



Prevalence and characterization of rotavirus from faecal samples of children and animals*

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ABSTRACT

Faecal samples (147; 60 bovine calf, 40 children, 47 piglets and pigs) were screened for the presence of rotavirus by RNA-PAGE and RT-PCR. The RT-PCR assay was standardized for the bovine and human rotavirus to amplify VP7 gene of the group A rotavirus. Out of 147 samples, 40 (27.2%) were found positive for rotavirus by RNA-PAGE, whereas only 15 turned out positive by RT-PCR. In bovine calves 21 (35%) and in children 19 (47.5%) cases were positive for rotaviral presence by RNA PAGE, on the contrary, RT-PCR was able to detect 7 cases of bovine (11.66%) and 8 cases of human (20%). We also showed G10 genotyping of rotavirus isolates of bovine origin by PCR. All the samples turned out to be PCR positive indicating that G10 is a predominant genotype responsible for bovine rotaviral diarrhoea in and around Bareilly region. The results indicated that direct detection of rotavirus by RT-PCR from faecal sample was not satisfactory as compared to RNA PAGE, probably due to large product size and sequence variations in rotaviruses.

Key words: Diarrhoea, dsRNA, Rotavirus, RT-PCR, RNA-PAGE

Food-borne diseases are a widespread and growing public health concern both in developed and developing countries (WHO 2002). An estimated 1.8 billion episodes of diarrhoea occur each year and 3 million children under the age of 5 years die of diarrhoea (Payne *et al.* 2008, WHO 1999). In India up to 17% of all death in indoor pediatrics patients are diarrhoea related (Banerjee *et al.* 2004). Rotavirus, *Salmonella*, *E.coli* seems to be more frequently implicated in diarrhoeal cases than other pathogens (Mathew *et al.* 1991). Rotaviruses, member of the family *Reoviridae* with a double-stranded RNA genome (Kalica *et al.* 1978), cause an estimated 130 million cases of gastroenteritis and 800,000 deaths in children in developing countries (Das *et al.* 2003). The prevalence of rotavirus diarrhoea in India varied from 5–71% in hospitalized less than 5 year -old children with acute gastroenteritis (Broor *et al.* 2003). Researchers used enzyme linked immunosorbant assay (ELISA), latex agglutination test (LAT), and polymerase chain reaction (PCR) etc. for rapid diagnosis of diseases or identification

of pathogens (Chapman 1989, Gouvea *et al.* 1990, Speirs and Akhtar 1991). PCR holds a great potential as a rapid, sensitive and specific diagnostic tool (Olsen and Rasmussen 1995). Several PCR assays were used for detection of *Salmonella*, *E. coli* and rotaviruses from the diarrhoeal patients (Pollard *et al.* 1990, Stacy-Phipps *et al.* 1995, Lin and Tsen 1996, Teng *et al.* 2004). For rotavirus detection from the diarrhoeal cases primers directed against VP7 and VP6 genes of various rotavirus types (A to G) have been widely used (Gouvea *et al.* 1990). Among these a set of primers for VP7 gene (Segment 8 or 9) of rotavirus A was reported to specifically amplify full length copies from any group A rotavirus strain (Gouvea *et al.* 1990). Keeping the above points in view, the present investigation was done to standardize the PCR for rapid detection of rotavirus and to study the comparative prevalence of rotavirus in diarrhoeal and non-diarrhoeal cases of animals and humans using PCR and RNA-PAGE.

MATERIALS AND METHODS

Reference strain of rotavirus (RRV) obtained from Christian Medical College, Vellore was used in the study. The RRV strain was maintained by periodical subculturing in MA104 cell line. MA104, an established cell line derived from fetal rhesus monkey kidney, was obtained from National Center for Cell Science, Pune, India. It was used for

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cultivation of RRV reference strain and isolation of rotavirus from the fecal samples.

Primers used in this study

Sl no.	Organism	Primer name	Primer sequence	References
1	Rotavirus	Bov9Com5	TGTATGGTATTGAATATACCAC	(Isegawa <i>et al.</i> 1993)
2	Rotavirus	Bov9Com3	TCACATCATACAACTCTAATCT	
3	Rotavirus	G10	TTCAGCCGTTGCGACTTC	(Falcone <i>et al.</i> 1999)
4	Rotavirus	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	(Gouvea <i>et al.</i> 1990)
5	Rotavirus	End9	GGTCACATCATACAATTCTAATCTAAG	

Sample collection

Diarrhoeal and non-diarrhoeal fecal samples (147) of animals (calves, piglets and adult pigs) and humans were collected from Bareilly and Pantnagar from December 2005 to March 2006 (Table 1). The calves were between 0 and 3 months of age, whereas piglets were 0–2 month-old. Human faecal samples were collected from children admitted to district hospital and various private hospitals of Bareilly district.

Detection of rotavirus from faecal sample

A 10% faecal suspension was made in Lysis buffer and centrifuged at 12 000 g for 20 min to remove coarse particles and cellular debris. The clarified supernatant was stored at –80°C till further use for extraction of rotavirus RNA. Three different methods were used for the extraction of rotavirus dsRNA from fecal sample, (a) phenol chloroform extraction method, (b) EZ-10 spin column, and (c) tri-reagent.

The extraction of RNA by phenol chloroform was accomplished as per the method described by Sambrook and Russell (2001). The extraction of dsRNA of rotavirus from faecal sample by EZ-10 spin column and tri reagent was done as per the manufacturer's instruction from 10% faecal extract.

Analysis of rotaviral dsRNA by RNA-PAGE technique followed by silver staining: The RNA extracted from the faecal sample was subjected to RNA-PAGE as per the procedure described by (Laemmli 1970) with little modification to determine the presence of rotavirus in the collected faecal samples. Briefly, the reagents of resolving gel (7.5%) and stacking gel (5%) were mixed sequentially as listed in Sambrook and Russell (2001). Subsequently, the

comb was put in the stacking gel solution and allowed to polymerize. The RNA pellet was dissolved in 20 µl of RNase free water. The microfuge tubes containing the RNA samples were heated at 56°C for 5–10 min to dissolve the pellet.

Subsequently, 10 µl of the sample was mixed with 5 µl of 6x loading dye on paraffin wax paper and loaded into the wells. The gel was resolved at constant voltage of 100 V for 2 h. The silver staining of the gel was done as described before (Svensson *et al.* 1986). The stained gel was photographed and stored in 10% ethanol.

Analysis of rotavirus by RT-PCR: The RT-PCR conditions were standardized for the bovine rotavirus and human rotavirus separately using oligonucleotide primers designed to amplify segment 8 or 9 (VP7 gene) of the group A rotavirus. The genotyping was also done for G10 genotype of bovine rotavirus.

RT-PCR for bovine group A rotavirus: RNA was transcribed to cDNA using VP7 gene specific primer by two step RT-PCR in a final volume of 20 µl reaction. 5 µl of RNA as template was used along with 2 µl each of forward and reverse primer (20 pmol). Following reactants were added carefully in 0.2 ml PCR tube which was placed on ice.

Component	Quantity of VP7 gene specific primer
Rotavirus dsRNA	5 µl
Bov9com5 (20 pmol)	2 µl
Bov9com3 (20 pmol)	2 µl
DMSO	1.5 µl
NFW	2.5 µl
Total	13 µl

The above mixture was centrifuged briefly, boiled at 95°C for 5 min and then snap cooled on ice. Separate master mix was prepared @ 6µl per reaction by adding following reagents in 0.2 ml PCR tubes on ice, 5X RT buffer –2.5 µl; 0.1 M dithiothreitol – 2 µl; 0.4 mM dNTP – 1 µl; RNAase inhibitor (40U/µl) –0.5 µl; M-MLV-RT –1 µl. The mixture was centrifuged briefly and RNA was reverse transcribed in thermocycler with the cycling condition of 25°C – 5 min, 42°C – 60 min, 70°C – 10 min. After completion of the reaction, cDNA formed was immediately used for PCR or stored at –20°C. PCR was carried out in a final volume of 25 µl using cDNA and VP7 gene specific primer along with the following reactant, nuclease free water (NFW) –12.5 µl; 10X

Table 1. Distribution of samples collected from different sources

Species	Source	Age	Number
Bovine Calves	IVRI Farm	0–3 M	60
Piglets	Farm, Pantnagar	0–2 M	20
Adult Pigs	Pig Farm, IVRI	1–3 Yrs	27
Humans	Private Hospitals and District hospital, Bareilly	0–5 Yrs	40
Total			147

PCR buffer –2.5 µl; 0.2 mM MgCl₂ –0.3 µl; 0.2 mM dNTP mix – 0.5 µl; cDNA template – 5 µl; forward and reverse primer (10 pmol/µl) – 2 µl each; Taq DNA polymerase (5U/µl) – 0.2 µl. The tubes were loaded on to thermalcycler and VP7 gene was amplified at 50°C annealing temperature.

RT-PCR for human group A rotavirus: The conditions for reverse transcription were same as described above except that the primers used were Beg 9 and End 9. For PCR the reaction was set in a total volume of 25 µl containing 2.5 µl of 10×PCR buffer, 20 pmol (2 µl each) of VP7 gene specific primer (Beg9 and End9), 0.3 µl of 0.2 mM MgCl₂, 0.5 µl of 0.2 mM dNTP mix, 5 µl of cDNA template, 1 unit of recombinant thermostable Taq DNA polymerase and 12.5 µl of NFW and the cycling conditions were set up with an initial denaturation of 1 min at 94°C, 30 cycles each of 2 min annealing at 55°C and 1 min extension at 72°C was carried out followed by a final extension for 7 min at 72°C.

PCR for genotyping of bovine group A rotavirus: Each RT-PCR product was subjected to a second round of amplification with the generic primer Bov9com5 and typing primer specific for G10. Production of 665 bp band was considered positive for G10 genotype.

RESULTS AND DISCUSSION

Comparative screening of rotavirus using RNA-PAGE and RT-PCR: Prevalence of rotavirus in diarrhoeal stool samples of human and animal origin by RNA-PAGE is depicted in Table 2. A total of 147 stool samples from diarrhoeal and non-diarrhoeal stool cases of man and animal origin were screened. Of which 40 (27.2%) were found to be positive for rotavirus by RNA-PAGE. Among the various sources, 19/40 (47.5%) human diarrhoeal samples (children between 1–5 yrs of age) (Fig. 1) and 35% (21/60) bovine calves (between 0–3 months of age) were positive for rotaviral diarrhoea (Fig. 2), whereas none of the piglet and adult pig stool sample were positive for rotavirus. Majority of the human and bovine rotavirus strains showed long pattern of electropherograms.

Reverse transcription-polymerase chain reaction assay was standardized for detection of human and animal rotavirus by amplifying VP7 gene. For standardization, cDNA was formed from the samples which were positive for rotavirus

Table 2. Prevalence of rotavirus in diarrhoeal cases of animals and human RNA-PAGE technique

Sl. no.	Source	No. of samples screened	RNA-PAGE positive (%)
1	Bovine calf	60	21 (35)
2	Human	40	19 (47.5)
3	Piglets	20	00 (0)
4	Adult pigs	27	00 (0)
Total		147	40 (27.2)

by RNA-PAGE. The annealing temperature of 50°C was found to give bright, expected product without any non-specific products. After standardization of different parameter of the cyclic conditions, an expected 1011 bp product was obtained from the samples in which specific primers were used for reverse transcription. Similarly RT-PCR was also standardized with Beg 9 and End 9 primers for detection of rotavirus from human stool samples. The standardized RT-PCR assays were utilized for detection of rotavirus from the extracted viral dsRNA's of human and animal faecal matrix. The results as shown in Tables 3 and 4 revealed that of the 19 samples which were positive for human rotavirus dsRNA by RNA-PAGE, only 8 got amplified by RT-PCR method (Tables 3, 4; Figs 3, 6). Samples, which showed negative results by RNA-PAGE were also negative by RT-PCR method. Similarly of the 60 faecal samples of bovine diarrhoeal cases only 7 showed amplification of VP7 gene by standardized RT-PCR (Tables 3, 4; Figs 4, 6). On the contrary RNA-PAGE revealed presence of rotavirus in 21

Table 3. Prevalence of rotavirus in fecal sample of animal and human using RT-PCR for VP7 gene

Sl. no.	Source	No. of samples screened	RT-PCR positive (%)	Genotype (G10) (%)
1	*Bovine calf	60	7 (11.66)	7 (100)
2	Human	40	8 (20)	ND
3	Piglets	20	0 (0)	ND
4	Adult pigs	27	0 (0)	ND

ND, Not done; *genotyping only done of bovine positive sample.



Mixed infection

Fig 1. Electropherotype migration pattern of human Group A rotavirus by RNA-PAGE technique.

Table 4. Comparison of RNA-PAGE and RT-PCR for detection of rotavirus in fecal sample

Sl. no.	Source	No. of samples screened	RNA-PAGE positive (%)	RT-PCR positive (%)
1	Bovine calf	60	21 (35)	7 (11.66)
2	Human	40	19 (47.5)	8 (20)
3	Piglets	20	00 (0)	0 (0)
4	Adult pigs	27	00 (0)	0 (0)
Total		147	40 (27.2)	15 (10.2)

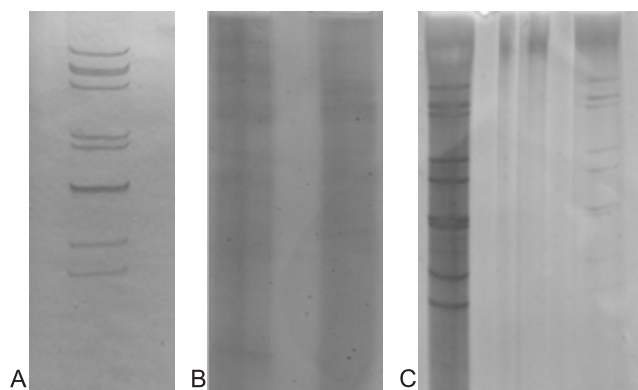


Fig. 2. SPS-PAGE showing 11 RNA segments of bovine group A rotavirus extracted from faecal sample.

bovine faecal samples. All the samples that were negative by RNA-PAGE also showed negative results by RT-PCR. Seven RT-PCR positive samples of bovine rotavirus were subjected to genotyping studies with primers of G10 genotype. As the number of positive samples for bovine rotavirus was very small by RT-PCR, genotyping was done for only G10 although G6 and G3 genotypes are also predominantly found. The results indicated that all the positive samples (7) belonged to G10 genotype (Table 3; Fig. 5).

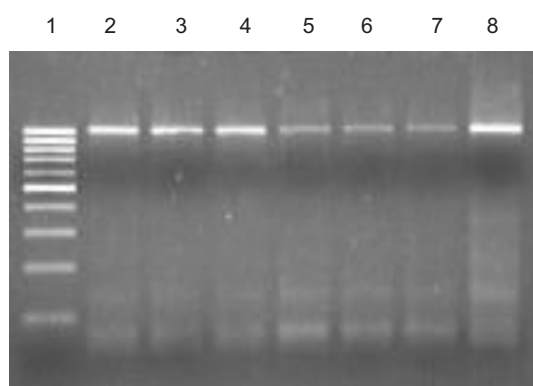


Fig. 3. PCR product (1062 bp) of VP7 gene of human group A rotavirus. Lane 1: DNA marker of 100 bp; Lane 2 to 8: PCR product of 1062 bp of VP7 gene of human group A rotavirus.

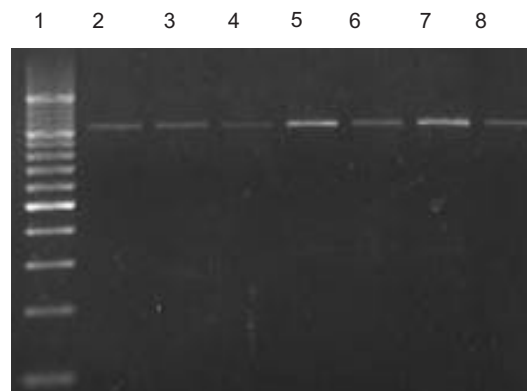


Fig. 4. PCR product of 1011bp of bovine group A rotavirus. Lane 1: DNA marker of 100 bp; lane 2-8: VP7 gene amplified (1011bp) of bovine group A rotavirus.

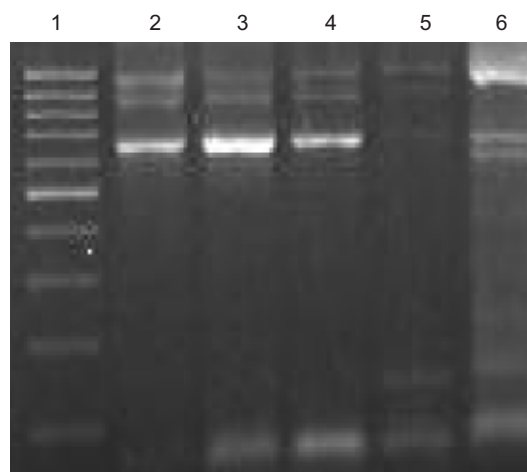


Fig. 5. PCR product of 665bp of G10 genotype of bovine group A rotavirus. Lane 1: DNA marker of 100 bp; lane 2, 3, 4 and 5: G-typing of bovine group A rotavirus with G10 primer (665bp); lane 6: Duplex PCR using G10 and Bov9com5 primer.

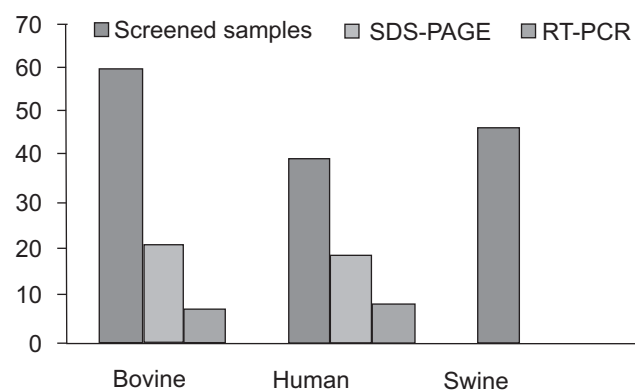


Fig. 6. Prevalence of rotavirus in different species screened by RNA-PAGE and RT-PCR.

Cross reactivity of primers used in human RT-PCR i.e. Beg 9 and End 9 was checked for amplification of VP7 gene for dsRNA extracted from positive cases of bovine faecal

material. Similarly primers of bovine RT-PCR, viz. Bov9com5 and Bov9com3 were used for amplification of human dsRNA material. The results showed that PCR primers of human and bovine did not cross-react with each other and only specific amplification was observed.

Our study indicated very high prevalence of rotavirus in bovines (35%) by RNA-PAGE. The prevalence rate observed in our study was in agreement with earlier reports from Haryana in which prevalence rates of 11–43% have been reported (Singh 1990, Grover 1998, Gulati *et al.* 1999). Similarly rotavirus had also been implicated as a causative agent of diarrhoeal episodes of calves in various parts of India and abroad (Brussow *et al.* 1992, Huang *et al.* 1992, Ghosh *et al.* 2007, Minakshi 2009). The RNA-PAGE migration pattern of rotavirus dsRNA segments in our study yielded long electrophoretic migration patterns. All the patterns were identical except one which showed different migration pattern. This clearly suggests that a single and similar type of rotavirus was responsible for the diarrhoeal cases of calves. Similar reports of majority of diarrhoeal cases with rotavirus showing single pattern had been reported by Minakshi (1999). The samples showing different migration pattern also showed more than 11 bands suggesting that there may be dual infection in this calf. Earlier workers have also reported presence of mixed infection or more than 11 segments in faecal samples of bovine rotavirus (Pocock 1987, Sukumaran *et al.* 1992, Legrottaglie *et al.* 1995, Minakshi 1999). The finding of extra viral RNA segments suggest possibility of simultaneous or sequential infection by more than one electropherotype or a modification in the length of RNA segments during infection (Minakshi 1999). In the present work, faecal sample from few non-diarrhoeal cases of bovines were also screened for rotavirus. However, none of the samples turned out to be positive. For initial RNA-PAGE screening, rotaviral RNA was extracted by 3 different methods, viz. (a) phenol chloroform method, (b) EZ–10 spin column total RNA miniprep super kit, and (c) tri reagent. The results of RNA-PAGE with RNA extracted by these methods indicated a clear superiority of results with the RNA extracted by phenol-chloroform method. Therefore, for further experiments phenol-chloroform method was used for RNA extraction. In present work RT-PCR was also used, targeting complete VP7 gene of segment 8 or 9, for rapid detection of rotavirus from diarrhoeal cases. The results indicated that 7 samples (11.66%) were positive by RT-PCR. As compared to RNA-PAGE (35%), lower % of samples turned out to be positive by RT-PCR (11.66%). Our results are in agreement with the previous findings of Wani *et al.* (2004) wherein they reported higher positivity by RNA-PAGE than RT-PCR. This lower prevalence by RT-PCR may be attributed to either non-specific inhibition of PCR by the components of faecal matrix that were carried through the extraction procedure (Xu *et al.* 1990, Wani *et al.* 2004) or the sequence variation in the rotavirus genome (Das *et al.* 1993,

Ushijima *et al.* 1994, Steele *et al.* 1995). RNA-PAGE positive samples of bovine rotavirus were subjected to RT-PCR with universal primers for VP7 gene as reported by Gouvea *et al.* (1990). In spite of their report that the primers can amplify VP7 gene from all group A rotavirus, in present work none of the bovine rotavirus showed positive results with these primers. This suggested variations in the sequence of rotavirus from various sources. Rotavirus is known to exhibit antigenic and genomic diversity throughout the world. This has led to the identification of several serotypes and genotypes of animal and human rotavirus. Due to segmented RNA genome, rotavirus is continuously changing and leading to emergence of new genotypes (Minakshi 1999, Minakshi 2009). Understanding the diversity of G and P genotypes of rotaviruses circulating in a geographical area is crucial for development of an effective vaccine. Therefore, in our study we also attempted partial genotyping of rotavirus isolates of bovine origin. For this, primers for G10 genotype as reported by Isegawa *et al.* (1993) were used. All the PCR positive samples of the present study turned out to be of G10 genotype indicating that G10 is a predominant genotype responsible for bovine rotaviral diarrhoeal cases. The previous reports from Jammu and Kashmir state (Wani *et al.* 2004) have also reported G10 to be predominant genotype of bovine rotavirus, whereas, Minakshi (1999) reported G10 and G6 genotypes of bovine rotavirus from calf diarrhoeal cases.

Screening of human diarrhoeal samples by RNA-PAGE revealed 19 (47.5%) rotaviral cases. In previous reports from different parts of India, the prevalence of rotavirus in acute diarrhoeal cases of children has been reported to vary from 6–45% from North India (Broor *et al.* 1993, Jain *et al.* 2001a, Chakravarti *et al.* 2010), 16–22% from South India (Bhat 1985, Brown *et al.* 1988, Saravanan *et al.* 2004), 41% from East India (Jain 2001, Chakravarti *et al.* 2010) and 28–30% from West India (Jain *et al.* 2001b, Kelkar 1999). Our results indicated that rotavirus is one of the most important causal agents of diarrhoea in children of Bareilly region and the prevalence is on the higher side than rest of the India. The electropherogram of human rotavirus isolate of the study showed long electrophoretic migration pattern in all the isolates. The pattern in majority of the isolates was similar, indicating a single type of rotavirus responsible for infection in children in this region. RT-PCR was also standardized for rapid detection of presence of rotavirus in children diarrhoeal cases. Of the 40 diarrhoeal cases screened with RT-PCR 8 were positive (20%). These results are on similar lines as that of observed in bovine rotavirus screening i.e. lower % of samples were positive by RT-PCR than RNA-PAGE.

Thus, our results revealed that rotavirus is one of the major causes of diarrhoea in human and bovine. The results of the direct detection of these pathogens by RT-PCR/PCR from the RNA extracted from faecal material were not satisfactory because of inhibition of PCR reaction. Therefore, modifications in the PCR protocols may be tried, such as

enrichment of samples followed by extraction of RNA, addition of bovine serum albumin to overcome PCR inhibitory substances.

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