PROGRESS REPORT

APPLICATION OF NOVEL METHODS FOR ROUTINE TESTING OF POTABLE WATER SAMPLES FOR BACTERIAL CONTAMINATION

INTRODUCTION

Water plays a vital role in human life. The purpose of water supply distribution system is to deliver safe potable water which is also adequate in quantity and acceptable in terms of taste, odor and appearance. A significant proportion of the world's population use potable water for drinking, cooking and personal and home hygiene (WHO 2004). However in the past decade there has been a growing concern among the general public with respect to the safety and aesthetic qualities of potable water supplies (Geldreinch 1996). Before water can be described as potable, it has to comply with certain physical, chemical and microbial standards which are designated to ensure that the water is potable and safe for drinking (Ihekoronye and Ngoddy, 1985). The lack of safe drinking water and adequate sanitation measures lead to a number of diseases such as cholera, dysentery, Salmonellosis and typhoid. Water that physically looks colorless, odorless and even tasteless is not sufficient to determine that the water is safe for consumption. In fact the drinking water should be examined on microbiological and physicochemical quality.

Potable water released into the distribution system becomes altered during its passage through pipes, open reservoirs, standpipes and storage tanks. Transient negative pressure and pipeline leak events provide a potential portal for the entry of ground water in treated drinking water and permit fecal indicators and microbial pathogens present in the soil and water exterior to enter the distribution system (LeChevallier et al. 2003).

The quality of water for drinking has deteriorated because of inadequacy of treatment plants and if inefficient management of the piped water distribution system (UNEP 2001). Bacteria may enter the distribution system through the failure to disinfect water to maintain a proper disinfection residual; low pipeline leakages; corrosion of parts; and inadequate sewage disposal (Lee & Schwab 2005).

Ideally primary water should not contain any microorganisms known to be pathogenic to any bacteria indicative of faced pollution. Detection of fecal indicator bacteria in drinking water provides a very sensitive method is quality assessment (WHO 1993). Over a billion people in India lack safe water. 80% of infectious diseases are water borne, killing millions of children each year. Several parts of India are facing immune challenges to meet the basic needs of safe water. These arises an urgent need for understanding the status of drinking water quality, the related problem and also the reason of the problem (Tambekar and Banginwar 2005).

The principle objectives of municipal water are the production and the distribution of safe water that is fit for human consumption. Thus, regular analysis of water at source must be carried out to determine the effectiveness of treatment process. Therefore this work is undertaken to evaluate the bacteriological quality of different water sources and hygienic practices of Mysore city.

Water has a major impact on the quality of life. However, in both developed and developing countries water quality continues to be a major health concern. Diarrheal diseases traceable to contaminated water worldwide has been estimated to cause 900,000,000 episodes of illness per year and approximately 2,000,000 deaths per year in children (World Development Report 1992).

Waterborne disease outbreaks are rising due to increasing vulnerable populations, political upheaval and high numbers of refugees in developing countries.

WATERBORNE PATHOGENS AND THEIR HEALTH EFFECTS

While waterborne diseases are typically considered to be problems of underdeveloped countries with inadequate sanitary practices, there is increasing recognition that industrialized, developed countries also have significant public health problems caused by use of untreated, partially treated, or inadequately treated domestic water supplies. The incidence of major outbreaks of the classical waterborne bacterial diseases, such as typhoid fever and cholera, has become very low in India since the initiation of chlorination of domestic water supplies. However, outbreaks of waterborne microbial diseases still occur as a result of consumption of untreated, inadequately treated, and conventionally treated domestic water supplies.

Outbreaks of waterborne disease are not currently reported in the Mysore. This makes it very difficult to track down the trends of emerging trends of water related diseases in the region. Contrarily, there is plenty information on water related diseases which indicate the trends of emerging pathogens.

Among the pathogens disseminated in water sources, enteric pathogens are the ones most frequently encountered. As a consequence, sources of fecal pollution in waters devoted to human activity must be strictly controlled. Entero-pathogens, such as *Escherischia coli* O157:H7, are generally present at very low concentrations in environmental waters within a diversified microflora. Complex methods are required to detect them, and these are extremely time-consuming.

coliforms

Most coliforms are present in large numbers among the intestinal flora of humans and other warm-blooded animals, and are thus found in fecal wastes. As a consequence, coliforms, detected in higher concentrations than pathogenic bacteria, are used as an index of the potential presence of entero-pathogens in water environments. The use of the coliform group, and more specifically *E. coli*, as an indicator of microbiological water quality dates from their first isolation from feces at the end of the 19th century. Coliforms are also routinely found in diversified natural environments, as some of them are of telluric origin, but drinking water is not a natural environment for them. Their presence in drinking water must at least be considered as a possible threat or indicative of microbiological water quality deterioration.

The coliform group includes a broad diversity in terms of genus and species, whether or not they belong to the *Enterobacteriaceae* family. Most definitions of coliforms are essentially based on common biochemical characteristics.

Health effects of Coliforms

Most coliform bacteria do not cause illness. However, their presence in a water system is a public health concern because of the potential for disease-causing strains of bacteria, viruses,

and protozoa to also be present. Waterborne disease from these organisms typically involves flu-like symptoms such as nausea, vomiting, fever, and diarrhea.

Microorganisms in the distribution system

Coliform bacteria do not occur naturally in the environment. The first barrier required to prevent microorganisms from entering drinking water is protection of the water source. Effective water source protection, including the construction of head works and the control of land use within the catchment or recharge area, will greatly reduce the numbers of pathogenic microorganisms in source water. This in turn reduces reliance on treatment processes to ensure water of acceptable quality. In many situations where groundwater is used, source protection measures can be designed to largely prevent contamination by pathogens.

Water treatment processes are capable of reducing heterotrophic microorganisms to less than10 cfu/ml, although waters from most treatment works typically contain higher numbers. Some viable organisms remaining in the water will multiply if nutrients are available, especially in waters that are above 15°C, and may lead to the formation of biofilms on internal surfaces. Biofilms typically contain numerous free-living heterotrophic bacteria, fungi, protozoa, nematodes and crustaceans. Older systems may contain deposits and sediments formed by the internal corrosion of metal pipes and insufficient water treatment; they may also contain many microorganisms. The multiplication of bacteria in a piped distribution system is driven by the availability of organic and inorganic nutrients in the conveyed water and in surface deposits.

MATERIALS AND METHODOLOGY

Study area

Mysore is located at 12°18′N 76°39′E12.30°N 76.65°Eand has an average altitude of 770 metres (2,526 ft). It is situated in the southern region of the state of Karnataka, at the base of the Chamundi hills and spreads across an area of 128.42 km^2 (50 sq mi). Mysore city is highly populated as compared to the other cities of Karnataka. Equipped with all the modern infrastructural amenities, Mysore offers its literate citizens with several opportunities to earn their livelihood. The present population of Mysore city is 1.1 million. The water demand is 135 Liters per Capita per Day (LPCD) and the water supplied is around 127 LPCD. The main sources of water to the Mysore city is river (Krishna Raja Sagara) Kauvery and the ground water. The water distribution in Mysore city includes 4 stages namely, Belagola 1st and 2nd stage, Hongalli 2nd stage, Hongalli 3rd stage and Melapura 1st & 2nd stage as shown in picture 1.

Name	**Available**
Belagola 1st stage	3.50 MGD
Belagola 2nd stage	6.40 MGD
Hongalli 2nd stage	6.50 MGD
Hongalli 3rd stage	12 MGD
Melapura 1st & 2nd stage	15 MGD
Bore wells	1.00 MGD

Name	**Nos**	**Capacity**
HLR – Yadavagiri	3 Nos	5 MGD
CSR – Vijayanagar	1 Nos	12 MGD
German press reservoir	1 Nos	2.50 MGD
Kuvempu nager	2 Nos	2.50 MGD
Devnuru	1 Nos	2.50 MGD

And

Over head tanks	20 Nos
Hand pump borewells	855 Nos
Gravity mains	1141 Km

Majority of water distribution is from over head tanks (OHT) and ground level reservoirs. Borewell water is distributed directly without treatment.

The raw water is treated in all the 4 stages and supplied to ground level reservoirs. There the water is again disinfected by chlorination and distributed to overhead tanks and to the consumer's points. The combinations of processes like pre-treatment, flocculation, sedimentation, rapid sand filtration and chlorination are used for municipal water treatment. But the bore-well water is distributed directly without the treatment. Both the surface and bore well water is supplied daily to the City but in many places the water is supplied on alternate days.

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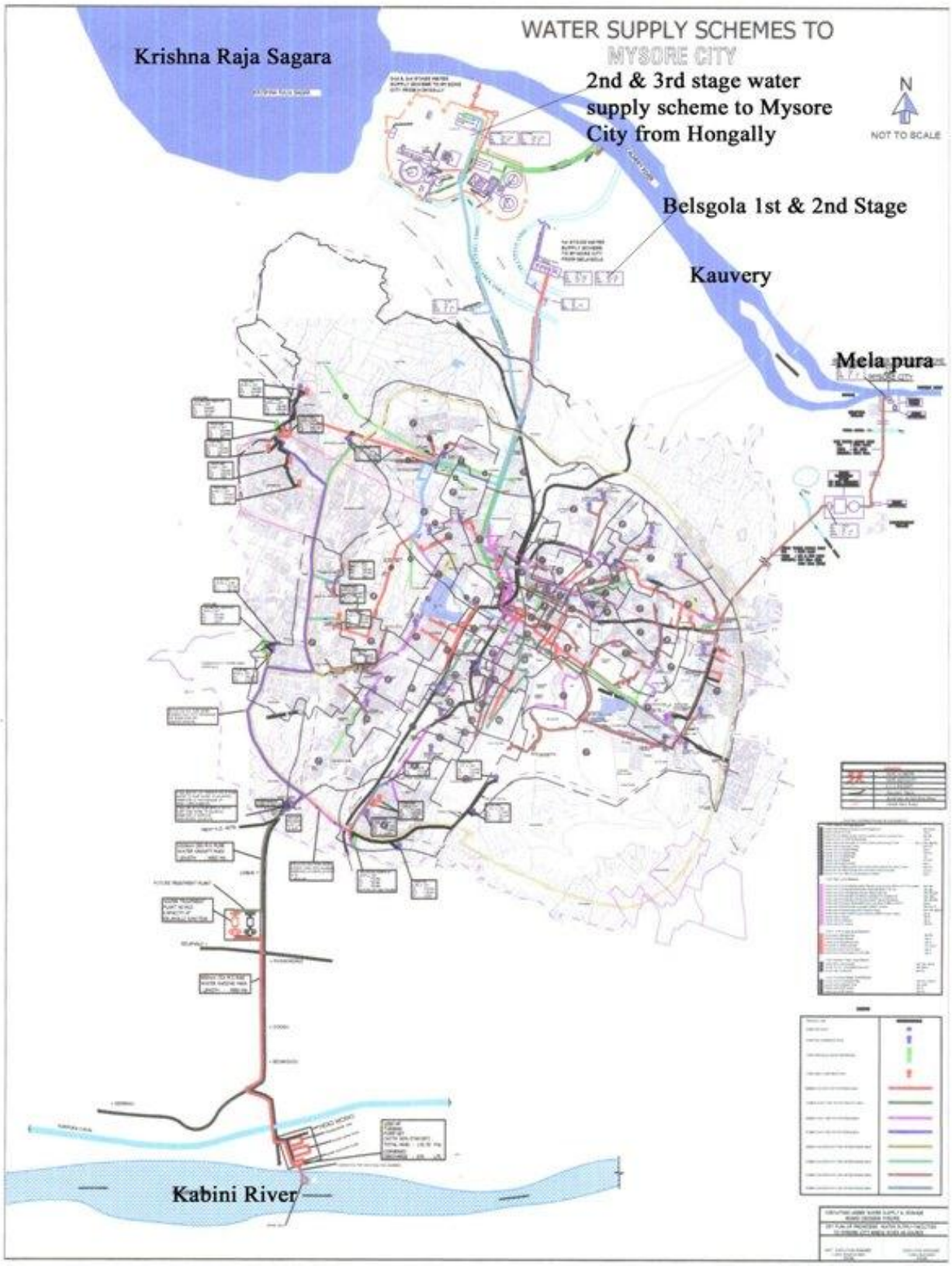

WATER SUPPLY SCHEMES TO
MYSORE CITY
Krishna Raja Sagara
2nd & 3rd stage water
supply scheme to Mysore
City from Hongally
N
NOT TO SCALE
Belsgola 1st & 2nd Stage
Kauvery
Mela pura
Kabini River

Sample collection

Sample site selection

Samples were taken from locations that were representative of the water source, treatment plant, storage facilities, distribution network, points at which water was delivered to the consumer, and points of use. In selecting sampling points each locality were considered individually and the following procedure were followed.

- Sampling points were such that the samples taken are representative of the different sources from which water is obtained by the public or enters the system.
- These points include those that yield samples representative of the conditions at the most unfavorable sources or places in the supply system, particularly points of possible contamination such as unprotected sources, loops, reservoirs, low-pressure zones, ends of the system etc.

Sampling points which are representative of the different sources from which water is supplied by public or system are selected. While sampling, size of the population is taken into account. The majority of samples were taken in potential problem areas like reservoirs, dead ends and poor hygienic zones. Samples are also collected from the storage system over head tanks, reservoirs.

Drinking water samples of tap, bore well and stored household water samples were collected at different distribution and consumer points. Residences located within the urban areas of Mysore city were choosen as the only criteria to participate in the study.

Sampling Procedure of Drinking Water

Plastic autoclavable bottles of 250 ml capacity were used for sampling. The clean bottles were added with 4-5 drops of aqueous Sodium thiosulphate solution (100g/lt) which immediately inactivate the chlorine residues and not the bacteriologic content of the water and a brown paper & aluminum foil cover was tied to the neck of the bottle. Then the bottles were autoclaved at 121^0C for 20 minutes.

While sampling from tap and borewell, the outlet was cleaned using alcohol soaked cotton and the tap was turned on to run at maximum flow for 1-2 minutes. The house hold water samples were taken from the drinking water storage vessel used by the family and containers used for collecting and transporting the water. A random sampling of the household water samples were carried out. The bottle was unscrewed the cap and immediately held under the water jet and filled with water sample. The bottles were recapped and placed in 4^0 C cooling boxes and transported to the laboratory & processed within 6 hrs of collection.

Traditional methods like multiple tube fermentation technique, Heterotrophic plate count, Presence and absence test etc. have been employed for the analysis of water samples.

Microbial examination of water samples

The samples collected in replicates were analyzed for microbiological quality according to the standard method.

Multiple tube fermentation technique.

The three basic tests to detect coliform bacteria in water are presumptive, confirmed and completed tests. The tests were performed sequencially on each sample under analysis.

The presemptive test is specific for detection of coliform bacteria. Measured aliquotes of the water to be tested were added to a single strength and double strength lactose fermentation broth containing an inverted gas vial. Tubes of this lactose medium were inoculated with 10-ml, 1-ml and 0.1-ml aliquots of the water sample. The series consists of at least three groups, each composed of three tubes of the specified medium. The inoculated tubes of lactose broth with water samples were incubated at 37^0C for 48 hrs. The tubes were examined for gas formation. Any gas observed was noted as a positive presumptive test for coliform organisms. The results were reported as MPN/100 ml.

The confirmed test was performed by using the selective and differential media such as EMB, Endo agar, McConkey agar and XLD agar media. The Positive presumptive tubes were streaked on differential agar media and incubated at 37^0C for 24 hours. The plates were then observed for the presence of coliform colonies.

The completed test was the final analysis of the water sample. It was used to examine the coliform colonies that appeared on the media used for confirmed test. The colonies isolated colony picked from confirmatory test plate and inoculated into a tube of Brillient green lactose bile broth containing inverted gas vial and incubated at 35^0C for 48 hrs. Following the incubation, tubes showing acid and gas in the lactose broth are indicative of a positive completed test. Further the gram staining was carried out by gram staining method.

HPC technique

The total plate count was conducted by heterotrophic plate technique on heterotrophic plate agar (HPA). HPC technique was performed by pour plate method by using Tryptone glucose extract agar media and the petriplates containing aliquots of water sample with the media were incubated at 37^0C for 24-48 hrs and all the colonies with different characteristics on specific media were identified on the basis of their colonial, morphological and biochemical properties.

Presence - Absence Test.

This test is based on the principle that coliforms and other pollution indicator organisms should not be present in a 100 ml water sample. In this test 50 ml of triple strength Presence absence broth was dispensed into screw cap autoclavable 250 ml plastic bottle and autoclaved at 121^0C for 20 minutes. The media was inoculated with 100 ml of water sample and incubated at 35^0 C for 24 – 48 hours and observed for production of gas. Any amount of gas and acid production was the indicative of positive P-A test.

Microscopic observation

Gram staining

Loop full of culture was spread onto the clean slide, air dried and heat fixed. The slide was then flooded with the crystal violet for one minute and washed with water. Gram's Iodine was gently flooded and let stand for 1 minute and rinsed with water. The slide was decolorized using 95% ethyl alcohol and immediately rinsed with water. The slide was then flooded with safranin

counterstain allowed for 1 min and washed with water, air dried and observed under the microscope.

Scanning Electron Microscopy (SEM)

1ml of bacterial culture broth was centrifuged at 8000 rpm for 10 minutes; the supernatant was discarded, and the cell pellets were washed with phosphate buffer (0.1M) for three times. One ml of 2.5% glutaraldehyde (in 0.1M Na-phosphate, pH 7.2) was added to the pellet, and all cultures were incubated overnight at -40C. The cell pellet was collected by centrifugation & washed with 1 ml 0.1M Na- phosphate buffer for three times (Each time centrifugation was done). Dehydrolysation of the sample was carried out by washing in different percent of ethanol varying from 30% -100%. The cell pellets were stored in 100% ethanol until the use. The cells were observed in SEM (LEO435VP, Cambridge, UK)

Biochemical Identification of Coliforms

Readily available biochemical kit (Himedia, Mumbai) was used. The wells were inoculated with 50 µl of 24 hr old culture by the surface method and incubated at 35° ± 2°C for 18-24hrs. Results were interpreted as per the instruction given in the result interpretation chart.

Antibiotic susceptibility testing.

Antimicrobial Susceptibility Testing has performed accordance with the Clinical Laboratory and Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing by standard Kirby-Bauer disc diffusion technique. Suspensions of *E. coli* strains grown in Tryptone Soya Broth were made in sterile 1x phosphate buffered saline (PBS) pH 7.2 to match a 0.5 McFarland Standard to achieve an inoculum density of approximately 1 x 108 Cfu/ml. *E. coli* from the standardized suspensions were inoculated on the Muller Hinton agar using sterile, nontoxic cotton swabs. Antimicrobial disks were placed on the surface of the inoculated plates.

Plates were incubated at 35^0C for 18-24 hours and zones of inhibition were measured in millimeters. The commercially available antibiotic discs (Hi-Media, Mumbai, India) used in the study were (potency in µg/disc): Amikacin (AK10), Ampicillin (AMP 25), Ampicillin (AMP 10), Aztreonam (AT 30), Cefotaxime (CTX 30), Cephalothin (CEP 30), Ciprofloxacin (CIP 5), Chloramphenicol (C 30), Erythromycin (E15), Gentamycin (GEN 10), Kanamycin (K30), Nalidixic Acid (NA 30), Nitrofurantoin (NIT 300), Nystatin (NS 50), Oxytetracycline (O30), Penicillin (P 2), Streptomycin (S 10), Tetracycline (TE30), Tetracycline (TE30), Trimethoprim

(TR 5), Vancomycin (VA 30). Minimum inhibitory concentrations (MIC) of antibiotics were determined by Antibiotic Zone Scale-(Hi-Media, Mumbai, India). An isolate was designated as antibiotic resistant (AR) if it was resistant to at least one of the antibiotics tested and multiple antibiotic resistant (MAR) if it was resistant to at least two antibiotics tested.

RESULTS AND DISCUSSION

A total of 226 drinking water samples were collected from different sources such as bore well water (hand pump), house hold tap water, house hold stored water from all over the Mysore city. Microbial analysis of all the water samples reveled that out of 226 samples, 80 samples were contaminated with enteric bacteria. The analysis of source wise contamination revealed that 20% of tap water samples were contaminated followed by bore well water 11% and stored house hold water 73% were contaminated with enteric bacteria. The greatest average concentration of HPC bacteria were observed in water collected from stored house hold water which exceeded 100 cfu/ml. The HPC bacteria of tap water samples of poor hygienic zones were more compared to the other water samples of same source which indicates the hygienic condition of the surrounding.

Totally 232 isolates of enteric bacteria were isolated and identified of which *E.coli* 61, *Salmonella* 21 and *Klebsiella* 76 & *Citrobacter* 74. The source wise analysis of bacterial isolates reveled that highest percentage of total Enterobacteria were present in stored water followed by tap and bore well water (fig 1). Further, source wise water sample were also analyzed to identify the different species of enterobacter and is given in the fig 2. It was found that *Klebsiella* and *Citrobacter* were found mostly in stored house hold water, compared with tap water samples where *Citrobacter* was the major enteric bacteria. In bore well water (hand pump) *Klebsiella* was detected. Among 232 isolates of enteric bacteria were identified of which 26% of E.Coli, 33% of Klebsiella, 32% of Citrobacter and 9% of Salmonella were isolated.

Biochemical Tests

All the isolates were Indole +ve, Methyl red +ve, Voges crossover –ve, and Citrate utilization –ve.

Microscopic observation.

All the isolates were rod-shaped and gram-negative when viewed under Microscope.

Hand pump water recorded zero level of *E. coli* count and the presence of coliforms in groundwater indicates that microorganisms from surface water have been able to reach the aquifer (Payment, 2011). The contamination of the household water was significantly greater when the bacteria count of source water was low. According to the drinking water standards, the bacteriological content of drinking-water leaving treatment plants should contain only very low levels of heterotrophic microorganisms. The coliform contamination in the few tap water samples but not in all the samples indicates the cross contamination of the water in the distribution system due to leaky pipes or the organic and inorganic nutrients present in pipeline supporting the bacterial multiplication. Most of the microorganisms developing within the distribution network are harmless except *Legionella* and *Mycobacterium avium* complex (WHO). And also the total coliform counts are not necessarily a measure of fecal pollution. Because it has other species of the four *Enterobacteriaceae* genera, *Escherichia, Klebsiella, Enterobacter* and *Citrobacter* which give positive coliform results (Cabral 2010), and these organisms are of less health concern comparable with *E. coli*. In the study though tap water had coliform contamination, few of the samples were detected with *E. coli*. Stored household water quality depends on the source, but with a comparison with the point of use the stored household water has a high density of bacterial contamination and the coliform contamination was above the WHO recommended limit. The low level of residual chlorine of both stored household water and tap water indicate the loss of chlorine level as the water travels in the pipeline to a distance till the point of use and increase in the storage duration. Free chlorine is unstable in aqueous solution, and may decrease rapidly, particularly at warm temperatures and exposure to intense light or agitation. Therefore the water samples should be analyzed for free chlorine immediately on sampling and not stored for later testing. Hence, when water leaves the treatment plant residual, free chlorine of about 1 mg/l is needed for health reasons, and such level should be maintained at points of consumption (Mamba *et al.,*2016).The lack of detectable levels of chlorine residuals in stored household drinking-water compared with piped water leads to the post-contamination. The presence of significant counts of coliforms in stored household water indicates post poor sanitation and existence of human activities. The temperature of the stored water was nearly 30^0C; the high temperature can also favor the growth of organisms in water resources (Muyima N, Ngcakani F, 1998)

Several previous reports were stating on the poorer bacteriological quality of stored household water than water from the source (Dissanayake *et al.,* 2004; Tabor *et al.,* 2011; Tambekar *et al.,*2006). In the study conducted in Ethiopia indicated that the number of total coliforms in household containers was higher compared to tap water (Agnew *et al.,*2007).Other studies are reporting on; higher compliance for piped water than from household water containers (Mamba *et al.,*2016). However, post water quality can be improved by promoting better water handling, storage, and treatment.

Antibiotic susceptibility testing.

Among the 15 *E. coli* isolates tested for antibiotic susceptibility, bout 54 % of *E.coli* were resistant, 16% of *E. coli* had intermediate and 30% susceptibility to the antibiotics used. The highest level of resistant shown by *E. coli*isolates was to Nystatin, Cephalothin, Cefotaxime, Penicillin, Ampicillin 10, (100%) followed by Vancomycin (93%),Aztreonam (93%) and Streptomycin (87%). There was a significant difference in the diffusion zone among the isolates. Chloramphenicol (C30) (100%) and Tetracycline (TE30) (53%) were active against most of the *E. coli*isolates with a large zone of inhibition. The isolates HS1, HS12, and NS1 from the household stored water showed 76%, 71%, and 71% resistance to the different antibiotics respectively. 100% resistant to more than nine antibiotics namely AMP10, CTX30, CEP30, NS50, E15, NA30, O30, AT30, CIP5, K30, P, S10, TE30, VA30, and AMP25. However, there were wide variations in the susceptibility between *E. coli*species of this study. *E. coli*is one of the most common pathogens of *Enterobacteriaceae* family responsible for nosocomial infections (Atul Khajuria, 2014). Several groups have reported the presence of antibiotic resistant *E. coli*isolated from environmental samples like sewage, ***waste water, urine samples,*** nursing samples (Urine and Feces) from human and animals, under grown water, surface water, drinking water at distribution system and point of use.Although several studies have detected ABR in drinking water systems (Armstrong *et al.,* 1981; Armstrong *et al.,*1982; Schwartz *et al.,* 2003; Zhang *et al.,* 2009).

Little is known about the fate of ABRs in stored household drinking water.The frequency of *E. coli*resistance to various antibiotics from different environmental samples have been extensively studied such as, Oxytetracycline and Cephalothin resistant (Kerketta *et al.,* 2015), Kanamycin and Tetracycline, tetracycline, ampicillin and erythromycin and susceptibility of a few isolates to streptomycin and kanamycin. In the study carried out by Paoli *et al.,* 2010, the isolated *E.coli*

from drinking water were resistant to nalidixic Acid (92.6%), ampicillin (88.89%), ciprofloxacin (37.04%), cefotaxime (18.52%), gentamicin (18.52%) and susceptibility to amikacin.In the present study, the percentage of resistant by the *E. coli*isolate is more towards more than five antibiotics namely, AMP10, CTX30, CEP30, NS50, P2 with 100%, AT30 (93%), S10 (86%) VA30 (93%) and K30 (60%).The emergence of resistance and decreasing levels of susceptibility of *E. coli*to a wide spectrum of antimicrobials is a matter of concern because it resistant (Satish *et al.,* 2004, Cernat, 2007) in drinking water, Trimethoprim resistant from natural water source (Park *et al.,* 2003), resistance to tetracycline, Cephalothin and streptomycin from live stacks and wildlife (Sayah *et al.,*2005), Cefotaxime resistance *Enterobacteriaceae* in hospital wastewater and activated sludge (Schwartz, 2003).In another study by Liu, 2013, found that the changes breakpoint in the CLSI guidelines resulted in higher resistance rates to cefotaxime**. *E. coli***isolated by Reinthaler and coworkers from sewage plant were resistant to several antibiotics such as ampicillin, piperacillin, cephalothin, cefuroxime, nalidixic acid, tetracycline, and sulfamethoxazole. A similar elimination rate was found for ciprofloxacin by With a n*et al.,* 2001. Walia *et al.,*2004 isolated two strains of *E. coli*resistant to six and one strain to seven antibiotics from drinking water. *E. coli*isolated from tap water by WoseKinge, *et al.,* 2010, was observed for 50%-90% resistance to chloramphenicolmay limit the availability of antimicrobials for clinical management of waterborne outbreaks in the future (Ram *et al.,* 2008). Routine monitoring of antibiotic resistance in pathogens provides data for antibiotic therapy and resistance control (O'Brien, 1997). The presence of antibiotic resistant E. coliin the sewage and its cross contamination with source and the distribution system through the pipeline is common, but contamination of household drinking water are purely on the hygienic practices of the individual when the tap water is without any contamination. Numerous studies have documented inadequate storage conditions and vulnerable water storage containers as factors contributing to increased microbial contamination and decreased microbial quality compared to either source waters or water stored in improved vessels (WHO, 2003).

The main object of our study was to evaluate the potability of water at different stages starting from the source till the storage in houses of Mysore city. Since the Mysore is fast growing city in Karnataka and the water consumption is increasing tremendously. People often collect and store water in large containers, increasing contamination events. Majority of diseases in developing countries are infectious diseases in nature caused by bacteria, viruses and other

microbes, which are shed in human faeces and pollute water supplies which people use for drinking and washing purposes. Unsafe water supply is a major problem and faecal contamination of water sources and treated water is a persistent problem worldwide (WHO, 2002).

Microbial contamination of water between source and point-of-use is widespread and often significant. Increased faecal and total coliform counts in household stored water container are generally high even when the source water is of good quality suggesting that contamination is widespread during collection, transport, storage and drawing of water (Write et al., 2004).

Many environmental strains of coliform bacteria such as *Citrobacter, Enterobacter and Klebsiella* may also colonise distribution systems (Martin et al. 1982). However it is generally agreed that water temperatures and nutrient concentrations are not elevated enough to support the growth of *E. colli* in biolofilms (Geldreinch and Lchevalier, 1999). Thus the presence of *E. coli* should be considered as evidence of recent faecal contamination of drinking water.

The present study showed contamination of majority of stored house hold water samples than the tap and bore well water. This indicates that lack of proper hygienic practices and types of container which play an important role on the quality of drinking water. The contamination may be due to large handling of the same storage water and withdrawing utensils as there are chances of water contamination with fingers or dirty dipper or glass wile withdrawing the water. Thus, fingertip-dipping may have resulted in the water becoming contaminated through contact with hands. Other studies have also found a similar deterioration in water quality between the source of drinking water and point of consumptions/use (Lindskog and Lindskog, 1988; Genthe and strauss, 1997). Thus contamination of drinking water after collection from the source may pose another risk for contracting diarrheal disease.

The most coli-forms are present in large number among intestinal flora of humans and other warm-blooded animals and are thus found in fecal wastes (Dorothy and Philip 1998). As a consequence coli-forms detected in higher concentration than pathogenic bacteria and used as an index of the potential presence of entero-pathogenic in water environments (Rompre et al. 2002). Coli-forms are also routinely found in diversified natural environment as some of these are telluric origin, but drinking water is not a natural environment for man. As a result their

presence in drinking water must be considered as harm to human health. Positive presence of coliforms in treated which is usually coliform free may indicate treatment infectiveness. A regular monitoring the water quality for improvement not only prevents disease and hazards but also checks the water resource from going further polluted (Trivedi and Goel 1986). From any study it is evident that contamination of stored water is more it could be attributed to the lack of proper sanitation and hygienic condition. Since the water samples were collected mainly from stress and areas of low income in addition that tap water samples were also contaminated since the Mysore city water supply system is the old hence these pipe-line may be corroded and sometimes damaged. Hence there is urgent need to replace the old pipe lines. Contamination of bore well (hand pumps) could be attributed to the percolations of drainage water to the underground.

The present data suggests that the water leaving the treatment plant meets bacteriological standards; the presence of coliforms indicator bacteria in some of the tap water samples and the stored household water samples suggests that the water is becoming contaminated in the journey through the distribution, plumbing systems (Cristobal et. al. 2008) and the poor hygienic storage practices. The periodical faecal pollution of container-stored water can be assaocated with poor personal hygiene practices as well as unhygeinci domestic environments (Mintz. et. al. 1995). The study recommended also that container with tap, long or short handle dipper used for withdrawal of water from storage container proper lid, daily washing of container, avoid addition of fresh water in residual water or residual water from dipper into stored water are few remedies to control transmission of contamination (Tambekar D.H. et. al. 2008). It is important to keep the source point clean so as to avoid the storage contamination and improvement in behavioral and water hygiene practices can improve the household water quality. Individual sanitary behavior, i.e. hand washing with soaps and use of clean vessels, also has an important role. Hygiene is key to improved health. People can protect themselves from diarrheal diseases and other infections if they are provided with the appropriate information and if they are encouraged to change their behavior. This may reduce the prevalence of diarrheal disease and promote the health of people.

The study recommended that container with tap, long or short handle dipper used for withdrawal of water from storage container, proper lid, daily washing of container, avoid

addition of fresh water in residual water or residual water from dipper into stored water are few remedies to control transmission of contamination (Tambekar D. H. 2008). It is important to keep the source point clean so as to avoid the storage contamination.

CONCLUSION

Inadequate and unsafe drinking water supplies are a continuing public health problem for most of the world's population. Improved water supply protection, wastewater and drinking water treatment using a multiple barrier approach and adequate protection of distribution systems will be needed to reduce the risk of infectious waterborne disease. We need to expand and coordinate surveillance systems for the early detection, tracking and evaluation of emerging waterborne pathogens. International scientists from government, academia and industry have recommended:

(1) New methods be developed utilizing molecular techniques for microorganism detection

(2) Establishing surveillance and occurrence data bases

(3) Risk assessment.

Improvements in Mysore city water distribution system

We should never become complacent about the vulnerability of drinking water systems to microbial contamination.

- Mysore water supply system has implemented many schemes for the distribution of good quality water.
    - JNNURM (replacement of old water pipe line)
    - Water will also be supplied by river KABINI
- It is important to keep the source point clean - to avoid the storage contamination.
- Improvement in behavioral and water hygiene practices can improve the household water quality.
- Health education is an important component to raise awareness and help people adopt correct behaviour.

Sl. No.	Place	E.Coli			Salmonella			Klebsiella / Entero bacter			Citrobacter		
		Tap water	Bore well water	Stored water	Tap water	Bore well water	Stored water	Tap water	Bore well water	Stored water	Tap water	Bore well water	Stored water
4	V.V. Mohalla									1			1
5	Paduvara halli	1		2	1		1	1		2	1	1	2
6	Jayalakshmipuram												
7	Vontikoppal												
8	Ashokpuram	1		2	1		2	2	1	2	1		2
9	Nachanahalli palya	1		2	1		2	2		2	2		2
10	Laskar Mohalla			2	1			1		2			2
11	Gayathri puram												
12	Jyothi Nagar			2						2			2
13	N.R.Mohalla												
14	Rajendra Nagar									1	1		1
15	Kurimandi	2		1	2			2		1	2		1
16	Gokulum			2						2			2
17	Vijayanagar		1	2					1	2		1	2
18	Hebbal												
19	Mahadevabadavane	1	1	2	1		1	1	1	2	1	1	2
20	Kuvempunagar			2			2			2			2
21	Ramakrishna nagar			2						2			2
22	Vivekananda nagar									1			1
23	Shrirampura				1			1		1			1
24	Kyathamarana hally			2			2			2	1		2
25	Raghavendra nagar			2						1			1
26	Shanthinagar									1			1
27	Kalyanagirinagar			2	1		2			2			2
28	Gousiya nagar			2			2		1	2	1		2
29	Ittigegudu			2	1		2			2		1	2
30	Sidhartha nagar			2			2			2			2
31	Vidyanagar												
32	Yaraganahalli			2						2			2
33	Vishweshwara nagar			2			1			2			2
34	Vidyaranya puram												
35	J.P Nagar		1						1	1			1
36	Chamundi puram			2	1			1		2			2
37	Bannimantap			2						2			2
38	Kesere	1		2	1		1	1		2	1		2
39	Metagalli	1	1	2	1			1	1	2	1		2
40	Brindavan Extn.									1			1
41	Bogadi			1						2			2
42	Vagdevi nagar									1			1
43	TK layout			2			1			2			2
44	Jayanagar			1						1			1

Sl. No.	Place	MPN number/100 ml			HPC count cfu/ml			Presence-Absence test		
		Raw		Treated	Raw		Treated	Raw		Treated
1	Belagola	1100		0	<300		Nil	Positive		Negative
2	Ramanalli	1100		0	<300		Nil	Positive		Negative
3	Hongalli	1100		0	<300		Nil	Positive		Negative
		Tap water	Bore well water	Stored water	Tap water	Bore well water	Stored water	Tap water	Bore well water	Stored water
4	V.V. Mohalla			7	4	1	11	-ve	-ve	+ ve
5	Paduvara halli	15	3	20	29	5	62	+ ve	-ve	+ ve
6	Jayalakshmipuram				2		9	-ve	-ve	-ve
7	Vontikoppal				5		10	-ve	-ve	-ve
8	Ashokpuram	11	7	120	12	12	<100	+ ve	-ve	+ ve
9	Nachanahalli palya	7		240	14	3	<100	+ ve	-ve	+ ve
10	Laskar Mohalla	7		23	13		47	-ve	+ ve	+ ve
11	Gayathri puram		4		2	11	52	-ve	-ve	+ ve
12	Jyothi Nagar			9	5		44	-ve	-ve	+ ve
13	N.R.Mohalla				1	2	16	-ve	-ve	-ve
14	Rajendra Nagar	3		7	10	10	19	-ve	-ve	-ve
15	Kurimandi	11		7	16	1	56	+ve	-ve	+ ve
16	Gokulum			20	3	1	<100	-ve	-ve	+ ve
17	Vijayanagar		3	11	5	16	39	-ve	-ve	+ ve
18	Hebbal				4		12	-ve	-ve	-ve
19	Mahadevabadavane	3	4	75	9	21	52	-ve	-ve	+ ve
20	Kuvempunagar			120	2		<100	-ve	-ve	+ ve
21	Ramakrishna nagar			23	4	12	33	-ve	-ve	+ ve
22	Vivekananda nagar			3	2	1	15	-ve	-ve	+ ve
23	Shrirampura	7		3	6		24	+ve	-ve	-ve
24	Kyathamarana hally	3		120	5		71	-ve	-ve	-ve
25	Raghavendra nagar			9	2		57	-ve	-ve	+ ve
26	Shanthinagar			3	3		23	-ve	-ve	-ve
27	Kalyanagirinagar	3		75	1	7	44	-ve	-ve	+ ve
28	Gousiya nagar	7		64	17	18	49	+ ve	-ve	+ ve
29	Ittigegudu	3	15	75	11	24	82	-ve	+ ve	+ ve
30	Sidhartha nagar			75	3	2	64	-ve	-ve	+ ve
31	Vidyanagar		3		2	6	13	-ve	-ve	-ve
32	Yaraganahalli			9	4		28	-ve	-ve	+ ve
33	Vishweshwara nagar			23	3		<100	-ve	-ve	+ ve
34	Vidyaranya puram				2		22	-ve	-ve	-ve
35	J.P Nagar		7	3	6	11	31	- ve	+ve	-ve
36	Chamundi puram	7		15	12		46	+ ve	-ve	+ ve
37	Bannimantap			11	5	2	31	- ve	-ve	+ ve
38	Kesere	7		39	14	1	52	+ ve	-ve	+ ve
39	Metagalli	7		11	8	6	34	+ve	-ve	+ ve
40	Brindavan Extn.			3	5		26	-ve	-ve	- ve
41	Bogadi			7	2		12	-ve	-ve	+ve
42	Vagdevi nagar			3	5	6	9	-ve	-ve	- ve
43	Tonachikoppal			11	2	1	49	+ve	-ve	+ ve
44	Jayanagar			7	3	2	12	-ve	-ve	+ ve

Table 2 : Coliform detected in different source of water samples of Mysore city by different methods.

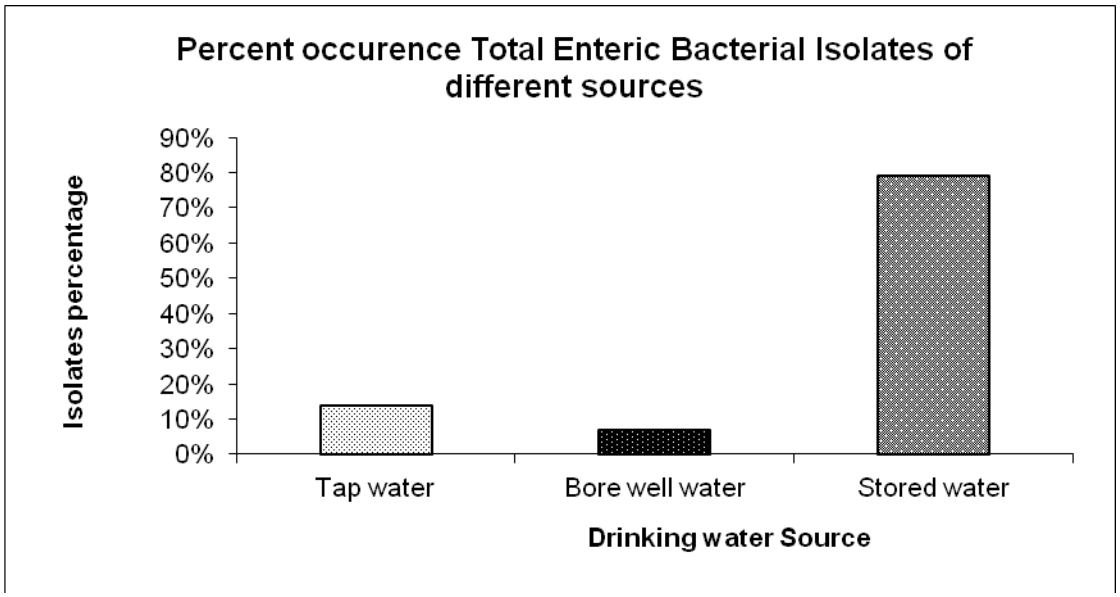


Fig 1

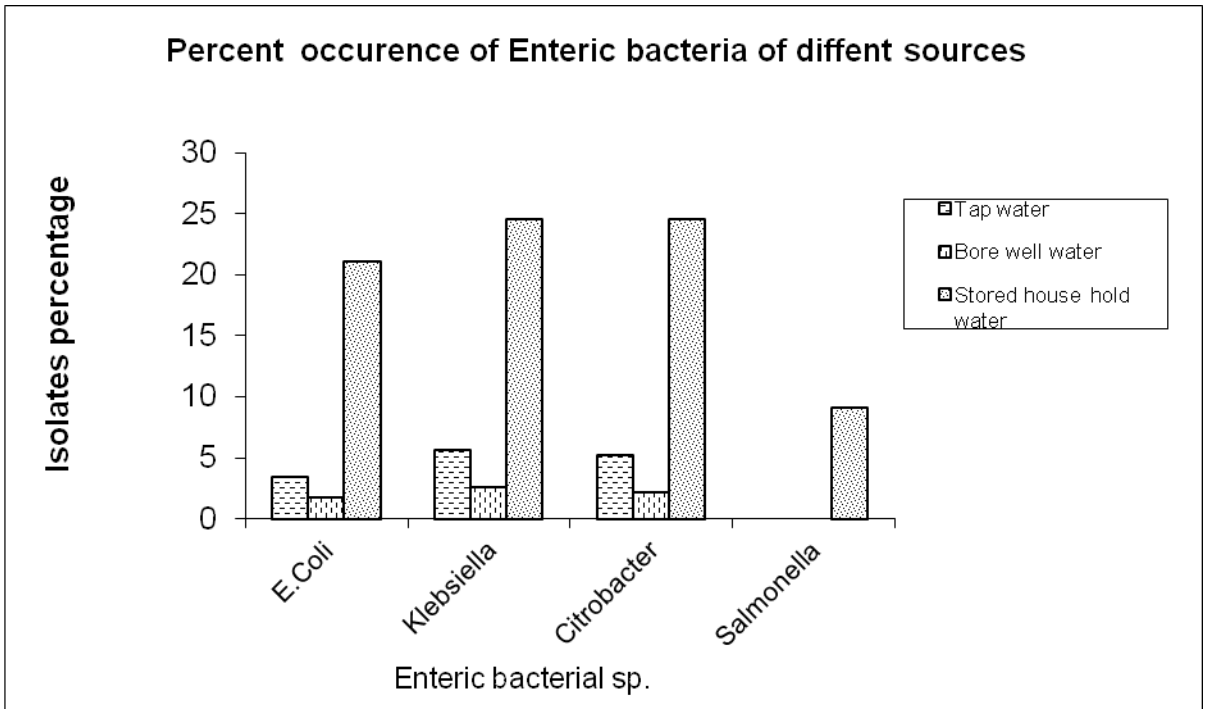


Fig 2

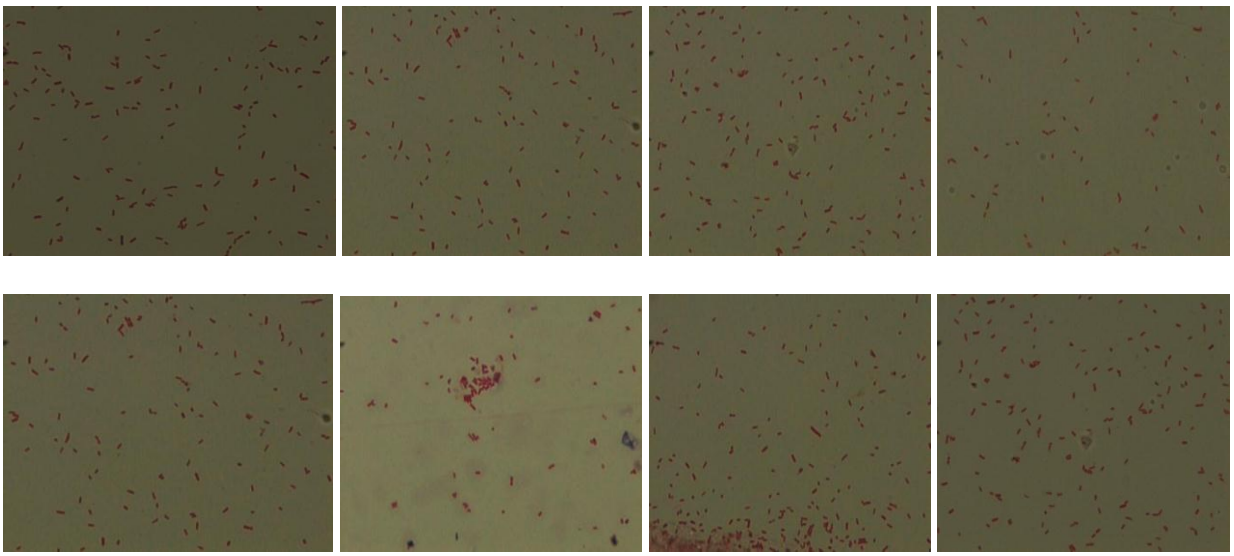
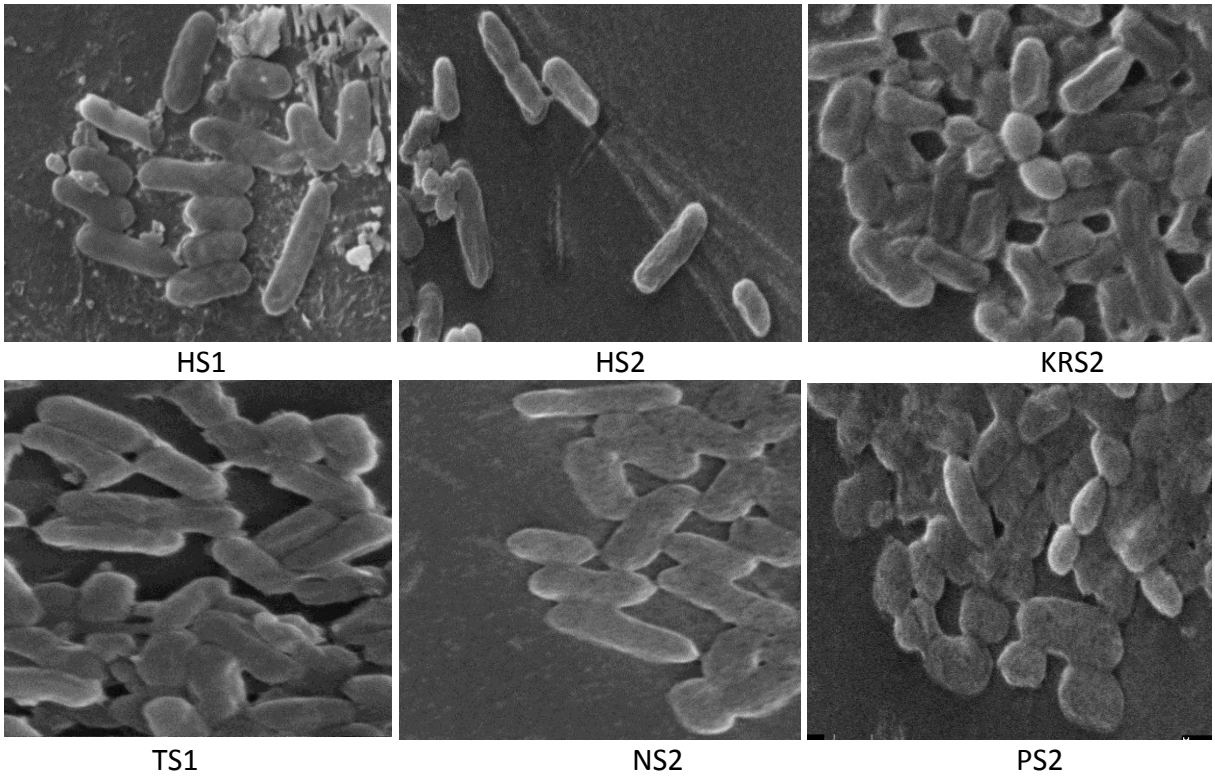
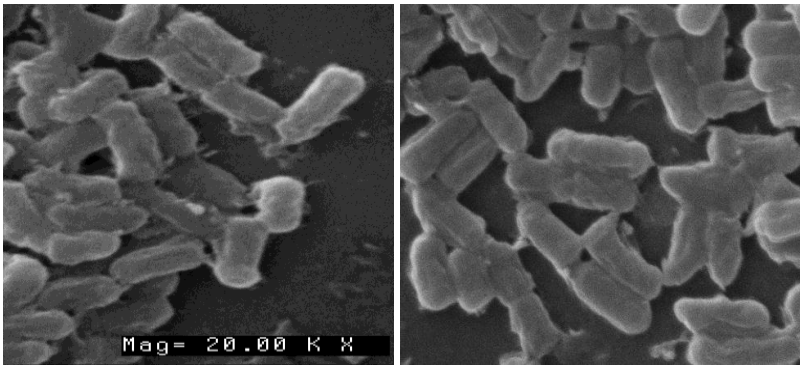


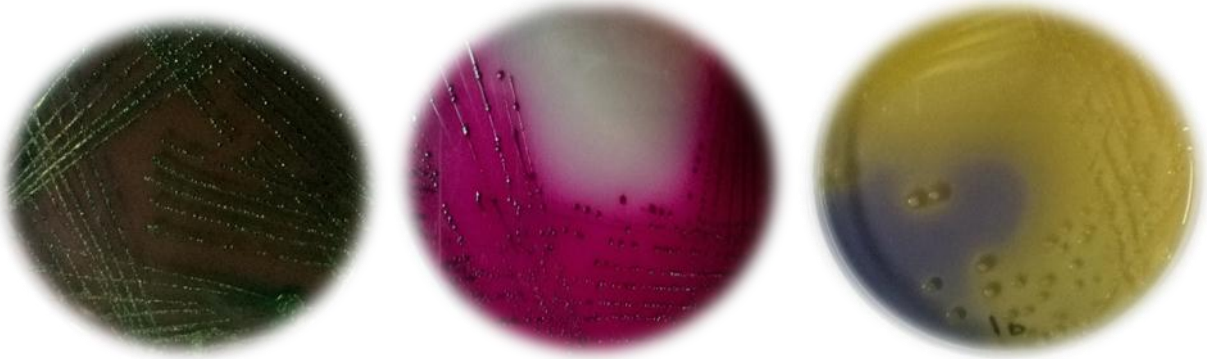
Figure: Gram staining of the isolates



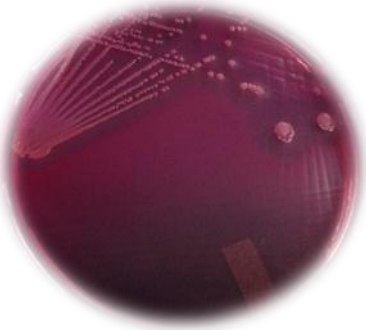


HDK3 HDK4

Figure: Scanning electron micro scopic image of the *e. Coli* isolates



EMB AGAR ENDO AGAR Mc Konkey Purple Agar

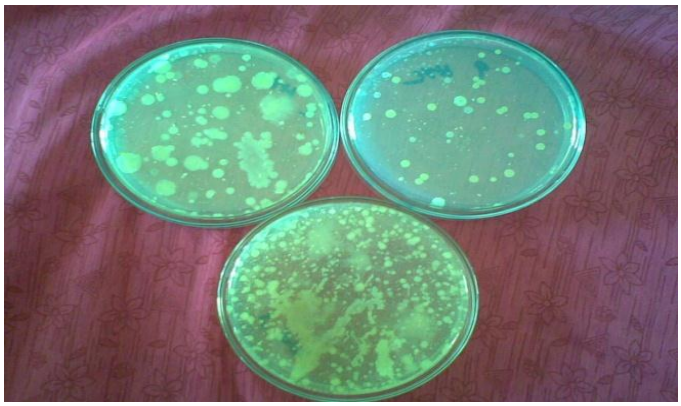


Mc Konkey agar

Figure: *E. coli* on different media

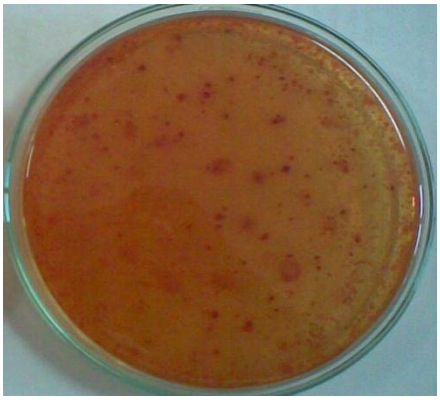
Positive negative control

Presence / Absence test.



Colonies on Tryptose glucose extract agar

HPC Method



Coliforms on McConkey agar

E.coli on EMB agar

Salmonella + ve on TSI

Control negative positive control

Indole test

Control positive negative

Citrate utilization test

positive

Lactose fermentation test

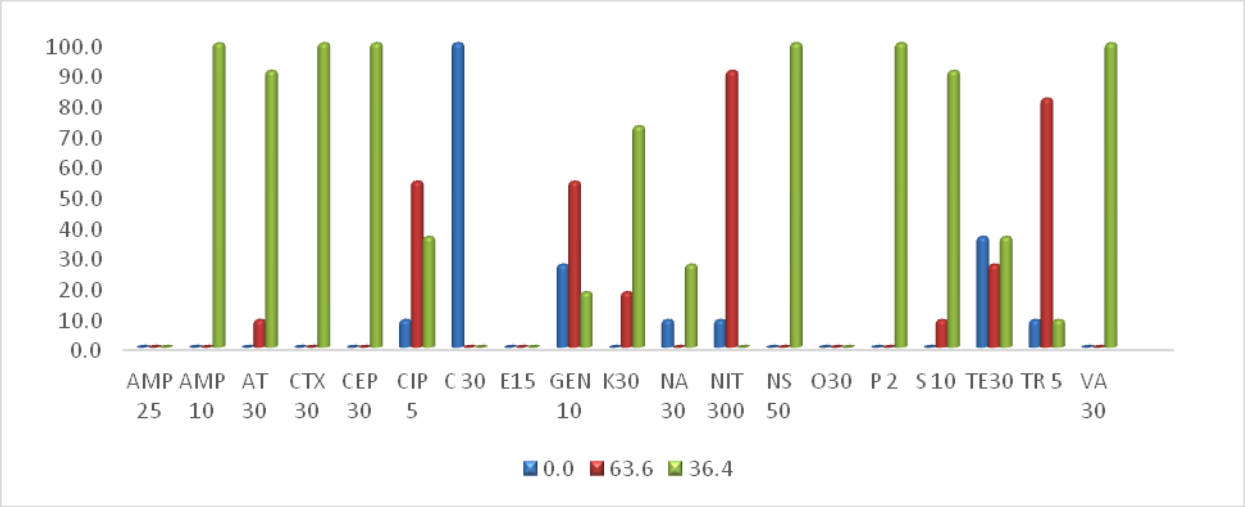
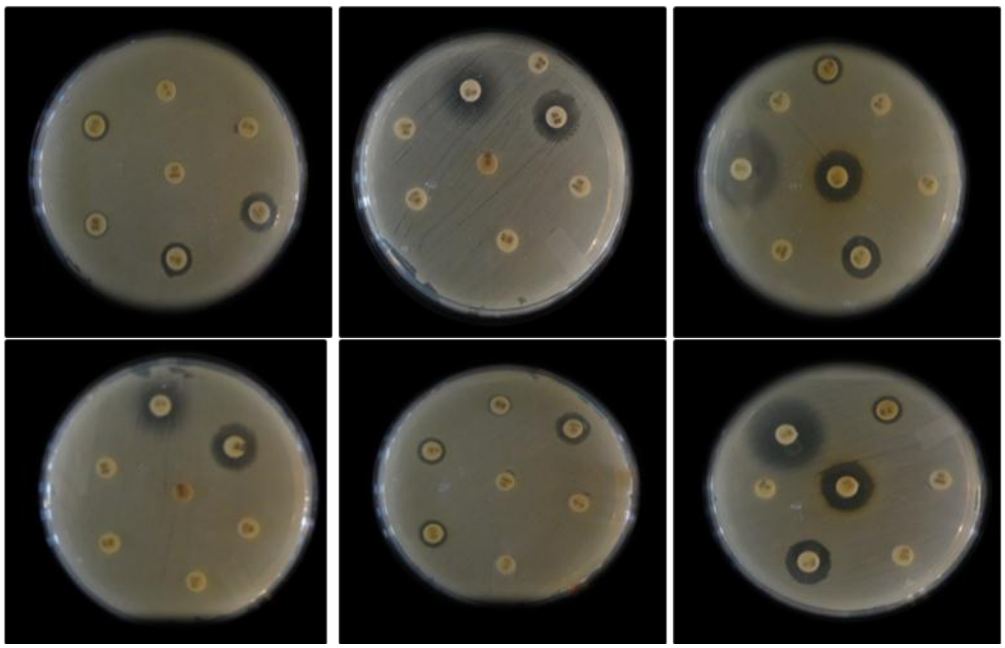


Figure: P**ercentage response of different E. *coli* isolates for various antibiotics tested**



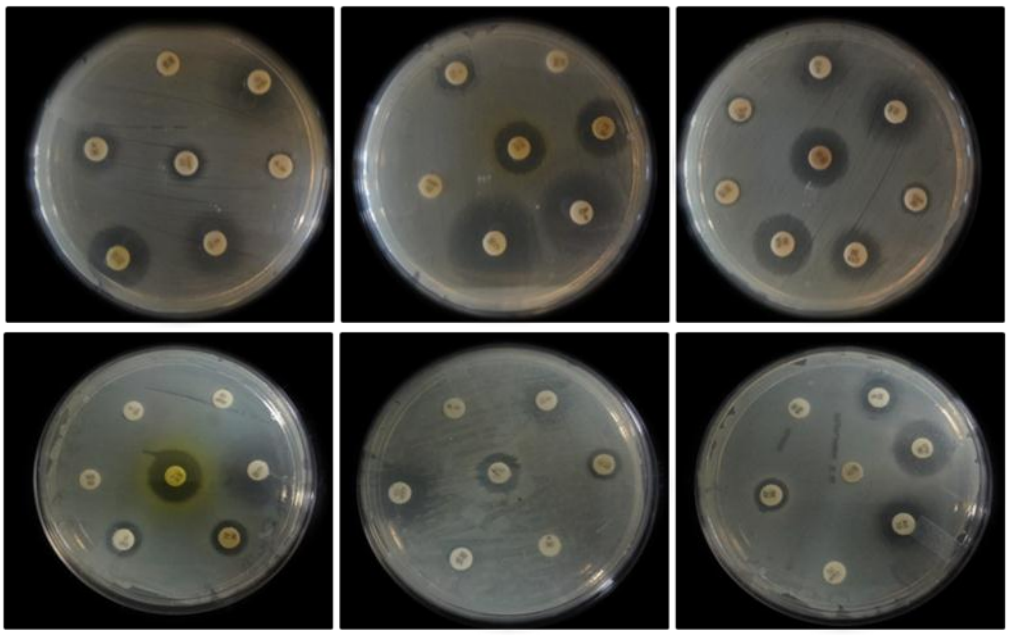


Figure: Antibiotic susceptibility test

Hygienic practices in potential problem areas

Hygienic practices in potential problem areas

Development of rapid methods for the detection of coliform contamination of drinking water

The determination of microbial water quality is a central activity in the control of total water quality. Efficient techniques for the detection of faecal pollution in aquatic environments are crucial for water monitoring programmes (Venter, 2000; Farnleitner et al., 2001; Rompre et al., 2002).

For more than 100 years, the microbial safety of drinking water has primarily been determined by testing for bacterial 'indicators' of faecal pollution, mainly *E. coli* and total coliforms. The coliform bacteria are one of the common contaminants present in drinking water. Therefore, detection of coliforms as indicators of human fecal contamination is very important to protect public health. These indicators are used to assess the potential public health risk of drinking water, and their absence or presence is key elements of most drinking water quality guidelines.

Coliforms belong to the family Enterobacteriaceae and they are facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C (APHA, 1998). Of the total coliform group, *E. coli* is the most numerous in mammalian feaces and is considered the most specific indicator of faecal pollution. According to the WHO guidelines for microbial quality of drinking water, in all water intended for drinking, *E.coli* or thermo tolerant Coliform bacteria must not be detectable in any 100 ml sample.

Multiple-tube fermentation technique, the membrane filter technique and the presence/absence test are some of the methods used for the detection of coliform contamination from decades. But these techniques have limitations, such as duration of incubation, antagonistic organisms interference, lack of specificity to the bacterial groups and a weak level detection of slow-growing or stressed bacteria. The proportion of non-culturable bacteria may be affected by unfavorable conditions for bacterial growth during culturing or by their entry into viable or active but non-cultruable states. (Shaban A.M *et al*., 2008).

The relevance of testing for total coliforms and *E.coli* has been questioned since its introduction and is again challenge. The limitations of the conventional methods led to the development of the rapid methods for determining the densities of total and fecal coliforms, particularly *E. coli.*

The development of more rapid detection methods

A variety of analytical approaches have been proposed for the rapid detection of bacteria in water. An essential requirement for the rapid methods should be the availability of data in the shortest time possible that means that these methods should be faster than the standard methods currently used. For bacterial indicators, the ideal for rapid methods should therefore be to have results within the same working day.

The tests rely on the detection of specific enzyme activities (β-D-galactosidase, β-D-glucuronidase, β-D-glucosidase) associated with the targeted indicator organism and no further confirmation tests are needed (Manafi *et al.*, 1991; Frampton and Restaino, 1993). The specific substrates allow the expression of these enzymes and their hydrolysis by the specific enzymes releases fluorophores or chromophores, providing a signal for detection. Since last ten years, enzyme substrates used in semi-quantitative methods have received more concern.

Coliforms secrete an enzyme, β-galactosidase (B-GAL) which can be monitored to measure activity of the coliforms. This ubiquitous enzyme β-galactosidase is oftern used as a general marker for total coliforms. The differential detection capacity of this enzyme prompted the development of rapid assays for monitoring their activity. The application of direct enzymatic detection of coliforms by monitoring β-D-galactosidase activity has been demonstrated by several authors including Davies and Apte (1999) and George et al. (2002).

Enzyme based methods

The biochemical tests used for bacterial identification and enumeration in classical culture methods are generally based on metabolic reactions. For this reason, they are not fully specific, and many additional tests are sometimes required to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted.

These methods are based on the fact that each genera, species or strain of microorganism has enzymes that participate in unique biochemical pathways. These unique enzymes are referred to as marker enzymes and could be employed in the design of selective assays. β-D-galactosidase (B-GAL) is a marker enzyme for total coliforms and β-D-glucuronidase (GUD) for faecal coliforms, specifically *E. coli* (Sartory and Watkins, 1999; Rompre et al., 2002). B-GAL catalyses the breakdown of lactose-based substrates to galactose and glucose-based products (Davies and Apte, 1999; George et al., 2002).

The enzyme substrate test utilizes hydrolysable substrate for the simultaneous detection of total coliform bacteria and *E. coli* enzymes. When the enzyme technique is used, total coliform group is defined as all bacteria possessing the enzyme β- D-galactosidase, which cleaves the chromogenic substrate, resulting in release of the chromogen *E. coli* are defined as bacteria giving a positive total coliform response and possessing the enzyme β- D-glucuronidase which cleave a fluorogenic substrate, resulting in the release of the fluorogen. The test can be used in either a multiple a multiple-tube, multi-well, or a presence – absence format.

β- D-galactosidase (B-GAL) is a marker enzyme for total coliforms and β-D-glucuronidase (GUD) for faecal coliforms, specifically *E. coli* (Sartory and Watkins, 1999; Rompre et al., 2002). B-GAL catalyses the breakdown of lactose-based substrates to galactose and glucose-based products (Davies and Apte, 1999; George et al., 2002). B-GAL from *E. coli* is a tetrameric metalloenzyme which performs two reactions with its disaccharide substrate lactose and exhibits broad specificity. The hydrolysis reaction produces the monosaccharides galactose and glucose. The transglycosylation reaction produces the disaccharide allolactose, which acts to induce the production of more β-galactosidase. By this approach, *E. coli* both responds to and utilizes lactose as a food source. The application of direct enzymatic detection of total coliforms by monitoring β-Dgalactosidase activity has been demonstrated by several authors including Davies and Apte (1999) and George et al. (2002). George et al. (2002) employed enzymatic reactions to detect pollution within 25-30 minutes. GUD catalyses the hydrolysis of glucuronide based substrates to corresponding aglycan and D–glucuronic acid (Rompre, et al., 2002). Substrates made up of glucuronide and galactose linked to a chromogen or fluorochrome have been designed for the detection of these enzymes (Frampton and Restaino, 1993). The use of

enzymatic reactions to detect coliforms provides a rapid, sensitive and less expensive alternative to the more traditional methods (George et al. (2002).

A major disadvantage of the enzyme-based method is the high levels of microbial contamination, hence enzyme, required to give activity, which may not be present in the samples under consideration and also the unspecific nature of the enzyme source. In addition, the use of more sensitive fluorogenic substrates is prone to interference by inherent compounds such as chemicals, cations and anions which may be present in the water samples. This has limited most of these techniques to laboratories for use on pure water samples. Coliform enzyme activity has been determined by different methods, by (i) either measuring the activity in the water sample itself by addition of the respective substrates or (ii) collecting the cells by filtration, followed by respective enzyme assays with the resuspended cells (Fiksdal et al., 1994; George et al., 2000). Either of these approaches will affect the results, since the enzymatic reaction conditions and sample manipulations will vary in each case. It is however, expected that the former situation will yield results close to what pertains in the environments, thus being more representative.

MATERIALS AND METHODS

Coliform enzyme activity has been determined by different methods, by (i) either measuring the activity in the water sample itself by addition of the respective substrates or (ii) collecting the enriched cells by filtration, followed by respective enzyme assays with the resuspended cells (Fiksdal et al., 1994; George et al., 2000). Either of these approaches will affect the results, since the enzymatic reaction conditions and sample manipulations will vary in each case. It is however, expected that the former situation will yield results close to what pertains in the environments, thus being more representative.

Enzymatic Tests

Spectrofluorometric method

Detections using chromogenic substances

Chromogenic compounds, which may be added to the conventional or newly devised media used for the isolation of the indicator bacteria. These chromogenic substances are

modified either by enzymes (which are typical for the respective bacteria) or by specific bacterial metabolites. After modification the chromogenic substance changes its colour or its fluorescence, thus enabling easy detection of those colonies displaying the metabolic capacity. In this way these substances can be used to avoid the need for isolation of pure cultures and confirmatory tests. The time required for the determination of different indicator bacteria can be cut down to between 14 to 18 hours.

This method makes use of the presence of the enzymes β-GAL and β-GUD in the coliforms, β-GUD catalyzes the hydrolysis of β-D-glucopyranosiduronic derivatives into D-glucuronic acid and aglycons indicating *E. coli.* β-GAL catalyzes the breakdown of lactose into galactose and glucose indicating total coliform bacteria. The detection of both these enzymes thus can be used for the enumeration of coliforms (Manafi et al., 1991).

Chromogenic or fluorogenic enzyme substrates are used to detect the enzymes β-GAL and β-GUD. The chromogenic enzyme substrates are phenol based, for example enzyme substrates for β-GUD ρ-nitrophenol-β-D-glucuronide (PNPG) and 5-bromo-4-chloro-3-indol-β-D-glucuronide (XGLU), where PNPG produces ρ-nitrophenol, a yellow colour and XGLU produces indolyl, which is blue.

The fluorogenic substrate used to detect β-GUD is 4-methylumbelliferyl-β-Dglucuronide (MUG) (Manafi, 1996; Manafi, 2000). β-GAL is detected by using chromogenic substrates such as chlorophenol red β-D-galactopyranoside (CPRG) and o-nitrophenol-β-D-galactopyranoside (ONPG) (Edberg et al., 1988; Cheng et al., 2002).

Total Coliform bacteria: Chromogenic substrate such as ortho-nitropheny β- D-galactopyranoside was used to detect the enzyme β- D-galactosidase which is produced by total coliform bacteria. The β- D-galactosidase enzyme hydrolyzes the substrate and produce a color change which indicates a positive test for total coliforms at 24 h (ONPG) without additional procedure.

***E. coli*:** A fluorogenic substrate, such as 4-methylumbelliferyl-β-Dglucuronide (MUG), is used to detect the enzyme β-D glucuronidase which is produced by *E. coli*. The β-D glucuronidase

enzyme hydrolyze the substrate and produce a fluorescent product when viewed under long-wavelength ultra light. The presence of fluorescence indicates a positive test for *E. coli*. Some strains of *shigella* also produce a positive fluorescence response.

Substrates and enzyme used

O-nitro beta d- galactopyranoside (ONPG), P-nitro beta-D-galactopyranoside (PNPG), 2-nitro beta-D- galactopyranoside, X-Gal, 4-Methyl umbelliferyl beta-D-galactopyranoside (MUGal), 4-Methyl umbelliferyl beta-D-galactopyranoside (MUGluc). isopropyl-b-D-thiogalactopyranoside (IPTG), MUG EC agar all the substrates were procured from the Himedia, Mumbai. Lactozyme 2600L (Sigma Chemical Co.)

MUG plate assay for identification of MUG utilizing *E. coli* isolates.

The *E. coli* strains isolated were inoculated onto the plates containing MUG EC agar (Himedia), and the plates were incubated at 37^0C for 24-48hrs. After the incubation, the plates were exposed to the UV light (360nm), and the blue fluorescence was observed.

Chromomeric substrate assay

Beta-D-galactosidase activity was measured in a 96 well flat bottom microtitre plate format (Tarson). 5 ml of overnight grown *E. coli* cultures (ATCC 19258 and ETEC (ATCC 31705) was taken in sterile centrifuge tubes, and the pellets were collected by centrifugation at 8000 rpm for 10 min.The cell pellets were suspended in 5 ml of Z – buffer (60 mM dibasic sodium phosphate, 40 mM of monobasic sodium phosphate, 10mMKCl, 1mM $MgSO_4$ and10mM beta-mercaptoethanol). Different concentration of pellets varying from 2.5µl to 25 µl was added to the microtitre plate wells in duplicate. Five µl of lysozyme (stock 15 mg/ml) was added to the pellets, mixed and incubated at 37°C for 10 to 30 min until culture becomes almost clear. 10 mM of the substrates PNPG, ONPG, and X-gal, was prepared in the Z- buffer and different concentration of the substrates varying from 50µM – 250 µM. The volume was made up to 150µl with the buffer. The plates were incubated at room temperature for one hour or until yellow color appears and the time of appearance of yellow color (**t**) was noted. The reaction was stoped by adding 50 µl of Na_2CO_3 (1M) and mixed. The reading at O.D $_{420}$ and O.D $_{550}$ was taken, Miller units were calculated, and the graph was a plot. Two sets of control were set up; an organism control (buffer + *E. coli*) and a substrate control (buffer + substrate) OD of each

control was measured 600nm wavelength. The assay for one substrate and one culture per plate was carried out.

Enzyme assay of the Isolates:

The assay was performed in the test tubes. 1 ml of overnight grown culture was taken in sterile centrifuge tubes, and the pellets were collected by centrifugation at 8000 rpm for 10 min. The cell pellets were suspended in 1ml of Z – buffer and the OD were measured at 600 nm. 15 µl lysozyme was added to the suspension, vortexed and incubate at 37°C for 10 to 30 min until culture becomes almost clear. 150 µl of freshly prepared ONPG (stock: 4 mg/ml) was added, vortexed for 5 to 10 min and Incubated at room temperature until yellow color appears and the time of appearance of yellow color (**t**) note was noted. The reaction was stoped by adding 0.4 ml of Na_2CO_3 (1M) and vortexed. Final concentration (**v**) was noted. All the remaining reaction was stopped after 1 hour. The reading at O.D $_{420}$ and O.D $_{550}$ was taken, and Miller units were calculated.

Enzyme assay of the Water samples:

100 ml of the water samples were filtered through the membrane filters and suspended in 1 ml of Z-buffer. The content was stirred well to release the microorganisms from the membrane and OD at OD_{600} nm was taken. 15µl of lysozyme was added and incubated for 10 min. 200 µM of PNPG was added to the assay mixture and incubated at room temperature for one hour. The reading was taken at O.D $_{420}$ and O.D $_{550}$, and Miller units were calculated.

Miller units : $1000 \times [Abs_{420} - (1.75 \times Abs_{550})]/t \times v \times Abs_{600}$

Abs_{420} = absorbance of yellow-p-nitrophenol

Abs_{550} = scatter from cell debris

(which when multiplied by 1.75 approximates the scatter observed at 420 nm)

t= reaction time in min.

v=volume of culture assay in milliliters

Fluorogenic substrate assay: The assay was adopted from Farnleitner *et al.,* (2001) with slight modification.

Standardization of the assay using enzyme:

Beta-D-galactosidase activity was measured in a 96 well flat bottom black microtitre plate format. The assay was perfumed as described in the section (0) using the MUGal substrate varying in the concentrations of 50µM to 300µM and the Lactozyme 2600L with different concentrations 0.2U, 0.4U, 0.6Um 0.8U, to 10U. The reaction was incubated for one hour, and Fluorescence emission was measured at 460 nm after excitation at 360 nm using an ELISA plate reader, the bandwidth was set at 12nm.

Standardization of the assay using *E. coli* (ATCC 19258):

Overnight grown *E. coli* cultures (ATCC 19258) was taken in sterile centrifuge tubes, and the pellets were collected by centrifugation at 8000 rpm for 10 min. The pellets were suspended in phosphate buffer and serially diluted to 10^{10} dilution. The dilution factor from 10^5 - 10^{10} were taken in microtitre plate in duplicates and the assay was followed as described above the without using lactozyme.

Fluorogenic assay using Water samples:

100 ml of the water samples were filtered through the membrane filters and suspended in 1 ml of Z-buffer. The content is stirred well to release the microorganisms from the membrane. 15µl of lysozyme was added and incubated for 10 min. 80 µM/ml of MUGal was added to the assay mixture and incubated at room temperature for one hour. Fluorescence emission was measured at 460 nm after excitation at 360 nm

Effect of Inducer on beta-galactosidase activity:

Water samples were inoculated into the sTSB media with and without IPTG (10mM) and the flasks were incubated for 6 hrs at two different temperatures i.e., 37^0C and 440C. The cells were collected by centrifugation and enzyme assay was performed as described in the section. The cell count was taken by serial dilution and pour plate method.

Enrichment media

mTSB media was selected as basal media and supplemented with protease casein 1%, Yeast Extract 0.5%, Soya Peptone 0.2%, Tryptone 1%, KCl 25µM, Ox Bile 0.1% and $MgSO_4$ 10mM. The media was compared with mTSB and TSB media by inoculating standard culture *E. coli* (ATCC 19258) and the water sample in separate 2sets of flasks. 100ml of water samples was

filtered through the membrane filter 0.2µm (Millipore) and inoculated into the sTSB medium. One set of the flask was incubated at 37^0C and the other set at 44^0C.

Chlorine stressing of *E. coli* (ATCC 19258):

The method was followed as described by Sen *et al.*, 2011 The chlorine stressing was carried out in 100 mL of (CDF) chlorine demand free buffer (0.05M potassium dihydrogen phosphate buffer at pH 7.0) and 1/200 diluted sodium hypochlorite. Chlorine concentration was adjusted to a final concentration of 2mg/l. The pellets of an overnight culture of *E. coli* (ATCC 19258) was collected by centrifugation at 5k x g for 5 minutes and washed twice in CDF buffer. The contents were then added to the conical flasks containing diluted sodium hypochlorite. After 20 seconds, 1 ml of a 10 % sodium thiosulphate solution was added to the flask to neutralize the sodium hypochlorite. Cell counts were taken before and after chlorine exposure.

Nutrient limitation/starvation stress experiments

For starvation stress experiments, *E. coli* (ATCC 19258) strain was grown on ½ strength TSB agar plates at room temperature for two days. Then the colony was picked and inoculated into sterile Butterfield's buffer (monobasic potassium phosphate 34g/l) and the flasks were incubated at dark for 15 days at room temperature. After 15 days cells were enriched and the cell counts after and before were taken.

Optimization of the enzymatic assays conditions

In order to establish an enzyme assay a number of steps are implemented to ascertain optimal conditions under which the enzyme will operate maximally. This process is called assay optimization. Assay optimization is usually based on previously published assay procedures and general characteristics of closely related enzymes.

In order to optimize the sample volume, varying volumes of 100ml to 1000 ml water samples were filtered and were reacted with the substrate and buffer. Buffer was used to adjust the volumes so as to maintain constant final reaction volume. Different temperature varying from 30 to 70^0C was performed in the B-GAL assay. Different time intervals of 30 to 8hs were performed to get a better identification of the sample.

Different enzyme substrate such as β-GUD ρ-nitrophenol-β-D-glucuronide (PNPG) and 5-bromo-4-chloro-3-indol-β-D-glucuronide (XGLU), where PNPG produces ρ-nitrophenol, a yellow colour and XGLU produces indolyl, which is blue were used with different concentrations.

Fluorogenic substrate

β–galactosidase activity was measured according to a modification of the method described by Apte and Batley (1994).

The assay involved the addition of pure 4-methylumbelliferyl-β-D-galactoside (MuGAL) substrate and 0.1 M sodium phosphate buffer (pH 7.2) to 5 ml of sample and incubation for 40-60 min at 44^0C. Activity was terminated with calcium carbonate buffer (pH 10) and the fluorescence was measured using a Fluorescence Spectrophotometer at 465 nm after excitation at 375 nm.

Optimization of the enzymatic assays conditions

The above assay procedures and selected volumes were used in an investigation to assess the effect of pH on the hydrolysis rates on the selected fluorogenic substrate. The buffer systems employed are sodium phosphate; pH 7.0-9.0. Controls to account for the spontaneous chemical degradation of the MuGAL substrate at the different pH values were run accordingly.

The same trials were followed for the fluorogenic substrate assay as it was followed for the chromogenic substreate.

Spectrophotometric Method

Coliforms isolated from Water samples are used for the Spectrophotometric method of identification of the presence of coliforms.

RESULTS AND DISCUSSION

Optimization of the enzymatic assays conditions

The volumes of assay components, including enzyme/sample volumes and/or concentrations of enzymes required have to be designed in such a manner to obtain a linear, stable and consistent reaction over time while rapid reactions that are inconsistent and difficult to monitor are avoided. Once these parameters are established, scaling up and scaling down of assay procedures can easily be performed. Temperature and pH play important roles in enzyme activities. pH affects the structural stability and solubility of, as well as the charge and charge distribution on both enzymes and substrates (Holme and Peck, 1998). These factors; in turn, alter bonds and bonding patterns, ultimately determining the rate of enzyme-catalysed reaction.

Temperature influences the rate of molecular collision and bond vibrations. Increasing temperature (usually up to 40^{o}C) increases the number of collisions between substrates and enzymes, thus increasing the reaction rate. An increase in temperature may also result in increased bond vibrations which could lead to bond disruptions, hence enhancing substrate breakdown. The bond disruption may, however, denature the enzymes under consideration. As a result, optimum temperature will be a compromise between maximum activity and enzyme denaturation (Holme and Peck, 1998). Each enzyme has a characteristic pH and temperature optimum. Enzyme and substrate concentrations, pH and temperature affect assay times. In order to accurately determine enzyme activity, a linear range of maximum product accumulation from progress curves of product formation or substrate consumption versus time is required. Tipton (2002) reported that different enzymes and those from different environments have varying shapes of progress curves. It is thus important to determine the characteristic shape of a progress curve of the enzyme in question. It is necessary to determine the location of the enzyme in question in order

Optimization of the parameters relating the enzymatic reactions was carried out on using the direct culture and several water samples variously contaminated by coliforms. The enzymatic activities were measured at various pH levels or temperatures.

Fluorogenic substrate

As expected, there was generally a direct relationship between B-GAL activity and the volume of water samples. Due to the random distribution of the coliforms, it was observed that, at higher water sample volumes the identity was early.

Enzyme activity fluctuated with changes in pH. The highest B-GAL activity was recorded at pH 7.2. With concern to the time as there is increase in time the color produced by the enzyme substrate reaction was more intensive.

Chromogenic substrate studies

A gradual increase in B-GAL activity was observed between pH 5.0 and 7.5 after which a sharp rise appeared at 7.8. Increase in activity occurred when the pH was increased from 7.5 to 7.8 and a similar level of reduction occurring between 7.8 and 8.0. Changes in the activity as a result of pH variation could be due to structural stability and solubility, charge and charge distribution on both enzymes and substrates. All subsequent B-GAL assays were thus performed at pH 7.8 for the remainder of the study.

A consistent increase in activity with increase in temperature was observed for environmental B-GAL up to 70^0C. Increasing the temperature from 35 to 40^0C resulted in increase in activity. The temperature optima at 40^0C were observed with the water samples.

Effect of environmental water sample on commercial B-GAL activity.

In order to establish the total contribution of compounds present in the environmental water samples on the enzyme assay, varying volumes ranging between 0-50 percent of the total assay volume were pre-incubated with commercial B-GAL assay for 30 min prior to determining the enzyme activity. At a 50% volume level, the environmental water samples reduced the activity of B-GAL about 15%. This implied that there was some level of underestimation of the amount of B-GAL determined in situ. The reduction of B-GAL activity by the environmental water samples could be due to the presence of metal ions or other pollutants which inhibit the activity of the enzyme or substrate or both.

Permeabilisation effects

All the chemicals used in the permeabilisation methods inhibited B-GAL activity at their higher concentration, The data reveals that Butanol, proponal, toluene, ethanol and benzene were effective in carrying out the permeabiliztion of coliform cells. The remaining chemicals Acetone, Chloroform, Tween 20 and Triton X-100 were found less effective. Different optimal concentration of chemicals was 10-40%. Amongst the various permeabiliztion agents used, the maximum permeabilization of *E. coli* cells were observed with 25% of butanol, 30% of Propanol and 20% of Ethenol. To find out the effect of temperature on the permeabilization of *E. coli* cells using butanol as permeabilization agent, the temperature was varied from 30-70^{0}C. The enzyme activity increased with increase in temperature up to 40^{0}C, further increase in temperature has shown a decrease in enzyme activity. Increase in temperature indicated the

It has been observed that permeabiliztion of the *E. coli* cells increases with the chemical concentrations up to critical value, where a maximum enzyme activity can be observed. At higher concentrations of the agent, the enzyme activity decreases which may be attributed to the leakage of the enzyme from the cells or cell lysis. At low concentration, the less enzyme activity may be due to the insufficient amount of the agent for effective permeabilization.

Spectrophotometric method Vs Spectroflourometric

The spectrophotometric method was less expensive than the spectrolourometic method but it could detect the coliform contamination only the cell concentration of >300 CFU/ml hence, non specific and sensitive. Hence, the spectrophotometric method is not suitable for the detection of the coliforms in drinking water. Whereas the detection by spectroflourometer is verey specific, sensitive, better and rapid which could detect coliform contamination even at low concentration in the water samples.

The overall results for the total coliforms and *E. coli* tests showed that enzyme method using chromogenic and flourogenic substrate can applied as an alternative method.

A number of chemical and physical factors involved in drinking water treatment, including disinfection, can cause sublethal injury to coliform bacteria. A free chlorine residual of 0.25 – 0.5 mg/l in drinking water is lethal to coliforms which could not be cultured or recovered from the culture method.

MUG plate assay for identification of MUG utilizing *E. coli* strains.

*E. coli*is olates from the water samples, have tested for the MUGluc test, all the strains except PS1 were positive for utilization of the substrate and giving blue fluorescence under the UV. The result indicates that the isolates have expression the β-D-glucurunidase enzyme which is a specificenzyme for the differentiation of *E. coli* from other bacteria. The inability of the utilization of the substrate may be due to the absence of the enzyme in the organism, or glucuronidase gene may be present but not expressed (Feng *et al.,* 1990).

Chromogenic substrate assay using standard cultures:

The substrates such as X-gal, ONPG and PNPG are the common chromogenic substrates used for the routine assay of the enzyme. The nitrophenol products released on hydrolysis of these substrates are usually measured at 420 nm. The assay was performed at constant pH 7 and room temperature (Juers *et al.*,2012). In the study, differentconcentrations of enzyme (2.5µl, 5µl, 10µl, 15µl 20µl, 25µl) against different concentrations of the different substrates were carried out. All the three substrates hade adifference in therate of enzyme activity with respect the different standard strains of *E. coli*. It has been observedthat the rate of enzyme activity increased withthe increase in the substrate concentration of both ONPG and PNPG of the standard *E. coli*ETEC (ATCC 31705) &*E. coli* (ATCC 19258) cultures. Andvisa verse.Whereas,in thecase of X-Gal, the enzyme activity increased to a certain level and then decreased, even in the presence of sufficient substrate. The maximumrate of enzyme activity was observed at 200mM substrate and 25µl of cell concentration of the strain*E. coli* ETEC (ATCC 31705) for PNPG where it contains 10^8CFU/ml. And25µl organism and 250mM substrate concentration for ONPG. The enzyme activity of the standard culture *E. coli*ATCC 19258 was maximum at 25µl *E. coli* and 250mM substrate concentration for both PNPG and ONPG Very low of rate of enzyme activity was observed from both the stains for the substrate X-gal Among the substrates used a low concentration of the PNPG (200µl) can sufficient to be used than the ONPG (250mM) in the cell concentration of more than 10^6 CFU/ml. The substrate X-gal has not efficient to impart the color in an one hour of the incubation. The study, reveals that the enzyme activity is greatly influenced by the concentration of the organism. The variation was also observed among the *E. coli*strains, where the response of strain*E. coli*ETEC (ATCC 31705) was earlier and more than the strain *E. coli*. ATCC 19258.

Chromogenic substrate assay of the isolates

E. coli isolated from different water samples of the study were tested for their enzymatic expression using ONPG as substrate and results are given in. All the isolates were positive for ONPG and the variation in the color intensity among the isolates were observed. The β-gal activity is controlled by the gene lacZ, and the level of enzyme activity depends on the level of gene expression. The each organism has a difference in the induction and the expression of the genes.

Chromogenic substrate assay of the water sample

6 Water samples of a hand pump, tap water, and stored household water have been tested for the enzyme activity using PNPG. All the water samples tested have not developed any characteristic yellow color of the assay in one hour of incubation but on further incubation for more than six hours low rate of enzyme activity of 3.2 MU activity was detected in the stored household water sample incubated at 37^0C. Whereas the other samples were negative for beta galactosidase enzyme.

Effect of Inducer on beta-galactosidase activity:

IPTG acts as an inducer which makes repressor protein into inactive form by forming conformational change in the shape of the repressor protein and prevents repressor from binding to the operator region. Thus, transcription of lac operon occurs by binding RNA polymerases to the promoter site without any obstacles. The production of the β-galactosidase enzyme is lac Z gene controlled process. IPTG was used as an alternative to lactose for the induction of the gene. The enzyme does not utilize IPTG, and the concentration remains unaltered throughout incubation period, but it acts as a substrate for bindingto the promoter and allowing gene transcription. From the results, it was noted that the use of IPTG as an inducer to enhance the enzyme is not significant for a short period of incubation. Similar to the study by Brenner *et al.,* 1991 the gratuitous lac operon inducer IPTG did not improve PNPG color development. In the study Essays, UK. 2013, IPTG has shown increased enzyme activity only after incubation for four days but in the present study, since the incubation period was only 6 hrs, it was found that IPTG had no effect on the enzyme induction. The weak growth of coliform was aslo been observed where,the it was one log CFU/ml. Theassay clearly states that the longer the IPTG induction time, the greater the units of beta-galactosidase per ml of bacterial culture produced.

Fluorogenic substrate assay:

Standard curve using enzyme beta-D-galactase:

The concentrations of enzyme,beta-galactosidase varying from 0.2 U to 10 U and the substrate MUGal concentration ranging from 10 μM to 250 μM was used. The result in the illustrates that very little enzymatic substrate hydrolyis is required to produce lage fluorescence signal. Low concentration of the MUG 10μM was able to be analyzed without any markedly changing the concentration of the MUG during the assay. 10μM of MUG hydrolyzed and large fluorescence signals were developed in all the concentrations of the enzyme ranging from 0.2U to 1U. The plates were aslo observed under long wavelength of UV at 360nm for the visualization of the fluoresecence.

Using standard culture *E. coli* (ATCC 19258):

The rate of fluorescence signal with different dilutions of *E. coli* (ATCC 19258) (from 10^3 to 10^8) to adifferent concentrations of substrate MUGal, indicated that there is a progressive decrease in the activity with the decline in the cell concentration. The increase of substrate concentration on the lower dilution factor has no effect and vice verse. Maximum fluorescence was observed at a 3rd dilution of the cell concentration with 80μM of the substrate concentration, and low-level fluorescence response was in the 8th dilution 50 μM concentration. Since the target of an experiment was to detect theminimum number of the *E. coli*cells in the water sample the substrate concentration of 80μM can significantly detect the cell number of 7 CFU/ml.

Fluorogenic substrate assay of water samples:

Among the water samples tested one of the waters samples of stored household dinking water was positive for coliform contamination with fluorescence range of 10MU at the substrate concentration of 50μM . The other water samples were negative by MUG substrate assay.

Enzyme assay of chlorine injured and nutritionally stressed *E. coli*

Both Chlorinated and stressed *E. coli* at lab level were performed for the detection by enzyme activity using ONPG, PNPG and MUGal all the substrates were not able to detect *E. coli* in one hour.

As enzymes in river water were estimated to be more diluted and behave differently due to the presence of the other substances, the time fixed to determine the enzyme reaction. Detection of coliforms in water by using enzyme substrate concept depends on the intensity of

the color produced by the reaction intern depends on the number of the organisms present in the water samples. Chromogenic and fluorogenic substrates have different advantages in testing procedures for *E. coli*. PNPG is hydrolyzed to release p-nitrophenol, and ONPG is hydrolyzed to release ortho-nitrophenol both had a yellow in color and measured at OD 420 nm. The β-galactosidase assay using X-gal occurs in two steps. Initially, the enzyme beta-galactosidase induces the release of a soluble, colorless indolyl group and then Subsequently to two indolyl moieties form a dimer which is oxidized to insoluble blue indigo precipitate (Pearson *et al.*,1963).

Both PNPG and ONPG are suitable for liquid media because of the solubility of the substrates (Manafi *et al.,* 1991). Where as the X-gal takes longer lag phase to develop a color. Bbecause of the precipitation X-gal is being used extensively to detect the presence of the lacZ gene in the molecular biology field. In this study bacteria from the drinking water was concentrated by filtration on smaller sized 0.2µm filter paper of dia 13 mm and tried to concentrate in one ml of the buffer, the cell captured were subjected to lysis using lysozyme enzyme for the release the target enzyme to utilize the substrate provided. This method has given a low rate of enzyme activity in one hour and detectable measurement by the further incubation in case of chromogenic substrate PNPG. The increased incubation time reduces the false-negative results (Clark *et al.*,1991). Enrichment of the filtered water sample in a formulated media of this study has also been carried out which has taken 6-7 hours for the complete detection of the coliform with the yield of color intensity slightly more compared to the direct water assay. The fluorogenic substrate MUGal used in the study by the same method has an efficiency of detecting *E. coli*in one hour of the incubation with few number of the cells, and is more sensitive than the chromogenic substrate which is agreeable with the previous findings (Manafi *et al.*,1991, Bej *et al.*,. 1990)

The fluorogenic substrate has been used by several authors for the rapid detection of coliform and *E. coli*in drinking water since many decades. The substrate method was combined with filtration technique and was incorporated into the different specific media for the detection of coliforms and *E. coli*(Dahlen and Linde, 1973; Brenner *et al.*,1993; Ciebin *et al.*,1995; Gaudet *et al.*,1996; Geissler, 1999; Davies and Apte, 1999,). Recently, Robert et al in 2015 developed a new low-cost growth medium, aquatest (AT). All these media and the kits based on this substrate takes 18 to 24 hours for the detection.

Berg and Fiksdal *et al.*, 1990, detected fecal coliform in 100 ml of water samples with a small number of 1 CFU in 6 h by incorporating the MUGal into the medium. Apte and Batley, 1992 detected coliform in one hour by modification of the media, Tryland and L. Fiksdal in 1997 in 25 min with high density of the cell concentration, all these techniques are based on the addition of the nutrients to the filtered rever water samples.A major concern with any assay based on enzyme activity for the detection of coliform and *E. coli*is the interference that can be caused by the presence of other bacteria (Koster *et al.*,) In addition, the use of beta-galactosidase in coliform detection has other disadvantages, that the enzyme can be found in numerous other organisms such as including *Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae* and *Neisseriaceae,* several Gram-positive, yeasts, protozoa, and fungi (Holt *et al.,*1989; Moberg, L. H. 1985; Robison, B. J. 1984). Beta-glucuronidase activity although produced by most *E. coli*strains the other Enterobacteriaceae including some *Shigella, Salmonella, Yersinia, Citrobacter, Edwardia Flavobacterium spp., Bacteroidesspp., Staphylococcus sp. Streptococcusspp., anaerobic corynebacteria, and Clostridium and Hafnia strains* (DAVIES *et al.,*1995; Tryland and Fiksdal, 1997) are also reported to produce the enzyme leading to false positive organisms. And the enzyme activity induced by these organisms were approximately same. To minimize the interference of the other on target organisms β-glucurinadase was used (Dahlen and Linde, 1973; Berg and Fiksdal, 1988; Brenner *et al.,*1993; Ciebin *et al.,*1995; Gaudet *et al.,*1996; Tryland and Fiksdal, 1997, Geissler, 1999; Davies and Apte, 1999, Wildeboer *et al.,*2010). But the disadvantage of using β-glucurinadase is the lack of the enzyme in some strains of pathogenic *E. coli*(Chang *et al.,*) especially O157:H7 which are significantly causing water bornd illness. George *et al.,*2000 reported the failure of the enzymatic method in detection of viable but nonculturable *E. coli*. Caruso *et al.,*1998, found that the insufficient production of the fluorescence intensity by the enzyme assay for the detection of *E. coli*in marine waters.

In spite of all the limitations, detection and enumeration of enteric bacteria using the metabolic activity of cellular enzymes are a well-developed and applied technique. (Coales, 2002, R&D Technical Report E1-105/TR R), Fluorogenic substrates can be used instead of the chromogenic substrate which is sensitive in detection of the coliforms. The limitation of the enzymatic assay suggests that the accuracy in identifying *E. coli*is not perfect. Nonetheless, the above problems generally result in fewer errors than traditional cultivation-based methods the

enzymatic method is easy to perform, can be detected by eye without the need for complicated instruments, cost-effective and rapid can be replaced with conventional method which takes several days for the confirmation.

Comparison of an enzymatic assay with standard methods techniques for detection bacteria showed more recovery of injured *E. coli* by enzymatic assay, resulting in a more realistic estimate of the actual population of indicator bacteria in public water supplies. The result indicated that enzymatic method is an alternative approach that could provide better and more rapid information for the assessment of microbial quality of drinking water. It could simultaneously detect total clifroms and *E. coli* from a water sample with the working day or the sampling day.

In conclution, addition of fluorogenic and chromogenic substrates to cultivation media to detect the enzymatic activity of coliforms have increased the sensitivity and the rapidity of the classical methods for estimating the microbial contamination of drinking water. Enzymatic method is easy to perform and gives more rapid and more realistic estimate of total coliforms and *E. coli* than multiple tube fermentation. This method might be more expensive in terms of consumables than the classical methods when the latter require no additional confirmation steps. In all cases however, enzymatic methods require less manpower and therefore their cost in terms of commercial value is lower. In conclusion, one could use total coliform β-D-galactosidase enzyme activity and CFU determination as a very good measure of microbial water quality assessment. Total coliform measurement may be considered as an imperfect but useful criterion of drinking water quality assessment and should find use at both small and large treatment facilities, as well as in routine analysis.

Based on the analysis of water samples and the result obtained, municipal office of the Mysore district has been informed about the risk. And also work shop has been conducted in the more contaminated low hygienic places to give then awareness about the handling and maintenance of the stored drinking water and how to keep the source pint clean to avoid the storage contamination. It also been educated about the improvement in behavioral and water hygiene practices which intern can improve the house hold water quality. It has also been informed to the municipal office about the contamination of the tap water samples in the distribution system which is far from the treatment point. To overcome the water crises Mysore

water supply system has implemented many schemes for the distribution of good quality water under JNNURM (replacement of old water pipe line) and supply of water from river KABINI

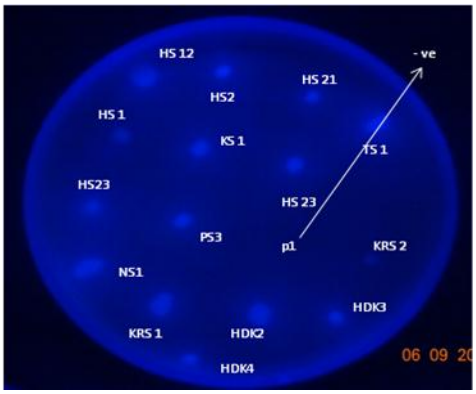


Figure: Cultures showing fluorescence on MUG EC agar plate under UV light

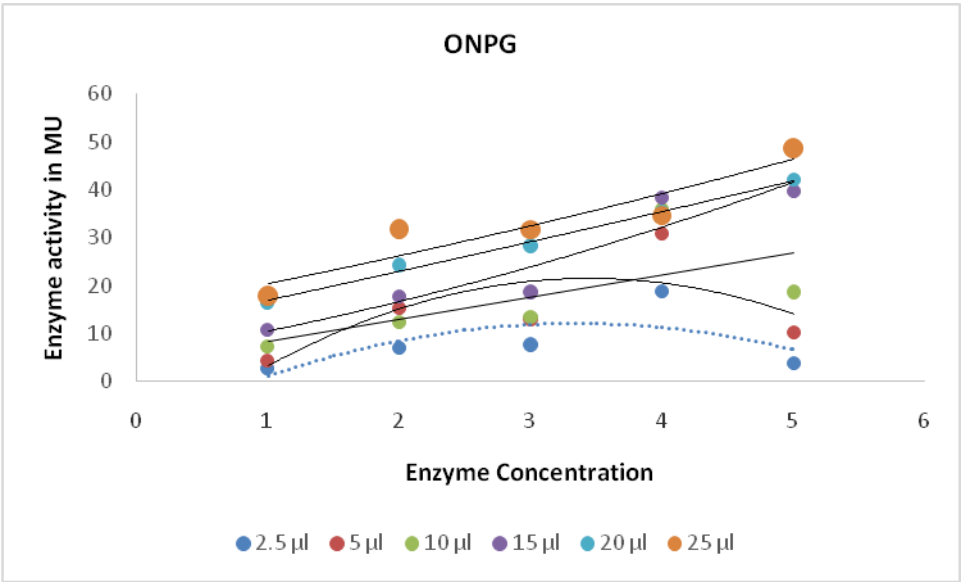


Figure: Effect of concentrations of *E. coli* ETEC (ATCC 31705) on Enzyme Activity to the substrate ONPG

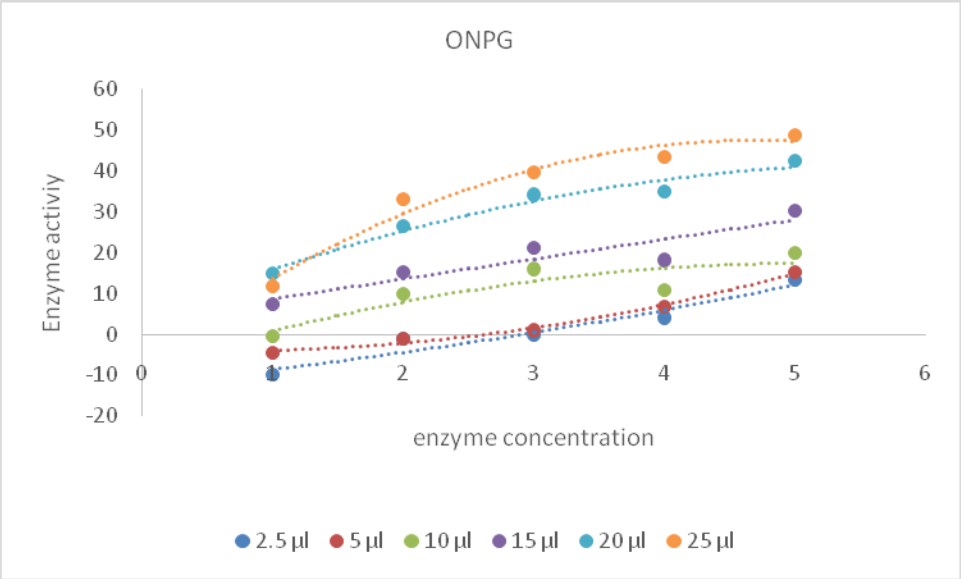


Figure: Effect of concentration of *E.coli* (ATCC 19258) on enzyme activity of the substrate ONPG

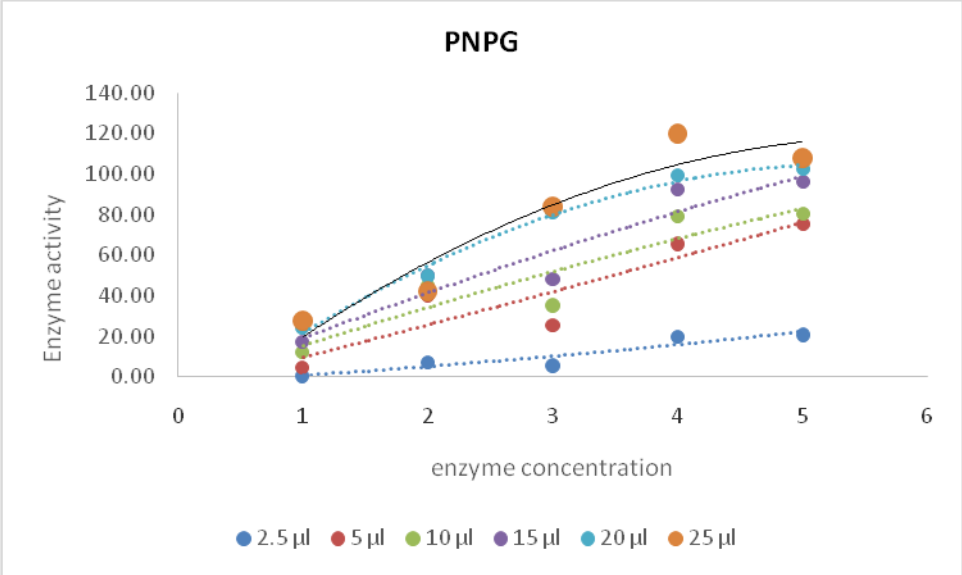


Figure: Effect of concentration of *E. coli ETCC* (ATCC 31705) to the substrate PNPG

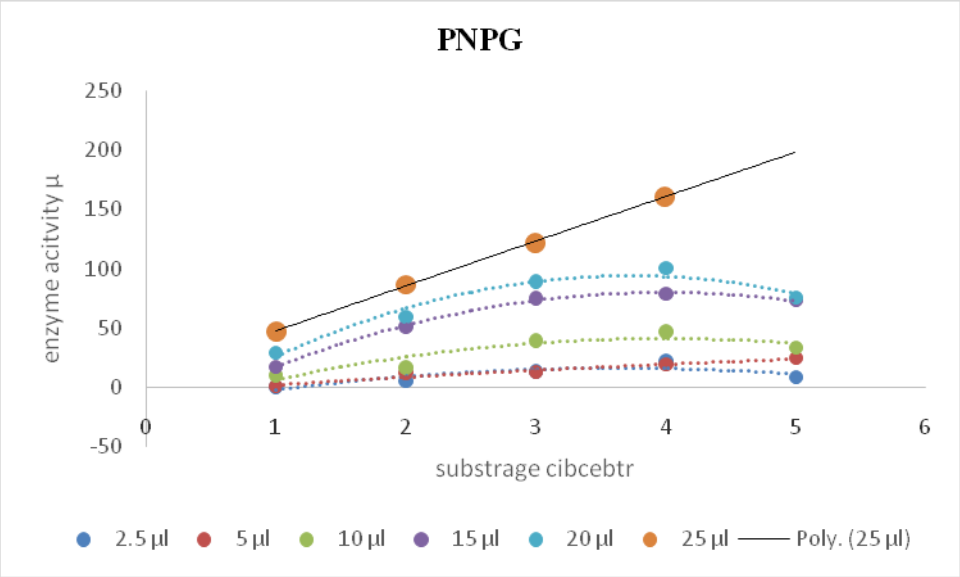


Figure: Effect of concentrations of *E. coli* (ATCC 19258) to the substrate PNPG

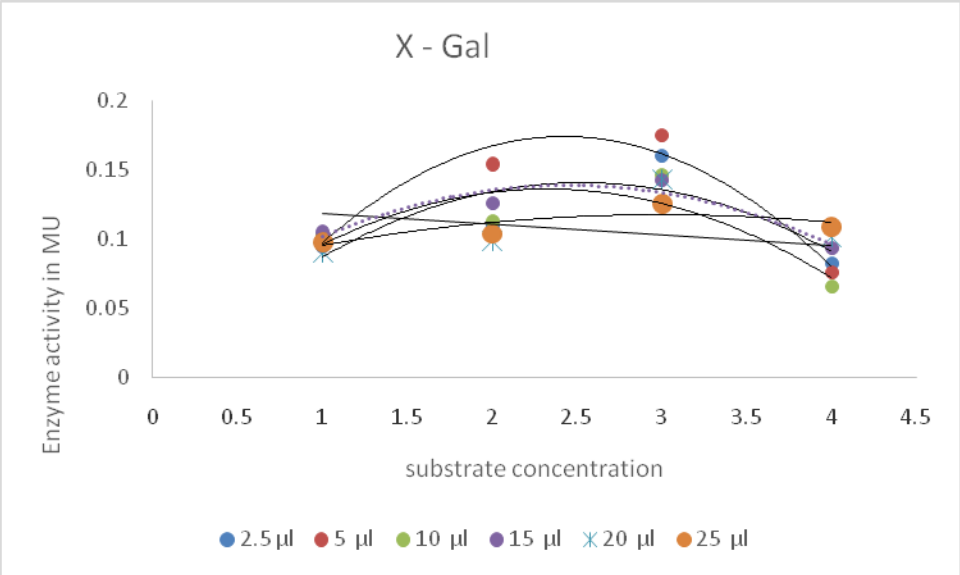


Figure: Effect of different concentrations of the *E. coli* ETEC (ATCC 31705) on enzyme activity of the substrate X-gal

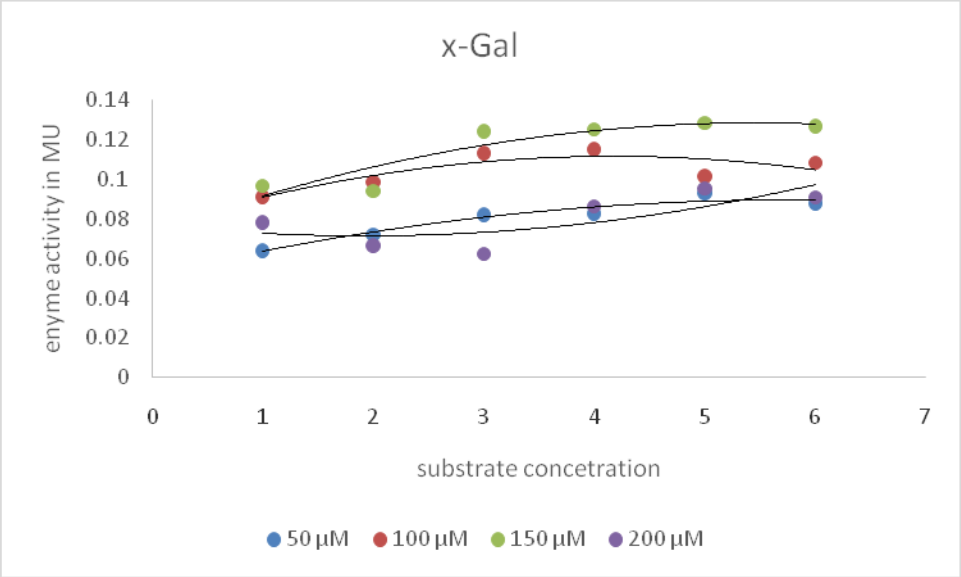
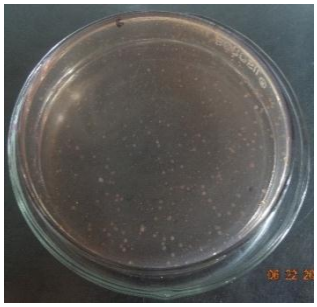
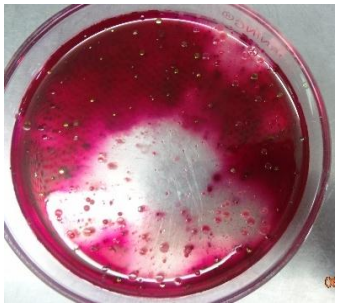


Figure: Effect of substrate X-gal concentrations on enzyme activity of *E. coli* (ATCC 19258)



Water samples treated with IPTG (37^0C)



Water sample without IPTG (37^0C)

Figure: Effect of IPTG on *E. coli* for the induction of the enzyme

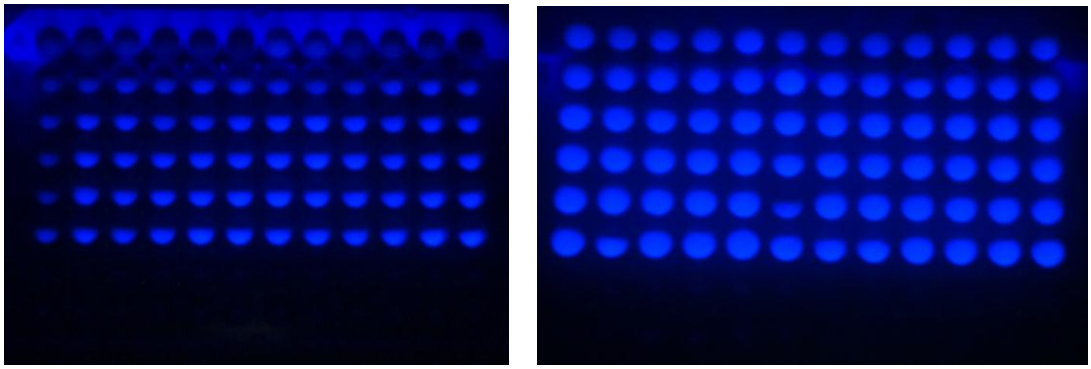


Figure: Fluorogenic substrate assay under UV light

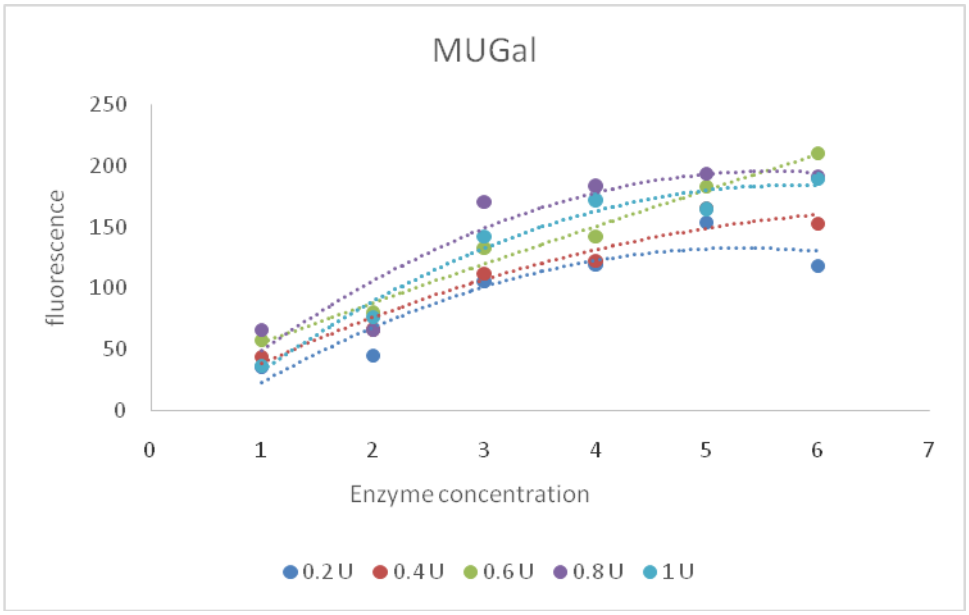


Figure: Fluorescence substrate assay of the enzyme beta D-galactosidase (Enzyme concentration)

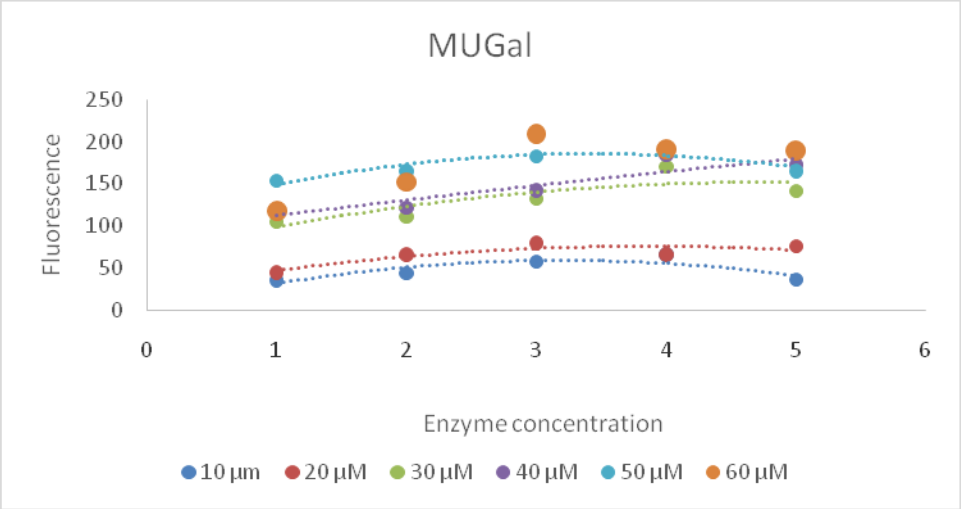


Figure: Fluorescence substrate assay of the enzyme beta D-galactosidase (Substrate concentration)

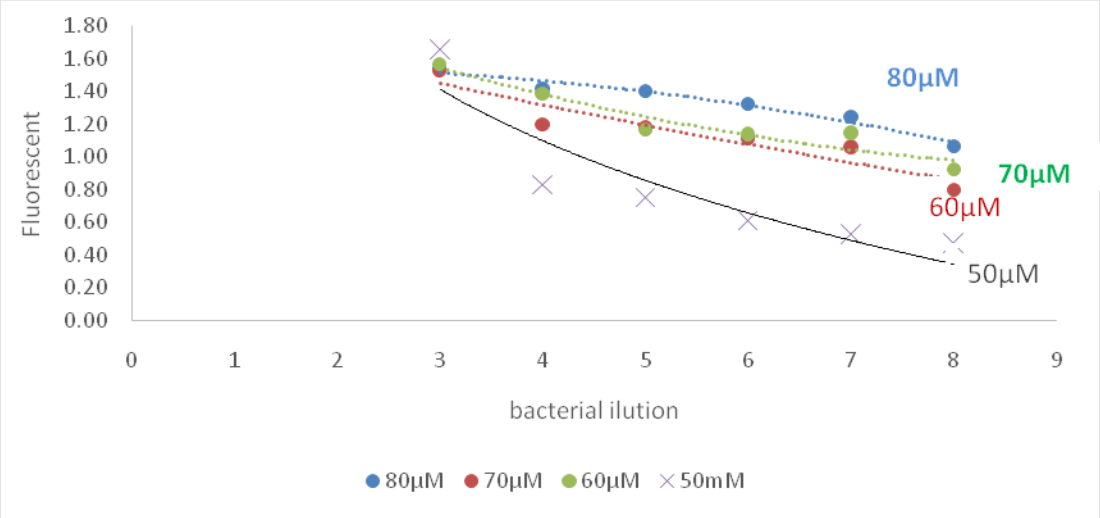


Figure: Enzyme activity of different concentrations of *E. coli* with a different concentration of the substrates

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