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Seasonal effect on the prevalence of virulence genes of non-O157 Verotoxic *E.coli* serogroups in faeces of cattle calves

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ABSTRACT

Calves faecal samples (n=216) were investigated to observe the effect of seasons on the prevalence of serogroups and virulence genes of non-O157 VTEC. A total of 177 (81.94%) *E. coli* were isolated and 32 (14.81%) were identified as VTEC and serotyping resulted in 13 different non-O157 'O' serogroups. The prevalence of serogroups and their virulence genes was found to be influenced by seasons and highest number were shed in summer (22.22%) followed by rainy (13.88%) and winter season (8.33%), respectively. A higher prevalence of O9 and O11 serogroups (25% each) was observed in summers. Molecular detection of virulence genes revealed the overall prevalence of vt_1 to be 37.5%, vt_2 43.8%, (vt_1+vt_2) 18.8%, *eae*A 21.9% and *hly*A 34.4% genes. Dominance of *hly*A 50% was observed in summers, whereas *vt*1 and *vt*2 were more prevalent during rain (50% each). The study revealed the link between the occurrence of *hly*A gene and O9, O11 serogroups in summers as both the serogroups were *hly*A gene bearer. This association might be responsible for more VTEC outbreaks in summers. So, faecal contamination of raw milk seems to pose greater threat of non-O157 VTEC outbreak during hotter and humid months.

Keywords: Characterization, STEC, Vero cell cytotoxicity assay (VCA), Vero toxin-producing *Escherichia coli,* VTEC

Verocytotoxin-producing *Escherichia coli* (VTEC) also known as Shiga toxin-producing *E. coli* (STEC) is an important zoonotic food-borne pathogen which can cause serious foodborne illnesses, and sometimes lead to fatal outcome in humans (Blankenship *et al*. 2020). The clinical spectrum of disease includes abdominal pain, watery diarrhea which may turn to haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombocytopenic purpura (Hoyle *et al*. 2021). The ruminants (especially cattle calves) are the major reservoirs of VTEC, carrying it asymptomatically in their gastrointestinal tract and shedding them in manure at the levels ranging from 10 to 10^5 CFU/g (Islam *et al.* 2014). More than 400 O:H serogroups of VTEC have been reported, of which 380 serogroups have been isolated from humans and more than 100 have been linked to human illness (WHO and FAO 2018). The serogroup *E. coli* O157:H7 was the first VTEC to be recognized as a major food safety threat but recently the other group of VTEC called as non-O157 VTEC has been increasingly implicated in human outbreaks (Tack *et al*. 2021). These main non-O157 VTEC serogroups are named as Gang of six or Top six or Big six (O26, O45, O103, O111, O121, and O145) (USDA/FSIS 2012). The VTEC can be transmitted through various routes, including consumption of contaminated meats, milk, fruits, juices, water and exposure of contaminated farm environments and farm animals (Parul *et al*. 2021). Virulence of VTEC is attributed to more than 40 different virulence factors, major being potent verotoxins (VT1 and VT2, encoded by phage mediated genes *vt1* and *vt2*). An intimin protein associated with attaching and effacing lesions (encoded by *eae*A gene) and a lytic toxin enterohaemolysin, (encoded by *hly*A gene) are found to be associated with VTEC causing human disease like HUS (Yoo *et al*. 2017).

The VTEC infection in human beings has been shown to be governed by factors such as seasonality, environmental effects on pathogen-host associations and characteristics of population (CDC 2018). While most studies on VTEC across the world mainly focus on O157 serogroup, the ecology of non-O157 VTEC is an area lesser studied due to lack of reliable detection and characterization methods (Hoyle *et al*. 2021). To fulfil this lacuna, a study was conducted mainly to focus on the seasonal prevalence of non-O157 VTEC serogroups and its virulence factors at Indian dairy farms.

MATERIALS AND METHODS

Faecal samples (n=216) were collected directly from rectum of cattle calves in three seasons, viz. summer, rainy and winter (n=72 per season) at three distantly located dairy farms (n=24 /season/farm) of Uttar Pradesh, India

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(Table 1). The samples were brought in chilled condition and processed within 24 h for the isolation and identification of *E. coli* as per standard microbiological techniques (Edward and Ewing 1972) with slight modifications. In brief, sample (1 g) was enriched in 10 ml of Trypticase Soy Broth (TSB) (HiMedia, India), containing acriflavin (10 mg/L) and incubated at 37°C for 24 h, then loopful broth was streaked over MacConkey agar (MLA) and further incubated at 37°C for 24 h. Further, single pink coloured colony was picked from MLA and streaked over Eosin methylene agar (EMB) (HiMedia, India) and after incubation, colonies showing green metallic sheen were picked as presumptive *E. coli* and biochemically confirmed by kit (KB010 Hi *E. coli* Identification kit-Hi Media, India). The isolation of O157:H7, was done as per the method of Johnson *et al.* (1996) and the enriched inoculum was also streaked on to CT-SMAC and plates were observed for colourless colonies produced by O157:H7.

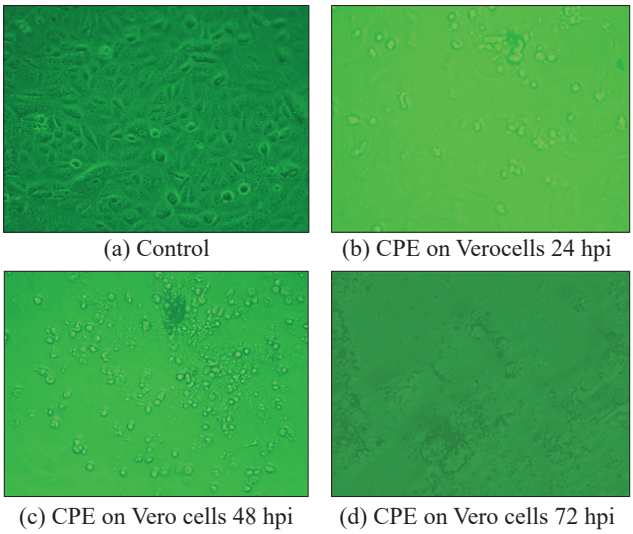
Vero cell line assay (VCA) and serotyping: Phenotypically, VTEC was identified by VCA as described by Konowalchuck *et al.* (1977) with slight modifications. Verotoxin was prepared in 4 ml TSB using shaker incubator at 200 rpm for 18 h at 37°C and VCA was performed in 96 wells microtitre plates (Nunc, USA). The confluent monolayers of vero-cells were challenged with 20 µl of the toxin (supernatant) in triplicates. The VTEC serotype O26 and non-pathogenic *E. coli* ATCC 25992 were used as positive and negative controls. The main cytopathic effect (CPE) observed was rounding of cells in more than 50% samples. These isolates were categorized as VTEC. The identified VTEC were sent to National *Salmonella* and *Escherichia* Center, Central Research Institute (CRI), Kasauli, Himachal Pradesh, India to be serotyped for available O antisera.

Molecular detection: VCA confirmed isolates were subjected to multiplex PCR using 4 sets of oligonucleotide primers for virulent genes *vt*1, *vt*2, *eae*A and *hly*A (Paton and Paton 1998). The template DNA was extracted from single colony of each isolate by kit method (Genei, Bengaluru) and its purity concentration were detected by nanodrop (Eppendorf, Germany). For amplification of virulent genes *vt*1 (180bp), *vt*2 (255bp), *eae*A (384) and *hly*A (534), PCR reaction was performed in a thermal cycler (Cyber lab) using standard cycling condition: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 6 min. Amplified products were separated by agarose gel (1% in 1× Tris-acetate -EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with Ethidium bromide (0.5 µg/ml). DNA of pathotype EHEC procured from Pennsylvania State University, USA and serotype O157 were used as positive control.

Resistance profile: The antibiogram of the non-O157 VTEC isolates was determined by disc diffusion method (Bauer *et al*. 1966) and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2012). All the isolates were tested against 15 antimicrobial with concentration: amikacin (AK, 30 µg), ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), cefuroxime (CXM, 30 µg), ceftriaxone (CTR, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 30 µg), co-trimoxazole (COT, 23 µg), gentamicin (GEN, 10 µg), nalidixic acid (NA, 30 µg), norfloxacin (NX, 10 µg), streptomycin (S,10 µg) and tetracycline (TE, 30 µg) and the outcomes were categorised as sensitive, intermediate, and resistant. The strain *E. coli* ATCC 25922 was used as a quality control strain. The antibiotic resistance patterns of isolates were observed for MDR (multiple drug resistance - resistance against three or more groups of antibiotics) as per Walsh *et al.* (2006) and MAR (multiple antibiotic resistances) index also calculated for MDR isolates (Sanjukta *et al*. 2019).

RESULTS AND DISCUSSION

A total of 177 presumptive *E. coli* were isolated from 216 calf faecal samples and phenotypically confirmed by production of pink coloured lactose fermenting colonies on MLA and the clear green metallic sheen on EMB agar and biochemical tests. The prevalence of *E. coli* was found to be 81.94% in calf faeces while isolation of *E. coli* O157:H7 serogroup was also attempted by streaking on the CT-SMAC, but none of the sample was able to produce the colorless colonies on this agar, indicating the absence of *E. coli* O157:H7 with zero prevalence. In VCA, 32 *E. coli* isolates were capable of producing rounding and shriveling of the Vero cells which is the landmark cytopathic effect to categorize the *E. coli* isolates as VTEC. Control of vero cells was depicted in (Fig. 1a) and rounding of 50% vero cells was observed in 24 h post inoculation (hpi) (Fig. 1b) than 90% after 48 hpi (Fig. 1c) and complete destruction of monolayer after 72 hpi (Fig. 1d). Phenotypically confirmed VTEC were serotyped into 13 different 'O'



(a) Control (b) CPE on Verocells 24 hpi

(c) CPE on Vero cells 48 hpi (d) CPE on Vero cells 72 hpi

Fig. 1. Vero cells, (a) Control, (b) at 24 hpi, (c) at 48 hpi and (d) at 72 hpi.

Table 1. Seasonal prevalence of VTEC and its virulent genes in calves faeces

Season	Tested sample	*E. coli* positive samples	Prevalence of *E. coli*	VCA positive	Prevalence of VTEC	Prevalence of *vt1*	Prevalence of *vt2*	Prevalence of *vt1+vt2*	Prevalence of *eaeA*	Prevalence of *hlyA*
Summer	72	62	86.11	16	22.22	31.25	43.75	43.75	6.25	50.0
Rainy	72	59	81.94	10	13.88	50.00	50.00	0.0	20.00	10.00
Winter	72	56	77.77	6	8.33	33.33	33.33	33.33	50.0	33.33
Total	216	177	81.94	32	14.81	37.25	43.75	18.75	18.75	34.37

serogroups and outcome of serotyping showed prevalence of non-O157 VTEC was 14.8% in faeces of cattle calves. On seasonal analysis, highest prevalence was found to be in summer followed by rainy and winters 22.22%, 13.88% and 8.33%, respectively (Table 1). Prevalence values of non-O157 VTEC in this study are concordant with values of non-O157 VTEC (0.42 to 74%) reported from cattle faeces worldwide (Hussein and Shakuma 2005, Stanford *et al*. 2016). In contrast to this study, much higher prevalence rates of non-O157 VTEC 43% and 62.7% were reported from Argentina in the studies of Fernendez *et al.* (2009) and Padola *et al.* (2004), in Brazil (43%) and Ethopia (33%) (Moreira *et al*. 2003, Ali *et al*. 2021) and in India (9.73%) and (18%) (Khan *et al*. 2002b, Wani *et al*. 2003) while almost similar findings, i.e. 19.0% to this study were obtained from USA (Wells *et al*. 1991).

A correlation between season and faecal shedding of O157 VTEC has been studied in several countries and its occurrence has been shown to follow a particular shedding pattern, increasing in spring, reaching to peak levels during the summer and then tapering off in the late autumn to very low in winter (Edrington *et al*. 2006, Sheng *et al*. 2016, Fink *et al*. 2018). Except, there is an apparent lack of such seasonal studies on the shedding of non-O157 VTEC serogroups across the globe except big six serogroups (O26, O45, O103, O111, O121 and O145). In this study, an attempt was made to analyze a seasonal pattern revealed in the shedding of non-O157 VTEC with highest prevalence recorded in summer. This finding corroborates with the work of Fernandez *et al.* (2009) who found that VTEC shedding in calves was significantly higher (50%) in warmer months compared with colder months (43%). In studies from Alberta, Canada and Scotland, similar seasonal shedding of non-O157 VTEC serogroups was observed (Renter *et al*. 2004, Stanford *et al*. 2016, Hoyle *et al*. 2021).

There are various hypotheses to support the seasonal shedding pattern of VTEC. It is assumed that warm weather plays a crucial role in the survival and maintenance of this organism outside the host, thus environment becomes a potent source of infection to animal and may cause more excretion from the host's body (Naumova *et al*. 2007, Lal *et al*. 2012). Thus it is apparent that the farm environment plays an important role in VTEC colonization and recirculation, as well as in direct and indirect transmission to human farm workers/visitors and consumers (Stacey *et al*. 2007, Lejeune and Kersting 2010, Smith *et al*. 2012). Most of the VTEC outbreaks and incidences in human diseases occur in summer months which can be correlated with the seasonal shedding of VTEC (Edrington *et al*. 2006). In contrast to findings of this study, increased incidence of VTEC shedding in cattle faeces during winter months was reported as 79.16% and 9.5%, respectively (Thran *et al*. 2001, Kobayashi *et al*. 2007). This could be due to fact that in winter, cattle are housed in overcrowded intensive conditions which would allow increased transmission among cattle. The relationship between day length and physiological responses within the animal may also affect the seasonal shedding (Callaway *et al*. 2013).

Higher prevalence of O9 and O11 sergroups (25% each) was observed from calves in summer season as compared to other serogroups, while in other seasons, no dominating pattern was observed (Table 2). Clinically important O26 and O156 serogroups shown to be associated with HUS cases in human being all over the world, were more prevalent in rainy and winter season as O26 (10%) and O156 (33.33%). In concordance with this study, some of top six serogroups in Scotland showed the highest herd prevalence during the autumn for O26, O103, O145 and lowest during the winter months for O103 and O145 and during spring for O26 (Hoyle *et al*. 2021). In Canada, all studied serogroups O26, O45, O103, O121 and O157 demonstrated seasonal variations in prevalence and were least prevalent ($P<0.001$) in cooler winter months (Stanford *et al*. 2016). Specific serogroups of VTEC and its seasonality also studied in Ireland (Patricia *et al*. 2016).

Virulence profile of serogrops was studied by mPCR and a perfect harmony was observed in outcome of VCA and mPCR as all the VCA positive isolates had *vt* genes either singly or in combinations with other virulence genes *eae*A and *hly*A. Prevalence of *vt*1, *vt*2, (*vt*1+*vt*2), *eae*A and *hly*A was found to be 37.5%, 43.8%, 18.8%, 21.9% and 34.4%, respectively and *vt*2 was most prevalent among these. The four different combinations of virulence genes were obtained in 16 VTEC isolates and rest 16 were single. The dominating combinations were (*vt*1+ *hly*A) and (*vt*1+ *eae*A) with prevalence of 15.6% each, while the prevalence of other two combinations was 12.5% (*vt*1+*vt*2+ *hly*A) and 6.25% (*vt*1+*vt*2+ *eae*A+ *hly*A). High prevalence of *vt*2 gene has been reported in several countries like USA, Canada, Europe and Australia (Dargatz *et al*. 2013, Bibbal *et al*. 2015, Mellor *et al*. 2016, Mainga *et al*. 2018). Several authors have reported the occurrence of *vt*1 gene to be more in cattle in their respective studies (Von Müffling *et al*. 2007, Cooley *et al*. 2013, Mir *et al*. 2015). In this study, the occurrence of other virulence genes *eae*A and

Table 2. Seasonal virulence profile of non O157 VTEC isolates

Place of collection	VTEC/ Season	Strains	Serotypes	Gene profile
Agra (A)	Summer VTEC (6)	DCA1	O11	*vt*1+ *hly*A
		DCA2	O81	*vt*1+*eae*A
		HCA1	O27	*vt*2
		HCA2	O27	*vt*2
		HCA3	O34	*vt*2
		HCA4	O52	*vt*2
	Rainy VTEC(3)	DCA3	O81	*vt*1+*eae*A
		HCA5	O52	*vt*2
		HCA6	O52	*vt*2
	Winter VTEC (2)	HCA7	O56	*vt*2
		HCA8	O156	*vt*1+ *vt*2 + *hly*A + *eae*A
Bareilly (B)	Summer VTEC(7)	DCB1	O11	*vt*1+ *hly*A
		DCB2	O11	*vt*1+ *hly*A
		HCB1	O9	*vt*1+*vt*2+*hly*A
		HCB2	O9	*vt*1+*vt*2+*hly*A
		HCB3	O9	*vt*1+*vt*2+*hly*A
		HCB4	O9	*vt*1+*vt*2+*hly*A
		HCB5	O34	*vt*2
	Rainy VTEC(4)	DCB3	O26	*vt*1+*eae*A
		HCB6	O56	*vt*2
		HCB7	O83	*vt*1
		HCB8	O84	*vt*2
	Winter VTEC (4)	DCB4	O81	*vt*1+*eae*A
		DCB5	O91	*vt*1+*eae*A
		HCB9	O134	*vt*2
		HCB10	O156	*vt*1 + *vt*2 + *hly*A + *eae*A
Mathura (C)	Summer VTEC (3)	DCM1	O11	*vt*1+ *hly*A
		HCM1	O52	*vt*2
		HCM2	O56	*vt*2
	Rainy VTEC(3)	DCM2	O11	*vt*1+ *hly*A
		HCM3	O83	*vt*1
		HCM4	O134	*vt*2
	Winter VTEC (0)	-	-	-

*hly*A was found to be 21.9% (7/32) and 34.4% (11/32), respectively whereas a few authors have shown that *eae*A gene which encodes for outer membrane protein intimin responsible for attaching and effacing of the bacteria is seldom found in healthy cattle, especially in non-O157 isolates (Beutin *et al*. 1997). The results are in agreement with finding of Von Müffling *et al*. (2007) and Scott *et al*. (2009) who reported *eae*A gene to be 28.0% and Cooley *et al*. (2013) who found *eae*A prevalence to be 29.0% in their works. These variations can be explained on the fact that genome of non-O157 VTEC from different geographical locations might vary significantly (Beraldo *et al*. 2014). Seasonality on gene carriage was also examined across the three seasons and it was found that *hly*A had the highest prevalence (50.0%) in summer followed by *vt2* (43.75%) although *vt*1 and *vt*2 genes were equally prevalent 50%

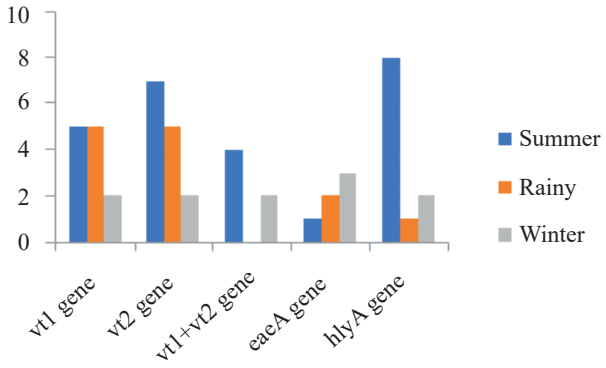


Fig. 2. Occurrence of virulence genes of VTEC.

in rainy season while in cooler months no such seasonal variation was evident (Table 2, Fig. 2). High carriage of (*vt*1+ *vt*2) combination was observed in warmer months and *vt*1 decreased in colder season in Argentina (Fernandez *et al*. 2009). In scottish cattle, *vt*1 showed highest prevalence in spring season (Hoyle *et al*. 2021). In Indian scenario, seasonal dominance of virulent genes of non-O157 VTEC is a new data and may help to improve the strategy adopted to control the transmission of VTEC from animal to human.

In vitro, the antibiogram of the 32 non-O157 VTEC isolates revealed that highest resistance was observed against ampicillin 96.87% while no resistance reported against co-trimoxazole (Table 3). The antibiotic resistance pattern of isolate were analyzed for multi-drug resistance (MDR) and found that 25 (78.13%) were resistant against three or more groups of antibiotics. The MDR isolates showed resistance to a minimum of 3 and maximum of 14 antibiotics. Thus, 12 resistance patterns were observed ranging from 3 to 14 antibiotics with maximum multiple antibiotic resistance (MAR) index of 0.93 and minimum MAR index of 0.20 in MDR isolates (Table 4).

In current scenario, the antimicrobial resistance along with MDR in *E. coli* is a very big issue and concerning to animal, human and environmental health. Although the use of antimicrobials in VTEC infected human patients is supposed to exaggerate the clinical condition via increased release of verotoxins, that is, the consequence of induced expressions of *vt* genes (Corogeanu *et al*. 2012). In spite of the controversies, there is no substitute of antibiotics to cure the VTEC infections in patients. In this work, the maximum resistance was evinced against ampicillin (96.87%) followed by streptomycin and, tetracycline (78.12%) each and these three antibiotics also constitute the resistance profile of multidrug resistant (MDR) isolates. The MDR-VTEC for streptomycin and tetracycline are already reported by many researchers (Aksoy *et al*. 2007, Momtaz *et al*. 2012). Thus, there is immense need for intense monitoring of all the factors which are contributing to the resistance in non-O157 VTEC subgroup.

The present study examined the seasonal prevalence of non-O157 VTEC and the effect of seasonal carriage of its virulence genes in faeces of cattle calves over a period of one year. The analysis of this data provides the useful

Table 3. Antibiogram of non-O157 VTEC isolates

Group	Antimicrobials (15)	non-O157 VTEC isolates (32)		
		Resistance (%)	Intermediate (%)	Sensitive (%)
Aminoglycosides	Amikacin (AK) 30	11 (34.37)	3 (9.38)	18 (56.25)
	Gentamicin (GEN)10	10 (31.25)	3 (9.38)	19 (59.38)
	Streptomycin (S)10	25 (78.13)	4 (12.50)	3 (9.38)
Amphenicols	Chloramphenicol (C)30	13 (40.63)	3 (9.38)	16 (50.00)
Cephalosporins	Ceftazidime (CAZ)30	5 (15.62)	2 (6.25)	25 (78.13)
	Cefotaxime (CTX)30	5 (15.62)	1 (3.13)	24 (75.00)
	Cefuroxime (CXM)30	5 (15.62)	2 (6.25)	25 (78.13)
	Ceftriaxone (CTR)30	3 (9.38)	3 (9.38)	27 (83.38)
Penicillins	Ampicillin (AMP)10	31 (96.87)	1 (3.13)	0 (0.00)
	Amoxicillin/Clavulinic acid (AMC)30	9 (28.12)	3 (9.38)	20 (62.50)
Quinolones	Ciprofloxacin (CIP)5	5 (15.62)	2 (6.25)	25 (78.13)
	Nalidixic acid (NA)30	6 (18.75)	2 (6.25)	24 (75.00)
	Norfloxacin (NX)10	7 (21.87)	3(9.38)	22 (68.75)
Sulphonamides	Co- Trimoxazole (COT)23	0 (0.0)	4 (12.5)	3 (9.38)
Tetracyclines	Tetracycline (TE)30	25 (78.13)	2 (6.25)	5 (15.62)

Table 4. Resistance patterns and MAR index of multi drug resistant strains

Resistance pattern of MDR strains	No. of MDR strains	No. of antibiotics	MAR index
AMP-S-T-C-AK-AMC-GEN-NX-NA-CIP-CAZ-CTX-CXM-CTR	3	14	0.93
AMP-S-T-C-AK-AMC-GEN-NX-NA-CIP-CTX-CTR	1	12	0.80
AMP-S-T-C-AK -GEN-NX-NA-CIP-CAZ-CTX-CXM	1	12	0.80
AMP-S-T-C-AK-GEN-NX-NA-CAZ-CXM	1	10	0.67
AMP-S-T-C-AK-AMC-GEN-NX	1	8	0.53
AMP-S-T-AK-AMC-GEN	1	6	0.40
AMP-S-T-C-AK-GEN	2	6	0.40
AMP-S-T-C-AK	1	5	0.33
AMP-S-T-AMC	2	4	0.27
AMP-S-T-C	3	4	0.27
AMP-S-AMC	1	3	0.20
AMP-S-T	8	3	0.20

information about the ecology of non-O157 VTEC in India which may prove useful in the prevention and surveillance strategies to control the future outbreaks. Furthermore, this seasonal analysis of non-O157 VTEC from Indian region is a well-placed effort in this area to enhance the knowledge of seasonal transmission of VTEC for better food safety in humans. This observation might be useful in devising effective control strategy and aim mainly towards control of the dominant serotypes in different seasons.

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