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Detection and Phylogenetic Analysis of Group A Rotaviruses in Faeces of Diarrhoeic Bovine, Caprine, Ovine, Porcine and Human Population from Central India

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ABSTRACT

Background: Rotavirus infection is a leading cause of acute dehydrating diarrhoea in humans, primarily affecting infants and young children and causes neonatal diarrhoea in the majority of domestic animals. However, there is a dearth of literature in India on geographic or temporal comparisons of rotavirus transmission between humans and animals.

Methods: Prior to analysis with one-step RT-PCR, RNA-PAGE confirmed 40 rotaviruses recovered from a total of 306 faecal and stool samples obtained from domestic animals, (245) suffering from watery diarrhoea and children (61) suffering from diarrhoea. The RT-PCR positive sample were subjected to sequence analysis followed by BLAST analysis to confirm the presence of VP7 gene specific to rotavirus. The sequences of rotavirus in study were aligned with Indian and global VP7 sequences and were further subjected for phylogenetic analysis.

Result: Only three samples of three different species (Human, cattle and buffalo) could show the positivity, while remaining 37 failed in RT-PCR. Besides, the similarity of the nucleotide sequence of one of the positive isolates recovered from cattle calf with one of the Indian human rotavirus sequences in phylogenetic analysis indicates the possibility of inter-species transmission of rota viral strains circulating in the area, indicating organism's zoonotic significance.

Key words: Phylogenetic analysis, Rotavirus, RT-PCR, VP7, Zoonosis.

INTRODUCTION

Rotaviruses causes significant gastrointestinal infections of both humans and a wide range of animal species, such as cattle, buffalo and pigs (Estes and Kapikian, 2007). According to estimates, rotavirus-associated diarrhoea causes around 125 million instances of infantile gastroenteritis and 600,000 child deaths annually, mostly in underdeveloped nations (Parashar *et al*., 2009). Rotavirus-induced neonatal diarrhoea causes significant economic losses due to high morbidity, mortality, treatment costs and decreased growth rate of infected animals (Maes *et al*., 2003). The viral transmission occurs through faecal-oral pathway. It causes gastroenteritis by infecting and harming lining cells of the small intestine. This virus encodes six structural proteins (VP1, VP2, VP3, VP4 and VP6) as well as six non-structural proteins (NSP1 to NSP6) (Matthijnssens *et al*., 2009). These viruses are divided into seven groups (A-G) based on the VP6 gene's antigen specificity. Group A is the most prevalent group that affects both humans and animals. Rotaviruses belonging to group A are further divided into several G and P types based on the antigenic and molecular characterisation of the VP4 and VP7 genes. G genotype is determined by VP7, whereas P genotype is determined by VP4 (Barbosa *et al*., 2013). Rotavirus A is endemic worldwide, accounting for more than 90% of rotavirus gastroenteritis in humans (Leung *et al*., 2005). Using RNA polyacrylamide gel electrophoresis (RNA-PAGE), rotaviral antigen or viral nucleic acid can be detected in faecal samples (Minakshi *et al*., 2009). A molecular technique of reverse transcriptase PCR (RT-PCR) is used to detect amplimers and identify specific G and P genotypes present in stool specimens (Van Doorn *et al*., 2009).

The literature provides an increasing amount of information supporting interspecies transmission and reassortment of rotaviruses between humans and animals,

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it may result in the emergence of a novel virus in the human population (Gentsch *et al*., 2005; Komoto *et al*., 2016). Other species, like the dog, cat, pig and cattle, provide more significant and frequent contributions to the genetic variety seen in humans (Martella *et al*., 2010). Several research studies have described the spread of rotavirus strains in humans or animals in India, but they haven't provided any geographic or temporal comparisons of transmission between humans and animals (Manuja *et al*., 2008; Minakshi *et al*., 2005). This is also comparable to the paucity of such reports globally and the shortage of investigations comparing strains simultaneously obtained from humans and animals in the same area (Steyer *et al*., 2008).

Considering the facts and circumstances, the current research was aimed at investigating the molecular characterization of PAGE positive rotaviruses recovered from human and animal species in Maharashtra, central India and to assess their co-relationship through sequencing and further phylogenetic analysis.

MATERIALS AND METHODS

Collection of faecal and stool samples

The present investigation was carried out with priorly RNA-PAGE confirmed rota viruses recovered from screening of 245 faecal samples collected from cattle calves (60), buffalo calves (55), sheep lambs (40), goat kids (40) and piglets (50) suffering with watery diarrhoea during period of years 2017-2020 at College of Veterinary and Animal Sciences, Udgir. Moreover, 61 stool samples of children suffering with diarrhoea from civil hospitals, primary health centers and district civil hospitals were also collected and processed with RNA-PAGE. On analysis there were 40 samples determined containing rota virus from different species (Table 1). Amongst these, 30 samples were from children, 9 from cattle and 1 sample was recovered from buffalo. The samples were transferred to the laboratory on ice and were stored at -20°C until processing.

Sample processing

The faecal and stool samples were suspended in 0.06 M phosphate buffered saline (PBS) at pH 7.2, then centrifuged at 12000 rpm for 30 min to remove coarse particles and debris. After that the supernatant was stored at -20°C until further use.

Extraction of RNA

The TRIzol method was used to extract rotavirus RNA, as described by (Gill *et al*., 2017) and (Gentsch *et al*., 2009). In brief, the protocol that was adopted was as follows: Before using the 10% faecal/stool suspension, it was vortexed and allowed to settle at room temperature for 30-60 min. The stool suspension was clarified by centrifugation at 5000 rpm for 5 min at room temperature in a mini centrifuge. The 250 µl supernatant from the stool and faeces was transferred to a sterile 1.5 ml Eppendorf tube and 750 µl of TRIzol reagent was added to it. The tube was vortexed for 30 seconds before being incubated at room temperature for 5 minutes. Following the incubation, 200 µl of chloroform was added to each sample and vortexed for 30 sec before incubating for 3 min. Centrifugation at 12000 rpm for 5 minutes at 40°C for phase separation. The 450 µl clear upper aqueous phase was transferred to a new sterile Eppendorf tube while avoiding the white interface and pink organic phase. 700 µl of ice cold isopropanol (isopropyl alcohol) was added to the above mixture and gently mixed 4-5 times by turning the tube upward and downward before incubating at -20°C for 20 min. The tube was then centrifuged at 12000 rpm for 15 min at 40°C to obtain the double-stranded (ds) RNA pellet. The supernatant was carefully discarded and the pellet was air dried at room temperature. The pellet was then resuspended in 20 µl of RNase-free water treated with Diethyl pyrocarbonate (DEPC). The samples were kept at -20°C.

One-step reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was conducted for PAGE positive samples as per Chitambar *et al*. (2011) with targeting VP7 gene-specific primers and SuperScript™ III One-Step RT-PCR Kit (Invitrogen, USA) in Mastercycler® nexus - PCR Thermal Cycler (Eppendorf, India). The RT was carried out at 45°C for 30 min followed by 94°C for 2 min. The RT was followed by PCR with cycling conditions of 95°C for 20 sec, 50°C for 20 sec, 72°C for a total of 30 cycles. The primers used for the PCR were as per Taniguchi *et al*., (1992).

Agarose gel electrophoresis

The amplified PCR products were analyzed in 1% agarose gel with ethidium bromide (0.5 µg /ml). About 5 µl of PCR product was mixed with 1 µl of 6X gel loading dye and loaded in the well. One well was loaded with 5 µl of 200 bp standard molecular weight DNA ladder (Himedia). Electrophoresis was conducted at 12 V/cm of gel till dye reached last third of gel. At the end of electrophoresis, the bands were visualized under UV transilluminator in gel documentation system (Biorad, USA).

Sequencing

Three rota positive sample each from cattle, buffalo and human were subjected to sequence analysis. The sequencing services were hired from BioResource Biotech Pvt. Ltd., 18/1, Madhukunj Society, Panchavati, Off Pashan Road, Pune-411008. The sequencing reactions of three PCR products were performed with ABI BigDye Terminator kit version 3.1 using an ABI automated genetic analyzer as per the method of (Araújo *et al*., 2007).

Sequence analysis of rotavirus

The obtained sequences from the rotavirus PCR positive samples were subjected to BLAST analysis with GenBank database sequences using BLASTn algorithm available at NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast) to confirm the presence of the gene specific to VP7 gene of rotavirus. The nucleotide sequences of VP7 gene fragment of rotavirus

were aligned using default parameters of muscle alignment implemented in MEGA 7.0 software (http://www.megas oftware. net/) as per the method of Kumar *et al*. (2016) with 53 sequences including sequences of Indian and foreign Rotavirus VP7 retrieved from GenBank (http://www. ncbi.nlm.nih. gov/genbank/index.html).

Phylogenetic analysis

The nucleotide sequences were aligned with 53 Indian and global VP7 sequences by using ClustalW program embedded in Mega7. The aligned sequences were subjected for phylogenetic analysis using the Neighbor-Joining method, associated taxa clustered together in the bootstrap test (1000 replicates) in MEGA7. The evolutionary distances were computed using the Kimura 2-parameter method.

RESULTS AND DISCUSSION

RT-PCR of PAGE positive samples

In present study, RNA-PAGE positive diarrheal faecal and stool samples were further subjected for screening by employing one step reverse transcriptase polymerase chain

Table 1: RNA-PAGE positive faecal samples for rotavirus (Cattle, buffalo, human).

Sample no.	Species	Age	Location
CF75	Cattle	1 M	Dairy Farm Solapur
CM76	Cattle	1 M	Dairy Farm Solapur
CM81	Cattle	1 M	Goshala Pandharpur Dist Solapur
CM118	Cattle	3 M	Dairy Farm Kolhapur
CM123	Cattle	3 M	Haroli Dairy Farm Jaisinghpur, Kolhapur
CF203	Cattle	2 M	Dairy Farm Wai, Dist. Satara
CF204	Cattle	1.5 M	Dairy Farm Shirwal, Dist. Satara
CM207	Cattle	1 M	Dairy Farm Shirwal, Dist. Satara
CM254	Cattle	2 M	Dairy Farm Nigadi, Pune
BF61	Buffalo	1.5 M	Goshala Solapur
HF40	Human	1 Yr	Pediatrics Private Hospital Miraj, Sangli
HM43	Human	2 Yr	Pediatrics Private Hospital Miraj, Sangli
HF45	Human	8 M	Pediatrics Private Hospital Miraj, Sangli
HM94	Human	10 M	Pediatrics Private Hospital, Solapur
HF95	Human	11 M	Pediatrics Private Hospital, Solapur
HM97	Human	1 Yr	Govt. Hospital, Solapur
HF98	Human	6 M	Pediatrics Private Hospital, Solapur
HM99	Human	8 M	Pediatrics Private Hospital, Solapur
HF106	Human	11 M	Pediatrics Private Hospital, Solapur
HM107	Human	8 M	Pediatrics Private Hospital, Solapur
HM180	Human	5 Yr	Pediatrics Private Hospital Kolhapur
HF182	Human	15 M	Govt. Hospital Kolhapur
HF183	Human	6 M	Pediatrics Private Hospital Kolhapur
HF186	Human	7 M	Pediatrics Private Hospital Kolhapur
HF189	Human	1 Yr	Govt. Hospital Kolhapur
HM190	Human	5 M	Pediatrics Private Hospital Kolhapur
HF238	Human	4 Yr	Pediatrics Private Hospital Karad, Satara
HF242	Human	5 M	Govt. Hospital Karad
HM243	Human	1 Yr	Govt. Hospital Karad
HM244	Human	3 Yr	Govt. Hospital, Satara
HM245	Human	2.5 Yr	Govt. Hospital, Satara
HF246	Human	1 Yr	Pediatrics Private Hospital, Satara
HM247	Human	6 M	Pediatrics Private Hospital Karad, Satara
HF283	Human	1Y Rr	Pediatrics Private Hospital Swargate,Pune
HF284	Human	1.5 Yr	Pediatrics Private Hospital Swargate,Pune
HM286	Human	7 M	Pediatrics Private Hospital, Swargate,Pune
HM287	Human	3 Yr	Pediatrics Private Hospital Swargate,Pune
HM293	Human	9 M	Govt. Hospital Pune
HM294	Human	2 M	Pediatrics Private Hospital Nigadi,Pune
HF295	Human	2 M	Pediatrics Private Hospital Nigadi, Pune

CM- Cattle male, CF- Cattle female, Buffalo female, HM- Human male, HF- Human female, M- Month, Yr- Year.

reaction (RT-PCR) using rotavirus VP7 gene specific generic primers. Amongst 40 RNA-PAGE positive samples screened, only three samples of three different species (human, Cattle and Buffalo) could show the positivity, while remaining 37 failed to reveal the amplicon. An analogous observation was recorded by Tiku *et al.* (2017), where they could amplify 50% of their isolate's VP7 gene. This could be attributed to the sequence variations detected in the circulating Indian rotavirus strains, though, novel methods like adaptor ligation amplification could resolve the problem. The problem of non-amplification of VP7 in most of the samples in present investigation can be correlated with Bhat *et al*. (2018) wherein they failed to amplify VP7 gene in any of their rotavirus positive isolates. Besides, it might be because of the existence of inhibitory substances in the fecal samples or mismatches in primer binding sites (Bhat *et al*., 2015; Manuja *et al*., 2008). Resembling findings were also reported by Gill *et al*. (2017) wherein, the researchers observed that only six out of nine RNA-PAGE positive samples could amplify VP7 gene in RT-PCR, attributing to the non-specific inhibition of PCR by the constituents of faecal matrix or strains non-typeable with the used primers.

The RT-PCR of CF75 (Cattle Calf), HF294 (Human) and BF61 (Buffalo calf) sample showed specific amplification of VP7 gene segments as evidenced by 1062 bp PCR amplicon in agarose gel electrophoresis (Fig 1). All RT- PCR positive samples of Human (HF294), Cattle (CF75) and Buffalo (BF61) were sent for sequencing. The recovered sequences were subjected to BLAST analysis with GenBank database sequences by means of BLASTn algorithm available at NCBI blast to ascertain that the sequences are specific to rotaviruses. The VP7 nucleotide identity of the obtained sequences was analyzed with available sequences from India, using NCBI BLASTn online tool. The results are indicated in Table 2.

The rotavirus sequences obtained from human and cattle showed maximum identity with G1 genotype, while, that of buffalo showed maximum identity with G3 genotype. The HRota/HF294 VP7 nucleotide sequence obtained from diarrheic child showed 92.05% identity with Indian bovine rotavirus (JX442769) isolate and 96.55% to 92.05% identity with Indian human rotaviruses (EU984109, JX411970). Similarly, CRota/CF75 VP7 nucleotide sequence obtained from diarrheic cattle calf showed 92.81% to 97.78% identity with Indian human rotaviruses (EU984109, JX411970) and 92.68% identity with Indian bovine rotavirus (JX442769). While, BRota/BF61 VP7 nucleotide sequence obtained from diarrheic buffalo calf showed 99.08% identity with Indian bovine rotavirus (MK043950) and 95.03% to 96.83% identity with Indian human rotaviruses (MF563923, MF621174).

Phylogenetic analysis of recovered rota viruses

The nucleotide sequences were aligned with 53 Indian and global VP7 sequences by using ClustalW program embedded in Mega7. The aligned sequences were subjected for phylogenetic analysis using the Neighbor-Joining method, associated taxa clustered together in the bootstrap test (1000 replicates) in MEGA7. The evolutionary distances were computed using the Kimura 2-parameter method. The optimal tree with the sum of branch length is shown in Fig 2.

The phylogenetic analysis based on VP7 sequences indicated genotype and origin wise clustering of the rotaviruses. All the Rotavirus VP7 sequences analyzed were placed into two major clads, upper and lower. The upper major clad consisted of the rotaviruses of human and animal origin belonging to the genotypes G1, G2, G4, G5, G6, G7, G8, G9, G10, G11 and G15. The lower major clad consisted of the rotaviruses of human and animal origin belonging to the genotypes G3, G12, G13 and G14. The Indian rotaviruses were placed in subclusters in every genotype separated from rotaviruses of foreign origin.

The CRota/CF75 and HRota/HF/294 VP7 sequences obtained from diarrheic cattle calf and child, respectively were clustered in subclad formed by G1 rotaviruses of human and animal origin in upper major clad. The BRota/BF61 sequence obtained from diarrheic buffalo calf was clustered in the subclad formed by G3 rotaviruses of human and animal origin in lower major clad. The CRota/CF75,

Table 2: Nucleotide identity of the rota virus isolates with G1 and G3 genotypes reported from India.

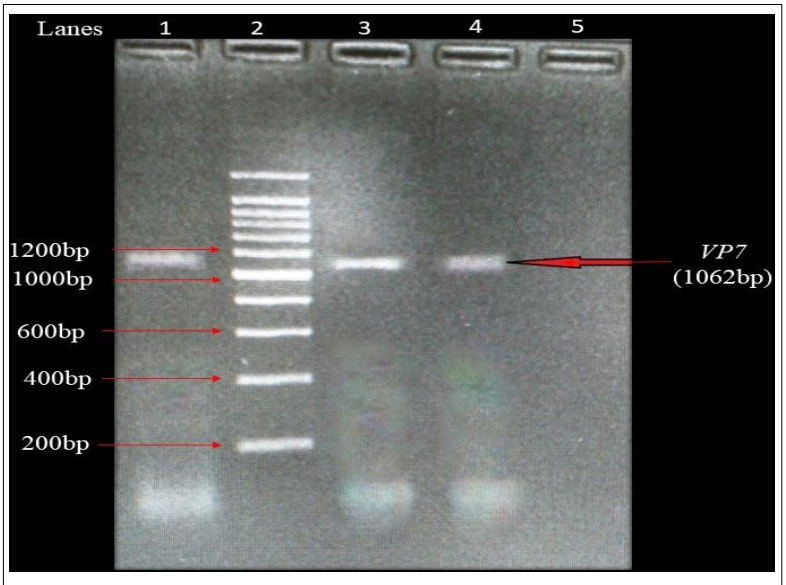
Sequence ID	HRota/HF294	BRota/BF61	CRota/CF75
HRota/HF294/G/MS/India/2019/VP7	100.00%	NS	91.44%
BRota/BF61/G/MS/India/2019/VP7	NS	100.00%	76.98%
CRota/CF75/G/MS/India/2019/VP7	91.44%	76.98%	100.00%
JX442769/BRota/G1/HR/India/2011/VP7	98.10%	77.26%	92.68%
EU984109/HRota/G1P8/MS/India/2006/VP7	96.55%	NS	92.81%
JX411970/HRota/G1/UK/India/2011/VP7	92.05%	76.18%	97.78%
JF689844/BRota/G3/UP/India/2009/VP7	76.57%	81.46%	77.62%
JX442779/BRota/G3/UK/India/2010/VP7	76.57%	81.46%	77.62%
AF386914/BRota/G3/KR/India/2002/VP7	76.54%	81.24%	77.66%
JF689835/BRota/G3/MP/India/2010/VP7	76.38%	81.05%	77.23%
MF621174/HRota/G3P4/WB/India/2016/VP7	76.01%	96.83%	NS
MK043950/BRota/G3/MS/India/2017/VP7	NS	99.08%	76.38%
MF563923/HRota/G3P8/WB/India/2016/VP7	73.81%	95.03%	NS

though obtained from diarrheic cattle calf, was closely placed with human rotavirus (JX411970) belonging to genotype G1. Similarly, HRota/HF/294 obtained from diarrheic child was placed closely with bovine rotavirus (JX442769) belonging to the genotype G1. However, BRota/BF61 was closely placed with bovine rotavirus (MK043950) belonging to the genotype G3.

In present study the isolates from RNA-PAGE positive stool samples from diarrheic cattle calf, buffalo calf and child were subjected for RT-PCR amplification followed by nucleotide sequencing and sequence analysis of VP7 gene. The cleaned sequences were blast with representative Indian isolates of rotavirus using GenBank BLASTn tool. The results showed that the HRota/HF294 isolate was most similar to the Haryana isolate JX442769, followed by the Maharashtra isolate EU984109 and the Uttarakhand isolate. Interestingly, out of these, JX442769 isolate from Haryana was isolated from bovines. All three isolates with the highest nucleotide identity to our isolate suggested that the HRota/HF294 isolate is a human rotavirus of the G1 genotype. This isolate shared 91.44% nucleotide similarity with CRota/CF75 recovered from cattle calf and no significant similarity with BRota/BF61 obtained from buffalo calf. Similarly, CRota/CF75 shared the highest nucleotide identity with the same group and hence belongs to the G1 genotype of human rotavirus, despite being recovered from a cow calf. The BRota/BF61 obtained from buffalo calf showed most identity (99.08%) with bovine rotavirus MK043950 recorded from Maharashtra earlier in 2017. This indicated that this isolate belongs to G3 genotype.

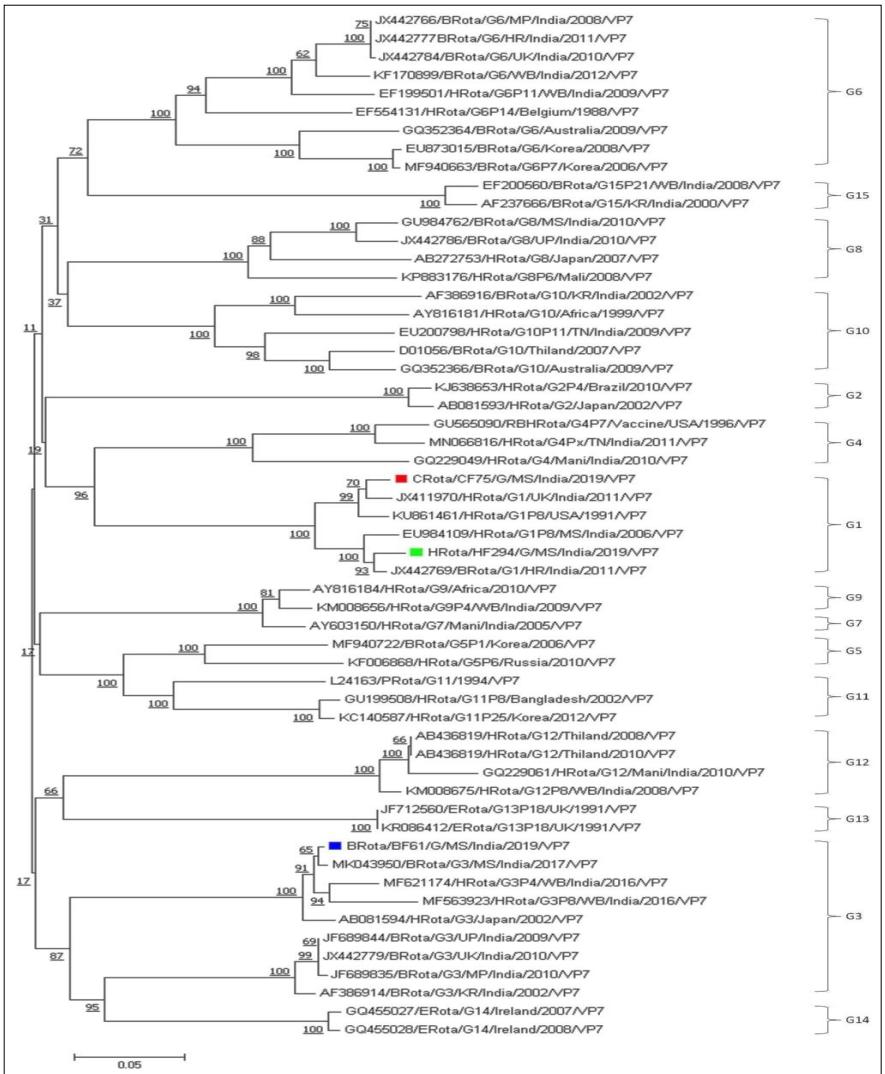
To support these findings, phylogenetic analysis was carried out using different Indian and global G genotypes of rotaviruses originated from human as well as animals. Since, VP4 amplification and sequence analysis was not considered in the present study, P typing of these isolates remains undetermined.

The phylogenetic analysis based on VP7 nucleotide sequences, revealed classification of the rotaviruses of Indian and foreign origin depending upon location and G genotypes. The HRota/HF294 and CRota/CF75 sequences were placed in a subcluster formed by G1 rotaviruses and closely with JX442769 and JX411970, respectively. This indicated that these isolates belong to G1 rotaviruses. Though, CRota/CF75 was obtained from cattle calf, we presume that this belongs to human G1 rotavirus as it was placed closely in the sub cluster of human G1 rotaviruses in the phylogenetic tree. Rotaviruses have a wide host range and are known to exchange hosts in natural situations (El-Attar *et al*., 2001). The study must be expanded to discover the P genotype of this isolate in order to ascertain whether this is an example of reassorting virus or inadvertent host switching (Bwogi *et al*., 2017). The host switching of the rotaviruses is considered as one of the most important factors for virus evolution as it may contribute to virus diversity (Estes *et al*., 2001; Desselgerger, 2014). The reassortment with changed virus diversity may lead to emergence of novel genotypes. As a result, we propose that this isolate be P typed. The reports also suggested that in same geographical area, the human and animal rotaviruses evolve separately (van der Heide *et al*., 2005). However,



Lane 1: HF294 (Human) sample positive for VP7.
Lane 2: L- 200bp DNA Ladder (Himedia, MBT050-50LN).
Lane 3: CF75 (Cattle) sample positive for VP7.
Lane 4: BF61 (Buffalo) sample positive for VP7.
Lane 5: NTC (Negative control template).

Fig 1: Amplification of VP7 gene in positive samples (Human, cattle and buffalo).



Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 gene of various group Rotaviruses from diverse geographical boundaries. The positive sequences of the current study are denoted by solid colored blocks. For each strain, the following data are given: Accession number/species/genotype and strain name/country origin/year/gene.

Fig 2: Phylogenetic tree.

possibilities of interspecies transmission can never be undermined because of prevailing conditions including close proximity, sharing of animals, open defecation particularly on grazing lands etc. which is common in low income rural community (Heylen *et al*., 2014).

The results also revealed that the BRota/BF61 isolate was belonged to G3 genotype of rotaviruses and was closely placed with bovine rotaviruses forming separate sub cluster from G3 human rotaviruses. This genotype was earlier reported in diarrheic calves by Varshney *et al.* (2002) from central and south India. The results suggested that the same genotype is circulating in calves of area under investigation.

CONCLUSION

To summarize, the similarity of the nucleotide sequence of one of the positive isolates recovered from cattle calf in this study, with one of the Indian human rotavirus sequences observed in phylogenetic analysis, suggests the possibility of a threat posed by inter-species transmission of rota viral strains circulating in the area, increasing the pathogen's zoonotic importance. From the standpoint of public health, it suggests an alarming picture that demands rigorous preventative intervention in the research region". Further P typing studies and regular sampling frameworks are warranted on molecular surveillance and cross species transmission of rotaviruses of animal and human origin.

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