

Original Research Article

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Multiplex PCR for Detection of *Escherichia coli* O157 : H7 in Faeces from Sheep and Goat

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ABSTRACT

E. coli O157:H7 is an emerging food borne pathogen having zoonotic significance. Though the primary reservoir of this serotype is cattle; Sheep and goat are also considered to be the main reservoir for *E. coli* O157:H7. Conventional cultural methods are time consuming method and also the specificity is very less. Multiplex PCR (mPCR) tends to be specific, more rapid and reliable. In the present study fecal samples collected from sheep (n=517) and goat (n=450) in different farms across Kalyana Karnataka region were analysed. The samples were processed and analysed for the cultural isolation, biochemical Characterization and latex agglutination test. An mPCR method for the detection and molecular Characterization of *E. coli* O157:H7 was standardized using primer pairs targeting six specific virulent genes; fliCh7, eaeA, rfbE, hly, stx1, and stx2. The mPCR produced species-specific amplicons of the six targeted genes of the size; 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively. The percent of sheep and goats that showed shedding of *E. coli* O157 in the faeces was 3.86% (20 out of 517) and 2.88% (13 out of 450) respectively. The results obtained show that the standardized mPCR protocol is a rapid, highly sensitive, species-specific and reliable method for the detection of the pathogenic *E. coli* O157:H7 and could be used for identification and molecular Characterization of *E. coli* O157:H7 in suspected food and water borne outbreaks, disease investigations and routine analysis etc.

Keywords

E. coli O157:H7;
Food borne
pathogen; Virulent
Genes; Multiplex
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Introduction

The *E. coli* which produce shiga toxins (*Stx*) have been referred as Enterohemorrhagic *E. coli* (EHEC) / Shiga toxin-producing *E. coli* (STEC). About 200 EHEC serotypes have been isolated from animal and food sources, (Denis *et al.*, 2012). The most significant EHEC of humans is *E. coli* O157:H7. After the first outbreak of bloody diarrhoea in

Oregon and Michigan, U.S.A. in 1982, *E. coli* O157:H7 has become the most widely known EHEC strain (Wells *et al.*, 1983). *E. coli* O157:H7 evolved pathogenic by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands. Because of the severity of these illnesses and the apparent low infective dose (< 10 cells, Bach *et al.*, 2002), *E. coli* O157:H7 is considered one of the most

serious of known food borne pathogens (Blanco *et al.*, 2003). Important reservoirs of pathogenic *E. coli* O157:H7 in the environment have been ruminants, particularly sheep and cattle which are asymptomatic carriers (Al-Saigh *et al.*, 2004).

These asymptomatic carriers normally shed the organism in faces contaminating soil and surface waters (Hilborn *et al.*, 1999). *E. coli* O157:H7 is mainly a food and water borne pathogen to humans (Soderlund *et al.*, 2012).

E. coli O157:H7 infections in humans produce Hemorrhagic Colitis (HC), post-diarrheal Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) (Karmali *et al.*, 2010 and Pennington, 2010). Hemolytic ureamic syndrome (HUS), a life threatening complication, is developed by about 10% of patients, mostly in elderly people and children (Faten and Afaf, 2013).

Conventional culture method using Soyabean bile broth with 20 mg/L novobiocin as enrichment media (OIE, 2006) and *Cefixime* and Tellurite - Sorbitol MacConkey (CT-SMAC) agar used as selective and differential medium is very useful for the identification of *E. coli* O157:H7. However, it is time consuming and needs a lot of media and supplement components (Visetsripong *et al.*, 2007). There is a possibility to get false results by these classic microbiological diagnostic procedures (Orth *et al.*, 2009).

Therefore, the present research work was undertaken to develop a rapid, sensitive, species-specific and reliable multiplex PCR procedure for the detection of pathogenic *E. coli* O157:H7, by targeting the six virulence genes; *fliCh7*, *rfbE*, *eaeA*, *hlyA*, *stx1*, and *stx2* genes which code for H7 flagellin, O157 endotoxin, intimin, heamolysin, shiga toxin 1 and shiga toxin 2 antigens, respectively.

Materials and Methods

Sample collections

The faecal samples were collected into sterile vials directly from rectum by sterile cotton swab stick (Hi Media, Mumbai, India) and also faecal pellet directly collected in sterile test tube. They were labelled properly and transported to laboratory in thermo-cool container jacketed with ice packs. The samples were processed and analyzed for the isolation of *E. coli* O157:H7 within 24 hours.

Cultural isolation of *E. coli* O157:H7 and biochemical characterization of isolates

1 grams of each faecal sample was inoculated into 9 ml (1:9 ratio) (modified tryptone soy broth (mTSB) supplemented with novobiocin and incubated overnight at 37°C for 24 hours. A loopful of inoculum from the enrichment medium was inoculated onto selective isolation medium: Sorbitol-MacConkey agar supplemented with cefixime (0.025 mg) and potassium tellurite (1.25 mg) (CT-SMAC). The agar plate was incubated for 24 h at 37°C. Growth of typical colourless colonies (sorbitol negative) indicates positive for *E. coli* O157:H7.

These isolates were further were subjected for inoculation on Eosin methylene blue (EMB) agar, to confirm the isolate as *E. coli* by observing for the colonies producing metallic sheen, gram's staining and biochemical tests. The biochemical tests performed were Indole test, Methyl Red test, Voges-Proskauer test, Citrate utilization test (IMVC), Nitrate test and Sugar fermentation test for Lactose, Sucrose and Dextrose. The typical colourless isolates on CT-SMAC agar showing metallic sheen on EMB agar, gram negative staining character, positive for in dole (pink red top line), methyl red (red colour), sugar fermentation (yellow slant and butt with gas

production) and nitrate tests (red colour) and negative for Voges-Proskauer test (no pink top line), Citrate utilization test (no change in colour of the medium from green to blue colour) were presumed as positive for *E. coli* O157:H7 and inoculated into modified tryptone soy broth for preservation and confirmative identification and molecular characterization by using multiplex PCR and latex agglutination.

DNA template preparation

The extraction of the genomic DNA from the grown *E. coli* O157:H7 culture was done by using the modified boiled cell method (Queipo-Ortuño *et al.*, 2008). For the extraction of the genomic DNA from broth, one millilitre of the culture broth was centrifuged at 15000 rpm for 2 min. The supernatant was discarded and the cell pellet was resuspended in 500 µl of sterile distilled water followed by vigorous vortexing. The homogenized cell suspension was boiled for 10 min. Then cooled at -20°C for 10 min and centrifuged again at 15000 rpm for 2 min. The supernatant, comprising DNA, was used to standardize the multiplex PCR.

For the extraction of the genomic DNA from agar, few colonies were picked up from the agar plate and resuspended in 500 µl of sterile distilled water by vigorous vortexing. The homogenized cell suspension was boiled for 10 min. Then cooled at -20°C for 10 min and centrifuged again at 15000 rpm for 2 min. The supernatant, comprising DNA, was used to standardize the multiplex PCR.

PCR amplification

The multiplex PCR protocol was standardized in a volume of 25 µl of reaction mixture containing 2 µl of *Taq* DNA Polymerase (1 unit/ µl), 2.0 µl of DNA template solution, 2.5 µl of 10 x reaction buffer, 0.5 µM of

deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 12 primers (6 primer pairs) and magnesium chloride (MgCl₂) (concentrations were standardized). Sterile nuclease free water was added accordingly to make up the 25 µl reaction mixture.

Q-Sat 96 Thermal Cycler (Quanta Biotech) was used to standardize the multiplex PCR protocol. Thermal cycling consisted of a 5 minute initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C (timing standardized), 1 min of annealing (temperature standardized), and extension for 1 min at 72°C, with a 10 min final extension at 72°C followed by maintenance at 4°C. The timings of denaturation temperature evaluated were 20 sec and 45 sec. The annealing temperature was evaluated between 58°C to 63°C by doing gradient mPCR.

The mPCR protocol has been developed using primer pairs targeting six specific genes of *E. coli* O157:H7; *fliCh7*, *eaeA*, *rfaE*, *hly*, *stx1*, and *stx2*. The primers were synthesised by Merck GeNei™. The sequences of the primers are shown in the Table 1.

Agarose gel electrophoresis

From each PCR product an aliquot of 5 µl was subjected to agarose (concentration standardized) gel electrophoresis containing 0.5 x TBE buffer (pH 8.0) and ethidium bromide was used to stain the gel. Electrophoresis was carried out at 80 Volt, 400 mA for 40 min with 5µl of 100 bp DNA marker. The DNA bands were observed under ultraviolet (UV) light using gel documentation system (Biorad). The agarose concentration was evaluated for 1% and 1.5%.

Results and Discussion

The extraction of the genomic DNA of *E. coli* O157:H7 was done by modified boiled cell

method. The parameters of multiplex PCR (mPCR) protocol standardized were time period of denaturation at 94°C (20 sec and 45 sec), annealing temperature (58°C - 63°C) and agarose concentration (1% and 1.5%).

The standardized mPCR consisted of volume of 25 µl of reaction mixture containing 12.5 µl of PCR master mix (DNA polymerase, dNTPs, MgCl₂ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 2.0 µl of DNA template solution, 0.5 µl of each forward and reverse primers (6 primer pairs) and sterile nuclease free water was added accordingly to make the volume to 25 µl.

The thermal cycling consisted of 5 minute initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 45 sec, 1 min of annealing at 60°C and extension for 1 min at 72°C, with a 10 min final extension at 72°C. The agarose gel electrophoresis was optimized at 1.5 % concentration of agarose. The mPCR produced amplicons of 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp sizes, respectively for the *fliCh7*, *eaeA*, *rfbE*,

hly, *stx1*, and *stx2* virulent genes. The size of the amplicons is presented in the Table1.

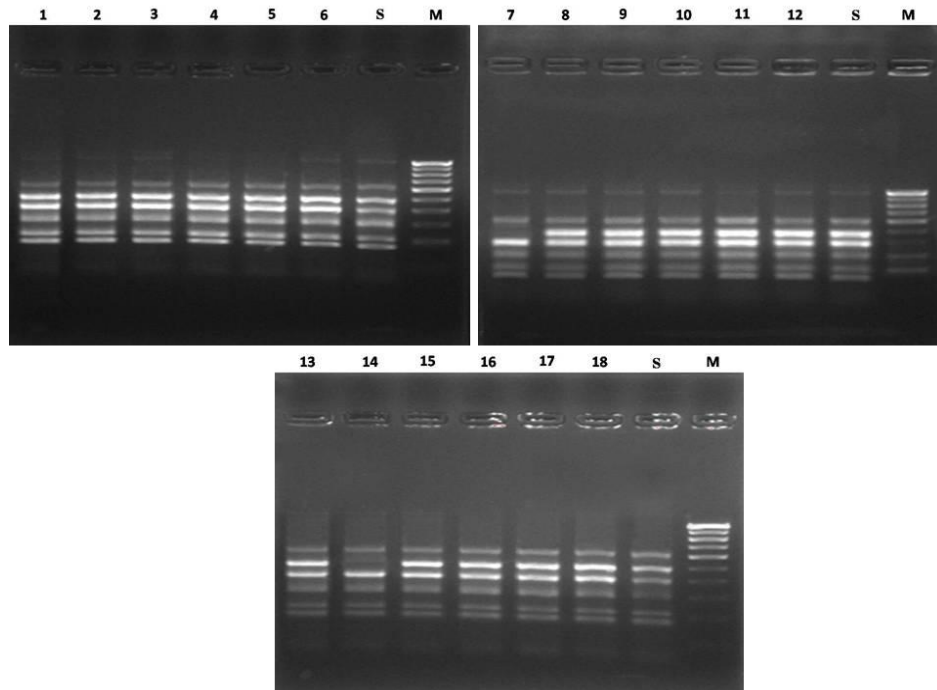
When isolates confirmed by Latex agglutination test subjected for standardized mPCR for the identification and molecular Characterisation of *E. coli* O157:H7, out of 517 samples analyzed in sheep, 18 samples were found to be positive for *E. coli* O157:H7 which accounts for 3.48 % (Figure I). Among the 18 positive samples, 16 samples showed presence of all the six virulent genes and two samples showed presence of only five virulent genes, viz; *fliCh7*, *rfbE*, *eaeA*, *hly* and *stx1* with the absence of *stx2* gene (as shown in Figure I [Lane 7 and 14]).

Out of 450 samples analyzed in goats, 12 samples were found to be positive for *E. coli* O157:H7 which accounts for 2.66 % (as shown in Figure II). Among the 12 positive samples, 11 samples showed presence of all the six virulent genes and one sample showed presence of only five virulent genes, viz; *fliCh7*, *rfbE*, *eaeA*, *hly* and *stx1* with the absence of *stx2* gene (as shown in Figure II [Lane 6]).

Table.1 Primer pairs used for the optimization of multiplex PCR

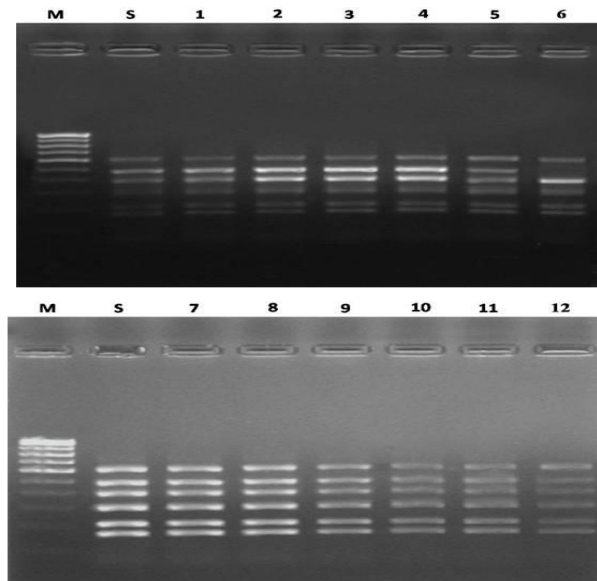
S. No.	Target gene	Primer	Sequence	Size of the Amplicons (bp)
1	<i>fliCh7</i>	FLICH-F	GCGCTGTCGAGTTCTATCGAGC	625 bp
		FLICH-R	CAACGGTGACTTTATCGCCATTCC	
2	<i>rfbE</i>	<i>rfbE</i> - F	CAGGTGAAGGTGGAATGGTTGTC	296 bp
		<i>rfbE</i> -R	TTAGAATTGAGACCATCCAATAAG	
3	<i>stx1</i>	SLTI-F	TGTA ACTGGAAAGGTGGAGTATACA	210 bp
		SLTI-R	GCTATTCTGAGTCAACGAAAATAAC	
4	<i>stx2</i>	SLTII-F	GTTTTTCTTCGGTATCCTATTCC	484 bp
		SLTII-R	GATGCATCTCTGGTCATTGTATTAC	
5	<i>eaeA</i>	AE22(F)	ATTACCATCCACACAGACGGT	397 bp
		AE20-2 (R)	ACAGCGTGGTTGGATCAACCT	
6	<i>hly</i>	MFSI-F	ACGATGTGGTTTATTCTGGA	166 bp
		MFSI-R	CTTCACGTCACCATACATAT	

Fig.1 Eighteen positive samples for *E. coli* O157:H7 by multiplex PCR in sheep faecal sample



Lane M : 100 bp DNA ladder
Lane 1-18 : Samples positive for *E. coli* O157:H7
Lane S : Standard culture- *E. coli* O157:H7 – US FDA strain

Fig.2 Twelve positive samples for *E. coli* O157:H7 by multiplex PCR in goat faecal sample



Lane M : 100 bp DNA ladder
Lane 1-12 : Samples positive for *E. coli* O157:H7
Lane S : Standard culture- *E. coli* O157:H7 – US FDA strain

The samples found positive for *E. coli* O157:H7 by cultural isolation, biochemical Characterisation and latex agglutination test were confirmed and characterized by using mPCR. The per cent of positive sample for *E. coli* O157:H7 in small ruminants using mPCR was found to be 3.20% (30 out of 967 samples). Of these, the per cent of sheep [3.48% (18 out of 517 samples)] shedding *E. coli* O157:H7 was higher than goats [2.66% (12 out of 450 samples)].

The incidence of the food borne outbreaks of *E. coli* O157:H7 is on the rise all over the world. In India too there could be food borne outbreaks. However, systematic diagnosis of the food borne outbreaks in India is not carried out. Also, the sensitive, specific and less time consuming diagnostic techniques are lacking. Conventional microbiological culture based method, though very useful for the identification of *E. coli* O157:H7, it is time consuming and needs a lot of media and supplement components (Visetsripong *et al.*, 2007).

There is a possibility to get false results by these classic microbiological diagnostic procedures (Orth *et al.*, 2009). The standardized multiplex PCR saves cost of chemicals and reagents which would be used for simple PCR targeting individual genes in separate PCR reaction mixture. And also the standardized multiplex PCR procedure produces distinct DNA bands for each targeted gene on agarose gel electrophoresis for the quicker detection of *E. coli* O157:H7.

In this study we standardized a multiplex PCR technique for the detection of *E. coli* O157:H7 by targeting 6 major specific virulent genes. These six virulent genes of *E. coli* O157:H7 would be a comprehensive set of genes and hence high sensitivity and specificity of the standardized multiplex PCR as discussed in the further paragraphs. The amplicons

observed on agarose gel electrophoresis were of the size, 625 bp, 397bp, 296 bp, 166 bp, 210 bp and 484 bp, respectively for *fliCh7*, *eaeA*, *rfbE*, *hly*, *stx1*, and *stx2* genes. A similar sized amplicons were obtained in the optimised multiplex PCR technique for the identification of *E. coli* O157:H7 (Jeshveen *et al.*, 2012).

In this standardization technique, modified boiled cell method was used (Queipo-Ortuño *et al.*, 2008). Many other researchers have used boiling cell method to extract genomic DNA for the detection of various bacteria by PCR; *Salmonella* (Park *et al.*, 2009) and *Campylobacter* spp. (Jeshveen *et al.*, 2012) have used boiling cell method to extract genomic DNA for the detection of *E. coli* O157:H7 by PCR. The results obtained in this standardized multiplex PCR technique proves that boiling cell method for the extraction of genomic DNA is simple, less harmful, quicker, has few steps and has produced optimum results of mPCR for the detection of *E. coli* O157:H7.

There are previous studies to develop multiplex PCR for the identification of *E. coli* O157:H7. Sahilah *et al.*, (2010) detected *stx1* and *stx2* Genes in *E. coli* O157:H7 Isolated from retail beef in Malaysia by multiplex PCR (2 gene multiplex PCR). Ahmad *et al.*, (2013) confirmed the presence of *E. coli* O157:H7 in 8% (of 200 samples) of ground beef hamburger samples using multiplex PCR that simultaneously detected three genes: *fliC* (encoding flagellar antigen), *rfbE* and *uidA* (encoding beta-glucuronidase). Puttalingamma *et al.*, (2012) confirmed the *E. coli* O157:H7 isolates by multiplex PCR using primers for four genes; *fliCh7*, *eaeA*, *stx2* and *stx1* genes. Divya *et al.*, (2013) characterized *Escherichia coli* O157 isolates by multiplex PCR targeting 5 virulence genes, namely; *eae*, *stx1*, *stx2*, *hlyA* and *fliCh7* genes. Pradeep Kumar (2017) characterized

Escherichia coli O157 isolates by multiplex PCR targeting 6 virulence genes, namely; *fliCh7*, *eaeA*, *rfbE*, *hly*, *stx1*, and *stx2* genes.

In the multiplex PCR standardized in this research work, the detection of the genes *fliCh7* (H7 antigen) and *rfbE* (O157 antigen) specifically confirms the identification of the organism *E. coli* O157:H7. Along with these two genes, detection of the other four virulent genes, viz; *eaeA*, *hly*, *stx1*, and *stx2* will further aid in confirmative identification and characterization of *E. coli* O157:H7 with higher sensitivity and specificity.

According to the USDA's Food Safety and Inspection Service (USDA-FSIS, 2002), ground beef is considered adulterated if as little as 1 CFU of EHEC O157:H7 is detected in 25 g of ground beef. The standardized multiplex PCR was very sensitive enough to detect up to 0.01 CFU emphasizing the sensitivity of the standardized multiplex PCR. The detection limit of the standardized multiplex PCR this study was better than that of other researchers. Bindu and Krishnaiah (2010) standardized three gene multiplex PCR for the identification of *E. coli* O157:H7 in beef samples where in the threshold sensitivity was 0.17 CFU/ml. Fode-Vaughan *et al.*, (2003) developed 2 gene multiplex PCR where in the threshold sensitivity was 20 cells / ml. Similarly Ibekwe and Grieve (2003) reported a minimum detection level of 20 cells of *E. coli* O157:H7 /ml with real time PCR.

In conclusion, the multiplex PCR standardized in this study is a rapid, sensitive, species-specific and reliable, as it targets 6 genes, method for the identification of the pathogenic *E. coli* O157:H7. The standardized multiplex PCR is open for further research work and could be used for the diagnosis of *E. coli* O157:H7 during emergencies of biological war, investigation of suspected food borne and water borne outbreaks.

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