Introduction

Rotavirus (RV) is recognized as the major viral etiological agent causing diarrhea in neonates of all species including animals, human and birds. It accounts for higher mortality in young animals and thus causes severe impediment in livestock sector growth. Rotavirus infection encountered in a herd is often difficult to manage because of the large number of other potential enteropathogens involvement, differences in individual animal’s immunity within the herd, population dynamics, environmental stresses, nutritional status, and difficulty in establishing an early etiological diagnosis.

Bovine rotaviruses (BoRVs) have been first described, characterized and confirmed in 1969 as a cause of diarrhea in calves (Mebus et al., 1969).

Rotavirus belongs to the genus Reoviridae. Its genome comprises of 11 segments of double stranded (ds) RNA, which encodes six structural (VP1-4, 6 and 7) and six non-structural proteins (NSP1-6) (Estes and Kapikian, 2006). Three out of the six structural proteins (VP7, VP6 and VP4) have major antigenic determinants and have been used for the basis of RV classification. Further, RVs have been divided into nine groups (A-I) and have been found to be the most common agents of diarrhea in animals and human beings on the basis of their distinct antigenicity of the capsid VP6 protein and dsRNA electropherotype pattern (Estes and Kapikian, 2006; Minakshi et al., 2015). Among all, group A is predominant serogroup in bovines and other mammalian species. Based on the sequences cut off percentage of outer capsid proteins, VP7 and VP4, genotyping systems, i.e. G (glycoprotein) and P (protease sensitivity) are used. Till date, about 32 G (G1-32) types have been reported, of which G6, G8, G10 belong specifically to bovine origin. There are 45 P (P[1-45]) types, among which P1, P5 and P11 are predominant in bovines (Park et al., 2006; Khamrin et al., 2007; Matthijnssens et al., 2011; Malik et al., 2012; Papp et al., 2013; Trojnar et al., 2013).

India has the large livestock population, where the dairy animals (cattle and buffalo) are the backbone of...
the rural economy. Rotaviruses possess a potential threat to this huge livestock sector and cause severe economic losses. Hitherto reports shows presence of RV and genotypes circulating in various parts of the country (Gulati et al., 1999; Wani et al., 2004; Minakshi et al., 2005; Saravananan et al., 2007; Ghosh et al., 2007 and Gaurav et al., 2009; Kaur et al., 2012; Malik et al., 2012; Minakshi et al., 2015; Malik et al., 2016). A constant surveillance network from time to time is required for the genotypes circulating in the Indian bovine population for the development of effective on-site diagnostics, interspecies transmission to determine the zoonotic potential of the BoRV strains and development of appropriate efficacious vaccine candidate. This study has been envisioned to identify the G and P genotypes present in Indian bovine population from different regions of country.

Materials and Methods

Sample collection and processing

A total of 218 diarrheic fecal samples were analysed for the presence of group A RVs (RVA) in bovine population (157 cattle, 61 buffalo) from six states (Uttar Pradesh, Uttarakhand, Madhya Pradesh, Haryana, Himachal Pradesh and Jammu and Kashmir) between 2010-2012. These samples were processed by making 10% fecal suspension (w/v) in phosphate buffered saline (0.01 M, pH 7.4; Sigma, USA), followed by centrifugation at 2000 g for 10 min. and filtration of upper aqueous layer through 0.22 µm syringe filter (MDI, India). The filtrates were archived and stored at -20°C until further use.

Nucleic acid extraction from the faecal suspensions

The viral RNA extraction was done using Tri Reagent RT (Molecular Research Center Inc, USA) following the manufacturer’s protocol. The extracted RNA pellet was eluted in 20 µL of nuclease free water (NFW) and stored at -80°C till further use. The purity of RNA was checked by measuring the OD value at 280 nm in Nanodrop Spectrophotometer.

RNA-Polyacrylamide gel electrophoresis (RNA-PAGE)

The extracted RNA (~500 ng) was mixed with an equal amount of 2X RNA loading buffer (Thermo Scientific, USA), loaded into the wells of the polyacrylamide gel and was electrophoresed at 100 volts in 12% resolving and 5% stacking gel with 1X tris–glycine buffer. Silver staining of gel was done as described by Savita et al. (2008).

RT-PCR for full length amplification

The full length amplification of VP7(1062 bp) and partial length of VP4 (864 bp) was done using 3 µl cDNA, 5 µL 10X PCR buffer, 2 mM MgCl₂, 2 µL DMSO, 10 mM dNTPs, 1.25 U of Taq DNA polymerase (Thermo Scientific, USA), 10 µM of forward and reverse primers using Beg9 (5’GGCTTTAAGAGAGAAAATTCCGTCTGG3’) and End9(R) -(1062-1) 5’GGTCACATCATACATTCTAATCTAAG3’ for VP7 and, Bov4com5(F) (1064-1085) 5’TTATATTTGCC- CGATTACA3’ and Bov4com3(R) (1897-1918) 5’CAACCGCAGCTGATATATCATC3’ for VP4. The total reaction volume was brought to 50 µL with NFW and the reaction was performed at an initial denaturation of 94°C for 4 min followed by 35 cycles of 1 min at 94°C, 2 min at 46°C, 48°C and 2 min at 72°C, and final extension at 72°C for 10 min. The PCR amplicons were gel purified using the Genejet Gel Extraction Kit (Thermo Scientific, USA), cloned into the pGEMT-Easy vector cloning system (Promega, USA) and outsourced for sequencing. The PCR amplified products were resolved on 1% agarose gel in Tris acetate EDTA (TAE) buffer (1X) and visualized under UV transilluminator (Alpha Imager EP, Alpha Inotech, San Leandro, CA, USA).

Multiplex PCR and genotyping

G and P genotyping was carried out by multiplex semi-nested PCR where one of the first round primers was used with the typing primers along with diluted first round amplified product. Briefly, G genotyping was performed using first round reverse primer used by (End9) Gouvea et al. (1990) along with typing primers for G3, G6, G8, G10 following procedures of Malik et al. (2013a), Gouvea et al. (1990), and Iturriza-Gomara et al. (2004). P genotyping was carried out using the forward primer (Bov4 Com5) along with the typing primers for P[1], P[5] and P[11] (Isegawa et al., 1993). A particular genotype for a sample was assigned based on the size of the amplified product on agarose gel electrophoresis.

Sequencing and bioinformatic analysis

Randomly selected five samples for full length VP7 and partial VP4 gene segments were sequenced in an Automated DNA Sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems, USA) using Big-Dye terminator sequencing chemistry. The sequence chromatogram was visualized in BioEdit visualization software. The sequences were annotated, matched in
NCBI database on BLAST analysis and submitted in NCBI GenBank.

The VP7 and VP4 sequences used in this study (n=5) along with other VP7 and VP4 sequences from India and across the world were retrieved from NCBI database. Multiple sequence alignment using the in-built ClustalW algorithm of MEGA6 software (Tamura et al. 2013) by Neighbour Joining (NJ) statistical method using the Maximum Composite Likelihood substitution model with 2000 bootstrap replicates was performed to evaluate the clustering authenticity of the taxa. The nucleotide and deduced amino acid sequence identities of VP7 and VP4 genes were analysed with VP7 and VP4 sequences of RVs from different species and geographical locations published in GenBank database. Furthermore, the RotaC v2.0 web based tool for RVA classification (http://rotac.regatools.be) was used to determine the genotypes. The VP4 (JF742653, JF689840, JF742655, JF720872, JF720876) and VP7 sequences of this study were submitted in NCBI GenBank (HM567170, HM591495, HQ199897, JF720877, JF742652).

Results

Electropherotyping

In viral PAGE analysis RV genome was detected in 67 out of 218 diarrheic fecal samples, wherein all positive samples showed a typical 4: 2: 3: 2 “long” electropherogram migration pattern. In majority of samples, genome segments 7, 8, and 9 co-migrated as a triplet, a typical characteristic of RVA. Region-wise distribution based on PAGE analysis revealed higher incidence of RVs in buffalo calves (39.34%) than cattle calves (27.38%). The percentage of RV positive samples in buffaloes was highest in Haryana (84.21%, 16/19), followed by Uttar Pradesh (43.75%, 7/16), where as only one sample was detected positive from Uttarakhand, while in cattle, the highest positive percentage of samples was from Uttar Pradesh (46.15%, 18/39) followed by Uttarakhand (33.33%, 15/45) and Madhya Pradesh (30.30%).

RT-PCR

Of the total 218 sample (157 cattle and 61 buffalo), 36.23% (50 samples from cattle, 29 samples from buffaloes) samples were found positive for 227bp amplicon of VP6 gene segment, where in highest positive samples were from cattle (51.28%, 20/39) from Uttar Pradesh, followed by Uttarakhand (44%, 20/45) and Madhya Pradesh (30.30%, 10/33). None of the sample from Jammu & Kashmir and Himachal Pradesh was detected positive, whereas in buffaloes from Haryana showed highest positive samples (94.73%, 18/19), followed by Uttarakhand (100%, 1/1) and Uttar Pradesh (62.55%, 10/16). No buffalo sample was detected positive from Madhya Pradesh, Himachal Pradesh and Jammu & Kashmir (Table 1). First-round amplification with generic primers of VP7and VP4 genes produced full-length 1062 bp amplicons for the VP7 gene and partial-length 864 bp products for the VP4 gene in all the 79 samples (50 cattle and 29 buffalo).

Distribution of G and P types

Overall, 42 G type and P type out of 79 positive samples could be characterized. The distribution of the G types in bovines showed occurrence of G3 was in 9 samples (21.42%, 6 in cattle and 3 in buffalo), G6 in 6 (14.28%, 4 in cattle and 2 in buffalo), G10 in 4 samples (9.52%, 2 each in cattle and buffal0), while 23 (54.76%) samples were having mixed G genotypes like G3G6 (11.90%) (3 in cattle and 2 in buffalo), G3G8 (11.90%) (3 in cattle and 2 in buffalo), G3G10 (9.52%) (3 in cattle and 1 in buffalo), G6G10 (7.14%) (2 in cattle and 1 in buffalo), G3G6G8, G3G6G10, G3G6G8G10 (4.76) (1 each in cattle and buffalo) (Table 2A). Distribution of P types showed P[11] in 35 (83.33%) (30 in cattle and 5 in buffalo), mixed P[1]P[11] in 2 (4.76%) (1 each in cattle and buffalo), P[1] in 4 (9.5%) (3 in cattle and 1 in buffalo), while 1 (2.38%) sample was non-typable, shown in Table 2(B).

Table 1. Region-wise distribution of rotavirus infection in bovines detected by RNA-PAGE and VP6 gene based RT-PCR

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Region</th>
<th>No. of samples</th>
<th>RNA-PAGE Positive (%) Prevalence</th>
<th>RT-PCR Positive (%) Prevalence</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Cattle</td>
<td>Buffalo</td>
<td>Cattle</td>
<td>Buffalo</td>
</tr>
<tr>
<td>1</td>
<td>Uttar Pradesh (Bareilly and Meerut)</td>
<td>39</td>
<td>16</td>
<td>18 (46.75)</td>
</tr>
<tr>
<td>2</td>
<td>Uttarakhand (Pantnagar and Mukteswar)</td>
<td>45</td>
<td>1</td>
<td>15 (33.33)</td>
</tr>
<tr>
<td>3</td>
<td>Haryana (Hisar)</td>
<td>0</td>
<td>19</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>4</td>
<td>Jammu &amp; Kashmir (Jammu)</td>
<td>30</td>
<td>21</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>5</td>
<td>Madhya Pradesh (Jabalpur)</td>
<td>33</td>
<td>0</td>
<td>10 (30.30)</td>
</tr>
<tr>
<td>6</td>
<td>Himachal Pradesh (Palampur)</td>
<td>10</td>
<td>4</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>61</td>
<td>43 (27.38)</td>
<td>24 (39.34)</td>
</tr>
</tbody>
</table>
Phylogenetic analysis

In VP7 phylogenetic analysis, 5 bovine Indian isolate sequences from this study showed three monophyletic clades as G3 lineage clade, G6 lineage clade and the G10 lineage clade (Fig. 1). One among the studied sequence (ME8- HM567170 from Meerut, Uttar Pradesh of this study) was clustering with G6 lineage and very close to Indian bovine strain B111-JX442777 from Haryana and G3 isolates of the study viz. (0970-HQ199897, BRV133-JF720877, P14-HQ199897) were clustering in the subclade of G3 lineage sequences closer to (C5-JX442779) and CC156 bovine strains among India and ERV316 from Australia. The studied sequence B72- HM591495 from Jabalpur (Madhya Pradesh) showed genetic relatedness with G10 lineage sequences of Indian origin RUB81 EF200547 and ERV2 DQ981473 and ERV316 strain from Australia (Fig 1). The phylogenetic analysis of VP4 gene sequences from the 5 bovine RVA isolates (Fig. 2) showed that the phylogram form two different clusters of P[1] and P[11] type. VP4 phylogenetic analysis of the five isolates when compared with other rotavirus isolates from India and the rest of the world, confirmed that the isolates 0970-JF42653, P14-JF742655, B-72 JF720872, and 15E-JF689840 belong to the P[1] cluster. While BRV133 was clustering with other isolates that belonged to the P[11] isolates. The isolates P14, 0970, B-72 and 15E were grouped together; which was closely related to caprine isolate G034 G4937880), respectively. Isolate, BRV133, clustered with Indian bovine B108 (JX442775) strain (Fig. 2).

Discussion

Enteric infections due to rotaviruses and other emerging viruses remains a concern for developing countries like India, albeit these possess significant public health concern also (Malik et al., 2011, Kaur et al., 2012, Kaur et al., 2013, Malik et al., 2014). The problem has been aggravated by the lack of awareness among vast majority of the Indian population; where there is close interaction of the human-livestock interface. The present study was undertaken to screen BoRVs from different geographical locations to ascertain genotype dynamics.
and molecular epidemiology of the disease. Some of the studies have been undertaken in India by various research groups to study molecular epidemiology and genotype profiling of RV in bovine population (Chitambar et al., 2011). In this study, the overall prevalence of RV infection in bovines as determined by RNA-PAGE was 30.73%, with higher incidence in buffalo calves (39.34%) than cattle calves (27.38%). All the BoRV samples showed a migration pattern of 4:2:3:2 which is typical of group A rotavirus. The samples showed long electropherotype based on the migration of the 10th and 11th segments. Gulati et al. (1999), Jindal et al. (2000) and Wani et al. (2007) also reported variation in electropherotypes of BoRVs. Malik et al. (2013b) also reported short and long electropherotype while screening samples from Madhya Pradesh. But, Haq (2011) only found long electropherotype in the bovine samples. The highest RV prevalence of 46.6% in bovines has been reported by Khattar and Pandey (1986) from Hisar (Haryana). However, Jhala and Raghavan (1997, Jindal et al. (2000), Manuja et al. (2008; 2010), Niture et al. (2009), Mondal et al. (2011) and Malik et al. (2013b) reported a lower prevalence of BoRVs than the present study. Very low prevalence of 3.3% has been recorded by Jhala and Raghavan (1997) from Karnataka and Udaykar et al. (2013) from Madhya Pradesh. The higher percentage of positive samples was detected from Uttarakhand (100%) and lowest from Uttar Pradesh (43.75%) region, whereas in cattle the highest positive percentage was seen from Uttar Pradesh (46.15%) and lowest from Madhya Pradesh (30.30%). In bovines, no sample from Jammu & Kashmir, and Himachal Pradesh was found positive, on account of not being truly diarrheic. However, Wani et al. (2007) and Beg et al. (2010) reported 18.7% and 15.5% prevalence of BoRVs from Kashmir valley and Sharma et al. (2009) recorded 43% and 30% RV infections in cattle and buffalo calves from Jammu.

RT-PCR has been greatly used for diagnosis of RVs since its first use by Gouvea et al. (1990) as it is very sensitive and rapid. Most of the conventional PCRs have targeted the conserved VP6 gene segment to identify group specific rotavirus. In case of bovines, a total of 79 samples (50 cattle and 29 buffalo) were found positive by RT-PCR with higher incidence in buffalo calves (47.54%) than cattle calves (31.84%). Highest percentage of RV positive samples in buffalo calves were detected from Uttarakhand (100%) and lowest from Uttar Pradesh (62.5%), whereas in cattle highest positive samples were detected from Uttar Pradesh (51.28%) and Madhya Pradesh (30.30%) the lowest. No buffalo sample was found positive by RNA-PAGE and RT-PCR from Madhya Pradesh, Jammu & Kashmir and Himachal Pradesh.

Lack of easy availability of monoclonal antibodies especially against the P serotypes, P and G serotyping were almost replaced by P and G genotyping based on the nucleic acid sequences. The P and G genotypes are

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. out of 42</th>
<th>No. in cattle</th>
<th>No. in buffalo</th>
<th>Prevalence (%)</th>
</tr>
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<tbody>
<tr>
<td>G3</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>21.42</td>
</tr>
<tr>
<td>G6</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>14.28</td>
</tr>
<tr>
<td>G10</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>9.52</td>
</tr>
<tr>
<td>G3G6</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>11.90</td>
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<td>G3G8</td>
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<td>11.90</td>
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<tr>
<td>G3G10</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>9.52</td>
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<tr>
<td>G6G10</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>7.14</td>
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<tr>
<td>G3G6G8</td>
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<td>4.76</td>
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<tr>
<td>G3G6G8G10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4.76</td>
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<tr>
<td>Total</td>
<td>42</td>
<td>26</td>
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<table>
<thead>
<tr>
<th>P type</th>
<th>No. out of 42</th>
<th>No. in cattle</th>
<th>No. in buffalo</th>
<th>Prevalence (%)</th>
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<tbody>
<tr>
<td>P[11]</td>
<td>35</td>
<td>30</td>
<td>5</td>
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<td>9.5</td>
</tr>
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<tr>
<td>Total</td>
<td>42</td>
<td>34</td>
<td>7</td>
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</table>
mostly determined by nucleic acid hybridization assay, RT-PCR assay and nucleotide sequence analysis (Estes and Kapikian, 2007). Quantitative detection of group A rotavirus in humans and animals by real-time RT-PCR was shown an effective way for its confirmatory diagnosis (Kaur et al., 2013).

In the present study, 42 samples from bovines (cattle and buffalo) from six states were genotyped (which were also positive for VP6 gene based PCR) by multiplex semi-nested PCR assay. The G genotyping PCR assay showed G3 as the most predominant G type in bovines and found in 9 isolates (21.42%, 6 in cattle and 3 in buffalo), followed by G10 in 4 (9.52%, 2 each in cattle and buffalo), G6 in 6 (14.28%, 4 in cattle and 2 in buffalo) and the mixed genotypes G3G6 in 5 (11.90%, 3 in cattle 2 in buffalo), G3G8 (11.90%, 3 in cattle and 2 in buffalo), G3G10 in 4 (9.52%, 3 in cattle and 2 in buffalo), G6G10 in 3 (7.14%, 2 in cattle and 1 in buffalo), G3G6G8, G3G6G10, G3G6GBG10, 2 in each (4.76%). In contrast to this study, the earlier reports detected G10 as the most predominant G-type in India (Varshney et al., 2002; Saravanan et al., 2006; Bardhan et al., 2007). Gulati et al. (1999) detected G10 (83%) as the most predominant G type in India followed by G6 (6%). Among P types P[11] is most predominant type found in 35 samples (83.33%) (30 in cattle and 5 in buffalo), P[1] in 4 (9.5%) (3 in cattle and 1 in buffalo) mixed type P[1]P[11] in 2 (1 each in cattle and buffalo) and one sample remained untypable.

In recent years, there are increasing reports of presence of mixed genotypes in the samples. The primers used in the genotyping assays have been used for many years now; there may be possibility of cross reactivity and non-specific binding of the primers and amplification of multiple genotypes (Malik et al. 2013a).

Phylogenetic analysis revealed individual monophyletic clade for the respective genotypic clusters. The isolates from the present study clustered with other isolates from India and shared common ancestors confirming the authenticity of the taxa analysed in the phylogenetic tree. The important information derived from the phylogenetic tree is that it enlightens the vaccine to be recommended for BoRV in the country.

In conclusion, the findings address some of the concern regarding the genotype dynamics and disease epidemiology of the circulating rotavirus in the bovine in Northern and central India. The genotype determination is paramount to prepare a vaccine formulations which contains the most prevalent genotype in particular niche for effective control of the rotavirus induced gastroenteritis. The vaccine is not only important in control of neonatal mortality in humans, but may also control calf mortality to improve livestock production enterprise in this country.

Acknowledgements

All the authors acknowledge the support from their respective institutions and universities.

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