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Comparative evaluation of coagulation pathway in Vechur and Frieswal cattle by analysing Protein S gene

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Abstract
The livestock sector in Kerala is a vital contributor to the rural economy, with cattle playing a major role. Native Vechur cattle are noted for disease resistance, whereas Frieswal, the newly registered synthetic breed of Indian milch cattle (62.5% Holstein Friesian × 37.5% Sahiwal) are recognised for their high milk yield. However, the molecular basis underlying breed-specific differences remains unclear. Comparative proteomic studies were conducted by Centre for Advanced Studies in Animal Genetics and Breeding, Kerala Veterinary and Animal Sciences University (KVASU), highlighted differential expression of immune-related proteins between these two breeds, particularly in the coagulation pathway. The coagulation pathway ensures haemostasis through platelet activation and fibrin clot formation. The present study compared bleeding time (BT), clotting time (CT) and expression profile of coagulation-related gene Protein S (*PROS1*) in Vechur and Frieswal cattle.
The BT and CT were evaluated in eighty animals (40 from Vechur and 40 from Frieswal) and relative gene expression of *PROS1* was analysed in blood from healthy heifers (n = 6 per breed) by qPCR using *β-Actin* as reference gene. Relative fold change was calculated using $2^{-\Delta\Delta Ct}$ method and results were analysed statistically using SPSS V.24.0. No significant differences were observed in BT and CT and expression analysis of *PROS1* in Vechur compared to Frieswal cattle. Polymorphism studies using targeted amplicon sequencing (TAS) revealed four single nucleotide polymorphisms (SNPs) in Vechur cattle. Although haemostatic parameters and *PROS1* expression were comparable between Vechur and Frieswal cattle, the identification of SNPs through targeted amplicon sequencing provides a basis for future association and functional studies in larger populations to elucidate their biological implications

Keywords: Coagulation cascade, Vechur, Frieswal, PROS1

1. Introduction
Vechur cattle, the smallest *Bos indicus* breed of India, showed strong adaptability to climate and disease resistance, whereas Frieswal cattle are selected mainly for milk production. Hence, comparative proteomic studies were conducted by Centre for Advanced Studies in Animal Genetics and Breeding, Kerala Veterinary and Animal Sciences University, which highlighted differential expression of serum proteins and downstream bioinformatics analysis revealed significant difference in coagulation pathway between Vechur and Frieswal cattle. Therefore, the present study aimed to evaluate the differences in BT, CT and expression of coagulation related gene *PROS1* in whole blood, between the two breeds. Haemostasis halts bleeding following vascular injury through three integrated phases- primary vascular-platelet response, coagulation cascade, and regulatory anticoagulant mechanisms [1,2]. Protein S is a vitamin K–dependent plasma glycoprotein with essential antithrombotic functions. It is encoded by *PROS1* gene (Location: Chromosome one; Exon count: 15). Target gene expression for elucidating the molecular basis of breed-specific disease resistance was studied [3]. The TAS focused on specific genes or loci, providing high sensitivity and specificity for variant detection [4,5]. In line with these approaches, the present study comparatively evaluated the expression and polymorphisms of the coagulation-related gene *PROS1* to elucidate breed-specific molecular differences underlying disease resistance.

2. Materials and methods

2.1 Animals and sampling

Eighty animals (40 Vechur and 40 Frieswal) were selected based on pedigree records for the study. For determination of BT and CT, samples were collected from 40 Vechur cattle maintained at Vechur cattle conservation unit, Mannuthy and 40 Frieswal cattle selected from different ICAR Field Progeny Testing (FPT) units, Cattle Breeding Farm, Thumburmuzhy and University Livestock Farm, Mannuthy. For gene expression analysis, blood samples were collected from apparently healthy heifers (18- 24 months old), six each of Vechur and Frieswal cattle, in EDTA vials and processed immediately.

2.2 Estimation of Bleeding and Clotting Time

Bleeding Time (BT) was measured using Duke's method and Clotting Time (CT) was assessed using the capillary tube method.

2.2.1 Duke's Test

The animal was restrained properly to avoid movement. The hairs around the ear pinna were clipped and cleaned with antiseptic. Then using a sterile needle, a shallow skin prick was made. As soon as the blood appeared, the timer was started and in every 30 sec, the drop of blood was blotted with filter paper gently, without touching the skin surface so that clotting was not disturbed. The timer was stopped when bleeding completely stopped at the puncture site. The bleeding time was recorded in minutes and seconds.

2.2.2 Capillary Tube Test

The animal was restrained properly to avoid movement. The site, usually ear vein was cleaned with antiseptic to prevent contamination. The vein was punctured using sterile needle. Blood was then allowed to enter the capillary tube via capillary action. The timer was started as soon as blood entered the tube. Small sections of the capillary tube were broken every 30 sec. The tube was separated gently to check for a fibrin strand bridging the broken ends. The timer was stopped as soon as visible fibrin strand appeared.

2.3 RNA isolation

Total RNA was isolated using TRIzol® LS reagent (Life Technologies, USA) and total RNA isolation kit (ORIonX Total RNA Isolation Kit, Cat. No. ODP419) followed by DNase treatment. RNA quality and concentration were assessed using NanoDrop spectrophotometer and agarose gel electrophoresis. cDNA synthesis was done using Origin cDNA synthesis kit.

2.4 Quantitative PCR

Quantitative PCR (qPCR) was employed to determine the relative expression of the candidate gene *PROS1*, using *β-actin* as the reference gene. Primers targeting short regions of these genes were designed from bovine mRNA sequences *(PROS1*: NM_174438.1; *β-actin*: NM_173979.3). Primer3 (v4.1.0) was used for primer designing, and primer characteristics such as Tm and GC content were evaluated using the PCR Primer Stats tool. Primers were positioned towards the 3′ end and across exon- exon junction to avoid genomic DNA amplification. Custom-synthesised primers (Sigma-Aldrich) were diluted to contain 10 pmol/µL, and details of sequences and properties are provided in Table 1.

Table 1: Sequences and properties of primers designed for qPCR

S. No	Primer Name	Accession number of the mRNA sequences	Primer Sequence (5'-3')	Product size (bp)
1	*PROS1* F	NM_174438.1	5'-CTTGGTTCCTGATTGCGCTT-3'	124
2	*PROS1* R		5'-TCCACAGAGACCATATGCCA-3'	
3	*β- Actin* F	NM_173979.3	5'-TGCGGCATTCACGAAACTAC-3'	147
4	*β- Actin* R		5'-CCAGGGCAGTGATCTCTTTCTG-3'	

Gradient PCR was performed and optimised conditions were applied for qPCR. The qPCR followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, which specified the minimum information necessary for evaluating qPCR experiments [6]. Reactions were performed in technical triplicates. Details of the reaction mixture, cycling conditions and primer-specific annealing temperatures are provided in Table 2 and 3. Following amplification, a melt curve analysis was performed to confirm amplification specificity.

Table 2: Reaction mixture used for qPCR

SI. No	Components	Volume (µL)
1	Template (cDNA) *	0.5
2	SsoAdvanced™ Universal SYBR® Green Supermix (2X)	5.0
3	Forward Primer (10 pmol /µL)	0.3
4	Reverse primer (10 pmol /µL)	0.3
5	Nuclease free water*	3.9
Total reaction volume		10.0
*Added individually based on concentration and adjusted accordingly		

Table 3: PCR conditions used for qPCR

SI. No	Steps	Temperature (°C)		Duration
1	Initial denaturation	95		10 min
2	Denaturation	95		15 sec
		PROS1	60.3	20 sec
		β-Actin	60.4	
4	Extension	72		30 sec
Steps 2 to 4 were repeated for 40 cyclesData acquisition was performed during the extension step				

Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [7], assuming equal amplification efficiencies. Fold changes reflected differences between treated and control groups. Statistical analysis was performed using independent t-tests followed by Duncan's multiple range test, using SPSS version 24.0 with significance set at $p < 0.05$.

2.5 Targeted amplicon sequencing

The TAS was performed using Oxford Nanopore Technology on Vechur cDNA. The quality and quantity of cDNA were assessed using NanoDrop One and samples with acceptable OD260/280 ratios were selected. PCR amplification was carried out in 25 µL reactions containing 100 ng cDNA, gene-specific primers (10 pmol/µL) and KAPA HiFi Hot Start Ready Mix. Amplicon quality was verified on 1.2% agarose gel. Libraries were prepared using the Ligation Sequencing Kit with Native Barcoding (SQK-LSK114), following end-repair, barcoding, pooling and AMPure XP bead purification. The final library was quantified using Qubit and sequenced on an Oxford Nanopore Mk1D platform. Basecalling was performed using Dorado to generate FASTQ files. Bioinformatics analysis included quality control, alignment using the minimap2-based EPI2ME pipeline, SNP calling with samtools and bcftools, and consensus polishing using Medaka. Neural-network-based correction was done to improve the accuracy of Nanopore-derived reads and produce a reliable final sequence output for each amplicon.

3. Results and discussion

The present study evaluated BT, CT and the expression of *PROS1* in Vechur and Frieswal cattle to understand potential breed-level differences in haemostatic regulation. Mean BT and CT values of both breeds are presented in Table 4. The study observed no significant difference in BT between Vechur and Frieswal cattle, indicating comparable primary haemostatic function in both the breeds. The BT reflected the overall efficiency of primary haemostasis, as it measured the period from induction of a standardised vascular injury to the cessation of bleeding and integrated the functional status of the vessel wall, platelets, and coagulation cascade [8]. The BT in healthy Hereford cattle was reported as 3.8 ± 1.6 minutes [9], which is lower than the results obtained for healthy Vechur and Frieswal cattle in the present study. The difference could probably be due to the difference in genetic constitution between these groups of animals.

Similarly, CT assessed using the capillary tube test also showed no significant difference ($p > 0.05$) between the two breeds. In the process of determining which animal models most adequately mimicked human clotting parameters, clotting parameters were assessed in Friesian calves (n=18) and it was reported that extrinsic and intrinsic clotting time were significantly prolonged in calves compared to humans (249.9 ± 91.3 and 376.4 ± 124.4 s vs. 63.5 ± 11.8 and 192.5 ± 29.0 s, respectively, $p < 0.01$) [10]. The present study observed CT values (Table 4) longer than those reported by Hasan and Alsaad (2018) who evaluated the blood coagulation indices in healthy vs bovine viral diarrhoea infected cattle (n= 494) in Iraq, wherein the CT averaged 3.38 ± 0.92 min for healthy adult cattle [11]. Though previous observations suggested that haemostatic profiles may vary across breeds, the present study did not show any significant difference between the breeds.

Table 4: Mean Bleeding time and Clotting time in Vechur and Frieswal cattle, min

Breed	Bleeding Time (min)	Clotting Time (min)
Vechur	4.20 ± 0.66 ^{ns}	5.03 ± 0.70 ^{ns}
Frieswal	4.49 ± 0.70	4.71 ± 0.74

^{ns} No significant difference at $p < 0.05$

The amplification plot and melt curve of *PROS1* and *β-Actin* are shown in Fig. 1 to Fig. 3. The *PROS1* expression did not differ significantly between Vechur and Frieswal cattle ($p > 0.05$), indicating comparable regulation of the anticoagulant arm of the haemostais in both breeds (Table 5). The free form of PROS1 provided anticoagulant activity while the bound form participated in complement regulation [12]. Natural anticoagulant genes like *PROS1*, counter balanced clot formation and maintained vascular integrity [13]. As a key anticoagulant, PROS1 functioned as a major cofactor for activated protein C and tissue factor pathway inhibitor [14] and directly inhibited the FVa–FXa prothrombinase complex [15]. Deficiency in PROS1 is associated with inherited thrombophilia [10] and acquired hypercoagulable states [16].

Table 5: Relative expression analysis of *PROS1* in Vechur and Frieswal cattle

Group	Mean C_t ± SE		ΔC_t ± SE	$\Delta\Delta C_t$ ± SE	Fold change from control ($2^{-\Delta\Delta Ct}$)	p value
	PROS1	*β-Actin*				
Vechur	24.79±0.06	17.96±0.11	6.83 ±0.13	0.15±0.16	0.89 ^{ns}(0.82- 0.98)	>0.05
Frieswal	24.37±0.05	17.69±0.08	6.68±0.10	0.00±0.10	1 (0.9 - 1.07)	

^{ns} No significant difference at $p < 0.05$

Targeted amplicon sequencing of cDNA of Vechur cattle for *PROS1* using Oxford Nanopore Technology, generated high-quality reads with adequate coverage (78%) across the target gene (Fig.4). Variant calling identified four SNPs in *PROS1*, each supported by high sequencing depth (~300×), indicating robust read support for the detected variants (Table 6). Though no significant difference was observed in *PROS1* transcript levels between Vechur and Frieswal cattle, the presence of sequence variation suggested underlying genetic diversity in the coagulation pathway. Functional studies demonstrated that missense mutations in *PROS1* led to Protein S deficiency by producing unstable or poorly secreted proteins with impaired anticoagulant activity, without significantly altering mRNA expression [17]. Multiple coding variants were identified in human *PROS1* associated with lower free Protein S levels and increased risk of venous thromboembolism in humans, illustrating how SNPs in *PROS1* can alter coagulation parameters [18]. A family carrying heterozygous *PROS1* mutations was associated with increased risk of thrombosis, highlighting the functional impact of *PROS1* genetic variation in coagulation [19]. The presence of SNPs in *PROS1* without baseline haemostatic differences align with the previous studies, suggesting underlying genetic variation that effects at the protein level during stress conditions rather than altering transcript abundance.

Table 6: Variant table showing the position of SNP

S. No.	mRNA Position	Reference Allele	Alternate Allele
1	182	G	A
2	962	T	C
3	995	T	C
4	1711	G	C

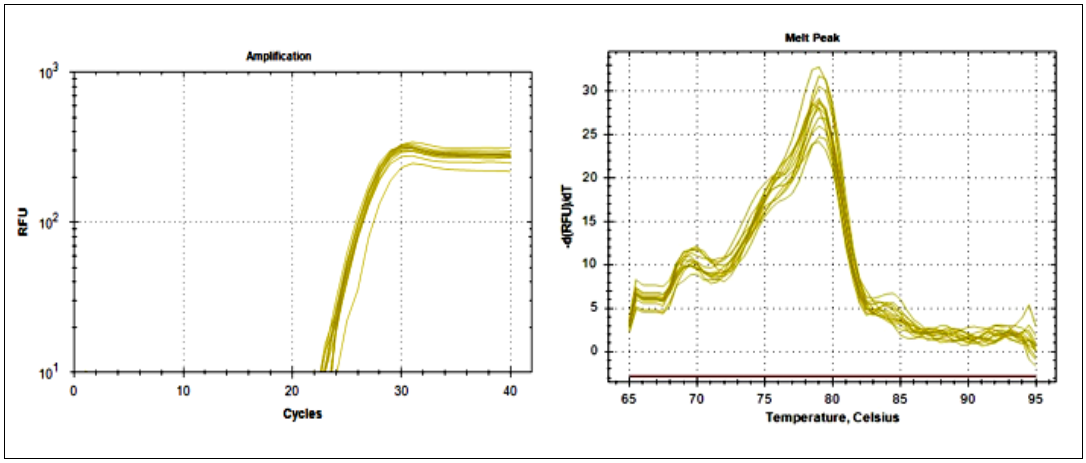

Fig. 1. Amplification plot and melt curve of *PROS1* in Vechur and Frieswal cattle

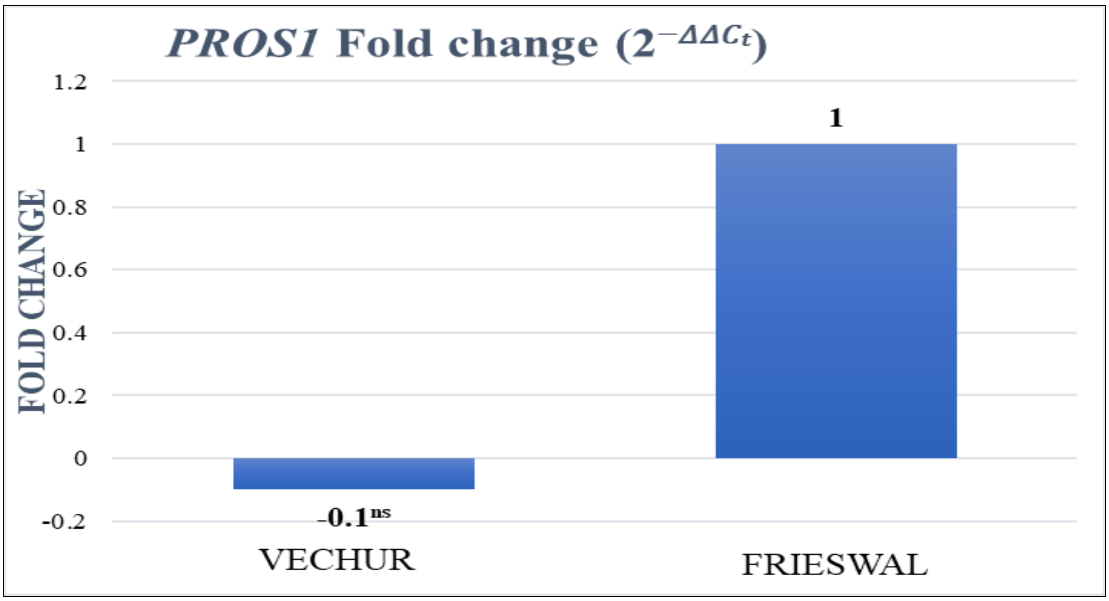

Fig 2: Relative expression of *PROS1* showing fold changes

ns Non-significant at p >0.05

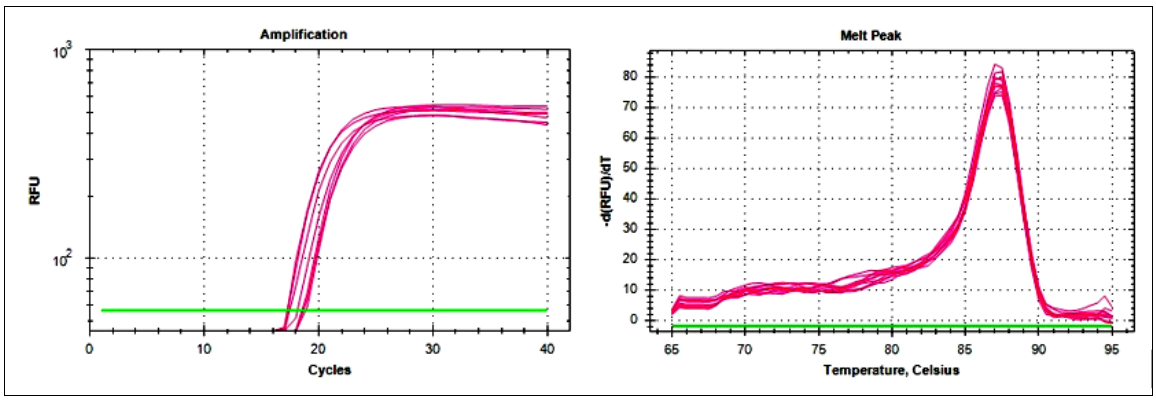

Fig 3: Amplification plot and melt curve of *β-Actin* in Vechur and Frieswal

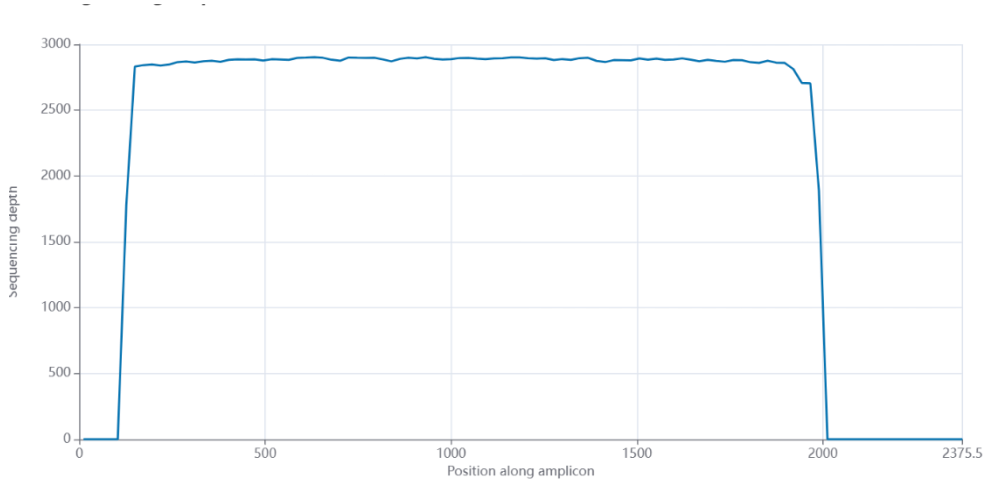

Fig. 4: Coverage plot of *PROS1* showing high sequence depth

4. Conclusion

The study revealed that BT, CT and *PROS1* gene expression were comparable between Vechur and Frieswal cattle, indicating similar haemostatic regulation at the phenotypic and transcriptional levels. In this context, the SNPs identified in Vechur cattle may represent cryptic genetic variation that does not affect baseline haemostatic parameters but could influence Protein S function under physiological stress, warranting validation in larger cattle populations. These findings provide a foundation for future large-scale population and functional studies to elucidate the potential biological significance of these polymorphisms in relation to breed-specific disease resistance and coagulation dynamics.

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6. Conflict of Interest

The authors have no conflict of interest to declare.

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