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Plasmid profiling and detection of LT and ST (enterotoxin) genes in *E. coli* isolates from diarrhoeic calves, kids and lambs

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Abstract. In a study on plasmid profiling 60 *E. coli* isolates obtained from diarrhoeic faecal samples from cattle calves, goat kids and lambs it was recorded that all the isolates harboured 10-15 kb plasmids. The PCR based detection of toxigenic gene showed presence of LT and ST gene in the reference strain with the specific primer sets producing amplicons of expected size i.e. 132 bp for LT (heat labile) enterotoxin and 171 bp for ST (heat stable) enterotoxin gene but out of randomly selected 35 *E. coli* isolates none showed presence of LT or ST toxin gene.
Key Words: *E. coli*, plasmid profiling, LT and ST enterotoxin, young animals.

Introduction. Enterotoxigenic *E. coli* (ETEC) has been the serious concern for the scientist to investigate its endemicity, pathogenicity and toxigenicity because it is the important category along with enteropathogenic *E. coli* (EPEC) which causes diarrhoea in first week of calving. Adherence to the intestinal mucosa and secretion of enterotoxins are important factors in the pathogenesis of ETEC (Butler & Clarke 1994; Al-Majali et al 2000). The main virulence factors of *E. coli* attributable to enteric infections are fimbriae, enterotoxin, haemolysin, invasiveness and colicin production. Two classes of enterotoxins, the heat-labile (LT) and the heat-stable (ST) are produced by ETEC (Levine 1987). Heat-labile enterotoxin is an immunogenic protein which loses its activity on heating at 60^{o}C for 10 min (Gyles 1971) and possesses enzymatic functions, namely adenosinediphosphorribosyl transferase activity (Fishman 1990). The other type of toxin, ST, is a heat-stable polypeptide with non-immunogenic properties and is resistant to heat at 100°C for 15 minutes (Lazure et al 1983). The ST toxin stimulates guanylatecyclase enzyme leading to increased intracellular cGMP level. This activity leads ultimately to stimulation of chloride secretion and/or inhibition of sodium chloride absorption, resulting in net intestinal fluid secretion that leads to diarrhoea (Sears & Kaper 1996). The genetic information for the synthesis of both ST and LT has proved to be plasmid mediated (Skerman et al 1972).

There are no suitable biochemical markers by which ETEC could be identified and distinguished from other *E. coli* strains producing diarrhoea. However, the PCR test is quite sensitive and specific and can be completed rapidly, giving results such as identification of genes of enterotoxins within one working day (Woodward et al 1992). Several PCR assays have now been developed for detection of ETEC strains.

The present work was undertaken with a view to carry our plasmid profiling in the *E. coli* isolated from diarrhoeic young ones of cattle, goat and sheep along with detection of LT and ST genes in respective isolates.

Material and Method

Isolation, identification and serotyping of isolates. Diarrhoeic faecal samples were collected from cattle calves (27), goat kids (17) and lambs (16) collected belonging to organized farms and private dairies located at different places in Bikaner city situated in the north-west part of Thar desert in Rajasthan state (India). From these samples 60 *E. coli* isolates were isolated and identified (Cowan & Steel 1975; Quinn et al 1994).

All the isolates had been serotyped from National Salmonella and *Escherichia coli* centre, Kasauli, H.P. (India). The isolates belonged to O2, O3, O5, O17, O21, O22, O25, O41, O45, O55, O60, O65, O70, O76, O114, O116, O147, O152, O158 and UT serogroups.

Plasmid DNA isolation. Plasmid DNA isolation was done as per the method described by Birnboim & Doly (1979), which was subsequently modified by Sambrook et al (1989). The plasmids of all processed isolates were eletrophoresed with 1kb ladder molecular weight marker for determining the plasmid size.

Detection of enterotoxin gene. Out of 60 *E. coli* isolates used for plasmid profiling only 35 isolates (15 from calves, 10 from goat kids and 10 from lambs) were subjected to PCR for detection of enterotoxin genes. The method described by Nishikawa et al (2002) was used for amplification of LT and ST genes using primers with sequence of 5'-AGC AGG TTT CCC ACC GGA TCA CCA-3' (Forward primer) and 5'-GTG CTC AGA TTC TGG GTC TC-3' (Reverse primer) for LT and 5'-TTT ATT TCT GTA TTG TCT TT-3' (Forward primer) and 5'-ATT ACA ACA CAG TTC ACA G-3' (Reverse primer) for ST.

Lyophilized powder of primers was initially added with 50 µl of TE buffer (pH 8.0) and then each primer was reconstituted in sterilized DNase free MiliQ water to arrive at a final concentration of 10 pmole μl^{-1}. The PCR mixture (total volume 50 µl), was prepared by mixing 1.0 µl (10 pmole μl^{-1}) forward primer, 1.0 µl (10 pmole μl^{-1}) reverse primer, 10.0 µl 5x PCR assay buffer containing 1.5 mM $MgCl_2$, 2 µl $MgCl_2$, 1.0 µl dNTP mix (10 mM μl^{-1}), 0.25 µl of Taq polymerase (5 U μl^{-1}), DNA template 3.0 µl and MiliQ deionised water 31.75 µl. The PCR was performed in 30 cycles. The denaturation, annealing and primer extension was carried out as mentioned in Table 1.

Table 1

Steps of PCR

Step No.	*Temperature*	*Time*
1	Initial denaturation (94°C)	5 min
2	Denaturation (94°C)	30 sec
3	Annealing (47°C)	1 min
4	Extension (72°C)	90 sec
5	Go to step 2	Repeat 25 times
6	Final extension (72°C)	10 min
7	Hold (4°C)	-

The PCR products (7µl) were electrophoresed along with 100 bp DNA ladder (Promega, USA) at 100 V/cm for 1-2 h (depending upon length of the gel or till the dye migrated more than half of the length of the gel) in 2% Agarose gel prepared in 1x TBE buffer containing 0.5 ng ml^{-1} of ethidium bromide. The amplicons were visualized under UV light and documented by Isogen Gel Doc Bioimaging system.

As a control for genetic detection of toxigenic genes in the test isolates, the standard strain of *E. coli* (MTCC No. 723) procured from Institute of Microbial Technology, Chandigarh (India) was used in the study. This strain is pathogenic having O78:K80:H11 serotype and CFA/I+ LT+ST+ properties (producing colonization Factor antigen I (CFX/I) and heat stable/heat labile entrotoxins).

Results and Discussion. Enterotoxigenic *E. coli* (ETEC) strains were first recognized as causes of diarrhoeal diseases in piglets, where the disease continues to cause lethal infection in new born animals (Alexander 1994). Enterotoxigenic *E. coli* from diarrhoeic calves, camel, goats, lambs, infants and milk have been isolated in India (Chauhan & Kaushik 1991; Dubey et al 2000).

In the present investigation all the 60 isolates were found to harbour single plasmid the molecular size of which was calculated to be approximately 10-15 kb (Figure 1). The presence of single plasmid in all the isolates in the study are in conformity to the earlier observation wherein more than 80% of *E. coli* harboured plasmid of varying molecular weights and molecular sizes (Rezina et al 2001; Wachsmuth et al 1983). Ahmadi et al (2009) did plasmid profiling of *E. coli* isolated from various animals and found all isolates to harbour a single plasmid of 22 kb.

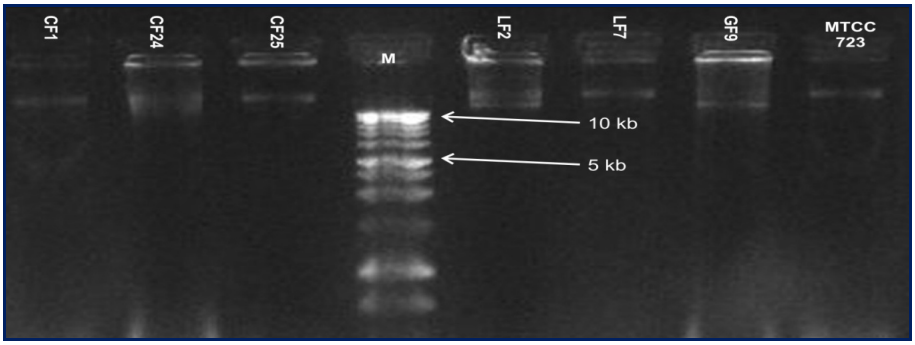

Figure 1. Plasmid profile of *E. coli* isolates.

Detection of LT and ST enterotoxin genes. The standard MTCC 723 produced amplicons of expected sizes i.e. 132 bp for LT and 171 bp for ST genes, respectively but none of the isolates in the present investigation showed presence of LT gene (Figure 2) and ST gene (Figure 3).

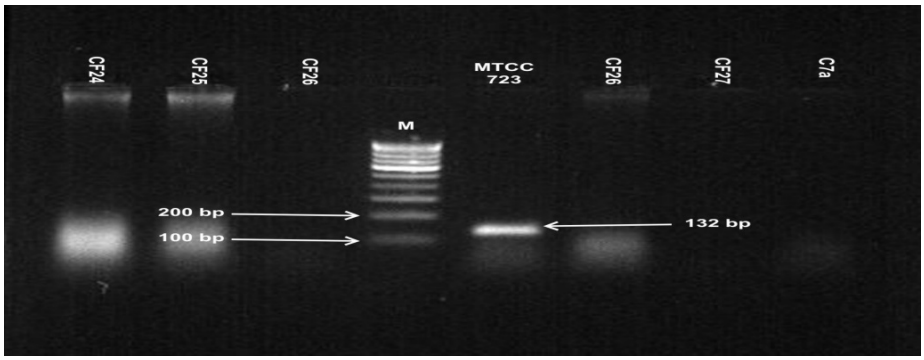

Figure 2. Amplification of LT gene from *E. coli* isolates.

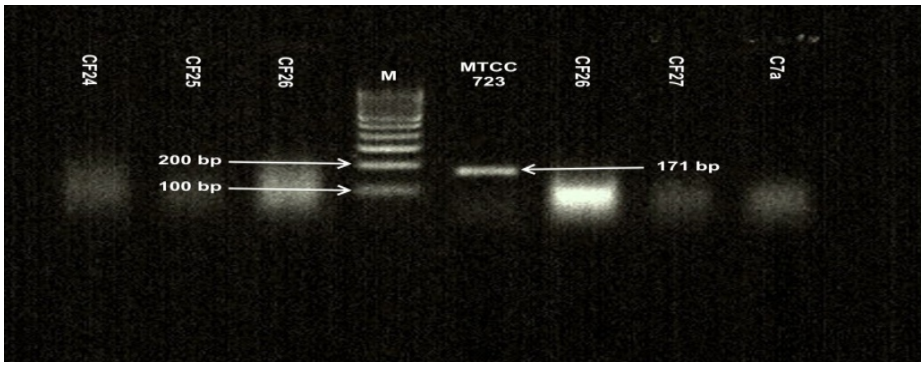

Figure 3. Amplification of ST gene from *E. coli* isolates.

The absence of LT or ST gene shows low frequency of enterotoxin gene among *E. coli* strains isolated from diarrhoeic young ones up to one month of age (Nishikawa et al 2002). Such a low frequency may be comparable to French (0%), Spanish (1.3%) and

Brazilian (3.9%) data (De Rycke et al 1986; Salvadori et al 2003). Similarly, Nishikawa et al (2002) from human diarrhoeal cases and Salvadori et al (2003) from diarrhoeic calves could detect presence of very low percentage of LT and ST genes using PCR technique. Mohammad et al (1985) isolated 273 *E. coli* strains from diarrhoeic calves and only reported VT (28%) and ST (18%) toxins whereas all of the isolates were negative for LT toxin gene. They found that LT toxins were not significantly associated with diarrhoea. The ETEC strains are often associated with diarrhoea in 2 to 3 days old calves (Gyles 1986).

Wani (2006) reported very low prevalence of LT toxin in children in Kashmir (India). He reported only 3 (0.92%) out of 326 *E. coli* isolates that were found to carry LT toxin gene of which two ETEC isolates belonged to O64 and O88 serogroups and one was untypable.

Acha et al (2004) obtained 1087 isolates from 47 diarrhoeic calves in Mozambique, of which they subjected 55 representative strains to PCR for detection of enterotoxin gene (ST and LT) and did not detect presence of enterotoxin genes in all 55 strains.

A very low prevalence of ST (2.4%) and LT (1.9%) toxin genes in various diarrhoeic animals in Turkey was reported by Gulhan et al (2009) in a study involving 695 *E. coli* strains from 2300 faecal samples collected from various diarrhoeic animals. Likewise, Rajkhowa et al (2009) reported very low number of LT (1) and ST (2) toxin producing *E. coli* strains in mithun calves by PCR. Similar findings were also reported by Mainil et al (1990) and Shin et al (1994). In another study carried out in lambs very low per cent of ST toxin (1.4%) and total absence for LT toxin gene was demonstrated by Orden et al (2002).

Darong (2010) investigated pathogenicity of *E. coli* and its association with concept of HPI (high pathogenicity island) in neonatal piglet diarrhoea. Wherein it was recorded that the HPI-harboring *E. coli* isolates were more frequently detected in the diarrhea samples and most HPI possessing isolates (95.0%) were toxin negative. The high-pathogenicity island has been identified in pathogenic *E. coli* strains causing diarrhea and dysentery in calves, rabbits, piglets and human (Paauw et al 2009). On the other hand, most *E. coli* are the normal inhabitants of the intestinal tracts of animals (Levine 1987).

The *E. coli* that causes diarrhoea in farm animals do not always act by elaborating toxins (Okerman 1987). These animal enteropathogenic *E. coli* attach to and efface the microvilli of the gut epithelium. In rabbits, only this type of *E. coli* enteritis is known to be important and is caused by enteropathogenic *E. coli* strains also called “attaching effacing *E. coli*” (AEEC). The AEEC may produce diarrhoea in calves also (Janke et al 1990). Further studies are necessary to know the possible role of AEEC and other types of enteropathogenic *E. coli* in lamb diarrhoea (Cid et al 1993). Blanco et al (1996) isolated 144 *E. coli* from 144 diarrhoeic lambs (5-21 days old) from 38 flocks in Spain and investigated for various toxins. They found only 10 toxigenic strains out of which only two were LT toxin producing. The outbreaks of neonatal diarrhoea investigated in this study, which typically affected lambs older than four days were not associated with ETEC. This is not surprising, since ETEC cause diarrhoea in lambs in the first three days of life (Tzipori et al 1981; Wray et al 1984). Thus diarrhoea is a common disease in lambs but evidence suggests that only a low proportion of cases could be attributed to toxigenic *E. coli.*

The fact that diarrhoea is not always associated with elaborated toxins is supported by the findings of Huasai et al (2012) also where they detected presence of ST and LT gene in 3.30% and 2.31% strains, respectively from normal faecal samples from adult dairy cattle.

Conclusions. In the present study all the 60 isolates from cattle claves, goat kids and lambs were found to possess plasmids of 10-15 kb molecular weight but none was detected to possess either LT or ST gene responsible for enterotoxigenic property of the *E. coli.*

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