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Determining genetic diversity of prevalent G and P genotype of *Bovine Rotavirus A* from neonatal calves of Gujarat, India

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ABSTRACT

Importance: Neonatal calf diarrhea is a major cause of mortality in newborn calves worldwide, posing a significant challenge in bovine herds. *Group A Bovine Rotaviruses* (*BRVA*) are the primary contributors to severe gastroenteritis in calves under two months old.
Objectives: This study examined the prevalence and molecular characterization of *BRVA* in neonatal calves in Gujarat, India.
Methods: Sixty-nine diarrheic fecal samples were collected and subjected to various molecular methods of *BRVA* detection, isolation, and characterization.
Results: The latex agglutination test (LAT), electropherotyping (RNA-PAGE), and reverse transcription polymerase chain reaction revealed positivity rates of 39.13%, 20.30%, and 37.70%, respectively. RNA-PAGE identified 11 bands with a 4:2:3:2 migration pattern, indicative of the segmented genome of *BRVA*. *BRVA* was successfully isolated from LAT-positive samples, with 26 samples exhibiting clear cytopathic effects upon passage in MA-104 cell lines. Genotyping identified G10 as the predominant G genotype, with P[11] genotypes comprising 76.92% of the isolates. The most common G/P combination was G10P[11], highlighting its zoonotic potential.
Conclusions and Relevance: These findings underscore the importance of molecular detection and genotyping for effective vaccine development. This study provides crucial insights into the prevalent G and P genotypes of *BRVA* in Gujarat, India, aiding in the development of targeted control measures.

Keywords: Diarrhea viruses, bovine viral; neonatal calves; cell culture; electropherotyping; genotyping techniques

INTRODUCTION

Neonatal calf diarrhea is a significant cause of morbidity and mortality in neonates of farm animals worldwide, which can exceed 20% in certain instances [1]. Several factors that influence the etiology of this syndrome include neonatal calf health, dietary and hygiene management, immunological aspects, and many infectious agents [2]. Worldwide, *Bovine*

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Conflict of Interest
The authors declare no conflicts of interest.

Data Availability Statement
No sequence data were generated during this study.

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rotavirus A (*BRVA*) is the most common viral pathogen associated with severe gastroenteritis and significant mortality in dairy calves before weaning [3]. In India, the prevalence of rotavirus diarrhea in calves younger than one month of age is 10%–52% [4].

Rotavirus is a member of the family Reoviridae, genus Rotavirus, with a distinct "cartwheel" structure in negative electron microscopy. It is a nonenveloped virion composed of a triple-layered icosahedral symmetry surrounding 11 dsRNA segments that encode six structural (VP1–VP4, VP6, and VP7) and six non-structural (NSP1–NSP5/6) proteins. The triple protein coat of the virus helps it escape unaffected from the acidic pH of the stomach and the digestive enzymes in the gut [5]. Rotaviruses mainly replicate in the mature villous enterocytes and destroy them. As the infected calf sheds a large amount of virus (up to 10^{11} viral particles per gram of feces), it can be diagnosed easily by electron microscopy, latex agglutination test (LAT), enzyme-linked immunosorbent assay, polyacrylamide gel electrophoresis (PAGE), and isolated in cell cultures [6].

The genetic and antigenic characteristics of the rotavirus internal capsid protein VP6 are used to distinguish rotaviruses into groups and subgroups. Rotavirus is classified into ten groups, from A to J. RVA is a major cause of rotaviral diarrhea in birds and animals, of which bats are believed to be the reservoir [7]. RVB, RVC, RVE, RVH, and RVI have been detected in mammalian hosts; RVD, RVF, and RVG have been detected only in birds, while RVI and RVJ were recently discovered in dogs and bats, respectively [8,9]. The sequence of the outer layer proteins VP7 (glycoprotein) and VP4 (protease-sensitive protein) are used for the binary classification of rotavirus strains in terms of their G and P genotypes, respectively. These proteins are responsible for the independent induction of neutralizing antibodies and are essential in host resistance against disease. Thus far, 41 G and 57 P types have been reported among rotaviruses [10]. In bovines, G6, G8, and G10 are the most predominant G types, while in P types, P[1], P[5], and P[11] have been recorded in the majority of bovine diarrheal cases.

The emergence of unusual G/P genotypes (due to genetic shifts/drifts) in cattle proves that humans and other animals may be a source of the evolutionary diversification of bovine rotaviruses. Nevertheless, data on the genetic characterization of bovine RVs from India are scarce, particularly those with atypical genotypes [11]. Determining the genotype(s) of the *BRV* strains that cause infections is one of the critical factors in developing effective vaccines and preventing severe dehydrating diarrhea in newborn calves. This study examined the incidence of *BRVA* using different diagnostic techniques to identify the prevalent G and P genotypes circulating in dairy calves in the Gujarat state of India.

METHODS

Ethical statement

This animal study did not require ethical review and approval because only diarrheic samples were collected from neonatal bovine calves.

Specimen collection

Sixty-nine diarrhoeic fecal samples of bovine calves of 0–9 weeks of age (Cattle = 53, Buffalo = 16; male and female) were collected from the Anand and Surat district (Gujarat, India) farms from November 2018 to February 2019. Metadata, such as age, sex, geographical regions, and species, were collected for each sample (**Supplementary Table 1**). The diarrheic fecal samples

were collected in sterile disposable plastic specimen vials, transported in refrigerated boxes, and stored at −80°C until laboratory testing.

Detection of *Bovine rotavirus A*

Different molecular methods were used to detect *BRV* from diarrheal samples of the bovine calf (**Fig. 1**).

Latex agglutination test (LAT)

The rotavirus antigen was detected from fecal samples using a HiRotavirus Latex Test Kit (HiMedia, India) according to the procedure described by the manufacturer. This kit is a rapid

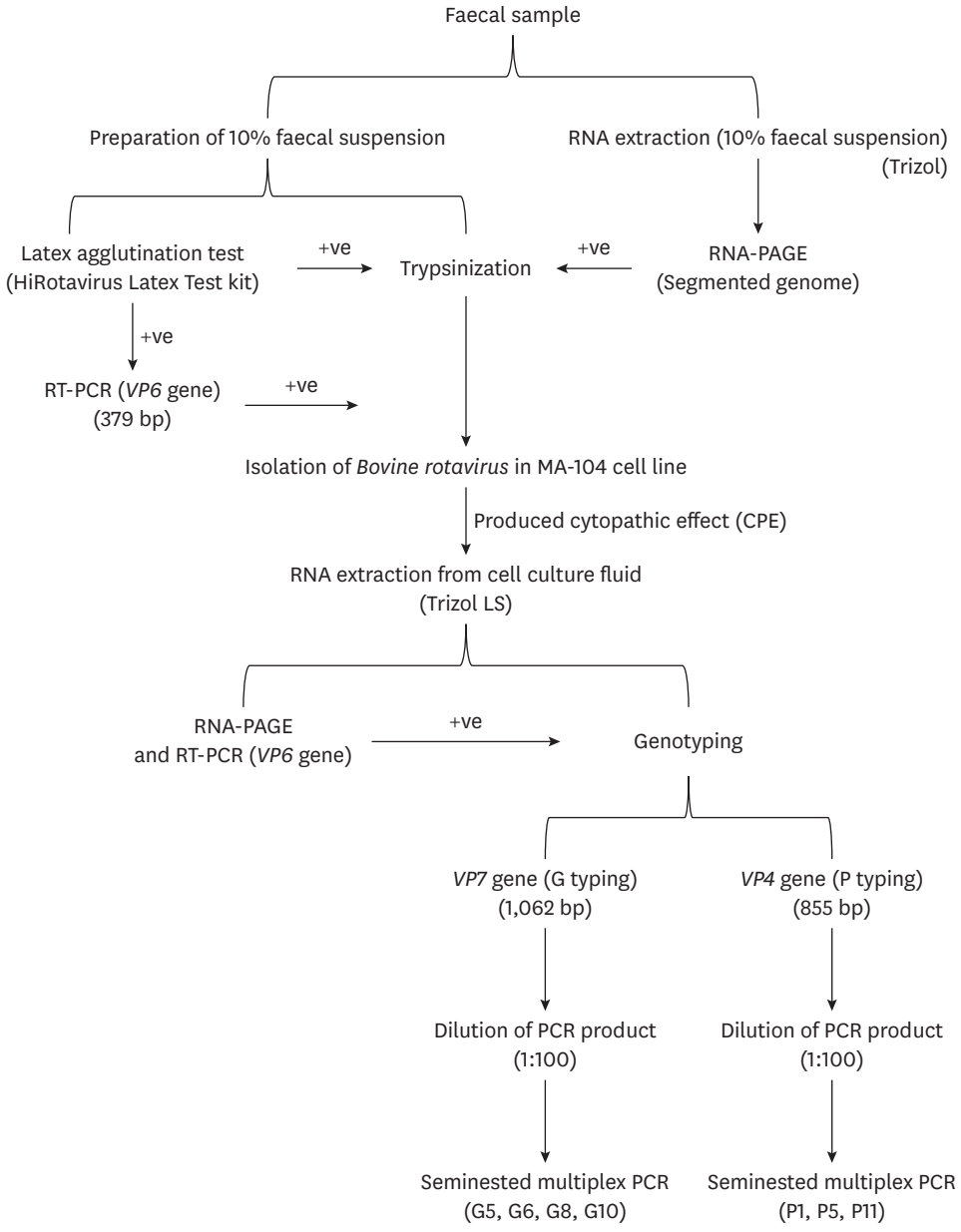

Fig. 1. Flow diagram of present research work for detection, isolation, and molecular characterization of *Bovine rotavirus A*.

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slide test in which latex particles are coated with antibodies raised against a pool of different rotavirus isolates, including humans. The visual results were observed within 3 min.

Extraction of rotaviral RNA from a fecal sample

At onset, all fecal suspensions (10% w/v) were prepared with phosphate buffer saline (PBS; pH 7.2), followed by centrifugation at 10,000 rpm (Eppendorf centrifuge 5804 R) for 30 min at 4°C. The clarified supernatant was stored at −20°C until further use. The viral RNA was extracted from the clarified supernatant using TRIzol reagent (Invitrogen TRIzol, USA) according to the manufacturer's instructions.

Electropherotyping of Bovine rotavirus A by RNA-PAGE

The extracted RNA was subjected to RNA-PAGE with slight modifications [12]. Finally, an 8% separating gel and 5% stacking gel were used to detect the rotavirus from the fecal samples by RNA-PAGE. The gel was stained with silver nitrate to detect the rotavirus genomic migration pattern.

Molecular detection of Bovine rotavirus A by reverse transcription polymerase chain reaction (RT-PCR) for VP6 gene

The extracted RNA template was denatured for 2 min at 95°C, followed by cDNA synthesis (cDNA: denaturation and annealing at 95°C for 3 min and 65°C for 5 min, respectively) using the components of the PrimeScriptTM RT-PCR kit (TaKaRa). The cDNA was amplified using VP6 gene (**Supplementary Table 2**) specific published primers [13]. The cycling conditions consisted of initial denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 30 sec, at 56°C for 30 sec, and at 72°C for 90 sec. This was followed by a final extension at 72°C for 10 min. The amplified product was visualized with UV light and documented using a gel documentation system (Genetix Biotech).

Reference *rotavirus* strains

Human rotavirus G1P[8] (Rotarix vaccine) was used as a positive control to standardize RT-PCR, RNA-PAGE, cell culture isolation, and G and P typing of *BRV* strain.

Isolation of *Bovine rotavirus A*

A seed culture of *Mycoplasma* free MA-104 cell line was provided by the National Research Centre on Equines (NRCE), Hisar, Haryana, India. MA-104 cells (10^5/mL) of the 17^{th} cell passage were cultured in sterile, minimum essential media (MEM) (Gibco MEM, USA) with sodium bicarbonate (2.2 g/lit) and antibiotics (100 µg/mL streptomycin and 500 units/mL penicillin) supplemented with 10% fecal bovine serum (FBS) (Gibco FBS, USA) in 25 cm^2 culture flasks with incubation of three days at 37°C with 5% CO_2. Filtered (0.2 µm) supernatant of centrifuged 20% PBS diluted LAT positive samples were mixed with an equal volume of Dulbecco's PBS (DPBS) (pH 7.4; Gibco, USA) supplemented with 500 µg/mL gentamicin (HiMedia) and incubated for 60 min at 25°C. In addition, trypsin-EDTA solution (Gibco, USA) was added to a final concentration of 100 µg/mL. A 500 µL sample of treated fecal suspension (or 1 mL of cell culture virus suspension) was used as the inoculum for the DPBS-washed MA-104 cells, followed by incubation at 37°C with 5% CO_2 for 60 min. The inoculum was replaced with 6 ml of MEM (without FBS; with 0.04 U/mL trypsin-EDTA solution) and incubated at 37°C with 5% CO_2 for two to three days. The cells were checked daily for the appearance of cytopathic effects (CPE) and morphological changes. After the incubation period, the lysate of freeze-thawed cells was passaged onto fresh monolayers. The monolayers showing approximately 90% CPE were harvested and stored at −20°C until further use.

Rotaviral RNA extraction from cell culture fluid and RT-PCR genotyping

Rotaviral RNA was extracted manually from the cells that developed a post-infection CPE using TRIzol LS (Invitrogen TRIzol LS, USA) according to the manufacturer's instructions. The extracted RNA was subjected to electropherotyping (RNA-PAGE), RT-PCR, VP6 to confirm the isolation of the virus in the cell line, and genotyping by multiplex RT-PCR. The G (1062 bp, VP7 gene) and P (855 bp, VP4 gene; **Supplementary Table 2**) typing primer sets used are described elsewhere [14,15]. The cycling conditions consisted of initial denaturation at 94°C for 5 min, 40 cycles of amplification (at 94°C for 1 min, annealing at 46°C (VP7), 48°C (VP4) for 2 min, and 68°C for 3 min) and final extension at 68°C for 10 min.

G and P genotyping of *BRVA* by semi-nested multiplexed PCR

One microliter of diluted (100-fold) first PCR products were then used for the second PCR amplification with Bov9Com5 and a mixture of four G-type specific primers (G5, G6, G8, and G10) for G typing. A mixture of three P-type specific primers (P1, P5, and P11) and Con3 was used for P typing (**Supplementary Table 2**) using the method described elsewhere [14,15] with the necessary modifications. The initial denaturation step was conducted at 94°C for 5 min, followed by 25 cycles (at 94°C for 1 min, at 54°C (G typing) and 52°C (P typing) for 2 min, and 72°C for 1 min), with a final extension at 72°C for 10 min. The positive and negative controls were used in the experiment, and the genotype was assigned according to the size of the amplified PCR products.

RESULTS

Overall Incidence of *Bovine rotavirus A* by LAT, RNA-PAGE, and RT-PCR

Of the 69 diarrheic fecal samples screened for *BRVA*, 27 (39.13%), 14 (20.30%), and 26 (37.70%) samples were found positive by LAT (**Supplementary Fig. 1**), electropherotyping, and RT-PCR, respectively (**Supplementary Table 3**). Of the 16 buffalo and 53 cattle calve samples tested, 6 (37.50%) and 21 (36.62%) were found positive by LAT, respectively.

In the case of cattle/buffalo calves, the overall incidence of *BRVA* by electropherotyping was 20.30% (14/69). RNA-PAGE was performed directly on the fecal samples from four (25%) buffalo calves and 10 (18.90%) cattle calves, and the results were positive. All the positive isolates on analysis exhibited a 4:2:3:2 classical long migration F pattern (Co-migration of 2 and 3, and 7, 8, and 9 as F, **Supplementary Table 4**) for *BRVA*.

RT-PCR-based amplification of the VP6 gene fragment of *BRVA* in 26 diarrheal samples indicated that calves were suffering from a rotaviral infection (**Supplementary Fig. 2**). RT-PCR revealed species-specific incidence rates of 37.50% (6/16) and 37.73% (20/53) for buffalo and cattle calves, respectively. The peak incidence of rotaviral gastroenteritis was recorded between 1–15 days of age, with an incidence rate of 47.40% (9/19), followed by 40.90% (9/22), 35.71% (5/14), and 21.42% (3/14) for the age group of 16–30 days, 31–45 days and 45–60 days, respectively. The overall higher incidence of *BRVA* was 39.50% (15/38) and 35.50% (11/31) in male and female calves, respectively.

Isolation of *Bovine rotavirus A* in cell culture

Confluent sheets of MA-104 cells (**Fig. 2A**) were used to isolate the rotavirus from the LAT-positive samples. The CPE consisted of obscure cell boundaries, cell fusion, cell rounding, and cell detachment, observed after 2–3 passages (**Fig. 2B and C**). Of 27 LAT-positive samples,

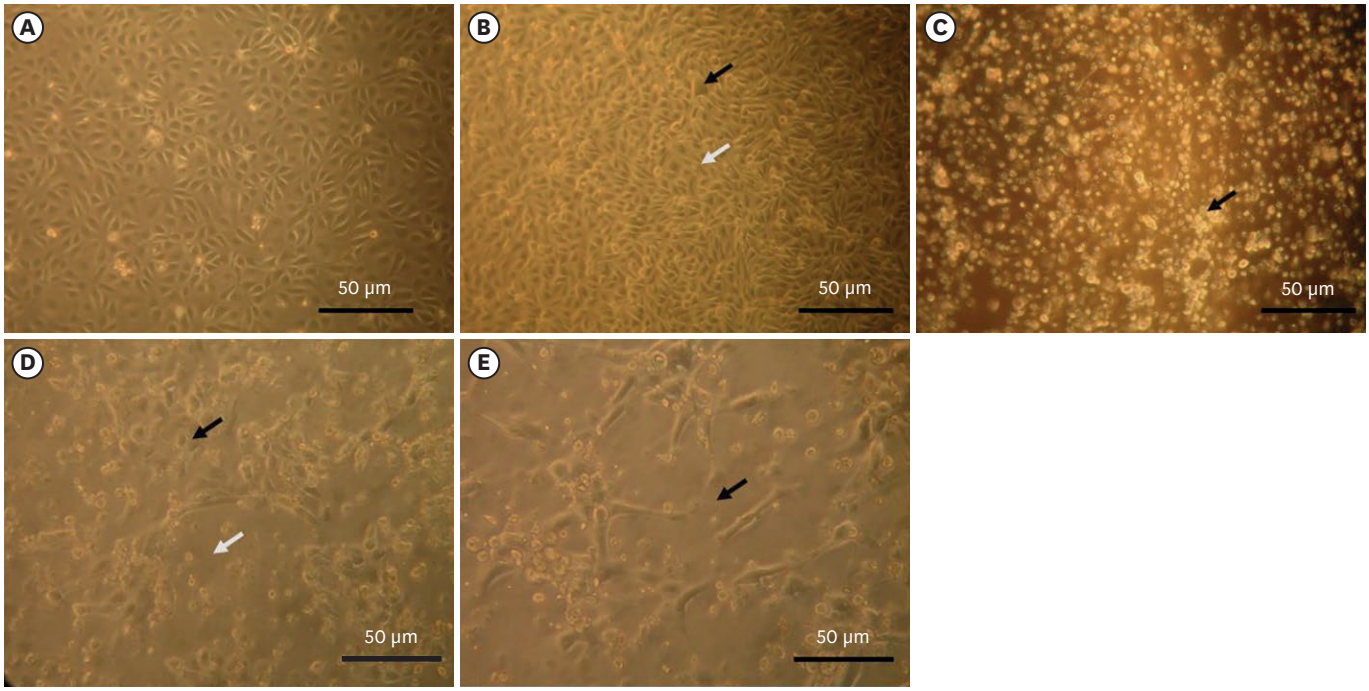

Fig. 2. (A) Non-infected/Uninoculated monolayer of MA-104 cells three days after. (B) Characteristic ballooning (black arrow) and spindle formation (white arrow) on passage 3 in infected MA-104 cells after 24 h. (C) Characteristic granulation (black arrow), rounding, and clumping occur singly or in clusters in MA-104 cells infected with rotavirus 36 h after infection (Scale bar: 50 μm; phase-contrast microscopy). (D) Characteristic rounding, necrosis (black arrow), and sloughing off (white arrow) of infected MA-104 monolayer cells after 48 h. of infection (Scale bar: 50 μm). (E) Later stage of infection with extensive cell degeneration, i.e., necrosis and sloughing off (black arrow) in the MA-104 monolayer cells infected with *Bovine rotavirus A* after 60 h. of infection. Scale bars: (A) 50 μm, without staining; (B-E) 50 μm.

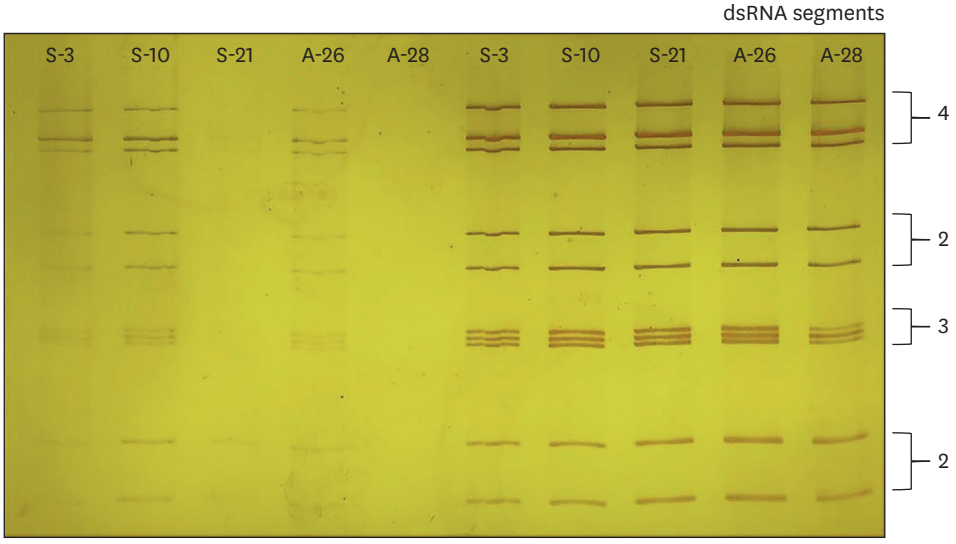

Fig. 3. Comparison of RNA-PAGE performed directly from fecal samples (left side) and RNA-PAGE after the isolation and propagation of *Bovine rotavirus A* in cell culture (right side) (Sample code: S-3, S-10, S-21, A-26, A-28).

26 (96.30%) were isolated in cell culture and showed a distinct CPE. All 26-cell culture isolates were positive for RT-PCR (VP6) and RNA-PAGE. RNA-PAGE detected only 14 positive samples directly from fecal samples, possibly due to the low virus titer in the feces not detected by RNA-PAGE (**Fig. 3**).

Four samples had a distinct CPE at the first passage, six at the second, fifteen at the third, and one became positive during the fourth tissue culture passage. Complete lysis could be detected two to four days after the infected cells had converted into a round, spindle-shaped form at the various passage levels (**Fig. 2D and E**). In some cases, however, 30%–50% of the cell sheet had disintegrated after 48 h, and no further increases in cell degeneration were seen. The earliest signal CPE was observed 16–18 h after inoculation during higher passages. The cell toxicity of the fecal samples and bacterial contamination played a minor role in this study.

Amplification of VP7 and VP4 Gene of *Bovine rotavirus A* strains

For characterization of the G and P genotypes of *BRVA*, all 26 cell culture isolates could be amplified for full-length VP7 and partial VP4 genes and gave the specific amplification product of 1062bp and 864bp, respectively (**Supplementary Fig. 2**, **Supplementary Table 5**).

'G' genotyping of Bovine rotavirus A by semi-nested PCR

In the present study, 24 out of 26 positive samples (92.30%) could be typed for the G genotypes, while two (7.70%) could not be typed with any of the four G serotype-specific primers selected in the present study. Thus, among the 24 RT-PCR positive samples, G10 was the predominant G type in 14 (53.84%) samples, followed by the type G8 found in seven (26.92%), and mixed genotype G10+G8 was found in three (11.53%) positive samples, while G5 and G6 type not detected in this study (**Supplementary Table 5**).

'P' genotyping of Bovine rotavirus A by semi-nested PCR

For P typing, 22 out of 26 VP4 gene-positive samples (84.61%) could be typed. Two G10 typed samples failed in P typing even after repeating the test, and two samples remained completely untyped for G and P typing. Nevertheless, the expected bands of the first-round PCR products were observed. The most common P-type among the diarrhoeic calves was P[11], accounting for 76.92%, followed by P[5] (7.70%), while P[1] was not detected in any of the positive samples (**Fig. 4**, **Supplementary Table 5**).

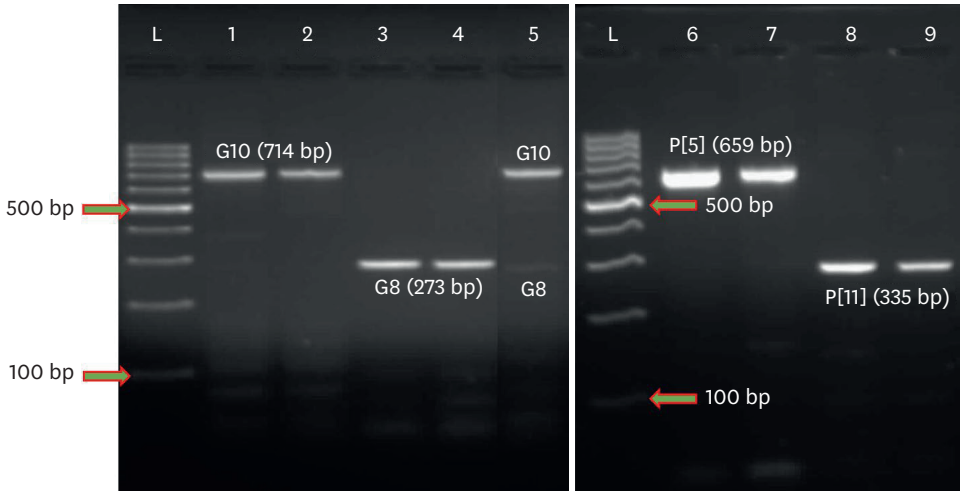

Fig. 4. Agarose gel shows amplified G and P genotyping products of *Bovine rotavirus A* isolates. L: DNA Molecular weight ladder – 100 bp, 1–2 lane: G10 (714 bp), 3–4 lane: G8 (273 bp), 5 lane: Mixed infection (G10 + G8), 6–7 lane: P[5] (659 bp) and 8–9 lane: P[11] (335 bp) (Sample code: A-9, S-14, A-25, S-18, A-16, A-9, A-30, A-16, S-23; lane: left to right side).

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Table 1. Overall G and P type combinations and relative frequency circulating in the bovine population

Genotype	P[5] genotype		P[11] genotype		Non-typable		Total	
	Positive samples	Positive %	Positive samples	Positive %	Positive samples	Positive %	Positive samples	Positive %
G8	0	0	7	26.92	0	0	7	26.92
G10	2	7.70	10	38.50	2	7.70	14	53.84
G8+G10	0	0	3	11.53	0	0	3	11.54
Non-typable	0	0	0	0	2	7.70	2	7.70
Total	2	7.70	20	76.92	4	15.40	26	100

Combination of G and P genotype of *Bovine rotavirus A*

Different combinations of G and P genotypes were observed (**Table 1**). G10P[11] was the most prevalent genotype 10/26 (38.50%) among the bovine calves, followed by G8P[11] 7/26 (26.92%), G10P[5] 2/26 (7.70) and mixed genotypes G8+G10P[11] in 3/26 (11.53%, **Fig. 4**) *BRVA* positive samples.

DISCUSSION

Overall incidence of *Bovine rotavirus A* in neonatal calf

BRV is considered an important disease for cattle and buffalo because of its significant economic impact on the dairy industries in India. Nevertheless, there is a lack of information on the prevalence and molecular characterization of *BRV* in Gujarat, India. In the present study, all bovine calves were less than two months old; the observed overall *BRVA* incidence rate by RT-PCR was 37.70%. The proportion of calves confirmed with *BRVA* was lower than in previous reports in India [16,17], which reported more than 50% of calves (below two months of age) infected with *BRVA*. The rotavirus detection rates by RT-PCR were different in the different regions of Gujarat (India): 7.89% in North Gujarat [18], 1.93%–22.22% in Middle Gujarat [19], and 26.60% in South Gujarat [20].

The incidence of rotaviral gastroenteritis in calves showed an inverse relationship with age. Age-wise, the incidence of *BRVA* was higher in the 1–15 days age group than in the other age groups. Similar findings were reported for 0–7 days [21], 1–10 days [19,20], and 4–14 days [22]. Various nutritional and environmental conditions (humidity and temperature) coincide with the appearance and initiation of rotavirus-induced diarrhea in the early weeks [23].

LAT is a highly sensitive and specific (94%–98%) test suitable for the rapid diagnosis of *BRV,* which can be performed in field conditions or at a patient's bedside [24]. A previous study in Gujarat and India showed a less than 30% incidence rate of *BRVA* [25,26]. In contrast, Muhammid et al. [16], Reidy et al. [27], and Al-Yousif et al. [24] reported a higher (more than 60%) incidence rate by LAT.

The migration pattern of a particular rotavirus in RNA-PAGE can be used to detect, characterize, and epidemiological investigate rotaviruses [28]. The incidence rate (20.30%) observed in the present study was distinct from previous studies in various states of India, i.e., 19.06% in Punjab, 15.50% in Kashmir, 10.52% in Kolkata, 7.14%–7.80% in West Bengal, 5.50%–12% in Haryana and Uttar Pradesh and 4.61% in North India [29-31]. In the present study, RNA-PAGE identified fewer positive samples than RT-PCR and LAT. This observation may be because of the lower sensitivity of electropherotyping, which can detect 10^{11} rotavirus particles per ml, compared to RT-PCR (10^4 particles) and LAT [32]. Thus, the present study showed that two or more methods should be used simultaneously to maximize the diagnostic potential/detection efficiency, e.g., LAT and RT-PCR in the present study [25].

G and P genotyping of *Bovine rotavirus A*

In India, the G and P genotypes of *BRV* are vastly diverse and have many combinations in circulation. Hence, uniform and widespread research or surveillance into the genotyping of *BRV* is needed. The present study results are in accordance with other studies, where G10 and G8 are the predominant G-types of *BRVA* detected in the field samples [33,34]. Previous studies have indicated that G8 is a less common genotype among bovine rotaviruses, but it is the third most common G-type worldwide after G6 and G10 [36]. Some studies have postulated that bovine G6, followed by G10, are the most common G types [3,34]. Thus, the resemblance and paradox of these findings suggest that the geographical location may limit the predominance of G6 and G10 genotypes. In India, the G10 genotype appears to be the dominant genotype in bovine rotaviruses [28].

Genotype P[11] is a common P-type found in cattle and infrequently in horses, pigs, and lambs. Interestingly, the P[11] genotype was detected more frequently than other P-types in the present study and other studies in India [11,28]. The significant incidence of the P[11] genotype reinforces the concept that *BRV* can pass the barrier of the host species and circulate among Indian infants [11]. The partially typed strain in this study could be a new variant of common *BRVA* with altered priming sites, or they might contain unusual types or reassortments [36].

Combination G and P genotype of *Bovine rotavirus A*

The *BRVA* G10P[11] genotype is one of the predominant rotavirus genotypes circulating in bovines throughout India and the world [37]. The occurrence of the G10P[11] combination strain in 38.50% of samples is in harmony with other studies in India [28,38]. G10P[11] has been detected in clinical and subclinical cases in Indian children and is assumed to have been acquired through zoonotic transmission [39]. This study found the common *BRVA* G10P[11] genotype in Gujarat and uncommon G and P types in combinations of G8P[11], which may be recognized as an essential contributor to the diversity of rotaviruses found in animal and human infections.

Molecular techniques, RT-PCR, RNA-PAGE, and LAT have revealed the presence of *BRV* in the tested samples. Characterization of the incidence of the *BRVA* genotype revealed the genotype, P genotype, and mixed genotype. This study contributes to a better understanding of the genetic diversity of *BRV* in bovine species and prevalent zoonotic potential genotypes in different geographical locations in Gujarat, India. Nevertheless, molecular epidemiological investigation and genome sequencing of rotavirus in close proximate human populations must be carried out to study circulating zoonotic genotypes in more detail.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Details of the fecal samples collected from bovine calves

Supplementary Table 2

Oligonucleotide primers used to amplify a part of the VP6, VP7, and VP4 genes and for the G and P typing polymerase chain reaction

Supplementary Table 3

Details of the samples that tested positive by LAT, RNA-PAGE, RT-PCR (VP6), and cell culture isolation of *Bovine rotavirus A*

Supplementary Table 4

Group, subgroup, and electropherotypes of *Bovine rotavirus A*

Supplementary Table 5

Details of the samples positive by RT-PCR for the VP6, VP7, and VP4 genes and G and P genotyping of *Bovine rotavirus A* isolates isolated in the cell culture

Supplementary Fig. 1

Screening of *Bovine rotavirus A* using Latex Agglutination Test kit (HiRotavirus Latex Test Kit; 1-4: diarrheal samples, 5: positive control, 6: negative control).

Supplementary Fig. 2

Representative image of an agarose gel showing approximately 379 bp, 1062 bp, and 855 bp amplified products of VP6, VP7, and VP4 gene of *Bovine rotavirus A* isolates, respectively. L: DNA molecular weight ladder – 100 bp, P: positive control, N: negative control, 1-2: isolates.

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