A DISSERTATION ON

High prevalence of Sulfonamide resistant bacteria in the Gomti river water

SUBMITTED TO THE

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FOR THE

DEGREE OF MASTER OF SCIENCE

IN MICROBIOLOGY

BY

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TO WHOM IT MAY CONCERN

This is to certify that **Katyayani Singh,** student of M.Sc. Microbiology (IV semester), Integral University has successfully completed her four-month dissertation entitled "**High prevalence of Sulfonamide resistant bacteria in the Gomti river water"** successfully. She has completed this work from March June 2022 under the guidance of **Dr. Mohd Ikram Ansari**. The dissertation was a compulsory part of her M.Sc.degree.

I wish her good luck and a bright future.

Prof.(Dr.) Snober S. Mir

Head Department of Biosciences

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Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Katyayani Singh** during the month of March-June 2022, reported in the present thesis, was under my guidance and supervision. The results she reported are genuine and the candidate herself has written the thesis script. The thesis entitled "**High prevalence of Sulfonamide resistant bacteria in the Gomti river water"** is, therefore, being forwarded for the acceptance in partial fulfilment 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 Bioscience

DECLARATION

I hereby declare that the present work on "**High prevalence of Sulfonamide resistant bacteria in the Gomti river water"** is a record of my original work under **Dr. Mohd Ikram Ansari, Assistant Professor**, **Integral University**, during the month of 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 Katyayani Singh

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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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My acknowledgment will be incomplete if I do not mention my parents, with whose blessing I was able to achieve my goal successfully. There are no words to express my feelings toward them. I silently acknowledge my debt to them.

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Table of Abbreviation

AMR : antimicrobial resistance..... 3

CFU : colony Forming Unit 22

DHFA : dihydrofolic acid..... 12

DHPS : dihydropteroate synthase 2

PABA : para-aminobenzoic acid 12

SJS : stevens-johnson syndrome..... 9

SNs : sulfonamides..... 07

THFA : tetrahydrofolic acid 12

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CHAPTER-1

INTRODUCTION

Introduction

Antibiotics have been routinely employed in human and animal medicine since their discovery in 1928.Residual antibiotics reach the environment through various routes, including wastewater discharge and runoff, and leaching from land fertilized with agricultural or human waste. Antibiotic contamination of wastewater has been documented. They have also been discovered in China's groundwater, animal waste, pond water, animal farm effluents, and rivers (Satoru,2012).

Sulfonamides have been widely used to treat bacterial and protozoan infections in humans, domestic animals, and aquaculture species. Sulfonamides impede folate manufacture by competing for binding to dihydropteroate synthase (DHPS), an enzyme in the folic acid synthesis pathway, with the natural substrate p-amino-benzoic acid(Satoru,2012).

Sulfonamide resistance is common among human and animal diseases worldwide; nevertheless, sul genes are not evenly distributed among bacterial populations. Hoa et al. (2008) found that among SMX-resistant bacteria isolated from VAC farms, municipal canals, and aquaculture locations in Vietnam, sul1 was the most common sul gene. Because most of these publications were based on separated bacteria, which may skew the interpretation of results owing to isolation techniques, we cannot get an accurate picture of antibiotic resistance gene distribution from culturable bacteria alone (Hoa and Nonaka et al.,2008).

Wastewater is a suitable medium for a wide range of microorganisms, including bacteria, viruses, and protozoa, and can be utilized as a reservoir for germs resistant to antibiotics. It transports resistant bacteria from human excretions and liquid waste released from household homes, agricultural and commercial sectors, pharmaceuticals, and hospitals into the sewage system (Houndt &Ochman et al., 2000). Bacteria are likely to face varied selective pressures for antibiotic resistance in different environments, resulting in different antibiotic resistance acquisition and evolution patterns. Antibiotic resistance determinants and organisms persist in the final effluent and are released into the environment from urban wastewater treatment plants. These are vital reservoirs of human

and animal commensal bacteria (Reinthaler and Feierl et al., 2003; Tennstedt 2003).

Antimicrobial resistance (AMR) is a global health issue causing an increase in the occurrence of both debilitating and fatal diseases. Understanding how microbes acquire and spread AMR can help develop new strategies to combat the problem. Antibiotic-resistant microorganisms pose health difficulties due to a lack of therapeutic options, particularly in underdeveloped nations with limited access to high-quality therapies. Infections continue to be a major source of morbidity and mortality.Antibiotic-resistant bacteria and unabsorbed antibiotic residues are discharged in urine and faeces, and eventually, make their way to wastewater treatment plants through home sewer systems. Antibiotic residues such as -lactam, macrolides, lincosamide, tetracyclines, sulphonamides, and fluoroquinolones have all been found in reclaimed municipal effluents (Martins et al., 2016)

The continuous rise in infections caused by numerous antibiotic-resistant pathogenic bacteria emphasizes the need to improve our understanding of the environmental dimension of antibiotic resistance. Because antibiotic resistance in infectious bacteria is frequently coupled with a plasmid, plasmids that carry antibiotic resistance genes may pose a public health danger. Furthermore, these plasmids are frequently mobilized and susceptible to acquiring antibiotic resistance gene cassettes on the move. Plasmid mobility is critical for the evolution and spread of antibiotic resistance in bacteria from various environments(Teddie 2014).Water contains many bacterial species; it is believed that 50% of all known bacterial genera contain species that can be classified as water bacteria. Antibiotics have been widely used for clinical and veterinary purposes since the late 1960s, so it is no surprise that antibiotic-resistant bacteria have grown common (Ansari and Grohmaan et al.2007).

The quality of wastewater effluents causes the degradation of receiving water bodies. This is because untreated or improperly treated wastewater effluent can promote eutrophication in receiving water bodies and generate conditions that encourage the expansion of toxin-producing cyanobacteria pathogens in the water. Recreational water users and anyone else who comes into touch with the infected water are at risk. Although diverse microorganisms serve a variety of

valuable roles in wastewater systems, many of them are thought to be critical contributors to a variety of waterborne epidemics. Careful planning, adequate and appropriate treatment, regular monitoring, and appropriate laws are required to achieve unpolluted wastewater discharge into recipient water bodies (Akpor et al., 2013). Considering all these following objectives were designed to be completed in this study.

Objectives:

1. Evaluation of the sulphonamide-resistant bacterial population in the Gomti river water near the wastewater disposal site of Lucknow.
2. Isolation of the sulphonamide-resistant bacteria from the Gomti river water of Lucknow.
3. Determination of multiple antibiotic resistance patterns in the bacteria isolated from the Gomti river water of Lucknow.

CHAPTER -2

REVIEW OF LITERATURE

Sulfonamides are a broad class of antibiotics with a variety of clinical uses. Sulfonamides, which have been present since the 1930s, were the first effective antibiotics to enter clinical use. They appear to work by blocking bacterial folic acid production, which is essential for cell growth. Sulfonamide resistance genes sul1, sul2, sul3, and sulA are known, and bacteria isolated from animal waste, natural water, and sludge have sul1, sul2, and sul3 but not sulA, according to previous investigations (Supuran 2003).

Structure And Nomenclature

Sulfonamides are made up of a sulphur atom (Fig.1) with two sets of double bonds to two oxygen atoms, a carbon-based side group, and a nitrogen atom linked to the sulphur. In organic chemistry, an amide is a carbonyl group attached to a nitrogen atom. Sulfonamides are identical to carbonylamides, except that a sulfone replaces the carbonyl group (a sulphur with two oxygen atoms). Because of this, the term 'amide' comes into the name.

FIGURE 7: AN AMIDE ; A SULFONAMIDE

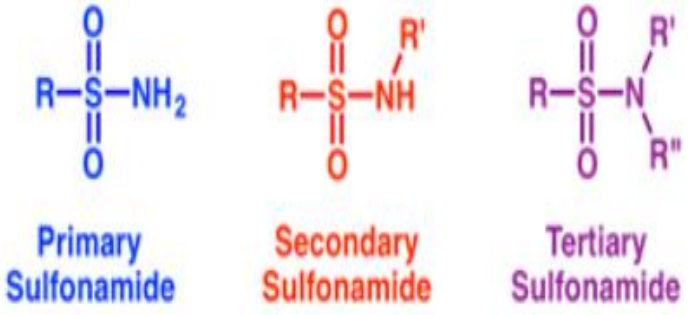


FIGURE 8:STRUCTURE OF PRIMARY, SECONDARY AND TERTIARY

The 'R' groups in figure 2 represent any generic carbon-based side chain, which might be anything. R could be a methyl group, a benzene ring, an alkane ring, or something else entirely. Sulfonamide is classed as primary if there are two hydrogens on the nitrogen atom, secondary if there is only one hydrogen, and tertiary if there are no hydrogens on the nitrogen atom.

A tertiary SN typically has a central sulphur atom with two doubly bonded oxygens, which is also connected to a nitrogen atom (existing as a substituted amine) and an aniline group, where R1/R2 might be hydrogen, alkyl, aryl, or hetero aryl groups. An organic molecule consisting of aniline derivatized with a sulfonamide group is another way of defining the basic SN drug structure (Pareek 2013; Sonu 2017).

SULFONAMIDE

4-aminobenzenesulphonamide is the IUPAC name for SN, and the two derivative drugs are 4-amino-N-(4, 6-dimethylpyrimidin-2-yl) benzene sulphonamide for SMZ and 4-amino-N-(pyrimidin-2-yl) benzene-1-sulphonamide for (Robertson and Moodie et al. 2020; "Sulfamethazine and Its Sodium Salt" 2001).

Classification

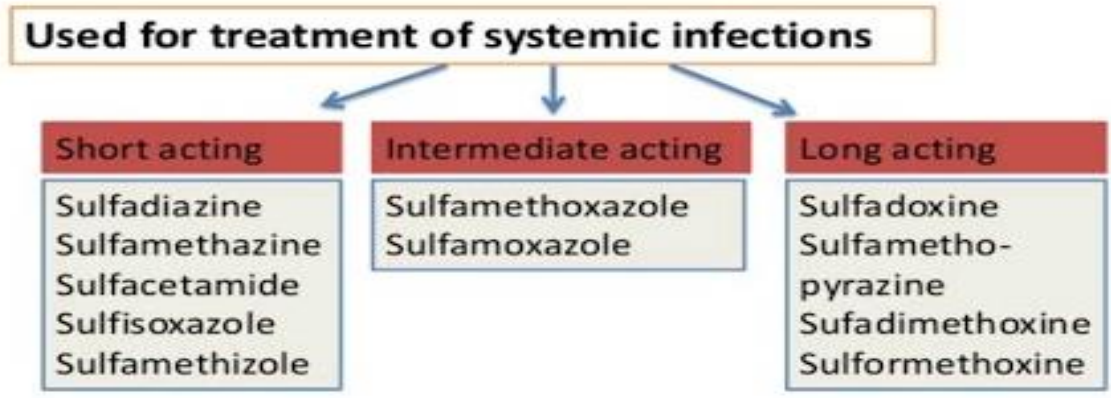
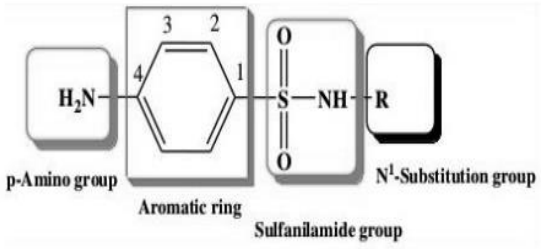
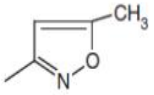


FIGURE 9:CLASSIFICATION

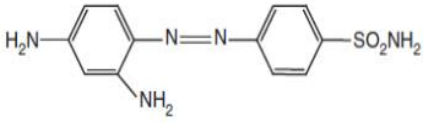
DERIVATIVES OF SULFONAMIDES



N-1 Substituted sulphonamides

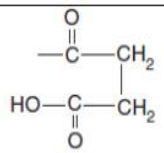
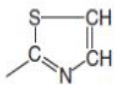
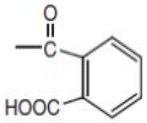
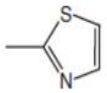
Name	R	R^1
Sulphanilamide	–H	–H
Sulphacetamide	–H	$-COCH_3$
Sulphadiazine	–H	
Sulphamethoxazole	–H	

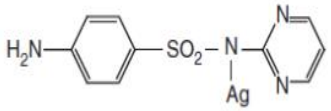
N-4 substituted suphonamides



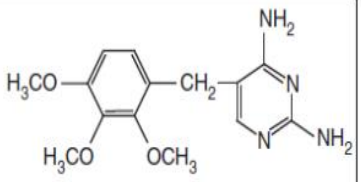
Prontosil

N-1 and N-4 substituted suphonamides

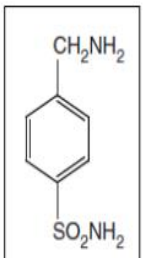
Name	R	R_1
Succinyl sulphathiazole		
Phthalylsulphathiazole		



Silver sulphadiazine



Trimethoprim



Mafenide

FIGURE 10: SULFONAMIDE DERIVATIVES

Uses

Sulfonamides represent a diverse range of medicines with a diverse range ofactions.Examples of some conditions that may be treated with sulfonamides include:

Bacterial infections: eg, sulfamethoxazole/trimethoprim, sulfisoxazole
Crohn's disease: eg, sulfasalazine
Diabetes: eg, glyburide, tolbutamide
Fluid retention: eg, chlorothiazide, furosemide, hydrochlorothiazide
Gout: eg, probenecid
High blood pressure: eg, chlorothiazide, hydrochlorothiazide
Pain and inflammation: eg, celecoxib
Rheumatoid arthritis: eg, sulfasalazine
Ulcerative colitis: eg, sulfasalazine. (sumanta and mondal 2017)

Side Effects

- Non-allergic reactions to SN drugs include diarrhoea, nausea, vomiting, dizziness, candidiasis, folate deficiency, and migraines.
- SNs are not readily biodegradable and have the potential to produce a variety of unfavourable side effects, including disorders of the digestive and respiratory systems (Sultan 2015; Mathews et al. 2015).
- They have the potential to cause side effects, including urinary tract problems, haemopoietic problems, and hypersensitivity reactions.
- When taken in large doses, it can cause severe allergic reactions.
- Stevens-Johnson syndrome (SJS) is a life-threatening skin disorder in which the epidermis separates from the dermis due to cell death.

Doses

The dose medicines in this class will be different for different patients.

1.For sulfadiazine (Tablet)

For bacterial or protozoal infections:

Adults and teenagers—2 to 4g for the first dose, then 1 gram every four to six hours.

Children 2 months of age and older—Dose is based on body weight.

The usual dose is 75mg/kg of body weight for the first dose, then 37.5 mg/kg of body weight every six hours, or 25 mg/kg of body weight every four hours.

Children up to 2 months of age—Use is not recommended.

2.For sulfamethizole (Tablet)

For bacterial infections:

Adults and teenagers—500mg to1g every six to eight hours.

Children 2 months of age and older—Dose is based on body weight.

The usual dose is 7.5 to 11.25 mg/kg of body weight every six hours.

Children up to 2 months of age—Use is not recommended

Antibacterial activity

Sulphonamides are a class of antibiotics with a broad spectrum of activity and are particularly efficient against gram-positive and gram-negative bacteria. *Klebsiella, Salmonella, Escherichia coli,* and *Enterobacter species* are among the sensitive gram-negative bacteria; however, sulfonamides have no inhibitory effect (bacterial resistance) against *Pseudomonas aeruginosa* and *Serratia species*. Sulfonamides have also been shown to impede the growth of fungus and protozoa. Many studies have shown that sulphonamide, sulfamethazine, and sulfa-diazine medicines have antibacterial properties. When used against bacterial infections caused by *Nocardia*, *Staphylococcus aureus*, and *Escherichia coli*, SN and its derivatives demonstrated significant antibacterial efficacy(Aben and Ovung et al. 2021).

Mechanisms of action and resistance of antibiotics

Antimicrobial drugs' mechanisms of action can be classified based on the function that the agents influence; they include inhibition of cell wall or nucleic acid synthesis, inhibition of ribosome activity, inhibition of cell membrane actors function, and inhibition of folate metabolism. There are two methods to define resistance:

A). Intrinsic resistance whereby microorganisms naturally do not possess target sites for the antimicrobials and the antimicrobial does not affect them. B). Acquired resistance is whereby a naturally susceptible microorganism acquires a mechanism not to be affected by the antimicrobial (Dowling and Dwyer et al., 2017).

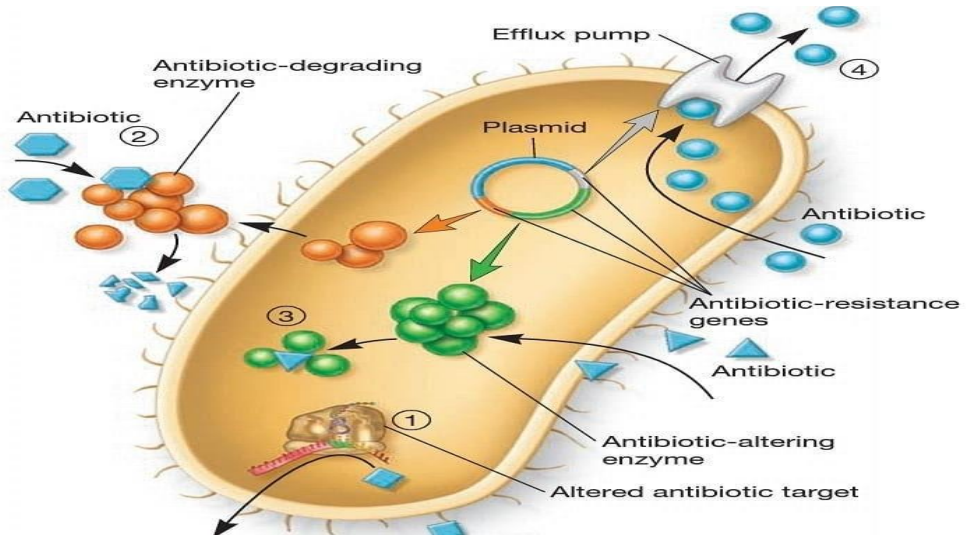


FIGURE 11: ANTIBIOTIC RESISTANCE MECHANISM

Mechanism of action of sulphonamides

Sulphonamides are among the earliest antibacterial chemicals still in use. Sulphonamides have been in clinical use for fifty years, and resistance is common. To broaden the spectrum and increase antibacterial activity against bacteria that would be resistant to either drug alone, trimethoprim (trimethoprim-sulphonamide) or ormetoprimare added. Diaminopyrimidines are what trimethoprim and ormetoprim are chemically. The chemical similarity between

sulphonamides and para-aminobenzoic acid (PABA) determines sulphonamide activity. Sulphonamides operate as a fake substrate, competing with PABA for the enzyme dihydrofolate synthase, blocking the synthesis of dihydrofolic acid (DHFA), which is then hindered by trimethoprim, which inhibits the formation of tetrahydrofolic acid (THFA) and the folate cofactor. For the production of nucleic acid, the folate cofactor works as a 1-carbon donor (DNA). Bacterial growth and division are halted when the manufacture of folate coenzyme is inhibited. Sulphonamides exhibit selective bacteriostatic toxicity because mammalian cells use preformed folates from the food while bacteria cannot use preformed folates and must synthesise their own folic acid (Dowling and Dwyer et al.2017).

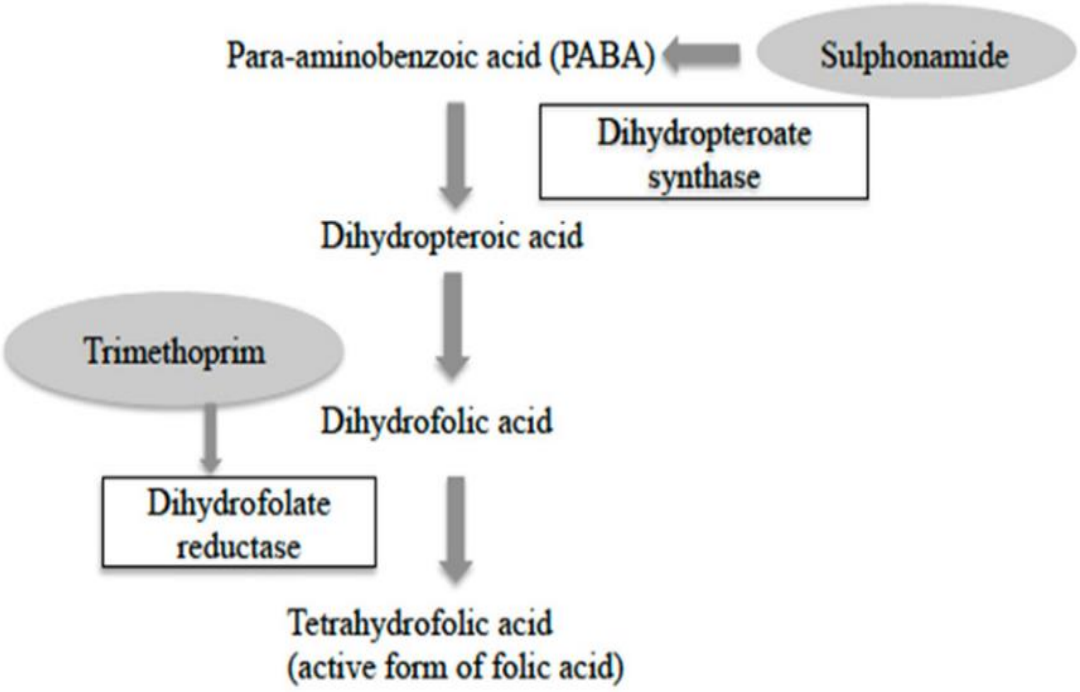


FIGURE 12:SIMPLIFIEDPATHWAY OF SULFONAMIDE AND TRIMETHOPRIM

Bacterial resistance to sulphonamides

The threat of antimicrobial resistance to the human, animal, and environmental health is growing. Overuse of antibiotics is a key contributor to antimicrobial

resistance; another aspect is the dearth of new treatments available to combat the problem. Bacteria can develop resistance to antibiotics in two ways:

Endogenous vertical evolution and Exogenous horizontal evolution. Vertical evolution covers the acquisition of resistance through spontaneous mutation within the bacterial genome, which then passes down to its children. In contrast, horizontal evolution describes the transfer of resistance genes between unrelated bacteria (Laws 2019).

Bacterial resistance to SN has been frequently reported, with some of the reported resistance cases being due to:-

(i) resistance bacterial genes to trimethoprim-sulfamethoxazole (used as prophylaxis for the treatment of severe respiratory tract infections)

(ii) resistance genes to SDZ resulted in phenotypic conversion showing a lack of sensitivity to polymyxin B for Serratia marcescen,

(iii) resistance bacterial genes to SN spread and distributed in soils and were detected around poultry farms in China,

(iv) resistance bacterial genes to SN discovered in the environment, and

(v) trimethoprim-SN resistance spread among pathogenic bacteria.With increasing antibiotic resistance to SN drugs, considerable effort needs to be directed to the development of new and effective medicines (Prestinaci, 2015; Taneja and Sharma, 2019)

Factors Influencing Sulfonamide Action

Their surroundings heavily influence all medications' strength of action. Sulfonamides are no exception when it comes to cell inhibitors. The effect of specific environmental factors easily altered on sulfonamide action has been the subject of much investigation. Several of the findings could have been predicted. However, the influence of pH has just recently been satisfactorily explained, and another, the effect of bacterial inoculum size on sulfonamide activity, continues to be a mystery. It is also crucial to remember that these studies and findings

are compatible with various ideas on how sulfonamides exert their inhibitory effects.

a) Drug concentration
b) Size of inoculum.

   In the presence of a constant amount of Sulfonamide, the inhibition of bacterial growth is inversely related to the number of organisms present; or in other words, as the size of the inoculum is increased, a greater amount of Sulfonamide is required to produce the same inhibition (Kohn and HARRIS et al., 1941).
c) Composition of the medium; In vitro vs. in vivo.
d) pH. Varying the environmental pH has a very definite effect on sulfonamide activity. This was first noted by those interested in sulfonamide therapy for urinary tract infections, an instance where the pH can be controlled within certain limits. Investigation showed that sulfonamide activity in urine increases as the pH increases, e.g., from the range 5.5-6 to 7.5-7.8. These investigations of sulfonamide activity at various urine acidities were made primarily in quest of an answer to an important clinical question and not to ascertain the nature of the effect of pH on sulfonamide activity(Fox and Rose et al.1942)
e) Temperature.

   A temperature rise significantly increases the sulfonamides' bacteriostatic and bactericidal effect in vitro. Several researchers compared the activity at 3700 degrees Fahrenheit (human body temperature) and several degrees above. It was discovered that the increase in temperature was not the primary cause of the observed effects. This is, of course, clinically relevant, as will be demonstrated further down. At slightly higher temperatures, a bacteriostatic concentration at 3700 turns antibacterial in some cases.At 37°C, 100 times the amount of Sulfonamide was necessary to provide the same impact as at 3900°C. The capacity of PABA to counteract sulfonamide activity reduces dramatically when the temperature rises over 3700°F. Increased sulfonamide activity is shown in vivo when the temperature is raised.

This has been shown in gonococcal infection of the chick's chorioallantoic membrane, rabbit pneumococcal septicemia, human gonococcal infection treated with artificial fever, and infected wounds (Green and Parkin et al. 1942).

f) Age of culture; length of experiments.Though it may be that, qualitatively, the action of sulfonamides on bacteria is the same regardless of the age of the culture, it would be expected that the action would vary quantitatively with age. Critical data on this particular problem are very scanty and conflicting.The ages of the cultures used by various investigators have differed considerably, and it seems probable that this factor has played an essential part in many of the quantitative differences observed (Bradbury and Jordan, et al. 1942).

STEPS TO DECREASE ANTIMICROBIAL RESISTANCE

(i) Decrease in selective pressures

Agriculture and medicine exert the most significant selected pressure. It is clear that reducing selective pressures that encourage and maintain the existence of resistant mutants over sensitive strains is necessary to regulate the development and spread of resistant germs. Antimicrobial misuse by health care professionals, unskilled practitioners, and patients can be reduced by auditing antibiotic use, limiting antibiotic choice, developing prescription guidelines, and emphasizing continuing medical and public education. In contrast, the misuse of antibiotics by health care professionals, unskilled practitioners, and patients can be alleviated by auditing antibiotic use, limiting antibiotic choice, developing prescription guidelines, and emphasizing continuing medical and public education. It is also desired to prohibit the selling of medications over the counter, but due to chronically inadequate health facilities in developing nations, this may be difficult to enforce.

(ii) Adoption of good infection control:

For an antibiotic-resistant infection to propagate, it must be carried, communicated, or moved to additional patients. The uneducated or irresponsible health care professional is frequently the vector for spreading the virus. To control multi-drug resistant infections, handwashing, primary contact control, disinfection of surrounding surfaces, and, when necessary, isolation or quarantining of patients are all required.

CHAPTER-3

MATERIALS AND METHODS

SAMPLING SITE:

The sampling site selected was the Gomti river water, located in Lucknow, Uttar Prades. 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. The city receives about 900 mm of annual rainfall between July and September, mainly 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 enormous pressure on land, water, housing, transport, health, education etc. This rising population significantly impacts the area's natural resources, especially water quality and quantity. Fresh water is the most critical natural resource for life, but overexploitation and unjustified water use have led to the deterioration of water quality.

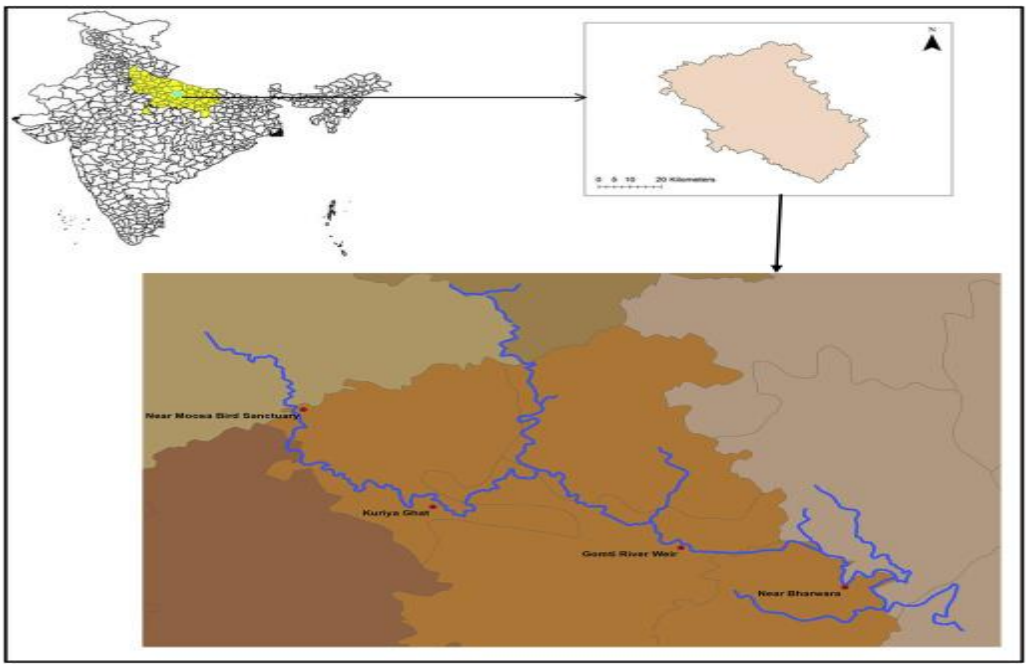


Figure 13: Map showing Gomti river and sampling location

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.

COLLECTION OF WATER SAMPLE:

Composited water samples were collected from two sampling sites in the Gomti river of Lucknow, UP (India), in 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 another wastewater was added to 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, appropriately labeled, and transported on ice to the laboratory for analysis.

Figure 14: Figure showing the actual site for collection of water sample

Culture media used for enumeration of the standard and sulphonamide-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 sulphonamide in the media. The Nutrient agar was amended with a sulfonamide to get a final concentration

of 100 µg/ml to enumerate the sulfonamide-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 was counted, and the population was evaluated using the formula

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

Isolation of sulphonamide-resistant bacteria from water

The 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 Sulfonamide, and incubated at 35°C for 24 h. Ten fast-growing bacterial isolates with distinct colony morphology were picked and purified by repeated streaking on nutrient agar (Table 2).

Subculturing for pure culture preparation.

Under the 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 cool. It was returned to the 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 the desired volume of double-distilled water in a conical flask and boiled to mix properly. The solution was mixed properly to make the uniform media solution, 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 cooled, the bacteria

were inoculated by the loop, and the slant test tubes were incubated at 37°C overnight. This process was done aseptically in laminar airflow. 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 using the disc diffusion method (Bauer et al. 1966). The following antibiotics (all from Hi-media, Mumbai, India) were used. The 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-trimoxazole (Co 25), doxycycline (Do 30), gentamicin (G 30), kanamycin (K 30), nalidixic acid (Na 30), neomycin (N 30), streptomycin (S 10) and tetracycline (T 30).

CHAPTER -4

RESULT 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 sulphonamide in the medium.

Sample	**Heterotrophic bacteria without antibiotic (A) (CFU/ml)**	**Heterotrophic bacteria with sulphonamide added in the medium (B) (CFU/ml)**
GRW1	$5.08X10^{8}±1.01X10^{8}$	$1.24X10^{3}±7.07X10^{2}$

The water samples collected from the Gomti River, Lucknow, show a high population of sulphonamide-resistant bacteria. The average heterotrophic bacterial count in the Gomti water was found to be $5.08X10^{8}±1.01X10^{8}$ CFU/ ml when no antibiotic was added to the growth media (Table 1). However, the bacterial population reduced to $1.24X10^{3}±7.07X10^{2}$CFU/ml when sulphonamide 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 sulphonamide-resistant bacteria. The analysis of the results revealed that the gomti water contains about 16% of sulphonamide-resistant bacteria (Figure 2). Similar to our results, Lye et al. (2019) reported sulphonamide-resistant bacteria in Larut River and SanggaBesar River receiving different wastewater. They found a high population of sulphonamide-resistant bacteria in river water. They further reported that the hospital wastewater contains the highest sulphonamide-resistant bacteria (10^{7} CFU/ml).

Similarly, Kergoat et al. (2021) reported that the presence of sulphonamide in the water inhibits bacterial growth and changes the water's microbial diversity. Water contamination by environmentally realistic concentrations of sulphonamide affected both the heterotrophic and autotrophic communities with

various effects according to the Sulfonamide and the exposure level. Thus, exposure to sulphonamide modified bacterial structure and impaired microbial enzyme functions. Moreover, sulfonamide exposure also had adverse effects on the autotroph component of the periphytic biofilm (Kergoat et al., 2021).

These results suggest first a direct effect of the sulfonamides on the exposed communities, resulting in impairment of the bacterial functions as already observed in soil microbial communities exposed to sulphonamide (Liu and Yang, et al., 2016) or in the biofilm which is exposed to river water contaminated by antibiotics (Proia, 2013). Further, sulfonamide exposure acted as a selection pressure on the microbial communities, selecting the most tolerant species able to maintain the reference level of extracellular enzyme activities on exposure to sulfonamides. Many studies observed the impact of antibiotics, including sulfonamides, on the bacterial structure in soil (Liu and Yang et al., 2016), sediments (Kergoat and Leremboure et al., 2021), or biofilm.

Figure1. Bacterial population on standard and sulphonamide amended plates

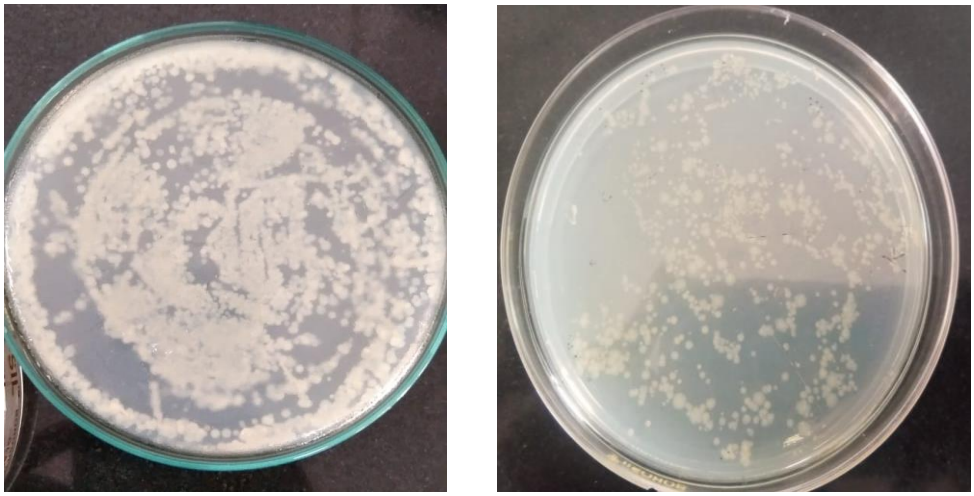
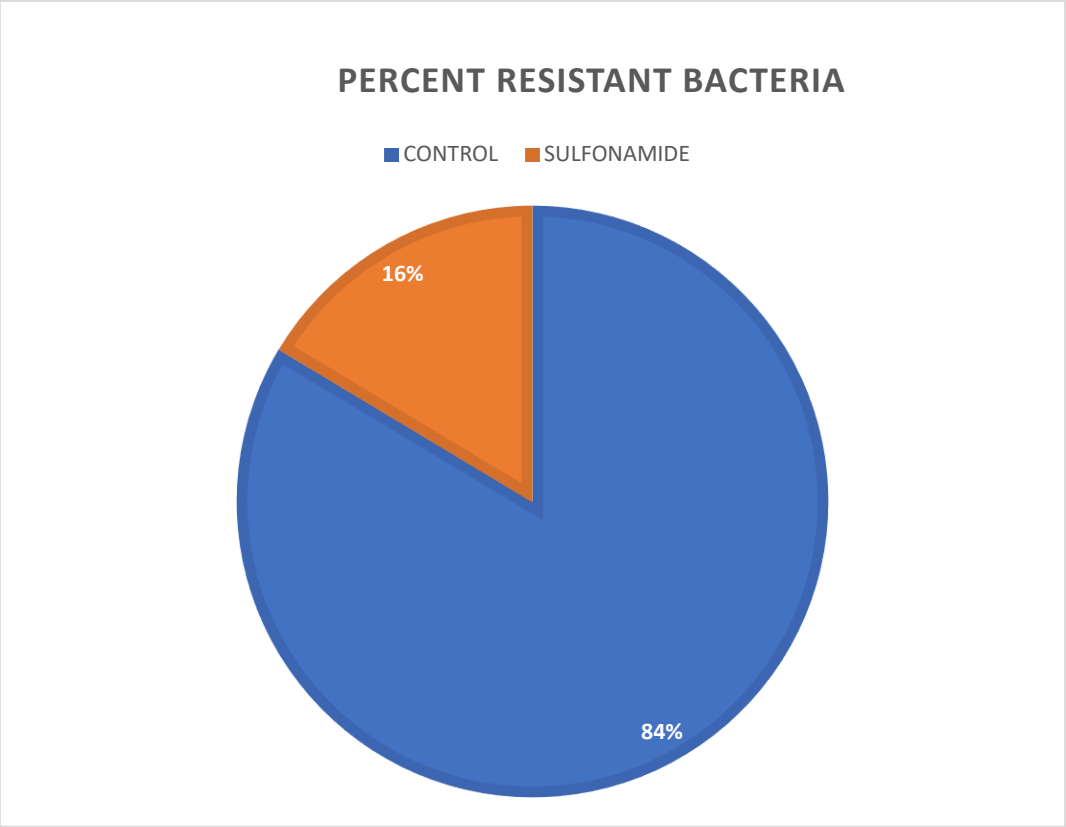


Figure 2. Percentage of sulphonamide-resistant bacteria in Gomti river water



OBJECTIVE 2:-

Isolation of antibiotic resistance Bacteria.

Sulphonamide-resistant bacteria were isolated on the LB agar containing sulphonamide 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 GSK-1, GSK-2, GSK-3, GSK-4, GSK-5, GSK-6, GSK-7, GSK-8, GSK-9, and GSK-1

Table 2. The morphological and physical appearance of isolated and purified isolates

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

OBJECTIVE3:-

(a) Multiple antibiotic resistance patternsof the isolated bacteria

Five antibiotic discs i.e., Methacilin (Met),Nalidixic acid (Na), Amikacin (Ak), Cefoxitin(Cx), Amoxicillin (Amx) were used to check the antibiotic resistance pattern.

Table 3. Antibiotic sensitivity test of the isolated bacteria

BACTERIA	ANTIBIOTICS				
	Met	Na	Ak	Cx	AMX
GSK 1	R	R	S (25mm)	R	R
GSK 2	R	S (24mm)	S (18mm)	R	R
GSK 3	R	R	S (22mm)	R	R
GSK 4	R	S (19mm)	S (18mm)	R	R
GSK 5	R	S (21mm)	S (20mm)	R	R
GSK 6	R	S (22mm)	R (19mm)	R	R
GSK 7	R	R	R (16mm)	R	R
GSK 8	R	R	S (20mm)	R	R
GSK 9	R	S (22mm)	S (20mm)	R	R
GSK 10	R	R	R (14mm)	R	R

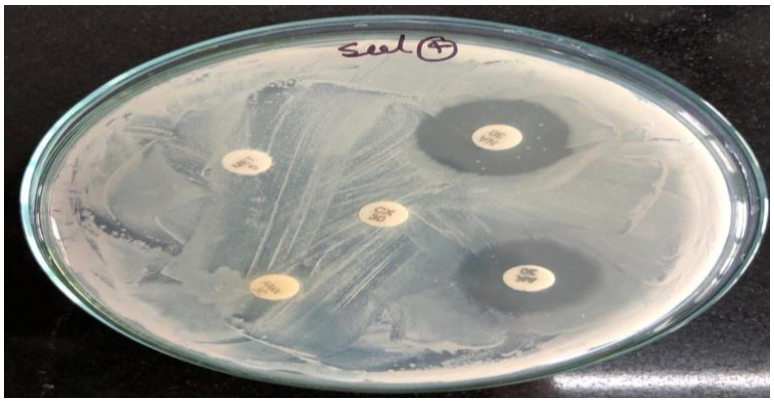


Table 4. Multiple antibiotic resistance among the isolated bacteria.

ANTIBIOTICS	CONCENTRATION	NO.OF ISOLATES	RESISTANCE ISOLATE(%)
Methicillin	5mcg	10	100
Nalidixic acid	30mcg	5	50
Amikacin	30mcg	3	30
Cefoxitin	30mcg	10	100
Amoxicillin	30mcg	10	100

Table 5. Antibiotic resistance pattern of 10 isolates from water of Gomti River

NO. OF ANTIBIOTICS	NO.OF ISOLATES	RESISTANCE PATTERN
3	4	Met, Cx, Amx
4	3	Met,Na,Cx,Amx
	1	Met, Ak,Cx,Amx
5	2	Met,Na,Ak,Cx,Amx

From table 4, we can conclude that 100% of bacterial isolates were resistant to Methicillin, Cefoxitin, and Amoxicillin; 50% were resistant to Nalidixic acid, and 30% were against Amikacin. Table 5 shows that from the ten bacterial isolates, four isolates were resistant to 3 antibiotics (Met, Cx, Amx). The other four isolates were resistant to four antibiotics in two different combinations. Three isolates were resistant to Met, Na, Cx, Amx, and the other one to Met, Ak, Cx, and Amx. The remaining two bacterial isolates were resistant to 5 antibiotics (Met, Na, Ak, Cx, Amx). Thus all the bacterial isolates show a different pattern of resistance. Correia et al. (2020) isolated 579 ampicillin-resistant bacteria and tested were resistance to 10 antibiotics. They found that 92.7% of the isolates were resistant to four or more antibiotic classes, indicating a high level of multi-resistance. They reported 143 resistance profiles among the isolated bacteria.

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.

References

Akpor, O.B. and Muchie, B., 2011. Environmental and public health implications of wastewater quality. African Journal of Biotechnology, 10(13), pp.2379-2387.

Ansari, M.I., Grohmann, E. and Malik, A., 2008. Conjugative plasmids in multi-resistant bacterial isolates from Indian soil. Journal of Applied Microbiology, 104(6), pp.1774-1781.

Bradbury, F.R. and Jordan, D.O., 1942. The surface behaviour of antibacterial substances: Sulphanilamide and related substances. Biochemical Journal, 36(3-4), p.287.

Campo, B., Vandal, O., Wesche, D.L. and Burrows, J.N., 2015. Killing the hypnozoite–drug discovery approaches to prevent relapse in Plasmodium vivax. Pathogens and global health, 109(3), pp.107-122..

Dowling, A., O'Dwyer, J. and Adley, C., 2017. Antibiotics: mode of action and mechanisms of resistance. Antimicrobial research: Novel bioknowledge and educational programs, 1, pp.536-545

Eduardo-Correia, B., Morales-Filloy, H. and Abad, J.P., 2020. Bacteria from the multi-contaminated tinto river estuary (SW, Spain) show high multi-resistance to antibiotics and point to Paenibacillus spp. as antibiotic-resistance-dissemination players. Frontiers in microbiology, 10, p.3071.

Fox Jr, C.L. and Rose, H.M., 1942. Ionization of sulfonamides. Proceedings of the Society for Experimental Biology and Medicine, 50(1), pp.142-145.

Green, H.N. and Parkin, T., 1942. Local treatment of infected wounds with sulphathiazole. The Lancet, 240(6208), pp.205-210.

Hoa, P.T.P., Nonaka, L., Viet, P.H. and Suzuki, S., 2008. Detection of the sul1, sul2, and sul3 genes in sulfonamide-resistant bacteria from

wastewater and shrimp ponds of north Vietnam. Science of the total environment, 405(1-3), pp.377-384.

Henry, R.J., 1943. The mode of action of sulfonamides. Bacteriological reviews, 7(4), pp.175-262.

Kergoat, L., Besse-Hoggan, P., Leremboure, M., Beguet, J., Devers, M., Martin-Laurent, F., Masson, M., Morin, S., Roinat, A., Pesce, S. and Bonnineau, C., 2021. Environmental concentrations of sulfonamides can alter bacterial structure and induce diatom deformities in freshwater biofilm communities. Frontiers in microbiology, 12, p.643719.

KOHN, H.I. and HARRIS, J.S., 1941. ON THE MODE OF ACTION OF THE SULFONAMIDES I. ACTION ON ESCHERICHIA COLI. Journal of Pharmacology and Experimental Therapeutics, 73(3), pp.343-361.

Lewis, K.H. and Snyder, J.E., 1940. The action of sulfanilamide in vitro on various species of Lactobacillus and Propionibacteria. J. Bacteriol., 39, p.28.

Liu, A., Cao, H., Yang, Y., Ma, X. and Liu, X., 2016. Combinational effects of sulfomethoxazole and copper on soil microbial community and function. Environmental Science and Pollution Research, 23(5), pp.4235-4241.

Negi, A.S., Kumar, J.K., Luqman, S., Shanker, K., Gupta, M.M. and Khanuja, S.P.S., 2008. Recent advances in plant hepatoprotectives: a chemical and biological profile of some important leads. Medicinal Research Reviews, 28(5), pp.746-772

Ovung, A. and Bhattacharyya, J., 2021. Sulfonamide drugs: Structure, antibacterial property, toxicity, and biophysical interactions. Biophysical Reviews, 13(2), pp.259-272.

Okoh, A.I., Sibanda, T., Nongogo, V., Adefisoye, M., Olayemi, O.O. and Nontongana, N., 2015. Prevalence and characterisation of non-cholerae Vibrio spp. in final effluents of wastewater treatment facilities in two districts of the Eastern Cape Province of South Africa: implications for public health. Environmental Science and Pollution Research, 22(3), pp.2008-2017.

Pareek, A., Rani, P. and Kishore, D., 2013. A short review on: Sulphonamides. Int J Pharm Bio Sci, 4(1), pp.812-820.

Parveen, B.R.S., Praveen, S. and Pal, H., 2017. A short review on Sulphonamides with antimicrobial activity. Int J Pharmaceut Chem, 7(05), pp.70-3.

Reinthaler, F.F., Feierl, G., Galler, H., Haas, D., Leitner, E., Mascher, F., Melkes, A., Posch, J., Winter, I., Zarfel, G. and Marth, E., 2010. ESBL-producing E. coli in Austrian sewage sludge. Water Research, 44(6), pp.1981-1985..

Robertson, L.P., Moodie, L.W., Holland, D.C., Jandér, K.C. and Göransson, U., 2020. Sulfadiazine masquerading as a natural product from scilla madeirensis (Scilloideae). Journal of natural products, 83(4), pp.1305-1308.

Sultan, E.A., 2015. Pathophysiologic mechanisms of immune-mediated drug hypersensitivity reactions to sulfonamides.

Mathews, S.M., Jiju, V., Thomas, I., Panicker, J.T. and Kuriakose, L.S., 2015. Sulfa drugs and the skin. World J Pharm Res, 4(10), pp.382-390.

Sustar, A., Perkovic, M.N., Erjavec, G.N., Strac, D.S. and Pivac, N., 2019. Association between reduced brain-derived neurotrophic factor concentration & coronary heart disease. The Indian journal of medical research, 150(1), p.43.

Zhang, X.X., Zhang, T. and Fang, H.H., 2009. Antibiotic resistance genes in water environment. Applied microbiology and biotechnology, 82(3), pp.397-414.