Development of a simple, rapid multiplex PCR tool kit by using 16S rRNA gene for the identification of faecal and non-faecal coli forms in the drinking waters
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ABSTRACT
A multiplex method for the detection of faecal and non-faecal coliforms in drinking water was developed using three primers from the V2, V3 and V9 variable regions of 16S rRNA gene. 194F, 474F and 1436R are the three primers designed for specific amplification of V2, V3, V9 hyper variable regions of 16S rRNA gene. Multiplex PCR allowed for differentiation of the total coliform from faecal coliform by specific amplicons: 1,285 bp of amplicon is specific for 6 non-faecal coliform genera and 1,009 bp of amplicon is specific for faecal coliform ie. E. coli. If the drinking water was contaminated with both faecal and non-faecal coliforms then two amplicons of 1,285 bp and 1,009 bp by combination of three primers are observed. The multiplex PCR assay based on 16S rRNA gene should be a beneficial tool kit for the rapid identification of the total coliforms in the large number of water samples compared with traditional methods. Results can be acquired within 3 hrs of time as compared with classic method of MPN (3–4 days). This assay will be useful in diversification and detection of seven genera of total coliforms by using variable regions of 16S rRNA.

Key words | 16S rRNA, coliform, E. coli, multiplex PCR, primer design

HIGHLIGHTS
- Rapid method to differentiate both faecal and nonfaecal coliforms in the water.
- Used V2, V3 and V9 variable regions of 16S rDNA for primer designing and amplified product with 1,009 bp band is specific for E. coli and 1,285 bp band is specific for 7 genera of total coliform, whereas two bands in the gel indicates the faecal and non faecal coliform.
- Accurate results when compared to other conventional methods.
Microbial safety of drinking water was assessed by the establishment of the presence of the microbial indicators like non-coli and faecal coliform bacteria such as *Escherichia coli* (*E. coli*). Faecal contamination in water was monitored by the existence of *E. coli* and entails that presence of pathogenic viruses, protozoa, bacteria, (Environment Agency 2002). Faecal contamination of water bodies may produce diarrheal illness and cause human mortality (Levy K. 2015). In the developing world, the major source of human illness is due to water related diseases (Magana-Arachchi, D. N et al. 2020). These vulnerable diseases to humans are caused due to drinking of untreated and contaminated water. *E. coli* O157:H7 is the most common type of Shiga toxin-producing *Escherichia coli* (STEC), but other types exist, which includes non O157; H7 strains include: O26:H11, O103:H2, O145:H28, and O111:H8, produces shiga toxin in the environmental waters, which are the major reservoirs of infectious *E. coli* (Bonetta et al. 2011, Iweriebor, B. C et al. 2015).

Global health issues have arisen due to waterborne diseases. Hence rapid identification of indicators of faecal contaminants, possibly the human pathogens is very critical in environmental samples. The identification and enumeration of bacteria from water are relevant, as it contributes to healthy public consumption of safe drinking water (Kaestli, M et al. 2019). Therefore, numerous methods for rapid detection and quantification of water quality indicators and waterborne pathogens have been established. Traditional approaches based on cultures are laborious, time-consuming. A rapid, simple protocol for the direct detection of microbes is therefore needed to be established (Cruz, C. D. et al. 2012).

Compared to the conventional approach, multiplex PCR is a valuable, quick and secure substitute for microbiological identification of drinking water organisms. For the concurrent amplification of target sequences in a single PCR, multiplex PCR (MPCR) uses one or more template and several sets of primers (Molina et al. 2015). For the differentiation and recognition of bacterial species, multiplex PCR uses several templates and special primers (Bourque et al. 1993; Geha 1994; Yoon et al. 1996b). When multiplex PCR was first applied it was used to effectively detect *Saccharomonospora* strains by means of the 16sRNS gene and was found t be capable of being species specific (Yoon et al. 1996b). The technique verified itself to be species-specific, quick and simple (Yoon et al. 1996b). Detection of 16S rRNA genes through PCR amplification is widely accepted method for the identification of bacteria (Sarvari et al. 2018). Sequence analysis of 16S rRNA can be used for taxonomic studies and bacterial species identification.
In this study, 16S rRNA sequences were obtained from 64 strains from 7 genera representing both total and faecal Coliforms. These sequences were used to develop a multiplex PCR assay targeting the 16S rRNA gene. The practicality of using multiplex PCR investigation for quick detection of total coliform strains in the contaminated water was examined rapidly (Lindsey R. L. 2017). 3 primers were previously designed to target variable regions (V2, V3, V9) in the 16S rRNA for the rapid detection of total coliform bacteria in the drinking water and shortened time to the time assay time significantly compared to traditional culture methods (Chakravorty et al. 2007; Ciara Willis et al. 2019).

**MATERIALS AND METHODS**

**Bacterial strains**

Isolates used in the present study were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India are tabulated in Table 1.

**Collection of water samples**

**Study location**

Mahabubnagar is one of the drought districts in Telangana state. The geographical area of the district is 18,432 Sq. kms, consists of 64 mandals with 1,549 villages. It lies between north latitudes 15° 55' 00” and 17° 20' 00” and east longitudes 77° 15' 00” and 79° 15' 00”. It is bounded on the north by rangareddy and nalgonda districts, on the east by guntur and nalgonda districts on the south by Krishna and Tungabhadra rivers and on the west by Raichur and Gulbarga districts of Karnataka state.

The main sources of potable water in the township of Mahabubnagar are the ramanpadu balancing reservoir and koilsagar reservoir (Figure 5). Ramanpadu is a balancing reservoir under the left-hand canal of the jurala project. A 64 km ramanpadu drinking water pipeline project started in 1999 for the town of mahabubnagar to reduce its drinking water problem. More than 2 lakhs population of the town requires 15 million liters of water every day, whereas the Ramanpadu water scheme was designed to lift 18 million liters of Krishna water every day.

Koilsagar dam is located at Koilsagar village of deverakadra mandal in mahabubnagar district. Koilsagar dam is built on a minor tributary of the krishna river. The proposal to construct a dam was put forward by the British rulers to collect and store the excess water in a catchment area of the krishna river for irrigation purposes.

Two separate lakes, i.e., Ramanpadu and Koilsagar, Mahabubnagar district, Telangana state, India were the study areas and a total of 64 water samples were collected in 1 L sterile bottles. Details of the source of water, time and date of sample collection were labeled on sample bottles (Volokhov et al. 2007; Shiva Shanker et al. 2019).

**Filtration for bacteria**

Bacterial cells were collected by filtering 100 mL of water sample using vacuum manifold through polycarbonate filter with 47 mm diameter, pore size 0.2 μm. To avoid possible contamination, it was performed within the laminar flow (Dehghan et al. 2014).

**Genomic DNA isolation from pure cultures and environmental samples**

Genomic DNA was extracted from the trapped bacteria as described previously (Pindi et al. 2013) using commercial kits (DNeasy Plant Mini Kit (cat. nos. 69104 and 69106).
Bioinformatic analysis of 16S rRNA gene and Primer designing

The PCR primers used in the study are shown in Table 2. The species-specific primer pairs targeting 16S rRNA gene variable regions were designed by using Primer 3 software (Andreas et al. 2012). Multiple sequence alignment was done by CLC sequence viewer 8.0 software to identify the high variable region to target primer within the gene. The designed primer were tested insilico by software available from www.bioinformatics.org. Oligonucleotide primers were procured from Eurofins MWG Operon (Bangalore, India).

PCR analysis

The MJ Mini Personal Gradient Thermal Cycler from Bio-Rad was used for all PCR analysis. The uniplex PCR reaction mixture consisting of 25 μl of KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Sigma Aldrich, City, India) or subsidiary in India. 1 μl 5 pmol of forward primer 194F, 1 μl 5 pmol of reverse primer 1436R, 1 μl 30–50 ng DNA template and finally 12 μl PCR grade water for a total volume of 50 μl were used for the amplification reaction.

The PCR gradient, with annealing temperature varying from 51 to 55 °C, was used to optimize the best annealing temperature (Fricker & Fricker 1994). The PCR loop, denaturation, annealing and extension were carried out for 5 min at 95 °C, 54.4 °C for 30 s, and 72 °C for 1 min, respectively. The final extension was carried out for 2 minutes at 72 °C. The PCR product was examined in 1.5% agarose gel Bio Rad (Jyothi kumar et al. 2003). The molecular weight marker of the 100 bp (NEB # B7025, Biolabs) DNA ladder (Figure 1) was used to classify the product bands. Using a PCR purification Kit (Bangalore Genei, India), the amplified product was purified and sequenced at Bioserve Biotechnologies Pvt, Hyderabad. Further, the 16S rRNA gene sequence was deposited in the NCBI GenBank and accessions numbers were allocated in Table 3.

Multiplex PCR

The multiplex PCR reaction mixture consisting of 25 μl of KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Sigma Aldrich, City, India) or subsidiary in India. The PCR conditions are similar for 1 μl 5 pmol of forward primers 194F, 474F and 1 μl 5 pmol of reverse primer 1436R, 1 μl 30–50 ng DNA template and finally 12 μl PCR grade water for a total volume of 50 μl were used for the amplification

| MTCC culture name | NCBI accession number |
|-------------------|-----------------------|
| Y. enterocolitica  | MW029934              |
| C. freundii       | MW029935              |
| E. aerogenes      | MW029936              |
| S. enterica       | MW029937              |
| S. marcescens     | MW029938              |
| E. coli           | MW029939              |
| K. pneumoniae     | MW029940              |

Table 2 | Primers for multiplex PCR

| Primer | Sequence | Sequence length | Tm | GC% | Target Origin | Product size (bp) | Remarks |
|--------|----------|-----------------|----|-----|---------------|-------------------|---------|
| 194F   | 5’-GTCGCAAGACCAAAGWGGGGA-3’ | 22 | 59 | 59 | V2            | 1285 bp           | Forward |
| 474F   | 5’-AAGTATAAATACCTTGTCTGATC-3’ | 26 | 53 | 35 | V3            | 1009 bp           | Forward |
| 1436R  | 5’-TGGTAAGCCTCCCTCAGGT-3’   | 22 | 59 | 62 | V9            | -                 | Reverse |
reaction (Figure 2). PCR product was examined by gel electrophoresis through a 1.5% agarose gel (Jyothi Kumar et al. 2005). Identification of the product bands was established by molecular weight marker of the 100 bp (NEB # B7025, Biolabs) DNA ladder. The fitness of the primer pairs for multiplex PCR system was checked using MultiPLX (Kaplinski et al. 2005) and FastPCR.

RESULTS

Identification of total coliforms using 16S rRNA markers through Uniplex PCR

Total coliform screening methods are mainly targeted to the 16S rRNA markers due to the presence of species-specific variable regions in total coliforms, V2 and V3 were most suitable for distinguishing all bacterial species to the genus level except for closely related enterobacteriaceae (Chakravorty et al. 2007). The efficiency of new designed primer pairs were tested separately to amplify their targets by uniplex PCR. Each uniplex PCR gave the expected one amplicon for non-faecal coliform and faecal coliform species of standard cultures. A 1,285 bp of PCR product was obtained, when the primer set 194F & 1436R was used for all 7 genera of total coliform group (Figure 1). No PCR fragment was observed for non-coli forms and water control (Figure 2) indicating would run a larger challenge set and actually calculate sensitivity and specificity using standardized methods. Moreover, the intensity of the DNA band increased correspondingly to use 194F & 1436R primer set the increased amount of specific template in the samples.

Multiplex PCR for differentiation of total faecal coli form and nonfaecal coliform

Specificity of multiplex PCR

PCR amplification with two sets of oligonucleotide primers (reverse primers common to both) resulted only in the presence of their respective DNA template in a detectable molecular weight fragment (1,285 bp for non-faecal coliform bacteria and 1,009 bp for *E. coli*) of a predicted molecular weight. These results indicate that each of the three primers namely 194F is a degenerated primer that targets the 7 genera of total coliform in combination with 1436R and in combination with 474F targets for *E. coli*. However, when both bands were observed 1,285 bp and 1009 it indicated that water was contaminated with faecal coliform bacteria that *E. coli* (Figure 2), thus by using these primers (194F, 474F, 1436R) we can differentiate the water contaminated

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**Table 4** List of isolates tested different reservoirs of Palamuru, namely, Ramanpadu, Koilsagar

| Isolated organism       | Positive/ negative |
|-------------------------|--------------------|
| Acinetobacter spp.      | –                  |
| Aeromonas spp.          | –                  |
| Azospirillum spp.       | –                  |
| Bacillus spp.           | –                  |
| Citrobacter spp.        | +                  |
| Cupriavidus spp.        | –                  |
| Enterobacter spp.       | +                  |
| Escherichia spp.        | +                  |
| Exiguobacterium spp.    | +                  |
| Klebsiella spp.         | +                  |
| Kocuria spp.            | –                  |
| Methylobacterium        | –                  |
| Pantoea spp.            | –                  |
| Psedomonas spp.         | +                  |
| Rhizobium spp.          | –                  |
| Salmonellaspp.          | +                  |
| Staphylococcus spp.     | –                  |
| Streptococci spp.       | –                  |
| Yersinia spp.           | +                  |

**Figure 2** Agarose gel (1.5%) electrophoresis of PCR amplified products from various pure bacterial DNAs, using multiplex PCR. M = Marker, 1 = Mixed DNA of 7 genera with 3 Primers (194F, 474F & 1436R), 2 = Mixed DNA of 7 genera with one set of primers (194F & 1436R), 3 = Mixed DNA of 7 genera with one set of primers (474F & 1436R), 4 = Mixed DNA of 6 genera with one set of primer (194F & 1436R) without *E. coli*, 5 = Negative control.
with faecal or non-faecal coliform bacteria. However, no PCR products were observed or other bacteria in negative control (Figure 3) that confirmed specificity of the assay.

**Bioinformatic analysis of 16SrRNA sequence analysis**

64 different group of bacterial 16S rRNA sequence and multiple sequence alignment were performed by CLC sequence viewer 8.0 software to identify the high variable region to target primer within the genes V2 and V3 and V9 are variables regions of 16S rRNA and primers were designed for these regions. V2, V9 has conserved regions specific for seven genera and these were grouped as total coliform showing nucleotide sequence identity (Figures 4 and 5). V3 regions are specific for E. coli. Primer 474 has 11 nucleotides variation from six genera of total coliform includes Citrobacter sp., Enterobacter sp., Klebsiella sp., Serratia sp., Yersinia sp. and Salmonella sp.

**Application of multiplex PCR to environmental isolates of E. coli**

The samples from different places were collected and validation was conducted of MPN positive sample for filed level conformation and later checked for the coliform contamination in the two major reservoirs of Ramanpadu and koilsagar reservoirs and total number of bacteria was estimated. The average total bacterial numbers of each sample ranged between 9.5 and 107 CFU/mL, 8.0 to 105 CFU/mL respectively in Ramanpadu, Koilsagar (Figure 2). The microbial diversity of water samples in Ramanpadu, Koilsagar were represented in (Figures 6 and 7).

64 isolates of faecal coli forms and non faecal coli forms from drinking water were subjected to multiplex PCR in the

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**Figure 3** | Agarose gel (1.5%) electrophoresis of PCR amplified products from environmental samples, using multiplex PCR. M – Marker, 1 – Negative control other than coli form DNA ie., Bacillus DNA, 2 – E. coli DNA, 3,4 MPN tested positive samples DNA, 5 – Negative control without DNA.

**Figure 4** | Nucleotide alignment and information from the 16S rRNA gene of total coliforms detection. The sequences in the boxes are the designed primers.
The present study. Each of *E. coli* isolates showed positive amplification of 16S rRNA fragments. These findings showed that the multiplex PCR method, based on the co-amplification of three primary DNA target sequences derived from 16S rRNA genes, allowed the unmistakable identification from drinking water for both faecal and non-faecal coli forms.

In order to validate the designed primers standard cultures of coliforms (7 genera) were procured from MTCC. Later the 1,285 bp gene sequence was confirmed and deposited in NCBI with accession No. (MW029934-MW029940). Meanwhile the non-coliforms by 16S rRNA and biochemical, phenotypic tests (Pindi PK et al. 2013), were found to be correctly identified with the newly designed primers. Thus accuracy of the primers for coliforms in the present study was found to be 99%.

**DISCUSSION**

Water-borne disease outbreaks continue to be a significant concern for global public health providers, claiming millions of lives each year (Jones et al. 2009; Cabral 2010; Gelting et al. 2011; Yang et al. 2011; Breathnach et al. 2012; Cheun et al. 2013; Pitkanen 2013; Walser et al. 2014). WHO reported in 2008 that, 2.5 million people died from diarrheal disease and that in 2011, the number of cases of cholera rose by 85%
compared to 2010 (WHO 2015). The coliform group includes the members of genera *Escherichia sp.*, *Klebsiella sp.*, *Citrobacter sp.*, and *Enterobacter sp.* and is termed as total coliforms regularly found in water. Faecal indicator bacteria are being used to predict the presence of pathogens in water intended for human consumption and bathing water by the European Commission (EC) (EC 1998, 2006, 2009), United States Environmental Protection Agency (USEPA) (USEPA 2002; Wade et al. 2003; Boehm et al. 2009) and WHO (WHO 2011). The conventional methods for the detection of pathogens takes a long processing period and is labor-intensive (Velusamy et al. 2010; Li et al. 2016; Pan et al. 2018). Newer techniques such as PCR are more adaptive, high-throughput, and many bacteria could be identified in a shorter time and with less reagent consumption without the repeated steps with multiplex PCR technology (De Freitas et al. 2010; Sánchez-Parra et al. 2019). Multiplex PCR has been successfully applied to rapidly detect various pathogens from environmental waters (Bej et al. 1991b). In this study, multiplex PCR was used for the identification of total coliform and non-faecal coliform bacteria in drinking water and the specificity of PCR was calculated for 64 bacterial strains. Only in the presence of their respective DNA templates did the findings of PCR yield a detectable DNA fragment of predicted molecular weight.

The input parameters for primer3 designing software includes the primer length of about 18–24 bp, G/C content 40–60%, start/end with 1–2 G/C pairs, primer melting point Tm 50–60 °C, F and R primer Tm value is within 5 °C and primers should not have complementary regions. One of the most critical parameters is the temperature for annealing. While many individual loci could be precisely amplified at 56–60 °C. In the present study the annealing temperature for multiplex PCR is 56–60 °C (Maheux et al. 2014; Thuy et al. 2016; Abada et al. 2019; Xie et al. 2020).

In the present study, 16S rRNA gene was used to detect both fecal coliform and non-faecal coliforms. Bacterial 16S rRNA consists of 9 hypervariable areas, which display considerable diversity of sequences among various bacterial species and can be used to classify species (Van de Peer et al. 1996). The hyper-variable regions of most bacteria are flanked with conserved stretches allowing the PCR to amplify target sequences with a universal primer (McCabe et al. 1999; Lu et al. 2000; Baker et al. 2003; Munson et al. 2004). 16S rRNA hypervariable area sequences have been found in various studies that classify a single bacterial species or distinguish between a small number of different species.
species or genera (Choi et al. 1996; Kataoka et al. 1997; Marchesi et al. 1998; Lu et al. 2000; Bertilsson et al. 2002; Rothman et al. 2002; Yang et al. 2002; Becker et al. 2004; Clarridge 2004; Maynard et al. 2005).

In the current study two primers of 194 F and 1436R were designed which amplifies 1,285 bp of PCR product specific for V2 hypervariable region of 16S rRNA gene and it is specific for the detection of coliform genera including Yersinia enterocolitica, Citrobacter freundii, Enterobacter aerogenes, Salmonella enteric, Serratia marcescens and Klebsiella pneumonia. 474F and 1436 R primers are specific for V3 hypervariable region specific for Escherichia coli and amplifies 1,009 bp of PCR product. (Chakravorty et al. 2007; Merkel et al. 2019; Sune et al. 2020). This method is more sensitive method than traditional culture-based methods because it depends on stable genetic parameters and detects accurate results with 3 hrs compared to the classic method for the regular monitoring of that takes 5–4 days. The presence of total coliforms from the environmental samples from Ramanpadu, Koilsagar reservoirs shows the specific amplicon size. The results are similar to Shankar et al. 2019.

CONCLUSION

Total coli form organism’s shows high genetic diversity hence they can be diversified by using molecular tools for their detection in drinking water. Among various methods available, the present study utilizes 16S rRNA gene proved to be simple and sensitive technique. 194F, 474F, 1436 R primer set is specific for amplification of V2, V3, V9 hyper variable regions of 16S rRNA gene. In multiplex PCR we will able to differentiate the total coliform with that of faecal coliform by specific amplicons includes 1,285 bp of amplicon specific for 6 genera of non-faecal coliform and amplifies 1,009 bp of amplicon specific for faecal coli form ie. E. coli. If the drinking water was contaminated with both faecal and non-faecal coli forms then we get two amplicons of 1,285 bps and 1,009 bps. This rapid method of molecular detection of total coliforms able to differentiate both faecal and nonfaecal coliforms in the water. We can get the accurate results with 3 hrs time duration when compare with classic method of MPN (3–4 days).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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