Analysis of increase in cell counts of *Escherichia coli* in groundwater of Rajasthan: Possible presence of VBNC cells

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Abstract
Escherichia coli is associated with the coliform group and is a more precise indicator of fecal contamination than other coliform bacteria; its existence indicates the possible presence of harmful disease-causing bacteria. A study is carried out to analyze the groundwater quality of the Rajasthan region under the BITS-UVA (University of Virginia) joint research project of costs and remediation of groundwater contamination in India. 1302 water samples were collected from 348 villages and cities during the year 2016-2019. Detection of E.coli bacteria in groundwater samples is performed using the culture-based method. No changes are observed in samples after 48 hours of the incubation period, but significant changes were observed in 99 water samples after 30 days of laboratory testing indicating the presence of E.coli bacteria with minimum cell counts of $4x10^2$ CFU/100ml and maximum cell counts of $132x10^2$ CFU/100ml.

Results show that there might be a possibility of viable but non-culturable (VBNC) E.coli cells present in groundwater samples that prevented the growth of bacteria and retained growth. Thereafter, sufficient conditions are achieved. It can be concluded that culture-based methods are not accurate for the detection of E.coli bacteria in water. Further research is needed to detect the VBNC cells of water-borne bacteria using sensitive, reliable and cost-effective methods. The study recommended that E.coli bacteria should not be used as an indicator organism when the cells are in a viable but non-culturable state.

Keywords: *Escherichia coli*, contamination, viable but non-culturable, groundwater, water quality.

Introduction

***E.coli*:** *E.coli* bacteria include gram-negative, non-spore, rod-shaped pathogenic bacteria that generate gas in the prescribed growth medium after fermentation within 48 hours at 35^0C[25]. In 1982, *Escherichia coli* was first recognized as a human pathogen[75]. It can be categorized into three classes of commensal, diarrheagenic and extraintestinal groups[83].

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The fecal coliform bacteria are Citrobacter, Enterobacter, Hafnia, Klebsiella and *Escherichia coli*, where *E.coli* is the most common bacteria that usually survive in the gastrointestinal tract of warm-blooded animals. Some bacterial strains are harmless like the commensal classes, but there are some infectious types[1]. Diarrheagenic strains may lead to diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, inflammatory colitis and dysentery diseases. Extraintestinal strains can cause urinary tract infections, septicemia and neonatal meningitis[21].

E.coli is a non-spore forming and rod-shaped bacteria with a diameter of around 0.5 µm and a length between 1.0 and 3.0 µm. *E.coli* bacteria are capable of surviving 4 to 12 weeks in water depending on the environmental conditions[56]. Fecal matter is the primary source of disease-causing agents in water and *E.coli* bacteria are commonly used as an indicator of water contamination that can impact rivers, sea beaches, streams, groundwater, surface water, natural water and the many varied activities associated with it[7]. According to WHO[87], USEPA[84] and IS 10500: 2012[13], *E.coli* bacteria shall not be detectable in 100 ml of the water sample.

VBNC: Laboratory-grown bacteria constitute only a minor part of the bacteria found in nature. It is found that on standard laboratory media, less than 1 % of environmental bacteria can grow[18]. The survival of microbial organisms depends mainly on their ability to exist in intimidating environments[9,31]. Bacteria should be able to withstand stress when environmental conditions are unfavorable and follow strategies that allow them to survive until sufficient conditions for growth are restored[9]. Clinical laboratories often grow enriched-media bacteria and are developed to upkeep the growth of specific pathogens. It is achieved by certain bacterial genera, for example, by evolving resistant structures such as endospores. While many bacterial cells enter a condition of deficient metabolic activity, it is generally called the viable but non-cultural condition (VBNC)[9,31].

Colwell and colleagues first described the VBNC condition in 1982[92].When bacterial cells can grow and form colonies on conventional culture media, they are said to be 'culturable' whereas if they are metabolically or physiologically active, they are 'viable'[22]. According to Oliver[68], "VBNC can be defined as a metabolically active bacterial cell that has crossed a threshold due to known or unknown causes and became unable to multiply in or on a medium that would normally support its growth". Under different stress

conditions, various bacteria including *E. coli* are known to enter a viable but non-cultural state (VBNC). Cells lose colony-forming units on Petri dishes during VBNC state while retaining the signs of viability.

Various environmental stresses like starvation stimulate the VBNC state. Bacteria in the VBNC condition cannot be grown on conventional media, usually escape plate count detection and pose a severe risk to drinking water safety and public health[67]; nevertheless, they maintain metabolic activity, respiration, membrane integrity and slow transcription of genes[14,28,32,67,76]. Despite the low metabolic rate of bacteria in this state, they may become culturable once again after specific resuscitation processes[65]. When exposed to adverse environmental conditions, many bacterial species use these conditions for long-term existence. Hence it can be recommended as a unique adaptation technique[36,73].

Review of Literature

The VBNC condition is defined as a state of dormancy in which certain bacterial strains may enter when encountered with severe environmental conditions[12,63]. Recent studies have shown that *E.coli* and certain bacteria may become viable but nonculturable (VBNC) under sublethal stress such as extreme temperature changes[10,12,14,22,64,73-76], starvation[12,14,22,45,58], high osmotic pressure[10,12,14,22,65], chlorine exposure[12,14,46], changes in pH[14,36,56], oxygen availability[38,39,53,60], heavy metals[54], or exposure to white light[14,22,55]. However, bacteria can resuscitate a culturable state under suitable conditions[13,16,24-27].

Apart from starvation, various severe environmental conditions such as changes in temperatures[34,35,44], salinity[37,60], nutrient scarcity[47], incubation outside the normal growth temperature range[48-50], osmotic pressure[51,52], UV radiation in combination with high salinity[57], low water availability[59], high concentration of copper[59] and severe environmental conditions[19] induced the VBNC state. Earlier studies showed the effect of temperature upshift with oxidative stress generation hindering the count of viable and culturable bacterial cells[40-43].

Such conditions could be lethal unless the organism has reached a VBNC state[22]. The non-culturability related to the VBNC state poses a possible problem to public health because of the methods commonly used to identify and count *E.coli* depending on culturing[23]. All non-pathogenic and pathogenic strains of *E.coli* have been shown to persist in sublethal conditions of environmental stress by entering the VBNC state[22,28-31]. Infectious bacteria, for example, pathogenic *E.coli,* is a crucial public health concern capable of entering a VBNC state[32]. Studies indicate that many pathogenic bacteria can persist and remain in pasteurized milk, processed food and drinking water, as well as in the environment[32]. There are various significant concerns regarding the involvement of cells in water in the VBNC environment, an example being that *E.coli* cannot be used as an indicator of fecal contamination when the cells are in VBNC state[33].

However, except for *E.coli* and *V. cholerae,* other pathogens such as *Aeromonashydrophila*[66,67], *Listeria monocytogenes*[48,68] and *Vibrio vulnificus*[69] are reported to have entered VBNC state[20]. Such pathogens present in the VBNC condition can easily evade testing by conventional plating methods while retaining or recovering toxic effects after achieving suitable conditions[49,70-72]. So monitoring of *E.coli* VBNC cells is particularly important in drinking water due to the possible transmission of pathogens in the distribution of water.

Leclair et al[34] studied the effects of time and temperature on the growth of *Escherichia coli* or *Listeria monocytogenes* and found that the storage type during transportation of water sample had a large and significant effect ($n2 = 0.70$, $p < 0.001$) on both pathogens. The refrigeration time also showed effect ($n2 = 0.43$, $p < 0.001$) on both pathogens. However, other factors like thawing and freezing did not show any effect on the pathogens ($p < 0.05$). The results showed that only *Listeria monocytogenes* bacteria were found to recover after 365 days of freezing. Tatangelo et al[82] studied the effect of different storage conditions on the structure of pathogens in the water sample.

The results showed that the concentration of bacteria with the Lifeguard solution was significantly lower than that of the water samples tested immediately after sampling. Lonsane et al[39] analyzed inoculated water samples for coliform counting by multiple tube dilution (MTD) and membrane filtration (MF) methods. The water samples were tested at different temperatures immediately after collection and after storage over a period of one and a half years. Growth of *E.coli* bacteria was observed at both room and refrigerated temperatures and was found to be decreased with storage time. Water samples inoculated with a pure culture of *E.coli* bacteria showed an increase in cell count when preserved at room temperature. The authors concluded that icing of the sample is essential to assess the extent of pollution.

Harmel et al[27] studied the effect of field storage time and temperature on *E.coli* concentration. The common holding time limit for *E.coli* bacteria was found to be 8 hours with a 10^0C storage temperature. Results showed that field preservation and temperatures had an effect on the growth of *E.coli*.

Pope et al[71] studied the effects of the sample holding time and storage conditions on *E.coli* concentration. Water samples were collected from 24 sites in three phases across the United States. Samples were analyzed for *E.coli* cell count at time 0, 8, 24, 30 and 48 hours after sample collection. Five out of seven sites showed no significant difference in *E.coli* concentration between 0 and 48 hours. Results showed that if samples are stored below 10^0C and

are not allowed to freeze beyond 8 hours of sampling, then it can generate *E.coli* counts. The study suggested that water samples be analyzed on the same day of sampling as soon as possible to reduce changes in bacterial densities.

Mason et al[46] studied the effect of time and temperature on *E.coli*. Water samples were collected from five sites across Hawkes Bay and analyzed for coliform counts for five days at 0, 24, 48, 72 and 96 hours after sample collection. IDEXX Colilert-18 test kit was used for the study. No noticeable difference was found in overall storage conditions from the initial zero-hour up to 72 hours. *E.coli* concentration showed a decrease in cells to 24 hours. This study is consistent with NIWA's work[83].

Lee et al[35] studied the effect of the incubation period on *E.coli* bacteria and observed to have a shorter mean incubation period of up to 12 hours. If this time is longer than one day or more, then there is a risk of other exposure in general. Jones et al[29] studied the behavior of *Escherichia coli*, cold-adapted log phase, exposed to different temperatures required for growth. Samples were incubated at a constant temperature of 2, 4, or 6^0C or temperatures permitted to rise from those temperatures to 10℃ at 6, 12 or 24 hours intervals and the spectrophotometer was used to calculate the optical absorbance value. Results showed that the minimum temperature for *E.coli* growth is around 7^0C. As temperatures rise from 6^0C to above 7 ℃ for < 45 min at an interval of about 12 hours, cells were able to divide.

In drinking water samples, McDaniels et al[47] examined the effects of holding time on bacterial growth. From August to December 1981, a total of 17 treated water samples from a public distribution system and 12 samples from January to May 1983 were collected. Standard analytical methods i.e. membrane filter, fermentation tube procedures and the pour-plate method were used for coliform counting and samples were analyzed at 0, 24, 30 and 48 hours and held at 5 and 22^0C.

Results showed that the plate counts of samples held at 22^0C during 30 and 48 hours increased slightly by 0.5 to 2.5 orders of magnitude and plate counts of the same sample held at 5^0C during 30 and 48 hours decreased.

Maier et al[42] compared the maximum holding time of 48 hours with a time of collection. Additionally, the acceptability of extending the holding time from 48 hours to 72 hours was investigated. No statistical correlation was found for all wild types between 0-48 hours and 48-72 hours.

Standridge et al[80] studied the effect of extending the storage time of water samples for coliform analysis for 24 hours at 4^0C and showed acceptable results. Results for 24 out of 28 samples were within 20% variation requirement of the study. Standridge et al[81] observed the effect of storage time on the bacterial concentration. Water samples were collected from the State of Wisconsin. Samples were stored at 20^0C for 24 and 48 hours and analyzed for the coliform count. The study showed that drinking water storage up to 48 hours had an impact on public health.

Dutka et al[20] studied the effect of storage temperature on the four parameters: total coliform, fecal coliform, fecal streptococcus and heterotrophic bacteria. In this analysis, the water sample was refrigerated instantly after collection by storage in crushed ice to around 1.5^0C. The sample was mixed at intervals of 2, 24, 30 and 48 hours and one subsample was selected and tested. Results indicated that 75 % of the samples analyzed were stable for at least 48 hours.

Water temperature, bacterial load and nutrient levels appear not to be consistent factors in sample preservation for bacteriological testing. Bacteria holding-time studies of up to 62 hours were conducted by Aulenbach et al[8] on five surface water samples collected from four sites near Atlanta, GA, USA with relatively high coliform bacteria densities. Membrane filtration and colilert were used to analyze water samples collected from urban streams with densities of bacteria > 126 CFU/100ml. It extended the processing time by as much as 62 hours.

Results showed that fecal coliform and total coliform remained consistent up to 27 hours while counts of *E.coli* remained consistent up to 18 hours. The results of *E.coli* differed slightly from those of the Pope et al study[71].

The Environmental Quality Commission adopted changes to the Oregon administrative rules on 11 January 1996, revising the State's water quality standards for bacteria[92]. In the rules, *E.coli* has been accepted as the indicator organism for microbiological water quality analysis.

Before this decision, bacterial standards were assessed using many organisms, including total coliform, fecal coliform and enterococci. The Department of Environmental Quality (DEQ) used a standard 30-hour holding time for the collection of water samples to test.

Material and Methods

Study area: Figure 1 shows the location of the study area. This study focused on costs and remediation of groundwater contamination in India with special reference to Rajasthan State. Groundwater samples were collected from 348 villages and cities in pre and post-monsoon season during the year 2016-2019. Water samples were analyzed for various physical, chemical and microbiological water quality tests in the laboratory.

These parameters are as follows; pH, total dissolved solids (TDS, mg/l), oxidation-reduction potential (ORP, mg/l), dissolved oxygen (DO, mg/l), electrical conductivity (EC, s/m), turbidity (NTU), fluoride (mg/l), nitrate (mg/l) and *E.coli* concentration (MPN/100 ml) determined in the laboratory using the titration and spectroscopy method.

Figure 1: Districts of Rajasthan state under study.

Water quality sampling and laboratory testing: The groundwater samples were collected from eight districts of the State of Rajasthan, India, under the BITS-UVA (University of Virginia) groundwater contamination project, containing 1302 water samples that are used in this study. Microbiological water quality analysis is done to identify the bacteria present in water using the gram staining culturing method. After identification, a viable count of *E.coli* bacteria is done to count the number of actively growing bacterial cells in terms of colony-forming units (CFU). The laboratory testing is carried out at Environmental Engineering Lab, Department of Civil Engineering, BITS Pilani, Rajasthan.

Present/Absent test (PA test): Detection of the presence or absence of *E.coli* bacteria in groundwater samples is performed. 100 ml water to be tested is added to the sterile disposable bottle and then powder medium (PA broth) is added to the water by swirling to completely dissolve the powder. Once dissolved, incubate the bottles for 24-48 hours at 35 ° C. Observe the transition in color of the medium from reddish-purple to yellow, indicating the presence of *E.coli*.

Identification of bacteria: The most significant bacteriological task is to classify the water-borne pathogens. Generally, bacteria display three basic shapes: round, rod-shaped, spiral. After the collection of water, bacteria have to be grown on culture media to be identified. Staining is the first step towards identifying bacteria. Gram staining is a common technique for the differentiation of bacteria in the cell wall based on their different constituents. Through coloring these cells in violet or red, the gram stain technique differentiates between gram-positive and gram-negative groups. Agar is a growth medium that is used for selective identification and differentiation of *E.coli* in water. Figure 2 shows a Petri dish containing *E. coli* bacteria.

Viable count: After the identification of bacteria, a viable count of *E.coli* bacteria is done to count the number of actively growing bacterial cells in terms of colony-forming units (CFU), it is a microscopically visible grouping of millions of bacteria from one single bacterial cell. The plate count method is used in which serial dilution of the water sample is done to count the number of bacterial cells present in water. The digital colony counter is used to count the number of colonies of bacteria on a Petri dish. The number of cells of *E.coli* bacteria presents per 1 ml of the water sample is then given by:

CFU/ml = Number of colonies * dilution factor

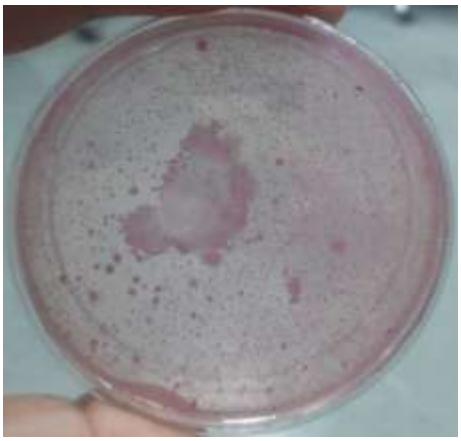

Figure 2: Petri dish containing *E. coli* bacteria

Results and Discussion

According to laboratory-based culture methods, 12-48 hours are required for bacteria to be reported. After plating, water samples are kept in an incubator at 35° C for 48 hours. The time of incubation depends on the organism and medium of growth, but every viable cell that has been spread on the Petri dish containing agar must grow and divide several times during the incubation to form a detectable colony of microorganisms. The growth of the bacteria is observed after 12, 24 and 48 hours of incubation. No changes are observed in any water sample after 48 hours of incubation. The sterile disposable bottles are stored in the laboratory under room temperature for preservation after analysis. After 30 days of water testing for detection of presence or absence of *E.coli* bacteria, changes are observed in 99 samples as colour change of the medium from reddish-purple to yellow, indicating the presence of *E.coli.*

According to WHO, USEPA and IS 10500: 2012, *E.coli* bacteria shall not be detectable in 100 ml of the water sample. The viable count analysis of water samples shows the presence of *E.coli* with minimum cell counts of $4x10^2$ CFU/100ml and maximum cell counts of $132x10^2$ CFU/100ml. It indicates that there may be a possible presence of viable but not culturable (VBNC) cells of *E.coli* induced by diverse environmental stresses that restricted the growth of bacteria under controlled laboratory conditions. When samples are kept at room temperature under anaerobic conditions, the bacterial cells become culturable once again after specific resuscitation protocols.

Both biotic and abiotic factors such as starvation, exposure to chlorine, pH, oxygen availability, heavy metals, exposure to white light, temperature changes, salinity, nutrient scarcity, incubation beyond normal growth temperature

range, osmotic pressure, copper, harsh environmental conditions, nutrient scarcity and many other factors induced the VBNC state. Specific parameters such as the method of storage, holding time and temperature also showed an influence on the concentration of *E.coli*.

Conclusion

It can be concluded that culture-based methods are not accurate for the detection of *E.coli* bacteria in water. Further research is needed to detect the VBNC cells of bacteria in water. *E.coli* entering the VBNC condition could have a detrimental effect on public health. The number of viable cells could be underestimated and at any time, the VBNC cells could still produce toxins or be resuscitated to become virulent again and again.

Various studies have found that resuscitation of *E.coli* post-VBNC may be possible. Some pathogenic *E.coli* strains can produce toxins in VBNC condition, while others are non-toxic, but are capable of regaining virulence after regeneration. The results showed that the units forming the colony grew over time. The cell wall of *E.coli* remained intact after one month of laboratory incubation.

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