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Characterization of buffalo, poultry and human rotaviruses in Western India

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ABSTRACT

The present investigation describes the detection of rotaviruses among buffalo, poultry and humans. A total of 83 buffalo, 54 poultry faecal and 81 human stool samples were screened by RNA-PAGE for the presence of rotavirus, of which 6 buffalo (12.5%), 4 poultry (7.84%) and 16 human (20.25%) samples were detected positive. All the buffalo and human rotavirus PAGE positive samples depicted a characteristic group A rotavirus migration pattern (4:2:3:2) of RNA segments, whereas a group D like migration pattern (5:2:2:2) was observed in poultry samples. Out of 26 rotavirus positive samples, all the buffalo (23.07%) rotavirus positive samples showed a long electropherotype, while a short migration pattern was revealed in avian samples (15.38%). Among human samples, the majority of the samples (60%) were long electropherotypes followed by short electropherotypes (40%). Hence, a total of 10 different electropherotypes were identified among the three host species, of which four belonged to buffaloes, one poultry, five human, and one human sample was of mixed infection. RNA-PAGE positive samples were further confirmed for the presence of rotavirus by VP4 & VP7 gene specific RT-PCR. The partial length amplification of the VP4 gene of buffalo and human rotaviruses yielded 856bp and 876bp products, respectively. The VP7 gene of both buffalo and human rotaviruses yielded 1062 bp products. On G genotyping of RT-PCR positive buffalo rotavirus samples, none of the samples revealed any G6, G8, and G10 type specific products. However, P genotyping of the same samples revealed the P[11] genotype in only 2 (33.33%) of the buffalo samples. Among the human rotaviruses, 6 (37.5%) were typed as G[1] genotype but remained untypeable for P genotypes. The VP4 and VP7 genes of avian rotavirus could not be amplified. However, the VP6 gene of all avian rotavirus yielded an amplicon of 493bp . The study reports probably the first ever detection of the group D avian rotavirus in Western India.

Key words: rotaviruses, buffalo, poultry, humans, India

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Introduction

Rotaviruses are recognized as the major cause of severe gastroenteritis in infants, young children, and the young of a variety of mammalian and avian species (KAPIKIAN, 1996). Rotaviruses are members of the *Reoviridae* family within the *Rotavirus* genus, having a segmented genome, which can be separated into discrete segments by RNA-polyacrylamide gel electrophoresis (RNA-PAGE). The segmented nature of the viral genome allows reassortment in a mixed infection in natural conditions, leading to the emergence of new serotypes of the virus. The viral genome is composed of 11 double stranded RNA segments surrounded by three protein shells, i.e. core, inner capsid and outer capsid. Seven groups of rotaviruses (A to G) have been described so far, of which group A rotaviruses have been found to be the most common agents of diarrhoea in humans, animals and avian species (HOSHINO and KAPIKIAN, 1996). The rotavirus has two distinct antigenic specificities: subgroup and serotype. The subgroup specificity is present on the inner capsid protein VP6, encoded by gene segment 6. The serotype specificity is determined by the VP7 antigen, encoded by gene segment 7, 8 or 9 depending on the virus strain and the VP4 antigen encoded by gene segment 4 (ESTES and COHEN, 1989). Viral surface proteins, VP4, a protease cleaved, or P, protein, and VP7, a glycoprotein, or G protein, are the targets of neutralizing antibodies. These proteins may mediate protection induced by a rotavirus vaccine and form the basis for the dual molecular classification scheme that indicates the G and P genotypes of the viruses (MARTELLA et al., 2006; RAHMAN et al., 2005).

Literature on combined studies of the prevalence of rotavirus associated diarrhoea in buffalo, avian and human species is limited in Western India. The present study was aimed at finding out the presence and genomic diversity of field rotaviruses of different host species, using RNA-PAGE and VP4 & VP7 gene based genotyping using reverse transcription polymerase chain reaction (RT-PCR) assays.

Materials and methods

Sample collection. A total of eighty-three samples from buffalo calves, fifty-four of poultry and eighty-one stool samples from children suffering from diarrhoea were collected over the period from October 2008 to March 2009 from various parts of Maharashtra State, India. A 10% faecal suspension was prepared in phosphate buffer saline (pH 7.2), mixed and centrifuged at 10000 x g for 15 min to remove coarse particles. The clear suspension was transferred to fresh tubes and stored at -20 ºC.

Rotavirus reference strains. Reference strains of Group A buffalo rotavirus of genotype G6P[1], group A avian and human rotavirus of G1P[8] provided by the Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of

Veterinary & Animal Sciences (Erstwhile CCS HAU), Hisar, Haryana, India were used as known positive controls.

Extraction of viral nucleic acid. The extraction of viral RNA from faecal suspensions of buffalo, avian and human species was done as described earlier (MINAKSHI et al., 2004) with minor modifications. The RNA pellet was air dried, dissolved in a RNA-PAGE sample buffer and stored at -20 °C. RNA of all the PAGE positive samples was also extracted by guanidinium isothiocyanate lysis method (CHOMOCZYNSKI and SACCHI, 1987) that was further used for RT-PCR amplifications of viral genes.

RNA-polyacrylamide gel electrophoresis. The segmented RNA genome of the rotavirus was analyzed by RNA-PAGE using the discontinuous buffer system without sodium dodecyl sulphate (SDS) as described by LAEMMLI (1970). RNA pellets dissolved in the RNA sample buffer were resolved in 5% stacking and 7.5% resolving gels at a constant voltage of 100 V until the dye just came out of the gel.

Silver staining of the gel. The gel was visualized after staining by the silver nitrate method of SVENSSON et al. (1986). The stained gel was photographed and stored in 10% ethanol for further studies.

Synthesis of cDNA by Reverse transcription and amplification. The cDNA was synthesized and amplified using RT-PCR with gene specific primers in a thermocycler (i-cycler, Bio-RAD, USA).

Buffalo. The RNA was transcribed into cDNA using a reverse transcriptase enzyme and amplified as described earlier for VP4 gene specific (MINAKSHI et al., 2001) and VP7 gene specific primers (MINAKSHI and PANDEY, 2002) using i-cycler (Bio-RAD, USA).

Avian. RT-PCR based on VP4, VP6 and VP7 gene was carried out as previously described by ITO et al. (1995).

Human. The RNA was transcribed into cDNA using the reverse transcriptase enzyme and further amplified using VP4 & VP7 gene specific primers as described earlier by GENTSCH et al. (1992) and TANIGUCHI et al. (1992) respectively.

All the amplified PCR products of buffalo, poultry and humans were analyzed by agarose gel electrophoresis and visualized under a UV transilluminator and photographed using a gel documentation system (Biovis Gel, USA).

Genotyping. The visible PCR products obtained as described above were diluted to 1:100 and used as the template for G (VP7) and P (VP4) genotyping by semi-nested PCR.

Buffalo. Genotyping of rotavirus isolates using G6, G8 and G10 and P[1], P[5] and P[11] typing primers was carried out following the method described earlier of MINAKSHI et al. (2005).

Human. Genotyping of rotavirus isolates using G1, G2, G3, G4, G8 and G9 and P[4], P[6], P[8] and P[10] primers was carried out as previously described by TANIGUCHI et al. (1992) and GENTSCH et al. (1992) respectively.

All the amplified products were visualized in 1% ethidium bromide stained agarose gel using a gel documentation system & photographed. G and P genotypes were assigned according to the size of PCR products of respective types.

Results

A total of 218 samples consisting of 83 buffalo (38.07%), 54 poultry (24.77%) faecal and 81 human stool samples (37.16%) were tested. On RNA-PAGE analysis, 6 buffalo (7.22% i.e. 6/83), 4 poultry (7.40%) and 16 human (19.75%) samples were positive for the presence of rotaviruses. All PAGE positive samples revealed eleven discrete RNA segments in buffalo, poultry and human viral genomes. The RNA migration pattern of 4:2:3:2 was observed in both buffalo and human samples (Figs. 1, 3), characteristic of group A rotaviruses. Poultry rotavirus showed a migration pattern of 5:2:2:2, which is characteristic of the group D avian rotavirus (Fig. 2). Out of 26 rotavirus positive samples from the three species, all 6 buffalo samples showed long electropherotypes whereas 10 (60%) human rotavirus samples showed a long RNA migration pattern and 6 (40%) had short electropherotypes. In this study, a total of 10 different electropherotypes were identified among the three different host species. Four types of electropherotypes were observed in buffalo, one in poultry and five in humans, and one human sample had a mixed infection with extra genomic segments (Fig. 4).

All the PAGE positive buffalo rotavirus samples were subjected to G and P genotyping. A PCR product of 856bp size was observed in all six PAGE positive samples (Fig. 5). On nested multiplex VP4 gene based P genotyping, two (33.33%) of six PAGE positive samples were of P [11] type (Fig. 6). Remaining rotaviruses could not be typed suggesting the presence of new P types other than P [1] and P [5] for which the typing primers were used in the study. Similarly, all the 6 samples yielded VP7 gene specific full length product of 1062bp (Fig. 7), but none of the samples subjected to G genotyping revealed any G6, G8, and G10 type specific products. Reference strain of buffalo rotavirus G6P[1] could be typed using the same set of typing primers.

As expected, when the human group A rotavirus samples were subjected to partial length amplification of the VP4 gene, they yielded a product of 876bp in all 16 samples (Fig. 8). Subsequently, when all the 16 samples were subjected to P genotyping, none of the samples could be typed into any P4, P6, P8, P10 types.

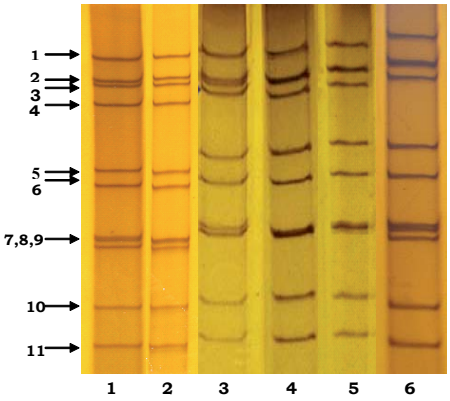

Fig. 1. Electrophoretic migration pattern of buffalo rotavirus isolates. Lane 1-6: Bovine rotavirus field isolates. The numbers on the left side indicate 1-11 RNA segments.

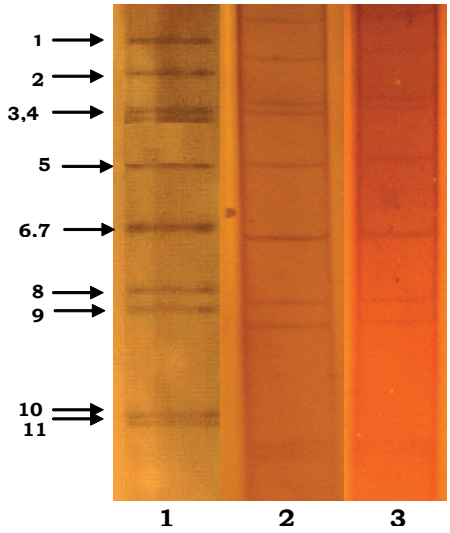

Fig. 2. Electrophoretic migration pattern of poultry rotavirus isolates. Lane 1-3: Avian rotavirus field isolates. The numbers on the left side indicate 1-11 RNA segments.

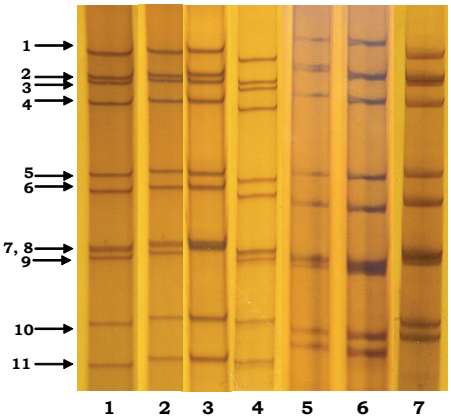

Fig. 3. Electrophoretic migration pattern of human rotavirus isolates. Lane 1-4: Long electropherotype. Lane 5-7: Short electropherotype. The numbers on the left side indicate 1-11 RNA segments.

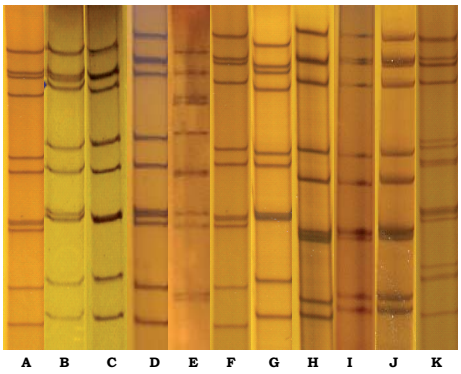

Fig. 4. Types of electrophoretic migration patterns of buffalo, poultry and human rotavirus isolates. Lanes A-D: Buffalo group A rotavirus. Lane E: Poultry group D rotavirus. Lanes F-J: Human group A rotavirus. Lane K: Mixed infection of group A human rotavirus.

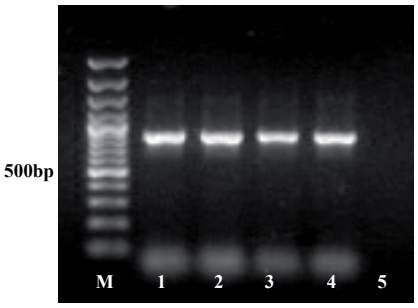

Fig. 5. Partial length amplification of VP4 gene of buffalo rotavirus. Lanes: M: 100bp DNA ladder (Fermentas). 1-4: Bovine rotavirus field isolates. 5: Negative control. Numbers on the side depict size of PCR amplicon in base pairs.

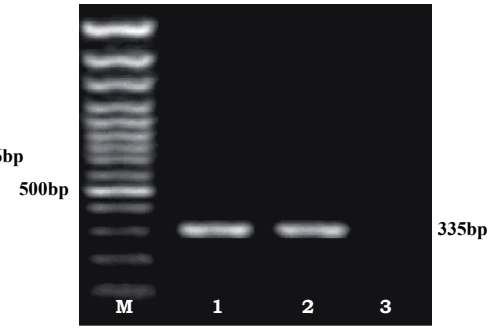

Fig. 6. Bovine P[11] genotype by semi-nested PCR. Lanes: M: 100bp DNA ladder, 1-2: Bovine rotavirus field isolates. 3: Negative control. Numbers on the side depict size of PCR amplicon in base pairs.

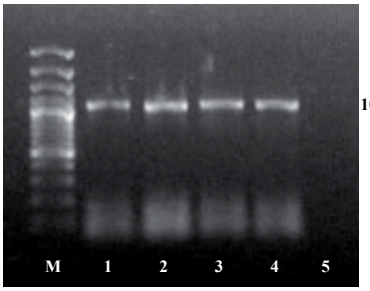

Fig. 7. Full length amplification of VP7 gene of buffalo rotavirus. Lanes: M: 100bp DNA ladder. 1-4: Bovine rotavirus field isolates. 5: Negative control. Numbers on the side depict size of PCR amplicon in base pairs.

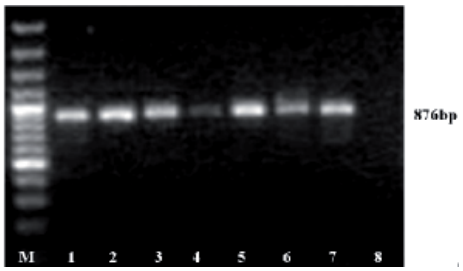

Fig. 8. Partial length amplification of VP4 gene of human rotavirus. Lane M: 100bp DNA ladder. 1-7: Rotavirus field isolates. 8: Negative control. Numbers on the side depict size of PCR amplicon in base pairs.

In the first amplification, the expected PCR product of 1062bp of full length amplification of the VP7 gene was seen in all sixteen RNA-PAGE positive human rotavirus samples, as observed in 1% agarose gel (Fig. 9). Using this PCR product, nested multiplex PCR for G genotyping was carried out and only G [1] was amplified in 6 (37.5%) samples showing a product size of 749bp (Fig. 10). The remaining rotaviruses remained untyped. These results could possibly be due to the emergence of new types. Reference strain of human group A rotavirus G1P[8] could be typed using the same set of typing primers.

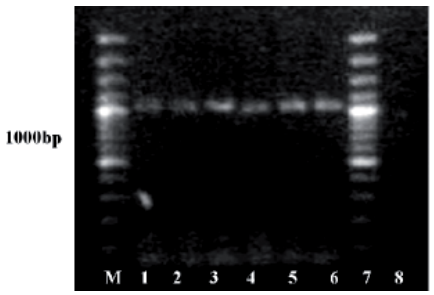

Fig. 9. Full length amplification of VP7 gene of human rotavirus. Lane M, 7: 100bp DNA ladder. 1-6: Rotavirus field isolates. 8: Negative control. Numbers on the side depict size of PCR amplicon in base pairs.

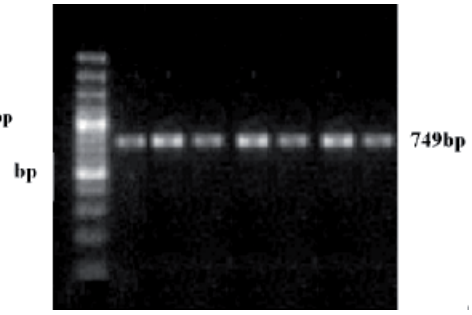

Fig. 10. G[1] genotypes of human rotavirus isolates. Lane M: 100bp DNA ladder. 1-6: G1 genotypes. Numbers on the side depict size of PCR amplicon in base pairs.

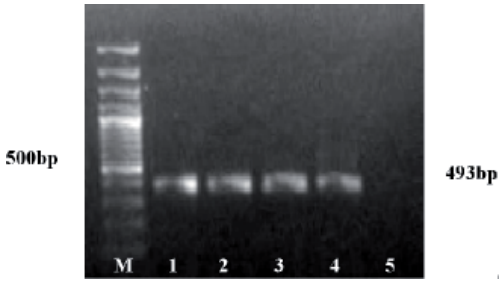

Fig. 11. Partial length amplification of VP6 gene of poultry rotavirus. Lane M: 100bp DNA ladder. 1-4: Poultry rotavirus field isolates. 5: Negative control. Numbers on the right side depict size of PCR amplicon in base pairs.

No amplification was observed in the four RNA-PAGE positive samples of poultry rotavirus when subjected to VP4 and VP7 gene based RT-PCR amplification, using the respective gene specific primers. However, the VP6 gene yielded a specific product of 493bp in all positive samples, as observed in 1% agarose gel (Fig. 11). This is probably the first ever report of the detection of group D avian rotavirus from Western India.

Discussion

RNA-PAGE has been used as a gold standard for identification of the segmented RNA of rotaviruses. The simultaneous investigation was carried out to find the actual prevalence of rotavirus in buffalo, avian and human species. The segmented viral RNA extracted from faecal samples of buffalo, poultry and human was resolved by RNA-

PAGE, followed by silver staining. The PAGE analysis revealed the presence of 11 segmented viral genome in buffalo and human rotavirus with a migration pattern of 4:2:3:2, characteristic of group A rotavirus. The virus causes severe diarrhoea in buffalo calves below three months of age, contributing to calf mortality and huge economic losses to farmers. Incidence of rotavirus associated diarrhoea in buffalo calves has been reported between 3% (GULATI et al., 1995) to 64.28% (MITTAL et al., 1986) in previous studies. The mortality due to rotavirus infection in buffalo calves may reach up to 25% (FAGIOLO et al., 2005). Interestingly, poultry rotavirus showed an electrophoretic mobility pattern of 5:2:2:2, characteristic of the group D avian rotavirus. RNA-PAGE analysis of poultry rotavirus showed close migration of segments 3 and 4, segments 6 and 7 were co-migrating, segments 10 and 11 also migrated closely, whereas segment 5 was distantly migrated. Avian rotaviruses have been isolated from diarrhoeic chickens, turkey poults and other avian species in various parts of the world (YASHON and SCHAT, 1985; ITO et al., 1995; AHMED and AHMED, 2006). The literature regarding avian rotavirus is very scanty in different parts of India. Only a few reports are available from the northern part of the country (WANI et al., 2003; MINAKSHI et al., 2004). SAVITA et al. (2008) reported detection of group D avian rotavirus in diarrhoeic faecal and environmental samples in central India. However, this could possibly be the first report of the detection of the avian rotavirus in this western part of the country.

In the present study, buffalo rotavirus positive samples revealed long electropherotypes, while poultry showed a short migration pattern. In human samples, both long and short types of electropherotypes were observed. For the study of genomic diversity, close observation of circulating electropherotypes becomes essential. In this study, a total of 10 different electropherotypes were identified among the three different host species. Four electropherotypes were observed in buffalo, one in avians, five in humans. SHARMA (2004) reported 5 different electropherotypes during a study on buffalo rotavirus. SINGH and PANDEY (1988) found only 2 electropherotypes in Chandigarh whereas SUKUMARAN et al. (1992) reported 10 electropherotypes in Bangalore. India being a vast country, it is essential to study the molecular epidemiology of rotaviruses in different parts of the country, in order to study the prevalence of different strains circulating in a particular time and space.

One of the human samples showed mixed infection with extra genomic segments. The analysis of the genomic segments showed two additional RNA bands above segments 5 and 10 respectively, indicative of the presence of more than one strain of rotavirus circulating in the host. Similar findings were reported in human samples (JAIN et al., 2001) and in buffalo rotaviruses (MINAKSHI et al., 2009).

Of the six buffalo rotavirus samples, when subjected to P genotyping, they yielded P[11] in two (33.33%) samples and the rest could not be typed into any P genotypes. This

could be due to the emergence of new genotypes because of the reassortment of the virus in nature. Similar findings were also observed earlier in the northern part of India, where the buffalo rotavirus remained untypeable and sequence based typing revealed a novel genomic constellation (G10 P[3]) of a group A rotavirus in buffalo calves (MANUJA et al., 2008). In previous studies P[11] has also been detected as the predominant P type among buffalos in northern India (GULATI et al., 1999; MINAKSHI et al., 2005).

None of the PAGE positive samples subjected to G genotyping revealed any amplification product. Non detection of G type may be due to the emergence of a new or unusual strain of virus circulating in this part of the country, or because of the reassortment of virus, which is frequently detected in developing countries due to the close contact of animals and humans. DNA sequencing of the part of VP4 and VP7 gene could be helpful to genotype such untypable samples (MANUJA et al., 2008).

RNA-PAGE positive 16 samples of human rotavirus did not reveal any P type. The untypeable samples possibly may lie outside the known types or may represent a new type or subtype (DAS et al., 2002). SOLBERG et al. (2009) suggested that a primer mismatch may be a widespread cause of genotyping failure, and might be particularly problematic in countries with greater rotavirus diversity.

The semi nested-multiplex PCR of human rotavirus for differentiating G genotypes revealed only G [1] type in 6 (37.5%) of 16 rotavirus positive samples. The remaining samples could not be typed in to any G genotypes. The G [1] genotype is the most prevalent type in the United Kingdom (ITURRIZA-GOMARA et al., 2000a), Brazil (KASULE et al., 2004), Canada (PANG et al., 2004), China (ZHANG et al., 2004). In India, the G1 genotype has also been reported to be the predominant type in different parts of the country, such as the north (DAS et al., 2004) and north-east (KANG et al., 2002). A failure to genotype or mistyping has already been reported from different parts of the world. These reports showed that nucleotide sequence differences between the target region of the respective genes and the primer sequences used for typing led to the genotyping failure (MAUNULA and VON BONSDORFF, 1998; ITURRIZA-GOMARA et al., 2000b). Further characterization of these isolates is required to reveal their true nature.

None of the four RNA-PAGE positive samples of poultry rotavirus could be amplified using VP4 and VP7 gene based primers. Similar difficulties were faced by DESWAL (2006), who could not even find a single PAGE positive sample of avian rotavirus showing amplification for VP4 and VP7 genes, which he described, which may be due to emergence of new strain. All the four positive samples yielded a specific product of 493bp on amplification with VP6 gene specific primers. This is probably the first ever report of the detection of a group D avian rotavirus from western India.

In conclusion, rotavirus is prevalent in buffalo, poultry and human species in this part of the country. RNA-PAGE was evaluated as a sensitive tool for the detection of rotavirus

from faeces. The electrophoretic migration pattern of a particular rotavirus may be used to characterize the virus strain, which may be used in epidemiological investigations. The results indicate that RT-PCR, based on VP4 and VP7 genes in buffalo and human samples while targeting the VP6 gene in avian species, can be employed as a sensitive and specific assay for the rapid detection of rotaviruses in faecal samples. In India, because of close contact with human children, backyard poultry and animals especially in rural areas, sometimes sharing the same space, contamination of water and food is possible. It may be responsible for interspecies transmission of rotaviruses. So, in order to obtain a much better perspective in terms of the circulating genotypes in different species and regions of the country, molecular surveillance of the circulation of rotaviruses in varied host species needs to be carried out.

Acknowledgements

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SAŽETAK

Istraživani su rotavirusa u bivola, peradi i čovjeka. Ukupno su na prisutnost rotavirusa bila pretražena 83 uzorka fecesa bivola, 54 peradi i 81 uzorak stolice čovjeka probirnim RNA-PAGE testom, od čega je pozitivnih bilo šest (12,5%) uzoraka bivola, četiri (7,84%) uzorka peradi i 16 (20,25%) uzoraka stolice čovjeka. Svi bivolji i ljudski uzorci rotavirusa pozitivni poliakrilamid gel elektroforezom pokazivali su profil RNA segmenata karakterističan za skupinu A rotavirusa (4:2:3:2), dok su uzorci karakteristični za rotaviruse skupine D (5:2:2:2) bili dokazani u peradi. Svi pozitivni uzorci iz bivola (23,07%) pokazivali su dugi elekroferotip, dok je kratak elekroferotip ustanovljen u uzorcima peradi (15,38%). Većina (60%) ljudskih uzoraka rotavirusa imala je dugi elektroferotip, a preostalih 40% kratki. Ukupno je 10 različitih elektroferotipova bilo dokazano u trima pretraživanima domaćinima od čega su četiri pripadala bivolu, jedan peradi i pet čovjeku, a jedan uzorak iz čovjeka dokazan je kod mješovite infekcije. Prisutnost rotavirusa u RNA-PAGE pozitivnim uzorcima bila je dodatno potvrđena RT-PCR-om specifičnim za gene VP4 i VP7. Djelomično umnožen odsječak gena za VP4 bivoljih rotavirusa sadržavao je 856 bp, a ljudskih rotavirusa 876 bp. Proizvod gena za VP7 i bivoljih i ljudskih rotavirusa sadržavao je 1062 bp. Genotipizacijom G RT-PCR-om pozitivnih uzoraka ni u jednom uzorku nije bio dokazan proizvod specifičan za ikoji od tipova G6, G8 i G10. Genotipizacijom P istih uzoraka ustanovljen je genotip P[11] samo u dvjema (33,33%) uzorcima bivola. Šest ljudskih rotavirusa (37,5%) bilo je tipizirano kao genotip G[1], ali se nisu mogli tipizirati na osnovi genotipa P. Geni za VP4 i VP7 ptičjih rotavirusa nisu se mogli umnožiti. Međutim gen za VP6 svih ptičjih rotavirusa dao je fragment od 493 bp. Ovo je prvi dokaz skupine D rotavirusa u Zapadnoj Indiji.

Ključne riječi: rotavirusi, bivol, perad, čovjek, Zapadna Indija