MOLECULAR DETECTION OF ENTEROHAE-MORRHAGIC *Escherichia coli* IN RAW MILK SAMPLES OF THRISSUR

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Abstract

The present investigation was undertaken to study the occurrence of Enterohaemorrhagic Escherichia coli (EHEC) in pooled and individual raw milk samples of milk co-operative societies belonging to Thrissur district. A total of 72 pooled and 230 individual raw milk samples were collected from six milk co-operative societies of Thrissur district, Kerala. Out of 72 pooled and 230 individual raw milk samples examined, E. coli was isolated from 27.78 and 26.52 per cent of samples, respectively. The isolation rate of EHEC was found to be 11.11 and 8.26 per cent for pooled and individual raw milk samples, respectively. For molecular confirmation of the isolates, a multiplex PCR assay targeting stx 1, stx 2, eae A and hly A genes of EHEC was standardised. The virulence genes encoding stx 1, stx 2, eae A and hly A were identified in 33.33, 88.89, 11.11 and 33.33 per cent of isolates, respectively. The results of the study suggest that raw milk could be an important source for EHEC infection and poses a high public health risk.

Key words: *Pooled milk, raw milk, EHEC, Milk co-operative society, Multiplex PCR*

Milk is a highly nutritious balanced diet and wholesome food for all mammals. Being rich in nutrients, it forms an inseparable component of the human diet, both in developed and developing countries. India is the largest producer of milk and contributes to 18.5 per cent of the world's total production. In spite of greater achievements in terms of production, Indian dairy industry is lagging in exports due to the quality of milk produced. Most of the domestically produced milk and milk products fail to meet microbiological quality standards of importing country and this impedes the trade.

Contamination of milk can occur at any stage starting from production in farm till the consumption point. Contaminated udder, environment and equipment are the major sources attributed for contamination of fresh milk with the pathogen. Much of milk produced in India is obtained from small holder production under poor hygiene and productivity which further increases the chance of microbial contamination.

There are many food borne illnesses associated with the consumption of raw milk. Among the food borne pathogens acquired

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through raw milk, the Enterohaemorrghagic *Escherichia coli* forms the major pathogen. Dairy cattle are the major reservoirs for EHEC and consumption of contaminated raw milk is a potential route for EHEC infection to human. In humans, EHEC infection results in infection ranging from uncomplicated diarrhoea to life threatening complications *viz*., haemolytic uremic syndrome and haemorrhagic colitis (Jaakkonen *et al*., 2017).

Besides various conventional culture techniques in place for isolation of EHEC, molecular confirmation of the pathogen is essential. Considering all these facts, the present study was undertaken with the objectives to know the status of raw milk contamination with EHEC in Thrissur district and to standardize a multiplex PCR for the detection of virulence genes of the EHEC isolates.

Materials and methods

Sample collection

A total of 72 pooled and 230 individual raw milk samples (100ml) were collected under aseptic condition from six milk co-operative societies of Thrissur district as described by International Dairy Federation guidelines.

Bacterial culture

Samples were subjected to isolation and identification of EHEC as per Meng *et al.* (2001) with certain modifications. To recover the stressed EHEC, initially 45 ml of milk samples were pre-enriched in 250ml of Tryptose Soya Broth and incubated for 24h at 37 ^{0}C. One millilitre of pre-enriched sample was transferred to EC broth for selective enrichment at 37 ^{0}C for 24h. A loopful of enriched sample was streaked onto Eosine Methylene Blue (EMB) agar and incubated overnight at 37 ^{0}C. For selective isolation of EHEC, three typical *E. coli* colonies with metallic sheen on EMB agar were streaked onto Cefixime Tellurite – Sorbitol Mac Conkey agar (CT-SMAC) supplemented with novobiocin, cefixime and potassium tellurite followed by incubation at 37 ^{0}C for 18h. To detect β-glucuronidase activity, the isolates were streaked onto 4-Methylumbelliferyl ß-D-Glucuronide (MUG EC) agar as described by Fujisawa *et al*. (2000). Non-fluorescent colonies observed under UV trans-illuminator were further subjected to primary and secondary biochemical tests.

Standardization of multiplex PCR

A multiplex PCR was standardized against virulence genes of EHEC using EDL 933 strain of *E. coli* O157:H7. Virulence gene profile of the isolated EHEC strains was analysed using multiplex PCR with primers specific against *stx* 1, *stx* 2, *eae* A and *hly* A genes, producing amplicons of size 180, 255, 409 and 526 bp, respectively. The primer for detection *eae* A gene was designed in the present study based on the sequence available at National Center for Biotechnology Information (NCBI) database operon (accession code: CP017446.1). The primers used and their corresponding product size are enlisted in Table 1 (Meng *et al*., 1998, Paton and Paton, 1998).

The bacterial DNA was subjected to multiplex PCR with a reaction volume of 30 µl consisting of 2.5 µl template, 3 µl 10X PCR buffer (200mM), 2 µl $MgCl_2$ (25 mM), 2.5 µl dNTP mix (2 mM each) 0.75 µl taq DNA polymerase (5 units/ µl), 1 µl each forward and reverse primers of *stx* 1, *stx* 2 and *eae* gene and 0.5 µl primers of *hly* A gene. The final volume was adjusted to 30 µl with nuclease free water. The multiplex PCR cycle consisted of initial denaturation at 95 ^{0}C for 5 min followed by 35 cycles of denaturation at 95 ^{0}C for 35 sec, annealing at 60 ^{0}C for 45 sec and elongation at 72 ^{0}C for 2 min. The final extension was carried out for 10 min at 72 ^{0}C. The primers used and their expected product size are enlisted in Table 1. The PCR products were separated on 1.5 per cent agarose gel stained with ethidium bromide.

Results and discussion

The overall occurrence of *E. coli* in pooled and individual raw milk samples was found to be 27.78 and 26.52 per cent, respectively. Enterohaemorrhagic *E. coli* was isolated from 11.11 and 8.26 per cent of pooled and individual raw milk samples, respectively. The standardized multiplex PCR was found

to be sensitive and specific in detection of virulence associated genes of EHEC (Fig. 1). On multiplex PCR, out of 27 isolates obtained 33.33, 88.89, 11.11, and 33.33 per cent of isolates harboured *stx* 1, *stx* 2, *eae* A and *hly* A genes, respectively (Table 2).

Similar findings were observed in US and Italy where the prevalence of EHEC in pooled raw milk was observed to be 15.2 and 12.5 per cent, respectively (Van-Kessel *et al*., 2011; Trevisani *et al*., 2014). Our findings significantly vary from the study conducted by Brenjchi *et al*. (2011) and Heuvelink *et al.* (1998) who found a lowest prevalence of EHEC in bulk milk samples of Masshad (0.77 per cent) and Netherlands (0.3 per cent).

Overall occurrence of EHEC in individual raw milk samples was found to be 8.26 per cent which is in accordance with the previous reports. In a study, Sethulekshmi (2016) had reported that 11.11 per cent of raw milk samples collected from Thrissur district was contaminated with EHEC. The most predominantly detected virulence gene in the present study was *stx* 2, followed by *stx* 1 and *hly* A, and *eae* A. The findings are in agreement with the previous study by Vendramin *et al.* (2014) where frequency of *stx* 2 occurrence in raw milk was more against *stx* 1.

The findings of the present study showed that the raw milk of Thrissur district was contaminated with EHEC and poses a serious health threat to the public. Presence of virulence genes increases the pathogenicity of pathogen. In this regard, raw milk consumption should be discouraged among the public through awareness programs. Further, the

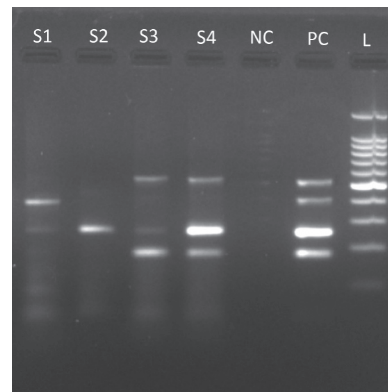

Figure 1. Multiplex PCR
S1 to S4 – EHEC isolates from samples, NC – negative control, PC – positive control (EDL 933 strain of EHEC), L- 100 bp ladder
(180bp – *stx* 1, 255bp – *stx* 2, 409bp- *eae* A and 526bp – *hly* A gene)

Table 1: Primer sequences and their respective product sizes

Genes	Primer	Primer sequence	Size (bp)	Reference
stx 1	F	5'ATAAATCGCCATTCGTTGACTAC - 3'	180	Paton and Paton (1998)
	R	5'- AGAACGCCCACTGAGATCATC- 3'		
stx 2	F	5'-GGCACTGTCTGAAACTGCTCC-3'	255	Paton and Paton (1998)
	R	5'-TCGCCAGTTATCTGACATTCTG- 3'		
hly A	F	5'-AGCCGGAACAGTTCTCTCAG -3'	526	Meng *et al*. (1998)
	R	5'-CCAGCATAACAGCCGATGT - 3'		
eae A	F	5'-ACGGTCTGGATCGTATCGTC -3'	409	Designed in the present study
	R	5'-GCATCCGTTTTGGCACTATT -3'		

Table 2: Occurrence of EHEC and associated virulence genes in raw milk

Sl No.	Source	Occurrence of *E. coli* (%)	Occurrence of EHEC (%)	Virulence genes (%)			
				stx **1**	***stx*** **2**	***eae*** **A**	***hly*** **A**
1	Pooled raw milk (n=72)	20 (27.78)	8(11.11)	5	6	1	2
2	Individual raw milk (n=230)	61 (26.52)	19(8.26)	4	18	2	7
Total = 302		81 (26.82)	27 (8.94)	33.33	88.89	11.11	33.33

source of contamination to raw milk should be studied. To curtail the occurrence of EHEC in raw milk, farmers should be trained on hygienic milking practices.

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