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Research Article

RT-PCR and nested RT-PCR for detection and genotyping of group A rotavirus from faecal samples of dairy cattle farms from Southern Karnataka

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Abstract

Rotaviruses are significant causes of severe diarrhoea in both humans and animals resulting in significant economic losses on local as well as global scale. The present study was carried out to determine the prevalence and characterization of group A rotavirus in cattle faeces. A total of 106 cattle faecal sample from four districts of Karnataka were subjected to RNA extraction, and cDNA was synthesized and subjected to RT-PCR by targeting the VP6 gene, in which 9 samples yielded an expected amplicon of 309 bp, giving an overall prevalence of 8.49%. Upon G and P genotyping, all 9 samples in the first round of RT-PCR yielded an expected PCR product of 1062 bp (VP7 gene) and 7 samples of 876 bp product for (VP4 gene). Further, second round nested RT-PCR revealed the presence of G6 (22.22%), G8 (11.11%), G9 (22.22%), P[4](11.11%), P[6](11.11%), and P[10](11.11%) genotypes. Higher prevalence was recorded in calves of less than 3 months of age (17.65%), followed by calves between 3 to 6 months of age (10.53%). Sex-wise prevalence, 7 females (77.78%) and 1 male (22.22%) were positive for group A rotavirus. The findings of this study showed the presence of common genotypes among cattle G6, G8, P[6]and uncommon genotypes G9, P[4] and P[10]. This will help for further surveillance and epidemiological research on rotavirus infection and related risk factors in cattle.

Keywords: Cattle, Genotyping, Group A rotavirus, RT-PCR, VP6 gene

Highlights

- RT-PCR was used to analyze 106 cattle faeces, and the total prevalence of group A rotavirus was 8.49%
- Highest prevalence of group A rotavirus was recorded in calves of <3 months of age (17.65%)
- Only 7 of the 9 positive samples showed presence of VP4 gene, but all 9 had the VP7 gene
- G and P genotyping revealed the presence of common genotypes among cattleG6, G8 and P[6]
- Three uncommon genotypes, G9, P[4] and P[10], were also detected

INTRODUCTION

Rotaviruses are enteric pathogens that cause acute and dehydrating diarrhoea in a variety of host species, including mammals and birds, across the world (Kaminjolo and Adesiyun, 1994; Malik *et al*., 1995). Rotaviruses belong to theSedoreoviridaefamily with 11 segmented double-stranded RNA as the genome, and each segment encodes for one or more proteins. Rotaviruses are divided into nine groups A to J (ICTV, 2024); among them, the highest prevalence and pathogenicity were shown by group A rotavirus. A number of studies have been carried out indicating the importance of bovine rotavirus with prevalence estimates ranging from 10% to 60% (Dhama *et al*., 2009; Kusumakar *et al*., 2010; Geletu *et al*., 2021). The infection is more common in neonates and young animals, which has a negative impact on the financial viability of dairy farms and, hence, dairy farmers. Neonatal calves get exposed to the virus through contaminated milk, water and feed material (Holland, 1990; Malik *et al*., 2005). The better prevention and control of rotavirus disease infection in calves is achieved through early laboratory diagnosis of rotavirus in dairy cattle farms and hence plays an important role. Rotaviruses that belong to Group A are categorized into G and P-genotypes based on two type-specific outer capsid proteins: VP4 (protease sensitive) and VP7 (glycoprotein). Up till now 42 G

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types and 58 P types have been identified (RCWG, 2024). Minakshi *et al*. (2005) reported that, out of 138 rotavirus positive calf faeces from Haryana state, 109 samples were typed as P11 (97.32%) and 3 as P1 (2.68%), and 97 as G10 (86.61%) and 15 as G6 (13.39 %). G1, G2, and G9 were found to be prevalent in Delhi, with overall rates of 25.8%, 24.9%, and 14.4%, respectively (Sharma *et al*., 2008). In another study, presence of G10P[11], G8P[11], G10P[5] and mixed genotypes G8+G10P[11] were reported from the neonatal calves of Gujarat, India. RT-PCR can be used as a powerful tool for screening as well as diagnosis of rotaviruses (Gentsch *et al*., 1992; Basera *et al*., 2010; Malik *et al*., 2013; Makwana *et al*., 2020). The present study was done to detect the group A rotavirus and characterize (genotype) rotavirus from the faecal samples of dairy cattle across farms using RT-PCR.

MATERIALS AND METHODS

Collection of samples: A total of 106 cattle faecal samples consisting of diarrhoeal (n=65) and non-diarrhoeal (n=41) were collected from the dairy farm of Veterinary College Shivamogga and dairy farms (HF cross, Jersey cross and non-descript cattle) in and around Shivamogga, Davanagere, Chitradurga, Chikkamagaluru districts of Karnataka. The samples were collected aseptically using sample collection vials or sterile zip lock pouches and transported as early as possible to the lab on ice at 4°C and stored in deep freeze (Samsung, India) at -20°C until further use.

Extraction of viral RNA: Faecal suspension (10%) was made in the phosphate buffered saline and centrifuged in the refrigerated centrifuge (Thermofisher Scientifics, USA) at 10000 rpm for 15 min to remove the coarse particles, and the supernatant was transferred to fresh sterile 1.5 mL tubes and kept at -20°C until further use. RNA was extracted using RNAiso Plus (Takara Bio Inc, India) reagent. In short, 300 µL of the faecal suspension was taken into a 1.5 mL tube, and 750 µL of RNAiso Plus reagent was added and mixed well. Later, it was vortexed for 15 seconds in a Vertex shaker (Tarson, India) and incubated for 10 min at room temperature. In this mixture, 200 µL of chloroform was added and vortexed for 15 seconds, and then it was incubated at room temperature for 5 min. Later, at 12000 rpm, it was centrifuged for 15 min at 4ºC, and then the supernatant aqueous phase was separated and transferred into a freshly labelled 1.5 mL tube. In this supernatant, 500 µL of isopropanol was added, mixed and incubated at room temperature for 10 min. Later, centrifuged at 12000 rpm for 10 minutes at 4ºC to form a pellet at the bottom. The supernatant liquid was discarded without disturbing the pellet. Further,1 mL of 75% chilled ethanol was added and mixed and centrifuged at 7500 rpm for 5 min at 4ºC. The ethanol was removed carefully using a micropipette to avoid dislodging of the pellet. The tubes were dried by keeping them on a paper towel for 10-15 min to remove residual ethanol. Finally, 30 µL nuclease-free water was added to this pellet, mixed well and incubated at room temperature for 10 min. The extracted RNA samples were stored in deep freeze (Samsung, India) at -20ºC until further use.

Reverse transcriptase – PCR (RT-PCR): RNA was transcribed to cDNA by using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc, India). Briefly, 1 µL of random 6 mers (50 µM), 1 µL of dNTP mixture (10 mM each), template RNA of 3 µL and 5 µL of nuclease-free water was added to make 10 µL template RNA primer reaction mixture and incubated at 65ºC for 5 min then cooled immediately on ice. Further to this, 10 µL of template, RNA primer mixture, 4 µL of 5X Prime Script buffer and 0.5 µL of RNase inhibitor (40 U/µL), 1 µL of PrimeScript RTase (200 U/µL) and 4.5 µL of nuclease-free water was added to make a final volume of 20 µL reaction mixture. This reaction mixture was subjected to a single cycle of 30ºC for 10 min in a thermal cycler (Biorad, USA), followed by 95ºC for 5 min and then cooled on ice. Then, the cDNA was immediately stored at -20ºC until further use. Then, PCR was performed in a 0.2 mL tube with a final volume of 25 µL using the cDNA as the template. The reaction mixture consisted of 10 pmol each of the forward primer (VP6F) 5'-AAAGATGCTAG GGACAAAATTG-3'and the reverse primer (VP6R) 5'-TTCAGATTGTGGAGCTATTCCA-3' (Elschner *et al*., 2002), 12.5 µL of Emerald Amp® GT PCR Master Mix (Takara Bio Inc, India), 2.5 µL of cDNA and nuclease-free water to make 25 µL. PCR was conducted in a thermal cycler (Biorad, USA) and the cycling condition consisted of initial denaturation at 95ºC for 3 min, 34 cycles of 30 sec at 95ºC, 1 min at 53ºC, and 30 sec at 72ºC, followed by a final extension at 72ºC for 5 min. The expected amplicon of 309 bp was analyzed by electrophoresis in 1.5% agarose gel in SBB (Sodium-borate buffer) containing ethidium bromide (1 mg/mL) and visualized over UV transilluminator (Genaxy Scientific Pvt. Ltd., India).

Genotyping of Rotavirus: The samples which were positive for group A rotavirus by RT-PCR were further characterized by G and P genotyping. G genotyping

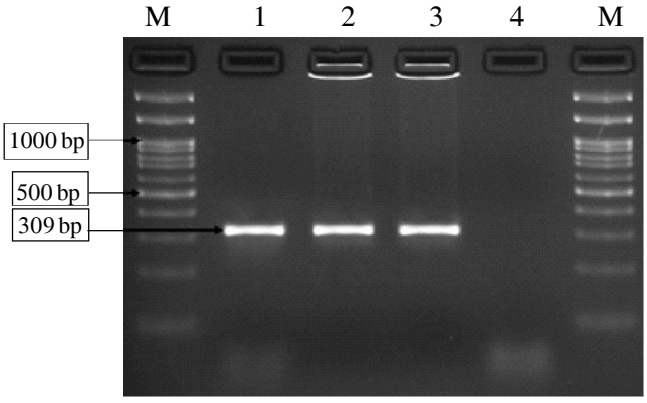
Table 1. Details of primers used in the present study

Primers	Sequence (5'-3')	Expected product size (bp)	Reference
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGGG	1062	Gouvea *et al*., 1990
RVG9	GGTCACATCATACAATTCT		
aBT1 (G1)	CAAGTACTCAAATCAATGATGG	749	
aCT2 (G2)	CAATGATATTAACACATTTTCTGTG	652	
aET3 (G3)	CGTTTGAAGAAGTTGCAACAG	374	
aDT4 (G4)	CGTTTCTGGTGAGGAGTTG	583	
G6	GATTCTACACAGGAACTAG	581	
aAT8 (G8)	GTCACACCATTTGTAAATTCG	885	
aFT9 (G9)	CTAGATGTAACTACAACTAC	306	
2T-1 (P4)	CTATTGTTAGAGGTTAGAGTC	483	Gentsch *et al*. (1992)
3T-1 (P6)	TGTTGATTAGTTGGATTCAA	267	
1T-1 (P8)	TCTACTTGGATAACGTGC	345	
4T-1 (P9)	TGAGACATGCAATTGGAC	391	

was carried out by RT-PCR targeting VP7 gene according to Gouvea *et al*. (1990) with slight modifications. The first PCR reaction was carried out in a final volume of 25 µL, which included 12.5 µL of Emerald Amp® GT PCR Master Mix (Takara Bio Inc, India), 2 µL of cDNA, 10 pmol of each Beg9 and RVG9 primers and nuclease-free water to make 25 µL. Semi-nested multiplex PCR was done to detect the G genotypes with the reaction mixture consisting of 12.5 µL of Emerald Amp® GT PCR Master Mix (Takara Bio Inc, India), 2 µL of 1:10 diluted PCR product of the first reaction, 1µL of each of the primers of RVG9, aBT1 (G1), aCT2 (G2), aET3(G3), aDT4 (G4), G6, aAT8 (G8), a FT9 (G9) and 2.5 µL of NFW. P genotyping was carried out with the primers developed by Gentsch *et al*. (1992) targeting VP4 gene with slight modifications (Table 1). First PCR reaction mixture consisted of 12.5 µL of Emerald Amp® GT PCR Master Mix (Takara Bio Inc, India), 2µL of cDNA, 10 pmol of each Con3 and Con2 primers and nuclease-free water to make 25 µL.In the semi-nested multiplex PCR 12.5 µL of Emerald Amp® GT PCR Master Mix (Takara Bio Inc, India), 2 µL of 1:10 diluted PCR product of the first reaction, 1µL of each of the primers of Con3, P4, P6, P8, P9, P10 and 4.5 µL of NFW. The PCR cyclic condition for the first PCR reaction for both G and P genotyping was standardized as initial denaturation at 95ºC for 3 min, then 34 cycles of 95ºC for 30 sec, 52ºC for 1 min, and 72ºC for 1 min, followedby a final extension at 72ºC for 7 min. The semi-nested PCR conditions for both G and P genotyping were standardized as 95ºC for 5 min initial denaturation followed by 34 cycles of 95ºC for 30 sec, 48ºC for 1 min, and 72ºC for 1 min and final extension at 72ºC for 10 min. The PCR products were analyzed by 1.5% agarose electrophoresis (Genaxy, India) in SBB (Sodium-borate buffer) containing ethidium bromide (1 mg/mL) and visualized over UV transilluminator.

RESULTS

A total of 106 faecal samples of cattle were analyzed for the presence of group A rotavirus, in which 9 samples were positive for VP6 gene of group A rotavirus by RT-PCR with an expected amplicon of 309bp (Fig.1), giving an overall prevalence of 8.49%. Five samples from dairy farms belong to Shivamogga (10.87%), two samples from Chikkamagaluru (7.41%)



[Lane M: 100 bp marker; Lane 1-3: VP6 gene positive samples showing 309bp; Lane 4: Negative Control]

Fig. 1. Agarose gel picture showing RT-PCR results for VP6 gene of group A Rotavirus

Table 2. Prevalence of group A rotavirus in dairy cattle faeces by RT-PCR

Sl. No.	Place of sample collection andno. of dairy farms (n)	No. of samples screened (population size)	No. of positive samples	Prevalence (%)
1	Shivamogga (n=9)	46 (224)	05	10.87
2	Davanagere (n=4)	18 (79)	01	5.56
3	Chikkamagaluru (n=7)	27 (118)	02	7.41
4	Chitradurga (n=5)	15 (71)	01	6.67
Total		**106 (492)**	**09**	**8.49**

and one sample each from Davanagere (5.56%) and Chitradurga (6.67%)districts were positive for Rotavirus (Table 2). Six young animals of less than 3 months of age were positive for group A rotavirus giving a prevalence rate of 17.65% and two young animals between 3 to 6 months of age and an adult cattle was positive for group A rotavirus with a prevalence rate of 10.53% and 3.23%, respectively, while none of the samples found positive for the age group between 6 months to one year (Table 3). Further, highest prevalence of group A rotavirus was found in

Table 3. Age-wise prevalence of group A rotavirus in dairy cattle

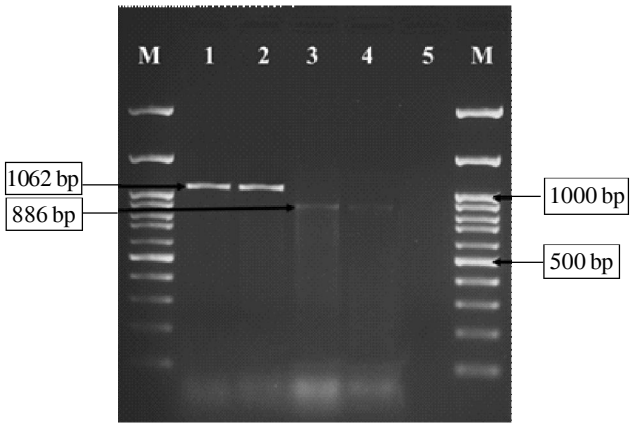
Sl. No.	Age group	No. of faecal samples screened	No. of positive samples	Prevalence (%)
1	<3 months	34	06	17.65
2	3-6 months	19	02	10.53
3	6 months -1 year	22	00	0
4	>1 year	31	01	3.23
	Total	**106**	**09**	**8.49**

Table 4. Season-wise prevalence of group A rotavirus in dairy cattle

Sl. No.	Season (months)	No. of faecal samples screened	No. of positive samples	Prevalence (%)
1	Summer (March to May)	24	03	12.5
2	Monsoon (June to September)	35	01	2.86
3	Post-Monsoon (October to December)	26	01	3.85
4	Winter (January to February)	21	04	19.05
	Total	**106**	**09**	**8.49**

the diarrhoeal faecal samples (7/65) compared to non-diarrhoeal faecal samples (2/41) with a prevalence rate of 10.77% and 4.88%, respectively. Out of 9 samples, 7 females (77.78%) and 2 males (22.22%) were positive for group A rotavirus. Season-wise wise in winter season 4 samples were positive (19.05%), followed by summer (12.5%), post-monsoon (3.85%) and monsoon season (Table 4).

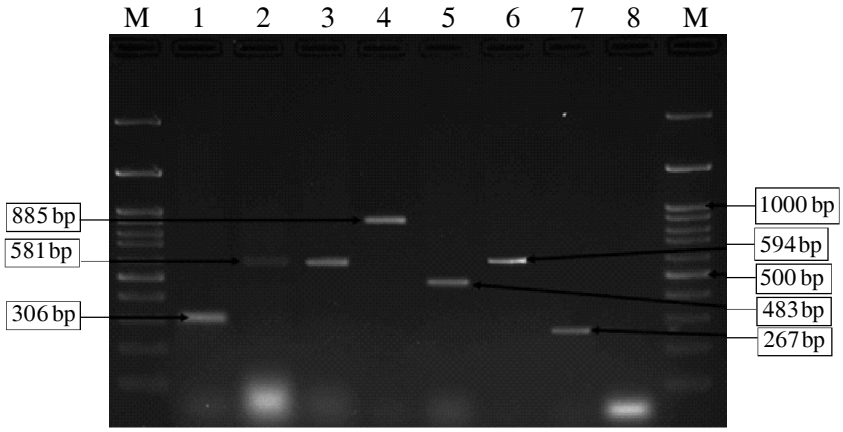
All the positive samples (n=9) were subjected to G and P Genotyping by RT-PCR by targeting VP7 gene and VP4 gene, respectively. All 9 samples in the first round of RT-PCR for G genotyping yielded an expected PCR product of 1062 bp for VP7 gene and similarly, P genotyping yielded 876 bp product for VP4 gene in 7 samples only (Fig. 2). Second round RT-PCR for G genotyping, only 5 samples shown the amplicons of 581 bp, 885 bp and 306 bp indicating the presence of G6, G8 and G9 genotypes. Similarly, P genotyping shows the amplicons of 483 bp, 267 bp and 594 bp, indicating the presence of P[4], P[6] and P[10] genotypes (Fig. 3 & Table 5). Two samples from



[Lane M: 100 bp marker; Lane 1-2: VP7 gene positive samples showing 1062 bp; Lane 3-4: VP4 gene positive samples showing 876 bp; Lane 5: Negative Control]

Fig. 2. Agarose gel picture showing RT-PCR results for VP7 and VP4 gene of group A rotavirus

Shivamogga and one from Chikkamagaluru showed the presence of both G and P genotypes, while one sample each from Shivamogga and Chikkamagaluru



[Lane M: 100 bp marker; Lane 1: G9 genotype showing 306 bp; Lane 2-3: G6 genotypes showing 581 bp; Lane 4: G8 genotype showing 885 bp; Lane 5: P[4] genotype showing 483 bp; Lane 6: P[10] genotype showing 594bp; Lane 7: P[6] genotype showing 267 bp and Lane 8: Negative Control]

Fig. 3. Agarose gel picture showing RT-PCR results for G & P genotyping of group A rotavirus

Table 5. Genotyping of group A rotavirus by RT-PCR

Sl. No.	Place of sample collection/ farms	Sample number	G genotype	P genotype
1.	Shivamogga	CF12	G6	-
		CF20	G9	P[10]
		CF25	-	-
		CF36	G8	P[6]
		CF33	-	-
2	Davanagere	CF47	-	-
3	Chikkamagaluru	CF64	G6	P[4]
		CF77	G9	-
4	Chitradurga	CF91	-	-
	Total	**09**	**05**	**03**

showed the presence of only the G genotype. A total of four samples (Two from Shivamogga and one each from Davanagere and Chitradurga) were untypeable.

DISCUSSION

Around the world, rotavirus infections are the most frequent cause of severe gastroenteritis in both animals and humans, indicating its potential of zoonotic and significant economic impact. Many factors, such as season, age, sex, geographical location, etc., are involved in causing diarrhoea in calves and adult cattle (Gulliksen *et al*., 2009). The present study describes the prevalence of group A rotavirus in cattle faecal samples. A total of 9 cattle faecal samples were positive for the group A rotavirus by RT-PCR by targeting VP6 gene, giving an overall prevalence rate of 8.49%. In concordance with the present study Rawat *et al*. (2012) has also reported slightly high prevalence of 13.3% group A rotavirus in diarrhoeic calves in northern India by targeting VP7 gene. Similarly, Rai *et al*. (2011) reported a prevalence of 15.68% in calves in Uttar Pradesh, and Malik *et al*. (2013) reported 13.33% in cattle in central India. A slightly low percentage of 5.02% prevalence was reported by Singh *et al*. (2021) in diarrhoeic calves from central and northern India. Langoni *et al*. (2004) reported a higher prevalence rate of 25.1% in diarrhoeic calves from Brazil. Similarly, Reidy *et al*. (2006) and Vende *et al*. (1999) reported 91% and 45.1% prevalence in diarrhoeic calves from Ireland and France, respectively. The prevalence studies carried out by various workers, including the present study, indicated that rotaviral gastroenteritis is a continued problem bothering dairy cattle farms.

In the present study, more samples were positive from calves of less than 3 months of age (n=6) and calves between 3 to 6 months of age (n=2), with a prevalence rate of 17.65% and 10.53%, respectively. Only one adult cattle of three and half years age from Shivamogga was positive for group A rotavirus with a prevalence rate of 3.23%, but its 1 month old calf was passing normal faeces and negative for group A rotavirus. More incidences of rotaviral diarrhoea in the age group of less than one month may be caused by their immature immune systems and insufficient maternal antibodies in their colostrums (Windeyer *et al*., 2014). Though several studies emphasized that rotaviral gastroenteritis is a disease of calves, its prevalence among adult dairy cattle is a cause for concern.

Genotyping results for the first PCR showed the presence of 1062 bp for VP7 gene and 876 bp of VP4 genes, respectively (Fig. 2). Many authors have

reported the presence of VP7 and VP4 genes with same set of primers or different set of primers (Isegawa *et al*., 1993; Reidy *et al*., 2006; Ahmed *et al*., 2022; Shin *et al*., 2023). Upon nested PCR for G genotyping, of the 9 positive rotaviruses, 5 rotavirus samples showed the presence of G6, G8 and G9 genotypes with a prevalence rate of 22.22% (2/9), 11.11% (1/9) and 22.22% (2/9), respectively and four rotavirus positive samples could not be typed. Similarly, upon P genotyping, only three rotaviruses could be typed, yielding P[4], P[6] and P[10] genotypes with a prevalence rate of 11.11% each. Babalola *et al*., 2020 reported the presence of G1, G5, G9, G10, G12, and P[6] rotavirus genotypes in cattle from Southwest Nigeria. Zaitoun *et al*., 2022 reported the presence of G6, G10, P[5] and P[11] genotypes among cattle from Egypt. Compared to other studies, the differences in the occurrence of different genotypes and their prevalence rate might be due to the differences in sample size and geographic locations of the study. Many other G and P genotypes of group A rotaviruses, such as G1- G6, G8, G10, P[1], P[3], P[5], P[6], P[7], and P[10] were found in humans and animals (Ghosh *et al*., 2007; Malik *et al*., 2013; Ghosh and Kobayashi, 2014; Vlasova *et al*., 2020; Geletu *et al*., 2021; Ahmed *et al*., 2022). The present study showed the presence of common genotypes among cattle, such as G6, G8, and P[6], and uncommon genotypes, including G9, P[4], and P[10]. In order to determine the evolving pattern of genotypic circulation in bovines throughout time, we need more thorough molecular epidemiology research in other species as well.

Rotavirus-induced diarrheal illness is a serious health concern for animals and humans as it disrupts the growth of individuals through decreased weight gain and higher mortality, as well as having the potential to spread zoonotic infections (Gelaw *et al*., 2018). In conclusion, the present study showed that the prevalence of group A rotavirus in young animals (bovine calves) indicates the importance of group A rotavirus as one of the etiological agents of diarrhoea in calves, which may result in loss of productivity and economy of farmers. An adult cattle was also positive for rotavirus. The presence of different genotypes among cattle indicates the evolving pattern of rotavirus across different species. RT-PCR can be used as an important tool for detection, characterization, and to get important epidemiological data regarding rotavirus. Further studies are required in these areas to explore the presence of different genotypes among cattle.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the present research, authorship, and/or publication of this article.

Author's contribution: PSB, AJB, MCB, PR, KLK: Design of research study and setting of objectives; MCB, AJB: Supervision of the research study; PSB: Wrote the manuscript with inputs from all the authors; PSB, UN, P: Sample collection and analysis; NSK, CSS, PR, KLK: Shaping of the research, verified the numerical analysis and the final version of the manuscript.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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