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Dairy farm investigation on Shiga toxin-producing *Escherichia coli* (STEC) in Kolkata, India with emphasis on molecular characterization

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SUMMARY

An investigation was conducted to determine the distribution, virulence gene profile and phenotypes of Shiga toxin-producing *Escherichia coli* (STEC) strains within a dairy farm in Kolkata, India by characterizing the STEC strains isolated from healthy dairy cow and calf stool samples, raw milk and farm floor swabs from July 2001 to March 2002. Primary screening by multiplex-PCR detected *stx1* and *stx2*, the common virulence genes of STEC, in 18·9% of cow faeces, 32·4% of calf stool samples, 21·6% of farm floor swabs and 4·5% of raw milk samples and viable STEC were recovered from 4·5, 9·9, 8·1 and 1·8% of the corresponding PCR-positive samples. Strains harbouring *stx1* (63·3%) and *hlyA* (53·3%) were frequently detected compared to *eae* (13·3%). Most of the strains harboured similar sets of reported virulence genes common among isolates from diarrhoea patients. Most of the strains also exhibited multidrug resistance, sorbitol fermentation and produced enterohaemolysin. The randomly amplified polymorphic DNA–PCR (RAPD–PCR) profile of the STEC strains isolated from the farm milieu revealed diverse banding patterns and clonal analysis demonstrated that the strains from different sources were not identical but showed some genetic relatedness. The study demonstrates the potential of dairy farm for housing virulent STEC.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) has emerged as an important global health threat and is recognized as an important pathogen of human diarrhoea capable of causing life-threatening sequelae like haemolytic–uraemic syndrome (HUS) [1, 2]. Implication of STEC in calf diarrhoea, oedema disease in pigs and 'Alabama rot' in greyhounds is well recognized, but not generally recorded in cattle [3]. The absence of illness in cattle, caused by STEC is well documented, and is probably due to the absence of a specific receptor for Shiga toxin [4]. Other reasons for the difference between outcomes in humans and cattle populations have been proposed [5]. Production of one or more Shiga toxins (Stx1, Stx2 or variants) mainly enable STEC strains to cause serious disease in

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humans [2]. The presence of a 43·4-kb pathogenicity island designated locus of enterocyte effacement (LEE) harbours a set of genes that contribute to STEC pathogenesis [6]. Moreover, the large virulence plasmid of STEC O157 (pO157) presumably harbours additional virulence factors such as the gene for enterohaemorrhagic *E. coli* haemolysin (*hly*) [7], the bifunctional catalase peroxidase (*katP*) [8], secreted serine protease (*espP*) [9] and the *etp* gene cluster [10].

More than 200 different O:H serotypes have been isolated from cattle, whereas in human cases the number exceeds 160, of which serotype O157:H7 accounts for 70–80% of STEC infection [11]. However, the O157:H7 serotype is far outnumbered in cattle by non-O157 serotypes that have the potential to cause zoonosis [3]. The role of non-O157 STEC as a cause of haemorrhagic colitis (HC) and HUS is regional in nature. Some of the non-O157 strains have been associated with outbreaks and sporadic cases of HC and HUS in European countries [12] and Argentina [13] but this is uncommon in the United States. Limited studies on ecology and epizootiology of STEC have been reported, particularly from developing countries like India [14–16]. The regional distribution of STEC serotypes associated with bovine reservoirs and possible human infection provides the basis for the current investigation.

MATERIALS AND METHODS

The study was conducted in a farm, located in a suburban area of the Kolkata metropolis which was selected with consideration of the appropriate herd size (~1000 cattle) for obtaining sufficient number of cows as well as proximate calf births for sampling. During July 2001 to March 2002, 111 samples were randomly collected, each from individual healthy dairy cows, fresh milk samples, calves (up to 1 year old), animal feed and farm floor swabs.

Sample collection

Cow and calf stool samples

Stool samples (~30 g) from healthy cows and calves were collected aseptically in a sterile McCartney bottle using a digital rectal retriever.

Milk samples

Milk samples were collected in sterile vials using disposable gloves. Before milking, the udder was cleaned well with water and swabbed with 70% ethanol. While collecting, the first two draws from the teat were allowed to drain and the subsequent flow was collected.

Farm floor swab

At the time of collection of the other samples, the floor swabs from different parts of the farm premises covering an area of ~1 sq. inch were collected in Cary–Blair transport medium.

Feed samples

Approximately 30 g of concentrate feed and the roughage used for feeding in batches were collected randomly in sterile polythene pouches.

All the collected samples were transported to the laboratory within 4 h of collection and processed immediately.

Processing of samples

Enrichment of samples

About 1 g of cow and calf stool sample was directly inoculated in 3 ml Bacto EC medium (Difco, Detroit, MI, USA) for enrichment. Similarly, 100 μl of each milk sample and floor swab were inoculated separately into the EC medium. For the feed sample, ~2 g of concentrate and the roughage were added to 20 ml EC medium. All the inoculated samples were inoculated overnight at 37 °C under shaking condition.

Strategies employed for screening

After incubation, each enriched culture was directly tested by the multiplex PCR assay using *stx1* and *stx2* specific primers (Table 1) following the method described earlier [14]. The target colony search was done, with the broth cultures of the samples that yielded positive PCR result, by way of dilution spread plating [16] and colony hybridization method [10, 17] using digoxigenin (DIG)-labelled probes corresponding to *stx1* and *stx2*. Probes used included fragments of *stx1* (905-bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN501) [18] and *stx*2-A (860-bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN502) [19]. The colony that yielded a positive result was further confirmed for the presence of *stx* by *stx*-PCR as mentioned above and was preserved in Luria–Bertani broth supplemented with 15% glycerol at −70 °C. While selecting the colony for preservation, we chose one colony as

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Table 1. *PCR primers and conditions used in this study*

Primer	Nucleotide sequence	Target	PCR conditions (35 cycles)			Amplicon size (bp)	Ref.
			Denaturing	Annealing	Extension		
EVT1/ EVT2	5′-CAACACTGGATGATCTCAG-3′ 5′-CCCCCTCAACTGCTAATA-3′	*stx1* family	94 °C, 60 s	55 °C, 60 s*	72 °C, 60 s	349	[14]
EVS1/ EVC2	5′-ATCAGTCGTCACTCACTGGT-3′ 5′-CTGCTGTCACAGTGACAAA-3′	*stx2* family	94 °C, 60 s	55 °C, 60 s*	72 °C, 60 s	110	[14]
hlyA1/ hlyA4	5′-GGTGCAGCAGAAAAAGTTGTAG-3′ 5′-TCTCGCCTGATAGTGTTTGGTA-3′	EHEC *hlyA*	94 °C, 30 s	57 °C, 60 s†	72 °C, 90 s	1551	[7]
D1/ D13R	5′-CGTCAGGAGGATGTTCAG-3′ 5′-CGACTGCACCTGTTCCTGATTA-3′	*etpD*	94 °C, 30 s	52 °C, 60 s†	72 °C, 70 s	1062	[9]
EAE1/ EAE2	5′-AAACAGGTGAAACTGTTGCC-3′ 5′-CTCTGCAGATTAACCTCTGC-3′	*eae*	94 °C, 60 s	55 °C, 90 s*	72 °C, 90 s	350	[16]

* Final extension step of 7 min at 72 °C was performed.
† Final extension step of 10 min at 72 °C was performed.

representative of the colonies that were identical with respect to the *stx* genotype to avoid the possibility of missing the strains belonging to different clones. A human O157:H7 strain (VTEC3) harbouring *stx1* and *stx2* genes and a K12 *E. coli* strain of DH5α were used as positive and negative controls respectively.

Serotyping

Serotypes of the STEC strains were determined by slide agglutination using an 'O' antisera kit (Denka Seiken Co., Tokyo, Japan).

Screening for genes by PCR

PCR for the detection of both chromosomal and plasmid virulence genes were performed using a thermal cycler (PerkinElmer Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl reaction mixture, containing 2·5 mM of each dNTP, 30 μM of each primer, 2 μl of 10 × PCR buffer and 1 U of r-*Taq* DNA polymerase (Takara Shuzo Co. Ltd, Otsu, Japan). The primer sequences of each virulence gene and PCR conditions are given in Table 1.

Detection of Stx production

A highly sensitive Bead-ELISA for detecting Stx1 and Stx2 and Vero cell assay for determining cytotoxic effect with cell-free culture filtrate and cell lysate of the strains was performed as described previously [16, 20].

Haemolysin activity and sorbitol fermentation

Haemolytic activity of the STEC strains was investigated by streaking the strains on tryptic soy agar (Difco) plates containing 5% washed and unwashed 'O' group human blood cells as described earlier [7]. Sorbitol fermentation ability of the STEC strains was detected by streaking the strains on sorbitol McConkey agar (Difco) plates.

Antibiotic resistance

An antibiotic susceptibility test was performed with all the STEC strains using commercial disks (Hi Media, Bombay, India) of ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), tetracycline (30 μg), cephalothin (30 μg), amikacin (30 μg), ceftazidime (10 μg), kanamycin (30 μg) and ceftriaxone (30 μg) by the disk diffusion method [21]. *E. coli* ATCC 25922 strain, sensitive to all the drugs, was used as a quality control strain. The characterization of strains as susceptible, reduced susceptibility, or resistant was done as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [21].

RAPD of STEC strains

Molecular typing of STEC strains was done by RAPD–PCR using a single primer 1247 (5′-AAGAGCCCGT-3′) [10] where genomic DNA (~100 ng)

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Table 2. *Isolation rates and phenotype of STEC strains from different sources in a dairy farm*

Source (*n*)	No. sample positive (% on *n*)		No. of viable strain isolated	Phenotype					
	PCR	Culture		Haemolysin	SF*	Vero†	Stx1‡	Stx2‡	Stx1 & Stx2‡
Cow (111)	21 (18·9)	5 (4·5)	5	3 (60)	5 (100)	5 (100)	—	—	1 (20)
Calf (111)	36 (32·4)	11 (9·9)	13	5 (38·46)	13 (100)	13 (100)	3 (23)	—	—
Milk (111)	5 (4·5)	2 (1·8)	2	1 (50)	2 (100)	1 (50)	—	—	—
Farm floor (111)	24 (21·6)	9 (8·1)	10	9 (90)	9 (90)	8 (80)	—	—	1 (20)
Total (444)	43 (23·2)	27 (6)	30	18 (60)	29 (96·6)	27 (90)	3 (10)	—	2 (6·7)

* Sorbitol fermentation.
† Vero cell assay.
‡ Toxin assay by Bead-ELISA.
n, No. sample screened. Values within parentheses indicate the percentage.

was used as template and amplified through the Gene Amp PCR system 9700 (PerkinElmer Applied Biosystems) in a volume of 50 μl containing 200 μM of each dNTP, 30 pmol of primer, 5 μl of 10-fold concentrated polymerase synthesis buffer, 3 mM $MgCl_2$ and 2·0 U of *Taq* DNA polymerase (Takara). After a hot start at 80 °C for 5 min, the DNA was subjected to 35 cycles of denaturing at 94 °C for 1 min, annealing at 40 °C for 1 min and extension at 72 °C for 2 min. A final extension step was done for 10 min at 72 °C. The gels were digitized for computer-aided analysis (Gel Doc system, Bio-Rad, Hercules, CA, USA).

Interpretation of RAPD–PCR results

Using Gel Doc 2000 (Bio-Rad) gel images were stored in a PC running on the Windows system. All the images were retrieved and aligned using Adobe software and analysed in the Diversity Database fingerprinting software (Bio-Rad). Calculation of the similarity matrix was performed with the Dice coefficient after defining each single band. The clustering was achieved with the weighted pair-group method with arithmetic averages. Comparison of differences in the patterns of RAPD–PCR bands were made to ascertain the clonal relationship between isolates.

RESULTS

Occurrence of STEC in a dairy farm

Positive PCR results for STEC were more common for calf stool samples (32·4%) than for other samples (Table 2). However, the isolation rate of STEC from the PCR-positive samples was low. In this study, 30 STEC strains were isolated; of these, five were from cow faeces, 13 from calf stool samples, two from fresh milk and 10 from farm floor swabs. No isolation of STEC was made from the feed samples.

Serotypes of STEC

Of the 30 STEC strains isolated in this study, 14 strains were O antigen-typable with somatic (O) antiserum. These were O136 (six strains, floor swab), O111 (three strains, calf stool and floor swab), O55 (three strains, calf stool) and O114 (two strains, calf stool). Serotype O111 was detected in both floor swab and calf stool samples.

Chromosomal and plasmid-encoded virulence traits

The virulence gene profiles of the STEC strains included in this study are shown in Table 3. Most of the strains harboured only *stx1* (36·7%) followed by *stx1*, *stx2* and *hlyA* (26·7%). Of the five strains identified from cows, three harboured only *stx1*, one each carried only *stx2* and both *stx1* and *stx2* and *hlyA* respectively (Table 3). Of 13 strains identified from calf stool samples, 11 carried only *stx1* and two had both *stx1* and *stx2* and among them four, five and two had *eae*, *hlyA* and *etpD* respectively. The two strains isolated from milk samples were positive for only *stx1*. Among the 10 strains isolated from farm floor samples, three harboured *stx1* and seven had both *stx1* and *stx2*; however, *hlyA* and *etpD* were detected in seven and one strains respectively (Table 3).

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Table 3. *Virulence gene profile of STEC strains*

Virulence gene(s)	No. of strains with indicated virulence gene(s)				Total (%)
	Cow ($n=5$)	Calf ($n=13$)	Milk ($n=2$)	Floor ($n=10$)	
stx1	3	6	1	1	11 (36·7)
stx2	1	—	—	—	1 (3·3)
stx1 and *stx2*	—	—	—	1	1 (3·3)
stx1 and *eae*	—	1	—	—	1 (3·3)
stx1 and *hlyA*	—	1	1	1	3 (10)
stx1, *stx2* and *hlyA*	1	1	—	6	8 (26·7)
stx1, *eae* and *hlyA*	—	2	—	—	2 (6·6)
stx1, *hlyA* and *etpD*	—	—	—	1	1 (3·3)
stx1, *hlyA*, *eae* and *etpD*	—	1	—	—	1 (3·3)
stx1, *stx2*, *hlyA* and *etpD*	—	1	—	—	1 (3·3)

Sixteen (53·3%) of the 30 STEC strains gave the 1551-bp amplicon corresponding to *hlyA* by PCR, eight of which were from farm floor swabs, six from calf and one each from cow and milk samples respectively (Table 3). However, two strains from calves and one strain from the farm floor swab harboured the *etpD* gene.

Phenotypic characterization of STEC strains

Eighteen (60%) out of 30 STEC strains showed the haemolytic activity that resembled the typical enterohaemolytic (Ehly) phenomenon (Table 2). The zone was narrow, clear and could be visualized after 8–18 h of incubation at 37 °C. Of these 18 strains, 13 revealed the amplicon of the *hlyA* gene in the PCR assay whereas the remaining five strains did not. On the contrary, three strains produced the *hlyA* amplicon but did not exhibit haemolytic activity. With one exception, all the STEC strains were positive for sorbitol fermentation (Table 2).

Detection for Stx production

Bead-ELISA assay detected Stx1 production in three *stx1*-positve strains from calf stool samples only (Tables 2 and 3). Of the 10 strains positive for *stx1* and *stx2* by PCR, only two (20%) (one each from a cow and a floor swab) were detected positive to produce both toxins in culture filtrate and cell lysate. However, the only strain which is *stx2*-PCR positive that was isolated from a cow was found to be negative in Stx2-specific ELISA (Tables 2 and 3).

Of the 30 strains examined, 27 (90%) were found to be cytotoxic to Vero cells whereas the remaining three strains failed to express the effect and were negative by Bead-ELISA (Table 2). Out of these 27 verocytotoxic strains, only five (18·5%) showed positivity for Stx in Bead-ELISA, however, 22 (81·5%) were detected as negative.

Antibiotic resistance

In the antimicrobial susceptibility assay, resistance was noted most frequently to cephalothin (80%), neomycin (46·7%), tetracycline and streptomycin (43·3% each) and ampicillin (40%) and less commonly to co-trimoxazole (16·7%) and kanamycin and amikacin (13·3% each) (Fig. 1). None of the strains were found to be resistant to gentamicin, ciprofloxacin, ceftazidime, ceftriaxone, nalidixic acid and norfloxacin. Twenty-one strains (70%) expressed multidrug resistance and no common resistance pattern among the strains was observed.

RAPD profile of STEC strains

The RAPD-PCR was performed for understanding the relatedness among the STEC strains. All the strains were typable with primer 1247 with the amplified fragment sizes ranging from 0·2 to 6·0 kb. The RAPD profiles of the two strains (C2 and C4) from two different cows were similar and both harboured only *stx1*. A similar RAPD profile was observed among the three strains (Ca10, Ca11 and Ca12) from calves where Ca11 and Ca12 were

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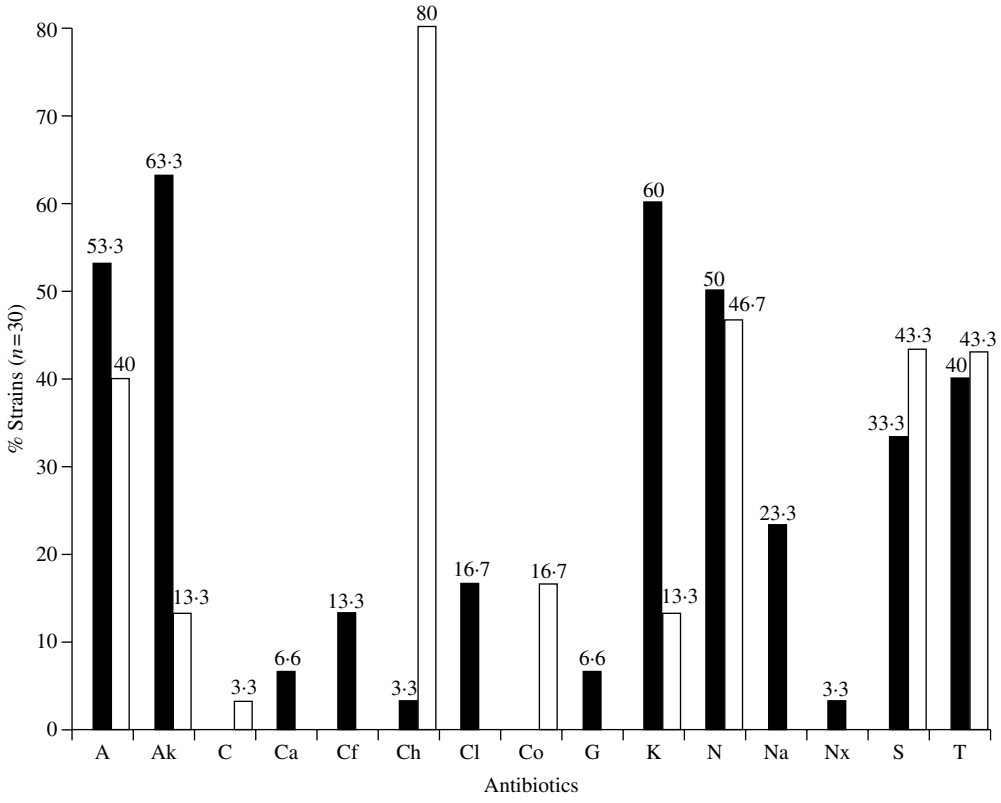

Fig. 1. Antibiotic resistance patterns of STEC strains. Abbreviations: A, ampicillin; Ak, amikacin; C, chloramphenicol; Ca, ceftazidime; Cf, ciprofloxacin; Ch, cephalothin; Ci, ceftriaxone; Co, co-trimoxazole; G, gentamicin; K, kanamycin; N, neomycin; Na, nalidixic acid; Nx, norfloxacin; S, streptomycin; T, tetracycline. ■, Reduced susceptibility; □, resistant.

obtained from the same sample. Except for *etpD*, the strains Ca10 and Ca11 had the same virulence gene profile (*stx1*, *stx2* and *hlyA*) and both were serologically untypable but strain Ca12 had a different virulence gene (*stx1*) profile and belonged to serotype O114. With the exception of minor band differences (~1·5 and 2·4-kb region), there was a similarity in RAPD banding pattern between Ca7 and Ca8 isolated from calf samples. Except for *etpD*, these two strains exhibited similarity in virulence gene profile (*stx1*, *eae* and *hlyA*) but belonged to different serotypes (O111 and O119 respectively). Among the floor swab strains, F1 and F2 showed a similar banding pattern, although they differed in virulence genotype (*stx1* and *stx1*, *hlyA* and *etpD* respectively) and were serologically untypable. Similarly, band matching was also observed among strains F6, F7, F8 and F9 where strain F6 belonged to the O125 serotype with virulence genotype (*stx1* and *hlyA*) and differed from the others (*stx1*, *stx2* and *hlyA*) which belonged to the O136 serotype.

The phylogenetic analysis of the STEC strains revealed four major clusters (clusters A–D) (Fig. 2) that combined many STEC strains representing diverse sources, although their RAPD banding patterns were not identical. STEC strains isolated from cow and calf samples were combined in clusters A and B. Similarly, the strains from calves, milk and floor and from calves and floor were found to be clustered in clusters C and D respectively.

DISCUSSION

The present study represents, for the first time in India, an extensive on-farm study to assess the prevalence of STEC within a dairy herd and the extent to which the dairy herd and its environment act as a reservoir for virulent STEC. In this study, 18·9% of cow faeces, 32·4% of calf stool specimens, 21·6% of farm floor swabs and 4·5% of raw milk samples produced positive PCR results for *stx*. The *stx*-PCR

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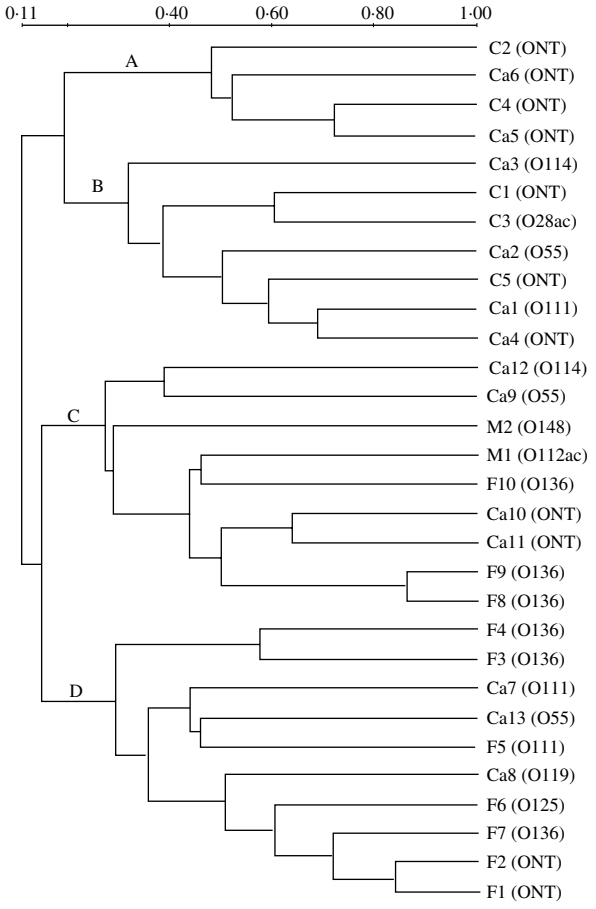

Fig. 2. Phylogenetic analysis of STEC strains. Unrooted WPGAMA method summarizes the similarity of RAPD–PCR profiles of STEC strains generated by the dendrogram. The similarity scale is above the dendogram. Four major clusters (A–D) are shown near the nodes of each cluster.

positive result for cattle was in accord with previous reports from Australia [22] and Germany [23] but differed from the results of other reports, e.g. ~70% of bovines were positive in France [24] and 69% of cows and 46% of calves in Japan [25]. Such variations could be attributed to differences in geographical factors that may have contributed to the perpetual prevalence of STEC in the cattle population [23] or due to various study designs adopted from time to time to screen STEC [25]. The infrequent detection of STEC in milk samples (4·5%) is in agreement with earlier studies conducted in Germany [26, 27] and The Netherlands [28]. However, the occurrence of STEC in milk highlights the potential for transmission of STEC through the food chain. In contrast, there was no apparent role of feed in the transmission of STEC in the farm under study. However, further studies employing improved sampling strategies aimed at this hypothesis are necessary.

We recorded low isolation of STEC from PCR-positive samples. Viable STEC were recovered from 24 to 40% of PCR-positive samples, depending on sample source. Lowest yields were from cow faeces, and highest from raw milk samples. This suggests some effect of competition from microflora in the samples as cow faeces have a richer microflora than the raw milk or perhaps Shiga toxin-producing bacteria other than *E. coli*. In addition, it was observed that shedding of STEC is less in well-fed animals than it is in populations under feeding restrictions [29]. This might be due to the presence of a very low number of the target organism present in sample [16]. Another possible explanation could be the absence of viable STEC in the sample although its DNA remains in the sample that served for the template in the PCR assay. When we cross checked the PCR products obtained from the samples by dot-blot assay using the labelled *stx* probe, all amplicons showed positivity, confirming the specificity of *stx*-PCR.

Analysis of the chromosomal virulence genes revealed that STEC from bovine sources in Kolkata mostly harbour *stx1* followed by both *stx1* and *stx2* but rarely with *stx2* which supports the observations recorded in Germany [10, 30]. However, abundance of *stx2* among STEC was common in France [31] and Japan [25]. The characteristic variation of *stx* in different countries may be attributed to the fact that *stx* is phage encoded and its propagation to the *stx*-negative *E. coli* population could generate a selective subset of STEC [23]. In this study, *stx1* was the predominant genotype in strains from cow and calf samples. However, both *stx1*- and *stx2*-positive STEC strains were recovered in a previous study of a suburban house dairy-cattle population in different locality in Kolkata [16]. This suggests that multiple subsets of *stx* (*stx1*-positive and both *stx1*- and *stx2*-positive) genotypes co-exist in the same geographical location as was recorded earlier in Germany [10, 23].

Relatively few STEC strains possessed the *eae* gene (13·3%), which extends the findings of other studies [16, 23, 24]. Interestingly, all the four *eae*-positive strains were from healthy calves without any overt disease, perhaps because the intimin is non-functional [32]. Another possibility is that in some cases intimin is involved in gut colonization but not directly in pathogenicity. Apparently virulent gene assortments were more common among strains from calves than

from cows and floor samples, supporting the role of young animals as an important reservoir of pathogenic STEC strains [33].

A good correlation was observed between the presence of *stx* genes and expression of cytotoxic effects on Vero cells. However, Bead-ELISA results showed that the majority (83·3%) of the STEC strains did not elaborate Stx. The results reflect that the STEC strains isolated in this study produce Stx which may not be complementary to the antibody as used and may constitute a set of new variants for *stx*. Another possibility could be that the amount of toxin produced by these strains may be low and go undetected by Bead-ELISA.

A strong correlation was also observed between the presence of *hlyA* and haemolytic activity. However, three *hlyA*-positive strains from calf samples did not show the haemolytic phenomenon and it could partly be explained that their haemolysin may remain cell associated because of a deficient transport system for the secretion of haemolysin [7]. On the contrary, five STEC strains exhibited haemolysin production although they did not give any positive amplicons in the *hlyA*-PCR. This could be attributed to the presence of *hlyA* alleles in these strains which were probably not recognized by the primers used in this study. The majority (70%) of the strains in this study expressed multidrug resistance as reported earlier [34, 35].

Human illnesses caused by STEC serotypes O114, O119, O112 and O125 have been reported [30], particularly due to serotype O111 which is placed in EHEC2 lineage ET group [36]. In the present study, verocytotoxin-producing STEC strains with serotype O111, O114, O119, O112 and O125 were recovered from calf, milk and farm floor samples and the strains harboured other virulence factors (*stx*, *eae* and *hlyA*). This indicates the potential for transmission of pathogenic STEC to humans from these sources.

Based on the relatedness in the RAPD–PCR dendogram, it could be postulated that the strains from calf and floor samples (clusters C and D) possibly result from a common source of dissemination. This aspect of herd management is worthy of scrutiny and may represent specific control points for reducing STEC spread within dairy units or other animal-rearing areas.

STEC strains from cows (C2 and C4) and calves (Ca5 and Ca6) with the same genotype and untypable serotype were closely clustered (Cluster A). These findings may suggest the probability of their transmission from cows to calves in this herd. Moreover, strains from different calves in the herd combined in cluster B and were considerably close which suggests that these strains may be similar in clonality and also suggest the possibility of transmission of STEC among calves housed in a pen. Adoption of segregated penning systems rather than group housing may reduce the prevalence of STEC within calf units that allude to the reduction of farm STEC prevalence and this practice may offer a credible control point [37].

The present study highlights the potential of healthy cows, calves and their environment to serve as a reservoir for virulent strains of STEC in Kolkata. As cattle act as the principal reservoir of STEC, it becomes of the utmost importance to reduce the number of STEC-positive animals as well as to reduce shedding thereby limiting the spread of STEC to humans. The present investigation may contribute as a *prima facie* reason towards the formulation of a strategy for the control of STEC throughout a dairy farm.

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