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Novel primer designing and PCR-AFLP approach for an expeditious detection of coliforms in potable waters

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Molecular techniques involving 16S rRNA gene have long been proved to be a mainstay of sequence-based bacterial analysis and enhance the competence of bacterial removal in drinking water and food. The main goal of this analysis was to reduce the time of detection of total coliforms by developing 16S rRNA based DNA markers by targeting variable region in the 16S rRNA gene position of V2 and V9. Coliform specific primers (189F and 1447R) were designed to amplify total coliform with an amplicon size of 1300 bp. The PCR product was later digested with Hind III and BseRI (restriction enzymes) to differentiate the type of contamination caused by fecal and non-fecal coliforms respectively. The digested amplicons were run on agarose gel electrophoresis and contamination levels were estimated based on the respective band pattern. This method can be applicable to know the coliform contamination levels of potable waters, in food and beverage industries within a short period of time. To our knowledge, this is the first report on newly designed primers which not only amplify coliform bacteria, followed by various restriction digestions of these amplicons but also provides unique band patterns to identify coliforms at genus level.

Keywords: 16S rRNA, Coliform, Molecular marker, Primer design, Restriction enzymes

Food and water contamination with coliform bacteria continues to be a major problem due to which global estimate of 1, 25 million deaths and 75 million life-years adapted to disabilities (Forouzanfar *et al*., 2016). The identification of indicator bacteria is one of the main ways of determining the efficacy of methods of water disinfection (Field *et al*., 2003). The most important indicator bacteria are *Escherichia coli* (*E. coli*) and other thermotolerant coliforms when present suggests inadequate disinfection process and also signify recent and regular contamination of water with humans and animal feces (Rodríguez *et al*., 2012). Coliforms resistant to thermos (Chandra *et al*., 2020), except for *E. coli* can reach drinking water through industrial waste water, pollute water and under soil and water degradation (Sahlstrom *et al*., 2004). Culture dependent methods are too laborious to sort out the pathogens from the bacteria surrounding them (Shanker *et al*., 2020), therefore, it is exceptionally necessary to establish a dependable technique for prompt identification and quantification of the fecal pollution source independent of fecal coliforms being cultivated (Moore *et al.*, 2001). A diversity of culture-independent molecular-based techniques has been developed that substantially boosts the speed, reliability, identification and quantification output of fecal sources (Okabe *et al*., 2007). Classically, the approaches used are based on the identification and quantification of different genome segments of pathogen (DNA or RNA) which allows researchers to exclusively and quickly detect pathogens in a single assay (Maynard *et al*., 2005; Straub *et al*., 2005; Marcelino *et al*., 2006). Molecular techniques that are mostly applied are based on protocols of nucleic acid amplification, of which polymerase chain reaction (PCR) is the most frequently used. Quantitative PCR (qPCR) and Quantitative reverse transcriptase PCR (qRTPCR) are rapidly becoming established in the environmental sector (He and Jiang, 2005). Besides PCR, other method available to amplify nucleic acids is Nucleic Acid Sequence-Based Amplification (NASBA) (Cook, 2003; Goodwin and Litaker, 2008). However, these methods do not provide information about the infectivity of pathogen or the indicator detected or the level of risk for population. Disinfection of water by UV and chlorine treatments may reduce the numbers of microbial particles quantified by qPCR and qRT-PCR, if severe treatments are applied. Nevertheless, the molecular techniques existing today are constantly being sophisticated to standardize and make them

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appropriate to a variety of matrices, improve their subtlety, time and steps that are required in analytical process.

As a result, a number of potentially more biased methods involving sequence analysis of bacterial 16S ribosomal RNA gene containing "hypervariable regions" with considerable sequence variation among different bacterial species have been used extensively to classify bacterial species and carry out taxonomic studies (Choi *et al*., 1996; Clarridge, 2004; Munson *et al*., 2004; Petti *et al*., 2005; Schmalenberger *et al*., 2001; Van de Peer *et al*., 1996). Generally, most bacteria contain some preserved stretches flanking these hypervariable regions, enabling PCR amplification of target sequences using universal primers (Baker *et al*., 2003; Lu *et al*., 2000; McCabe *et al*., 1999; Munson *et al*., 2004). The nine hypervariable regions spanned nucleotides 69-99, 137-242, 433-497, 576-682, 822-879, 986-1043, 1117-1173, 1243-1294 and 1435-1465 for V1 through V9 respectively numbering based on the *E. coli* system of nomenclature (Brosius *et al*., 1978). Chakravorty *et al*., (2007) demonstrated that the hypervariable regions V2, V3 and V6 contain maximum nucleotide heterogeneity among which V6 is the shortest hypervariable region with maximum degree of sequence heterogeneity. They have also reported that V1 is the best target for differentiating *S. aureus* with potentially pathogenic CONS. V2 and V3 appeared to be excellent targets for speciation among the common *Staphylococcal* and *Streptococcal* pathogens as well as *Clostridium* and *Neisseria* species, with V2 especially useful for speciation of *Mycobacterium* sp. and for designing specific probes to detect *E. coli* O157:H7. V3 appears to be especially useful for speciation of *Haemophilus* sp. V6 is the best target for the development of specific probe-based PCR assays to identify and distinguish the CDC select agents that are potential bio-terrorism agents.

Techniques involving single variable region that can categorized bacterial species when present in single or insufficient are most commonly employed (Becker *et al*., 2004; Bertilsson *et al*., 2002; Kataoka *et al*., 1997; Marchesi *et al*. 1998; Maynard *et al*., 2005; Rothman *et al*., 2002; Yang *et al*., 2002; Stohr *et al*., 2005; Varma-Basil *et al*., 2004). In the present research, focus has been put on rapid detection of coliforms by three main steps, First, collection and trapping the bacteria cells by membrane filter method, then the isolation of total genomic DNA from the trapped bacterial cells followed by PCR amplification by newly designed coliform specific primers and the third step includes restriction of digestion of amplified products for genera-level detection of coliform bacteria. The proposed study would be useful in evaluating the drinking water quality in India's rural and urban areas in terms of bacterial pollutants that are responsible for high risk of infection. Observations on the assessment of drinking water quality and data on human health scenarios in this area have been reported and further research on the health issues of people consuming polluted drinking water is needed to monitor the effect of such water on people. Accordingly, given the importance of these hypervariable regions in 16S rRNA in the detection of coliforms, the current study was used to develop new coliform specific primers and AFLP analysis for both rapid detection of pathogens in water and food that would minimize the detection time.

Materials and Methods

Standard Cultures

Standard strains used for the present study were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. These include *Escherichia coli* MTCC 1687, *Enterobacter aerogenes* MTCC 111, *Klebsiella pneumoniae* MTCC 3384, *Citrobacter freundii* MTCC 1658, *Serratia marcescens* MTCC 97, *Yersinia enterocolitica* subsp. *enterocolitica* MTCC 4857 (Fig. 1).

Collection of water samples

Water samples were collected from two different reservoirs *i.e*. Ramanpadu and Koilsagar of Mahabubnagar, Telangana state, India. The samples were collected in 1 L sterile water bottle, labeled with all required details like the source of water, time and date of sample collection (Shanker *et al*., 2019; Volokhov *et al*., 2007). The collected samples were

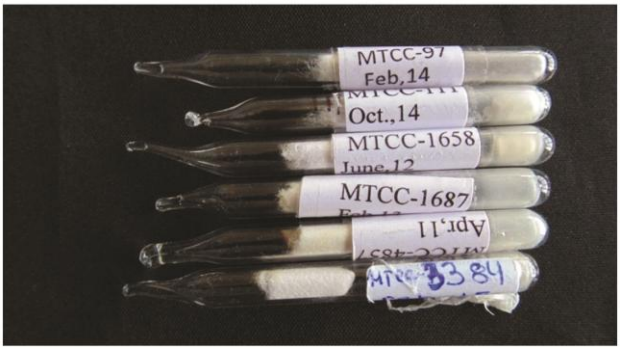

Fig. 1 — Standard cultures of coliform bacteria from MTCC

then transported to research laboratory, Department of Microbiology, Palamuru University, Mahabubnagar under cold stored conditions,–protected from light within 6 h of its collection for further studies.

Membrane filtration

Bacterial cells were collected by filtering 100 mL of water sample by using vacuum manifold through polycarbonate filter with 47 mm diameter, pore size 0.2 µM. To avoid possible contamination, the entire process was performed in a laminar flow unit (Fatemeh *et al*., 2014).

Extraction of DNA from Cultures

Trapped bacterial cells were used to extract genomic DNA using commercial kits (Hi Media column based DNA extraction kit) as described previously (Pindi *et al*., 2013). To ensure efficiency and suitability, the Nano Drop (thermofisher) method was used to test 5 µL of the extracted DNA by electrophoresis on 1.5 % agarose gel in 1X TAE buffer and the consistency and quantity of the DNA was determined.

Primer Designing and PCR amplification

Primers were designed by using 150 different group of bacterial 16S rRNA sequence downloaded from Ribosomal data base and multiple sequence alignment was performed by a CLC sequence viewer 8.0 program to define the area of high variable target priming within the gene. *In silico* the designed primer were tested by software www.bioinformatics.org. The oligonucleotide primers Coliform Specific Forward (CSF) 189F– 5′ AAYSTCGCAAGAAGWGG 3′ and Coliform Specific Reverse (CSR) 1447R 5′ TGAATCACAAGTGGTAGCGC 3′ have been used to assess the specificity of the PCR primers (as shown in Fig. 2) and coliform and non coliform bacterial by using standard cultures. PCR reaction, each 20 µL PCR reaction contains 10 µL of Master Mix (KAPA SYBR FAST qPCR Master Mix (2X) Kit), PCR grade water 7 µL, 5 pmol of forward primer 1 µL, 5 pmol of reverse primer 1 µL, DNA template 30-50 ng 1 µL were used for the amplification reaction (Martineau *et al*., 1998).

Optimization of annealing temperature of primers

Gradient PCR with annealing temperatures ranging from 51°C to 57°C were performed to optimize the annealing temperature (Fricker and Fricker., 1994). PCR cycle, denaturation, annealing and extension were carried about at 95°C for 5min, 51.4°C for 30 sec, 72°C for 1min respectively and this was repeated for 30 cycles. Final extension for 2 min was performed at 72°C. The PCR sample (product) was

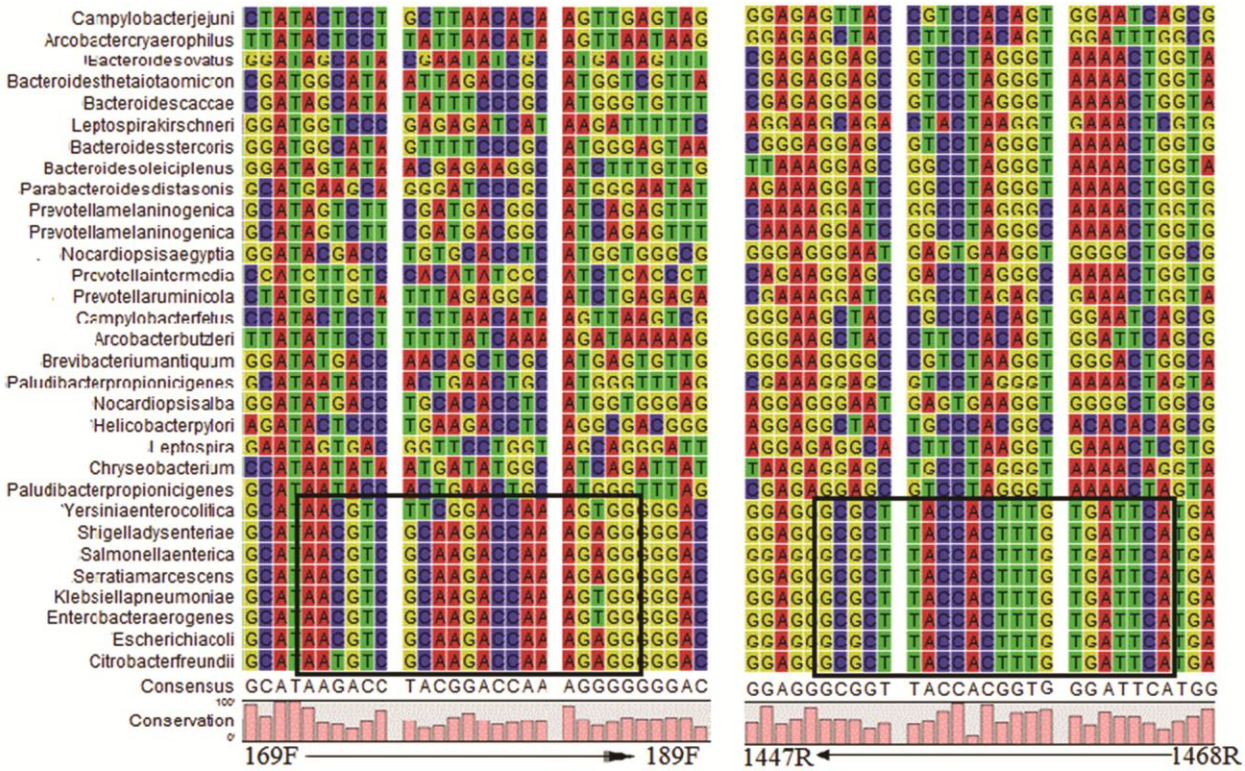

Fig. 2 — Coliform specific primers (A) Forward; and (B) Reverse

analyzed in 1X TAE buffer by gel electrophoresis of 5 µL of the amplified DNA *via* a 2 % agarose gel Bio Rad (Jothikumar *et al*., 2003). The drug band identification was developed using the 100 bp (NEB # B7025, Biolabs) DNA ladder molecular weight marker (Figs 3-6).

2.7 Restriction digestion of the amplified products (Total AP with RE):

Restriction digestion of the amplified PCR products was performed at a volume of 10 µL of PCR product, 2.0 µL of 10X digestion buffer, 1-2 µL HindIII (Thermo Scientific, India) and BseRI (Bio Labs, New England) Restriction enzymes and 7.0-8.0 µL Nuclease Free Water, total volume 20 µL. Then the samples were incubated for the digestion at 37°C in a water bath for 1-2 h. They were loaded on to the 2.0 % (*w/v*) agarose gel electrophoresis gel after the PCR products restriction digestion and operated at 60 V for 1-2 h. Finally, the bands solved under the BioRad gel doc method were observed.

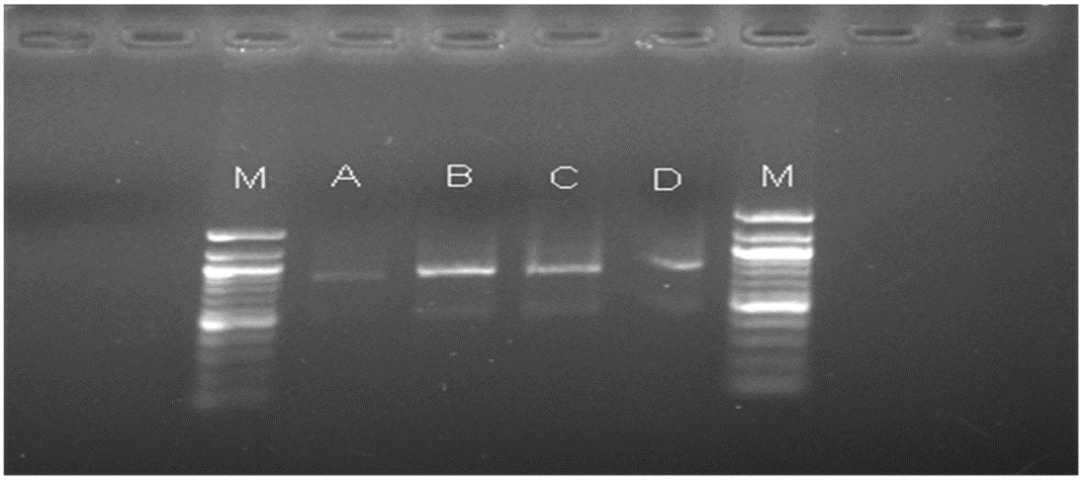

Fig. 3 — Optimization of primer annealing temperature M= Marker, A=51.6°C, B= 51.4°C, C=51.2°C, D= 51.0°C. *In Silico* finding of restriction sites

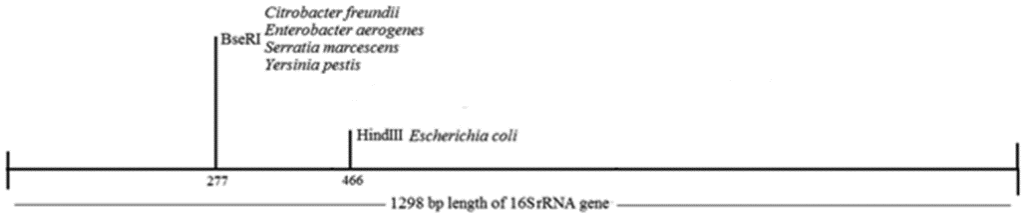

Fig. 4 — Restriction map of PCR product

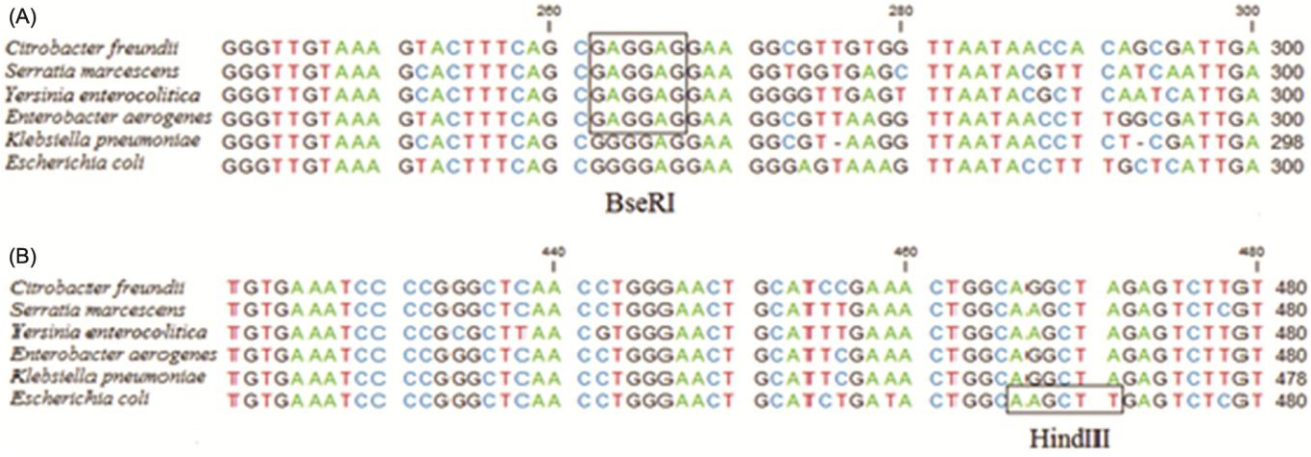

Fig. 5 — Alignment of 5 coliform bacteria with (A) BseRI Restriction site; and (B) HindIII Restriction site

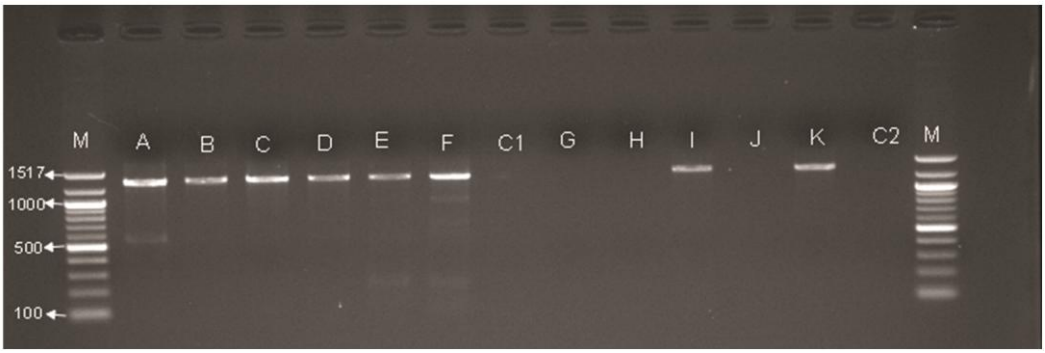

Fig. 6 — Testing of new coliform primers with standard cultures: M= Marker, A= *Citrobacter freundii*, B= *Enterobacter aerogenes*, C= *Escherichia coli*, D= *Klebsiella pneumonia*, E=*Serratia marcescens*, F=*Yersinia enterocolitica*, C1 & C2=Negative Control, G, H, I & J=Field water samples-1, 2, 3 & 4, K= Positive control

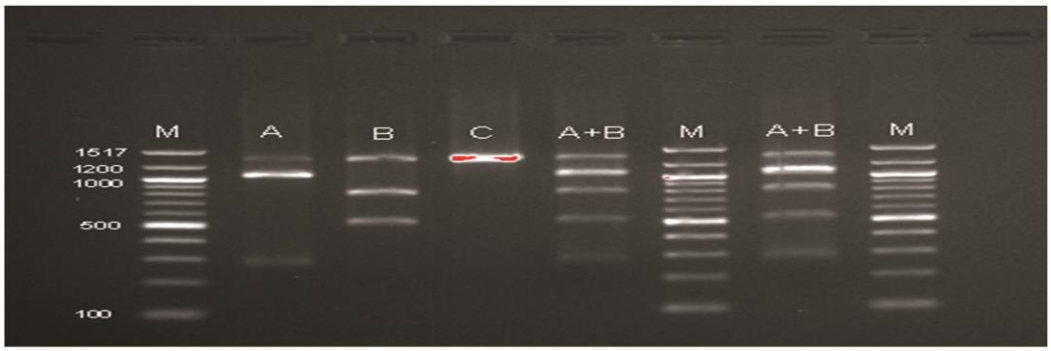

Fig. 7 — Restriction digestion of PCR product of 1300 bp: M=Marker, A=BseRI, B=HindIII, C= Control, A+B=Restriction digestion of A & B

Results

PCR-AFLP

The coliform specific PCR method was used for six genera of MTCC standard coliform cultures. Genomic DNA of standard strains and the heterologous DNA isolated from 4 different potable samples were subjected for PCR amplification and purified by using gel elution kit. Genomic DNA was isolated (Pindi *et al*., 2013) from the six pure cultures of *Escherichia coli* MTCC 1687, *Enterobacter aerogenes* MTCC 111, *Klebsiella pneumoniae* MTCC 3384, *Citrobacter freundii* MTCC 1658, *Serratia marcescens* MTCC 97, *Yersinia enterocolitica* subsp. *enterocolitica* MTCC 4857 of MTCC culture bank, Chandigarh.

The extracted DNA was amplified with newly designed coliform specific universal primers 189 F` and 1447 R` and the produced PCR product was run on an agarose gel of 1 percent and the expected product size of 1298 bp was observed using WEB 100 bp ladder (Fig. 6). Six-sample PCR products were purified with the use of gel elution kit (HiPurATM fast gel purification kit-MB539-20PR). The purified products were then digested with respective restriction enzymes like (NEB) BseRI, HindIII, to determine the amplified fragment length polymorphism (AFLP) that can be used to identify specific coliform bacteria. The coliform specific PCR products were digested with several restriction enzymes (Abtahi *et al.*, 2008; Khatib *et al.*, 2002) which is in accordance with the present work. Restriction enzyme HindIII showed specific restriction digestion with *E.coli* whereas, BseRI is present in *C. freundii*, *E. aerogenes*, *S. marcescens*, *Y. enterocolitica* and absent in *E. coli* (FigS 7 and 8).

Discussion

PCR method has been frequently documented for the identification and discernment of microbes in foods, soils, and sediments, while its application in drinking water is very recent (Trevors and van Elsas, 1995). Sequence analysis of preserved "household" genes *i.e*., 16S rRNA sequences of bacteria is currently well known for the study of phylogenetic relationships and the identification of bacterial species according to their divergence and the creation of determinative hybridization

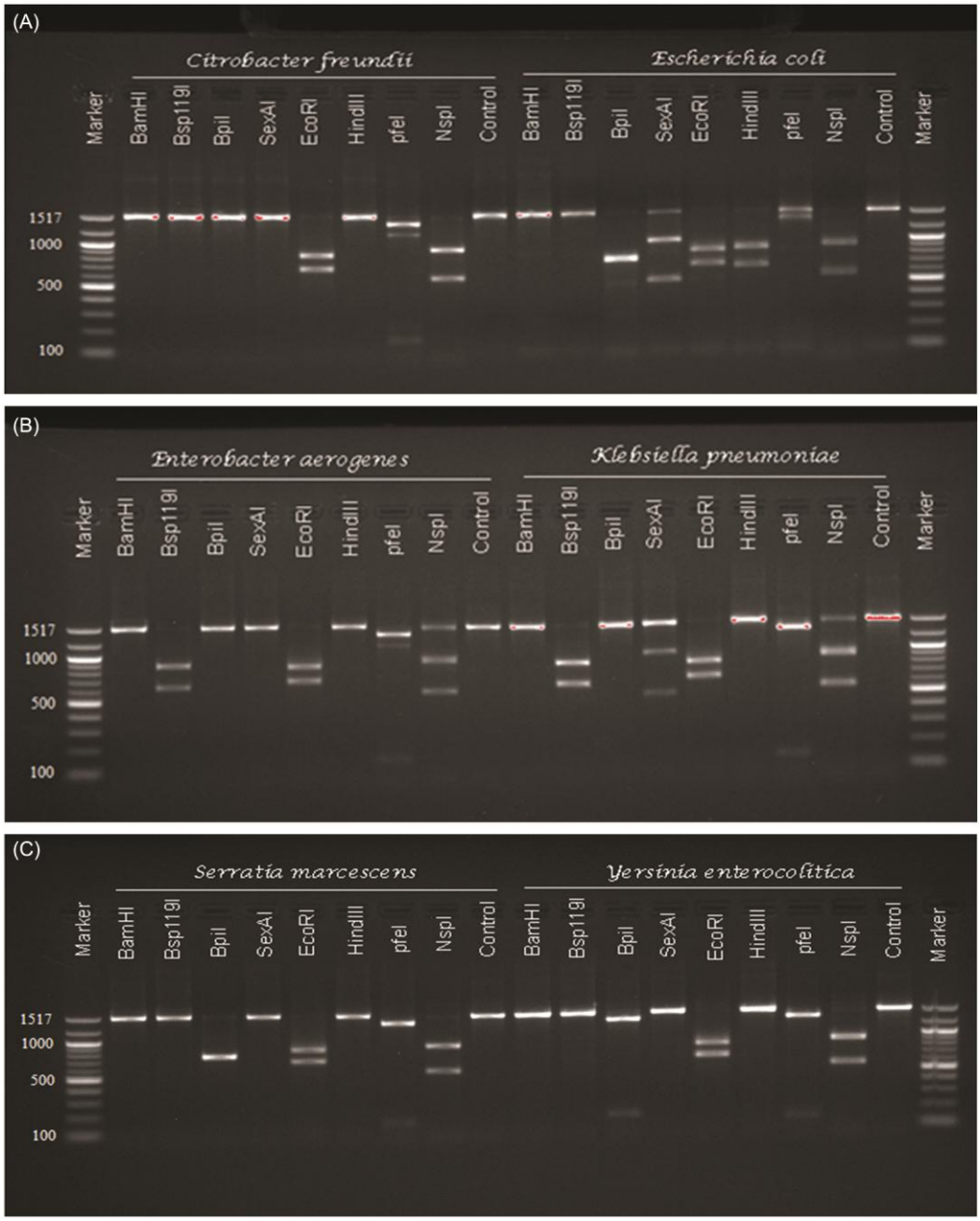

Fig. 8 — Restriction digestion of 1500bp PCR product of (A) *Citrobacter freundii* and *Escherichia coli*; (B) *Enterobacter aerogenes* and *Klebsiella pneumonia*; and (C) *Serratia marcescens* and *Yersinia enterocolitica*

samples in clinical practice and scientific research (Amann *et al*., 1995, Clarridge, 2004, Petti *et al*., 2005). It has been difficult to develop coliform primers since this category, as described by the water industry, is large, containing several genera that excludes some closely related groups. As a result, the primers must be accurate enough not to identify non-coliforms relevant to phylogenetics (Bej *et al*., 1990, 1991).

The present study was aimed to reduce the detection time of coliforms by developing 16S rRNA based DNA markers by targeting variable region in the 16S rRNA gene position of V2 and V9 coliform specific primers (189 F and 1447 R) and to amplify total coliform with an amplicon size of 1300 bp. These primers were developed by using the tool CLC sequence viewer 8.0 and has shown unique specificity towards coliforms when compared with coliform and

non coliform bacterial species by using online bioinformatics software www.bioinformatics.org. There are enough reports that show the importance of these variable regions in the identification of microbes. It is known that *E coli* are characterized by single nucleotide polymorphism (SNP's). O157 Sakai coli strains K-12 MG1655 were present in the V1, V2, V6 and V9 vector regions (Johnson *et al*., 2019). Kataoka *et al*., and Stackebrandt *et al*., previously described that such hypervariable regions are ideally suited for the design of Streptomyces sp. recognition probes. (Kataoka and others, 1997; Stackebrandt and others, 1991). V2 region average length was 100 bp which was the target region for primer designing for coliform, V2 can distinguish between the closely research showed that the initial 500-1500 bp sequence of 16S rRNA was adequate to differentiate between bacteria (Clarridge, 2004). These 16S rRNA hypervariable regions were also useful in detecting bacterial species by PCR techniques and concluded that the V4 and V5 region was functional (Schmalenberger *et al*., 2001). Similarly in other studies V3 and V6 regions were amplified to identify *Escherichia coli* O157:H7, *Klebsiella pneumoniae, Serratia marcescens, and Yersinia pestis,* when distinguishing between the closely related enterobacteriaceae, V3 seemed better than V2. *Pneumoniae*, E. Chakravorty *et al*. (2007). Kerrigan *et al*. amplified the hypervariable V4 and V6 regions of the 16S rRNA gene and clustered 97 percent associated sequences into taxonomic operating units (OTUs) to check for diversity and population composition. The OTU richness with the V6 tag is much higher than with the V4 tag, and consequently the OTU-level group composition between the two tags is very different (Kerrigan *et al*., 2019).

Every mismatch, irrespective of its location within the primer sequence, will result in a decreased thermal stability of the primer-template duplex, thus potentially affecting PCR specificity. However, mismatches located in the 3′ end region (defined as the last 5 nucleotides of the 3′ end region) of a primer have significantly larger effects on priming efficiency than more 5′ located mismatches, (Kwok *et al*., 1999, Bru *et al*., 2008, Christopherson *et al*., 1997, Klein *et al*., 2001, Whiley and Sloots 2005) since 3′ end mismatches can disrupt the nearby polymerase active site (Johnson and Beese 2004; Beard *et al*., 2004).

Amplification of conserved sequence region of 16S rRNA in different coliform bacteria by newly designed coliform specific PCR technique was sent to DNA sequencing. However, in this method only one set of primers and eight restriction enzymes were used for the detection of 6 genera of coliforms instead of using probe or sequencing. Restriction enzymes were selected so that they should have unique restriction site length that differ from coliform bacteria, moreover these should not possess common restriction sites for coliform bacteria. Restriction site for BseRI RE enzyme for coliform 451 but same site for *Bacillus licheniformis* 677, *Clostridium perfringens* 643, *Campylobacter fetus* 1156, *Erwinia amylovora* 847, *Proteus mirabilis* 1024, *Mycobacterium tuberculosis* 754, baceroid cysticercosis 1449, *Lactobacillus delbrueckii* 688, *Ralstonia mannitolilytica* 79, *Azotobacter chroococcum* 76, *Micromonospora halophytica* 733, *Acetobacter pasteurianus* 201, *Agrobacterium vitis* 81. HindIII site 466 for *Escherichia coli*, *Clostridium botulinum* 62, *Bacillus subtilis* 542, *Enterococcus faecalis* 1 296, *Morganella morganii* 70, *Staphylococcus epidermidis* 1117, *Mycobacterium smegmatis* 199, *Bacteriod esoleiciplenus* 577, *Corynebacterium diphtheria* 437, *Bacillus megaterium* is at 68, *Arthrobacter citreus* 195, *Campylobacter fetus* 65 position. Restriction digestion of this region by different restriction enzymes showed different restriction patterns with MTCC standard 6 genera of coliforms as well as local isolates of different drinking water samples. No specific changes have been observed in restriction patterns of local isolates in comparison with MTCC bacteria of coliforms, and not amplified any other bacteria as taken as controls. Therefore, coliform specific PCR followed by restriction digestion could be considered as a simple, sensitive, and swift method for the detection and identification of coliform bacteria (Girones *et al.*, 2010; Martineau *et al.*, 1998). Nested PCR protocols have been used to detect *E. coli.* in drinking water, (Juck *et al*., 1996) and some pathogens (Delabre *et al*., 1997), while Waage *et al*. (1999a, b) used them to detect low numbers of *Salmonella* spp. and *Y. enterocolitica* cells in water.

Interestingly, when BseRI and HindIII restriction enzymes were able to differentiate the contamination level of water by fecal coliform and non fecal coliform contaminants, HindIII shown only one specific band patterns of *Escherichia coli,* a fecal coliform and BseRI cut the bands in 4 genera of non fecal coliforms such as *Citrobacter freundii, Enterobacter aerogenes, Serratia marcescens,*

Table 1 — Band pattern length

	Citrobacter freundii	*Enterobacter aerogenes*	*Escherichia Coli*	*Klebsiella pneumoniae*	*Serratia marcescens*	*Yersinia enterocolitica*
BseRI (gaggag)	277/1023	277/1023	X	X	277/1023	277/1023
HindIII (aagctt)	X	X	466/834	X	X	X

Table 2 — Band pattern length with 8 restriction enzymes

	Citrobacter freundii	*Enterobacter aerogenes*	*Escherichia Coli*	*Klebsiella pneumoniae*	*Serratia marcescens*	*Yersinia enterocolitica*
BamHI (ggatcc)	X	X	X	X	X	X
Bsp119I (ttcgaa)	X	628	X	623	X	X
Bpil (gaagac)	X	X	741	X	742	X
SexAI (gaattc)	X	X	972	969	X	X
EcoRI (gaattc)	662	665	663	660	664	647
HindIII (aagctt)	X	X	637	X	X	X
pfeI (gawtc)	1326 & 1463	1329 & 1466	1327 & 1464	1324 & 1461	1328 & 1465	1312 & 449
NspI (rcatgy)	47 & 939	50 & 942	46 & 1464	47 & 937	47 & 941	30 & 924

Yersinia enterocolitica as shown in the (Figs 3 & 4 and Tables 1 & 2). However, it is noted that the combination of restriction enzymes such as AflIII, BssHII, ClaI, DraI, DraIII, HpaI, NdeI, NsiI, and SalI differentiated the amplified products successfully, including *E. coli*, *S. marcescens* showed identical AFLP patterns which reflected the similarity of the 16S rDNA gene sequences for these two species (Okhravi *et al*., 2000).

The present study showed that it is possible to use molecular assays to detect total coliforms in drinking water despite the high genetic variation of the total coliform community. Molecular assay techniques involving 16S rRNA have demonstrated to be precise and highly suggested over culture dependent methods (Maheux *et al.*, 2008). The overall time required to perform the entire process including direct DNA extraction from filters was found to be in 3-4 h which is remarkable compared to the conventional methods which takes two to three days for the identification of bacteria. These obtained results and this approach can be used by the personnel who are responsible for water quality to inform and take decisions about public health and water quality management (USEPA.1989).

Conclusion

Detection of coliforms in water and food is an important environmental issue because of health problems that can be involved. In the present study we have designed novel coliform specific primers which could prove to be a rapid and sensitive approach (PCR-AFLP) based on gene amplification. Investigation of microbial communities at taxonomic levels aims to provide a quite different viewpoint to that given by abundance estimates at the genus level. Use of these newly designed primers and specific restriction digestion enzymes will provide a great platform for rapid detection, identification of coliforms with high sensitivity, specificity and its cost effectiveness.

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Conflicts of interest

All authors declared no conflicts of interest.

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