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Isolation and Characterization of Coliform Bacteria and Bacteriophages from Ganga River in Northern Himalayan Regions

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ABSTRACT

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River Ganga with its source in Himalayas is considered to be the most sacred and holy river in Hinduism. Anthropogenic activities in the Ganga is deteriorating the quality of water and raising a serious question about purity of the water of river Ganga and its suitability to be used for drinking and other purposes. The present study is aimed to check the bacteriological quality of the Ganga water by analyzing the coliform bacteria and Bacteriophages in the Ganga. The sample were processed and analyzed for their quality with respect to various microbiological aspects. Investigation Method used for detection of coliform bacteria is Most Probable Number Technique and Surface plating and detection of bacteriophages are Isolated and characterized by plaque assay method.

Introduction

The water is the container of life and strength. The quality of water is very important to the health. The Ganga is the sacred fluid and an essential element for all the Hindu rites and rituals (Buhtiani *et al.*, 2016). The main river stream of Ganga originates from the Garhwal Himalayan river called Bhagirathi, which joins with Alaknanda (originates nearby) to form Ganga at the craggy canyon carved town of Devprayag. Therefore, the sacred Ganga is a confluence of rivers Bhagirathi and Alaknanda. As the Ganges flows out of the Himalayas, it creates a narrow, rugged

canyon. The Ganges River emerges from the Himalayas at the town named Rishikesh where it begins to flow onto the indo-Gangetic plain, Rishikesh is known as the gateway to the Garhwal Himalayas. From here, the Ganga travels to the Haridwar, which is a Hindu pilgrimage Centre. Till Haridwar, the route of Ganga is little southwest, from here it begins to flow southeast through the plains of northern India. Ganga flows southeast through Indian states of UP, Bihar and West Bengal (Ashbolt, 2001). The Ganges passes some of the most popular cities of India including Kanpur, Allahabad, Varanasi, Patna and Kolkata.

The Ganga is not only a holy river, but also a lifeline of a large population of India, as it covers more than 26% of the country's area in its basin in the north and drains 25% of the annual run-off. Fast urbanization and steep demand for water have led to serious problem of water quality degradation (Baghel, 2005). One of the main reasons of the pollution of Ganga River is faecal coli forms bacteria and organic pollution which is due to open defecation and discharge of waste water directly through small drains into the river (Singh, 2010).

The value of coliforms indicating sewage is directly dumped into the river (Sood A. Singh, 2008). Almost all the lodges, hotels and residential building dump their waste water into the Ganges in Rishikesh, Haridwar and other cities situated upstream of Ganga river.

Despite of pollution created by human, the river Ganga supports a wide diversity due to its self-cleaning and regeneration properties. Studies in 1896 by British bacteriologist Ernest Hankin showed the antimicrobial properties of river Ganga against *Vibrio cholera*, causative agent of cholera which gets destroyed in Ganga water but thrived in tap water. He proposed that a heat labile unknown substance which Passed through fine porcelain filters was responsible for preventing the spread of Cholera epidemic near downstream villages.

After a decade in 1916 *Felix d' Herelle*, a French Canadian microbiologist proposed that it was virus that parasitized bacteria based on the appearance of small, clear zones in the lawn of bacterial culture and the lysis in liquid culture.

He initially named it as taches, thentaches verges and later plaques. *Felix d' Herelle* officially named it as "bacteriophage" that formed from "bacteria" and "phagein" which

meansphages "eat" or "devour" bacteria (Sulakvelidze *et al.*, 2001).

Bacteriophage is the prokaryotic viruses that solely infect and/ or destroy the bacteria. Bacteriophage were associated with the special property of river Ganges (Hankin, 1896; D'Herelle, 1917) and it always present in the water and can survive thousands or lakhs of years even without having food (Adams, 1959). The source of the bacteriophage in Ganga is not yet known, right from the Gangotri from where it originates to the Sundarban delta where it merges into sea. Bacterial viruses (Bacteriophage) occupy all those habitats of the world, where bacteria thrive (Mulani, *et al.*, 2015). It has been estimated that for each bacterial cell there are ten bacteriophage particles. Viruses are considered as obligate intracellular parasites requiring a specific host cell for its replication (Guttman *et al.*, 2005) these are selfish replicators that use bacterial genome for their replication.

Materials and Methods

Sample collection

The sample was collected onsterile screw capped container from the various sites of Uttarakhand Ghats safely. The various sites are Badarinath, Devprayag, Uttarkashi, Rishikesh & Haridwar. All the samples are collected from Northen Himalayan Region, Uttarakhand, India (Fig. 1 and Table 1).

Media and reagents

All the media like Nutrient agar, MacConkey agar, MacConkey broth, Tryptone broth, LB Agar, Citrate agar, MR-VP Medium, Gelatin hydrolysis medium and reagent like Gram stain, indole reagent which are used in this experiment are manufactured by Hi-Media.

Microbial analysis of water sample

Most Probable Number (MPN) technique

The most probable number (MPN) is a statistical method to detect the number of coliform bacteria in a sample, most commonly for water. This test involves a multiple series of Durham fermentation tube and is divided into three parts- the presumptive, confirmed and completed tests (Fig. 2 and Table 2).

Presumptive test

This test is a screening method to sample water for the presence of coliform organisms. If presumptive test is negative no further test is done.

Procedure

The tubes containing 10ml of single strength MacConkey broth (SSMB) and double strength MacConkey broth (DSMB) were prepared. A Durham's tube was placed in each tube in an inverted position. The tubes were labeled according to the amount of water sample to be dispensed into it i.e. 10ml, 1ml and 0.1ml. The water sample was homogenized by shaking the bottle containing the water vigorously several times. 10ml of the water sample were transferred to each of the 5 DSMB tubes using a 10ml pipette, with a micropipette, 1ml and 0.1ml of the water sample were transferred to batches of 5ml SSMB tubes and Incubated at 37°C for 24 hours and the number of tubes in each set that produced 10% or more gas with a change in color from purple to yellow was recorded. MPN was determined by referring to the MPN table by "*Mackie and McCartney*".

Confirmatory test

Some microorganism other than coliform bacteria also produces acid and gas thus to

confirm presence of coliform bacteria, confirmatory test was performed.

Procedure

A loopful of culture from positive tubes was transferred to a fermentation tube containing brilliant green lactose bile broth tubes were incubated for 48±3 hours at 35±0.5°C.

Number of tubes that produced gas was recorded.

Completed test

Since some microorganism show false positive results, so it is desirable to do completed test.

Procedure

The sample from all positive BGLB tube is streaked onto eosin methylene blue agar and incubated at 37°C for 24 hours. Colonies produced greenish metallic sheen is differentiated from non-coliform bacteria.

Isolation and enumeration of bacterial pathogen

The direct plating procedures were followed for the isolation and enumeration of bacterial pathogen on Nutrient agar medium by the spread plate technique. When the growth was observed the separate colonies was sub cultured on MacConkey agar medium by surface plating. After the observation of growth, the Biochemical tests are used for identification of organisms.

IMViC test

This is a series of group of four individual tests used to identify coliform bacteria. The four reactions are: Indole test, Methyl-red test, Voges Proskauer test and Citrate test.

Indole test

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan into metabolic product indole, which accumulates in the medium. Using Kovac's reagent, indole combine with p-Dimethyl amino benzaldehyde which in acidic pH produce red-violet compound.

Procedure

5 ml media was poured in culture tubes and autoclaved at 121°C for 15-20 minutes for sterilization. Then 5 ml of media were inoculated with pure culture and incubated overnight at 37°C. To this 0.5 ml of Kovac's reagent were added and observed for color formation. Cherry red color show positive test and original color indicates negative result.

Methyl red test

Methyl red (MR) test is a biochemical test performed on bacterial species to detect the ability of an organism to produce stable acids end products (mixed acid fermentation) from supplied glucose.

Procedure

5 ml media was poured in culture tubes and autoclaved at 121°C for 15-20 minutes for sterilization. Then 5 ml of media were inoculated with pure culture and incubated overnight at 37°C. To this 5drops of methyl red indicator were added and observed for color formation. A distinct red color show positive test and distinct yellow color indicates negative result.

Voges proskauer (VP) test

The Voges proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. If

present, acetyl methyl carbinol is converted to diacetyl in the presence of α -Naphthol, strong alkali (40% KOH), and atmospheric oxygen. This is indicated by pinkish red color.

Procedure

5 ml media was poured in culture tubes and autoclaved at 121°C for 15-20 minutes for sterilization.

Then 5 ml of media were inoculated with pure culture and incubated for 24-48 hours at 37°C. To this 0.6 ml of α Naphthol added and observed for color formation. Cherry red color show positive test and original color indicates negative result.

Citrate test

Citrate agar is used to test an organism's ability to utilize citrate as the carbon source.

Procedure

Pure culture was streaked on Simmon's Citrate agar with the help of inoculating loop. The slants were incubated for 48 hours at 37°C. Growth on slants changes the color of slants from green to blue. No color change indicates negative result.

Hanging drop method (motility test)

Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), often is used in dark illumination to observe the motility of bacteria.

Procedure

Dab Vaseline on cover slip edges using a toothpick then place loopful of culture broth in the Centre of cover slip, turn the concavity

slide upside down over the cover slip and observe the motility on slide under microscope.

Catalase test

Catalase is an enzyme, which is produced by microorganism that lives in oxygenated environments to neutralize toxic forms of oxygen neutralizes the bactericidal effects of hydrogen peroxide and protects them.

Anaerobes generally lack the Catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce Catalase enzyme, a small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles.

Bacteriophage isolation and purification

After isolation of coliform bacteria from Ganga River their respective Bacteriophages were isolated.

Removal of contaminants of water

1.5 ml Ganga sample was taken out from each Ganga water sample in Eppendorf tube by the help of micropipette. Sample was treated with 1% chloroform for 20 minutes then centrifuged at 10,000 rpm for 10 minutes (Seaman P.F, Day M.J, (2007)). After centrifugation the upper part of the water decanted into a new sterile conical tube and was taken out for processing.

Plaque assay for phage concentration

Plaque is a clear lysed area in a monolayer displaying acytopathic effect. Plaque is formed when a virus infects a cell (Yang *et al.*, 2010).

Procedure of plaque assay

Enrichment of coliform bacteria

Species of coliform bacteria was inoculated into 50 ml of nutrient agar medium then incubated overnight at 37°C.

Amplification of bacterial virus

Isolated bacteria were inoculated in 5 ml sterile nutrient agar medium for 24 hours at 37°C. Following day, 5 ml of the pre-incubated bacterial inoculum, 5 ml of (10X) nutrient broth, 40 ml of Ganga sample was mixed and was incubated for 24 hours at 37°C.

Bacteriophage isolation and plating

After incubation, bacteria-bacteriophage culture was centrifuged for 5 minutes at 2000 rpm and supernatant was separated. Then supernatant was filtered, this step removes remaining bacteria. Prepare a series of microfuge tube for making bacteriophage dilution. Distribute 0.5ml of coliform bacterial species into each of six microfuge tube, labeled 1-6. Incubate at 37°C for 10 minutes to allow the phage to adsorb (attach) to the bacteria. The cell phage mix is prepared. In the meantime, label six warm, dry, nutrient agar plates 1-6 (one for each infection). Write on the bottom plate along the late edge. Keep the plates in the 37°C incubator until used. Add the content of cell-phage tube 1 to a vial containing 3ml of top agarose (molten, at 45°C). Quickly cap the tube, and mix it by gently inverting it three times. Quickly pour the mixture into warmed plate 1. Slightly tip the plate to spread the top agarose. Push the plate aside, but do not pick it up until agarose solidify. Repeat this step for each of the remaining 5 samples, 2-6 then allow the plates to cool without being disturbed for approximately 10 minutes after the top

agarose has solidified, incubate the plates, inverted, at 37°C for 24 hours. Plaques were observed on the agar medium plates (Ellis and Winters, 1969).

Results and Discussion

Most Probable Number (MPN) of coli forms

Coli forms count performed by multiple normal tubes to determine the most probable number (MPN) is commonly used as indicator of portability of water.

It gives us statistical estimate of coli forms population and measured the extent of coli forms in 100ml water. Coli forms produce gas and acid hence the production of gas in Durham tube, constituted positive test (Vass, 2010).

It is generally believed that more is the MPN of coli forms, higher is the extent of pollution in a given sample indicating heavy bacterial load and contamination.

The main source of organic burden of sewage in most communities is human body waste or faeces and microorganisms themselves are a part of human faeces.

When water sample is tested, it is not possible to identify all water borne pathogens. The solution is to look for indicator organism i.e. coli forms group of bacteria which contain *E. coli*, *Proteus spp*, *Klebsiella spp*. During the study plates were incubated at 37°C and 44.5°C.

All the water samples collected during the study some are positive and some are negative with respect to the coli forms occurrence well above the permissible limits, though the counts were variable. It is generally believed that more is the MPN of coli forms, higher is the extent of pollution in a given sample.

Bacterial characteristics on media

On nutrient agar media

White, creamy and fairly circular colonies were observed on Nutrient agar medium plate and shows swarming in the subculture as seen in figure 3.

On MacConkey agar

Lactose and non-lactose fermenting colonies was observed. *E. coli* colonies were observed as dark pink, dry on MacConkey while *Klebsiella* were observed as large shiny, and dark pink in color. *Proteus* was observed as pale, colorless and translucent colonies as shown in figure 4.

Morphological studies

Gram's staining

Gram staining reactions were studied with all the isolated culture to determine their morphology. Some isolates were gram negative bacilli while some were gram negative cocci. Some isolates are gram positive cocci, shown in the figure 5.

Biochemical characterization of isolates from various Ganga water sample

The sample were spread on nutrient and MacConkey medium according to the test required and the colonies that appeared after 24-48 hours were picked up based on their characterization and Biochemical test like: IMViC, catalase and motility test are performed.

Indole test

The isolates were screened for indole test. Red colored ring is observed in the tryptone broth containing bacterial isolates indicating presence of *E. coli* (Fig. 6 and Table 3).

Methyl red test

The isolates were screened for M-R test. A distinct red color is observed in the M-R media of bacteria isolates indicating presence of *E. coli* and *Proteus*.

Voges Proskauer test

The isolates were screened for VP test. A cherry red color change is observed in the isolates containing VP media indicating presence of *Klebsiella* and *Proteus*.

Citrate test

The isolates were screened for citrate test. Green color is observed in the citrate media containing bacterial isolates indicating presence of *Klebsiella* and *Proteus* (Fig. 7).

Motility test

The isolates were screened for motility test. Brownian movement is shown by bacteria when observed under microscope.

Catalase test

All isolates were screened for Catalase test. Bubbles are produced which indicates breakdown of hydrogen peroxide by bacteria, indicating presence of *E. coli* and *Klebsiella* as shown in figure 8.

Biochemical characterization of bacterial isolates

Isolation of bacteriophage

All the plates showed positive for the isolation of bacteriophage, round, clear and transparent plaques are observed in the plates, indicating that the samples collected from different Ganga river contained bacteriophage that were infectious against Coliform bacteria (Vitaliano *et al.*, 2007). The phages produced clear plaques with well-defined edges in bacterial lawn, showing that the isolated phages have

lytic effect against coliform bacteria (Fig. 9).

Natural water bodies are extensively polluted with the life-threatening biological and toxic organic and inorganic pollutants. In India 70% surface water resources have already been polluted.

The current study has been taken up to isolate the bacteriophage of coliform bacteria from river Ganga, due to its holiness large number of people as well as pilgrim use the Ganga water for drinking and bathing purposes. The project aims to check the bacteriophage presence in Ganga River as increase in pollution of Ganga River directly affects the quantity of Bacteriophages as these are the viral indicators of coliform bacteria. The Ganga sample were processed and analyzed for their quality with respect to coliform bacteria and then its bacteriophages are isolated.

The results obtained from the microbial analysis of the Ganga River water signified the occurrences of the bacterial pathogens at the sites indicated fecal contamination in the holy river. The high load of coliform bacteria in the river sites is possible, as the human population in northern region has grown considerably due to presence of Ganga River, due to which the holy river became polluted. Bacteriophages present in River Ganga kill or infect bacteria which gave Ganga River its bactericidal affect and help Ganga River possess its medicinal qualities.

To isolate coliform bacteria and their bacteriophages various sample were collected from different sites of Northern Himalayas. These sample were processed and analyzed to detect coliform bacteria by microbial and biochemical methods and then plaque forming methods and general enrichment methods are employed for detection of bacteriophage of these isolated coliform bacteria.

Table.1 Sampling sites and date of collection

S.no	sampling code	Sampling Sites	Date of Collection
1.	G1	Badarinath	17.04.19
2.	G2	Devprayag	17.04.19
3.	G3	Uttarkashi	20.05.19
4.	G4	Rishikesh	17.05.19
5.	G5	Haridwar	22.04.19

Table.2 MPN value/100ml

S.No	Sample No.	Date of Processing	MPN Value/100ml
1.	G1	19.04.19	8
2.	G2	19.04.19	12
3.	G3	22.05.19	17
4.	G4	19.05.19	70
5.	G5	24.04.19	220

(Mackie & McCartney, *Practical Medical Microbiology*, 14th edition)

Table.3 Biochemical characterization of obtained isolates

Bacterial isolates	Gram stain	Indole Test	MR Test	VP Test	Citrate Test	Motility Test	Catalase Test
<i>E.coli</i>	-ve	+ve	+ve	-ve	-ve	+ve	-ve
<i>Klebsiella</i>	-ve	-ve	-ve	+ve	+ve	-ve	+ve
<i>Proteus</i>	-ve	+ve	+ve	-ve	+ve	-ve	-ve

Fig.1 Sample collection from various sites



Fig.2 MPN for coliform detection



Fig.3 Isolation of bacteria in nutrient agar medium



Fig.4 Isolation of bacteria in MacConkey agar medium

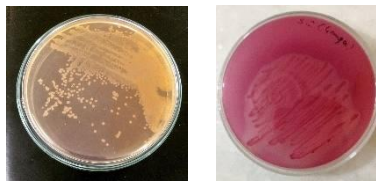


Fig.5 Gram staining of isolates

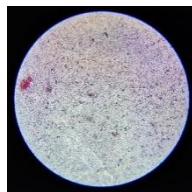


Fig.6 Indole test



Fig.7 Citrate utilization test

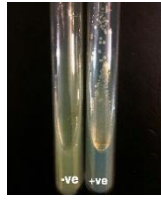


Fig.8 Catalase test

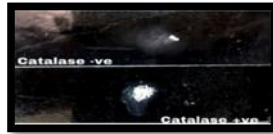


Fig.9 Isolation of bacteriophage from plaque assay method



These viruses help in cleaning of the holy River by eradicate the coliform bacteria. Other than that Bacteriophages are the alternative to antibiotics resistant bacteria as the evolution of pathogenic bacteria against commercially available antimicrobial agents has become a critical problem in modern era. Phage therapy could be used as potential alternative to antibiotics.

Despite unabated disposal of waste, river Ganga finds its own way to survive with its antimicrobial attributes due to the presence of bacteriophages. These Bacteriophages are viruses that infect bacteria that can be used as a potential tool for cleaning microbial based pollution from holy River Ganga as these viruses' combat bacteria without a harmful effect on the human body. In Ganga River, Bacteriophages were detected to be approximately 3 times more in proportion and the species of Bacteriophages which are present in river are unique giving the river its holy property. Bacteriophages are protein -

based biological agents (viruses) can potentially interact with the body's immune system. It can actively replicate and evolve after administration. Its higher replication rate has been also beneficial at the commercial production of phage. Bacteriophages have several characteristics that make them potential therapeutic agents i.e., they are highly specific and very effective in lysing targeted pathogenic bacteria, safe, and have ability to rapid modification to combat emergence of newly arising bacterial threats.

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