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A Biotic Way ToControl Antibiotic Resistance Water Borne Pathogenic Bacteria

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Abastract

In recent times, humans are easily infected with antibiotic resistance waterborne pathogens So, in this context there should be analternative method to control these pathogens so that is negligible or no harm water as well as tocreatures dependent on these water bodies. Therefore, an easy cleaning can be applied by using aneconomic biological agent called phages which attack only bacteria kill them even if they are resistantto chemicals and do not cause any harm to humans or normal flora and funa or chemical nature ofwater for domestic use. In our work also disease resistance strains of *Enterobacter* and*Klebsiella* were susceptible to phage cocktail from Ganga Water which can be use it in future can be use in large scale toeliminate pathogens.

Key words: Antibiotics, Phage, Plaques, Pond water

1.Introduction

Water security is very crucial for global health and economic development. Due to unsafe drinking water, approximately 1.8 million people die each year (Liu et al. 2012). The co-relation between fecal contamination and serious waterborne diseases is widely reported (Herrador et al. 2015Microbial contamination and its check both are crucial due to its adverse effect on public health and industry. Antibiotics play the significant roles to eradicate bacterial diseases in humans and animals for a long time (Saga and Yamaguchi 2009). Some microorganisms are becoming resistant against common disinfectants so their use is being restricted (Waak et al. 2018). There is quest for alternative therapies or biocontrol agents due to emergence of antibiotic resistant rrmicroorganisms. Bacteriophages are considered as an alternative therapeutic agent (Myelnikov 2018). Mixture of phages could be useful as use of single phages led to resistant strains against individual phages (Safwat et al. 2017).

Bacteriophages are one type of viruses that infect and kill bacteria. They are everywhere and keep microbial balance in every ecosystem (Kutter and Sulakvelidze 2005). Phages are present in bacteria enriched environments like animal farms, slaughterhouses, wastewater, soil and aquatic environments. It had been seen that sea water contains upto 9 X 10^8 virions per milliliter (Wommack and Colwell 2000), and up to 70 % of marine bacteria may already be infected by phages (Jassim and Limoges 2013). Phages are isolated from several bacterial pathogens like *E. coli*, *Bacteroides fragilis*, and Salmonella serotypes in high concentration (10^5 pfu/10^{-2} g of feces) (Havelaar et al. 1986).

There are several reports on pages used as an indicator in wastewater treatment systems (Charles Fan et al. 2012)discussedon elimination of human pathogens by bacteriophage in the water systems like river, swimming tanks, ponds, and lakes. Not only waterbodies, microbial load could be reduced from food sample by applying phages (Sulakvelidze 2013). So phages have potency to eliminate pathogens from the waterbodies and thus the following study has been initiated to use phages as a biocontrol agent against pathogens in a pond.

2. **Materials and methods**:

2.1 **Collection,Transportation and analysis of pond water**

The water sample was collected from pondwater (Latitude 22.89^0N and Longitude 88.14^0E)and transported to our laboratory by standard method and physical and biochemical parameters of the sample was measured. The samplewasthensubjected to viable count studies by spreading 100 µl of 10^{-1} to 10^{-7} dilution prepared in sterile saline over the nutrient agar plate. The plates were incubated overnight at 37°C and plates showing 30 to 150 colonies were used for expressing the total viable bacterial count. The bacteriological analysis like the number of bacterial colonies, number of total coliform, and faecal coliform were measured by standard plate count (SPC), most probable number (MPN) and faecal coliform count (FCC) respectively. The samples were also plated in specific media to isolate the potentially dreadful pathogens (Microbiological Analysis of Food and Water 1998) and subjected to further characterization to identify the organisms as per the standard procedures.

2.2 ***Invitro*** **Antibiotic Sensitivity Analysis:**

The antibiotic sensitivity assay was performed using disk diffusion test (Bradshaw 1979). Selected microorganisms were spreaded into Mullarhinton(Himedia) agar plates. Antibiotics discs of Tetracycline, Chloramphenicol, Amphilicillin and Streptomycin of 25µg to 30µg were used in this test.

2.3 **IDENTIICATION OF SAMPLE B BY 16S rDNA**

Bacterial 16S rDNA sequences are attractive targets for developing identification methods because they represent conserved regions in all bacteria and species having 70% orgreater DNA similarity usually have more than 97% sequenceidentity (Stackebrandt and Goebel, 1994).Bacterialidentificationbased on % similarity of 16S rDNA has been using PCRtechnique, DNA sequencing and similarity analysis of rRNA genes. A direct comparison of 16S rDNA sequence is probablythe most powerful tool for the identification of many bacteria(Stackebrandt and Goodfellow, 1991). 16S rDNA was amplifiedand sequenced using oligonucleotide primers complementary tohighly conserved regions of bacterial rRNA gene. For identification ,DNA was isolated from the Sample A & B. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-

molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from theabove isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. (Figure1).The PCR amplicon was purified to remove contaminants.Forward and reverse DNA sequencing reaction of PCR amplicon (Figure 2) was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzerand consensus sequence was generated by Aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database (Pruitt et al., 2005).

2.4**Isolation of specific bacteriophages against the target**

The isolation ofphages was done bymixing a phagecontaining Ganga Water with *E.coli* in 2X Luria Broth with modifications along with 0.2%Maltose and 0.2% MgSO4 and following incubation for stipulate time of 48 hours bacterial debris were removed by filtration followed by centrifugation (Hyman et al. 2019).

2.5**Utilization of the bacteriophages as biocontrol agents against potential pathogen in water**

Enumerated bacteriophages were tested for the biocontrol efficacy(Hennes and Simon 2005). in controlling the target pathogens. The test organism selected for the study was *E. coli*. The target pathogens were inoculated separately as well with specific bacteriophages and time course study was done to know about the survival rate of pathogens.

3. Results and discussions

3.1 Characterization and bacteriological analysis of pondwater

Phyicalparameters(Table 1) of Pondwater like color, transparency,MPNpH.TDS(Total Dissolved Solid)valueand BOD of pondwaterconfirms presence of biological and pollutantadulterants in it . Moreover, biochemical tests(Table2) like Indole(I), Methyl Red(MR) and VogasProskauer(VP) test revealed that presence of both fecal and non fecal coliforms (Table2**).** Bacterial analysis directs us to determine the microbial pollution, which is a paramount in assessing the associated health risks. The huge amount of offaecal coliform clearly directed the presence of faecal material from warm blooded animals. All the water samples were contaminated with more number of faecalcoliforms(Rajurkar et al. 2003). The reason for the high number of faecal streptococci might be due to addition of human and warm blooded animal's excretae (Joshi et al. 2002). According to WHO estimate about 80% of water pollution in developing country, like India is carried by domestic waste and about 95% of rural population living in India depends on ground water for domestic use (Moharir et al. 2002). Untreated wastewater conistsof numerous disease causing microorganisms and toxic compounds that dwell in the human intestinal tract may contaminate the land or water body.

3.2**Identification of potential pathogens and Antibiotic Sensitivity Results**

Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clust16s rRNA sequencing(Figure 3 &4) was done for both the strains present in the pond water. and it was found that *Enterobacterhormaechei*and*Klebsiellapneumoniaea* . From these results, it was clearly revealed that Gram negative pathogens dominate this pondwater and it poses threat not only health risk, but also poses threat to other marine organisms but Both these strains showed the antibiotic resistant to common antibiotics (Table.3) and compared with standard chart (Sarker 2014)

3.3 Utilization of bacteriophages as antibacterial agents against potential pathogens in pond water

Biological hazard in water resources (Ahiwale et.al. 2012) in the form of pathogenic organisms are responsible for major outbreak in most of the developing countries. In this situation, every effort leading to reduction in sewage pollution and pathogenic microbes has to be promoted and implemented. This is required to healthy and sustainable environment. Entry of antibiotic resistant pathogens into the sewage is inevitable as survival is the key for existence. Development of multidrug resistant bacteria and exit of many antibiotic companies necessitates to search for novel approaches to tackle the multidrug resistant bacteria. Phage therapy(Lin et.al 2017) is a promising tool to treat these menacing organisms. It is essential for the success of any phage therapy; suitable phage should be isolated, enriched to produce sufficient numbers for the application. Phage enrichment normally involves the inoculation of mixed environmental samples. In this case phage cocktail in triplet test also showed promising result with Sample A(Figure 5) is more suseptable than Sample B(Figure6) with avergage of $3.8x10^7$pfu/ml and $1.8x10^7$pfu/ml respectively compared to test sample with 4.610^7pfu/ml.

Conclusion

Our experiment revealed that pond water of college contains several waste that affect negatively on the microbiological and physiochemical parameters on the environment. This finding suggests that the canteen wastes pose a serious environmental threat to our college. This study highlights the role of phages to control potential pathogens in the pond water. Pathogen specific phage has the ability to eliminate the dreadful pathogens. Thusphage treatment could be a alternative method of controlling pathogens in pond water. Though it has many limitations, phage application to wastewater treatment deserves attention.

Conflict of interest

There is no conflict of interest

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Tables & Figures

Table

Table1 Physical Parameters of Pond Water*

Parametres	Inference(s)
Temperature	$26^0 \pm 2^0$C
Colour	Green
Transparency	Turbid
Odor	Odorless
pH	6.8±0.2
TDS	523±20ppm
BOD	9±0.3ppm
MPN	≥2400

+Done in triplicate sets

Table2BiocnemicalChracterization of bacterial Sample*

TEST	RESULT	
	A	B
Lactose Fermentation	+	+
EosinMethylene-Blue Agar(Himedia)	Golden	Dark pink
Gram Stain	-	-
Indole	+	-
Methyl Red	+	-
Voges Proskauer	+	+

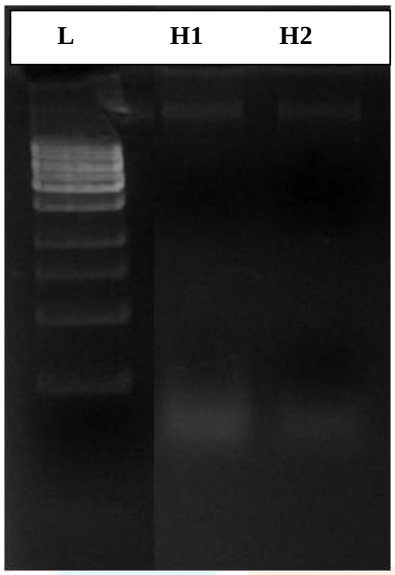
***Done in triplicate sets with incubation of 48 hours**

Table 3 *Invitro* Antibiotic Test*

Antibiotics(µg) disk	Zone of Inhibition(mm)	
	A	B
Ampicillin(AMP25)	13.3±0.3	12.1±0.3
Streptomycin(S25)	9.4±0.3	8±0.2
Chloramphenicol(C30)	12±0.2	11.6±0.3
Tetracycline(TE 30)	11.4±0.5	10.5±0.4

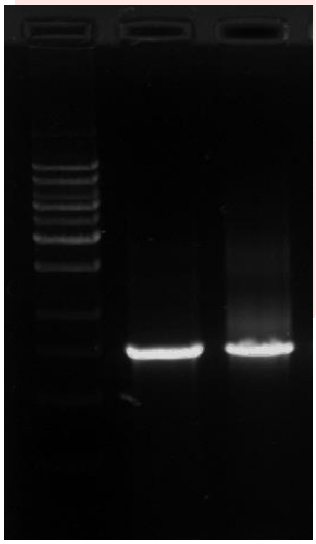
***Done in triplicate sets and compared with standard chart**
≥18mmsesitive, 15-16mm- intermediate and ≤11mm resistance

Figures:



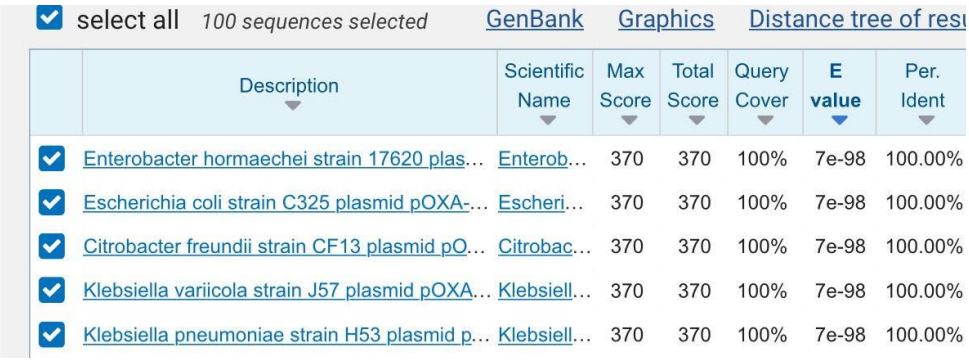
A- 1% Agarose gel stained with EtBr
B-Position of sample
Lanes H1, H2: Representative gel image of genomic DNA (3ul loaded)
Lanes L: 1kb DNA marker (3ul loaded)

Figure 1 Isolation of g DNA of Sample A(H1) and Sample B(H2)



A- 1% Agarose gel stained with EtBr
B-Position of sample

Figure 2 Isolation of PCR Product of Lane 2 Sample A(H1) and Lane 3 Sample B(H2)



select all 100 sequences selected GenBank Graphics Distance tree of resu

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
Enterobacter hormaechei strain 17620 plas…	Enterob…	370	370	100%	7e-98	100.00%
Escherichia coli strain C325 plasmid pOXA-…	Escheri…	370	370	100%	7e-98	100.00%
Citrobacter freundii strain CF13 plasmid pO…	Citrobac…	370	370	100%	7e-98	100.00%
Klebsiella variicola strain J57 plasmid pOXA…	Klebsiell…	370	370	100%	7e-98	100.00%
Klebsiella pneumoniae strain H53 plasmid p…	Klebsiell…	370	370	100%	7e-98	100.00%

Figure 3. NCBI BLAST of Sample A

select all 100 sequences selected GenBank Graphics Distance tree of re

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
Klebsiella pneumoniae strain 12 16S ribosomal RNA gene, partial sequence	Klebsiella pneu...	163	163	96%	7e-36	89.17%
Klebsiella pneumoniae strain IAUK 8737 16S ribosomal RNA gene, partial sequence	Klebsiella pneu...	161	161	93%	3e-35	89.74%
Klebsiella sp. A9-kP5 16S ribosomal RNA gene, partial sequence	Klebsiella sp. A9...	161	161	93%	3e-35	89.74%
Enterobacter sp. UIWRF0539 16S ribosomal RNA gene, partial sequence	Enterobacter sp....	161	161	93%	3e-35	89.83%
Klebsiella pneumoniae subsp. ozaenae strain H39 16S ribosomal RNA gene, partial sequence	Klebsiella pneu...	161	161	93%	3e-35	89.74%

Figure 4. NCBI BLAST of Sample B

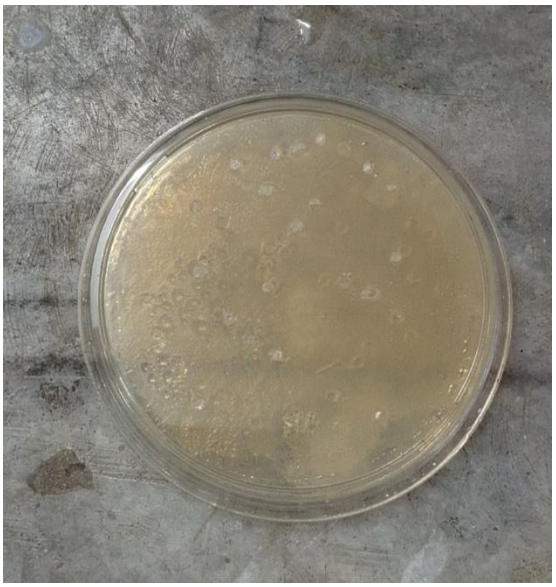


Figure 5 Clear Plaque fornation on Sample A

Figure 6 Clear and Cloudy Plaque formation on Sample B