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Immunological and molecular detection of *Bovine rotavirus* from diarrhoeic calves

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Abstract
This study was conducted to detect *Bovine Rotavirus* in diarrhoeic calves using immunological and molecular diagnostic techniques, namely Lateral Flow Assay (LFA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). A total of 50 faecal samples were collected from diarrhoeic calves in the Junagadh region, considering factors such as age, sex, species, season, feeding practices and colour of diarrhoea. All samples were initially screened using a commercial LFA kit (Anigen Rapid Rota Ag Test Kit, BioNote) for rotaviral antigen detection. Viral RNA was extracted using the RNA Sure® Virus Kit, followed by cDNA synthesis and RT-PCR targeting the VP6 and VP7 genes of group A rotaviruses. Results showed that 4 out of 50 samples (8%) were positive by both LFA and RT-PCR. The study confirms the presence of *Bovine Rotavirus* in diarrhoeic calves of the Junagadh region. LFA proved useful for rapid field screening, while RT-PCR remains the preferred confirmatory diagnostic method due to its higher sensitivity and specificity.

Keywords: *Bovine Rotavirus*, Diarrhoea, Lateral Flow Assay, RT-PCR, VP6 and VP7 genes

1. Introduction
Rotavirus is a major cause of acute gastroenteritis in calves, humans, and several other species (Dhama *et al.*, 2009; Martella *et al.*, 2010) [6, 17]. In calves, *Bovine Rotavirus* is the leading infectious agent of neonatal calf diarrhoea (NCD), commonly termed "white scour," and it most frequently affects animals between 1-2 weeks of age (Dhama *et al.*, 2009) [6].
Rotavirus, a member of the *Reoviridae* family, is a non-enveloped virus with a triple-layered capsid and an 11-segment double-stranded RNA genome (Estes and Kapikian, 2007) [7]. These segments encode structural proteins VP1-VP7 and non-structural proteins NSP1-NSP6. VP6, a highly conserved inner capsid protein, is used to classify rotaviruses into groups A-H, with evidence of a potential ninth group (Matthijnssens *et al.*, 2012; Mihalov-Kovács *et al.*, 2015) [18, 19]. The outer capsid proteins VP4 (P type) and VP7 (G type) are key immunogenic markers, and their reassortment results in diverse circulating genotypes (Kadam *et al.*, 2019) [12].
Group A *Bovine Rotavirus* is the primary cause of NCD and contributes substantially to economic losses, estimated at around USD 9.5 million annually (Silva *et al.*, 2012) [25]. Infection leads to 5-20% mortality in neonatal calves and shows a global prevalence of 30-40% (Dhama *et al.*, 2009; Swiatek *et al.*, 2010) [6]. In India, *Rotavirus* accounts for 10-52% of diarrhoeal cases in calves younger than one month (Nataraju *et al.*, 2009) [20].

2. Materials and Methods
2.1 Sample collection from calves
A total of 50 faecal samples or rectal swabs were collected from diarrhoeic calves. The samples were collected following the standard method described by Yilmaz (2016) [30]. An aliquot of each faecal sample was diluted in phosphate-buffered saline (PBS, pH 7.2). A 10% (w/v) faecal suspension was prepared using PBS. The suspension was centrifuged at 10,000 rpm for 30 minutes at 4 °C to remove coarse debris and bacteria. The resulting clarified supernatant was stored at -20 °C until further use for RNA extraction.

2.2 Lateral flow assay test (LFA)
The Anigen Rapid Rota Ag test kit used in the study was manufactured by BioNote, Inc. (South Korea).

2.3 Reverse transcriptase Polymerase Chain Reaction (RT-PCR)
2.3.1 Isolation of viral RNA
The extraction of viral RNA was a critical step in the detection of RNA viruses. Viral RNA was extracted from the collected faecal samples using the RNA Sure® Virus Kit (Genetix, Asia Biotech Pvt. Ltd., New Delhi), following the manufacturer's protocol. The extracted RNA was stored at -20 °C until further use.

2.3.2 Complementary DNA (cDNA) Synthesis
cDNA was synthesized using the First Strand cDNA Synthesis Kit following the manufacturer's instructions.

2.3.3 Amplification of VP6 & VP7 Gene of *Rotavirus*

Table 2.1: Nucleotide sequences of primers used for identification of *Bovine Rotavirus*

Primers	Sequence (5'- 3')	Product size	Reference
VP6 - F	GACGGVGCRACTACATGGT	379bp	(Falcone *et al.*, 1999)
VP6 - R	GTCCAATTCATNCCTGGTGG		
VP7 - F	GATCCGAATGGTTGTGTAATCCAAT	304bp	**(**Husain *et al.*, 1995)
VP7 - R	AAT TCG CTA CGT TTT CTCTTGG		

Table 2.2: Components of reaction mixture for amplification of VP6 & VP7 gene

Sr. No	Components	PCR Reaction
1	PCR Master Mix (2x)	12.5 µl
2	Forward Primer (10pmol/ µl)	1 µl
3	Reverse Primer (10pmol/ µl)	1 µl
4	Template DNA	3 µl
5	NEW (Nuclease Free Water)	7.5 µl
Total Reaction volume		25 µl

Table 2.3: Thermocycling conditions used for PCR to amplify the VP6 & VP7 gene

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	03 minutes	1
Denaturation	94 °C	30 Second	35
Annealing	51 °C	01 minutes	
Extension	72 °C	01 minutes	
Final extension	72 °C	10 minutes	1

3. Results and Discussion
The samples were screened for the presence of *Bovine Rotavirus* using both Lateral Flow Assay (LFA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR), targeting the VP6 and VP7 genes. Out of the 50 samples, 4 (8.00%) tested positive for *Rotavirus* by LFA, and the same number of samples (4/50, 8.00%) were confirmed positive by RT-PCR.

3.1 Detection of *Bovine Rotavirus* by Lateral flow assay test (LFA)
Out of the 50 diarrhoeic faecal samples tested for *Rotavirus* using the Lateral Flow Assay (LFA), 4 samples (8.00%) tested positive. The positive results were indicated by the presence of clear lines in both the test and control areas of the device, while negative samples showed only the control line (Figure 1).

The overall prevalence of *Bovine Rotavirus* detected by LFA in the present study aligns with the findings of Uddin-Ahmed *et al.* (2022) [29], who reported an overall prevalence rate of 8.54%. However, Malla *et al.* (2024) [16] observed a slightly lower prevalence of 7%. In contrast, several other studies have reported significantly higher prevalence rates: Klein *et al.* (2009) [14] - 71.87%, Içen *et al.* (2013) [11] - 25%, Hassan *et al.* (2014) [9] - 36%, Abouelyazeed *et al.* (2021) [1] - 16.2%, and Barua *et al.* (2021) [2] - 10%.

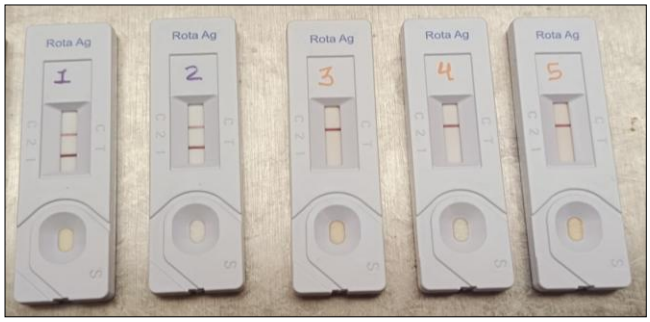

Fig 1: Lateral flow assay kit showing isolate 1 and 2 observed both test and control line that indicate positive and isolate 3,4 and 5 observed only control line that indicate negative

3.2 Detection of *Bovine Rotavirus* by Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)
For determining the overall prevalence, RT-PCR results were considered more definitive. The successful amplification of VP6 and VP7 gene fragments in these 4 samples confirmed the presence of *Rotavirus* infection in the calves (Figure 2 and 3).

The overall *Rotavirus* prevalence of 8.00% observed in this study is comparatively lower than the findings of several earlier studies. For instance, higher prevalence rates have been reported by Basera *et al.* (2010) [3] - 10.15%, Pourasgari *et al.* (2016) [23] - 49.4%, Makwana *et al.* (2020) [15] - 13.11%, and Chen *et al.* (2022) [4] - 46.00%. In contrast, several studies have reported lower prevalence of *Bovine*

Rotavirus, including Udaykar *et al.* (2013) [28] - 4.3%, Debelo *et al.* (2021) [5] - 3.64%, Singh *et al.* (2021) [26] - 5.02% and Nayak *et al.* (2018) [21] - 1.93%.

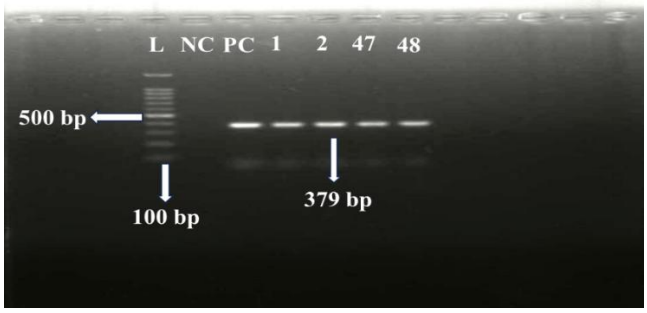

Fig 2: RT-PCR of *Bovine Rotavirus* for VP6 gene (379 bp)

(L: 100 bp plus ladder; PC: Positive control; Well characterized *Rotavirus* sample 1, 2, 47, 48: Samples positive for *Bovine Rotavirus*; NC: Negative control)

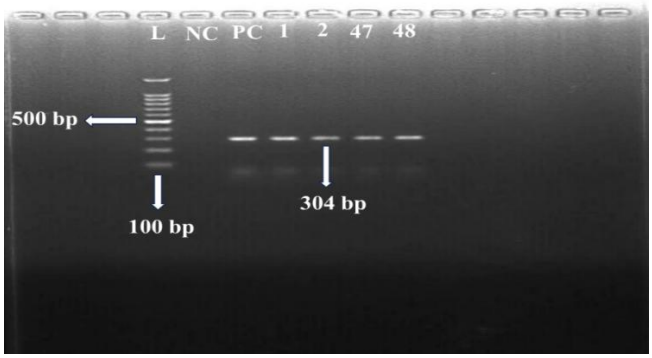

Fig 3: RT-PCR of *Bovine Rotavirus* for VP7 gene (304 bp)

(L: 100 bp plus ladder; PC: Positive control; Well characterized *Rotavirus* sample 1, 2, 47, 48: Samples positive for *Bovine Rotavirus*; NC: Negative control)

3.3 Comparison of LFA & RT-PCR for the detection of *Bovine Rotavirus*

In this study, 50 faecal samples from bovine calves were tested for *Bovine Rotavirus* using Lateral Flow Assay (LFA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Both methods detected *Rotavirus* in 4 samples (8%), and statistical analysis showed no significant difference between the two techniques (Chi-Square test value: χ^2 = 0.00, Degrees of Freedom: df = 1, P-value = 1.00). This indicates complete agreement in the number of positives identified by LFA and RT-PCR.LFA demonstrated 100% sensitivity and 100% specificity when compared with RT-PCR, suggesting excellent diagnostic accuracy. However, the small sample size (n = 50) limits generalization, and larger-scale studies are needed to confirm the reliability of LFA in field conditions.

The findings differ from previous studies, which reported lower LFA sensitivity while maintaining high specificity. Reported sensitivities and specificities include: 81.9% and 98.2% (Nemoto *et al.*, 2010) [22], 78.7% and 100% (Khamrin *et al.*, 2011) [13], 83% and 100% (Sakli *et al.*, 2019) [24], and 74.54% and 98.44% (Malla *et al.*, 2024) [16]. These variations indicate that LFA performance may differ across populations and study conditions, emphasizing the need for broader evaluation.

3.4 Species-wise prevalence of *Bovine Rotavirus*

Table 3.1: Species-wise prevalence of *Bovine Rotavirus*

Species (calves)	No. of samples tested	No. of samples positive	Prevalence %
Buffalo	02	00	00
Cattle	48	04	08
Total	50	04	08

The species-wise prevalence of *Bovine Rotavirus*, as detected by RT-PCR, was found to be higher in cattle calves compared to buffalo calves. The findings of the present study are consistent with those of Ade *et al*. and Makwana *et al.* (2020) [15], who also reported a higher prevalence of *Bovine Rotavirus* in cattle calves than in buffalo calves. However, the present results contrast with the observations of Malik *et al.* (2013) [28], Udaykar *et al.* (2013) [28] and Sthevaan *et al.*, who reported a slightly higher infection rate in buffalo calves compared to cattle calves, with prevalence rates of 22.01% in buffalo calves vs. 13.33% in cattle calves, 4.76% vs. 3.77%, and 24.24% vs. 10.34%, respectively.

3.5 Age-wise prevalence of *Bovine Rotavirus*

Table 3.2: Age-wise prevalence of *Bovine Rotavirus*

Age groups (Days)	No. of samples tested	No. of samples positive	Prevalence %
I (1-15)	10	1	10.00
II (16-30)	26	2	7.70
III (31-45)	4	1	25.00
IV (45-60)	5	0	00
V (> 60)	5	0	00
Total	50	4	8.00

The highest incidence was observed in the 31-45 days age group, with a prevalence rate of 25.00% (1/4), followed by 10.00% (1/10) in the 1-15 days group, 7.70% (2/26) in the 16-30 days group, and 0.00% (0/5) in both the 45-60 days and >60 days age groups. This indicates that *Rotavirus* infection was most prevalent in calves aged 31-45 days, followed by those in the 1-15 day and 16-30 days groups.

However, these findings contrast with those reported by Patel *et al.* (2019), Makwana *et al.* (2020) [15], and Golaviya *et al.*, who observed higher prevalence rates of *Bovine Rotavirus* infection in the youngest age group (1-15 days), with reported rates of 38.46%, 11.58%, and 47.40% respectively.

3.6 Sex-wise prevalence of *Bovine Rotavirus*

Table 3.3: Sex-wise prevalence of *Bovine Rotavirus*

Sex	No. of samples tested	No. of samples positive by LFA & Prevalence	No. of samples positive by RT-PCR & Prevalence
Female	21	2 (9.50%)	2 (9.50%)
Male	29	2 (6.90%)	2 (6.90%)
Total	50	4 (8.00%)	4 (8.00%)

The prevalence among females was found to be 9.5% (2/21), while in males it was slightly lower at 6.9% (2/29). Although the prevalence was marginally higher in females, the difference was not statistically significant due to the limited sample size.

Similar findings were reported by Makwana *et al.* (2020) [15], who observed a higher susceptibility to *Bovine Rotavirus* among female calves (10.61%) compared to male calves (6.81%). In contrast, Dash *et al.* (2011) reported a higher prevalence of *Rotavirus* in male diarrhoeic calves (20.40%) compared to female diarrhoeic calves (12.80%).

3.7 Season -wise prevalence of *Bovine Rotavirus*

Table 3.4: Season -wise prevalence of *Bovine Rotavirus*

Season	No. of samples tested	No. of samples positive by LFA & Prevalence	No. of samples positive by RT-PCR & Prevalence
Winter	15	2 (13.33%)	2 (13.33%)
Summer	29	0 (0.00%)	0 (0.00%)
Monsoon	6	2 (33.33%)	2 (33.33%)
Total	50	4 (8.00%)	4 (8.00%)

The seasonal analysis revealed the highest prevalence of *Bovine Rotavirus* during the monsoon season (33.33%), followed by winter (13.33%), with no cases detected in the summer. Similar findings were reported by Fernandes *et al.*, who observed an increased detection of *Rotavirus* in calves during the rainy season in Brazil—a period comparable to the monsoon in India—likely due to higher humidity and favorable environmental conditions for viral survival. In contrast, Singh *et al.* (2021) [26] reported a higher prevalence of *Rotavirus* A (RVA) infection during the winter season compared to other seasons. Likewise, Ahmad Malla *et al.* (2022) [16] observed the highest prevalence in winter (28.6%), followed by autumn (12.5%).

3.8 Feeding -wise prevalence of *Bovine Rotavirus*

Table 3.4: Feeding -wise prevalence of *Bovine Rotavirus*

Feeding	No. of samples tested	No. of samples positive by LFA & Prevalence	No. of samples positive by RT-PCR & Prevalence
Milk (Colostrum)	21	2 (9.52%)	2 (9.52%)
Milk + Fodder	25	2 (8.00%)	2 (8.00%)
Only fodder	4	0 (0.00%)	0 (0.00%)
Total	50	4 (8.00%)	4 (8.00%)

Samples categorized based on feeding practices, the highest prevalence was observed in calves fed exclusively on colostrum, with 2 out of 21 samples (9.52%) testing positive. A similar number of positive cases (2 out of 25; 8.00%) was recorded among calves that received a combination of milk and fodder. Notably, no positive cases were detected among the four calves that were fed only fodder.

This finding is consistent with the observations of Hou *et al.*, who reported a higher prevalence of *Bovine Rotavirus* in milk-fed calves, with a positivity rate of 14.58%, which was significantly higher than the rate found in older or weaned calves (approximately 8.75%).

3.9 Symptoms (Colour of diarrhoea) - wise prevalence of *Bovine Rotavirus*

Table 3.4: Symptoms (Colour of diarrhoea) - wise prevalence of *Bovine Rotavirus*

Symptoms (Colour of diarrhoea)	No. of samples tested	No. of samples positive by LFA & Prevalence	No. of samples positive by RT-PCR & Prevalence
White	12	3 (25.00%)	3 (25.00%)
Yellow	16	1 (6.25%)	1 (6.25%)
Green	17	0 (0.00%)	0 (0.00%)
Bloody	5	0 (0.00)	0 (0.00)
Total	50	4 (8.00%)	4 (8.00%)

In the present investigation involving 50 diarrhoeic calf samples, the prevalence of *Bovine Rotavirus* varied based on the colour of the diarrhoeic faeces. The highest prevalence was observed in cases of white-coloured diarrhoea, where 3 out of 12 samples (25.0%) tested positive using both LFA and RT-PCR. Yellow-coloured diarrhoea showed a lower prevalence, with 1 out of 16 samples (6.25%) testing positive. No positive cases were detected among the samples with green (17 samples) or bloody (5 samples) diarrhoea.

These findings contrast with those reported by Singh *et al.* (2021) [26], who observed a higher prevalence of *Rotavirus* infection in calves exhibiting pasty yellow-coloured faeces compared to those with watery diarrhoea. Similarly, Debelo *et al.* (2021) [5] reported the highest prevalence of *Rotavirus* (30.8%) in calves with watery diarrhoea.

4. Conclusion

The present study highlights the importance of Rotavirus as one of the major etiological agents of diarrhoea in bovine calves, contributing to considerable economic losses in both the dairy and meat industries. For the detection of *Bovine Rotavirus* antigen, diarrhoeic samples should be screened using LFA and RT-PCR. The findings indicated that there was no significant difference in the diagnostic performance of these two tests.

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