single copy was susceptible to tetracycline, whereas the strains carrying multiple tandem duplications of this chromosomal region were resistant. Cloning of the region on a multicopy plasmid produced the same tetracycline resistance. The expression of the gene is too low to confer resistance if the gene is present in a single copy. Sequence analysis of the gene has shown that it belongs to class L. All strains of B. subtilis do not have any hidden tetracycline efflux gene. Although B. subtilis is not a human pathogen, finding a hidden resistance gene in this strain raises the possibility that such genes also occur in pathogenic species. Efflux resistance structural genes from different classes share considerable DNA sequence similarities. Classes A and C share 74% DNA sequence identity. In comparison, class B shares 45% identity with class A and class C. Similarly, the two gram-positive efflux structural genes, tet(K) and tet(L) share 69% DNA sequence identity. The tetracycline efflux proteins share homology with other proton-dependent transport proteins such as sugar transporters, especially in their amino-

terminal regions. Thus, the efflux-type resistance genes may have evolved from transport genes.

3. Ribosome Protection

Ribosome protection is a less familiar type of tetracycline resistance mechanism than tetracycline efflux. Although this mechanism is less familiar, it is probably more widespread than tetracycline efflux (Salyers, Shoemaker;1990). The resistance gene product migrates as a 68-kDa protein on sodium dodecyl sulfate_polyacrylamide gel electrophoresis. However, DNA sequence analysis reveals that the protein size is 72 kDa. This cytoplasmic protein interacts with the ribosome, making it insensitive to tetracycline inhibition (Fig. 2). The exact mode of interaction of the resistance protein with the ribosomes is not understood. Burdett(1991) has purified one of the ribosome protection resistance proteins (TetM) and has shown that it can bind to ribosomes. Manavathu et al.(1990) showed that in the presence of TetM, the binding of tetracycline to ribosomes is not altered. The specific purpose of the ribosome protection protein is yet unknown. It will not be determined until more research is done to establish its binding locations on the ribosome and its role in protein synthesis. Three classes of ribosome protection resistance genes have been characterized and sequenced: tet(M),tet(O), and tet(Q). Burdett et al.(1992) originally identified a second class of ribosome protection genes in streptococci and designated it class N, but this class has subsequently been shown not to exist. Tet(M) was originally discovered in gram-positive cocci but has now been found in many bacteria, including Neisseria, Haemophilus, Mycoplasma, Ureaplasma, Streptococcus, Staphylococcus, Peptostreptococcus, Bacteroides, Kingella, and Bacillus spp. Tet(O) found initially in Campylobacter spp., has now been found in Streptococcus, Peptostreptococcus, Enterococcus, Lactobacillus, and Mobiluncus spp. (So far, tet(Q) has been found only in Bacteroides species. These distributions may be misleading. Hybridization probes for identifying tet(M) and tet(O) have been available for some time and have been used in many surveys. In contrast, probes identifying tet(Q) have only recently

become available. Thus, tet(Q) may be found in many more species than indicated above when surveys including this class are made of tetracycline-resistant strains. TetM and TetO are closely related and share 75% amino acid sequence identity. TetQ is much more distantly related and shares only 40% amino acid identity with TetM and TetO. All three resistance classes confer resistance to minocycline and other tetracycline derivatives. An exciting feature of the ribosome protection resistance proteins is that they share considerable amino acid homology to elongation factor G. This homology is concentrated in the region of elongation factor G that contains the GTP-binding site. Recently, Burdett has shown that Tet(M), like elongation factor G, has ribosome-dependent GTPase activity. Thus, the ribosome protection genes may have evolved from genes encoding bacterial elongation factors. Strains of bacteria carrying combinations of efflux and ribosome protection resistance genes have been found. Bismuth et al. reported that many strains of Staphylococcus aureus carried both tet(K) and tet(O), while some isolates carried tet(K),tet(L), and tet(M). Roberts found various combinations of classes K, M, and 0 in some strains of Streptococcus spp. and Peptostreptococcus spp.

4.Tetracycline Inactivation

The third type of tetracycline resistance mechanism has been discovered recently: enzymatic inactivation of Tetracycline (Fig. 2). The gene encoding this resistance was found on two closely related Bacteroides transposons that also carry a gene for erythromycin resistance. The tetracycline resistance gene was first identified by its ability to confer resistance to E.coli. However, the gene worked only in aerobically grown E.coli cells and did not confer resistance on anaerobically grown E. coli or Bacteroides spp. The gene product is a 44-kDa cytoplasmic protein that chemically modifies tetracycline in a reaction that requires oxygen and NADPH. However, Speer and Salyers (1990) showed that efflux activity was not linked with the resistance phenotype and that increasing the efflux activity did not make tetracycline-resistant strain. The finding that a strain exhibiting tetracycline efflux was not tetracycline-resistant raises further questions about how efflux of tetracycline

confers resistance. The clinical significance of tet(X) is unclear. Not only does it not confer resistance on the Bacteroides strains in which it was originally found, but it requires such high levels of aeration to function as a resistance factor in E. coli that it probably could not confer meaningful levels of resistance in the microaerophilic environment found in most sites on the human body. At this point, the possibility cannot be ruled out that some interaction with hemoglobin or other oxygen-bearing molecules allows it to function in the human body. It will be interesting to see whether further examples of this class of antibiotic resistance gene are found in aerobic or facultative clinical isolates.

ARGs related to tetracycline

Tetracycline-resistant microorganisms were detected in the environment, especially after the introduction of tetracycline. (Dancer et al. 1997). Today at least 38 distinct tetracycline resistance genes have been identified, as well as three oxytetracycline resistance (otr) genes. There are 23 genes that code for efflux proteins (efflux pump mechanism), 11 genes that code for ribosome protection proteins (target modification mechanism), three genes that code for an inactivating enzyme, and one gene with an unknown resistance mechanism among these genes (Levy et al. 1999; Roberts 2005). More than 22 tet or otr genes have been found in bacterial isolates from aquatic environments. Most environmental tet genes code for transport proteins that help ribosomes function by pumping antibiotics out of the bacteria cell and keeping intercellular concentrations low. The efflux genes of tetA, B, C, D, and E frequently appeared in various environmental compartments, including activated sludge of sewage treatment plants, fish farming ponds, surface water, and swine lagoon. Recently, the tetracycline resistance genes, including tetM, O, S, Q, and W, coding for ribosomal protection proteins, have also been detected in microbial communities of sewage treatment systems, hospitals, or animal production wastewaters, and even in natural water environments. Although many tet genes are found on nonmobile plasmids or incomplete transposons in the chromosome, genes encoding efflux enzymes

(tet A, B, C, E, H, Y, Z, and 33) and ribosomal protection proteins (tetM and O) have been found in a variety of environmental bacteria, including both Gram-negative and Gram-positive species. Agers and Petersen (2007) recently discovered that tetE is frequently found on large horizontally transferable plasmids of Aeromonas spp. isolated from fish farm pond water. The gene is capable of interspecies transfer to Escherichia coli. TetA, D, and M can also be transferred horizontally from ambient bacteria to E. coli strains recovered from chicken, pigs, and humans via an oxytetracycline resistance plasmid, indicating the tet ARGs' potential environmental risks.

Habitats of ARGs in the water environment

ARGs are found in a variety of water bodies. The first step is to identify the main habitats of ARGs in the environments. Hospital wastewater and livestock manure are regarded major sources of environmental ARGs as a result of widespread usage of human and veterinary antibiotics. ARGs can enter aquatic environments by either discharging untreated wastewater directly into the environment or entering STPs via wastewater collection.(Auerbach et al. 2007). ARGs can be introduced into soils by enriching farmland with animal manure and processed sludge from STPs. This can be dissolved into water and may be carried to surface water by runoff and erosion. ARGs can pass through drinking water treatment facilities and into water distribution systems since surface water and deep groundwater are commonly used as drinking water. Antibiotic resistance genes occur in aquatic environments due tointense antibiotic usage in hospitals, swine production areas, and fish farms, and genes in surface water and groundwater near such locations can transfer antibiotic resistance to bacteria in drinking water or the food chain.

Genetic mechanisms involved in the horizontal transfer of ARGs among environmental bacteria may include the following:

1. Conjugative transfer by mobile elements including plasmids, transposons, and integrons on plasmids.

2. Transformation by naked DNA, in the case of naturally competent state of some bacteria, or an environmentally induced competence such as the presence of calcium;

3. Transduction by bacteriophage. Most environmental bacteria develop antibiotic resistance due to the acquisition of additional genes typically used in conjunction with mobile components. Plasmids are key pools of plasmids with transportable ARGs that were first discovered in the aquatic environment, and STPs are considered significant pools of plasmids with transportable ARGs. STPs' activated sludge has yielded a variety of plasmids that provide resistance to aminoglycosides, quinolones, erythromycin, and various other drugs.

Transposon and integron are two mobile components that play crucial roles in the horizontal transfer of environmental ARGs.

Transposons and integrons containing diverse ARGs have been found in animal production or aquaculture areas, STPs, surface waters, and sediments. These elements are not self-replicating and must be transported from one cell to another by phage or a plasmid. Insertion sequences, which are a form of tiny transposons, only encode recombinase and transposase. Transposons and insertion sequences on genomes and plasmids frequently bounce around randomly, resulting in new or multiple resistances (Naas 2007). Integron cannot move, but it can grab, integrate, and express resistance gene cassettes in its variable sections, and it can be passed on through transposons and conjugative plasmids. The acquisition of ARGs is facilitated by high selective pressure, which may improve the fitness of certain bacteria and allow for rapid emergence and spread on a global scale. Antibiotics at low sub-inhibitory doses can enhance environmental ARGs' horizontal transfer and spread (Kümmerer 2004). It was discovered that retaining the antibiotic concentration in the mating medium at a subinhibitory level improves conjugal transfer mediated by plasmid or transposon. In contrast, UV disinfection did not affect tet gene removal in wastewater effluent. Auerbach et al. (2007) observed that the average loss of tetM, O, P, and W in aquatic environments is directly related to UV exposure. Rasmussen and Sorensen (1998) discovered that Tetracycline and Mercury resistance genes were more frequent in conjugative plasmids in a contaminated

environment. A new tetracycline resistance gene, tetA, was recently discovered in *Serratia marcescens* isolated from a heavy metal-contaminated stream, providing indirect evidence of co-resistance.

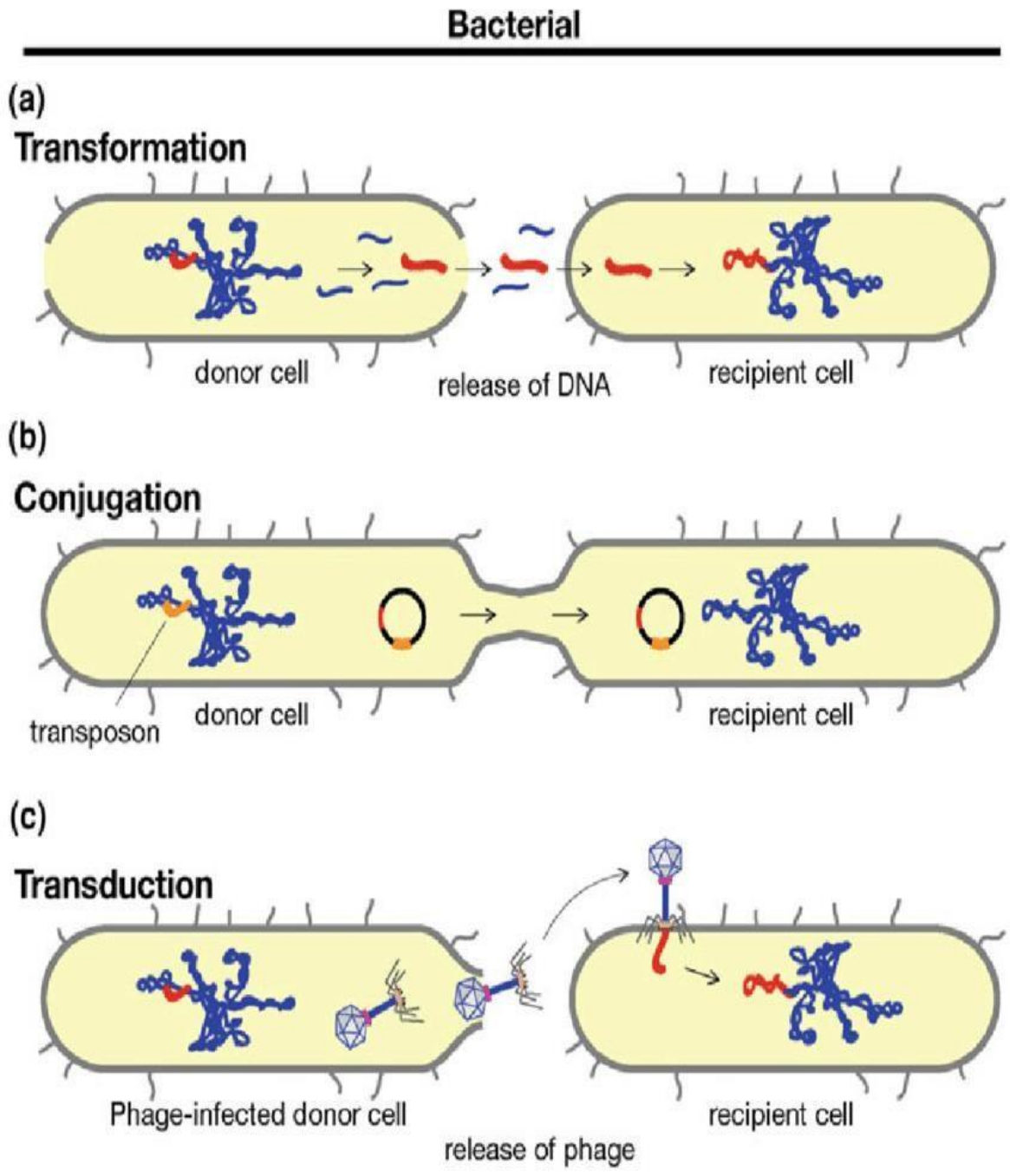
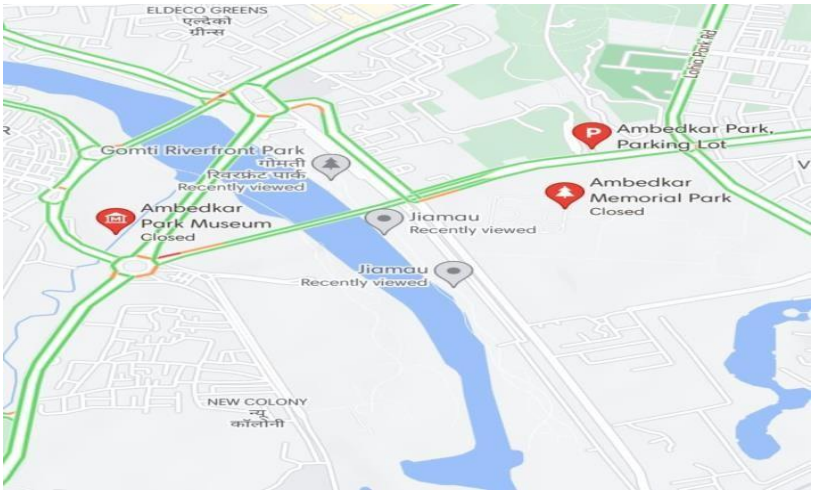


Figure 3 : Horizontal Gene Transfer Mechanism

CHAPTER 3 - MATERIAL AND METHODS

SAMPLING SITE:

The sampling site selected was the Gomti river water, located in Lucknow, Uttar Pradesh. Lucknow, the capital of Uttar Pradesh (India), is located in the part of the central Gangetic plain between North latitudes 26°30′ and 27°10′ and East longitudes 80°30′and 81°13' (Fig. 3). The city has a humid subtropical climate with a cool, dry winter from December to February and a hot summer from April to June. The temperature extremes varied from 48.9 °C in the summer to 1.67 °C in the winter. Between July and September, the city receives about 900 mm of annual rainfall, mostly from the southwest monsoon. The city's elevation varies from 100 to 130 m above mean sea level and generally slopes to the east. Lucknow is one of the fastest-growing cities in the country, with a population projection of 4.7 million in 2031 from 2.8 million in 2011. Rapid unplanned urbanization has created many problems as it places huge pressure on land, water, housing, transport, health, education etc. This rising population has a major impact on the area's natural resources, especially water quality and quantity. Fresh water is the most important natural resource for life, but overexploitation and unjustified use of water has



led to the deterioration of water quality.

Figure 4: Map showing Gomti river showing sampling location

Several streams cut across Lucknow. Gomti, the major river, flows from North-West to South-East through the city's center. It is one of the city's major public water supply sources, along with groundwater. Generation of sewage and proper treatment and disposal of this waste is the major problem in the

city. Poorly drained sewerage systems and lack of treatment capacity of sewage treatment units have severely degraded the quality of river water.

1.COLLECTION OF WATER SAMPLE:

Composited water samples were collected from two different sampling sites of Gomti river Lucknow, UP (India), during February 2022 (Fig. 5). The first sampling site (site I) was selected near the localities where effluents were poured directly into the open channel. The second sampling site (site II) was selected about 1 km from the first site, during which other wastewater is added in the river at several points. The sample was composited by mixing 2 L of water collected at two different points at each sampling site to make a 4 L composite sample. Samples were aseptically collected, labelled properly and transported on ice to the laboratory for analysis.

Figure 5:Figure showing the actual site for collection of water sample

Culture media used for enumeration of normal and tetracycline resistant bacterial population

Total numbers of culturable heterotrophic aerobic bacteria and colony-forming units (CFU) were determined by serial dilution and plating on Nutrient Agar. The nutrient agar plates were prepared with and without tetracycline in the media.

The Nutrient agar was amended with tetracycline to get final concentration of

100 µg/ml to enumerate the tetracyclin resistant microbial population. Serial dilutions of river water(10 mL) were made in 90 mL of normal saline solution. Bacteria were counted at 35°C after 3–5 days of incubation on nutrient agar (peptic digest of animal tissue, 5 g/L; sodium chloride, 5 g/L; beef extract, 1.5 g/L; agar, 15 g/L). The number of colonies were counted and the population was evaluated using the formula

CFU= Number of colonies X Dilution factor/volume of culture plated

Isolation of tetracyclin resistant bacteria from water

Water sample was made by vortex mixing 10 ml of water for 30 min. in 90 ml saline solution (0.86%). The supernatant was then serially diluted (up to 10^7), plated on nutrient agar containing 100 µg/ml of tetracycline and incubatedat35°C for 24 h. Ten different fast growing bacterial isolates with distinct colony morphology were picked and purified by repeated streaking on nutrient agar (Table 2).

Sub culturing for pure culture preparation

Under aseptic technique, the inoculating loop was sterilized in the Bunsen burner by putting the loop in the flame until it was red hot. It was allowed to cool. An isolated colony was picked from the agar plate culture and stroked over the first quadrant using close parallel streaks the loop was flamed again and allowed it cooled. Gone back to edge of area one that just streaked over the second quarter of the plate. This process was continued three to four times. The streaked plate was incubated at 37°C for 24hrs.the colonies grown were observed on the plate carefully.

Preparation of agar slant:

The nutrient agar was weighed and dissolved in desired volume of double distilled water in conical flask and boiled to mix properly. The solution was mixed properly to make the uniform media solution and dispensed in the test tubes to a volume of 5 ml and autoclaved at 121°C for 30 minutes. The agar was allowed to cool with the tube lying in a slant position resulting in a large surface area for inoculating a culture. After the slant agar tubes got cooled, the bacteria were inoculated by the loop and the slant test tubes were

incubated at 37°C for overnight. This process was done aseptically in laminar air flow. Finally it was used for storing pure cultures for a moderately long term and can be used to culture bacterial cells for other experiments.

Antibiotic sensitivity test

All the isolates were tested for sensitivity to antimicrobial agents by means of disc diffusion method (Bauer et al.1966). The following antibiotics (all from Hi-media, Mumbai, India) were used. Concentration of the antibiotics used is given in μg／disc. The abbreviations and concentrations of the respective antibiotics are given in parentheses: ampicillin (A 25), chloramphenicol (C 25),ciprofloxacin (Cf 30), co-tri moxazole (Co 25), doxycycline(Do 30), gentamicin (G 30), kanamycin (K 30),nalidixic acid (Na 30), neomycin (N 30), streptomycin (S10) and tetracycline (T 30).

CHAPTER 4 - RESULTS AND DISCUSSION

Enumeration of microbial population in the Gomti River water

Table 1. Heterotrophic bacterial population in the Gomti River water; (A) without added antibiotic; (B) with added Tetracycline in the medium.

Sample	**Heterotrophic bacteria without tetracycline**	**Tetracycline resistant heterotrophic bacteria**
GRW2	$9.25X10^{8}±1.77X10^{8}$	$5.53X10^{4}±8.33X10^{6}$

The water samples collected from the Gomti River, Lucknow, show a high population of tetracycline-resistant bacteria. The average heterotrophic bacterial count in the Gomti water was found to be$9.25X10^{8}±1.77X10^{8}$ CFU/ml when no antibiotic was added to the growth media (Table 1). However, the bacterial population reduced to $5.53X10^{4}±8.33X10^{6}$CFU/ml when tetracycline was added to the growth media (Table 1). There was a reduction in the total bacterial population when antibiotic was added to the medium. Heterotrophic bacterial analysis on the growth media shows that the Gomti river water contains a significant population of tetracycline-resistant bacteria. The analysis of the results revealed that the gomti water contains about 24% of tetracycline-resistant bacteria (Figure 2).

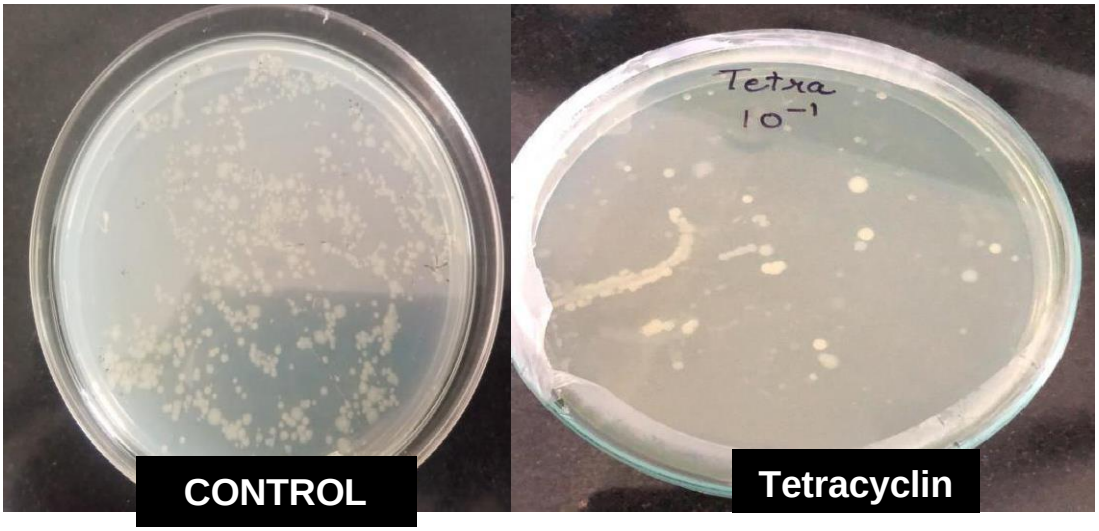


Figure1.simple microbial plate antibiotic microbial plate

The emergence of antibiotic resistance in microbes from humans and animals is a global concern. It is due to the overuse of antibiotics (Grenni et al., 2018; Laxminarayan, 2014; Sousa et al., 2018), suggesting an input of large amounts of ARGs in the environment, including in surface and river waters.

Inputs in these ecosystems mainly occur through livestock waste discharges (He et al., 2020), effluents from hospitals, and wastewater treatment plants (Osińska et al., 2020; Rizzo et al., 2013). Wastewater treatment plantscannot eliminate antibiotics because they are not designed to remove them (J. Wang & Chen, 2020). Tao et al. (2010) reported multiple drug resistance in bacterial isolates from variousPearl River sites, possibly due to sewage discharge and input from other anthropogenic sources along the rivers. The presence of antibiotics in rivers may also negatively affect natural microbial communities, which areessential for key cycles/mechanisms/processes and the maintenance of water quality, compromising fundamental ecological processes (Grenni et al., 2018).

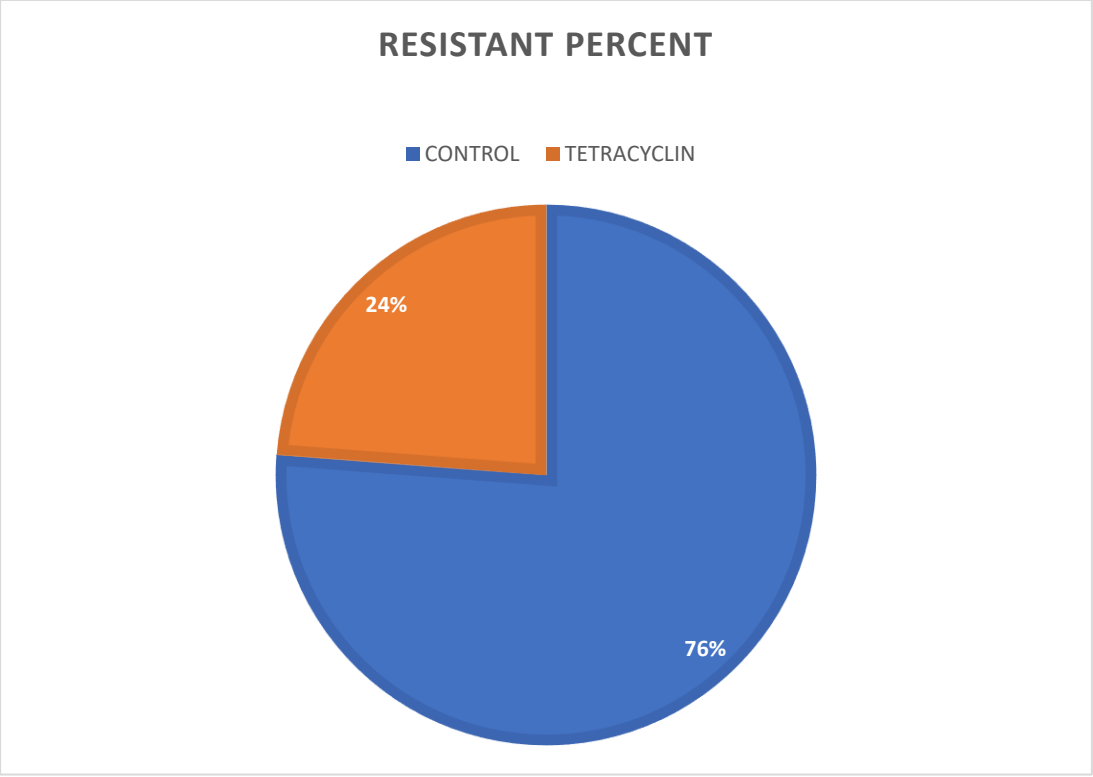


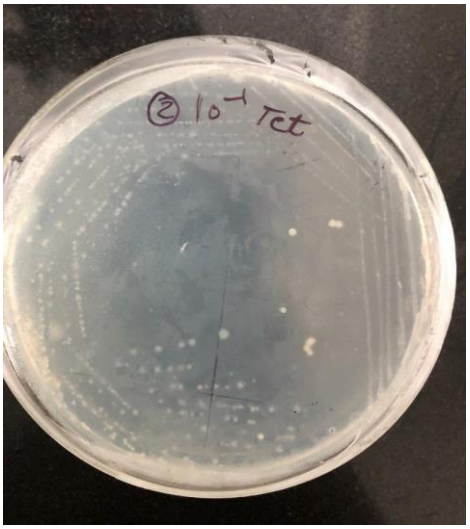
Figure 2. Percentage of Tetracycline resistant bacteria in Gomti river water

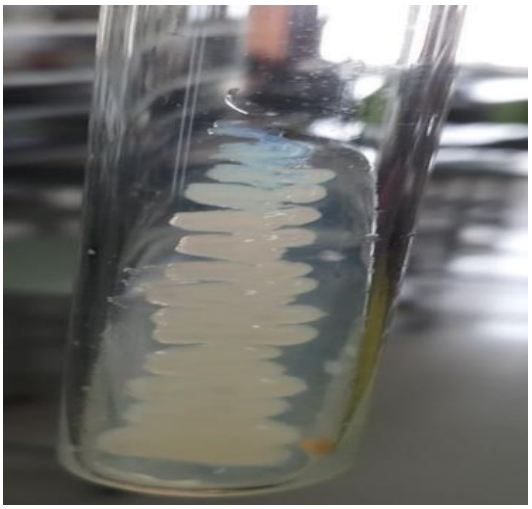
Isolation of antibiotic resistance Bacteria

Tetracycline-resistant bacteria were isolated on the LB agar containing tetracycline at a 100 µg/ml concentration. From which ten bacterial isolates were selected based on morphology and colour. The well-separated colonies were selected and purified by repeatedly re-streaking (Table 2). The bacterial isolates were named as GTR-1,GTR-2,GTR-3,GTR-4,GTR-5,GTR-6,GTR-7,GTR-8,GTR-9,and GTR-10.From the ten streaked plates, slants were prepared in 10 test tubes in order to preserve the culture.

Table 2. Morphological and physical appearance of isolated and purified isolates

Isolate	Colony morphology		
	Colour	Shape	Size
GTR-1	white	Round	Small
GTR-2	White	Round	Small
GSK-3	Yellow	Irregular	Small
GTR-4	Grey	Oval	Large
GTR-5	White	Round	Large
GTR-6	Brown	Regular	Large
GTR-7	Cloudy	Round	Small
GTR-8	Cream	Regular	Large
GTR-9	Yellow	Irregular	Small
GTR-10	White	Round	Small





Multiple antibiotic resistance in the isolated bacteria

All the ten isolated bacteria were tested for the resistance to the other five different antibiotics by the disc diffusion method. Five antibiotic discs, i.e., Amoxicillin-30mcg (AMX),Kanamycin-30mcg (K), Amikacin-30mcg (AK), Methacilin-5mcg (MET), and Nalidixic acid-30mcg (NA)were used to check the antibiotic resistance pattern (Table 3).

Table 3. Antimicrobial susceptibility of the ten isolated bacteria

BACTERIA	ANTIBIOTICS				
	Amx	K	Ak	Met	Na
GTR 1	R	R	R	R	R
GTR 2	R	R	R	R	R
GSTR3	R	R	R	R	R
GTR 4	R	S (20mm)	S (22mm)	R	R
GTR 5	R	R	R	R	R
GTR6	R	R	S (18mm)	R	S(19mm)

GTR 7	R	R	R	R	S(24mm)
GTR 8	R	R	S(18mm)	R	S(19mm)
GTR 9	R	R	R	R	R
GTR 10	R	R	R	R	R

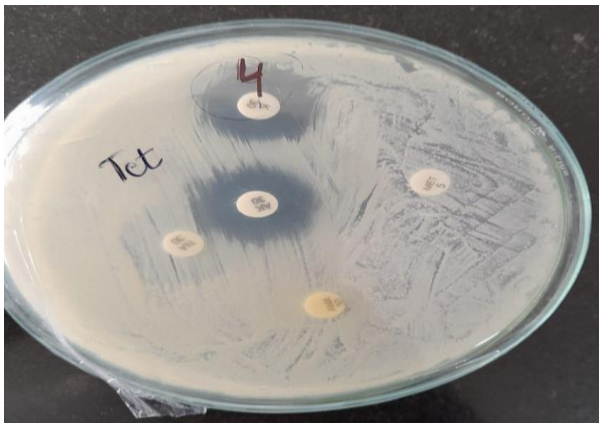


Figure 4. Antibiotic-resistance of the GTR-4 isolate using the disc diffusion method

Table 4. Percent antibiotic-resistant bacteria in the Gomti river water

ANTIBIOTICS	CONCENTRATION	NO.OF ISOLATES	RESISTANCE ISOLATE(%)
Amoxicillin	30mcg	10	100
Kanamycin	30mcg	9	90
Amikacin	30mcg	7	70
Methicilin	5mcg	10	100
Nalidixic acid	30mcg	7	70

The percentage of antibiotic-resistant bacterial strains from Gomti river water is shown in Table 4. Among the ten isolated bacteria, 100% bacterial isolates showed resistance against Methicilin and Amoxicillin. While 90% of the bacteria showed resistance against Kanamycin, only 70% were resistant to Amikacin and Nalidixic acid (Table 4). Sixty percent of the isolates were resistant to five different antibiotics, and10% were resistant to four different antibiotics. At the same time, 30% of the isolates were resistant to three different antibiotics in two different combinations (Table 5).

The bacterial strains isolated from Gomti river water receiving wastewater showed high antibiotic resistance. Our results are in agreement with those of earlier works. Biswas et al. (2015) reported the prevalence of multiple antibiotic resistant bacteria in Ganga river, Serampore, West Bengal,India. Presence of antibiotic resistance bacteria in a given environment may be an indication that an area is contaminated with antibiotics. The feacal coliform bacteria are numerous in this riverine water might be due to the presence of anthropogenic wastes particularly sewage.Shafiani and Malik (2003) isolated 64 bacteria(40 Pseudomonas spp., 12 Azotobacter and 12 Rhizobiumspp.) from wastewater irrigated soil. They tested all the isolates for their antibiotic susceptibility against different antibiotics i.e. nalidixic acid, cloxacillin, chloramphenicol, tetracycline, amoxycillin, methicillin and doxycycline and reported that 100% of the Pseudomonas isolates were resistant to cloxacillin and 57.7% to methicillin. 7.5% of the isolates exhibited multiple resistance to five different antibiotics in three different combinations, whereas 25%of the isolates showed multiple resistance to four antibiotics in seven different combinations.

Table 5. Antibiotic resistance pattern of the ten isolates from water of Gomti River

NO. OF ANTIBIOTICS	NO.OF ISOLATES	RESISTANCE PATTERN
3	2 (20)	Amx, K, Met
	1 (10)	Amx, Met, Na
4	1 (10)	Amx, K. Ak, Met
5	6	Amx, K, Ak, Met, Na

Conclusion

The culturable bacterial population from the Gomti river water contains highly-multi-resistant bacteria, some of which have been isolated and characterized for multi-drug resistance profiles to 5 antibiotics, which show a diversity of combinations of resistances. The bacterial population isolated from river water is resistant to multiple antibiotics, and it can be concluded that the river water contains pathogenic bacteria having multi-drug resistance. Multi-drug resistance in pathogenic bacteria is a significant challenge that leads to high morbidity and mortality. The sample collection sites have anthropogenic inputs of fecal origin, and the site's location near the anthropogenic source may be the reason for high antibiotic resistance. The microbial population with multi-drug resistance can be managed by restricting the usage of antibacterial drugs and making people aware of the ill effects. Also, the patients should be encouraged to complete the required dosage of the medicines so that the gut microbes cannot develop drug resistance. Further, decentralized domestic wastewater treatment should be encouraged to reduce the pathogenic bacteria reaching the river.

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