

## Molecular epidemiology and antibiotic resistance pattern of Enteropathogenic *Escherichia coli* isolated from bovines and their handlers in Jammu, India

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### ABSTRACT

The study was aimed to investigate the molecular epidemiology and antibiotic resistance pattern of Enteropathogenic *Escherichia coli* (EPEC) in bovines and their handlers in Jammu, India. A total of 173 samples comprising of 103 fecal samples from bovines (60 from cattle and 43 from buffaloes), 28 stools and 42 fingertip rinses from bovine handlers were collected during August 2011 to March 2012. The isolated 126 *E. coli* strains (86 from bovines and 40 from handlers) belonged to 25 different serogroups in addition to rough and untypeable strains. Using multiplex polymerase chain reaction, four EPEC strains were isolated; two each from bovines and their handlers, of which two possessed the hemolysin (*hlyA*) gene. The prevalence of EPEC was recorded as 1.66% (n=1/60) in cattle, 2.32% (n=1/43) in buffaloes, and 2.85% (n=2/70) in their handlers. Antibiogram studies with the EPEC revealed the presence of multi-drug resistant *E. coli*. The isolates were mostly resistant to Amikacin, Amoxicillin, Cefixime and Streptomycin, and sensitive to Chloramphenicol. This study indicates that bovines as well as their handlers in Jammu region harbor EPEC, many of which being multi-drug resistant and carrying the hemolysin gene could be of high pathogenic potential for humans.

### Keywords

Antibiotic resistance pattern, Bovines, EPEC, Handlers, Prevalence, Serogroups

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### INTRODUCTION

Enteropathogenic *E. coli* (EPEC) are an important cause of diarrhea worldwide especially in developing countries (Chen and Frankel, 2005; Alikhani et al., 2007). These extracellular pathogens intimately attach to the epithelial cells of intestine resulting in a severe lesion on the epithelial layer called attachment-effacement (A/E) lesion that destroys the absorptive villi resulting in malabsorption and diarrhea (Schmidt, 2010). The attachment is carried with an outer membrane protein intimin, encoded by the *eaeA* gene that exists as a part of 35 kb Pathogenicity Island called locus for enterocyte effacement (LEE). The latter encodes a type III secretion system (T3SS) that translocates multiple effector proteins into the host cells and disrupts the cytoskeleton to produce the A/E lesion (Clarke et al., 2003; Garrido et al., 2006).

Cattle are regarded as potential sources of EPEC that possess the virulence machinery to be pathogenic to humans (Monaghan et al., 2013; Bolton et al., 2014). Transmission to human occurs via the food chain (Trabulsi et al., 2002), but direct contacts with the ruminant feces and their environment may represent an increased risk factor for human disease (Aidar-

Ugrinovich et al., 2007). In India, several EPEC strains isolated from cattle belong to serogroups found associated with severe disease in humans (Wani et al., 2003). Taking this into consideration and the increasing concern of resistance of pathogenic bacteria to antibiotics among animals and humans, the present study was undertaken on the epidemiology and antibiotic resistance pattern of EPEC from bovines and their handlers in Jammu, India.

## MATERIALS AND METHODS

**Collection of samples:** A total of 173 samples were collected from bovines and bovine handlers. Of these, 103 fecal samples were collected from bovines comprising 60 from cattle and 43 from buffaloes per rectally at organized farms (Cattle farm Belicharana and Cattle farm, RS Pura), as well as from the household farms of the areas of Sidher, Khanachak, and Kotli, RS Pura, Jammu during the period from August 2011 to March 2012. Samples from cattle and buffalo calves were obtained by rectal swabs (Hassan et al., 2014). Twenty seven (27) stool samples and forty three (43) fingertip rinses were obtained from the persons handling or rearing the animals at these farms. Samples were collected in plastic containers and transported on ice to the laboratory.

**Selective plating:** The samples were enriched using MacConkey broth followed by selective plating (Khan et al., 2002). Two to three lactose fermenting colonies from each MacConkey Agar plate were streaked on Eosin Methylene Blue agar for appearance of characteristic metallic sheen. Presumptive *E. coli* after isolation were subjected to further biochemical identification (Hitchins et al., 1992; Quinn et al., 1994; Roy et al., 2012), and the purified cultures were maintained in 0.75% nutrient agar media slants in triplicate.

**Serogrouping:** The *E. coli* isolates were submitted to National Salmonella and Escherichia Centre, Central Research Institute Kasauli, HP, India for serogrouping on the basis of their "O" antigens.

**Multiplex polymerase chain reaction (mPCR):** DNA extraction was carried out by the heat lysis (snap chill) method. The *E. coli* isolates were first revived in MacConkey agar to obtain fresh isolates and re-suspended in 100 µL of nuclease free water in separate microcentrifuge tubes, which were subjected to heat lysis by keeping in boiling water bath for 10 min, and quickly placed in ice. The bacterial lysates were centrifuged at 10,000 rpm for 10 min, and the supernatant was taken

as template DNA for mPCR. Previously reported primers (Paton and Paton, 1998) were used in this study as mentioned in **Table 1**. Briefly, the mPCR was carried out in a final reaction volume of 25 µL using 2 mM MgCl<sub>2</sub>, 0.6 mM concentrations of each 2'-deoxynucleoside 5'-triphosphate (dNTPs), 5 µL of 5x assay buffer, 0.5 µL of forward and reverse primers, 2.0 µL template DNA and 1.0 U of GoTaq DNA Polymerase (PROMEGA CORPORATION, MADISON, USA) in a Thermocycler (APPLIED BIOSYSTEMS GENE AMP PCR SYSTEM 2400, USA). The amplified PCR products were analyzed by gel electrophoresis in 2% agarose containing ethidium bromide (0.5 µg/mL), visualized with UV-illumination, and imaged with gel documentation system.

**Table 1:** Primers used in the multiplex PCR (mPCR) reaction.

Primer	Sequence (5'-3')	Target size (bp)
<i>stx1</i> -F	ATA AAT CGC CAT TCG TTG ACT AC	180
<i>stx1</i> -R	AGA ACG CCC ACT GAG ATC ATC	
<i>stx2</i> -F	GGC ACT GTC TGA AAC TGC TCC	255
<i>stx2</i> -R	TCG CCA GTT ATC TGA CAT TCT G	
<i>eaeA</i> -F	GAC CCG GCA CAA GCA TAA GC	384
<i>eaeA</i> -R	CCA CCT GCA GCA ACA AGA GG	
<i>hlyA</i> -F	GCA TCA TCA AGC GTA CGT TCC	534
<i>hlyA</i> -R	AAT GAG CCA AGC TGG TTA AGC T	

The oligonucleotide sequences were described by Paton and Paton (1998).

**Antibiotic resistance testing:** The EPEC isolates were examined for their antimicrobial drug susceptibility/resistance pattern against 15 antibiotics by disc diffusion technique (Bauer et al., 1966; Roy et al., 2012). The antibiotic discs were obtained from HI MEDIA LABORATORIES PVT. LTD. (Mumbai, India) (**Figure 1**). Culture and sensitivity test (CST) was done by inoculating 3-4 colonies of *E. coli* in 5 mL nutrient broth followed by incubation at 37°C for 4h till light to moderate turbidity develops. After inoculation with 100 µL of the broth culture using sterile cotton swabs, plates of Mueller-Hinton Agar (MHA) (HI-MEDIA, Mumbai, India) were allowed to dry, antibiotic discs were placed 2 cm apart on the MHA plates and incubated at 37°C for 16-18 h. The diameter of the zones of inhibition was measured and interpreted with HI MEDIA antibiotic zone scale.

## RESULTS AND DISCUSSION

A total of 126 *E. coli* strains were isolated; 86 from bovines and 40 from their handlers. The isolates belonged to 25 different serogroups based on "O" antigen in addition to rough and untypeable strains

**Table 2:** Serogroups of *E. coli* isolated from organized and unorganized farms.

Farm	Species	Type of sample	Serogroup
Cattle Farm Belicharana	Cattle	Feces	O2 (1), O8 (1), O45 (1), O76 (1), O123 (1)
	Buffaloes	Feces	O2(1), O3 (1), O8 (1), O17 (1), O25 (1), O36 (2), O43 (2)
	Handlers	Stool	O68 (2), O55 (2), O106 (1)
Fingertip rinse		O106 (2)	
Cattle Farm, RS Pura	Cattle	Feces	O110 (1), O120 (1), O88 (1), O139(1), O64 (1), O14 (2), O58 (1)
	Buffaloes	Feces	O106 (2)
	Handlers	Stool	O21 (1), O25 (1), O37 (1)
Fingertip rinse		O106 (2)	
Rural Household Farms (unorganized)	Cattle	Feces	O25 (2), O106 (1)
	Buffaloes	Feces	O52 (1), O60 (1), O91 (1), O106 (2), O8 (1)
	Handlers	Stool	O60 (2), O68 (2), O106 (1)
		Fingertip rinse	O60 (2)

Figures in parenthesis indicate number of isolates.

**Table 3:** Prevalence of Enteropathogenic *E. coli* in cattle, buffaloes and their handlers.

Species	No. of samples	<i>E. coli</i> isolated	<i>E. coli</i> with virulence gene(s)			Prevalence (%)
			<i>eaeA</i>	<i>hlyA</i>	<i>eaeA</i> + <i>hlyA</i>	
Cattle	60	51	1	3	0	1 (1.66)
Buffaloes	43	35	0	1	1	1 (2.32)
Handlers	70	40	1	0	1	2 (2.85)
Total	173	126	2	4	2	4 (2.31)

(Table 2). Most of these serogroups were described by Wani et al. (2003) and Orden et al. (2002) isolated from bovines. Serogroups O3, O8, O17, O25 and O76 were reported by Vagh and Jani (2010) in cattle and buffalo calves, whereas O8, O17 and O25 were reported in cattle and buffalo calves by Joon and Kaura (1993) and Kaura et al. (1991). The presence of other serogroups could be due to their varied environmental distribution. Certain *E. coli* strains with serogroups O25, O106 and O60 were isolated from bovines as well as their handlers at the same farm indicating their possible transmission (Table 2).

#### Prevalence of EPEC in bovines and their handlers:

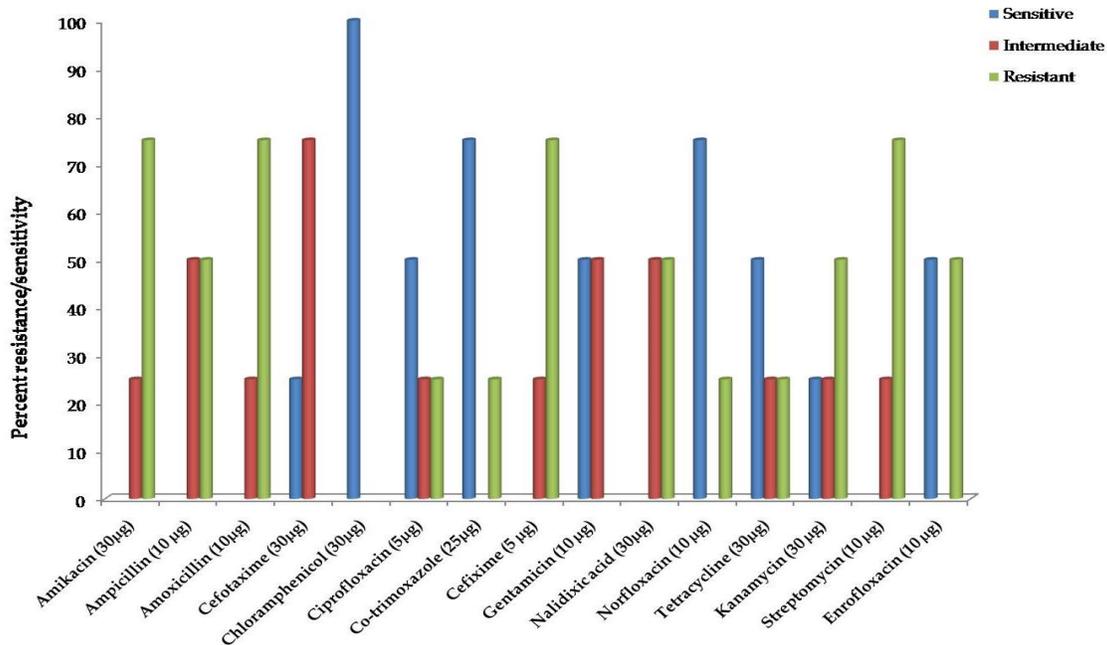
Multiplex PCR revealed that only 8 *stx*<sub>1</sub> and *stx*<sub>2</sub> negative *E. coli* isolates were carrying the intimin (*eaeA*) and/or hemolysin (*hlyA*) genes. Two out of 4 (50%) *eaeA* positive, *stx* negative *E. coli* isolates (EPEC) carried the hemolysin (*hlyA*) gene (Table 3). Earlier, a high proportion of *hlyA* gene (80%) among the EPEC and other *stx* negative *E. coli* strains was observed among calves in Brazil (Aidar-Ugrinovich et al., 2007) and from cattle (95.3%) in Australia (Hornitzky et al., 2005), indicating that bovine EPEC frequently carry the hemolysin gene.

All the four EPEC isolates were obtained from one farm i.e., Cattle Farm Belicharana, and were untypeable. The prevalence of EPEC in cattle, buffaloes and bovine handlers was 1.66%, 2.32% and 2.85%, respectively (Table 3). Several other researchers have reported EPEC prevalence close to these values. In Kashmir

India, it was found to be 1.53% in diarrhoeic calves (Kawoosa et al., 2008), 2.7% from calves in Sao Paulo, Brazil (Aidar-Ugrinovich et al., 2007), 3.7% in bovine feces from Ireland (Monaghan et al., 2013), 5.8% in cattle feces from a cluster of twelve farms in Netherland (Bolton et al., 2014), and from 1.8% of diarrheic stool samples in Kolkata, India (Dutta et al., 2013). However, the prevalence of EPEC from rural household farms was nil in our study, which is in contrast to other findings from Kashmir, India reported in calves at unorganized farms (Kawoosa et al., 2008). The isolation of EPEC strains of bovine and human origin from one farm where the handlers had an association with large number of animals suggests that risks of transmission to the handlers could be higher in larger intensive farms as compared to small and household farms.

#### Antibiotic resistance pattern of the EPEC isolates:

Resistance to three or more antibiotics was observed in one EPEC isolate of bovine origin and among two human isolates. Highest resistance (75%) was observed against Streptomycin, Amoxicillin, Amikacin and Cefixime (Figure 1). However, all of the EPEC isolates were sensitive to Chloramphenicol, followed by Norfloxacin (75%) and Co-trimoxazole (75%). Interestingly, an EPEC isolate obtained from the stool sample of a bovine handler at Cattle Farm Belicharana was resistant to twelve of the fifteen antibiotics used, but showed sensitivity to Chloramphenicol and intermediate resistance to Gentamicin and Cefotaxime.



**Figure 1:** Antibiotic resistance pattern of EPEC (n=4) against the 15 commonly used antibiotics.

Multi-drug resistance among EPEC from human patients was reported from North India (Vaishnavi and Kaur, 2003). Higher resistance of EPEC to Streptomycin, Ampicillin, Tetracycline and Sulphonamides was also reported from children in Brazil, with 43% of typical EPEC isolates being resistant to three or more of the antibiotics tested (Scaletsky et al., 2010). Resistance of EPEC to Nalidixic acid and Tetracycline isolated from bovine feces at different bovine farms was reported from Ireland (Bolton et al., 2014). However, the isolates showed intermediate resistance to Ciprofloxacin unlike our study in which the isolates in general showed sensitivity to Chloramphenicol, Norfloxacin, Co-trimoxazole and Ciprofloxacin. The higher antibiotic resistance among EPEC of both human and animal origin isolated in this study could be a cause of concern for the public health.

## CONCLUSIONS

This study indicates that cattle, buffaloes as well as their handlers in Jammu region harbor EPEC. A high prevalence of hemolysin gene among the EPEC and other *E. coli* strains and the multiple antibiotic resistance warrant of their high pathogenic potential to humans. However, further epidemiological studies are suggested involving large populations especially among the unorganized farms of this region.

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