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Short communications

Determination of bovine rotavirus genotypes (G and P) circulating in Uttarakhand (2010–2012) by polymerase chain reaction

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Rotavirus (RV), a prime cause of enteric infections in animals and humans (Kapikian *et al*. 2001, Parashar *et al*. 2006), possess a segmented RNA genome and is thus more prone to mutations leading to advent of newer genotypes (Estes and Kapikian 2007). For genotyping a dual classification system is used which classifies glycosylated VP7 protein gene (G types) into 27G genotypes and outer capsid protease-sensitive VP4 protein gene (P types) into 35P genotypes (Matthijnssens *et al*. 2011). In India, predominantly rotavirus G6, G8 and G10 genotypes are reported (Gulati *et al*. 1999, Wani *et al*. 2004, Bhat *et al*. 2005, Saravanan *et al*. 2006, Manuja *et al*. 2008, Minakshi *et al*. 2009, Singh and Jhala 2011, Malik *et al*. 2013). Moreover, recently rotavirus isolates of G3 genotype specificity were identified as emerging genotypes in bovine population (Varshney *et al*. 2002, Ghosh *et al*. 2007, Malik *et al*. 2012). Amid the P genotypes, P[1] and P[11] are most prevalent genotypes (Gulati *et al*. 1999, Bhat *et al*. 2005, Saravanan *et al*. 2006, Manuja *et al*. 2008, Minakshi *et al*. 2009). Exploring the knowledge on circulating genotypes in susceptible animal population from different geographical locations of India is essential to understand the virus evolution, interspecies transmission, especially when we are in a phase of launching the prophylactic vaccine for bovine rotaviruses. Though, epidemiological data on the prevalence of rotavirus is available for many parts of the country, still the information on bovine RVs from Uttarakhand state is sporadic, in particular from high altitude areas. In view of which, the present work was started to check the existence and circulating genotypes in bovine RV isolates from high altitude areas of the state.

Faecal samples (87) were collected from diarrheic cattle calves below 6 months of age from organized crossbred dairy farm at Mukteswar, Nainital (n=20) and Tunja, Bajwar, Dharanaula, Bhaishori villages of Almora districts (n=67) of Uttarakhand during winters of 2010–2012. All the 67 samples from Almora were from hill cattle calves. Faecal suspension (10% w/v) was prepared in 0.2 mM phosphate buffer saline (PBS) (pH 7.4) and centrifuged at 2,000 g for 20 min and the upper aqueous layer was then filtered through 0.22 µm syringe filters and stored at –20°C till its further use. The viral RNA was extracted from 500 µl of 10% faecal suspension in PBS using an equal volume of TriReagent-LS. RNA was eluted in 25 µl nuclease free water (NFW) and assessed qualitatively and quantitatively using nanodrop spectrophotometer. At first, RNA from all faecal samples were electrophoresed in polyacrylamide gels and impregnated with silver nitrate following the procedure described previously (Malik *et al*. 2012).

Reverse-transcription of viral RNA was performed with 0.2 µg/µl random hexamer. Initially, 50–100 ng of viral RNA, 0.1 µg random hexamer, and 2 µl of dimethyl sulphoxide (DMSO) were added to thin wall 0.2 mL PCR tube followed by incubation of the reaction mixture at 95°C for 5 min for denaturing the RNA strands. The mixture was immediately snap chilled on ice followed by the addition of 4 µl of 5X RT buffer, 2 µl of 10 mM dNTPs, 40 U RNase inhibitor and 200 U MMLV-RT to make a final volume of 25 µl in NFW and it was kept at 37°C for 90 min. The RT enzyme was denatured at 80°C for 5 min and first round of PCR was carried out for the amplification of full length VP7 gene (1062 bp) and partial length VP4 gene (864 bp) following the optimized conditions and primers as described earlier (Kusumakar *et al*. 2010, Malik *et al*. 2012, 2013). The second round PCR was performed for genotyping of RV isolates for predominating bovine genotypes reported in India, viz. G3, G5, G6, G8, G10 and P[1], P[5] and P[11] using 1:100 dilution of first round PCR amplicons as template and reported primers. The amplicons were resolved on 2% agarose gel after electrophoresis at 100 V for 1 h in 1X TAE buffer with 0.5 µg/ml ethidium bromide and viewed under gel documentation system-ImageMaster® VDS.

Previous reports are suggestive of regular temporal shifts

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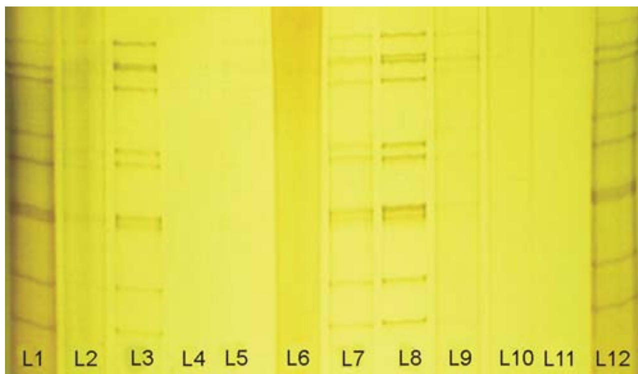

Fig. 1. RNA-polyacrylamide gel electrophoresis of diarrhoeic faecal samples for detection of bovine rotavirus. The genomic segments migration pattern of 4:2:3:2, typical of group A rotavirus was observed, where segments 7, 8, and 9 moved in a triplet. Lane (1-3; 7-9 and 11) showing long electropherotypes based on relative migration of 10th and 11th segments.

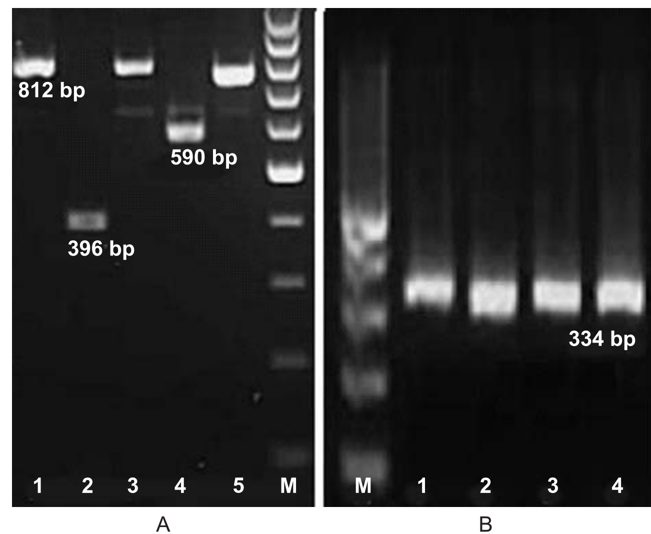

Fig. 2. PCR amplification of VP7 and VP4 genes using cocktail of primers for genotyping of group A rotaviruses. Specific amplicon size (812 bp, 590 bp, 396 bp and 334 bp for G3, G8, G10 and P[11], respectively) were visualized in 1.5% ethidium bromide stained agarose gel. A. Lanes: 1–2: G3G10 genotypic combination (M10 isolate); 3–4: G3G8 genotype combination (M3 isolate); 5: G3 genotype (M1 isolate) and M: 100 bp DNA ladder (Fermentas). B. Lanes: M: 100 bp DNA ladder (Fermentas); 1–4: P[11] genotype with specific amplicon of 334 bp.

in the circulation of G and P genotypes of rotavirus in different locations (Gulati *et al*. 1999, Ghosh *et al*. 2008, Manuja *et al*. 2008, Malik *et al*. 2012). Though studies were undertaken to define specific genotypes of RVs circulating among cattle and buffalo calves for their eventual control in different parts of India, still several parts remain unscathed. The rate of prevalence of group A rotaviruses in bovine varied between the regions and reports from different parts of the country showed this in the range of 4.61% to 24.03% in North India (Minakshi *et al*. 2005, 2009, Manuja *et al*. 2008, Basera *et al*. 2010, Dash *et al*. 2011, Rai *et al*. 2011), 12.5% to 16.9% in western India (Niture *et al*. 2011, Singh and Jhala 2011), 8.7% to 23.6% in central India (Kusumakar *et al*. 2010, Malik *et al*. 2012), and 22.6% to 23.15% in eastern India (Ghosh *et al*. 2008, Nataraju *et al*. 2009). In this study, association of RV with clinical cases of diarrhea was established for the first time and genotypic diversity of RVs circulating in bovine population of hill areas of Uttarakhand region was confirmed. During the study a prevalence of 8.04% (7/87) was noticed in Mukteswar, Nainital while none of the samples from Almora district was detected positive for RV infection either in RNA-PAGE or RT-PCR.

In RNA-PAGE all the positive samples (7) exhibited 4:2:3:2 migration pattern of its genomic segments, where segments 7, 8, 9 moved in a triplet, typical of group A rotaviruses (Fig. 1). Basera *et al*. (2010) reported prevalence of RVs in the range of 8.7 to 10.15% in bovine population in *tarai* areas of Uttarakhand. Further, all the PAGE positive samples were genotyped (G and P) using the multiplex PCR assay. The nested PCR revealed the existence of a single genotype in 3 samples (M1, M7 and M8) while mixed infection with more than 1 genotype was seen in 4 samples (M3, M5, M9, M10) (Fig. 2 A, B). M1 and M8 samples were genotyped as G3, while the sample M7 exhibited G10 genotype specificity. Out of the 4 samples showing mixed infection, 3 samples (M3, M5, M9) revealed dual G3G8 genotypes and remaining 1 (M10) possessed the G3G10 genotype combination. All the 7 positive samples exhibited a single P[11] genotype with amplification of 334 bp specific amplicon (Fig. 2B). Overall, RVs existed in 4 genotypic combinations, i.e. G3P[11], G10P[11], G3G8P[11] and G3G10P[11] in bovine population of the region. Occurrence of more than 1 genotypes of RV in a single host reassures that reassortment events might be more frequent (Minakshi *et al*. 2009). In the present study we noted mixed infection in 4 out of 7 positive bovine RV samples. Similar results were perceived earlier in which following genotypic combinations were detected, viz. G6G10 (Minakshi *et al*. 2009); G8G10, G6G10 (Sharma *et al*. 2009); G8G10 (Beg *et al*. 2010) and G3G8, G3G10 (Malik *et al*. 2012). The mixed genotypes reported in our previous studies occurred at the prevalence rate of G3G10 (55%), G3G8 (45%) and P[1]P[11] (6.1%) in different ecoregions of India (Malik *et al*. 2012). Genotyping results from this region confirms the adaptability of G3 genotype as a more common genotype in bovines, which was ignored for a longtime before its first report from central India in 2002 (Varshney *et al*. 2002).

In conclusion, results of present study confirmed the adaptability of G3 genotype as a more common genotype in bovines which either exist singly or in combination with G8/G10. The similarity trend matches with our previous report from tarai area of the state (Malik *et al.* 2012). However,

still studies are required to characterize the RV isolates recovered from the area at genomic level to see the evolution of these emerging rotavirus isolates which are dominating in bovine population of the state.

SUMMARY

The study describes the prevailing bovine rotavirus (RV) genotypes in high altitude areas of Uttarakhand state of India. The results confirmed the dominance of G3 genotype, which is responsible for diarrhea either alone or together with G8 and G10 genotypes. The P typing results on the other hand showed prevalence of only P[11] genotype in all bovine rotavirus isolates, confirming that least diversity exists in the major outer capsid protein gene (VP4) of bovines. The rotavirus existed in 4 types of genotypic combinations i.e. G3P[11], G10P[11], G3G8P[11] and G3G10P[11] in cattle calves of the region.

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