

ORIGINAL ARTICLE

Serogroups, Atypical Biochemical Characters, Colicinogeny and Antibiotic Resistance Pattern of Shiga Toxin-producing *Escherichia coli* Isolated from Diarrhoeic Calves in Gujarat, India

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Impacts

- *Escherichia coli* (*E. coli*) from neonatal diarrhoea in calves carry genes and have biochemical properties (urease production) responsible for human disease and thus may be an important source of infection to humans as they are present in the faeces of the calves.
- Different *E. coli* serogroups showed multiple antibiotic resistance so that the indiscriminate use of antibiotics may lead to the development of drug resistance in bacteria thus making treatment difficult.
- Certain serogroups like O2, O55, O86, O128 and O157 isolated in this study have been found to be associated with human diseases and thus may pose a public health hazard.

Keywords:

Shiga toxin-producing *E. coli*; calf diarrhoea; India; colicinogeny; antibiotic resistance

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Summary

This study was designed to investigate the antibiotic resistance, colicinogeny, serotyping and atypical biochemical characteristics of 41 Shiga toxin-producing *Escherichia coli* (STEC) strains detected using polymerase chain reaction from 90 *E. coli* strains isolated from 46 diarrhoeic calves. The STEC strains belonged to 14 different serogroups. Seventeen per cent of the STEC strains carried the *eaeA* gene while 14.28% of the 49 non-STEC strains were *eaeA* positive. Twenty eight (68.29%) of the 41 STEC strains were rhamnose non-fermentors. All the STEC strains revealed resistance to at least three of the antibiotics tested. 100% resistance was found against kanamycin and cephalexin followed by cephaloridine, enrofloxacin, amikacin, ampicillin, tetracycline, ceftiofur, ciprofloxacin, colistin and co-trimoxazole. Eighteen (44%) of the STEC strains produced colicin and all these colicinogenic strains were resistant to three or more antibiotics. Eleven STEC strains (26.82%) showed urease activity. The results of this study suggest that diarrhoeic calves are an important reservoir of STEC strains that are potentially pathogenic for farm animals and humans. Moreover, rhamnose fermentation, colicinogeny and atypical biochemical behaviour, such as urease activity, may serve as important markers or diagnostic tools for epidemiological surveys to trace the source of infection in disease outbreaks.

Introduction

The emergence and dissemination of antimicrobial-resistant Shiga toxin-producing *Escherichia coli* (STEC) zoonotic bacteria in food-producing animals pose a global

public health hazard because of potential transmission from animals to humans. The recently published WHO guidelines regarding control of antimicrobials in food animals (WHO, 2000) echo the grave concerns pertaining to antibiotic resistance in food animals. These guidelines aim

at the judicious use of antimicrobials in food animals and at establishing mechanisms to monitor the antimicrobial resistance in food-borne pathogens of zoonotic importance.

Escherichia coli (*E. coli*) is one of the leading causes of neonatal calf diarrhoea which frequently results in substantial economic loss in the dairy industry (Bradford et al., 1999; Wray et al., 1993). Shiga toxin-producing *E. coli* are well recognized in colibacillosis in neonatal calves. Even though many studies showed that both healthy and diarrhoeic calves harbour STEC in their intestine, natural outbreaks and experimental conditions have demonstrated the association of STEC with diarrhoea and dysentery in calves (Hall et al., 1985; Schoonderwoerd et al., 1988; Sandhu and Gyles, 2002). Cattle, both dairy and beef herd, have been implicated as the principal reservoir of STEC (Cobbold and Desmarchelier, 2000; Thran et al., 2001). In humans, STEC can cause severe diseases, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) which is the main cause of acute renal failure in children (Griffin and Tauxe, 1991). In addition to Shiga toxin production, intimin, encoded by the *eaeA* gene, is another virulence-associated factor that has been implicated in patients with HC and HUS (Paton and Paton, 1998). Indeed, enteropathogenic *E. coli* (EPEC) that result in attaching and effacing (AE) lesions but do not produce Shiga toxin, have been isolated as the causal agent of diarrhoea in humans and different animal species (Mainil, 1999; Cid et al., 2001). Although O157:H7 is the dominant serotype associated with human disease worldwide, sporadic cases of HC and HUS are increasingly being attributed to non-O157 STEC serotypes in many countries (Mercado et al., 2004; Brooks et al., 2005; Aidar-Ugrinovich et al., 2007). In addition, several authors have found that STEC isolated from diarrhoeic calves frequently share virulence-associated traits, i.e. genes with human enterohaemorrhagic *E. coli* (EHEC) strains (Maidhof et al., 2002).

In the past few years, *E. coli* strains isolated from diarrhoeic calves have shown resistance to multiple, structurally unrelated antibiotics, such as aminoglycosides, fluoroquinolones and expanded-spectrum cephalosporins (White et al., 1997, 2000; Bradford et al., 1999). Moreover, some of the quinolones can enhance the production of Shiga toxin by induction of bacteriophage via bacterial SOS response and induce stx-phages to lytic cycles thereby facilitating their dissemination in the environment (Zhang et al., 2000). The emergence of such multi-drug-resistant pathogenic strains of *E. coli* poses an increasing threat to the successful management of calf scour (Donaldson et al., 2006). Some researchers have speculated that the indiscriminate use of antibiotics in food-producing animals may lead to the development of

antimicrobial resistance in pathogenic bacteria infecting humans (Aarestrup and Wegener, 1999; Van den Bogaard and Stobberingh, 1999; Wegener, 2003).

However, data on antimicrobial resistance of STEC in food-producing animals is still scarce and an efficient survey of antimicrobial resistance in STEC would be helpful in the treatment and management of disease both in cattle and humans. Although *E. coli* strains are usually negative for urease (Edwing, 1986), phenotypic variants of *E. coli* serotypes O157:H7 and non-O157, STEC urease-producing strains have been reported (Tschape et al., 1995) and urease activity can be considered as a putative virulence factor of increasing importance in STEC infecting cattle and humans (Heimer et al., 2002). A positive correlation in production of colicins and pathogenicity of *E. coli* from diarrhoeic calves has also been reported (Harnett and Gyles, 1984; Singh et al., 1989). Here, we discuss the prevalence of STEC and non-STEC *eaeA*-positive strains in diarrhoeic calves in Gujarat, a leading dairy industry state of India, together with atypical biochemical characteristics and antimicrobial resistance. This is the first survey of STEC strains in diarrhoeic calves in Gujarat state of India.

Materials and Methods

Calves

This study was carried out from November 2004 to February 2005. In this period, around five hundred calves (below 2 months of age) from four different locations viz. two Livestock Research Stations one each from the Anand Agricultural University (AAU) and the Junagadh Agricultural University, clinical cases reported at veterinary clinics of AAU and small household farms around the Anand city of Gujarat state, India, were investigated, so that isolates represent the actual prevailing animal husbandry practices across the state. Calves were screened for diarrhoea and clinical cases from different settings, i.e. 30 from two Livestock Research Stations of the universities and 16 from veterinary clinics AAU and small household farms were further selected for faecal sampling. A total of 46 calves showing symptoms of diarrhoea were selected.

Samples

Samples ($n = 46$) consisted of rectal swabs or faeces collected from each diarrhoeic calf using Hiculture (HiMedia, Mumbai, India) sterile swabs. Inoculated swabs were transported on ice to the laboratory.

Isolation and identification of *E. coli*

All faecal samples were streaked onto MacConkey's agar and incubated for 24 h at 37°C. Five to six lactose

fermenting pink colonies per sample were picked and subcultured on eosin methylene blue (EMB) agar plates for preliminary characterization. Colonies showing the characteristic metallic sheen on EMB agar (two well isolated colonies) were picked and considered presumptive *E. coli*. The purified cultures of *E. coli* were inoculated on phosphate buffer agar slants. *E. coli* isolates were preliminarily identified by biochemical tests; indole, methyl-red, Voges-Proskauer and citrate utilization. The isolates were further characterized for their biochemical activity by the following tests; carbohydrate fermentation, urea hydrolysis, production of hydrogen sulphide on TSI agar as per Edwing (1986). Thus in total 90 *E. coli* strains were isolated.

Detection of *stx1*, *stx2* and *eaeA* genes by PCR

All the *E. coli* strains were screened for the presence of the *stx1*, *stx2* and *eaeA* genes as described by Pollard et al. (1990) and Nishikawa et al. (2002) with some modifications. PCR was performed in a thermal cycler (Eppendorf thermal cycler, Hamburg, Germany). Oligonucleotide primers were procured from Sigma Aldrich Chemicals, Bangalore, India. The primers and the predicted lengths of PCR amplification products are listed in Table 1.

The PCR assay was carried out in a 15 µl volume containing 7.5 µl of 2X PCR Mastermix (4 mM MgCl₂, 0.4 mM of each dNTPs, 0.05 units/ml of Taq DNA polymerase, 150 mM Tris-HCl PCR buffer) from Fermentas Life Sciences, 0.5 µl of each primer (10 pmol/µl), 5.5 µl Dnase-free distilled water and 1.0 µl of template DNA (30 ng/µl). A known *stx1* and *stx2* positive *E. coli* O157 strain maintained at the department of Veterinary Microbiology, AAU, Anand, Gujarat served as a positive control for the PCR. The thermal cycling programme for the PCR used in this study is shown in Table 1. Amplified PCR products were analysed by gel electrophoresis in 2% agarose containing ethidium bromide (0.5 µg/ml) as described earlier (Sambrook and Russell, 2001).

Serogrouping of *E. coli*

Isolated *E. coli* strains were serogrouped on the basis of their 'O' antigen by the National Salmonella and Escherichia Centre at Central Research Institute (Kasauli, India).

Carbohydrate fermentation tests of *E. coli*

The ability of the *E. coli* isolates to ferment dulcitol, raffinose, rhamnose, salicin, starch and sucrose was assessed using 1% of each sugar in a peptone water base with Andrade's indicator. Isolates grown in peptone water were used to inoculate each sugar medium. The tubes were

Table 1. PCR primer sequences, sizes of amplified DNA fragments and amplification conditions used to detect virulence genes of *E. coli* strains isolated from diarrhoeic calves in Gujarat (India)

Target gene	Primer sequences (5'-3')	Conditions	Product size	Reference
Shiga toxin 1 (<i>stx1</i>)	F- GAAAGATCCGTGGGATTACG R- AGCGATGCAGCTATTAATAA	94°C 5 min, 94°C 2 min, 55°C 1 min, 72°C 1 min, 72°C 7 min, 35 cycles	130 bp	Pollard et al. (1990)
Shiga toxin 2 (<i>stx2</i>)	F- TTTACGATAGACTTCTCGAC R- CACATATAAAATTTTCGCTC	94°C 30 s, 47°C 1 min, 72°C 1.5 min, 25 cycles	228 bp	Nishikawa et al. (2002)
<i>E. coli</i> attaching and effacing gene (<i>eaeA</i>)	F- ACGTTGCGATGGGTAACCTC R- GATCGGCAACAGTTTCACCTG	94°C 1 min, 55°C 1 min, 72°C 2 min, 35 cycles	815 bp	Nishikawa et al. (2002)

F, forward primer; R, reverse primer.

incubated at 37°C for 7 days and observed every 24 h. Production of pink colour was considered a positive reaction.

Colicin production of *E. coli* strains

The colicinogenicity of the isolated *E. coli* strains was assessed according to the method of Barker and Old (1979) with some modifications. Dextrose free bile salt agar (1.3%) was inoculated with the isolated strains. After 48 h of incubation at 37°C, the organisms were killed by treatment with chloroform vapours for 30 min and the plate overlaid with a thin layer of soft agar lightly inoculated with an overnight broth culture of the indicator strain (*E. coli* K-12 Row, maintained at department of Microbiology, College of Veterinary Science, AAU Gujarat, India). After solidification the plate was again incubated overnight at 37°C. The production of colicin was marked by a distinct inhibition zone in the lawn of the indicator bacteria around the macro colony of the producer.

Atypical biochemical characterization of *E. coli* strains

Urease production: 2% of urea agar slants were inoculated and incubated at 37°C and observed for 7 days. A positive reaction was observed by the development of the pink colour in the slant (Barrow and Feltham, 1993).

Antibiotic resistance testing

Sensitivity to antibiotics was determined by the agar diffusion technique recommended by Bauer et al. (1966). Disks (HiMedia) containing the following agents were used: ampicillin (10 µg), amikacin (30 µg), ceftiofur (0.2 µg), cephalexin (30 µg), cephaloridine (10 µg), colistin (10 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), enrofloxacin (10 µg), kanamycin (30 µg) and tetracycline (30 µg). A sterile cotton swab was dipped into the bacterial suspension and spread uniformly on the entire surface of a Muller-Hinton agar plate. The inoculum was allowed to dry for 5 min. Four antibiotic disks were placed on the plate and after incubation for 24 h at 37°C the diameter of the inhibition zone was measured in millimetres with calipers. Zones of growth inhibition were evaluated according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997) guidelines.

Results

Among the 90 *E. coli* strains isolated, a total of 41 strains (45.55%) were positive for *stx* genes. As shown in Table 2, from the *stx*-positive strains, three strains harboured the

Table 2. Virulence genes profile and biochemical characteristics of *E. coli* strains isolated from diarrhoeic calves in Gujarat (India)

Strain no.	Serogroup	<i>stx</i>	<i>eaeA</i>	Urease	H ₂ S	Citrate
AU3	O22	<i>stx1-stx2</i>	-	+	-	-
AU4	O22	<i>stx1-stx2</i>	-	-	-	-
AU5	O24	<i>stx1-stx2</i>	-	-	-	-
AU10	O55	<i>stx1-stx2</i>	-	-	-	-
AU11	O55	<i>stx1-stx2</i>	-	-	-	-
AU13	O62	<i>stx1-stx2</i>	+	-	-	-
AU15	O86	<i>stx1-stx2</i>	-	+	-	-
AU16	O86	<i>stx1-stx2</i>	-	-	-	-
AU17	O86	<i>stx1-stx2</i>	-	-	-	-
AU18	O86	<i>stx1-stx2</i>	-	+	-	-
AU19	O86	<i>stx1-stx2</i>	-	-	-	-
AU20	O86	<i>stx1-stx2</i>	-	-	-	-
AU23	O110	<i>stx1-stx2</i>	-	-	-	-
AU25	O128	<i>stx1-stx2</i>	-	-	-	-
AU27	O128	<i>stx1-sxt2</i>	-	+	-	-
AU28	O128	<i>sxt1-stx2</i>	-	-	-	-
AU29	O131	<i>stx1-stx2</i>	-	+	-	-
AU30	O157	<i>stx1</i>	-	+	-	-
AU32	O171	<i>stx1-stx2</i>	+	-	-	-
AU33	O171	<i>stx2</i>	+	-	-	-
AU34	O171	<i>stx2</i>	-	-	-	-
AU35	O171	<i>stx1-stx2</i>	-	-	-	-
AU36	O172	<i>stx1-stx2</i>	-	-	-	-
AU37	O172	<i>stx1-stx2</i>	-	-	-	-
AU38	O172	<i>stx1-stx2</i>	-	+	-	-
AU39	O172	<i>stx1-stx2</i>	-	-	-	-
AU40	NT	<i>stx1-stx2</i>	-	+	-	-
JU13	O128	<i>stx2</i>	+	-	-	-
JU15	O131	<i>stx2</i>	-	-	-	-
JU16	O168	<i>stx1-stx2</i>	-	-	-	-
JU17	O171	<i>stx1-stx2</i>	+	+	-	-
JU18	NT	<i>stx1-sxt2</i>	+	-	-	-
OF1	O5	<i>stx1</i>	-	+	-	-
OF7	O86	<i>sxt1-stx2</i>	-	-	-	-
OF16	O157	<i>stx1-stx2</i>	-	-	-	-
OF21	R	<i>stx2</i>	-	-	-	-
OF24	NT	<i>stx1</i>	+	+	+	-
OF26	NT	<i>stx1-stx2</i>	-	-	-	-
VC1	O2	<i>stx2</i>	-	-	-	-
VC5	O167	<i>stx1-stx2</i>	-	-	-	-
VC6	O167	<i>stx1-stx2</i>	-	-	-	-

stx, Shiga toxin genes; R, rough strain; NT, non-typable, -, negative for particular character; +, positive for particular character.

stx1 gene (3.3%), six strains (6.66%) carried the *stx2* gene and 32 strains (35.55%) harboured both *stx* genes. In total, 14 (15.55%) of 90 *E. coli* strains were positive for the *eaeA* gene, of which seven were STEC strains and seven strains were *eaeA*-positive non-STEC. Eleven STEC strains (26.82%) were positive for the production of urease. Among the isolated STEC strains, two belonged to the O157 serogroup. Out of 41 STEC-positive strains, 18 strains (44%) were positive for colicin production

(Table 3) and 27 strains (66%) showed the ability to utilize one or more sugars while 14 strains (34%) failed to ferment any sugar. All the STEC strains revealed multiple drug resistance to 3–10 different antibiotics (Table 3). The most frequent resistance types were kanamycin (100%), cephalixin (100%) followed by cephaloridine (95%), enrofloxacin (85%), amikacin (80%), ampicillin (73%), tetracycline (63%), ceftiofur (34%), ciprofloxacin (22%), colistin (12%) and co-trimoxazole (5%).

Discussion

This study demonstrates the presence of *stx* and *eaeA* genes among *E. coli* isolated from diarrhoeic calves. As this study targeted only diarrhoeic calves most likely to excrete the pathogen, this prevalence rate cannot be extrapolated to healthy calves or cattle on farms. Unlike in developed countries, dairy cattle farming in India is a household practice. Considering routine contacts with

Table 3. Antibiotic resistance and colicinogeny patterns of 41 shiga toxin-producing *E. coli* strains isolated from diarrhoeic calves in Gujarat (India)

No. resistances	Strain no.	Serogroup	Resistance pattern	Colicinogeny
3	JU13	O128	Ak, Cp, K	++
5	JU17	O171	Ak, Cp, Cr, Ex, K	+
	OF26	NT	Ak, Cp, Cr, Ex, K	–
	OF21	R	Ak, A, Cp, Ex, K	–
	OF16	O157	Ak, Cp, Cr, Ex, K	+
	OF1	O5	Ak, Cp, Cr, Ex, K	–
	AU39	O172	A, Cp, Cr, Ex, K	+
	AU38	O172	A, Cp, Cr, Ex, K	+
	AU37	O172	A, Cp, Cr, Ex, K	–
	AU36	O172	A, Cp, Cr, Ex, K	–
	AU35	O171	A, Cp, Cr, Ex, K	–
	AU34	O171	A, Cp, Cr, Ex, K	+++
	AU33	O171	A, Cp, Cr, Ex, K	+
	AU32	O171	A, Cp, Cr, Ex, K	+
	AU5	O24	Ak, Cp, Cr, K, T	+
	AU29	O131	Ak, Cp, Cr, K, T	+
	6	JU15	O131	Ak, Cp, Cr, Ex, K, T
VC1		O2	Ak, Fur, Cp, Cr, K, T	–
AU11		O55	Ak, A, Cp, Cr, K, T	–
AU10		O55	Ak, A, Cp, Cr, K, T	–
7	JU16	O168	Ak, A, Cp, Cr, Ex, K, T	+
8	OF7	O86	Ak, A, Cp, Cr, Cf, Ex, K, T	–
	AU40	NT	Ak, A, Cp, Cr, Cl, Ex, K, T	–
	AU28	O128	Ak, A, Cp, Cr, Cl, Ex, K, T	–
	AU27	O128	Ak, A, Cp, Cr, Cl, Ex, K, T	+
	AU25	O128	Ak, A, Cp, Cr, Cl, Ex, K, T	–
	AU23	O110	Ak, Fur, Cp, Cr, Cf, Ex, K, T	+
	AU20	O86	Ak, A, Fur, Cp, Cr, Ex, K, T	–
	AU19	O86	Ak, A, Fur, Cp, Cr, Ex, K, T	–
	AU18	O86	Ak, A, Fur, Cp, Cr, Ex, K, T	–
	AU17	O86	Ak, A, Fur, Cp, Cr, Ex, K, T	–
	AU16	O86	Ak, A, Fur, Cp, Cr, Ex, K, T	–
8	OF24	NT	Ak, A, Cp, Cr, Co, Ex, K, T	++
	AU13	O62	Ak, A, Cp, Cr, Cf, Ex, K, T	++
9	JU18	NT	Ak, A, Fur, Cp, Cr, Cf, Ex, K, T	+
	VC6	O167	Ak, A, Fur, Cp, Cr, Cf, Ex, K, T	+
	VC5	O167	Ak, A, Fur, Cp, Cr, Cf, Ex, K, T	+
10	AU3	O22	Ak, A, Fur, Cp, Cr, Cf, Ex, K, T	–
	AU4	O22	Ak, A, Fur, Cp, Cr, Cf, Ex, K, T	–
	AU30	O157	Ak, A, Fur, Cp, Cr, Cf, Cl, Co, Ex, K	–

Ak, amikacin; A, ampicillin; Fur, ceftiofur; Cp, cephalixin; Cr, cephaloridine; Cf, ciprofloxacin; Cl, colistin; Co, co-trimoxazole; Ex, enrofloxacin; K, kanamycin; T, tetracycline.

cattle in day-to-day life, a large human population in this region may be at risk.

In this study we found a total of 41 strains (45.55%) positive for *stx* genes. Similar results of a high percentage (40% or more) of *stx* gene positive *E. coli* strains have been reported by Khan et al. (2002) and Salvadori et al. (2003), but this is in contrast to the findings of Wani et al. (2003) from India who reported STEC-positive strains only in 9.73% of the diarrhoeic calves. Isolation of STEC O157 serogroup from calves with diarrhoea, in the present work, is in agreement with findings of Dean-Nystrom et al. (1999), Wani et al. (2003) and Sharma et al. (2004) who also found O157 serogroup as the cause of severe diarrhoea and attaching and effacing mucosal lesions in neonatal calves. The prevalence of *stx* genes among 39 non-O157 (42.85%) strains was measured in this study. Similar findings have been reported by Rahman (2002), Blanco et al. (2003) and Mercado et al. (2004). The majority of the STEC strains isolated in this study did not belong to the serogroups (O8, O20, O26, O111, O113, O126, O145), which have previously been found associated mainly with diarrhoea and enteritis in calves outside India (Mainil, 1999). The serogroups most commonly reported in India from calf diarrhoeal cases are O24 (Kaura et al., 1991), O2, O5, O9, O62, O55, O86, O131, O157 (Wani et al., 2003) and O172 (Sharma et al., 2004). Furthermore, certain serogroups like O2, O55, O86, O128 and O157 isolated in this study have been found to be associated with infantile diarrhoea among neonates and adult human patients suffering from bloody diarrhoea/HC and/or HUS as reported by Beutin et al. (1989) and Nishikawa et al. (2002). Thus these serogroups have a great importance in public health. Four of the 41 STEC strains serotyped in this study were considered O non-typable (ONT) with all available O antisera (O1 to O185). Similarly Aidar-Ugrinovich et al. (2007) found six ONT of 55 STEC strains. This highlights the need of extending the serotyping scheme to include new and emerging STEC serogroups. Interestingly, in this study, we have identified three new serogroups (O167, O168 and O172) previously unreported in either human or animal STEC strains.

In this study, we observed that the *stx2* gene was more prevalent than the *stx1*, while the *eaeA* gene, which encodes intimin, a 94- to 97-kDa outer membrane protein necessary for attaching and effacing activity (Paton and Paton, 1998), was almost uniformly associated with the distribution of *stx1* and *stx2* genes in bovine STEC strains. This is in contrast with earlier reports that most STEC strains from calves with diarrhoea harbour the *stx1* gene along with the *eaeA* gene (Mainil et al., 1993; Wieler et al., 1996) but in agreement with the findings of Wani et al. (2003) from India who have reported a similar

pattern in diarrhoeic calves. Several other investigators have underlined the strong association between the presence of the *eaeA* gene and the capacity of STEC to cause severe human disease, especially HUS (Paton and Paton, 1998; Oswald et al., 2000; Beutin et al., 2004). This important virulence gene was detected in 17% of the STEC strains assayed in this study and is in contrast with the results described for bovine isolates by Aidar-Ugrinovich et al. (2007). Nevertheless, the production of intimin is not essential for pathogenesis because a number of sporadic cases of HUS have been caused by *eaeA*-negative non-O157 STEC strains. Thus, STEC O104:H21 and O113:H21 strains lacking *eaeA* gene were responsible for an outbreak and a cluster of three HUS cases in the USA and Australia respectively (Paton and Paton, 1998; Paton et al., 1999).

The virulence gene *stx2* has been found to be significantly associated with an increased risk of HUS in humans infected with non-O157 STEC in the USA and UK (Brooks et al., 2005). So the high prevalence of *stx2* genes alone or in combinations with *stx1* may be a potent threat to the public health of farm workers and people living in the vicinity of animal farms. Further studies in this direction are needed.

In this study, non-STEC *eaeA*-positive strains were detected in seven (14.28%) of 49 non-STEC strains, which is close to the findings of Orden et al. (1998) and Wani et al. (2003) who, respectively, detected non-STEC *eaeA*-positive strains in 8.1% and 9.73% of calves with diarrhoea. Non-STEC *eaeA*-positive strains in this study belonged to O18 (2 strains), O26 (2 strains), O76 (1 strain), O126 (1 strain) and ONT (1 strain) serogroups with predominant prevalence of O18 and O26 (28.57% each). This corroborates the observation of Orden et al. (1998) that the O26 serogroup was the most prevalent (29.4%) among EPEC from calves with diarrhoea in Spain. Moreover, Mainil et al. (1993) found that all EPEC strains isolated from calves with diarrhoea, except for one that could not be typed, belonged to this serogroup. Similarly, the O26 serogroup has been detected more frequently in calves with diarrhoea than in healthy cattle suggesting a pathogenic role in neonatal calf diarrhoea (Orden et al., 1998).

In this study, a multiple drug resistance pattern (at least against three antibiotics) was observed in isolated strains. All the strains were resistant to at least three antibiotics tested, with 20 of 41 isolates (49%) being resistant to at least eight of 11 antibiotics. A high degree of resistance to kanamycin, cephalixin, enrofloxacin, amikacin, ampicillin and tetracycline was observed. Such a high degree of antibiotic resistance of STEC strains has been reported by many researchers. Donaldson et al. (2006) reported a similar degree of resistance in *E. coli* isolates

from healthy calves against ampicillin, ceftiofur, chloramphenicol, florfenicol, gentamycin, tetracycline, etc. Werckenthin et al. (2002) also reported resistance rates of 70–90% in *E. coli* isolates from healthy and diarrhoeic calves for ampicillin, tetracycline, streptomycin, chloramphenicol, kanamycin and neomycin. The multiple drug resistance pattern most often found in this study was resistance to kanamycin–cephalexin–cephaloridine–enrofloxacin–amikacin–ampicillin–tetracycline.

The multidrug resistance of *E. coli* isolates against cephalosporins and fluoroquinolones is a matter of great concern regarding public health. Studies have shown that a high level of resistance to enrofloxacin as observed in our study conferred a similar level of resistance to enoxacin (a fluoroquinolone available for human clinical use). This has been observed by many researchers especially in Europe (Bywater et al., 2004). Threlfall et al. (1998) have suggested that the emergence and spread in the UK of *Salmonella typhimurium* DT F04 isolates, a *Salmonella* prevalent in humans with reduced sensitivity to ciprofloxacin, followed the licensing of enrofloxacin for veterinary use in that country in 1993. Because of this, they have recommended a restriction of the veterinary use of fluoroquinolones. Zhao et al. (2001) reported the presence of extended spectrum cephalosporin-resistant *E. coli* and *Salmonella* species in retail ground meat, signifying the public health importance of this issue. Moreover, the pathogenic multidrug-resistant strains of *E. coli* in the intestinal microflora serve as an important reservoir of mobile resistance genes which can be transferred to other bacterial species in the intestines, including pathogens, such as *Salmonella* species (Winokur et al., 2001). This can be an important mechanism for acquiring antibiotic resistance in pathogenic bacteria that pose a challenge for effective antibiotic therapy. The findings of our study suggest that STEC-positive *E. coli* isolates may play a dynamic role in the ecology of multidrug resistance in the dairy environment and perhaps pose an important public health hazard.

Another intriguing issue of using quinolones, such as ciprofloxacin, as well as other antibiotics (trimethoprim-sulphamethoxazole) in food-producing animals is their ability to induce the bacterial SOS response with resulting *stx* prophage induction and enhanced intrainestinal Shiga toxin production. The pervasive and indiscriminate use of these antibiotics in animals may result in the dissemination of bacteriophage-encoded virulence factors, such as Shiga toxins in the environment. Zhang et al. (2000) have demonstrated that *stx*-encoding phages are able to move from one strain of *E. coli* to another in the intestine of mice and that antibiotic-mediated prophage induction enhances this process. It is possible that the use of phage-inducing antimicrobials in farm animals may

inadvertently enhance the dissemination of bacteriophage-encoded toxins. This could contribute to the emergence of new pathogens and the spread of *stx* genes to other members of the Enterobacteriaceae family, such as *Citrobacter* and *Enterobacter* species, both of which have been associated with Shiga toxin related HUS in humans (Tschape et al., 1995; Paton and Paton, 1996).

Urease production can contribute to the virulence of several Gram-negative bacteria and enhance their acid resistance but in general, ureolytic *E. coli* strains are rarely found among clinical isolates. In our study, 11 (26.82%) STEC strains expressed urea hydrolysis. Phenotype variants of *E. coli* serotypes O157:H7 producing this enzyme have been isolated from patients with bloody diarrhoea and HC (Public Health Laboratory Service, 1991). Urease-positive STEC strains from cases of calf diarrhoea and from slaughtered beef cattle have been reported (Tomimaga et al., 1989; Conedera et al., 1997). Among the STEC of serotype O5:H-, urease production has also been reported (Hall et al., 1985). Remarkably, Mercado et al. (2004) also reported urease-producing STEC isolates (37.5%) from diarrhoeic calves in Argentina. Moreover, one of the two O157 STEC strains isolated in our study showed ureolytic activity similar to that reported by Friedrich et al. (2005) in STEC O157 of bovine origin. It has been postulated that urease production could offer protection from transient digestive activity during an oral route and perhaps would contribute to a higher virulence in those strains (Heimer et al., 2002). Urease activity could, therefore, be considered as a putative virulence factor of increasing importance when present in STEC infecting cattle and humans.

In this study, we found that 28 (68.29%) of 41 STEC strains were rhamnose non-fermentors while 33 (66%) of 49 non-STEC strains were rhamnose non-fermentors. Murinda et al. (2004) also reported similar findings of a higher percentage of STEC O26 (100%) and a lower percentage of non-STEC O26 (40%) rhamnose non-fermentor strains. Thus the rhamnose fermentation reaction may serve as a diagnostic tool for the detection of STEC from the cases of diarrhoeal illness.

A number of investigators have reported a correlation between colicinogenicity and pathogenicity (Papavassiliou, 1962; Yadava and Gupta, 1971; Singh et al., 1989). They reported that the incidence of colicinogenicity among bovine strains ranged from 32% to 61% and pathogenic strains were more frequently colicin producers than were non-pathogenic strains. In our study, we also found 18 (44%) of 41 STEC strains positive for colicinogeny while 14 (28%) of 49 non-STEC strains were colicinogenic. This frequency of colicinogeny among STEC strains of diarrhoeic calves in our study is very close to that in humans (41%; Smarda and Obdržálek, 2001). So colicinogeny

may be an important factor in the colonization of intestinal tract and may also dynamically regulate the colonization pattern of Shiga and non-Shiga toxin producing *E. coli*.

We concluded that in Gujarat, the leading milk-producing state of India, STEC and non-STEC *eaeA*-positive strains are prevalent in calves with diarrhoea and could be the cause of disease. Atypical biochemical characters and colicinogeny of STEC strains along with multiple drug resistance have been found in this study, indicating that the development of such genetic combination in STEC strains may increase the virulence and pathogenicity of host strains. However, molecular characterization of plasmids in these colicinogenic drug-resistant STEC strains is required to know the significance of such genetic combinations. A high rate of prevalence of STEC and non-STEC *eaeA*-positive strains has been found in this small-scale study, but screening of larger numbers of cattle and humans with diarrhoeal disease should be carried out to confirm the distribution of the *stx* and *eaeA* genes. Further studies regarding the epidemiological and zoonotic potential of these strains are required with improved diagnostic methods for better surveillance and control measures in the state of Gujarat, India.

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