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Characterization of *E. coli* pathotypes of bovine and livestock farm environment origin

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ABSTRACT

In the environment of the farms, feed, fodder and water could be contaminated with fecal material especially which could constitute a reservoir of *Enterobacteriaceae bacteria*.Total of 80 fecal samples collected from diarrheic calves, normal calves, diarrheic cattle, healthy cattle and shed of cattle were included in this study. Of which 67 (83.75%) isolates were biochemically identified as *E.coli.* Among 67 *E.coli* isolates, 12 (17.91%) isolates were of diarrheic cases (10 isolates from 1 month-6 months calf and 2 isolates from diarrheic cow), 51 (76.11%) isolates were of healthy cows (14 isolates from 1 month-6 months calf and 37 isolates from normal cows), 2(2.98%) from water samples, one isolate (1.49%)each from cow manure and air sample at farm respectively, from cow shed. Eleven (16.41%) out of 67 isolates were found to cause lysis on sheep erythrocytes and 55 (82.089%) out of 67 isolates were found to be biofilm producers on Congo red. Twenty-four (35.82%) isolates out of 67 were positive for *bfpA* gene, eight (11.94%) for *eaeC* gene, while five (7.46%) for both the genes of *E.coli* strain.

Key words: *Enterobacteriaceae,* EPEC, ETEC, Pathotypes, Virulence.

INTRODUCTION

Escherichia coli is one of the members of the family *Enterobacteriaceae*, which resides as a commensal flora in the intestinal lumen of animals and humans but can cause diarrhea by different mechanisms (Liu *et al*.,2012). *Escherichia coli* is an important pathogen in bovine neonates, capable of causing intestinal and extra intestinal infections which constitute a public health hazard. These are disseminated in other environment as farm animals and derived foods, domestic and even in wild animals,healthy humans, waste water, vegetables and other sources (Ben Sallem *et al.,* 2011).Three general clinical syndromes associated with infection from inherently pathogenic *E.coli* strains includes Urinary tract infection, sepsis/ meningitis and enteric/diarrheal disease (Nataro,2005).Calf diarrhea is one of the most economic and pervasive concern in veterinary industry all around the world (Fernandez *et al.,* 2009). Along with mixed infections with other enteric pathogens, many additional parameters, such as nutritional factors, hygiene conditions and environmental factors, also contribute to the final outcome of the disease (Shams *et al.*, 2010; Croxen and Finlay, 2010). Currently there are at least eight recognized enterovirulent *E. coli* pathotypes that cause gastrointestinal disease Enteropathogenic *E.coli* (EPEC), Enteroaggregative *E.coli* (EaggEC), Enteroinvasive *E.coli* (EIEC), Enterotoxigenic *E.coli* (ETEC), Enterohaemorrhagic *E.coli* (EHEC), Shigatoxin producing *E.coli* (STEC) Diffusely adherent *E.coli* (DAEC), Necrotizing factor producing *E.coli* (NTEC) Uropathogenic *E.coli* (UPEC) are important causes of diarrhoeal diseases in developing countries(Moxley and Smith, 2010).

EPEC strains are eae harbouring diarrhoeagenic *E.coli* that possess the ability to form attaching- effecting (A/E) lesions on intestinal cells and that do not possess shigatoxin encoding genes (Moxley and Smith, 2010). Typical EPEC strains produce bundle forming pili (BFP), which are long, flexible, rope-like structures composed of intertwining fibers. BFP are polymers of bundlin, a pilin protein that is encoded by the *bfpA* gene found on a large EPEC plasmid of 60 Mda called pMAR2, which is influencing the expression of chromosomal genes for *eaeA* and intimin, an outer membrane protein. BFP probably plays a role in the bacterial colonization process *in vivo* because of the intertwine, creating a fibrous network connecting individual bacteria. The result consists in localized adherence, the capacity of EPEC to form defined microcolonies upon the epithelial cells and human intestinal tissue.

The best-studied virulence factor is *eaeC*, the master regulator of EAEC virulence, which controls expression of adherence factors, a dispersin protein, and a large cluster of genes encoded on the EAEC chromosome (Nataro, 2005).

Cattle are a key reservoir for EAEC, which is a highly infectious A/E pathogen that colonizes the distal ileum and large bowel in humans and is often the causative agent

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of outbreaks of severe gastroenteritis in developed countries (Kaper, *et al.,* 2004).The EPEC strains could be further classified as typical on basis of *eae* the *bfp* genes and exhibiting a localized adherence pattern on cultured epithelial cell in more frequent developing countries while atypical (eae+ ,bfp-) in more frequent in industrialized countries (Blanco *et al*., 2006)

There are few studies about the prevalence of EPEC and EAEC among bovine fecal samples and livestock farm environment. The aim of this study was to isolates the *E.coli* and to characterize the recovered isolates for by virulence attributes.

MATERIAL AND METHODS

A total of 80 samples from different origin livestock farm were collected in sterile containers, labeled and transported to laboratory for immediate analysis. Out of 80 samples, 12 fecal samples were from diarrheic cases (2 from cow and 10 from 2-6 months calves), 54 fecal samples were from healthy cattle (1-19 years), 5 water samples (from water tank at farm), 7 manure samples (from farm) and 2 air samples which were collected by keeping EMB agar plates open for 1 min (Yassin and Almouqatea ,2010) in cattle shed. The isolation and identification of *E.coli* were performed as per the guidelines of Cowan and Steel (1970) and Cruickshank *et al.*(1975) and Rappaport *et al.*(1953). *E.coli* isolates were screened for hemolysin production on 5% sheep blood agar

The isolates were further analysed for biofilm production on Congo red medium, prepared as per the Berkhoff and Vinal (1986) and *E.coli* isolates were streaked on the CR medium and incubated at 37°C for 3 days. The colonies were examined daily for color change. The *E.coli* isolates which produced intense orange or brick red colonies were considered as CR positive and those which produced grayish—white colonies and remained so throughout the incubation period were recorded as CR negative.

Detection of *bfpA* and *eaeC* genes using Polymerase Chain Reaction

Preparation of DNA template: Genomic DNA was prepared as per standard Phenol: Chloroform method. The DNA pellet obtained was resuspended in 50mL TE buffer and the DNA was stored at -20°C until futher use .The DNA was quantified using spectrophotometer.

The primers for detection of the virulence genes *viz*. *bfpA* and *eaeC* used in study were synthesized from Sigma Aldrich Pvt. Limited, New Delhi. The details of the primer sequences are presented in Table 1.

Standardization of Singleplex Polymerase Chain Reaction (PCR): PCR was standardized for the detection of *bfpA* and *eaeC* genes (Obi *et al*., 2004).Briefly, PCR was set for 25µl reaction volume, for detection of *bfpA* and *eaeC* gene in *E.coli*, Reaction mixture for PCR was optimized as follows:

2.5µl of 10X PCR buffer (consisting of 100 mM Tris-HCl (pH 8.3), 500mM $MgCl_2$ and 0.01% gelatin), 0.5µl dNTP mix (10mM, with final concentration of 0.2mM), 1µl of 50mM $MgCl_2$ and 1µl of forward and reveres primer of each set (final concentration 0.1 µM each), 1 unit of Taq polymerase, 3.5µl of DNA and 14.5µl milliQ water to make up the reaction volume.

The cycling conditions conditions for *bfpA* and *eaeC* gene included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles each of 30 seconds denaturation at 94°C, 1 minute annealing at 56°C and 53°C and 2 min and 1 min extension at 72°C respectively . It was followed by final extension of 10 minutes at 72°C.

PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

RESULTS AND DISCUSSION

Escherichia coli isolates were biochemically identified and studied for haemolysis, biofilm production, and presence of virulence marker genes viz, *bfpA* and *eaeC* by single-plex polymerase chain reaction. Out of 80 samples collected frombovine fecal, water, manure and air samples from various farms, 67 (83.75%) isolates were biochemically identified as *E.coli*. Biochemically characterized strains were screened for haemolysis, biofilm production and presence of virulence marker genes viz. *bfpA* and *eaeC.*

Among 67 of *E.coli* recovered 12 (17.91%) isolates were of diarrheic cases (10 isolates from 1 month-6 months calf and 2 isolates from diarrheic cow), 51 (76.11%) isolates were of healthy cows (14 isolates from 1 month-6 months calf and 37 isolates from normal cows), 2(2.98%) from water samples, one isolate (1.49%) each from cow manure and air sample at farm, respectively (Table 2).

Phenotypic characteristic of *E.coli* isolates

Biofilm production: In the present study, *E.coli* isolates were screened for biofilm production on 0.03% Congo red agar.Fifty five (82.08%) out of 67 *E.coli* isolates were found to be biofilm producers on Congo red Agar (Table 3).

Table 1: Details of Primer

Primer		Sequence	Amplicon size (bp)	Reference
bfpA	F	5'ATTGGTGCTTGCGCTTGCTGC3'	326	Yatsuyanagi *et. al.,* (2002)
	R	3'GCCGCTTTATCCAACCTGGTA5'		
eaeC	F	5'CTGGCGAAAGACTGAATCAT3'	630	Schmidt *et. al.,* (1995)
	R	3'CAATGTATAGAAATCCGCTGTT5'		

Table 2: Details of *E.coli*isolated from different source

Sample/Source	Sample Screened	No. of samples positive for *E.coli*
Fecal sample from diarrheic calves(1-6 months calves)	10	10 (100%)
Fecal samples from normal calves(1-6 months calves)	17	14 (82.35%)
Fecal samples from diarrheic cattle (1-19 years)	2	2 (100%)
Fecal samples from healthy cattle(1-19 years)	37	37 (100%)
Water sample	5	2 (40%)
Manure sample	7	1 (14.28%)
Air sample	2	1 (50%)
Total No. of samples	80	67 (83.75%)

Table 3: Phenotypic virulence profile of *E.coli*

Sample/Source	No.of strains isolated	Haemolysis	Biofilm production
Fecal sample from diarrheic calves(1-6 months calves)	10	1 (10%)	7 (70%)
Fecal samples from normal calves(1-6 months calves)	14	4 (28.57%)	11 (78.57%)
Fecal samples from diarrheic cattle (1-19 years)	2	2 (100%)	2 (100%)
Fecal samples from healthy cattle (1-19 years)	37	2 (5.40 %)	35 (94.59%)
Water sample	2	1 (50%)	Nil (0%)
Manure sample	1	1 (100%)	Nil (0%)
Air sample	1	Nil (0%)	Nil (0%)
Total No. of samples	67	11 (16.41%)	55 (82.08%)

Hemolysin Production: Eleven (16.41%) out of sixty seven *E.coli* isolates were found to cause lysis on sheep erythrocytes (Table 3).

In Singleplex PCR out of total 67 isolates twenty-four (35.82%) were positive for *bfpA* gene(326bp), eight (11.94%) for *eaeC* gene(630 bp), while both genes were observed in five (7.46%) *E.coli* strains.(Table 4 and Figure 1)

Virulence factors associated with strains of *E.coli* includes adhesions, invasions and capsule production (Gyles, 1993).Twenty four (35.83%) out of 67 *E.coli* isolates possessed *bfpA* gene, amplicon of 326 base pairs produced by enteropathogenic *E.coli.* Result suggests that prevalence of virulence gene is more in diarrheic cases and comparatively lower than Bardiau *et al*. (2009) study.

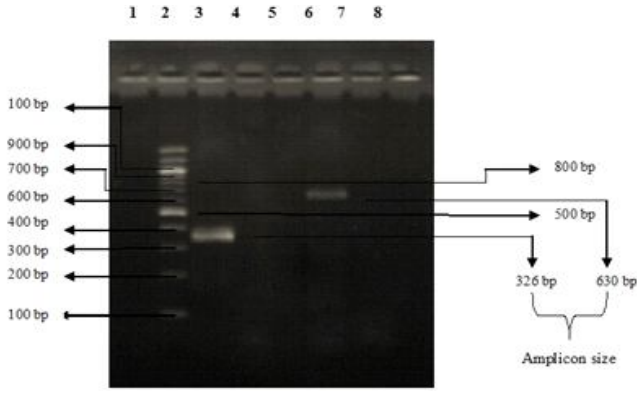
Shahrani *et al.*(2014) found that 630 out of 824 samples (76.45%)were positive for *E. Coli* from diarrheic calves in Iran. Herrera-Luna *et al.* (2009) reveales that 17% of all diarrheic and healthy calves of Australian herds were infected by *E.coli*, showed that 15.2 % *E. coli* strains harbored the shiga toxin genes *eae* and stx1, stx2 and hly. Nguyen *et al*., 2011 , revealed that the incidence of *eae* gene was 31.48% while in our study 11.94% isolates were positive for *eae* gene. Unno *et al.*(2011) studied 269 fecal sample reveals percentage of eaec A gene 10.8% (9 out of 83) from beef cattle and 1 (1.8%) (1 out of 55) from dairy cattle.

Prevalence of Enteroaggregative *E.coli*

Many putative virulence genes and EAEC strains that produce cytotoxins, biofilm. Although not all EAEC infections result in symptomatic illness (Adachi *et al*.,2002), eight (11.94%) out of sixty seven *E.coli* isolates possessed *eaeC* gene, amplicon of 630 base pairs produced by Enteroaggregative *E.coli.* Seventy of 156 isolates were positive for at least one virulence factor: ten (14.3 %) from diarrhoeic animals and 60 (85.7 %) from healthy calves while in our data eaeC was isolated from (8 of 67 11.94%) healthy cattle. The virulence factors identified were eae (17 of 70, 24.3 %), In diarrhoeic animals, eae (28.3 %), was detected in isolates from healthy calves. EPEC was detected in one isolate from a healthy animal (Andrade *et al.*,2012).

Table 4: Details of prevalence of virulence marker gene in *E.coli* isolates

Source	No. of strains isolated	Virulence marker gene		
		bfpA	***eaeC***	***bfpA+eaeC***
Fecal sample from diarrheic calves(1-6 months calves)	10	4 (40%)	NIL (0%)	NIL (0%)
Fecal samples from normal calves(1-6 months calves)	14	4(28.57%)	3(33.33%)	2 (22.22%)
Fecal samples from diarrheic cattle (1-19 years)	2	2 (100%)	NIL (0%)	NIL (0%)
Fecal samples from healthy cattle (1-19 years)	37	13(54.16%)	5(55.55%)	3(33.33%)
Water sample	2	NIL (0%)	NIL (0%)	NIL (0%)
Manure sample	1	1(100%)	NIL (0%)	NIL (0%)
Air sample	1	NIL (0%)	NIL (0%)	NIL (0%)
Total No. of samples	67	24(35.82%)	8(11.94%)	5(7.46%)



Lane 1 = Blank
Lane 2 = 100kb ladder
Lane 3 = Positive sample for *bfpA* gene
Lane 4 = Negative sample for bfpA gene
Lane 5 = Bank
Lane 6 = Positive sample for eaeC gene
Lane 7 = Negative sample for eceC gene
Lane 8 = Bank

Fig 1: Amplicon of bfpA and eceA gene of E.coli by singleplex PCR

CONCLUSION

Based on present findings it is concluded that diarrheic calves are major source of EPEC and EAEC than compared to healthy cattle. The two pathotypes studied reveals emerging molecular and cellular basis of pathogenesis for both pathotypes.

Out of sixty seven *E.coli* isolates, 24 (35.83%) were *bfpA* gene, characteristic of EPEC while 8 (11.94%) out of 67 *E.coli* isolates possessed *eaeC* gene, indicative of EAEC.Five (7.46%) out of sixty seven *E.coli* strain possessed both *bfpA* and *eaeC* genes. Out of sixty seven *E.coli* isolates, 55 (82.08%) were found to be biofilm producers on Congo red Agar and 11 (16.41%) out of 67 *E.coli* isolates were found to be haemolytic. The most frequent category of diarrheagenic *E. coli* detected was EPEC, such high prevalence of EPEC and EAEC in animals at farms shows the necessity to adapt proper management measures in the farm.

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