RESEARCH ARTICLE

Bovine Rotavirus in Diarrhoeic Calves: A Combined RNA-PAGE and RT-PCR Approach to Study the Burden on Neonatal Calf Health

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Abstract

Diarrhoeal diseases in neonatal calves pose significant health and economic challenges, with rotavirus, particularly group A rotavirus (RVA), being a major causative agent of calf diarrhoea globally. This study was aimed to determine the prevalence of bovine rotavirus (BRV) in diarrhoeic calves from dairy farms in and around Bareilly, Uttar Pradesh, India. A total of 116 diarrhoeic faecal samples were collected from calves and analysed using RNA-PAGE and RT-PCR targeting the *VP6* gene. RNA-PAGE identified 5.17% of samples as positive, while RT-PCR identified 10.34% of samples as positive. The study highlights the presence of BRV as a significant viral causative agent of calf diarrhoea in the region and underscores the importance of a combined diagnostic approach to enhance its detection accuracy.

Keywords: Bovine rotavirus, Neonatal calf diarrhoea, Prevalence, RNA-PAGE, RT-PCR

Ind J Vet Sci and Biotech (2025): 10.48165/ijvsbt.21.3.10

Introduction

Neonatal calf diarrhoea poses a significant challenge to the cattle industry, impacting both dairy and beef herds. It incurs economic losses through substantial mortality rates and high treatment costs, as well as production losses due to impaired growth and diminished production. Its etiology is complex, involving multiple pathogens, including bacteria, viruses, and parasites. Rotavirus, particularly rotavirus group A (RVA) is a major cause of neonatal diarrhoea in calves under six weeks of age specifically within the first two weeks of life. Infected calves typically exhibit clinical signs such as anorexia, dehydration, depression, and yellowish-watery diarrhoea (Geletu *et al*., 2021) and shed virus in large quantities in the faeces. Moreover, subclinical infections in older animals can serve as reservoirs for the rotavirus (Cho and Yoon, 2014). In the animal kingdom, rotaviruses are especially notorious for affecting a diverse range of domesticated and wild mammals, notably calves, goats, lambs, pigs, and horses, as well as various avian species (Otto *et al*., 2015; Althof *et al*., 2023).

The laboratory diagnosis of the specific causative agent of diarrhoea is crucial for its timely treatment and effective management , as clinical symptoms alone are insufficient to distinguish between the various pathogens involved. Moreover, prompt diagnosis facilitates the implementation of targeted preventive as well as control actions. A range of techniques has been utilized to detect rotaviral antigens or nucleic acids in faecal samples. These methods include electron microscopy, immune electron microscopy, immunofluorescence staining, immunohistochemical staining, RNA polyacrylamide gel electrophoresis (RNA-PAGE), enzyme-linked immunosorbent assays (ELISA), latex agglutination tests (LAT), and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (WHO, 2009). The detection of rotavirus using RNA-PAGE followed by silver staining, a technique first introduced in 1982 (Herring *et al*., 1982), is highly specific, and relatively simpler. In recent years, RT-PCR has become one of the most adopted laboratory techniques for rotavirus detection and confirmation owing to its high sensitivity and specificity. In India, calf diarrhoea primarily attributed to bacterial and protozoan pathogens receives significant attention, while viral causes are often neglected. Therefore, this study was aimed to evaluate the prevalence of one of the principal viral agents of diarrhoea in neonatal calves, bovine rotavirus (BRV), on dairy farms in and around the city of Bareilly in the Uttar Pradesh state of India utilizing the RNA-PAGE and RT-PCR techniques.

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How to cite this article: Saini, S., Dubal, Z. B., Malla, B. A., Rajak, K. K., Dhanze, H., & Bhilegaonkar, K. N. (2025). Bovine Rotavirus in Diarrhoeic Calves: A Combined RNA-PAGE and RT-PCR Approach to Study the Burden on Neonatal Calf Health. Ind J Vet Sci and Biotech, 21(3), 48-52.

Source of support: Nil

Conflict of interest: The authors declare that there is no conflict of interest.

Submitted 04/02/2025 **Accepted** 14/03/2025 **Published** 10/05/2025

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Materials and Methods

Sample Collection

A total of 116 diarrhoeic faecal samples from calves (both cattle calves and buffalo calves) of age ranging between 0-3 months were collected in sterile collection vials (M/s. Hi Media Lab.) from October 2022 to March 2023 from dairy farms and Gaushalas located in and around Bareilly, Uttar Pradesh (India) after getting formal consent from the respective farm in-charges/caretakers. Approximately 10 gm/mL diarrhoeic faecal sample was collected in each sterile collection vial using disposable latex gloves after cleaning the anal area and all other necessary precautions. The collected faecal samples were labelled and brought to the laboratory as soon as possible in ice-packed containers maintaining a temperature of 4-8°C and stored at -20°C till used for RNA extraction followed by screening with RNA-PAGE and RT-PCR by targeting the *VP6* gene of RVA.

Extraction of Rotavirus dsRNA and its Detection by RNA-PAGE and RT-PCR

Faecal samples were allowed to thaw at room temperature. A cell culture-propagated rotavirus sample that had been confirmed by RNA-PAGE and RT-PCR targeting the *VP6* gene was used as a control sample for both procedures. Extraction of dsRNA of RVA was performed from a 10% suspension of faecal samples (10% w/v in PBS, pH 7.4) using Trizin RNA extraction reagent (M/s. GCC Biotech, India) in accordance with the WHO manual of rotavirus detection and characterization methods (WHO, 2009). The extracted dsRNA was analysed using RNA-PAGE following the protocols outlined by Herring *et al.* (1982). A 7.5% resolving gel and a 5.0% stacking gel were employed to separate the 11 distinct segments of rotavirus dsRNA, which were subsequently visualized through silver staining (Herring *et al.*, 1982).

The RNA extracted from each faecal sample was further analysed for detection of RVA by RT-PCR targeting *VP6* gene using GEN-VP6 F (5'-TTTGATCACTAAYTATTCACC-3') and GEN-VP6 R (5'-GGTCACATCCTCTCACTA-3') primers with an expected amplicon size of 227 bp (Kattoor *et al.*, 2013). For reverse transcription, firstly the complementary DNA (cDNA) was synthesized using 1 µL of each forward and reverse primer (10 pmol concentration), 1.5 µL of dimethyl sulfoxide (DMSO), 5 µL of dsRNA template, and 7 µL of nuclease-free water (NFW; M/s. GCC Biotech, India), resulting in a final volume of 15.5 µL. This reaction mixture was denatured at 95°C for 5 min in a thermocycler and immediately snap chilled on ice. The remaining master mix, consisting of 5 µL of 5X RT buffer, 1 µL of 10 mM deoxynucleotide triphosphates (dNTPs), 2 µL of 100 mM dithiothreitol (DTT), 0.5 µL of RNAse inhibitor (40 U/mL; M/s. Fermentas, USA), and 1 µL M-MuLV- Reverse Transcriptase (200 U/mL; M/s. Fermentas, USA), giving a volume of 9.5 µL was then added to the tube containing the denatured dsRNA yielding a final reaction volume of 25.0 µL. The mixture was incubated at 37°C for 1 h to activate the reverse transcription activity of the M-MuLV RT enzyme, and the reaction was stopped by heating to 65°C for 10 min to inactivate the residual M-MuLV-RT enzyme. The cDNA synthesized was either stored at -20°C or used immediately for RT-PCR.

In RT-PCR, the reaction mixture consisted of 6.25 µL of master mix (M/s. Thermo Fisher Scientific Inc., USA), 1 µL of both GEN-VP6 forward and reverse primer (10 pmol concentration for each), 2 µL of cDNA synthesized earlier, and the remaining 2.25 µL of NFW to make a total volume of 12.5 µL. The PCR was performed in a thermal cycler (M/s. Eppendorf, Germany) with the following cyclical conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles each of 95°C for 30 sec (denaturation), 50°C for 30 sec (annealing) and 72°C for 30 sec (extension), and one cycle of final extension at 72°C for 10 min followed by holding at 4°C.

The cDNA of the cell culture propagated rotavirus sample was used as a positive control, while NFW served as a negative (non-template) control. The PCR products were analysed by agarose gel electrophoresis on a 1.5% agarose gel containing 5 µL/100 mL ethidium bromide, visualized, and photographed using a gel documentation system (M/s. Vilber, France). A PCR product size of 227 bp (for the *VP6* gene) was considered to be positive for RVA detection.

Statistical Analyses

Statistical analyses were performed using R software version 4.2.1. McNemar's test was used to compare detection rates between RNA-PAGE and RT-PCR at a 5% level of significance (α) and 1 degree of freedom (df). P-values <0.05 were considered to be statistically significant. Furthermore, Cohen's Kappa statistic was used to assess agreement between the two methods.

Results and Discussion

Out of 116 diarrhoeic faecal samples collected from calves, 12 tested positive for rotavirus based on the combined results of RNA-PAGE and RT-PCR techniques, yielding an overall positivity rate of 10.34% (12/116)(Table 1). Notably, all RNA-PAGE-positive samples were also detected by RT-PCR, whereas RT-PCR identified additional cases that were negative by RNA-PAGE.

For individual assay results, screening by RNA-PAGE identified 6 samples positive for the rotavirus genome, each displaying the characteristic 11-segmented genome pattern of rotavirus (Fig. 1). The overall prevalence of rotavirus detected by RNA-PAGE was 5.17% (6/116) (Table 1). Among the 103 faecal samples from cattle calves, 6 (5.83%) were positive, while no samples from buffalo calves tested positive for rotavirus by RNA-PAGE (Table 2).

Table 1: Prevalence of rotavirus in calves by RNA-PAGE and RT-PCR testing

S.No.	Source of sample/ Location of farm	Samples collected	Samples positive by RNA-PAGE (%)	Samples positive by RT-PCR (%)
1.	Dairy Farm, Izatnagar	64	5 (7.81 %)	10 (15.63 %)
2.	Gaushala, JogiNawada	21	1 (4.76 %)	1 (4.76 %)
3.	Gaushala, City Station	21	0	0
4.	Livestock Farm, Cantonment	10	0	1 (10.00 %)
Total		**116**	**6 (5.17 %)**	**12 (10.34 %)**

Table 2: Details of farm-wise sample collection and positivity of rotavirus therein

S. No.	Source of sample/ Location of farm	Total no. of samples collected		Samples positive by RNA-PAGE (%)		Samples positive by RT-PCR (%)	
		Cattle calves	Buffalo calves	Cattle calves	Buffalo calves	Cattle calves	Buffalo calves
1.	Dairy Farm, Izatnagar	56	8	5 (8.93 %)	0	9 (16.07%)	1 (12.5 %)
2.	Gaushala, JogiNawada	19	2	1 (5.26 %)	0	1 (5.26 %)	0
3.	Gaushala, City Station	20	1	0	0	0	0
4.	Livestock Farm, Cantonment	8	2	0	0	1 (12.5 %)	0
Total		**103**	**13**	**6 (5.83 %)**	**0**	**11 (10.68%)**	**1 (7.69 %)**

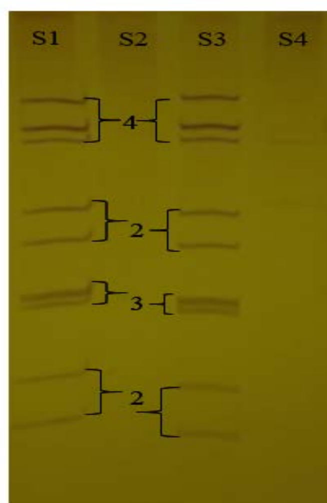

Fig. 1: RNA-PAGE showing characteristic 4:2:3:2 pattern of electrophoretic migration for group A rotaviruses: Lane 1, 3: Positive samples; Lane 2,4: Negative samples

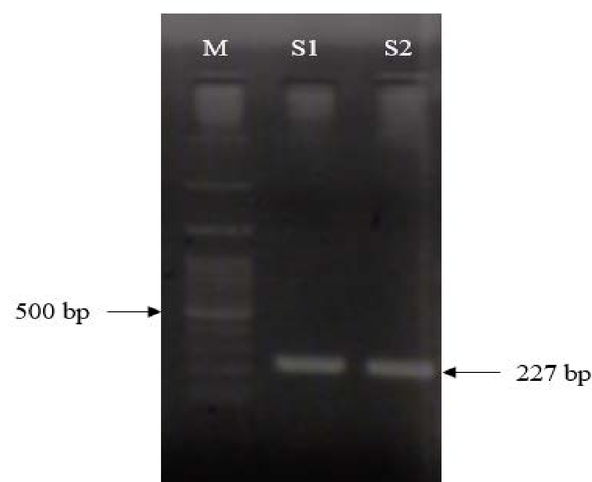

Fig. 2: PCR confirmation of rotavirus by amplification of *VP6* gene (227 bp): Lane M: 100 bp DNA ladder; Lane 2,3: Positive samples

Furthermore, the RNA-PAGE gel revealed the classical electropherogram of the 4:2:3:2 migration pattern corresponding to that of the 11 segments of the RVA in the positive faecal samples. In addition, on the account of the relative migration of the 10th and 11th segments all positive samples displayed a long electropherotype (Fig. 1).

Rotavirus dsRNA can be detected in faecal specimens by extracting the viral RNA and analyzing it by polyacrylamide gel electrophoresis (PAGE), followed by its silver staining. Using the RNA-PAGE technique, all 11 segments of rotavirus RNA can be readily separated based on their molecular weight, producing a characteristic visual pattern after silver staining known as the RNA electropherotype. Following electrophoresis, group A, B, and C rotaviruses display distinct patterns of gene-segment distribution, hence have been with designated different electropherotypes, *viz*., 4:2:3:2 for group A rotaviruses, 4:2:2:3 for group B rotaviruses, and 4:3:2:2 for group C rotaviruses (Dhama *et al*., 2009). These patterns are useful for diagnosis and differentiation among the different rotavirus groups.

RNA-PAGE detected 5.17% (6/116) rotavirus-positive samples in the dairy herds of the study area. Additionally, all 6 isolates detected in this study by RNA-PAGE were identified as typical group A rotaviruses, displaying the characteristic 4:2:3:2 (Class I, II, III, IV) migration pattern. Electropherotypes can be classified as long or short, depending on the relative

migration rates of the 10th and 11th segments. Faster migration of the 11th segment relative to the 10th results in the distinctive long electropherotype, while slower migration results in the short electropherotype (Gulati *et al*., 1998). In our study, all 6 RNA-PAGE-positive samples exhibited the long electropherotype.

For the detection of group A rotavirus, RT-PCR amplification of the partial length *VP6* gene was done using GEN-VP6F and GEN-VP6R primers. The expected 227 bp amplicon of the partial *VP6* gene was successfully amplified in 12 samples (Fig. 2), hence revealing an overall prevalence of 10.34% (12/116) (Table 1). Among the 103 faecal samples from cattle calves, 11 (10.68%) tested positive, while one faecal sample (7.69%) out of 13 from buffalo calves was positive for rotavirus by RT-PCR (Table 2).

RT-PCR is capable of detecting low copy numbers of rotavirus RNA in clinical samples and has thus become a widely used technique for laboratory detection of group A rotaviruses worldwide as well as for genotyping the rotavirus strains (WHO, 2009). Consequently, numerous RT-PCR assays have been developed using primers specific to various rotavirus genes. In the present study, of the 116 diarrhoeic faecal samples screened for the presence of the group-specific *VP6* antigen, twelve samples (12/116; 10.34%) tested positive for group A rotaviruses, amplifying the 227 bp *VP6* gene product.

Previous studies conducted in the same region have reported a similar prevalence of bovine rotavirus in calves. In a study, Malla *et al*. (2022) reported that 6.3% and 12.5% of diarrhoeic calf faecal samples tested positive for rotavirus by RNA-PAGE and RT-PCR respectively, aligning with our findings. Similarly, a study by Rawat *et al*. (2012) revealed positivity rates of 12.4% and 11.4% in diarrhoeic faecal samples from calves under 3 months of age using RNA-PAGE and RT-PCR, respectively, with an overall positivity rate of 13.3%.

There was a statistically significant difference in detection rates between RNA-PAGE and RT-PCR ($p=0.0143 < 0.05$), with RT-PCR showing higher sensitivity in detecting the samples. This is attributed to the relatively higher sensitivity of RT-PCR ($p=0.0143 < 0.05$), which requires a lower viral particle count for detection than RNA-PAGE (WHO, 2009; Vlasova *et al*., 2020). This is supported by findings from a similar study by Dubal *et al*. (2014), where RT-PCR was shown to be more sensitive than RNA-PAGE for detecting rotavirus in faecal samples from both humans as well as animals. Moreover, the substantial agreement between the two methods ($\kappa=0.632$) supports their complementary use in order to obtain a better and more accurate detection rate, which can aid in revealing the true picture of the burden of bovine rotavirus on neonatal calf health in India.

Conclusion

The study confirms the presence of RVA as a significant viral etiological agent of calf diarrhoea in the Bareilly region of Uttar Pradesh, India. Further research is needed to investigate the prevalent genotypes to understand their epidemiological as well as zoonotic implications. In the wake of the substantial agreement between the two different diagnostic techniques (RT-PCR & RNA-PAGE), this study also highlights the importance of using a combination of detection techniques rather than relying on a single method to improve the detection rate and diagnostic efficacy for rotavirus in clinical samples.

Acknowledgement

The authors gratefully acknowledge the Director and Joint Director (Research) of the ICAR-Indian Veterinary Research Institute, Izatnagar, for providing the necessary facilities to conduct this research work. They also thank the Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India, for funding this study, and the farm in-charges/personnel for their consent and cooperation with sample collection.

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