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## Detection of rotavirus associated with diarrhea in bovine calves by RNA-PAGE and RT-PCR

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### Abstract

Bovine fecal samples of cattle calves (180), buffaloes calves (31) between the age group of 0-1yrs were collected from diarrheic animals from different regions of Maharashtra during the year 2016-17. Of them a total of 58 fecal samples comprising of 48 (cattle calves), 10 (buffaloes calves), screened for the presence of rotavirus by rapid *Rota virus* Ag detection Biochrom kit & RNA-PAGE. The rotavirus was detected in 4 out of 58 bovine fecal samples by rapid *Rota virus* Ag detection and one sample of cattle calf found positive for the RNA-PAGE assay. The bovine rotaviruses were positive for electrophoretic migration pattern of 11 segments with a typical pattern of 4:2:3:2. In a long run, segment 10<sup>th</sup> and 11<sup>th</sup> of all bovine rotavirus migrated faster as compared to short run electropherotype, while no sample from buffalo calves was found positive for both the test. RNA-PAGE positive sample of calf was subjected to RT-PCR of VP7 & VP4 gene which yielded amplicon of 1011 & 864bp RT-PCR targeting group specific VP7 & VP4 gene confirmed Group A rotavirus.

**Keywords:** Group a rotavirus, VP7 & VP4 gene, diarrhea, RNA-PAGE, RT-PCR

### Introduction

Neonatal diarrhea is considered as one of the important diseases of several species of domestic animals and human beings, associated with huge economic loss worldwide (Holland 1990) [4]. Rotavirus is one of the important causative agents of diarrhea in animals and human (Rajendran *et al.*, 2014.) [13] which account for 25% mortality in young animals (Mukhtar *et al.*, 2016) [12]. 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) [17].

Genus Rotavirus belongs to the family *Reoviridae*. *Rotaviruses* are non enveloped double-stranded RNA viruses having distinct wheel like appearance by negative staining electron microscopy (EM) and thus the name *rota* which in Latin means "wheel". The virus particles are about 70 nm in diameter and possess icosahedral symmetry. They have triple layer capsid, the innermost layer of which, the core, contains the viral genome. The genome consists of 11 segments of dsRNA of molecular weight ranging from  $2.0 \times 10^5$  to  $0.2 \times 10^6$ , that code for 6 structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 non-structural (NSP1-NSP6) protein. However, gene segment 11 encodes both NSP5 and NSP6 proteins. The structural proteins are located in the core (VP1-VP3), inner shell (VP6) and outer shell (VP4, VP7). To date, at least 27 G-types, 35 P-types and 42 different G-P type combinations have been detected. Matthijnssens *et al.*, (2011) [8]

Rotavirus with 30 kbp sized genome with 11 gene segments are separated by polyacrylamide gel electrophoresis and generally recorded as RNA pattern after staining the RNA in gel. The rotavirus patterned differently according to their type of strain present. Four subgroups from class I (1-4), class II (5-6), class III (7-9) and class IV (RNA segment 10-11) have been recognized among RNA A rotaviruses. There is difficulty in propagation of rotaviruses in the tissue culture the comparison of the migration pattern of the RNA during PAGE becomes an important laboratory technique for characterization of strains. RNA-PAGE procedure provides a rapid, simple and reproducible method of obtaining rotaviral double stranded RNA preparation suitable for electrophoretic analysis in polyacrylamide gels. Saravanan *et al.*, (2006) [14]

Similarly there is serotype based classification based on outer capsid layer proteins i.e. VP4 and VP7. VP7 is a glycoprotein and designated as G protein while VP4 is a protease sensitive protein designated as P protein. To date based on the sequence analysis of the VP7 and VP4

genes at least 27 G, 35 P genotypes and 42 different G and P type combinations have been detected in group A rotaviruses in humans and animals (Matthijssens *et al.*, 2011) [8]. Considering the importance of the disease the present study was aimed for the detection of Rota viral A infection in diarrheic calves by RNA-PAGE and RT-PCR amplify VP4 and VP7 gene of group A bovine rotavirus.

## Materials and Methods

### Collection of samples

Bovine fecal samples of cattle calves (180), buffaloes calves (31) between the age group of 0-1yrs were collected from diarrheic 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

### Detection of rotavirus in fecal samples

Initial screening of the processed fecal samples for rotavirus was done by RNA- PAGE followed by RT- PCR of VP4 and VP7 genes.

### Ribonucleic acid polyacrylamide gel electrophoresis (RNA PAGE)

Viral RNA was extracted from the fecal suspensions by using Trizol method as described by Jadhav *et al.* (2009) [6]. The extracted total RNA was quantified by using nanodrop instrument (Nano Drop 1000 Thermo Scientific USA). The extracted RNA was then subjected to native RNA PAGE. The electrophoretic run was carried out at voltage 100V till the dye run out from the gel (6-7hrs) using vertical gel

electrophoresis apparatus (Hoeffar, USA). The genomic migration pattern was detected by silver-staining of the gel (Svensson *et al.* 1986) [15].

### RT-PCR for detection of Rota viral nucleic acid

The VP4 and VP7 gene were amplified as per method of Isegawa *et al.*, (1993) [5]. The cDNA synthesis was carried as per High-Capacity cDNA Reverse Transcription Kits of Applied Biosystems.

### Amplification of VP4, and VP7 gene of rotavirus

The VP4 and VP7 region in the c-DNA samples were amplified by RT-PCR using gene specific primers. The sequence of the primer set F 5 TGT ATG GTA TTG AAT ATA CCA C 3/- R-TCA CAT CAT ACA ACT CTA ATC T for VP4 and F 5 /- TTCATTATTGGGACGATTCACA -3/, R 5/ - CAACCGCAGCTGATATATCATC -3/ for VP7 were used for gene amplification producing 1011 and 864 bp sized amplicon on 1% agarose gel. For PCR, the reaction mixture was prepared by mixing of 5X buffer 2.0 µl, 25mM MgCl<sub>2</sub> 1.5 µl, 25 dNTP 0.4 µl, Forward primer (10 pmols/ µl) 0.12 µl, Reverse primer (10pmols/ µl) 0.12 µl, Taq DNA polymerase (IU/ µl) 0.4µl, Template (c-DNA) 1.0 µl and Nuclease free water 14.46 µl for both the genes. The properly mixed 20µl reaction mixture was initially denatured at 95 °C for 8 minute. Thirty cycles at 94 °C (45 sec), 52 °C (45 sec), 72(90sec), Final extension 72 °C for 10 min 1cycle for VP7 gene and initially denatured at 94 °C for 4 minute. Thirty cycles at 94 °C (1min), 48 °C (2 min), 72(2 min), Final extension 72 °C for 10 min 1cycle for VP4. Both the positive and negative controls were run parallel along with the test samples. To confirm the targeted PCR amplicon which was obtained, the PCR products were subjected to electrophoresis in 1% agarose gel containing ethidium bromide (10 mg/ml) with 1X TAE buffer 100 volts (V) for 60 minutes @ 200 mA. The image was finally captured under gel documentation system. The sequences and nucleotide position of oligonucleotide primers are shown in Table 1.

**Table 1:** Sequences and nucleotide position of oligonucleotide primers

Primer	designation Sequences (5'-3')	Amplicon size	Reference
Bov9Com3 (+)	TGT ATG GTA TTG AAT ATA CCA C	1011 bp	Isegawa <i>et al.</i> (1993) [5]
Bov9Com5 (-)	TCA CAT CAT ACA ACT CTA ATC T		
Bov4Com5	5'TTCATTATTGGGACGATTCACA 3'	864bp	
Bov4Com3	5'CAACCGCAGCTGATATATCATC 3'		

## Results and Discussion

As resolved on RNA PAGE, 1out of 4 fecal samples found to be positive for rotavirus typical RNA-PAGE with 11 segments having typical migration pattern 4:2:3:2 distribution indicated that positive sample belonged to group A rotavirus with long electrophoretic migration pattern as shown in (fig.1). None of the buffalo calf sample was positive for rotavirus. The bovine rotaviruses were positive for electrophoretic migration pattern of 11 segments with a typical pattern of 4:2:3:2. In a long run, segment 10<sup>th</sup> and 11<sup>th</sup> of all bovine rotavirus migrated faster as compared to short run electropherotype. However Similar findings were reported by Ahmed *et al.*, (2017) [1]who showed that of 196 fecal specimens collected from both diarrheic and non-diarrheic calves, 26 (13.26%) samples from diarrheic calves were found to be positive for Rota virus in standard RNA-PAGE assays. Saravanan *et al.*, (2006) [14] utilized RNA-PAGE to detect rotavirus in calves fecal samples with 09 diarrheic fecal

samples collected from calves in nine dairy cattle herds located in different areas in Tamil Nadu State, India found to be positive 4.30% out of 209 calves tested for group A rotavirus by polyacrylamide gel electrophoresis (RNA-PAGE) and there was no difference in migration pattern by RNA-PAGE.

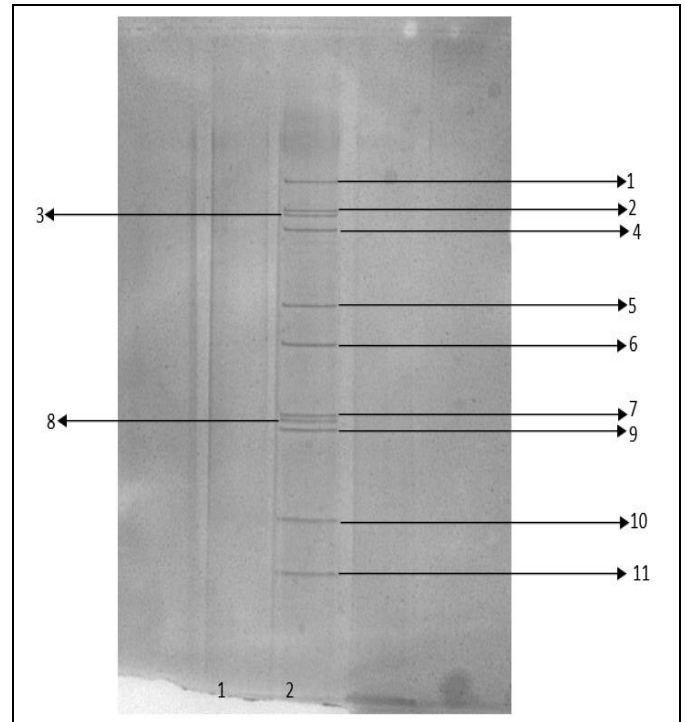
Basera *et al.*, (2010) [2] screened 110 cattle calves fecal samples by RNA-PAGE, and found that 13(11.81%) were positive while no sample from buffalo calves was found positive. All samples were found to have long electropherotype and two distinct electropherotypes having segment variation were observed.

In this study one diarrheic fecal sample from cattle calf which was positive in RNA-PAGE was also positive for RT-PCR of VP4 & VP7 gene. An amplicon of 864 bp & 1011bp resolved on agarose gel along with molecular marker (100 bps) indicated the sensitivity of PCR for detection of bovine rotaviruses (Fig.2). Similar findings were reported by Wani *et al.*,

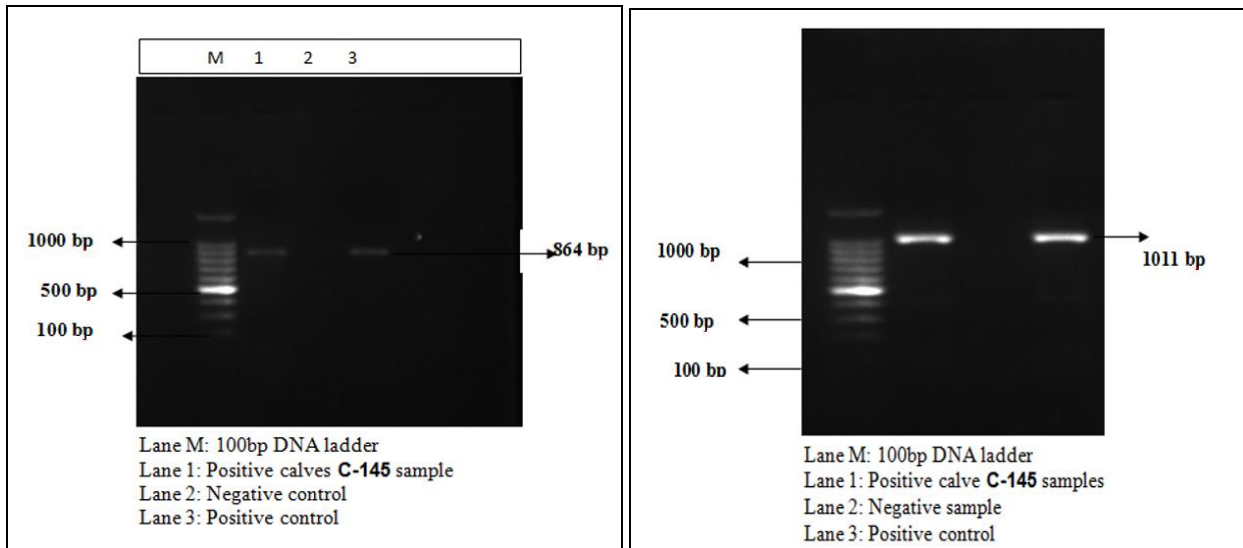
(2004) [16] who screened 10 diarrheic fecal samples from four to forty-five-day old calves positive for group A rotavirus. Of them 6 rotavirus were successfully amplified using VP7 genes based RT-PCR of the expected size (1,011 bp). Ahmed *et al.*, (2017) [11] found that 36.22% (71/196) of fecal samples 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. Hassan (2014) [3] employed RT-PCR based genotyping assay to characterize 34 bovine, 8 ovine field rotavirus samples and 37 stool samples from hospitalized children for G and P typing. Out of 34 bovine positive samples, 29 (85.2%) samples could be typed for both G (VP7) and P (VP4) types. Mondal *et al.*, (2011) [11] reported that Among the cow calves 1 (4%) sample and among the buffalo calves 4 (7.89%) samples showed migration pattern of 4:2:3:2 typical for RVA. Of the RV positive buffalo calf samples, 3 samples yielded a specific product of 1013 bp of VP7 gene by RT-PCR. Mondal *et al.* (2012) [10] examined 113 diarrheic fecal samples of cattle and buffalo calves by RNA-PAGE. Eleven (9.73%) samples were detected Group A rotavirus positive with long electropherotype and showed prevalence of G10 genotype by RT-PCR G typing. Minakshi *et al.*, (2015) [9] found that out of total 103 fecal samples from diarrheic calves of 0 to 1 month of age only 11 samples show amplicons of 864 bp VP4 gene by RT-PCR.

In the present study also, four selected samples, only one were identified by RT-PCR while three failed to produce an amplified viral band despite being known to contain sufficient rotaviral particles by RNA-PAGE and any amplification with primers specific to VP4 and VP7 genes by RT-PCR. This might be due to the presence of inhibitory substances in the

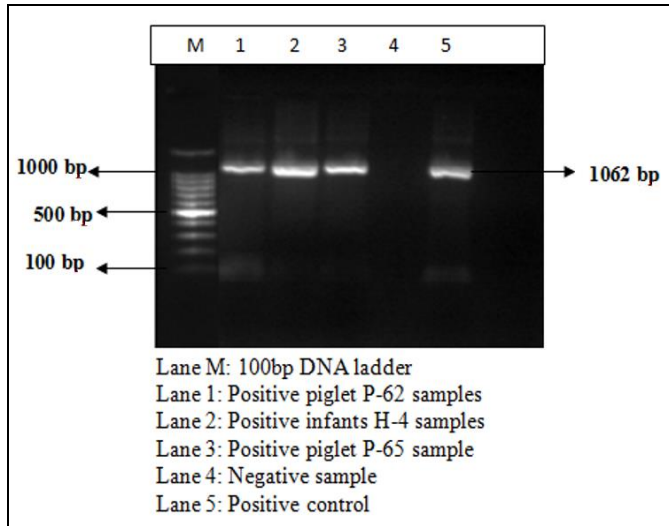
fecal samples or mismatches in primer binding sites (Manuja *et al.*, 2008) [7].



**Fig 1:** Electrophoretic Migration Pattern 4:2:3:2 distributions of cattle calve (1-11 segments of RNA) 1-Negative sample 2-Positive cattle calve sample



**Fig 2:** VP4&VP7 RT-PCR showing positive cattle calve sample resolved on agarose gel along with molecular weight marker



**Fig 3:** VP7 RT-PCR showing positive piglet and human infants sample resolved on agarose gel along with molecular weight marker

### Conclusion

In conclusion, the findings of this study have confirmed the occurrence of group A rotavirus in fecal samples of calves. The positive sample when amplified for partial length amplification of VP4 and VP7 gene based RT-PCR indicating the sensitivity of PCR for detection of bovine rotaviruses. However, in order to know the genotypes of the circulating rotavirus strains and their interspecies transmission from one animal species to others, further investigation is required

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