International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Molecular Characterization of Human and Bovine Rotaviruses in Marathwada Province of Maharashtra State

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Abstract: ***Rotavirus is the major etiologic agent associated with diarrhoeal diseases of young ones of many farm animals and human infants. Due to segmented nature of the RNA genome and wide host range, vast genetic and antigenic diversity exist amongst different isolates of rotavirus, keeping this point in view, the present study was undertaken to characterize bovine and human rotavirus circulating in Marathwada province of Maharashtra state. Total 211 faecal samples from bovines calves and 55 stool samples from children were collected from various regions of Marathwada. 21 (9.95%) and 6 (10.90%) samples were positive for rotavirus in bovine calves and children respectively. The characteristic RNA migration pattern of group 'A' rotavirus was observed in all bovine and human samples through RNA PAGE showing long electropherotype. All the PAGE positive samples tested by RT-PCR for VP7 and VP4 genes of bovine and human were amplified as evidenced by an expected PCR product of 1062bp of VP7 gene, 856bp of bovine VP4 gene and 876bp of human VP4. Nucleotide sequences retrieved after sequencing of PCR products were subjected to sequence analysis of VP7 and VP4 genes for further confirmation which showed 85% to 99% homology with other group 'A' rotaviruses after examined by nucleotide BLAST revealing the interspecies transmission.***

Keywords: Rotavirus, RNA, RT-PCR, VP4, VP7

1. Introduction

Neonatal calf transience due to diahhorea is one of the most common animal health concerns for dairy business leads to not only major financial losses but also creation of farm animals for Dairy and Beef (Kapikian, 1996, Tamilmani Suresh *et al.* 2012**,** Lorenz *et al.*, 2011**)**. Neonatal calf mortality occurs majorly between 3rd week to one month of age due to calf diarrhoea (Jenny BF *et al*. 1981). In case of human infants 800,000 deaths occurs between ages of 6 months to 2 years in developing countries a lead to major loss. In India, one of every 250 children death is attributed to rotavirus diarrhea every year. Prevalence of rotavirus diarrhoea in India has been found to vary from 5-71% in hospitalized children below 5yrs of age with acute gastroenteritis (Shoobha broor *et al.*, 2003.)

Rota is a latin word means wheel, As rotavirus has individual wheel like appearance by negative stain electron microscopy and thus have been named *Rotavirus* (Shobha broor *et al.*, 2003). Rotavirus belongs to *Reoviridae* family, characterized by non enveloped triple layered viral particles with a viral genome having 11 double stranded RNA segment (ds RNA). Rotaviruses are classified in 7 groups (A-G) according to the antigenic variability of the inner capsid protein VP6. Group A Rotavirus are further classified into G & P types based on the genetic and antigenic variation of the 2 outer capsid proteins VP7 (Glycoprotin) & VP4 (protease sensitive protein) respectively (Estes, 1996**).** In India Group A bovine rotaviruses are responsible for neonatal calf diarrhoea, and having characteristic 4:2:3:2 pattern of 11 monocistronic dsRNA segments where segments 7, 8, and 9 were grouped as a triplet, typical of group A rotaviruses. RNA polyacrylamide gel electrophoresis (RNA PAGE) has been employed for detection of rotavirus antigen or viral nucleic acid in faecal samples (Minakshi *et al*., 2009) and molecular methods by reverse transcriptase PCR (RT-PCR) for the amplification of rotaviruses. As segmented nature viral genome allows re-assortment in mixed infection situation leading to emergence of new strains of the virus. There are also few reports in other countries noticed mixed infection in human infants (Nakagomi *et al.*1991*)* and bovine calves. The present study was undertaken with objectives: as screening of faecal samples from calves and children for rotavirus by employing RNA polyacrylamide gel electrophoresis (PAGE) and molecular characterization of rotaviruses.

2. Material and Methods

Total two hundred and sixty six faecal samples were collected in and around Marathwada province in Maharashtra state. 101 samples from cow calves and 110 from buffalo calves less than one month of age. The samples were collected from veterinary dispensaries, Dairy farms, Animal market, Small dairy farmers of various districts in Marathwada region. Along with fifty samples of human neonates were collected from both private and government pediatric hospitals were admitted for acute gastroenteritis in and around marathwada region during winter and prior to summer season in year 2014. All samples are collected in a sterile screw cap vials and keep it at 4^0C and The faecal samples were diluted in phosphate buffer Saline (P^H 7.2) to make 10% suspension, followed by centrifugation at 10,000xg for 15 mins to remove course partials and cellular debris. The clarified supernatant was stored at -20^0C until further use (Minakshi *et al*., 2009).

Extraction of Viral RNA: RNA extraction were done with TRI reagent directly from 10% faecal suspensions, described by World health organization Department of Immunization, Vaccines and biological (www.who.int/vaccines-documents/

2009) with minor modifications. Finally RNA pellet was air dried and suspend in 20 µl nuclease free water and keep at -20^{0}C till further use for RNA-PAGE and RT-PCR (Minakshi *et al*., 2009 , Yashpal S. *et al.*2013).

RNA- Polyacrylamide gel electrophoresis: The discrete segmented RNA genome was analyzed by RNA-polyacrylamide gel electrophoresis (RNA-PAGE) discontinuous buffer system as per (Herring *et al.* 1982) with minor modifications. The gel was run at a constant 120V for 2 hrs. After electrophoresis was done unload the gel from electrophoresis chamber and take out the gel from gel caster and carry on gel staining with silver nitrate method (www.who.int/vaccines-documents/ 2009).

Silver staining of dsRNA in gels*:* The RNA-PAGE gel was stain with Silver Nitrate Method described by (Herring *et al. (*1982). The PAGE positive Samples observed typically pattern of group A rotavirus (4:2:3:2) (Minakshi *et al*., 2004).

Reverse Transcription Polymerase Chain Reaction (RT-PCR): All the RNA-PAGE positive samples were subjected to RT-PCR for further conformation and amplification of VP4 (P) and VP7 (G) genes.

Table 1: List of oligonucleotide primers of VP7 and VP4 gene for bovine and human rotaviruses for RT-PCR.

Sr. no.	Primer	Sequence	Position
1	FalcBeg9	5'-gctttaaaagagagaatttccgtttgg-3'	(1-28)
2	FalcEnd9	5'-ggtcacatcatacaactctaatct-3'	(1039-1062)
3	C1	5'-ggctttaaaagagagaatttccgtctgg-3'	(1-28)
4	C2	5'-cacatcatacaattctaatctaag-3'	(1039-1062)
5	Falc FP	5'-ttcattattgggacgattcaca -3'	(1064-1085)
6	Falc RP	5'-caaccgcagcggatatat atc -3'	(1897-1918)
7	CON 3	5'-tggcttcgccattttatagaca- 3'	876bp (11-32)
8	CON 2	5'-atttcggaccatttataacc- 3'	(868- 887)

For RT-PCR primers as described by (Falcone et al. 1999, Taniguchi *et al*.1992, Gouvea *et al*. 1990) were used for bovine and human samples, respectively.

Reverse transcription polymerase chain reaction (RT-PCR) for VP4 and VP7 gene segments: The RNA was transcribe into template cDNA Synthesis by Superscript III RT *Taq* polymerase reverse transcriptase enzyme (Invitrogen). The Reaction condition for RT and PCR were standardized to get desired specific product by one step RT-PCR kit following manufactures instructions (Invitrogen) . The amplified PCR products were analyzed by 1% agarose gel electrophoresis. The gel was visualized using Gel documentation system (Biorad).

Sequence analysis of rotavirus: The RT-PCR positive samples were subjected to nucleotide sequencing. DNA sequencing was performed by the di-deoxynucleotide chain-termination method, using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Irene Trigueiros Arau *et al.*, 2007).

The sequences obtained were subjected to BLAST analysis with GenBank database sequences using BLASTn algorithm available at NCBI blast (*http://blast.ncbi.nlm.nih.gov/Blast*) to conform the presence of genes specific to rotavirus (Yashpal S. *et al.* 2013). The nucleotide sequences of VP7 and VP4 gene fragments of rotavirus were aligned using default parameters of muscle alignment implemented in MEGA 7.0 software (*http://www.megasoftware.net/*). The sequences were further analyzed for identity and differences using Sequence Identity and Similarity Tool available at (*http://imed.med.ucm.es/Tools/sias. html*).

3. Results and Discussion

This study was mainly aimed at distribution, detection, molecular characterization and sequence analysis of rotaviruses isolated from bovines and humans in faecal samples of diarrheic neonates using RT-PCR.

Detection of bovine and human rotavirus in RNA- PAGE
As shown in plate 1, 21 (9.95%) out of 211 faecal samples tested positive for rotavirus by RNA-PAGE resolved 11 segments with typical migration pattern of 4:2:3:2. This confers that all of them belongs to Group A rotavirus.

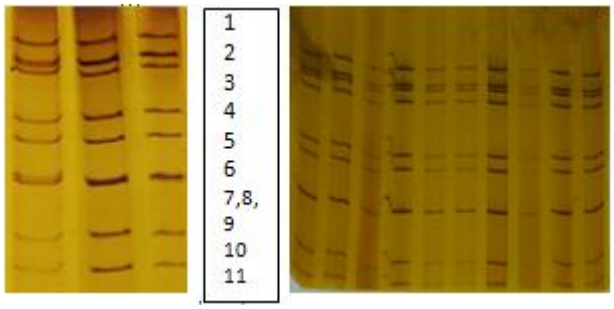

Plate 1: Electrophoretic migration pattern of bovine *rotavirus isolate from faecal samples analyzed by RNA-PAGE*

All of the positive bovine rotaviruses showed 'long' electropherotic migration pattern. Similar migration pattern was recorded by (Broor *et al.*1993, Kasule *et al.*2004, Urbina *et al* 200, Zuridah *et al.*2004). During the study, out of 55 human stool samples tested, 6 (10.90%) showed a typical migration of dsRNA segments in 4:2:3:2 pattern, indicative of group A rotaviruses shown in plate 2**.**

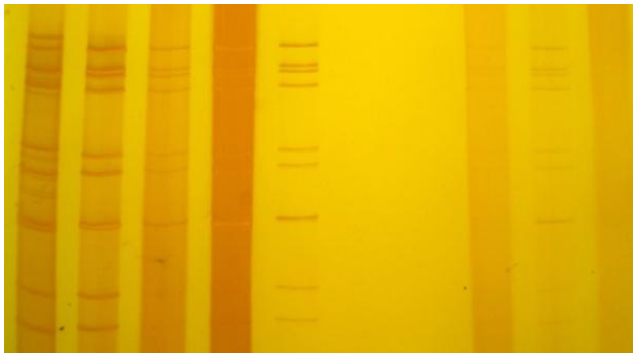

Plate 2: Electrophoretic migration patter of human rotavirus isolate from faecal samples analyzed by RNA-PAGE

However, in a study of rotaviruses in Kolkata, (Barman *et al.* 2004) detected a total of 18 (10.3%) Group A (4:2:3:2) rotavirus from 175 diarrheic stool samples of calves between 1 to 6 months of age. Group A rotaviruses have been detected worldwide. In India, Group A rotavirus has been

reported by (Kelkar *et al.*2000, Siwach 2005 and Premsagar 2008).

Amplification of VP7 gene of bovine and human Rotavirus: Full length (1062 bp) amplification of VP7 gene was transcribed into cDNA by reverse transcription using FalcBeg9 and FalcEnd9 primers. The cDNA was amplified by PCR using same gene specific terminal sequence primers (Table 1).

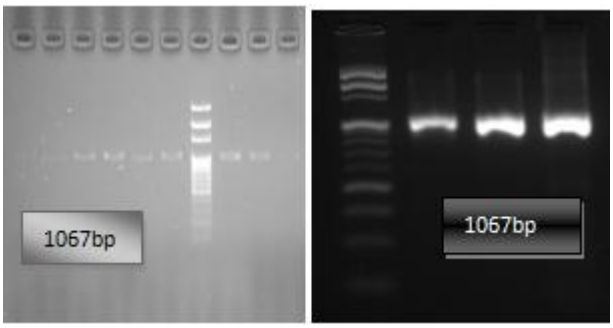

Plate 3: Agarose gel electrophoresis showing full length amplification of VP7 gene of bovine rotavirus isolates by RT-PCR.

Samples subjected to full length amplification yield a specific product of 1062 bp as observed in 1% agarose gel (Plate no 3) as per (Isegawa *et al.*1993 and Deswal *et al.* 2006). for bovine samples (Gentsch *et al.*1992, Siwach 2005 and Deswal 2006) for human samples.

Amplification of VP4 gene of bovine and human rotavirus: For partial length Bovine (856bp) and human (876 bp) amplification of VP4 gene was transcribed into cDNA by reverse transcription using gene specific (Falc FP and Falc RP) primers for bovines and (CON 3 and CON 2) primers for human. The cDNA was amplified by PCR using terminal sequence primers (Table 1).

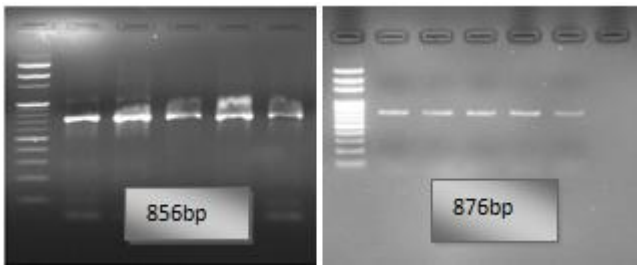

Plate 4: Agarose gel electrophoresis showing partial amplification of VP4 gene of bovine and human rotavirus isolates by RT-PCR

Samples subjected to partial length amplification, yield a specific product of 856 bp for bovine and 876 bp for human as observed in 1% agarose gel. Representative sample are shown in Plate no. 4. Similar findings were recorded by Gentsch *et al.(*1992), Kumar Harsh Bardhan (2007); Siwach (2005), Deswal (2006).

Nucleotide sequence analysis

Nucleotide sequence analysis of VP7 for bovine and human

The VP7 sequence homology analysis of bovine and human rotavirus isolates of Marathwada region revealed maximum homology 99% and 97% at nucleotide level in India and around countries for both bovine and human with Accession number (KJ701395, KJ701394.1) and (AB905458.1, JN192109.1 JN192100.1, JN192098.1, JN192096.1, JN192059.1) respectively. Similar findings were recorded by Yashpal S. *et al.*(2013)., Masako Abe *et al.(2009*), V. Martella *et al* (2009).

Nucleotide sequence analysis of VP4 for bovine and human

The VP4 sequence homology analysis of bovine rotavirus isolates of Marathwada region didnot revealed homology with any bovine species so it may be because of re-assortment of virus which is frequently detected in developing countries due to close contact of animals and human and inter-species transmission of rotavirus. The maximum homology recorded 87% and 86% at nucleotide level around Asian countries with human rotavirus G4P6 VP4 gene isolated from Argentina (KC412048) and from Japan (AB770153.1). But in human rotavirus isolates of Marathwada region revealed maximum homology 87%, 86% and 86% at nucleotide level with the Rotavirus A isolates G4P6 from (KC412048.1), VP4 gene,G1P[10] from Japan (AB770153.1) and G6P[6] VP4 gene from Belgium (EF554085.1). Similar findings were recorded by William A. Rodríguez *et al.*(2009)., Tung Gia Phan *et al.*(2007). Masako Abe *et al.(2009*), V. Martella*et al* (2009). This indicates cross species transmission among bovine and human rotavirus types (Souvik *et al.* 2007)

4. Summary and Conclusion

Out of 211 samples collected from cattle calves and buffalo calves, 21(9.95%) samples were positive showing typical long electropherotype pattern (4:2:3:2) indicating group A Rotaviruses. So present study reveals that there was Group A rotavirus was prevalent in Marathwada region and the prevalence was about 9.95% in case of bovines. In case of human neonates 55 stool samples are collected in that 6(10.90%) samples are positive showing long electropherotype pattern (4:2:3:2) indicating of group A rotaviruses. For further conformation with RT-PCR by amplifying VP4 and VP7 gene for both bovines and humans were amplified as evidence by an expected PCR product of 856bp of bovine and 876bp of human for VP4gene and 1062bp for VP7 gene. Representative sample from bovine and human ware analyzed by nucleotide sequencing for VP7 and VP4 genes further conformation which showed 85% to 99% homology at nucleotide level with other group 'A' rotaviruses when subjected to nucleotide BLAST reveals cross species transmission of bovine rotavirus VP4 gene reveals 87% homology at nucleotide level with human G6P[6] VP4 gene.

In future, extensive evaluation of serogroup specific nested RT-PCR assays with isolates of different serotypes from varied geographical locations or regions would strengthen

their serogroup specificity. Large scale testing of field samples would be useful in better extending application of these assays at field level. Moreover, due to the ability of the genomes of virus strains to re-assort, other genome segments should also be targeted, to obtain a truly comprehensive picture, which will in turn help to formulate comprehensive control strategies including effective vaccines development.

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