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Genetic Characterization of an Emerging G3P[3] Rotavirus Genotype in Buffalo Calves, India

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ABSTRACT

The present study describes characterization of a novel rotavirus (B29) isolated from a buffalo calf causing severe diarrhea. It was confirmed by VP4 and VP7 gene based RT-PCR and in sequence analysis, the VP4 gene showed up to 99.3% sequence identity at nucleotide as well as amino acid levels with P[3] rotavirus genotypes. Similarly, VP7 gene showed a maximum nucleotide and amino acid identity of 99.1 and 98.7%, respectively with G3 genotypes of group A rotavirus (RVA) from several host species and places. The phylogenetic analysis of VP4 gene also revealed close relatedness with other P[3] genotype of rotaviruses from bovine, goat, canine and feline origin. Similarly, VP7 gene revealed close relation with several G3 rotaviruses from bovine and equine origin. The buffalo rotavirus isolate B29 was genotyped as G3P[3]. This is the first report of rotavirus isolate with a G3P[3] genotypic constellation in buffalo calvesfrom this region of India. The detection of buffalo rotavirus of G3P[3] genotypic combination reveals an unexpected epidemiological situation and diversity of bovine rotaviruses in India.

Key words: Bovine rotavirus, RNA-PAGE, RT-PCR, Sequencing, VP7 gene, VP4 gene, G3P[3] constellation in buffalo

INTRODUCTION

Rotaviruses are major etiologic agents of severe, acute dehydrating diarrhea in the young ones of many mammalian species, including humans, calves and foals (Dhama *et al*., 2009; Estes and Greenberg, 2013; Derby *et al*., 2014). Bovine group A rotavirus (BoRVA) is the main cause of diarrhea in neonatal calves causing severe economic loss due to morbidity and mortality in calves (Badaracco *et al*., 2012). The rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA). They encode 6 structural and 6 nonstructural proteins. The viral genome is enclosed in a triple-layered protein capsid. Outer capsid is made up of VP4 and VP7 protein which are encoded by gene segments 4 and 7, 8 and 9 (depending on the strain), respectively. The VP6 encoded by gene segment 6, constitutes the intermediate capsid and VP2, encoded by segment 2, forms the innercapsid (Estes and Greenberg, 2013).

Based on the antigenic epitopes present on the intermediate capsid protein VP6, rotaviruses are classified into groups and subgroups. The 8 groups, termed A-H have been identified, of which group A rotaviruses (RVA) are the major pathogens of humans and animals. An estimated 17.4% of deaths in children (1-11 months old) and 11.9% of deaths in children (1-4 years old) has been reported globally due to diarrhea (Derby *et al*., 2014). Rotaviruses have also been classified into G and P serotypes based on the antigenic specificity of outer capsid proteins VP7 (glycoprotein) and VP4 (protease sensitive), respectively (Esona *et al*., 2015). So far, 27 G genotypes have been recognized and of these, several serotypes are shared between humans and animals (El-Attar *et al*., 2002; Matthijnssens *et al*., 2011). Bovine rotaviruses most commonly belong to G types 6, 8 and 10 and have P types [1], [5] or [11]. Serotype G3 strains appear to have the broadest host range and were observed in humans and many animal species (Malik *et al*., 2013; Gauchan *et al*., 2015). The 3 widely separated regions, A (amino acids 8-101), B (amino acids 143-152) and C (amino acids 208-223) have been identified as major antigenic determinants on VP7. These regions were suggested to form complex, functionally related and operationally overlapping conformational epitopes that determine the serotype and neutralization specificities of rotaviruses (Esona *et al*., 2015). It has been reported that at least 27 G and 37 P genotypes have been identified and 73 G/P genotype constellations of GAR have been reported (Matthijnssens *et al*., 2011; Trojnar *et al*., 2013). Most of the bovine rotaviruses exhibit an RNA electrophoretic migration pattern of4:2:3:2 characteristic of RVA (Minakshi *et al*., 2005; Malik *et al*., 2012). To date, there is no information on G3P[3] buffalo group A rotaviruses. However, G3P[3] combination of bovine rotavirus have been characterized in cow calf in India (Ghosh *et al*., 2007). This limited epidemiological study was undertaken to determine the genotypic nature of rotaviruses circulating in diarrheic buffalo calves. Here, we describe for the first time the identification and molecular characterization of unusual G3P[3] buffalo rotavirus that is likely to be a reassortant bovine strain.

MATERIALS AND METHODS

Sample collection: A total of 85 fecal samples were collected from young buffalo calves suffering from severe diarrhea below the age of 6 months during June 2003-2004 from different animal farms of Haryana, India.

RNA extraction: Fecal samples were diluted with 9 volume of phosphate-buffer saline (pH7.4) and clarified by centrifugation at 1000Xg for 10 min at room temperature. Viral RNA was extracted from supernatant using phenol-guanidinium isothiocyanate-chloroform extraction method with minor modifications as described in the previous study (Minakshi *et al*., 2005; Minakshi *et al*., 2013). The extract was subjected to polyacrylamide gel electrophoresis for the detection of viral genomic RNA and characterization of RNA electrophoretic pattern (Malik *et al*., 2013).

Reverse Transcriptase-PCR (RT-PCR) and cloning of VP4 and VP7 gene: Viral genomic dsRNA purified from fecal samples was used for reverse transcriptase PCR using MMLV reverse transcriptase and *Taq* DNA polymerase (MBIFermentas, USA). Gene-specific primers were used for cDNA synthesis and PCR amplification. The VP4 gene was amplified using VP4 gene specific primers, Bov4Com5 (1067-1088): 5'-TCATTATTG GGACGATTCACA-3' and Bov4Com3 (1930-1909):

5'CAACCGCAGCTGATATATCATC3', respectively (Manuja *et al*., 2008). Similarly, the VP7 gene sequences were amplified using VP7 gene specific primers, Beg9 (1-21 bp): 5'GCTTTAAAAGAGAGAATT3' and End9 (1136-1062 bp): 5'GGTCACATCATACAATCTAAT CTAAG3' (Manuja *et al*., 2008). The PCR amplified DNAs were cloned into pBluescript (KS+) vector (Stratagene, USA) as per manufacturer's instruction. The DH5*α* strain of *E. coli* was used as host system. The positive clones were selected and insert sequence was confirmed by colony touch PCR. The plasmids having gene of interest were allowed for nucleic acid sequencing from both direction. The vector sequences were removed from the desired nucleotide sequences using vecScreen programme (http://www.ncbi.nlm.nih.gov/tools/vecscreen).

Nucleic acid sequencing and phylogenetic analysis of VP4 and VP7 genes: The PCR products of VP4 and VP7 gene were allowed for nucleic acid sequencing using automated DNA sequencer ABI PRISM^{TM} 3130xL at Department of Animal Biotechnology, LUVAS Hisar (Haryana). Nucleotide sequence data obtained was analyzed using BLASTN 2.2.31+, multiple sequence alignment and calculation of percent identity matrix of nucleotide as well as deduced amino acid sequences of VP4 and VP7 gene of buffalo rotavirus and representative isolates of all well characterized genotypes of group A rotavirus isolates were done using Bioedit 7.2.5 (Manuja *et al*., 2008).The phylogenetic analyses of VP4 and VP7 gene sequences of rotavirus from various species from different regions of the world were done using neighbor joining method of MEGA 5 software with 1000 bootstrap values (Tamura *et al*., 2011).

RESULTS

Detection of Rotavirus from diarrheic faecal samples by RNA-PAGE: Rotavirus in the specimens was first detected by RNA-electrophoresis of genomic dsRNA. Out of 85 specimens, 11 (12.94%) were detected positive with characteristic migration patterns (4:2:3:2) of long electropherotype of group A rotavirus (data not shown).

RT-PCR and cloning of VP4 and VP7 gene: Viral dsRNA of RNA-PAGE positive samples were transcribed into cDNA and allowed to PCR amplification using VP4 and VP7 gene specific primers of RVA. One out of eleven samples (B29 isolate) showed amplification in VP4 and VP7 gene specific RT-PCR. The agarose gel electrophoresis of VP4 and VP7 gene PCR products of B29 isolate showed 864 and 1062 bp amplification, respectively (Fig. 1 and 2). The VP4 and VP7 gene PCR products were cloned into pBlue script (KS+) vector (Stratagene, USA). On sequencing of cloned PCR products of VP4 and VP7 gene 864 and 1062 bp of nucleotide sequence were obtained.

Analysis of nucleotide sequence data: The nucleotide sequence of B29 isolate was submitted in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers DQ487204 and DQ478582 for VP4 and VP7 gene, respectively. The nucleotide sequences were compared with the sequences available in NCBI database using BLASTN+2.2.31 search. The VP4 gene sequences of B29 isolate showed maximum identity of 99.3% nucleotide and amino acid sequence identity with P[3] genotype of GAR isolated from various species (Table 1). Similarly, VP7 gene of B29 isolate showed a maximum nucleotide identity of 99.1 and 98.7% amino acid with G3 genotype of group A rotavirus from different species (Table 2). Thus, the B29 isolate was confirmed as G3P[3]

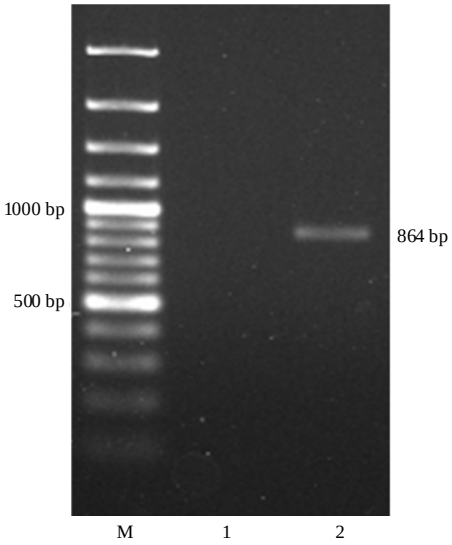

Fig. 1: VP4 gene amplification of buffalo rotavirus samples yielding 864 bp products in 1% agarose. Lanes: M: 100 bp DNA ladder (MBI Fermentas), 1: Negative control (Nuclease free water), 2: B29 isolate

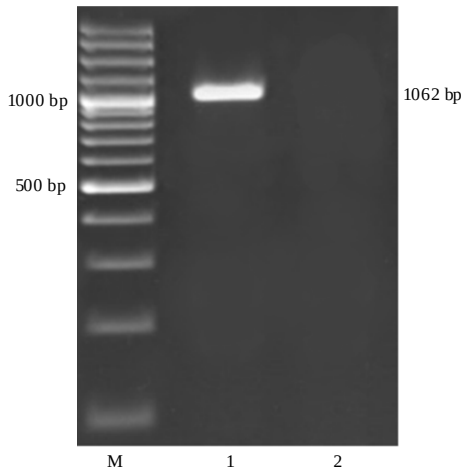

Fig. 2: VP7 gene amplification of buffalo rotavirus samples yielding 1062 bp products in 1% agarose. Lanes: M: 100 bp DNA ladder (MBI Fermentas), 1: B29 isolate, 2: Negative control (Nuclease free water)

genotype. The phylogenetic analysis of VP4 gene of B29 isolate showed close cluster with other P[3] genotype of buffalo origin from India (Fig. 3). Similarly, VP7 gene of B29 isolate showed close cluster with several G3 genotype of bovine and equine origin from India (Fig. 4).

DISCUSSION

Rotaviruses are major cause of diarrhoea in young animals and humans. Group A rotaviruses are most important etiological agent of gastroenteritis and severe diarrhoea in younger age group of human and animals of various species (Minakshi *et al*., 2005; Malik *et al*., 2014; Gauchan *et al*.,

Table 1: Comparison of the nucleotide and deduced amino acid sequence of VP4 gene of B29 (G3P[3]) strains with P[3] genotypes from different geographical regions

Rotavirus VP4 gene Accession No./strain/host/country	Genotype	VP4 gene identity	
		Nucleotide 1	Amino acid 1
DQ487204.B29.Buffalo.India	P[3]	100	100.0
EU311198.BRV116.Buffalo.India	P[3]	99.3	98.6
EU311197.BRV73.Buffalo.India	P[3]	99.1	98.6
JF720870.Ind/MP/B48.Bovine.India	P3	98.6	99.3
HQ440223.cow/B85/MP/India/2008.Bovine.India	P3	98.6	99.3
AB971758.JPN/SG33/2010/G27P[3].Sugar glider.Japan	P[3]	82.9	93.3
AB055967.GRV.Goat.Japan	P[3]	82.5	91.2
D13401.CU-1.Canine.Japan	P 13	77.9	87.1
JF712569.ARG/E30/1993/G3P[12].Horse.Argentina	P[12]	74.8	84.3
D13402.Cat97.Feline.Japan	Unknown	76	86.1
D14723.FRV64.Feline.Japan	Unknown	77.2	88.1
D14725.K9.Canine.Japan	P 13	77.4	88.1
DQ288661.CMH2221.Human.Thailand	P[3]	77.5	87.1
DQ841262.SA11-H96/1958/G3P5B[2].Simian.USA	P5B[2]	73.3	85.4

Table 2: Comparison of the nucleotide and deduced amino acid sequence of VP7 gene of B29 (G3P[3]) strains with G3 genotypes from different geographical regions

Rotavirus VP7 gene Accession No./strain/host/country	Genotype	VP7 gene identity	
		Nucleotide 1	Protein 1
DQ478582.B29.Buffalo.India	G3	100	100.0
EF200549.RUBV3.Bovine.India	G3	99.1	98.7
HQ199897.Bov/India/UKD/PTN/P-970.Bovine.India	G3	99.0	98.7
DQ487203.B31.Bovine.India	G3	98.3	98.1
HQ440224.cow/B85/MP/India/2008.Bovine.India	G3	98.9	98.7
JF720882.Ind/HR/B54.Bovine.India	G3	98.9	98.4
JF689845.Bov/Ind/UKD/09/P9.Bovine.India	G3	98.9	98.4
DQ981477.Erv80.Equine.India	G3	98.6	97.5
AF386914.J63.Bovine.India	G3	97.6	96.3
JX036370.RVA/Horse-wt/ARG/E3198/2008/G3P[3].Equine.Argentina	G3	88.2	94.8
EU636932.RVA/Simian-tc/USA/RRV/1975/G3P[3].Rhesus.USA	G3	87.3	93.9
DQ981479.Erv105.Equine.India	G3	87.0	93.9
EU791924.CMH079/05.Human.Thailand	G3	86.9	93.9
HQ661117.RVA/Human-tc/ITA/PA260-97/1997/G3P[3].Human.Italy	G3	86.6	94.2
AY707792.CMH222.Human.Thailand	G3	86.6	93.6
KJ639017.RVA/Human/JPN/S13-30/2013/G3P[4].Human.Japan	G3	86.5	94.2
AF271090.RV52/96.Canine.Italy	G3	86.5	93.9
AB046466.JE75.Equine.Japan	G3	86.5	92.0
KJ639028.RVA/Human/JPN/S13-45/2013/G3P[4].Human.Japan	G3	86.4	93.9
HQ661128.RVA/Dog-tc/ITA/RV52-96/1996/G3P[3].Canine.Italy	G3	86.4	93.9
KM454497.H2.Equine.United Kingdom	G3	86.0	92.0
AY740736.RVA/Human-wt/BEL/B4106/2000/G3P [14].Human.Belgium	G3	84.3	93.3
AB792651.RVA/Cat-tc/JPN/FRV64/1989/G3P[3].Feline.Japan	G3	84.2	93.9

2015). Rotavirus infection in calves is more common in 5-10 days of age. Infected calves used to excrete rotavirus in their faces up to the 6-8 weeks of age. There are number of assays for rotavirus diagnosis such as RNA-PAGE, dot blot hybridization using radio labeled c-DNA and non radiolabel probes and RT-PCR, multiplex real time RT-PCR (Minakshi *et al*., 2005; Minakshi *et al*., 2011; Malik *et al*., 2013; Esona *et al*., 2015), rapid but less sensitive latex agglutination test (Singh and Jhala, 2011) for detection of rotavirus infection in animals and humans.

Various genotypes of rotaviruses are specific to different host species. The common bovine rotavirus strains are G6, G8 and G10. However, recent epidemiological studies in developing countries have shown increasing diversity of bovine rotaviruses (Minakshi *et al*., 2011;

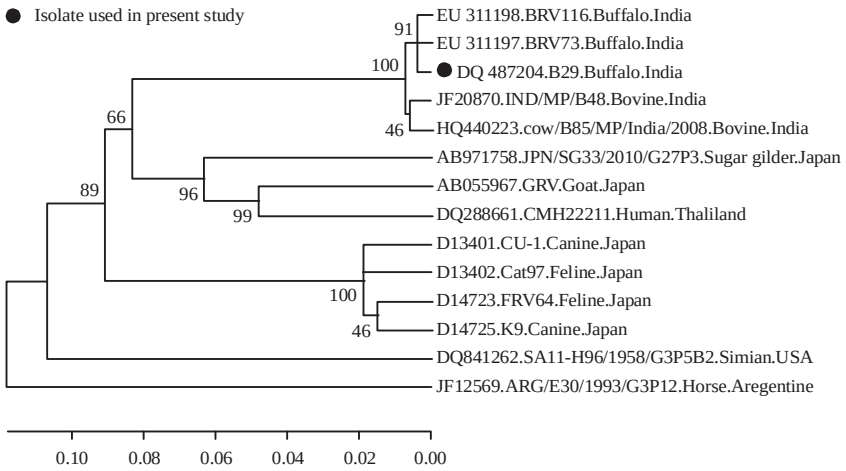

Fig. 3: VP4 gene nucleotide sequence based phylogenetic analysis of B29 isolate along with other global isolates of group A rotavirus

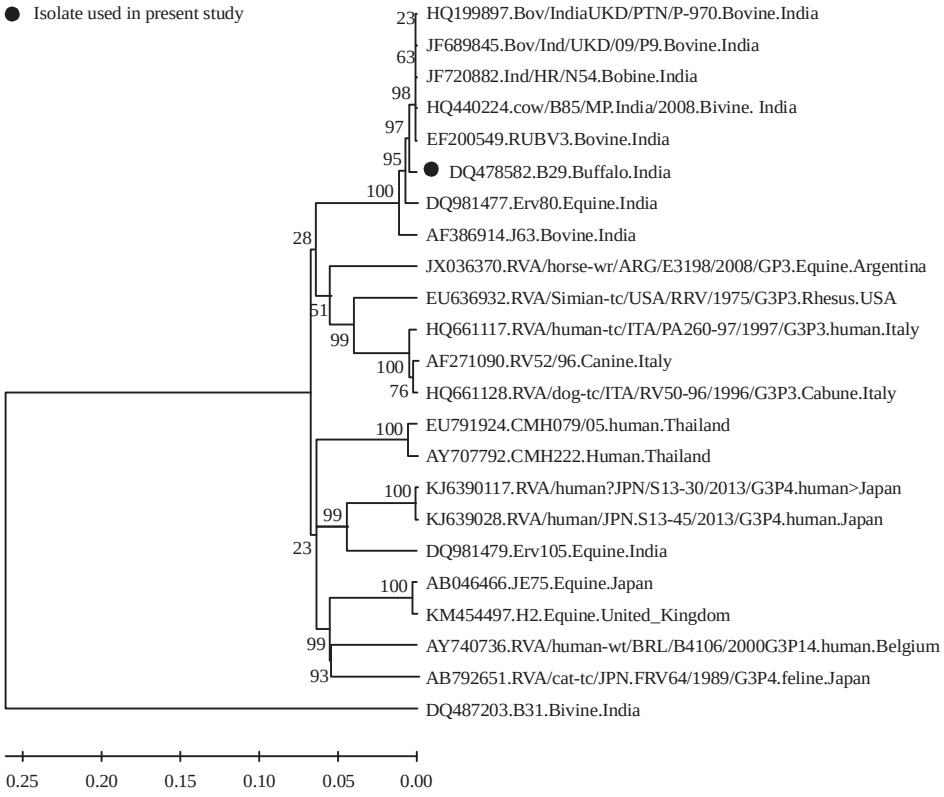

Fig. 4: VP7 gene nucleotide sequence based phylogenetic analysis B29 isolates along with other global isolates of group A rotavirus

Badaracco *et al*., 2012; Malik *et al*., 2013, 2014). In addition, unusual G types, P types and G-P combinations have also been reported (Manuja *et al*., 2008). Accordingly, introduction of reassortant and interspecies transmission of rotavirus gene alleles may have occurred on different occasions.

Out of 85 fecal samples of buffalo calves, 11 (12.94%) were detected positive for BoRVA by RNA-PAGE. All the positive samples belonged to long electropherotype of GAR. The RT-PCR assay has been proved as the most useful assay for diagnosis of common rotavirus types with higher sensitivity over RNA-PAGE (Minakshi *et al*., 2015). The RT-PCR based on VP7 gene for G-typing and VP4 gene for P typing (Manuja *et al*., 2008) has been in wide use. The RT-PCR of B29 isolate showed specific amplification of VP4 and VP7 gene segments as evidenced by 864 and 1062 bp PCR amplicon in agarose gel electrophoresis.

However, other isolates did not produce any amplification with primers specific to VP4 and VP7 genes. This might be due to the presence of inhibitory substances in the fecal samples or mismatches in primer binding sites (Manuja *et al*., 2008).

The multiple sequence alignment using Bioedit 7.2.5 revealed that VP4 gene sequences of B29 isolate showed nucleotide/amino acid (nt/aa) sequence identity of 77.5-99.3/87.1-99.3% with P[3] genotype of group A rotavirus from buffalo and other species originated from different geographical region of world (Table 1). Similarly, VP7 gene showed a maximum nucleotide identity of 84.2-99.1/92-98.7% nt/aa identity with G3 genotype of group A rotaviruses from different parts of the world (Table 2). Thus, the B29 isolate was assigned genotype as G3P[3]. Although, previous study have shown the presence of G3P[3] genotype of rotavirus from human and several species of animals such as Human, cow, dog, equine, cat, monkey and goat (Ghosh *et al*., 2007; Lee *et al*., 2003; Mino *et al*., 2013; Matthijnssens *et al*., 2011; Gauchan *et al*., 2015) but this was the first report of G3P[3] genotype of group A rotavirus from buffalo calf from this part of Haryana, India.

Earlier G3 strains have been detected in India and United Kingdom (UK) in bovine (El-Attar *et al*., 2002; Ghosh *et al*., 2007; Malik *et al*., 2012). Age-old traditions, the extensive use of cattle waste as manure and firewood and the close proximity of the majority of the Indian population with cattle appear to have played a facilitating role in the evolution and persistence of a reassortant virulent genotype G3P[3] in buffalo calves in India. This could be the first report of genotype G3P[3] strains in buffalo calf and considering the unique epidemiological scenario in this part of Haryana, India. It is likely that the genotype G3P[3] strains evolved by reassortment in nature between bovine strains and adapted to growth in foals, causing diarrhea. The animal-human or animal-animal reassortant rotavirus strains have also been reported from humans and cattle (Malik *et al*., 2012). The epidemiology of buffalo rotaviruses is still widely unknown. Herd management as well the strict species affinity between cows and buffalo might explain the detection of buffalo rotaviruses with G6, G8 or G10 associated with P6[1], P7[5] or P8[11] specificities in India and abroad (Malik *et al*., 2012, 2013, 2014; Minakshi *et al*., 2015). Thus, it will be of great interest to understand the origin of the B29 strain of genotype G3P[3]. Similar case has been reported in a surveillance study conducted in Eastern India where G3P[3] genotype was detected in diarrheic cow calf (Ghosh *et al*., 2007).

Moreover, the phylogenetic analysis of VP4 gene revealed that B29 isolate cluster more close with P[3] genotype of buffalorota viruses, BRV116 (Accession No. EU311198) and BRV73 isolate (Accession No. EU311197) (Fig. 3) (Manuja *et al*., 2008). The B29 isolate was also found closer to P[3] genotype of bovine origin from India, Ind/MP/B48 (Accession No. JF720870) and cow/B85/MP/India/2008 (Accession No. HQ440223). Moreover, the B29 isolate was found also closer to several group A rotaviruses originated from goat (Accession No. AB055967), canine (Accession No. D13401 and D14725.K9), Feline (Accession No. D13402 and D14723). Similarly, VP7 gene based phylogenetic analysis showed close relation of B29 isolate to several other G3 genotype

of bovine origin, J63 isolate and RUBV3 (Ghosh *et al*., 2007), Bov/India/UKD/PTN/P-970 (Malik *et al*., 2012), Bov/Ind/MP/10/MF10 (Accession No. JF689835), cow/B85/MP/India/2008 (Accession No. HQ440224), Ind/HR/B54 (Accession No. JF720882) and Bov/Ind/UKD/09/P9 (Accession No. JF689845) (Fig. 4). It is also closer to an equine isolate Erv80 (Accession No. DQ981477).

Strains that are reassortant between animal-human or animal-animal strains have also been reported for both humans and cattle in India (Varghese *et al*., 2006; Malik *et al*., 2012). Cross-species transmission of rotaviruses has been documented and represents 1 mechanism for genetic diversity of rotaviruses. The similarity of B29 to caprine, canine and feline strains suggests that the flux of genetic material between animal and animal rotaviruses under natural conditions might be more common than expected. Since the VP4 sequence of the B29 strain is located in a feline and caprine cluster of the P[3] phylogenetic tree, it is likely that the infection was the result of feline to bovine or canine to bovine or caprine to bovine transmissions, rather than bovine to human interspecies transmission event. The discovery and surveillance of novel bovine and non-bovine rotavirus G or P types or of novel G/P combinations is essential for the design of future rotavirus vaccines and for our understanding of rotavirus diversity and evolution.

CONCLUSION

Rotaviruses are likely to spread through different routes in developed and developing countries. Evidence for genetic reassortment between human and animal rotaviruses has been obtained from animal species, cows, buffalo and pigs, which are in close contact with humans. Extensive surveillance of rotaviruses in humans as well as animals is therefore warranted. In developing countries, people and domestic farm animals, especially domestic pigs, buffalos, cows, sheep and goat live in close proximity. The identification of a buffalo G3P[3] rotavirus supports a dynamic interaction between animal-animal and animal-human rotaviruses. In this regard, further investigations are required to elucidate whether the B29 strain represents a natural reassortment between buffalo and caprine or feline viruses and is able to stabilize and spread successfully in bovine. Simultaneous surveillance of animal and human rotavirus infections is therefore, of paramount importance for studying the evolution of these viruses.

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