Recent Research in Science and Technology 2013, 5(2): 57-63
ISSN: 2076-5061
Available Online: http://recent-science.com/

Isolation, biochemical and molecular characterization of strains of coliforms from the water sample collected from Shivnath river

R. Senthil Kumar, M.G. Roymon and Jyothika Ann Ipe*

*Department of Microbiology, St. Thomas College, Bhilai 490006, India.

Abstract
Coliform bacteria include organisms like *Escherichia coli, Enterobacter sp., Klebsiella sp. and Citrobacter sp.*and are gram negative, facultative anaerobic, non-sporulating and lactose fermenting organisms. *E. coli* is used as the indicator organism for detection of faecal contamination of water. Conventional methods for the detection of Coliforms in water include microbial culture technique in lactose containing media, Biochemical characterization, study of cell morphology, colony morphology etc. These methods are time consuming with limited specificity. DNA based molecular techniques like 16S rRNA gene sequencing is highly specific. In this research work, the16S rRNA gene sequencing technique was used to characterize the two isolates from water samples of Shivnath River after the above said preliminary tests were conducted. The two strains of coliforms identified by this technique were *Escherichia coli* strain GA and *Enterobacter cloacae* strain AB6.

Keywords: Coliforms, 16S rRNA gene sequencing technique, indicator organism.

INTRODUCTION

Testing for individual disease causing agents are possible and is often done when there is a known or suspected occurrence of a waterborne disease. However in some cases officially unreasonable, to routinely observe for all disease causing bacteria, viruses, and protozoan that may be found in impure surface water. For routine water quality monitoring, nontoxic bacteria that occur in higher figures and originate from the same sources as the diseases causing bacteria are typically calculated.

Even though the presence of indicator bacteria does not demonstrate that pathogenic bacteria are present in the environment, the presence does explain the contamination by faecal material has occurred. The organic pollution is especially severe in the river waters due to the large density of population in river banks (Scialabba, 1998).

Direct release of domestic waste and leaching from poorly maintained septic tanks and improper management of farm waste are implicit as the major sources of waterborne diseases (Huttly, 1990). Sewage effluent has a wide range of pathogenic microorganisms which may pose a health hazard to human population, when they are discharged into the river waters (Borrego and Figueras, 1997).

Shivnath River present in Durg district, Chhattisgarh is one of the important rivers from domestic utilization point of view. The slum areas as well as urban areas consume the water from both the rivers either directly or in a distilled manner. It is a common mode of practice in these locations for the cattle to use these water bodies for their livelihood. Hence any mode of pollution at any level puts a direct impact on the populations.

Keeping in mind the above situation and importance of monitoring the pollution level of the river from indicator organism point of view, the present work was carried out with the following objectives:

- To enumerate Coliform bacterial population in domestic waste polluted water sample by employing MPN test method.
- Isolation of Coliform bacteria from domestic waste polluted water sample.
- Screening of Coliform having pathogenic nature.
- Antibiotic sensitivity test of the environmental isolate.
- Biochemical test of the isolates.
- Molecular Identification of strains of Coliform.

MATERIALS AND METHODS

Collection of water samples from the sites

The water samples were collected aseptically in autoclaved sample container from four different sites of Shivnath River, Durg, Chhattisgarh.

The Physical Properties such as Temperature and pH of water at the sites were noted. The water samples were processed immediately for the determination of Coliform bacterial population count and isolation of microorganisms.

Total Coliform count by Most Probable Number (MPN) Test

In order to assess the domestic pollution level in the study sites the Most Probable Number (MPN) Test was conducted for total Coliform count. The technique involves three successive steps namely, presumptive test, confirmatory test and completed test.

*Corresponding Author

Jyothika Ann Ipe
Department of Microbiology, St. Thomas College, Bhilai 490006, India.

Email: jyo_131@rediffmail.com

Isolation of Coliform by Membrane Filtration Technique

In the Membrane Filtration Technique 100 ml of the collected water sample was filtered through membrane filter with 0.22µm pore size filter paper by using vacuum filtration system and the filter paper was transferred on to 100ml of sterile Nutrient Broth and was incubated for 24 hours for enrichment. 0.1 ml of the enriched culture was then inoculated on EMB agar plate and incubated for 24hours at 37ºC. Plates were checked for bacterial colonies with greenish metallic sheen which is the characteristic feature of Coliform bacteria.

Fifteen Bacterial colonies with greenish metallic sheen were picked up from the mixed culture and were streaked on to the EMB agar plates to isolate pure culture. Each isolate was then streaked on Mac Conkey Agar to check lactose Fermentation by the isolates. Pure culture of the fifteen isolates was maintained on Nutrient agar medium for further characterization.

Biochemical characterization of the isolates

The biochemical tests performed include Indole production test, Methyl red test, Vogues-Proskauer test, Citrate Utilization test, Oxidase production test, Catalase production test, Triple Sugar Iron Agar test, Carbohydrate Fermentation test namely Fructose, Maltose, Dextrose and Sucrose. These biochemical tests were performed as per standard Microbiological methods (Cappuccino and Sherman, 2007).

Study of the nature of the environmental isolates on the basis of their pathogenicity

The pathogenicity nature of the environmental isolates was tested by streaking the isolates on Blood Agar plates. The plates were incubated for 48 hr at 37ºC and observed for the alpha, beta or gamma pattern of haemolysis of Red Blood Cells (RBC) by the bacterial isolates..

Antibiotic Sensitivity Pattern of the isolates

All the isolates were tested for antimicrobial resistance by the method of (Bauer *et al*., 1966) with antibiotic impregnated discs (Hi-Media Laboratory, Bombay, India). The following antibiotic discs with conc. of the drug as stated in the observation were used, Amoxicillin (AMX, 10µg, 25 µg and 30 µg), Nalidixic Acid (NA, 30µg), Chloramphenicol (C, 10 µg, 25 µg and 30µg), Tetracyclin (TE, 10 µg and 30µg), Gentamycin (GEN, 10µg and 30 µg) and Ampicillin (AMP, 10µg and 25 µg). The antibiotic test was performed as per CLSI guidelines.

The zones showing complete inhibition were measured and the diameters of the zones were measured to the nearest millimetre. By the antibiotic zone scale, the area of inhibition was measured for each antibiotic. Sensitivity of the isolates to each antibiotic was determined according to the chart provided by Himedia, Mumbai.

Molecular characterization of the two isolates (12 and 14)
Isolation of Genomic DNA

DNA was isolated and spooled onto a glass rod (or Pasteur pipette with a heat-sealed end). DNA was washed by dipping end of rod into 1 ml of 70% ethanol for 30 sec. DNA was resuspended in 100-200 µl TE buffer.

PCR amplification of 16s r-DNA

PCR amplifications of the 16S rRNA gene, from the purified genomic DNA, were carried out using the primer sets
27Forward (5´-AGAGTTTGATCCTGGCTCAG-3´) and
1492Reverse (5´-GGTTACCTTGTTACGACTT-3´)
PCR Conditions: (in MJ Research DNA Engine Tetrad)

95ºC	94ºC	55ºC	72ºC	72ºC
5 min	1:00 min	1:00 min	1:00 min	1:00 min
1 Cycle	30 Cycles			1 Cycle

Agarose gel electrophoresis

Electrophoresis was performed in a horizontal sub-marine apparatus. TE buffer was used as the tank buffer and electrophoresis was carried out for 30 minutes at constant voltage. The gel was visualized under UV transilluminator and photographed.

PCR product purification

The unpurified DNA sample (atleast 10-15 µl) was purified and the DNA pellet was dried and dissolved in 10-15 µl of Milli Q water.

Sequencing

The sequencing of the target gene was done using BigDye Chemistry, and performed as per the manufacturer's protocols (Applied Biosystems 3730xl DNA Analyzer) in our lab. Sequence data analysis was done using ChromasPro and Sequencing Analysis software.

16SrRNA gene Sequence Analysis

Sequence alignments provide a powerful way to compare novel sequences with the previously characterised genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments, regions of similarity may provide important clues to the function of uncharacterized nucleotides and proteins.

Phylogenetic tree Analysis

Phylogenetic tree Analysis is the technique of systematically demonstrating an evolutionary relationship between species. This method is useful to determine whether group of genes are related through a process of divergent evolution from a common ancestor or the result of convergent evolution. In the present study a Phylogenetic tree was constructed using BLAST tree tool.

RESULTS
Physical properties of water at the collection sites

The physical properties of the water at the four collection sites of the river Shivnath are recorded as follows:

Physical properties of water at the collection sites

Physical property	Site 1	Site 2	Site 3	Site 4
Temperature	29.5 ºC	29.7 ºC	29.9 ºC	29.8 ºC
pH	7.4	8.3	8.9	9.1

Total Coliform count by Most Probable Number (MPN) Test
Presumptive test

After performing the MPN test the Coliform bacterial populations in the four study sites were found to be in the range of 910-1600 CFU/ml for Shivnath river site by comparing the value of the positive test tubes (test tubes showing Acid and Gas production) with the standard MPN chart.

Presumptive test result

Sample No.	10ml	1ml	0.1ml	MPN INDEX
1	5	5	3	910
2	5	5	4	1600
3	5	5	4	1600
4	5	5	4	1600

Confirmatory test

When the positive tubes from the MPN test were streaked on EMB plates the Coliform characteristic features of greenish metallic sheen was found in the isolated colonies, thus confirming the presence of Coliform bacteria in the test sample.

Completed test

The positive isolates giving greenish metallic sheen on EMB Agar plates were inoculated on brilliant green lactose bile broth. All the tubes gave positive result, thus completing the Coliform test for the water samples.

Isolation of Coliform by Membrane Filtration Technique

The water samples collected from Shivnath River were used for this study. In Sample 1 the total number of colonies present in filter paper was 184 CFU per 100 ml, in sample 2 it was found to be 168 CFU per 100ml, in sample it was 172 CFU per 100ml and in sample 4 it was 187 CFU per 100ml.

Gram staining of the isolates

All the fifteen isolates gave the result of gram negative rods which is the characteristic feature of Coliform under oil immersion microscope.

Colony characteristics on MacConkey Agar

Out of the 15 isolates, 14 samples gave pink coloured colonies on MacConkey Agar showing lactose fermentation. While sample no. 11 showed negative result.

Biochemical characterization of the isolates
IMViC and TSIA Test result

Isolate no.	Indole Production Test	Methyl red test	Vogues-Proskauer test	Citrate Utilization test	Triple Sugar Iron Agar test A / G
1	+ ve	+ ve	- ve	- ve	+ ve/+ve
2	+ ve	+ ve	+ ve	- ve	+ ve/+ve
3	+ ve	+ ve	- ve	- ve	+ ve/+ve
4	+ ve	+ ve	- ve	- ve	+ ve/+ve
5	+ ve	+ ve	- ve	- ve	+ ve/+ve
6	+ ve	+ ve	- ve	- ve	+ ve/+ve
7	+ ve	+ ve	- ve	- ve	+ ve/+ve
8	+ ve	+ ve	- ve	+ ve	+ ve/+ve
9	+ ve	+ ve	- ve	- ve	+ ve/+ve
10	+ ve	+ ve	- ve	- ve	+ ve/+ve
11	- ve	- ve	+ ve	+ ve	- ve/-ve

12	+ ve	+ ve	- ve	- ve	+ ve/+ve
13	- ve	- ve	+ ve	+ ve	+ ve/+ve
14	+ ve	- ve	+ ve	+ ve	+ ve/+ve
15	- ve	+ ve	- ve	- ve	+ve/+ve

Sugar Fermentation, Oxidase and Catalase Test result

Isolate no.	Fructose utilization test A / G	Maltose utilization test A / G	Dextrose utilization test A / G	Sucrose utilization test A / G	Oxidase production test	Catalase production test
1	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve	+ ve
2	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve	+ ve
3	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/-ve	- ve	+ ve
4	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/-ve	+ ve	+ ve
5	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve	+ ve
6	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve	+ ve
7	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve	+ ve
8	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve	+ ve
9	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve/-ve	+ ve	+ ve
10	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve/+ve	+ ve	+ ve
11	- ve/-ve	- ve/-ve	- ve/-ve	- ve/-ve	+ ve	+ ve
12	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve/+ve	+ ve	+ ve
13	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve	+ ve
14	+ ve/+ve	+ ve/+ve	+ ve/+ve	+ ve/+ve	- ve	+ ve
15	+ve/+ve	+ve/+ve	+ve/+ve	+ve/+ve	+ ve	+ ve

Haemolysis pattern of the bacterial isolates on Blood Agar

All the 15 isolates showed gamma pattern of haemolysis of Red Blood Cells (RBC).

Antibiotic Sensitivity Pattern of the isolates

Isolate no.	AMX 10µg	AMX 25µg	AMX 30µg	NA 30µg	AMP 10µg	AMP 25µg	G 10µg	G 30µg	C 10µg	C 25µg	C 30µg	TE 10µg	TE 30µg
1	I	S	S	I	S	I	I	I	I	S	S	I	I
2	I	S	S	I	S	S	I	I	I	S	S	I	I
3	I	S	S	I	S	S	R	R	I	I	I	I	I
4	I	S	S	S	S	S	I	S	I	S	S	I	I
5	I	I	S	S	S	S	I	S	S	S	S	I	I
6	I	I	S	I	S	S	I	I	S	S	S	R	I

7	I	S	S	S	S	S	I	S	I	S	S	I	I
8	S	S	S	I	S	S	I	I	I	I	I	I	I
9	I	S	S	S	S	S	R	R	I	I	I	I	I
10	R	R	R	S	R	R	R	R	R	R	R	R	R
11	R	R	R	I	R	R	I	S	R	I	I	R	I
12	R	R	R	I	R	R	R	I	R	R	R	R	R
13	I	S	I	I	I	S	R	R	R	R	R	R	R
14	R	R	R	R	R	R	R	R	R	I	I	R	I
15	I	S	S	I	S	S	R	R	R	R	I	I	R

AMX - Amoxicillin, NA - Nalidixic Acid , C - Chloramphenicol , TE - Tetracyclin, GM - Gentamycin, AMP – Ampicillin, S – Sensitive, I – Intermediate, R – Resistant

Molecular characterization of the isolates

Identification of Bacterial Strain by 16SrRNA Gene Sequencing

Genomic DNA of Isolate No. 12 and 14 were isolated and Polymerase Chain Reaction was performed in Thermocycler to produce multiple copies of the 16SrRNA gene. The PCR product was run on 1.2% agarose gel electrophoresis along with DNA ladder mix and visualized under UV light.

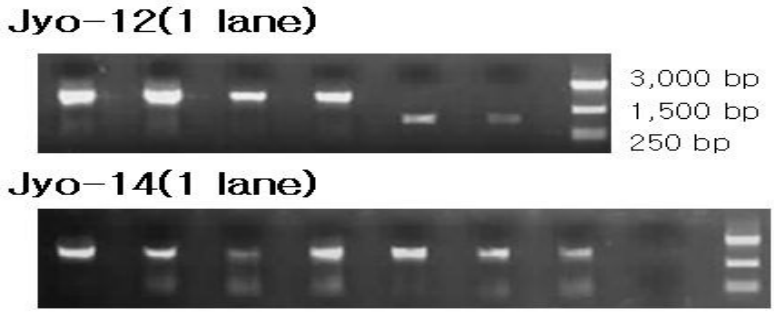
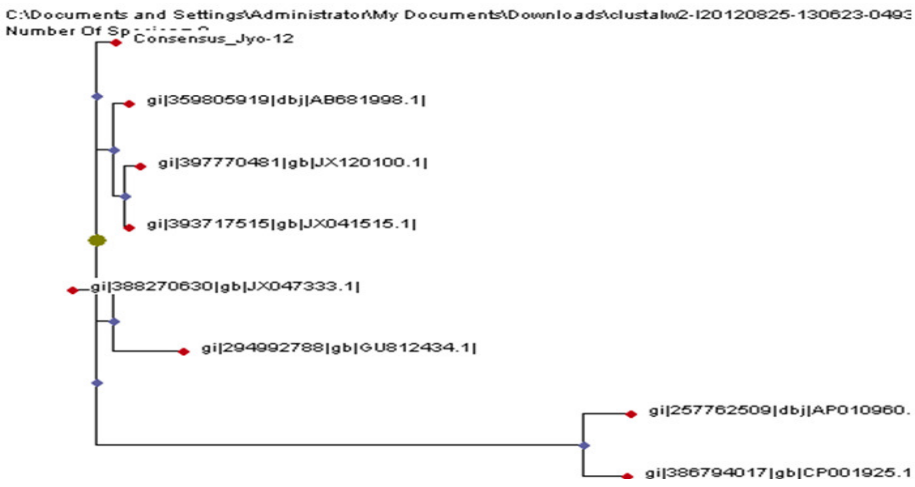
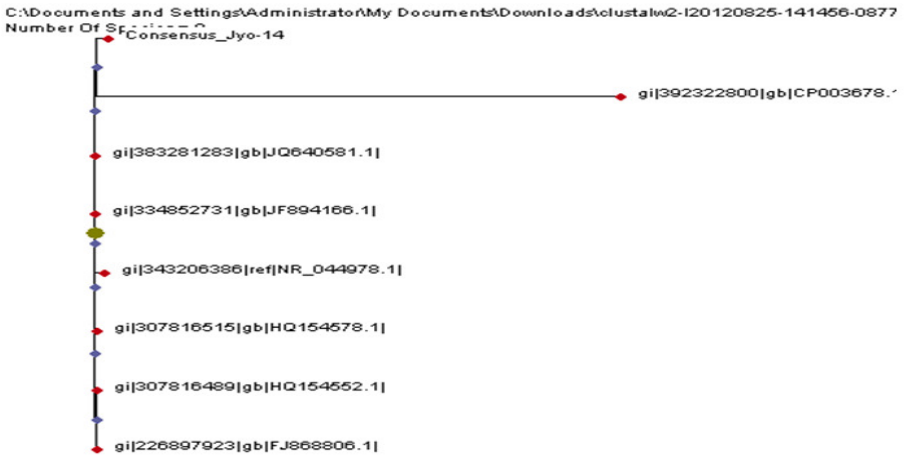


Fig. Amplified 16SrRNA gene of sample 12 and 14

From sequence obtained, the two Bacterial strains were ascertained its systematic position based on 16SrRNA gene sequence analysis and with the aid of computational programme, BLAST homology analysis was also carried out to compare with other 16SrRna sequences available in the GenBank of NCBI. The sequence analysis of 16SrRNA gene for the two isolates shows maximum homology (100%) with other *Escherichia coli* strain GA and *Enterobacter cloacae* strain AB6, respectively, from the database. The phylogenetic trees of the organisms were constructed in accordance with previous reports.



BLAST tree result for *Escherichia coli* strain GA



BLAST tree result for *Enterobacter cloacae* strain AB6

DISCUSSION

The water samples collected aseptically in autoclaved sample container from four different sites of Shivnath River, Durg, Chhattisgarh, were processed immediately for the determination of Coliform count and isolation of microorganisms. The Physical Properties such as Temperature and pH of water at the sites showed a slight alkalinity and a slightly higher temperature in the river water as compared to the result quoted by Shittu, *et. al,* 2008 and Olatunji *et.al.* 2011.

The MPN (Most Probable Number) test showed a count of 910-1600 CFU/ml of coliforms in the water sample which was less than the result shown by Shittu, *et. al,* 2008 and Olatunji *et.al.* 2011, for river water. The presence of coliforms were confirmed by plating from positive tubes of presumptive test on to the EMB (Eosin Methylene Blue) Agar plates which showed green metallic sheen and was further completed by inoculation into BGLB (Brilliant Green Lactose bile Broth). Using MFT (Membrane Filtration Technique) the number of coliforms was counted on EMB plates and was found to be in the range of 168 - 187 CFU per 100ml which was similar to the results obtained by Ouseph *et. al.,* 2009 . Comparing the CFU/ml obtained from MPN and MFT, MPN shows higher detection which is similar to the result obtained by Jacobs *et.al.,* 1986.

All the fifteen isolates gave the result of gram negative rods in the Gram's staining. It was observed that except Isolate no. 11 all the fourteen isolates were able to ferment lactose in MacConkey Agar plate. The Biochemical Tests performed to characterize the Isolates were Indole production test, Methyl red test, Vogues-Proskauer test, Citrate Utilization test, Triple Sugar Iron Agar test Sugar Fermentation (i.e. Fructose, Maltose, Dextrose and Maltose), Oxidase and Catalase Test. All the 15 bacterial isolates showed positive result for catalase production test similar to the results obtained by Seema *et.al., 2009* and gamma pattern of haemolysis for all the 15 Isolates on Blood Agar while the results of other tests varied among the Isolates.

The Antibiotic Sensitivity Pattern of the isolates were tested against antibiotic discs with concentration of the drug as Amoxicillin (AMX, 10µg, 25 µg and 30 µg), Nalidixic Acid (NA, 30µg), Chloramphenicol (C, 10 µg, 25 µg and 30µg), Tetracyclin (TE, 10 µg and 30µg), Gentamycin (GEN, 10µg and 30 µg) and Ampicillin (AMP, 10µg and 25 µg). The Antibiotic Sensitivity Pattern was determined by comparing the zones of inhibition (measured in mm) with the reference chart as Sensitive, Intermediate and Resistant. The isolates were sensitive to Amoxicillin and Ampicillin, Intermediate to Nalidixic Acid, Gentamycin, Chloramphenicol and Tetracycline while resistant to Gentamycin which was a different antibiotic susceptibility pattern as compared to the results obtained from Prakash, 2008.

The two isolates – Isolate no. 12 and 14 were further characterized by the molecular technique 16SrRNA sequencing since these two Isolates showed no sensitivity to the antibiotics tested. The 16SrRNA sequencing was carried out in the way similar to Kenneth *et.al.,* 2007 and Leonid *et.al.,* 2002, i.e. by first isolating the Genomic DNA then amplifying the 16SrRNA gene by PCR technique and sequencing it in automated sequencer and finally identified as *Escherichia coli* strain GA and *Enterobacter cloacae* strain AB6, respectively, by the BLAST homology analysis from the database available in GenBank of NCBI. The evolutionary relationship of the isolates with the previously established strains were analysed by constructing phylogenetic tree.

CONCLUSION

The characterization of organism by the molecular technique 16SrRNA sequencing is specific and reliable method. It is carried out by first isolating the Genomic DNA then amplifying the 16SrRNA gene by PCR technique and sequencing it in automated sequencer and finally identifying by the BLAST homology analysis from the database available in GenBank of NCBI. The evolutionary relationship of the organisms with the previously established strains can be analysed by constructing phylogenetic tree. In the above work Sample no. 12 and 14 were identified as *Escherichia coli* strain GA and *Enterobacter cloacae* strain AB6, respectively.

ACKNOWLEDGEMENT

I wish to extend my earnest and profound thanks to Dr. R. SENTHIL KUMAR, M.Sc., Ph.D., Head, Department of Microbiology, J.J. College of Arts and Science, Pudukottai and Dr. M.G.Roymon, Head, Department of Microbiology, St. Thomas College, Bhilai, C.G., for giving me such a wonderful opportunity to learn and carry out this study and also for all his guidance and consistent support.

REFERENCES

[1] Bauer A. W., Kirby W. M. M., Sherris J. C. and Turck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 36:493-496.

[2] Borrego J.J. and Figueras M.J.1997. Microbiological quality of natural waters. *Microbiol*. 13: 413–426.

[3] Huttly S. R.1990. The impact of inadequate sanitary condition on health in developing countries. *World Health Stat*. 43: 118-126.

[4] James Cappuccino and Natalie Sherman. 2007. *Microbiology- A laboratory manual* 8th Edition, Benjamin-cummings Publisher.

[5] Kenneth T. Belt, Christina Hohn, Aiah Gbakima and James A. Higgins. 2007. Identification of culturable stream water bacteria from urban, agricultural, and forested watersheds using 16SrRNA gene sequencing. *Journal of water and health*, 5(3), 395 – 406

[6] Leonid A. Kulakov, Morven B. McAlister, Kimberly L. Ogden, Michael J. Larkin and John F. O'Hanlon. 2002. Analysis of Bacteria Contaminating Ultrapure Water in Industrial Systems. *Applied and Environmental Microbiology*. 68(4) 1548–1555

[7] Olatunji M. Kolawole, Kolawole T. Ajayi , Albert B. Olayemi and Anthony I. Okoh. 2011. Assessment of Water Quality in Asa River (Nigeria) and Its Indigenous *Clarias gariepinus* Fish. *Int. J. Environ. Res. Public Health*, *8*, 4332-4352

[8] Ouseph P.P., Prasanthan V., Abhilash P.P., Udayakumar P.2009. Occurrence and distribution of some enteric bacteria along the southern coast of Kerala. *Indian J. Mar. Sci*. 38(1), 97–103.

[9] Prakash K.P.2008. Epidemiology and Antimicrobial Resistance of EntericPathogens in Dhahira Region, Oman *Iranian J Publ Health,* 37(3), 60-69

[10] Scialabba N. 1998. Integrated Coastal Area Management and Agriculture, Forestry and Fisheries. *FAO Guidelines*.

[11] Seema Tharannum, Sarah Sunitha, Nithya J., Chandini M., Vanitha J., Manjula T.S. and Shyam Sundar C.2009. Molecular Confirmation of the presence of coliforms in drinking water using Polymerase Chain Reaction. *Kathmandu University Journal of Science, Engineering and Technology*. 5(II), 130- 136.

[12] Shittu O.B., Olaitan J.O. and Amusa T.S. 2008. Physico-Chemical and Bacteriological Analyses of Water Used for Drinking and Swimming Purposes in Abeokuta, Nigeria. *Afr. J. Biomed. Res. 11: 285- 290*