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The evaluation of antibiotic resistant genes in bacteria isolates of drinking water of Bhopal District of Madhya Pradesh

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***Abstract*---**Antibiotic resistance is becoming a major problem all over the world. The spread of antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) in the environment is a major health related problem. The aquatic ecosystem is an important source for ARBs and ARGs. The antibiotic resistance in bacteria is the emerging issue in the drinking water industry, Therefore, this study was done to study the presence of ARB and ARG in drinking water of Bhopal district of Madhya Pradesh. The 200 drinking water samples were collected from the point of domestic use (POU) into an autoclaved vial. The samples were applied on nutrient agar medium which is used for cultivating microorganisms. Each randomly appearing bacterial colony was taken into LB broth and further incubated. Extracted DNA were used for the PCR amplification for the detection of Gyr A and Tet A. Antibiotic resistance carrying genes were observed in the studied samples, the prevalence of Gyrase A was found to be 7%, while in Tet(A) gene it was reported to be 4.5%, therefore, the present study confirmed the presence of antibiotic resistance genes “Gyrase A and Tet A" in high prevalence in bacterial isolates of drinking water from Bhopal district, Madhya Pradesh.

***Keywords*---**GyrA, Tet A, Antibiotic resistance, Drinking water, PCR.

Introduction

Bacterial viral, fungal, and parasitic pathogenicity is a global phenomenon. Infectious diseases is responsible for over 14.9 million deaths. According to National Centre for Health Statistics, diarrheal infections kill 1.8 million people each year, while tuberculosis kills 1.5 million. Around 50,000 people die from pneumonia and over 130,000 die from septicemia in the United States alone

Antibacterial agents are compounds with a low molecular weight (usually less than 1,000 daltons) [Walsh C et al, 2020]. Antibiotics can be produced by a variety of ways, including as chemical weapons or as signaling molecules to close relatives in eukaryotic plants and insects [Clark et al, 2013, Klassen JL et al, 2014].

Antibiotic-producing bacteria have been used to prevent disease for millennia with moldy bread healing potions being used to treat open wounds in various part of the world for more than 2000 years ago [Zasloff M et al 2002]. Antibiotics were the biggest medical breakthroughs of the 20th century. MRSA (methicillin-resistant Staphylococcus aureus) was found to be killed by an Anglo-Saxon recipe from 1000 years ago [Truman A et al 2019]. The advent of antibiotics marks the beginning of the antibiotic era in human clinical medicine, with the discovery of penicillin 75 years ago and the development of the first commercially viable antibiotics 50 years later [Scholar EM et al 2000]. Antimicrobial peptides typically install themselves specifically into bacterial membranes, disrupting their barrier function [Behroozian S et al 2016].

Antimicrobial resistance (AMR) has risen rapidly as a result of the overuse of these beneficial substances, with some infections being effectively untreatable. Bacterial resistance is the ability of bacteria to withstand the bacteriostatic or bactericidal effects of antibiotics [Munita JM et al 2016]. Antibiotic overuse, both intentional and unintentional, contributes to bacterial resistance development [Kraemer SA et al 2019]. Genes encoding drug resistance mechanisms, such as antibiotic inactivating enzymes, efflux pumps, and alteration of the antibiotic action target, may be found in the DNA of bacteria, regardless of their chromosomal, plasmidic (episomal), or phagic form [Schaechter M 2010, Todar K et al 2011, Hawkey PM et al 1998]. Antibiotic resistance in bacteria can be acquired naturally (intrinsic, innate) or acquired by modifying endogenous genes or integrating exogenous resistance genes [Decoster A et al 2009, Schaechter M 2010].

Certain pathogenic organisms, such as Pseudomonas aeruginosa, E. coli, and Enterobacter cloacae, have a low permeability of the external lipopolysaccharidic membrane, which acts as a barrier to antibiotic diffusion inside the bacteria, resulting in natural resistance to a variety of antibiotics [Schaechter M 2010, Todar K et al 2011]. Pseudomonas aeruginosa, for example, has inherent resistance to ampicillin and tetracycline, while E. coli has natural resistance to vancomicyn and other antibiotics [Hawkey PM et al 1998]. The bacterial chromosome supports natural resistance genetically [Mihăescu G 2007, Decoster A 2008]. Antibiotic-producing bacteria must defend themselves against the antibiotics they produce [Todar K, Mihăescu G 2007].

Raw water sources have been linked to antibiotic resistance genes in drinking water. Six ARGs were discovered in Michigan and Ohio's raw water sources. In a separate investigation conducted in Louisiana, sul1 and tetA genes were discovered in raw water source, Nigeria (Adesoji et al., 2017), Canada (Fernando et al., 2016), Korea (Son et al., 2018), Japan (Nguyen et al., 2019), Tanzania (Lyimo et al., 2016), Poland (Koniuszewska et al., 2020), Germany (Stoll et al., 2012; Voigt et al., 2020).

Methodology

1. Collection of water sample: The drinking water samples were collected from the point of domestic use (POU) in Bhopal district in an autoclaved vial.
2. Screening of microorganism in water samples using serial dilution and NAM media: Bacteria were isolated using Nutrient Agar Media (NAM). NAM media was prepared by suspending 28g of NAM in 1000 ml of distilled water and was autoclaved at 121^0C for 15 min at 15 psi. Approximately 25 ml of the sterilized media was poured in each plate and cooled down until solidified. Serial dilution of water sample was carried out as follows: five test tubes were labelled and filled with 9 ml of sterile distilled water. 1 ml of water sample was poured in first tube containing 9 ml distilled water and mixed well (dilution- 10^{-1}). After that, 1 ml from the previous tube was taken and poured into second tube containing 9 ml distilled water (dilution- 10^{-2}). Similarly, the steps were carried out till the dilution was 10^{-4}. 100 µl from the 10^{-4} dilution tube was spread in the NAM plate. This process was done for each water sample and the plates were incubated at 37^0C for 24 hours. The CFU were counted and reported for each sample.
3. Culturing the selected colonies in LB broth: Luria Broth (LB) was prepared by mixing 20g in 1000 ml distilled water and was autoclaved at 121^0C for 15 min at 15 psi. Approximately 25 ml of LB broth was transferred in each culture tube and loopful of selected colonies were inoculated in the broth. The culture tube were incubated at 37^0C for 2-3 days for optimum growth for genomic DNA extraction.
4. DNA extraction using phenol chloroform method: Genomic DNA of the bacterial cell was extracted by phase separation method using phenol chloroform and isoamyl alcohol (PCI). The incubated culture broth was centrifuged at 5000 RPM for 15 min to obtain bacterial pellet. 800 µl Sol. B and 20%, 50 µl SDS (Sodium dodecyl sulphate) was added in the pellet at incubated at 56^0C for one hour. After that, 200 µl 5 M sodium acetate and 500 µl PCI [phenol-chloroform-isoamyl alcohol (25:24:1)] was added and mixed well. The tube was centrifuged at 10000 RPM for 10 min. Upper aqueous layer was collected from the obtained three layers. Into the aqueous layer 500 µl of chilled Isopropyl alcohol (IPA) was added and centrifuged at 10000 RPM for 10 min. Supernatant was discarded and pellet was washed with 70 ethanol by centrifuging at 10000 RPM for 5 min. Pellet obtained was dried and dissolved in 20-25µl Mili-Q water. The DNA extracted was confirmed by agarose gel electrophoresis.
5. Detection of antibiotic resistant genes using PCR: The antibiotic resistance was confirmed against Gyrase A, and Tet (A) gene. Reaction mixture for each sample was made according to the table given (1 &2). The components were mixed and run up to 36 cycles for optimum amplification. Later, The PCR product was confirmed by agarose gel electrophoresis using ladder.

Table 1: Composition of PCR maser mix for the PCR of tet (A) & gyr (A)

S. No.	Content	Volume (20 µl)
1.	Primer (forward)	0.5 µl
2.	Primer (reverse)	0.5µl
3.	DNTPs	0.5 µl
4.	Mgcl2	0.5 µl
5.	Buffer	2 µl (10X)
6.	Taq polymerase	0.2 µl
7.	DNA Sample	1 µl
8.	D/W	14.8µl

Table 2: Modified PCR conditions for tet (A) & gyr (A) applied in presents study.

S.No.	Treatments	Temperature	Duration
1.	Pre-denaturation	95°C	5 min
2.	Denaturation	95°C	30 sec
3.	Annealing	61 °C for tet (A) 50 °C for gyr (A)	30 sec
4.	Extension	72°C	30 sec
5.	Final extension	72°C	5 min

Table 3: Present table showing the Primer details of tet (A) & gyr (A) applied in presents study

S. NO.	Resistant against	Mutant gene	Primer sequence	PCR product	Reference
1	Tetracycline	tet(A)	(F) GCGCGATCTGGTTCACTCG	164	Aminov et al., 2002
			(R) AGTCGACAGYRGCGCCGGC		
2	Oflaxocin and Ciprofloxacin	gyrA	(F) TACCGTCATAGTTATCCACGA	313	Wasylet al., 2014
			(R) GTACTTTACGCCATGAACGT		

Results and Discussion

In present study, successfully grown the bacterial colony from water samples on Nutrient Agar Medium (Figure1.) The isolated bacterial colonies were subjected to use for downstream process as per standard protocol (Gholami et al.,2016: Mulamattathil et al., 2014)

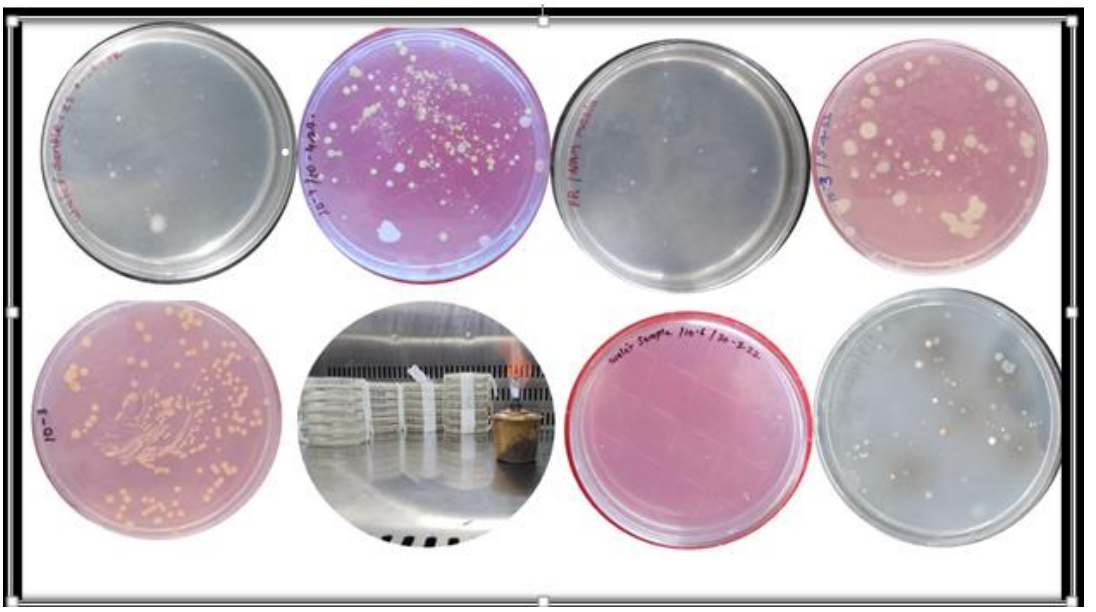

Figure 1: The figure showing selected images of isolated bacterial pure cultures during present study

DNA extracted from all the selected bacterial colonies using Phenol chloroform methods, and The DNA extracted was confirmed by agarose gel electrophoresis as shown in the Figure 2.

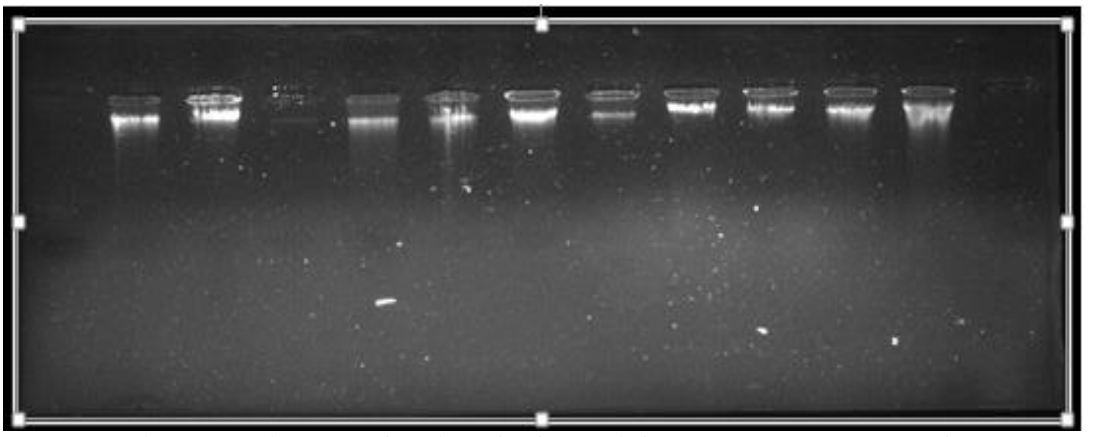

Figure 2: Figure A showing the bacterial genome on agarose gel

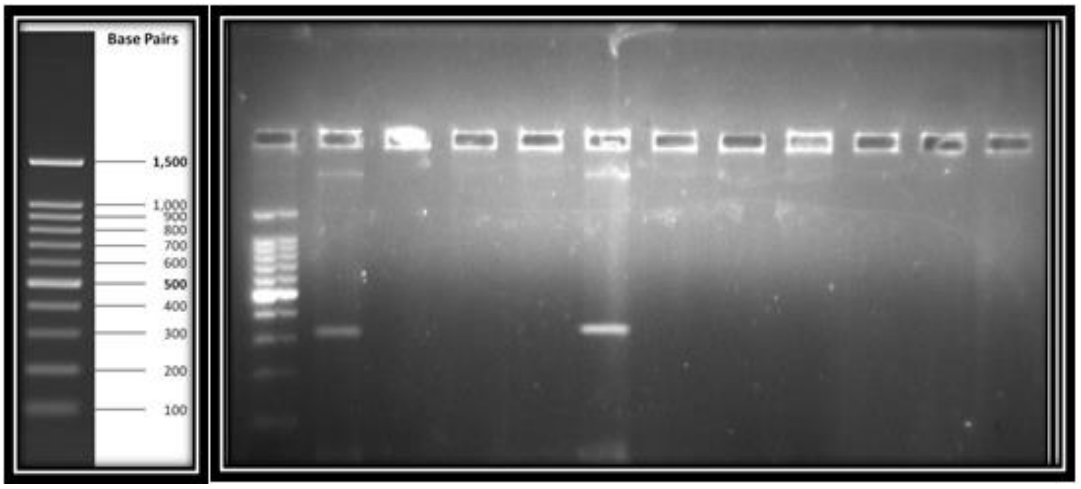

Figure 3: Figure showing the gyr (A) gene detection through PCR using established primer set

Figure 4: Figure showing the Tet (A) gene detection through PCR using established primer set

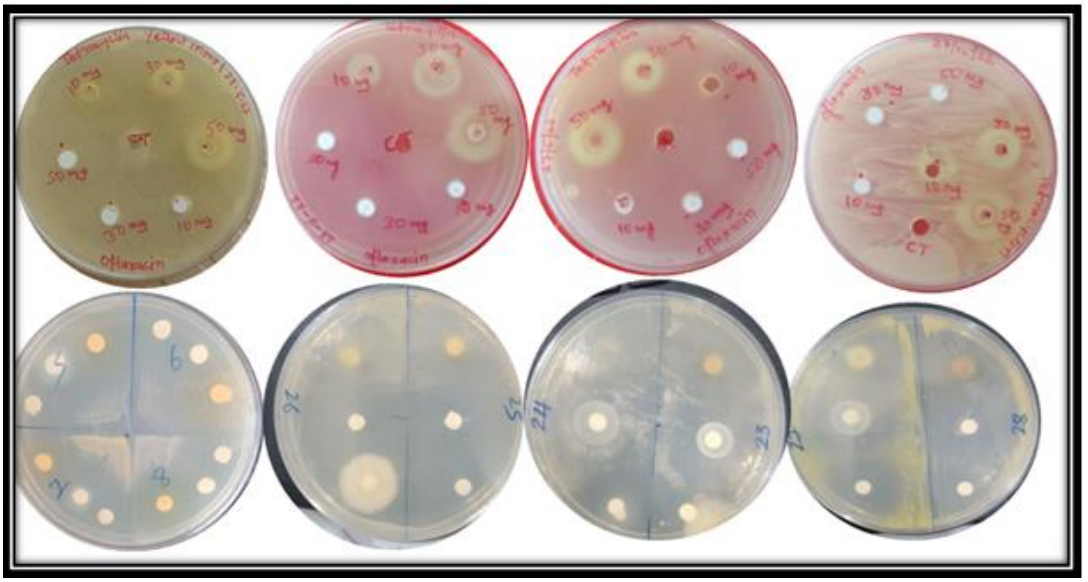

Figure 5: Figure showing the antibiotic sensitive and resistant bacterial species against Tetracycline and Oflaxocin

The antibiotic resistance was confirmed against Gyrase A, and Tet (A) gene using PCR techniques (Figure 3 & 4), 9 bacterial species were reported positive for the Tet (A) while 14 isolates were found positive for the Gyrase A (table 4).

The genetic analysis revealed that spontaneous resistance to Tetracycline and Oflaxocin in some studied samples caused by a punctiform mutation in each of the two genes gyrA and gyrB, which code for the two protein subunities of the enzyme DNA gyrase, resulting in enough conformational modifications of the gyrase to reduce or eliminate the gyrase's affinity for fluoroquinolones [Willmott et al., 1993; Heddle et al., 2002].

Table 4: The table showing the frequencies of tet(A) and gyrA in 200 bacterial isolates

S. NO.	Resistant against	Mutant gene	Gene reported in 200 colonies of Bactria species
1	Tetracycline	tet(A)	9
2	Oflaxocin	gyrA	14

In present study we have obtained the antibiotic resistance at the significant level in the collected water samples. Earlier research reports also found the considerably higher quantities of bacterial resistance in the water samples (Schwartz et al., 2003). Raw water sources have been linked to antibiotic resistance genes in drinking water. Six ARGs were discovered in Michigan and Ohio's raw water sources. In a separate investigation conducted in Louisiana, sul1 and tet(A) genes were discovered in raw water sources (Bergeron et al., 2015).

Conclusion of the study

The present study confirmed the presence of antibiotic resistance genes "Gyrase A and Tet A" in high prevalence in Bacterial Isolates of drinking water of Bhopal District of Madhya Pradesh.

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