Epidemiology of diarrhoea caused by rotavirus and *Escherichia coli* in lambs in Kashmir valley, India

S.A. Wani[*], M.A. Bhat, I. Samanta, S.M. Ishaq, M.A. Ashrafi, A.S. Buchh

Rotavirus Laboratory, Division of Veterinary Microbiology and Immunology, SK University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Shuhama, Srinagar 190006, India

Abstract

One hundred and twenty-nine faecal samples, collected over a period of 1 year, from 96 diarrhoeic and 33 non-diarrhoeic lambs aged between 0 and 3 months were examined for presence of rotavirus and *Escherichia coli* (*E. coli*). Group A rotavirus was detected in 24 (25%) of diarrhoeic lambs using sandwich enzyme linked immunosorbent assay (ELISA) and ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE). Statistically no significant relation was found between rotavirus infection and age of the lambs. The prevalence of group A rotavirus was more related to meterological changes than age of the lambs as the number of diarrhoeic lambs with rotavirus infection was found to increase in spring months during which temperature and humidity ranged between 7.34 and 28.9 °C and 34.28 and 82.58%, respectively. The migration pattern of ovine rotavirus RNA in PAGE was typical of mammalian group A rotaviruses. O25, O26, O30, O43, O75, O76, O102, O113, O132, O153 and O157 *E. coli* serogroups were isolated from diarrhoeic lambs positive for rotavirus infection while as O8, O20, O21, O26, O39, O43, O45, O69, O75, O82, O104, O107, O113, O120, O127, O139, O141, O143, O153 and O157 serogroups of *E. coli* were isolated from diarrhoeic lambs without rotavirus infection. O8, O21, O43, O82, O104, O113, O120, O127, O132 and O139 serogroups, recovered from diarrhoeic faecal samples with or without rotavirus, were positive for congo red dye binding activity. O88, O113, O157 and O168 serogroups were isolated from non-diarrhoeic faecal samples out of which O88 and O168 were congo red positive. None of the lambs without diarrhoea carried rotavirus infection. Group B rotavirus infection commonly reported in diarrhoeic lambs outside India was not detected in any of the faecal samples screened.
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1. Introduction

The aetiology of diarrhoeal syndrome is quite complex involving many infectious agents of bacterial, viral and protozoan nature. Among the bacterial and viral agents *Escherichia coli* and rotavirus are notable one. In general *E. coli* plays predominant role during first week and rotavirus is more important between 7 and 30 days of age (Lucchelli et al., 1992) However, the precise role of each pathogen in different geographical conditions is not known. Rotavirus was first discovered to be an animal pathogen in 1969 by Mebus et al. (1969). Since then it has been accepted as an important aetiological agent of diarrhoea in neonates of almost all animal species, humans and even in birds. These viruses are subdivided into seven antigenically distinct groups designated A through G

* Corresponding author. Tel./fax: +91-194-2262211.
E-mail address: microlab@sancharnet.in (S.A. Wani).

(Estes, 2001). Most of rotaviruses detected in clinical specimens of domestic animals belong to group A (Theil et al., 1995). Ovine rotaviruses, in contrast to the globally distributed rotaviruses infecting other livestock, have been reported from few countries like UK, Japan, Morocco, Australia, USA (Chasey and Banks, 1984; Makabe et al., 1985; Fassi-Fehri et al., 1988; Ellis and Daniels, 1988; Theil et al., 1995). Both groups A and B rotaviruses infect lambs (Snodgrass et al., 1976) but group B rotaviruses have been shown to be more common cause of rotavirus diarrhoea in neonatal lambs (Chasey and Banks, 1984; Snodgrass et al., 1984). In India, the work on ovine rotavirus infection was initiated in 2000 only (Anon, 2001). Wani et al. (in press) are first to report the presence of rotavirus in diarrhoeic lambs in India.

From standpoint of pathogenic mechanisms four major categories of *E. coli* recognised are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC), which are represented by different serotypes based on O (cell-wall lipopolysaccharide), H (flagellar protein) and K (capsular polysaccharide or envelope) antigens (Collee et al., 1996). There are meagre reports of isolation of *E. coli* from diarrhoeic lambs. Cid et al. (2001) isolated attaching and effacing *E. coli* belonging to serogroups O2, O4, O26, O80 and O91 from diarrhoeic lambs. However, O4, O5, O8, O75, O84, O85, O91, O123, O128, O136, O157 and O166 are the most commonly reported ovine STEC serogroups in healthy sheep all over the world out of which O5, O91, O128 and O157 are implicated in serious diseases of human beings (Djordjevic et al., 2001). Serogroup O78 was isolated from haemorrhagic gastrointestinal outbreak in sheep in India (Sharma et al., 2003). The isolation and distribution of various *E. coli* serogroups has been studied in India in diarrhoeic calves and goats by several workers (Patil et al., 1999; Dubey et al., 2000a,b; Hussain and Saikia, 2000). But there seems to be no report except from this lab (Wani et al., 2003) available on isolation of *E. coli* from lambs with diarrhoea in India.

Pathogenic strains of *E. coli* are known to produce several virulence factors that can be demonstrated by different in vitro and in vivo methods under laboratory conditions. The ability to bind congo red (CR) dye in agar medium has been proposed as a marker for the invasive ability of several enteropathogens including *E. coli* (Payne and Finkelstein, 1977; Berkhoff and Vinal, 1986). Yoder et al. (1989) provided evidence that CR binding might be directly related to virulence. Similarly, Singh and Gupta (1996) reported a good correlation between CR binding and pathogenic potential. Since precise role of each pathogen in diarrhoea in different geographical conditions is not known, the present study was carried out to reveal the first ever epidemiology of rotavirus and *E. coli* infection in diarrhoeic lambs in Kashmir valley in India.

2. Materials and methods

One hundred and twenty-nine faecal samples from lambs were collected from Sheep Breeding Farm (SBF), Gaowbal, SBF, Dachigam and SBF, Shuhama, Srinagar and from private flocks. Out of them 96 were from diarrhoeic lambs and 33 were from non-diarrhoeic lambs. The samples were collected from lambs of different age groups as shown in Table 1. The samples were collected in May 2001 and during December 2001 to May 2002. From June to November 2001, the samples could not be collected as lambs were over 3 months as well as due to migration of sheep to highland pastures. The samples were collected in polythene bags and transported to laboratory on ice. The samples were immediately processed for presence of rotavirus and *E. coli*.

Table 1
Agewise distribution of rotavirus infection in faecal samples collected from lambs in Kashmir valley (India)[a]

S. no.	Age	Healthy		Diarrhoeic	
		Screened	Positive	Screened	Positive
1	1 week	4	0	10	0 (0.00)
2	2 weeks	2	0	20	4 (20.00)
3	3 weeks	5	0	15	3 (20.00)
4	1 month	1	0	10	0 (0.00)
5	2 months	8	0	23	7 (30.43)
6	3 months	13	0	18	10 (55.55)
Total		33	0	96	24 (25.00)

[a] Figures in parenthesis indicate percentage of rotavirus positive samples.

2.1. Detection of rotavirus

Detection of rotavirus was carried out by sandwich enzyme linked immunosorbent assay (ELISA) and ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) followed by silver staining as detailed below.

2.1.1. Twenty percent faecal suspension

Twenty percent faecal suspension was prepared in PBS and clarified by centrifugation at $10,500 \times g$ for 20 min at $4\,^\circ$C. The supernatant was used as source of rotavirus antigen as well as basic material for extraction of rotavirus RNA. The supernatant was kept at $-20\,^\circ$C till further use.

2.1.2. Extraction of RNA

For extraction of rotavirus RNA, the technique of Svensson et al. (1986) was followed with minor modifications. In brief, 450 μl of clarified 20% faecal suspension were vortex mixed with 50 μl of 10% sodium dodecyl sulphate (SDS) and 4 μl of 20 mg/ml proteinase K (Bangalore Genei Pvt. Ltd., Bangalore, India) and incubated at $56\,^\circ$C in a water bath for 1 h. Clarified faecal extract was treated with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution. It was vortexed twice and centrifuged at 12,000 rpm for 15 min at $4\,^\circ$C. Upper aqueous layer was collected in a fresh tube and the extraction was repeated. Next extraction was done with equal volume of chloroform:isoamyl alcohol (24:1) solution. The aqueous phase thus obtained was precipitated by 3 M Sodium acetate and 1 ml of cold ethanol at $-20\,^\circ$C overnight. Next day the RNA was sedimented at 12,000 rpm for 15 min. The dried pellet was dissolved in 10 μl of $2\times$ Laemmli's sample buffer (0.12 M Tris–HCl, 0.1% SDS, 20% glycerol, 0.002% bromophenol blue; pH 6.8). This served as source of rotavirus RNA for RNA-PAGE.

2.1.3. RNA-PAGE

Electrophoresis of RNA samples was performed in 1.5 mm thick, 7.5% polyacrylamide gel of 10 cm × 10.5 cm dimension using Laemmli's discontinuous buffer system. Electrophoresis was done at 15 mA in Hoefer miniVE electrophoretic unit (Amersham Pharmacia Biotech, USA) for 6 h. Silver staining of the gel was done according to the method described by Svensson et al. (1986).

2.1.4. Sandwich ELISA

Ninety-six-well micro-ELISA plate (Maxisorp-Nunc) was coated with 50 μl of rabbit anti-rotavirus serum diluted 1:100 in coating buffer (sodium carbonate 0.015 M, sodium bicarbonate 0.034 M in distilled water). The plate was incubated overnight at $4\,^\circ$C. Next day the plate was washed five times using washing buffer PBS-T (PBS, pH $7.2 + 0.05\%$ Tween-20). Non-specific binding was prevented by adding 100 μl of a blocking solution (5% skimmed milk in PBS-T) to each well. The plate was incubated in a humid environment at $37\,^\circ$C for 2 h followed by washing as before. Then, 50 μl of the 20% faecal suspension in PBS was added to duplicate wells. The plate was incubated as before at $37\,^\circ$C for 1 h and the washing again repeated. Then, 50 μl of diluted (1:500 in PBS) Guinea pig anti-rotavirus serum was added to each well as tracing antibody. The plate was incubated as before at $37\,^\circ$C for 1 h followed by further washing. Rabbit anti-guinea pig HRPO conjugate (Sigma, Missouri, USA) diluted 1:5000 in PBS was added to all wells and plate was incubated as before. After again washing the plate, 50 μl of freshly prepared substrate solution was added to all the wells and the plate was left at room temperature for 5–10 min to allow the reaction to develop. The substrate solution was prepared by adding 6.0 mg of OPD (Sigma, Missouri, USA) and 20 μl of 30% Hydrogen peroxide to 10 ml of substrate buffer (citric acid monohydrate 0.012 M, di-sodium hydrogen phosphate 0.031 M in distilled water). The reaction was stopped by adding 50 μl of 1 N H_2SO_4 to the wells. The OD of wells was read with a Multiskan ELISA reader (ThermoLabsystems, Helsinki, Finland) using a 492 nm filter. Appropriate positive and negative control wells were also included.

The development of a lemon-yellow colour showed a positive test. Absorbance (A_{492}) of test column was compared to that of negative column to decide the positive reaction.

2.2. Bacterial examination

For detection of *E. coli*, 96 diarrhoeic and 33 non-diarrhoeic faecal samples were directly inoculated on MacConkey's agar plates and incubated overnight at

37 °C. The rose pink colonies were picked up and subcultured on eosin methylene blue (EMB) agar plates to observe the characteristic metallic sheen. The well-separated colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological, biochemical tests as described by Edwards and Ewing (1986). The confirmed *E. coli* isolates were got serogrouped from National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India. The pathogenicity of *E. coli* strains was determined by congo red binding assay.

2.2.1. Congo red binding assay

The test was carried out in trypticase soya agar (Hi-Media, Mumbai, India) enriched with 0.05% of congo red dye. All 60 *E. coli* isolates were streaked on these plates and incubated at 37 °C for 24–72 h. The congo red binding isolates produced brick red colonies while congo red negative isolates produced white colonies.

2.3. Statistical analysis

The data on agewise distribution of rotavirus infection was subjected to *T*-test for difference of two means where by $T_{cal} = 0.000775$ which is less than the table value of *T* at five degrees of freedom.

3. Results

Out of 96 faecal samples collected from diarrhoeic lambs, 24 samples revealed the presence of group A rotavirus by sandwich ELISA and PAGE. Age wise distribution of rotavirus in diarrhoeic calves is shown in Table 1. From Table 1 it is clear that rotavirus incidence was more in 3-month-old lambs (55.55%) as compared to other age groups. Farmwise distribution revealed higher incidence (30.64%) of rotavirus infection in SBF, Goawbal followed by SBF, Dachigam (Table 2). The monthwise distribution of rotavirus in three sheep breeding farms is given in Fig. 1. From the figure it is evident that the peak prevalence of rotavirus infection in sheep breeding farms was in the months of April and May. The minimum and maximum temperature and humidity in these months ranged between 7.34 and 28.9 °C and 34.28 and 82.58%, respectively. Out of 24 rotavirus positive diarrhoeic faecal samples, 21 were positive by sandwich ELISA of which seven

Table 2
Distribution of rotavirus infection in faecal samples collected from lambs at various organised sheep breeding farms and private flocks of Kashmir valley (India)[a]

S. no.	Source	Diarrhoeic	Healthy	Total
1	SBF, Gaowbal	62 (30.64)	30	92
2	SBF, Dachigam	12 (16.66)	0	12
3	SBF, Shuhama	10 (10.00)	0	10
4	Private flocks	12 (16.66)	3	15
Total		96 (25.00)	33	129

[a] Figures in parenthesis indicate percentage of rotavirus positive samples.

were positive by PAGE as well. Three samples positive by PAGE could not be detected by sandwich ELISA. Migration pattern of ovine rotavirus in RNA-PAGE is shown in Fig. 2. The electropherotypic pattern of all the rotavirus isolates was typical of mammalian group A rotaviruses, i.e. 4-2-3-2 migration pattern and the bands co-migrated with that of bovine rotavirus. None of the faecal samples from healthy lambs was found positive for rotavirus.

Ninety-six diarrhoeic and 33 non-diarrhoeic faecal samples on bacteriological examination revealed presence of 56 and four strains of *E. coli*, respectively. Out of 56 strains from diarrhoeic samples five (8.92%) were untypable, four (7.14%) strains were rough and remaining 47 strains were identified as belonging to 25 different antigenic groups (Table 3). O26 and O113 *E. coli* serogroups were found most prevalent (7.14% each) followed by O21, O25, O82 and O157 (5.35% each). O25, O26, O30, O43, O75, O76, O102, O113, O132, O153 and O157 serogroups were isolated from diarrhoeic lambs positive for rotavirus infection while as O8, O20, O21, O26, O39, O43, O45, O69, O75, O82, O104, O107, O113, O120, O127, O139, O141, O143, O153 and O157 serogroups were isolated from diarrhoeic lambs without rotavirus infection. Only 15 strains belonging to O8, O21, O43, O82, O104, O113, O120, O127, O132 and O139 serogroups were found positive for congo red dye binding activity while majority, i.e. 41 (73.21%) were found negative for congo red binding activity. From non-diarrhoeic faecal samples four strains of *E. coli* belonging to O88, O113, O157 and O168 serogroups were isolated from four non-diarrhoeic faecal samples, out of which O88 and O168 serogroups were found positive for congo
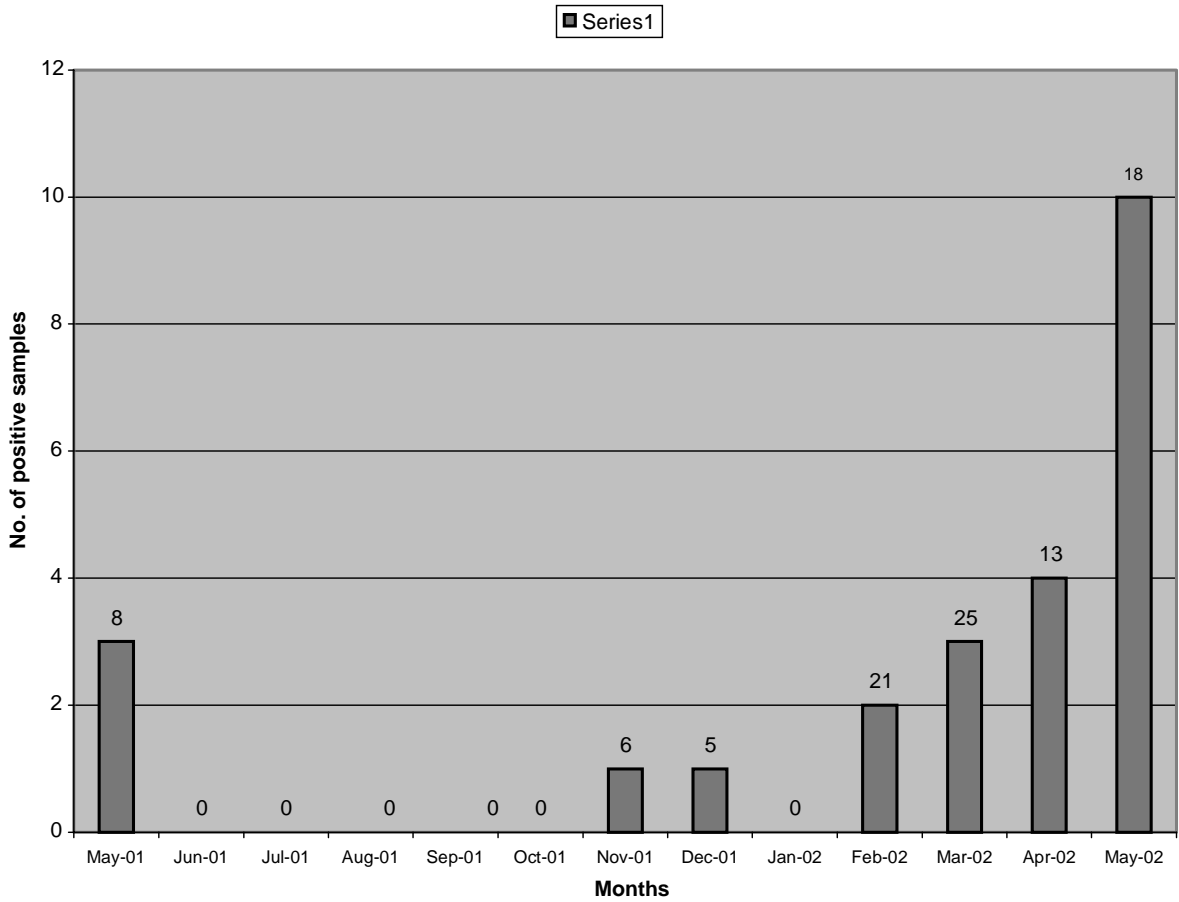
Fig. 1. Monthwise distribution of rotavirus infection in diarrhoeic lambs in organised sheep breeding farms of Kashmir, India. Values at the top of each bar indicate the total number of samples processed.

Table 3
Details of *E. coli* serogroups isolated from lambs in Kashmir valley (India)[a]

Diarrhoeic lambs		Non-diarrhoeic lambs	Congo red positive	Classified as
Positive for rotavirus	Negative for rotavirus			
O25 (3), O153	O8, O139 (2), O141 (2), O153	–	O8, O139 (2),	Enterotoxigenic *E. coli* (ETEC)
O26, O157	O26 (3), O157 (2)	O157	–	Enterohaemorrhagic *E. coli* (EHEC)
–	O127	–	O127	Enteropathogenic *E. coli* (EPEC)
O30 (2), O43, O75, O76, O102, O113, O132	O20, O21 (3), O39, O43, O45, O69, O75, O82 (3), O104, O107 (2), O113 (3), O120 (2), O143, UT (5), R (4)	O88, O113, O168	O21 (2), O43, O132, O82 (3), O113, O88, O104, O120 (2), O168	Others

[a] Figures in parenthesis indicate the number of isolates, if more than 1. UT, untypable; R, rough.
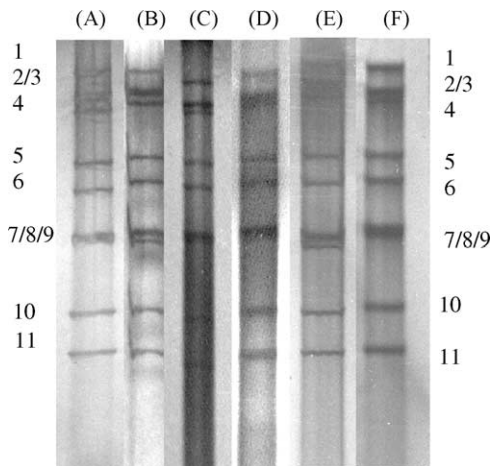
Fig. 2. Electropherogram of ovine group A rotaviruses in poly-acrylamide gel. Numbers on left and right indicate genomic segments in decreasing order of molecular weight. Lanes A–E: ovine samples. Lane F: bovine sample.

red dye binding activity. O113 and O157 serogroups were isolated from both diarrhoeic and non-diarrhoeic lambs.

4. Discussion

Only a few investigators, across the globe, have examined the epidemiology of ovine rotavirus (Saif et al., 1994). The present work was undertaken to investigate some of the epidemiological features of diarrhoea caused by rotavirus and *E. coli* in lambs in Kashmir, India. Association of rotavirus with lamb diarrhoea was known in India after 2000, only (Anon, 2001; Wani et al., in press). This is the second report on ovine rotavirus from India. During the investigation 96 diarrhoeic and 33 non-diarrhoeic faecal samples were screened by sandwich ELISA and PAGE. Twenty-four diarrhoeic faecal samples found positive for rotavirus revealed presence of only group A antigen/electropherotype. This is contrary to the findings of other workers (Chasey and Banks, 1984; Snodgrass et al., 1984; Theil et al., 1995) who reported the predominant involvement of group B rotaviruses in lamb diarrhoea in United Kingdom and USA. However, these findings are in agreement with those of Snodgrass et al. (1976) and Munoz et al. (1996) who

have also reported group A rotavirus infection in diarrhoeic lambs. Eleven bands of genomic RNA revealed a migration pattern of 4-2-3-2, which is typical of mammalian group A rotaviruses (Saif et al., 1994). Among the different strains of ovine rotaviruses no difference was observed in migration pattern by PAGE, which indicated their close genetic relatedness. Over all incidence of rotavirus in diarrhoeic lambs was 25%. This corroborated with the observations of Fassi-Fehri et al. (1988) who reported a rotavirus incidence of 30% in lambs in Morocco. Similarly, in a survey of rotavirus in lambs in Scotland, ovine rotavirus was isolated from 22 of the 87 diarrhoeic lambs (25%) and on 13 of 28 farms (46%). Though the agewise distribution revealed more number of cases in 3-month-old lambs but statistically no significant relationship was found between age of lambs and rotavirus infection. To our wisdom rotavirus infection in diarrhoeic lambs was related to meteorological changes as the number of diarrhoeic lambs was found to increase in spring months. These findings are in agreement with those of Theil et al. (1995, 1996) who observed the repeated occurrence of severe outbreaks of neonatal lamb diarrhoea in early spring with high morbidity and mortality in Ohio, USA.

In the present study ELISA detected rotavirus in 21 faecal samples while as PAGE detected it in only 10 samples. These findings are in agreement with Arguelles et al. (2000) who reported the sensitiviness of ELISA over PAGE as 10^6 and 10^{11} rotavirus particles per ml were required to be detected by ELISA and RNA-PAGE, respectively. For unknown reasons, ELISA could not detect three samples positive by PAGE. However, Khattar and Pandey (1986) also encountered similar situation while detecting group A rotavirus from buffalo calves with diarrhoea by PAGE and sandwich ELISA.

Fifty-six strains of *E. coli* recovered from lambs with diarrhoea were found to belong to 25 different serogroups like O8, O20, O21, O25, O26, O30, O39, O43, O45, O69, O75, O76, O82, O102, O104, O107, O113, O120, O127, O132, O139, O141, O143, O153 and O157. The pathogenic significance of these serogroups in lamb diarrhoea is to be established as serogrouping of *E. coli* isolates though useful is not definitive (Barlow et al., 1999). The information about the isolation of *E. coli* in sheep is scanty. However, Cid et al. (2001) reported the association of O2, O4,

O26, O80 and O91 STEC serogroups with lamb diarrhoea. None of the serogroups, except O26, isolated in the present study belonged to these STEC serogroups. Similarly, 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). Very recently, we have also detected association of *E. coli* O4:NM serotype with calf diarrhoea (unpublished data). In the present study two strains belonging to O157 serogroup were isolated from lambs with diarrhoea. There seems no report available on isolation of O157 serogroup from lambs with diarrhoea to compare the results. However, Dean-Nystrom et al. (1999) reported O157 serogroup as cause of severe diarrhoea and attaching and effacing mucosal lesions in neonatal calves. Similarly strains belonging to O8, O9, O20 and O101 ETEC serogroups have been found commonly associated with diarrhoea in calves less than 1 week of age and could be a problem in calves as old as 2–3 weeks (Acres, 1985; Holland, 1990). In the present study O8 and O20 serogroups were isolated from diarrhoeic lambs. During the present investigation O88, O113, O157 and O168 serogroups were isolated from healthy lambs. Except O157 serogroup none of the serogroups isolated belonged to the most commonly reported ovine STEC serogroups in healthy sheep all over the world (Djordjevic et al., 2001). Similarly, Chapman et al. (2001) reported isolation of *E. coli* O157 from 7.4% of healthy sheep and 0.7% of lamb carcasses in UK and most of the strains were verocytotoxigenic. Diarrhoeic faecal samples negative for rotavirus and *E. coli* suggested that diarrhoea in these lambs might be due to other enteropathogens (salmonella, campylobacter, protozoa, coronavirus, etc.).

To determine the invasive property, all the *E. coli* isolates were subjected to congo red binding assay. Out of 56 strains of *E. coli*, from diarrhoeic faecal samples, only 15 were found positive for congo red binding activity. Even two strains of *E. coli* recovered from two non-diarrhoeic faecal samples were also found congo red positive. These findings are contrary to those of Berkhoff and Vinal (1986) who reported a strong correlation between the expression of CR phenotype and virulence of *E. coli*. However, our findings correlate with those of Gowda et al. (1999) who also did not find association between the expression of CR phenotype and virulence of *E. coli*. Similarly, Panigrahy and Ling (1990) attributed CR binding property to certain structural and functional properties expressed in a suitable growth medium in vitro.

The isolation of O8, O26, O113 and O157 was alarming, as strains belonging to these serogroups have been found to cause life-threatening infections in humans, viz. bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombocytopenic purpura (Willshaw et al., 1992; Schmidt et al., 1999). Isolation of O157 has been so far rare in India that too from cattle, pigs, poultry and humans. The isolation of many pathogenic serogroups of *E. coli*, especially O157 serogroup, from sheep suggests that it could pose a serious public health problem in future as well as challenge to animal food industry.

5. Conclusion

The present investigation revealed an incidence of 25% of group A rotavirus infection in lamb diarrhoea in India. Number of rotavirus associated diarrhoeic cases was found to increase in spring months. There is need to study the virulence attributes of rotavirus isolates as well as to know their genotypic character. Isolation of many pathogenic serogroups of *E. coli* especially O26, O113, O157, etc. may pose serious public health problems in future as well as challenge to food industry. Further characterisation of *E. coli* isolates will confirm their pathogenic potential or otherwise.

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