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Association Between Virulence Genes and Serogroups of *Escherichia coli* Isolates from Calves

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ABSTRACT

Escherichia coli associated calf diarrhoea is a major economic concern for the dairy producers in this geographic area. The aim of this study is to investigate various pathotypes, virulence genes and their association with serogroups of *E. coli* isolated from diarrhoeic and healthy calves**.** A total of 194 rectal swabs were collected from diarrhoeic (129) and healthy (65) calves of different age groups from various districts of Andhra Pradesh and Telangana States. The *E. coli* were isolated and confirmed by cultural, biochemical and molecular testes. The virulence genes of *E. coli* pathotypes were detected using PCR with specific primers. Serogrouping was carried out at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh. Out of 129 and 65 faecal samples (194) collected 60 (46.51%) and 21 (32.31%) samples were found positive for *E. coli* in diarrhoeic and healthy calves, respectively. Among different age groups, high rate of *E. coli* was isolated from 1-7 day old calves. Among the virulence genes of STEC, *eaeA & hlyA* genes were most prevalent in diarrhoeic (20%) and healthy calves (14.28%) and only *stx1* was detected in diarrhoeic (6.66%) and healthy (4.76%) calves. The *cnf2* (5%) and EAST1(3.33%) genes were detected only in diarrhoeic calves. The serogroup O157 which is having zoonotic significance was detected in STEC isolates from diarrhoeic and healthy calves. This study concluded that *E. coli* isolates from diarrhoeic calves are potential source of virulence genes and both diarrhoeic and healthy calves are the reservoirs of O157 serogroup in this geographic area.

HIGHLIGHTS

- *E. coli* isolation rate was high in calves of below one week age.
- *eaeA & hlyA* genes were most prevalent among the STEC isolates from diarrhoeic and healthy calves.
- Calves are reservoirs of O157 serogroup in this geographic region.

Keywords: *E. coli*, pathotypes, virulence genes, serogroups, calves

Calf diarrhoea is one of the leading causes of calf morbidity and mortality resulting economic loss to the dairy producers. Among the etiological agents of calf diarrhoea, *Escherichia coli* has been identified as a significant infectious cause (Nguyen *et al*., 2011). *E. coli* is a gram-negative, rod-shaped, flagellated, non-sporulating and facultative anaerobic bacterium of the family Enterobacteriaceae. The pathogenic *E. coli* can be categorized based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper *et al.,* 2004). The diarrheagenic *E. coli* are classified into enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) (Nagy and Fekete, 2005). Various pathotypes of *E. coli* have been known by the presence of certain

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virulence genes. The virulence factors such as heat-labile enterotoxins (LT) and heat-stable enterotoxins (STa or STb) are able to causes severe diarrhoea in calves (Nguyen and Vu-Khac, 2011). The Shiga toxin 1 (*stx1*) and Shiga toxin 2 (*stx2*), the protein intimin (*eae*) and the plasmid-encoded enterohaemolysin or enterohaemorrhagic *E. coli* haemolysin (*ehly*) are related to the pathogenesis of STEC strains (Law, 2000). The NTEC are able to produce two types of cytotoxic necrotizing factors (*cnf1* and *cnf2*).

E. coli was serogrouped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Nataro and Kaper, 1998). Currently there are 186 different *E. coli* O-groups and 53 H-types (Fratamico *et al.,* 2016) were recognized. Several serotypes of EHEC such as O26:H11, O91:H21, O111:H8, O157:NM, and O157:H7 are frequently associated with human diseases (Paton and Paton, 1999). Therefore, pathogenic *E. coli* constitutes a genetically heterogeneous family of bacteria, and they continue to evolve. Hence, the present study was carried to find out various pathotypes, their virulence genes and serogroups of *E. coli* isolates from diarrhoeic and healthy calves.

MATERIALS AND METHODS

Collection of faecal samples

A total of 194 rectal swabs were collected from diarrhoeic (129) and healthy (65) calves of different age groups viz. 1 to 7, 8 to30, 31 to 60 and 61 to 90 days from organized dairy farms and individual farmers of East Godavari, West Godavari, Krishna, Chittoor Districts of Andhra Pradesh and Ranga Reddy District of Telangana State.

Isolation and identification of *E. coli*

The faecal samples were streaked on to MacConkey agar plates and incubated overnight at 37 ˚C for 24h. The pink colonies obtained were again inoculated on EMB agar. The colonies showing green metallic sheen were selected and confirmed as *E. coli* by standard biochemical tests (Cruickshank, 1970). The *E. coli* were further confirmed by PCR amplification using *E. coli* 16s rRNA gene specific primers quoted by Sun Dong-bo *et al*. (2011). The PCR reactions were carried out in an Eppendorf thermal cycler. The amplification conditions were 5 min of denaturation at 95°C, followed by 35 cycles of 95°C for 1 min, 50°C for 50 s, and 72°C for 1 min, and a final extension step of 72°C for 10 min. DNA amplified by PCR was subjected to 2% agarose gel electrophoresis as described by Sambrook and Russel (2001).

Multiplex PCR for detection of virulence genes (*stx*1, *stx*2, *eae*A, *hly*A)

The primers used in the present study for the detection of shiga toxin producing *E. coli* were as described by Paton and Paton (1998).

Table 1: Details of the primers used for the detection of *stx*1, *stx*2, *eae*A and *hly*A genes

Sl. No.	Primer	Sequence (5'---- 3')	Target gene	Expected Amplicon size (bp)
1	*Stx*1F	ATAAATCGCCATTCGTTG ACTAC	*stx*1	180
	*Stx*2 R	AGAACGCCCACTGAGAT CATC		
2	*Stx*2 F	GGCACTGTCTGAAACTG CTCC	*stx*2	254
	*Stx*2 R	TCGCCAGTTATCTGACAT TCTG		
3	*eaeA* F	GACCCGGCACAAGCATA AGC	*eae*A	384
	eaeA R	CCACCTGCAGCAACAAG AGG		
4	*hlyA* F	GCATCATCAAGCGTACG TTCC	*hly*A	534
	hlyA R	AATGAGCCAAGCTGGTT AAGCT		

Standardization of multiplex PCR protocol for detection of *stx*1, *stx*2, *eaeA* & *hlyA* virulence genes

PCR for amplification of the *stx*1, *stx*2, *eae*A, *hly*A genes was set up in 25 µL reaction. Following initial trails with varying concentration of components the reaction mixture was optimized as below:

Component	Quantity
Primer F (20 p mol)	0.12 µL× 4
Primer R (20 p mol)	0.12 µL× 4
Master mix	12.5 µL
Template	5.00 µL
NFW water	6.54 µL
TOTAL	**25 µL**

PCR tube containing the reaction mixture was flash spun in a microcentifuge to settle the reactants at the bottom. PCR assay was performed in Eppendorf thermal cycler with heated lid. Samples were subjected to 35 cycles as per the procedure of Paton and Paton (1998). The cycle consisting of one min of denaturation at 95ºC, two min of annealing at 65ºC and 1.5 min of elongation at 72ºC for the first 10 cycles, decrementing annaealing temperature to 60ºC by cycle 15, one min of denaturation at 95ºC, two min of annealing at 60ºC and 1.5 min of elongation at 72ºC, incrementing to 2.5 min from cycles 26 to 35. On completion of the reaction, tubes with PCR products were held at 4ºC.

Detection of STa, LT, *cnf2,* EAST1 genes: The primers and PCR conditions used for the detection of STa, LT, *cnf2,* EAST1genes is presented in Table (2 &3).

Standardization of PCR for the detection of STa, LT, *cnf2,* EAST1 genes

PCR for amplification of genes was set up in 25 µL reaction (Eppendorf thermal cycler). Following initial trials with varying concentration of components the reaction mixture was optimized as below.

Serogrouping of the *E. coli* isolates: The isolated samples which were confirmed as *E. coli* by cultural, Gram's staining, biochemical procedures and molecular detection were submitted for serogrouping to National *Salmonella* and *Escherichia* Centre, Central research institute, Kasauli, Himachal Pradesh on nutrient agar slants.

RESULTS AND DISCUSSION

Prevalence of *E. coli*

Out of 129 and 65 faecal samples (Total 194) collected 60 (46.51%) and 21 (32.31%) samples were found positive for *E. coli* in diarrhoeic and healthy calves, respectively. Similar trend of high prevalence in diarrhoeic calves than in healthy calves was also reported by Begum *et al*. (2014) in Assam and Luna *et al*. (2009) in Austria. Compared

Table 2: Details of the primers used for the detection of virulence genes

Primer	Sequence (5'---- 3')	Target gene	Expected Amplicon size(bp)	Reference
LT-*F*	ATTTACGGCGTTACTATCCTC	LT	281	Nguyen *et al.* (2011)
LT-*R*	TTTTGGTCTCGGTCAGATATG			
STa-*F*	GCTAATGTTGGCAATTTT TATT TCTGTA	STa	190	Nguyen *et al.* (2011)
STa-*R*	AGGATTACAACAAAGTTCACAGCAGTAA			
Cnf2-F	A AATCTAATTAAAGAGAAC	*Cnf2*	543	Blanco *et al.* (1996)
Cnf2-R	CATGCTTTGTATATCTA			
EAST1-*F*	CCATCAACACAGTATAT	EAST1	111	Nishikawa *et al.* (2002)
EAST1-*R*	GGTCGCGAGTGACGGCTTTGT			

Table 3: Composition of PCR reactions for virulence genes

Gene	Mastermix µL	Forward primer (20 picomoles/ µL) µL	Reverse primer (20 picomoles/µl) µL	Template µL	Mgcl2 µL	NFW µL
LT	12.5	0.62	0.62	3.00	—	8.26
STa	12.5	0.62	0.62	3.00	—	8.26
cnf2	12.5	0.62	0.62	3.00	—	8.26
EAST1	12.5	0.62	0.62	3.00	—	8.26

Table 4: PCR conditions for detection of virulence genes

Gene	Initial denaturation Min (°C/min)	Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Final extension (°C/min)	No of cycles
LT	94/10	94/40	60/40	72/60	72/7	30
STa	94/3	94/40	50/45	*72/90	72/7	30
cnf2	94/2	94/60	48/60	72/60	72/7	30
EAST1	94/3	94/60	55/60	72/120	72/7	35

*For STa extension time was ramped for an additional 3 seconds per cycle.

Table 5: Distribution of virulence genes in *E. coli* isolates from diarrhoeic and healthy calves

Pathotype	Virulence gene	No. of *E. coli* isolates from diarrhoeic calves with the virulence gene (N=60)	%	No. of *E. coli* isolates from diarrhoeic calves with the virulence gene (N=21)	%
STEC	*stx1*	4	6.66	1	4.76
	stx2	0	0	0	0
	stx1, stx2 & hlyA	0	0	0	0
	stx1, *eaeA* & *hlyA*	0	0	0	0
	stx1& hlyA	5	8.33	0	0
	eaeA & hlyA	12	20.00	3	14.28
NTEC	*cnf2*	3	5.00	0	0
EAEC	EAST1	2	3.33	0	0

with present study, higher prevalence *E. coli* in diarrhoeic calves was observed by Begum *et al*. (2014) in Assam (88.57); Pourtaghi *et al*. (2013) in Iran (86.7%); Haggag and Khaliel (2002) in Egypt (82%); and Islam *et al.* (2015) in Bangladesh (57%); and in healthy calves Begum *et al*. (2014) in Assam (60%); Picco *et al*. (2015) in Argentina (63.7%). On the other hand, lower prevalence of *E. coli* than the present study was reported in diarrhoeic calves by Oporto *et al*. (2008) in Northern Spain (35.9%), Luna *et al.* (2009) in Austria (18.9%) and Izzo *et al*. (2011) in Australia (17.4%) and in healthy calves by Luna *et al*. (2009) in Austria (15.7%). The differences in prevalent rates may be due to differences in calf management in different study areas.

Among different age groups, high rate of *E. coli* was isolated from 1-7 day old diarrhoeic (51.52%) calves followed by 46.67, 44.00 and 36.36% from 8-30, 61-90 and 31-60 day age groups. In healthy calves also *E. coli* isolation rate was high (41.18%) in 1-7 day old calves followed by 34.61, 28.57 and 12.5% from 8-30, 31-60 and 61-90 days age groups, respectively. Consistent with the results Shahrani *et al.* (2014) also found highest incidence of *E. coli* in calves of 1 to 7 day old compared to the old calves. The increased isolation rate in young calves may be due to lower immunity particularly in those calves received insufficient colostrum (Abdulgayeid *et al.*, 2015).

The STEC isolates from diarrhoeic and healthy calves carried 6.66 and 4.76% of *stx1* gene. However, *stx2* gene was not detected either from healthy or diarrhoeic calves. Similar to present findings Luna *et al*. (2009) and Mercado *et al.* (2004) reported higher frequency of *stx1* gene than *stx2* gene in diarrhoeic and healthy calves in Austria and Argentina, respectively. However, Wani *et al.* (2003) reported more prevalence of *stx2* than *stx1* gene in diarrhoeic calves and lambs in India. Production of *stx2* toxin is an index for serious clinical consequences in infected patients, as there is a strong association between the presence of the *stx2* gene and the capacity of STEC strains to cause severe human disease (Bielaszewska *et al*., 2006).

Among the virulence genes of STEC studied, *eaeA & hlyA* genes were most prevalent in diarrhoeic (20%) and healthy cow calves (14.28%) (Table 5). Similar results of higher frequencies of *eaeA & hlyA* genes than *stx1* and *stx2*

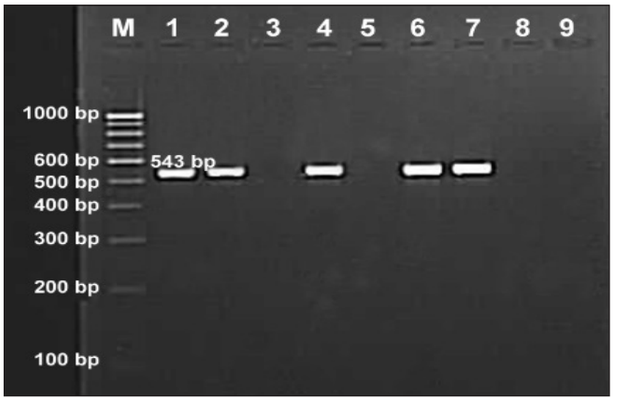

Fig. 1: Amplified product of *cnf*2 gene

Lane M: 100 bp DNA ladder; Lanes 1,2,4,6,7: *E. coli* isolates carrying *cnf2* gene; Lanes 3, 5,8 & 9: Negative isolates.

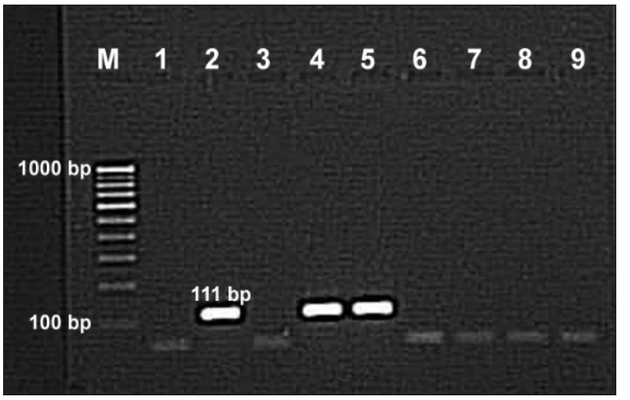

Fig 2 : Amplified product of EAST1 gene

Lane M: 100bp DNA ladder; Lane 2,4 & 5 *E. coli* isolates carrying EAST1 gene; Lane 1, 3 & 6 to 9 Negative isolates.

Table 6: Association between virulence genes and serogroups of *E. coli* isolates from diarrhoeic cow calves

Serogroups	No. of *E. coli* isolates	*stx1* (n=4)	*stx1 & hlyA* (n=5)	*eaeA & hlyA* (n=12)	*cnf2* (n=3)	EAST1 (n=2)
O2	1				+	
O8	2		+			
O9	3			+		
O49	1		+			
O63	1					+
O84	1			+		
O101	2	+				
O118	1				+	
O120	1	+				
O126	3			+		
O128	1			+		
O157	2			+		
UT	1	+				
UT	1				+	
UT	1					+
UT	2		+			
UT	2			+		

Table 7: Association between virulence genes and serogroups of *E. coli* isolates from healthy cow calves

Serogroups	No. of *E. coli* Isolates	*stx1* (n=1)	*eaeA & hlyA* (n=3)	*cnf2* (n=0)
				-
O116	1		+	
O91	1		+	
O157	1		+	
UT	1	+		

genes were reported by Wani *et al.* (2004) in diarrhoeic calves in India. Several earlier reports revealed a strong association between the carriage of *eaeA* gene and the capacity of STEC to cause severe human disease (Blanco *et al*. 2004). The combination of *stx1 & hlyA* genes were detected only in diarrhoeic calves (8.33) but not in healthy calves. Shahrani *et al.* (2014) also observed the combination of *stx1 & hlyA* genes in calves of Iran. This study was not detected *stx1, stx2 & hlyA* and *stx1, eaeA & hlyA* combinations either in diarrhoeic or in healthy calves in this geographic region.

In NTEC pathotype only *cnf2* gene was detected in diarrhoeic calves but not in healthy calves. Parallel with present findings, Borriello *et al*. (2012) also detected the increased frequency of *cnf2* (83%) genes in diarrhoeic calves in Italy. The *cnf2* positive *E. coli* strains were usually associated with septicaemia, diarrhoea, pneumonia, mastitis, abortions, urinary tract infections and metritis in cattle, goat and horses and these strains were also isolated from healthy cattle (Pohl *et al.,* 1993).

In India *cnf2* are found to be associated with childhood diarrhoea (Kavitha *et al.*, 2010). EAST1 gene was detected (3.33%) only in isolates from diarrhoeic calves. The EAST1 toxin has been commonly found in *E. coli* strains associated with diarrhoea in suckling and weaning piglets (Vu-Khac *et al*., 2004).

Association between virulence genes and serogroups

In diarrhoeic calves, the STEC belongs to O8, O49, O84, O101, O120, O126 O128, and O157 serogroups, while O91, O116 and O157 serogroups were identified in healthy calves (Table 6&7). This study also detected that the NTEC pathotypes belongs to O2 and O118 and EAST1 belongs to O63. Detection of O157 serogroup in diarrhoeic as well as healthy calves in this study indicated zoonotic significance. Several authors earlier reported that O157 is associated with hemorrhagic colitis and hemolytic-uremic syndrome in humans (Beutin *et al.,* 2001; Willshaw *et al.,* 2001; WHO, 1998). Several studies also earlier indicated that cattle are important reservoirs of O157 (Paiba *et al*., 2002; Zhao *et al*., 1995). Similar to the present results, the STEC from diarrhoeic calves in Srinagar, India, belongs to O8 serogroup (Wani *et al.,* 2007). Manna *et al*. (2010) and Islam *et al*. (2008) also detected O157 serogroup from STEC in faecal samples of slaughtered cattle and diarrhoeic calves in West Bengal and buffaloes in Bangladesh, respectively.

CONCLUSION

This study concluded that *E. coli* isolates from diarrhoeic calves are potential source of virulence genes than healthy calves. Detection of O157 serogroup in STEC isolates indicated calves are the potential reservoirs of this serogroup.

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