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Detection of bovine rotavirus (BRV) infection in neonatal calves of in and around Navsari district of South Gujarat, India

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Abstract
The present study reports the incidence of bovine rotavirus infection in bovine calves of in and around areas of Navsari district. 157 faecal samples comprising (122 diarrheic and 35 non diarrheic) from 104 cattle and 53 buffalo calves of < 45 days' age were collected. All the samples were screened by LAT, ELISA and VP6 gene based RT-PCR and 17 (10.82%), 11 (7.0%) and 16 (10.19%) samples were found positive respectively. Diarrheic sample wise 13.11% incidence was recorded while all the non diarrheic samples were found negative. Species wise higher incidence was observed in cattle calves (10.58%) than buffalo calves (9.43%). Sex wise higher incidence was observed in female calves (10.61%) than male calves (6.81%). Age wise higher incidence was recorded in 0-15 days age group (11.58%) followed by 15-30 days age group (10.26%) and 30-45 days age group (4.35%).

Keywords: LAT, ELISA, BRV, TLP, RT PCR

1. Introduction
Diarrhea is one of the important causes of calf mortality, morbidity and economic losses in the dairy industry, especially in developing countries [1]. Calf diarrhea is attributed to both infectious and non-infectious factors. Multiple gastro-enteric pathogens (e.g., viruses, bacteria, and protozoa) are involved in the development of this disease. Amongst gastro enteric infectious agents, Rotavirus, Coronavirus, *Cryptosporidium* and *Escherichia coli* are collectively responsible for 75-95% of infection in neonatal calves worldwide and Rotavirus alone accounts for about 27- 36% [2, 3]. Rota-viral diarrhea are common in calves and affected young calves may die as a result of severe dehydration or secondary bacterial infections which in turn causes major economic losses [4].
Rotavirus belongs to the genus *Rotavirus* of the *Reoviridae* family possess distinct cartwheel structure while viewed in electron microscopy. The fully infectious RV particle is also termed as triple-layered particle (TLP) which is similar to wheels (latin word *Rota* means wheel) and this has led to the name of rotavirus for the genus [5]. The virus particles are about 80-100 nm in diameter, have icosahedral symmetry and has a segmented genome with 11 segments of double stranded (ds) RNA. The virus particle possesses 6 structural (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 non-structural (NSP1, NSP2, NSP3, NP4, NSP5, NSP6) proteins. The virus consists of three concentric proteinaceous layers, the inner most viral core is formed of VP2 molecules, the VP1, VP3 and the dsRNA genome lie inner to the VP2 protein layer. The middle layer is composed of VP6 molecules and outer layer (outer capsid) is composed of VP4 and VP7 protein molecules.
Many enteric pathogens are responsible for neonatal calf diarrhea and therefore confirmatory diagnostic tests are necessary for specific agents. From Navsari district, few studies pertaining to bacterial neonatal calf diarrhea has been carried out, but study regarding virus association is significantly low, so present study was aimed to detect incidence of rotavirus infection in bovine neonatal calves this region.

2. Materials and Methods
2.1 Sample collection from calves
A total of 157 faecal samples comprised of 122 diarrhoeic and 35 non diarrhoeic from 104 cattle and 53 buffalo calves were aseptically collected.

Calves ageing from birth to 45 days of age were selected and grouped as < 15 days, 15- 30 days and 30 -45 days from different regions in and around regions of Navsari district. Collected samples were immediately transported with ice pack to the department and then initially screened by LAT and then stored at -20 ^{0}C for processing by other tests.

2.2 Latex agglutination test (LAT)
LAT was performed to detect rotavirus antigen from faecal samples for initial screening of all the samples using HiRotavirus Latex Test Kit (HiMedia Laboratories, Mumbai, Cat. No LK08-50NO) as per the kit instructions.

2.3 Enzyme Linked Immunosorbent Assay (ELISA)
For antigenic diagnosis of bovine rotavirus, Bio X Bovine Rota virus ELISA kit (Bio X diagnostic, Belgium, Product ID - BIO K 343/2) was used and performed as per the kit instructions. The 96 well plate is coated with by specific antibodies for the rotavirus which allows specific capture of the rotavirus antigens present in the fecal samples. The net optical density of each sample was calculated at 450 nm by formula given by kit and value that obtained ≥ 22% considered as positive sample.

$$\text{Value} = \frac{\text{Delta OD of Sample}}{\text{Delta OD of Positive}} \times 100$$

2.4 Reverse transcriptase Polymerase Chain Reaction (RT-PCR)
2.4.1 Extraction of Viral RNA
Pure quality viral RNA was extracted from faecal supernatants by QIAamp® Viral RNA Mini kit (Cat. No. 52904) as per the kit protocol. The extracted total RNA was quantified using nanodrop (NanoDrop 1000, Genaxy) at the ratio of 260/280 and stored at -20 ºC until further use.

2.4.2 Processing of RNA samples for one step RT PCR
The pure quality RNA (3.0 µl**)** was taken in the RNAse free PCR tube and kept at 95ºC for 5 minutes in thermal cycler and immediately snap chilled on ice. Then it was used for one step RT-PCR technique.

2.4.3 Method of one step RT- PCR
One step RT- PCR was performed by using one step RT PCR kit (SuperScript™ III one step RT-PCR kit with platinum™ *Taq* DNA polymerase) as per the kit manufacturer instructions. Reaction mixture was prepared for RT- PCR (Table 1) and immediately added into the PCR tube containing snap chilled RNA. Primers (Table 2) for the present study were send for custom synthesis from Eurofins Genomic Pvt. Ltd., Bangalore.

Table 1: Reaction mixture for RT PCR

Component	Volume
2X Reaction Mix	25 µl
Forward primer (VP6)	1 µl
Reverse primer (VP6)	1 µl
SuperScript™ III RT	2 µl
Autoclaved distilled water	18 µl
RNA sample	3.0 µl
Total reaction volume	50.0 µl

Table 2: Primers used for detection of VP6 gene in RT-PCR technique

Gene	Primers Sequence (5´–3´)	Amplicon size
VP6- F	5'GAC GGV GCR ACT ACA TGG T3'	379bp
VP6- R	5'GTC CAA TTC ATN CCT GGT GG3'	

Table 3: Thermal cycler condition for Reverse Transcriptase PCR (RT-PCR)

cDNA Synthesis and pre-denaturation		Denaturation	Annealing temp according to primer	Extension	Final extension
1 cycle		35 Cycles			1 cycle
55 º C	94 ºC	94 ºC	47 ºC	68 ºC	68 ºC
30 minutes	2 minutes	15 seconds	30 seconds	1 minute	5 minutes

Thermal cycler condition to amplify VP6 gene was set/ followed as per the kit instructions. The annealing temperature was set 10ºC than the melting temperature of primer according to kit instructions. Hence, final thermal cyclic condition used for RT-PCR described in Table 3.

For confirmation of desired amplified gene, 8.0 µl of PCR product was mixed with 5X gel loading dye (2.0 µl) using 1 X TBE (Tris Borate EDTA) buffer along with 100 bp DNA molecular weight marker (6.0 µl) and analyzed on 1.5% agarose gel containing 0.5µg/ml ethidium bromide and Electrophoresis was run at 90 V for 1 hour. The amplified gene was examined under UV transilluminator and photographed in gel documentation unit (SynGene, Gene Genius Bio Imaging System, UK).

2.5 Statistical analysis
Species wise, sample wise, sex wise age wise, season wise statistical analysis were calculated by WASP - Web Agri Stat Package Developed by Ashok Kumar Jangam and Pranjali Ninad Wadekar at ICAR Research complex, Goa.

3. Results and Discussion
All the 157 samples were screened for the detection of bovine rotavirus. Out of these 17 (10.82%), 11(7.00%) and 16 (10.19%) samples were found positive by LAT, ELISA and RT- PCR respectively.

3.1 Detection of bovine rotavirus by Latex agglutination test (LAT)
Out of 157 samples tested total 17 (10.82%) samples were found positive by LAT that showed clear visible agglutination in faecal samples (Figure 1). Similar to this 10.86% positivity of bovine rotavirus was recorded [6] by other researchers. Compared to this higher positivity 12.80% [7], 23.93% [8], 63% [9], 65% [10], and 91% [11] as well as lower positivity 9.60% [12], 6.75% [13] observed by various workers.

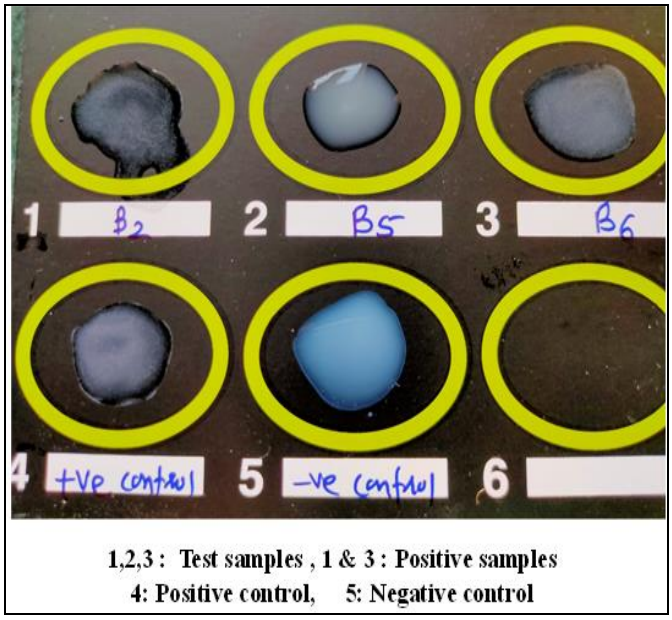

Fig 1: Detection of bovine rotavirus by Latex agglutination test

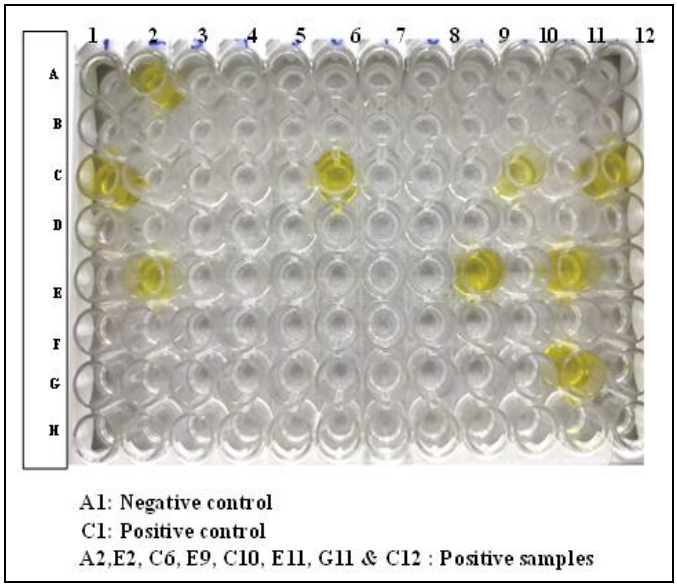

Fig 2: Detection of bovine rotavius by ELISA

3.2 Detection of bovine rotavirus by ELISA

In the present study total 11 (7.00%) samples found positive by antigen capture ELISA (Figure 2). Value of each sample was calculated as per kit formula and value that obtained ≥ 22% considered as positive sample. Similar to this findings various workers have used antigen detection based ELISA and reported lower positivity 7.0% and 4.61% by ELISA respectively [14, 15]. While others detected higher positivity 24.10%, 9.59%, 32.5%, 43.5%, 29.4%, 15.38% [16-20, 8] respectively, compared to present study.

3.3 Detection of bovine rotavirus by Reverse Transcriptase Polymerase Chain Reaction (RT- PCR) by detecting VP6 gene

In the present investigation out of 157 samples 16 (10.19%) samples amplified for VP6 gene specific amplicon at 379 bp and revealed 10.19% positivity (Figure 3). Contrast to this lower infection rate 6.89% was recorded by others [21]. While others detected [22, 23, 4, 8] 24.10%, 36.22%, 22.28%, 22.22% higher infection rate by VP6 gene based RT-PCR.

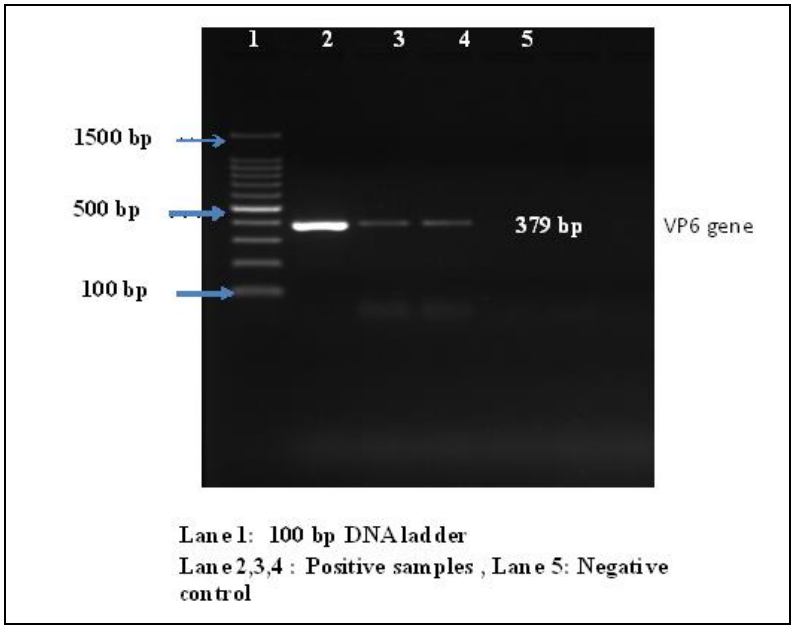

Fig 3: Detection of bovine rotavirus by RT - PCR

3.4 Overall detection of bovine rotavirus

As for confirmation of virus either virus isolation or nucleic acid based technique is more reliable, overall detection of rotavirus was considered on the basis of RT PCR.

In the present study, overall positivity of rotavirus was detected 10.19%. Similar to this other workers [24] detected 10.0% positivity. Compared to this higher prevalence 63.50% [25], 91% [11], 32.07% [26], 43.5% [19], 19.16%[27], 65%[10], 13.10% [28], 39.13% [8] as well as lower incidence 3%, 6.75%, 8.92%, 6%, 6.89%, 5.11% detected by various other workers [29, 13, 30, 31, 21, 1] respectively.

For the present study, species wise, sample wise, sex wise, age wise and season wise positivity of rotavirus was considered on the basis of positivity of RT PCR (Table 4).

Table 4: Detection of bovine rotavirus in bovine calves

Parameter		Total number of samples screened	Number of samples positive	%	χ^2 value
Species	Cattle	104	11	10.58	0.050 (*P*>0.05)
	Buffalo	53	05	9.43	
Type of sample	Diarrhoeic	122	16	13.11	5.111*(*P*≤0.05)
	Non diarrhoeic	35	00	00	
Sex	Male	44	04	9.09	0.081 (*P*>0.05)
	Female	113	12	10.61	
Age	<15 days	95	11	11.58	1.058 (*P*>0.05)
	15-30 days	39	04	10.26	
	30-45 days	23	01	4.35	
Season	Summer	12	01	8.33	0.061 (*P*>0.05)
	Rainy	34	03	8.82	
	Winter	111	12	10.81	

3.4.1 Species wise incidence of bovine rotavirus
In the present study, cattle calves showed 10.58% higher infection as compared to buffalo calves 9.43%. Similar to this, others have [32] detected more prevalence in cow calves (41.6% by RNA PAGE and 33.3% by ELISA) than buffalo calves (20% by RNA PAGE and 24% by ELISA). In contrast to this lower prevalence in cattle calves than buffalo calves (22.73% and 77.78%) and (13.33% and 22.01%) was reported [26, 33] respectively.

3.4.2 Sample wise incidence of bovine rotavirus
In the present study, 16 (13.11%) diarrhoeic samples found positive while from 35 non diarrheic samples all the samples were found negative. This result shows significant difference between diarrheic and non diarrheic samples for detection of rotavirus. Similar to this, others [23, 34] have also found positive samples from diarrheic calves while all the non-diarrheic samples were found negative.

3.4.2 Sex wise incidence of bovine rotavirus
Sex wise, female calves showed higher incidence 10.61% than male calves 9.09%. This finding showed agreement with other researchers [35, 36] who recorded higher infection rate in female than male. In contrast to this others [34, 8] found higher positivity in male than female calves.

3.4.3 Age wise incidence of bovine rotavirus
In the present study, highest infection rate was observed in age group pertaining to 0-15 days 11.58% followed by 15-30 days 10.26% and then in 30-45 days 4.35%. These findings are in accordance with various workers [8, 19, 35] who also observed higher infection rate in early days of age group i.e. 1-15 days. The age wise higher infection in present study in earlier age (0-15 days) might be due to immune system of very young calves is not fully mature to fight against infection. Other workers [37-39] also revealed that diarrhea in calves from 5 to 10 days of age is commonly due to rotavirus infection. Similarly other workers [34, 40-42] reported that the susceptibility of bovine calves to rotavirus infection decreases with age, probably due to loss of receptors on enterocytes. In the present study, lower infection rate in 30-45 days age group compared to 0-15 days age group recorded that may be due to with progression of age calves acquire increased natural resistance against enteropathogens [43, 44, 35, 19].

3.4.4 Season wise incidence of bovine rotavirus
In the present investigation, highest positivity (10.81%) found during winter month followed by rainy season (8.82%) and summer (8.33%). In accordance with this, others [1, 31] also detected higher prevalence in winter season compared to summer season. Various other workers also stated that diarrhea in calves due to rotavirus mostly occurs during winter periods and it has been observed that the morbidity also increases during adverse weather conditions [45-47]. This may be due to overcrowding of population and cold temperature leads to stress to younger calves.

4. Conclusion
Present study reveals importance of Rotavirus as a one of etiological agent of diarrhea in bovine calves and has been associated with significant economic losses to dairy industry. For detection of bovine rotavirus antigen diarrhoeic samples should be screened by LAT and ELISA for initial screening of samples while for confirmation of rotavirus nucleic acid detection based technique should be used. As there is variation was seen in positivity by different tests used combination of two or more teats should be used.

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