Molecular characterization and phylogenetic analysis of rotavirus of human infants, calves and piglets

UM Tumlam, VC Ingle, Dhruv Desai and SR Warke

Abstract
Rotavirus has been registered as one of the common and worldwide prevalent causative agent of enteric illness in newborn of most mammals and poultry species. The infection brings about severe symptoms of diarrhea in lambs, pigs and calves. In the present study out of 58 cattle & buffaloes calves fecal sample 04 (6.89%), 50 human infants stool samples 18 (36.00%), 71 piglets fecal samples 07(9.85%) were screened by rapid antigen detection kit. It was observed that out of 4 faecal samples from calves, 1 sample and out of 18 human stool samples, 07 samples out of 7 piglets 3 samples were found positive for RNA-PAGE. Representative PAGE positive fecal and stool samples, were selected for RT-PCR. The VP4 & VP7 genes of one calf sample were successfully amplified by RT-PCR with expected size of1011bp & 864bp. out of seven only six human infants samples amplify for VP7 gene, 3 out of 7 human infant sample amplify for VP4 gene and 3 out of 3 piglet samples amplify for VP4 & VP7 gene with the expected size of 1062 bp & 876 bp were confirmed positive for Rotavirus infection. One sample from calf and one from human infants, one from piglets were sent for sequencing. The phylogenetic analysis was carried out and it was confirmed that the positive samples were showing relatedness with the Rotavirus sequences taken from genebank and after sequence analysis, it was found that the piglets positive sample showed its relation with human rotavirus. This is indicative of trans-species transmission of rotavirus can occur. This work indicates that the migration of rotavirus throughout the country is possible.

Keywords: Rotavirus, human infants, calve, piglets, phylogenetic analysis

Introduction
Diarrheal diseases are mainly attributed to enteric viruses like rotavirus, norovirus, astrovirus, adenovirus and bacteria like Salmonella, E. coli and Campylobacter. Among the enteric viruses, rotavirus has been identified as the most common cause of severe gastroenteritis in children and young ones of animals. According to World Health Organization estimates 5.27 million children aged below 5 years die every year from vaccine-preventable rotavirus infections, most of these children are from low-income countries (WHO, 2012). Rotavirus is the most common cause of severe gastroenteritis in animals and is recognized as the single most significant cause of severe gastroenteritis, malnutrition and diarrhea, affecting a wide range of mammalian and avian species (Estes MK and Kapikian AZ, 2007). The situation remains stern in India with rotavirus diarrhea leading to an estimated 1.22-1.53 million deaths, 4.57-8.84 million hospitalizations and 2 million hospital visits in children below 5 years of age, every year. India pays out Rs.2.0-3.4 billion annually to treat rotavirus infections (Jacqueline et al., 2009). Likewise, rotavirus-associated enteritis is a major problem in young calves. Besides causing infection in cattle and buffalo calves, rotaviruses also affect piglets, foals, lambs, and young ones of pet animals and poultry. The virus affects young animals resulting in huge economic loss to farmers in terms of cost of treatment and mortality. Genus Rotavirus belongs to the family Reoviridae. Rotaviruses are non-enveloped double-stranded RNA viruses about 70 nm in diameter and possess icosahedral symmetry. The genome consists of 11 segments of dsRNA of molecular weight ranging from 2.0 × 10^6 to 0.2 × 10^6, that code for 6 structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 non-structural (NSP1-NSP6) protein. To date, at least 27 G-types, 35 P-types and 42 different G-P type combinations have been detected. Matthijssens et al., 2011) Therefore, the present study was conducted considering the zoonotic importance of Rotavirus, targeting VP4 & VP7 gene and phylogenetic analysis of the positive PCR samples of calves and human infants, piglets was performed and thereby confirmed Rotavirus infection.
Materials and Methods
Sample collection
Bovine fecal samples of cattle calves (180), buffaloes calves (31), human infants (50) and piglets (104) between the age group of 0-1yrs were collected from diarrhoeic animals from different regions of Maharashtra during January 2016 to October 2017. Approximately 5-10gms of sample was collected in a sterile, screw capped containers. At the time of sample collection, date of collection, age, clinical signs, important clinical history was recorded. The samples were transported to the laboratory in container containing ice bag and stored at -20 °C till processing. Each of the fecal samples was suspended in 10% phosphate buffered saline (PBS, pH 7.2), clarified by centrifugation at 8000 x g for 10 min at 4 °C and supernatants were collected and stored at – 20 °C till further use.

Extraction of Rota viral ds RNA
The dsRNA of Rotavirus was extracted from faecal and stool samples using TRIzol method Jadhav et al. (2009) [6] as per the manufacturer’s protocol. The isolated RNA was then used for further downstream applications.

Polyacrylamide Gel Electrophoresis
The RNA extracted from the faecal sample was subjected to ribonucleic acid-poly acrylamide gel electrophoresis (RNA-PAGE) as per the procedure described by Laemmli (1970) [8] & Sambrook and Russel (2001) [10] in separating gel of 8.0% and stacking gel of 5.0% concentration were used for the detection of rotavirus by RNA-PAGE.

Table 1: The sequences and nucleotide position of oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>designation Sequences (5’-3’)</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Bov9Com3 (+)</td>
<td>TGT ATG GTA TTG AAT ATA CCA C</td>
<td>1011 bp</td>
<td>Isegawa et al. (1993) [4]</td>
</tr>
<tr>
<td>Bov9Com5 (-)</td>
<td>TCA CAT CAT ACA ACT CTA ATC T</td>
<td>864bp</td>
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<tr>
<td>Bov4Com5</td>
<td>5’TTCATTATTGGGACGATTCACA 3’</td>
<td>1062bp</td>
<td>Gentsch et al. (2005) [3]</td>
</tr>
<tr>
<td>Bov4Com3</td>
<td>5’CAACCGCAGCTGATATATCATC 3’</td>
<td>876bp</td>
<td></td>
</tr>
<tr>
<td>BEG9 1-28</td>
<td>5’GGCATTAAAGAGAGAGAAATTCCGGTCTGG3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>END9 106-136</td>
<td>5’GGTCACATCATACATACATCTCAATCTAAGG3’</td>
<td></td>
<td></td>
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<tr>
<td>Con3 11-32</td>
<td>5’GGCTTCTGCCAT TTT ATA GAC G3 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con2 868-887</td>
<td>5’ATT TCG GAC CAT TTA TAA CC 3’</td>
<td></td>
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</tbody>
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Agarose gel electrophoresis
The PCR amplified products were analyzed on agarose gel electrophoresis (AGE). The amplified products were analyzed by electrophoresis on 1% agarose, and analyzed and photographed by a gel documentation system (BIO-RAD, USA). RT-PCR positive, one calve sample & one human infants sample, one piglet sample was sent for sequencing. The sequences were analyzed using BLAST (Basic Local Alignment Search Tool) and the Clustal-W (CLUSTAL2.1多重sequence alignment) to generate sequence alignment reports. Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 was used for construction of phylogenetic tree. The bootstrapped phylogenetic tree was constructed using Neighbor-joining method.

Results and discussion
In the present study out of 58 cattle & buffaloes calves fecal samples screened, 04 (6.89%) were found to be positive. The per cent positivity of 10.26% (4/39) was recorded in early days of 0-1 month and Out of 50 human infants stool samples tested 18 (36.00%) found to be positive for rota virus infection. The per cent positivity of 34.88% (15/43) was found in day of 1 month and Out of 45 human infants stool samples tested 15 (33.33%) found to be positive. The per cent positivity of 10.26% (4/39) was recorded in early days of 0-1 month and Out of 45 human infants stool samples tested 15 (33.33%) found to be positive. The per cent positivity of 10.26% (4/39) was recorded in early days of 0-1month and Out of 50 human infants stool samples tested 15 (30.00%) found to be positive.

Silver staining of dsRNA in polyacrylamide gel
For rapid detection of sample positive for Rotavirus, the typical pattern of migration of 11 different segments of rotavirus RNA were observed via silver staining as described by Svensson et al. (1986) [11] The gel was constantly run for 15 min at 60V followed by 110 V for approximately 4 hours.

Reverse transcriptase-polymerase chain reaction
The dsRNA was subjected to reverse transcription as per the protocol given by Isegawa et al., (1993) [4] The synthesized cDNA was stored at -20°C till further use. For the detection and confirmation of group A rotavirus, amplification of partial length VP4 & VP7 gene was carried out. The reverse transcriptase polymerase chain reaction (RT-PCR) was conducted for PAGE positive samples. The cDNA synthesis was carried as per the following protocol using cDNA synthesis kit from High-Capacity cDNA Reverse Transcription Kits of Applied Biosystems. The clyclic conditions for VP7 &VP4 gene were Initial denaturation at 95 °C for 8 min 1cycle respectively followed by 35 cycles of denaturation at 94 °C for 45 sec, Annealing at 52°C for 45 sec, extension at 72 °C for 90 sec and one cycle of the final extension at 72 °C for 10 min 1cycle. The samples were hold at 4 °C. Initial denaturation at 94 °C for 4 min 1 cycle 1 cycle respectively followed by 35 cycles of denaturation at 94 °C for 1 min, Annealing at 48 °C for 2 min, extension at 72 °C for 2 min and one cycle of the final extension at 72 °C for 10 min cycle. The PCR products were stored at 4 °C and run on agarose gel for the checkup of the amplicon size and amplicon quality. The sequences and nucleotide position of oligonucleotide primers are shown in Table 1.
rotavirus. Of them 6 rotavirus were successfully amplified using VP7 genes based RT-PCR of the expected size (1,011 bp). Ahmed et al., (2017) [1] found that 36.22% (71/196) of fecal samples of both diarrheic and non-diarrheic calves with age group up to 4 months from different places of Assam, India found to be positive for both VP7 and VP4 genes of group A bovine rotavirus by RT-PCR. Kumar et al., (2011) [7] reported an increased false positive rate with RNA-PAGE (40) as compared to only (15) for RT-PCR when tested fecal samples of bovine calf, children, piglets and pigs together. But overall result indicated that it is tough to put RT-PCR in direct detection method as compared to RNA-PAGE simply because of large product size and sequence variations in rotavirus for VP7 gene. The PCR product of one human infants and one calf representative samples were sent for sequencing and sequences were brought. (fig.4) Using BLAST, the sequences of the both samples (one human and one calf) obtained were confirmed and compared to the sequences available in the GenBank database. The resulted sequences were also deposited in the GenBank database with the Accession numbers LC377483, LC377479, LC377480, LC377484, LC377485, LC377486 respectively.

Fig 1: VP4&VP7 RT-PCR showing positive cattle calve sample resolved on agarose gel along with molecular weight marker

Fig 2: VP7 RT-PCR showing positive Piglets and Human infants sample resolved on agarose gel along with molecular weight marker

Fig 3: RT-PCR showing VP4 positive Human infants sample resolved on agarose gel along with molecular weight marker
Fig 4: Phylogenetic tree of Partial gene sequence of Calf, Piglets & Human infants
Conclusion
The present study records the detection and molecular characterization of VP7 and VP4 genes of group A rotaviruses (GARV) circulating in different animal species viz. cattle and buffalo calves, piglets, and human infants there is evidence of the same strain of this viruses circulating in India. From the phylogenetic study it was confirmed that the phylogenetic analysis of VP4 gene of cattle calf (C-47) and piglet (P-371) came under same cluster showed 100% homology with human and bovine with Accession Number KJ873130|India|2012/12,KY888948|Bangladesh|2016/12/26, KX904819|Bangladesh|2015/06/26 The phylogenetic analysis of VP7 gene of piglet (P-368) VP7-BEG9 in separate node with one cluster showed homology with cattle calf sequence reported from China, India, Japan, South Korea which showed possibility of zoonotic transmission. Phylogenetic analysis showed not much sequence variation between rotavirus from human infant (H-122) and cattle calf (C-145). The piglets and human rotavirus samples were clustered together and after sequence analysis, it was found that the piglets positive sample showed its relation with human rotavirus. This is indicative of trans-species transmission of rotavirus can occur. This work indicates that the migration of rotavirus throughout the country is possible. VP4, VP7 RT-PCR positive samples showed interspecies cross relationship. Sequencing of some of the genotypes and subsequent analysis revealed varying degree of similarity with world and Indian isolates.

Acknowledgement
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References
6. Jadhav MB. Detection and molecular characterization of bovine and porcine rotavirus from Nagpur region, M.V. Scthesive, MAFSU, Nagpur, 2009