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Genetic polymorphisms in *TLR4* exon 1 and their association with antibody response following lumpy skin disease vaccination in Hallikar and crossbred Holstein Friesian cattle

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Abstract

Toll-like receptor 4 (*TLR4*) is an important pattern-recognition receptor involved in innate immune activation and the development of adaptive immune responses. The present study investigated polymorphisms in *TLR4* exon 1 and their association with antibody response following Lumpy Skin Disease (LSD) vaccination in Hallikar and Crossbred Holstein Friesian (CB-HF) cattle. A total of 154 cattle (86 Hallikar and 68 CB-HF) were screened, and 100 seronegative animals (50 per breed) with indirect ELISA percentage positivity (PPV) < 35 and Serum Neutralization Test (SNT) titre < 1:8 were enrolled. Animals were vaccinated with the live attenuated Goat pox vaccine (Uttarkashi strain). Serum samples collected on day 0 and 28 days post-vaccination were evaluated by indirect ELISA and SNT. Based on the quartile distribution of fold change in log₂-transformed SNT titres, animals were classified as higher and lower antibody responders. A total of 48 animals (12 higher and 12 lower responders from each breed) were selected for molecular analysis. *TLR4* exon 1 was amplified using exon-specific primers, followed by bidirectional Sanger sequencing. Consensus sequences were generated using CLC Genomics Workbench and aligned with the bovine reference sequence using CLUSTAL W in BioEdit (version 7.2.5). Sequence analysis identified two polymorphic positions in Hallikar cattle and one polymorphic position in CB-HF cattle. In Hallikar cattle, position 413 showed a G→A transition that was not significantly associated with antibody response whereas, position 534 showed an A→C transversion that was significantly ($P = 0.0084$) associated with responder category. In CB-HF cattle, a G→C transversion at position 245 was not significantly associated with antibody response. These findings indicate breed-specific variation in *TLR4* exon 1.

Keywords: *TLR4*, lumpy skin disease, vaccination, antibody response, single nucleotide polymorphism

Introduction

Lumpy skin disease (LSD) is an economically important transboundary viral disease of cattle caused by Lumpy skin disease virus, a member of the genus *Capripoxvirus* under the family *Poxviridae*. It is an acute, subacute, viral disease of cattle which is genetically similar to the goat and sheep pox virus. The disease is characterized by fever, anorexia, and distinctive nodules of 2-5cm on the skin and mucous membranes of nostrils, mouth, udder, rectum, and genitals (Moda *et al.*, 2024, Manjunatha Reddy *et al.*, 2025) [1, 2]. The virus is primarily transmitted mechanically by blood-feeding arthropod vectors, including biting flies, mosquitoes, and ticks. LSD has emerged as a major threat which leads to significant reductions in the productivity and deaths of the animals. The impact of LSD on milk yield differs by region, producing per-animal losses of roughly 8-90 litres (Naidu *et al.*, 2025) [3]. In recent years, LSD outbreaks in India have caused severe economic damage with losses estimated at approximately USD 2.2 billion (Manjunatha Reddy *et al.*, 2023, Tadakod S *et al.*, 2026) [4, 5]. Vaccination remains the principal strategy for preventing and controlling the disease, particularly in endemic and high-risk regions (Tuppurainen *et al.*, 2017, Reddy GB *et al.*, 2026) [6, 7].

Although vaccination can produce protective immunity, considerable animal to animal variation is often observed in the magnitude of the antibody response. Such variation may arise from the combined effects of breed, age, sex, nutritional status, physiological condition,

management, and genetic variation in immune response genes (Meeusen *et al.*, 2007; Glass *et al.*, 2012; Bayyappa *et al.*, 2025) [8, 9, 10]. Candidate genes involved in antigen recognition, antigen presentation and cytokine regulation may contribute to differences in vaccine responsiveness. Genetic differences in genes involved in pathogen recognition, antigen presentation and cytokine signalling may therefore contribute to variation in vaccine responsiveness among cattle (Leach *et al.*, 2010; Glass *et al.*, 2012) [11, 9].

Toll-like receptors are germline encoded pattern-recognition receptors that constitute an important component of innate immunity. These receptors recognize conserved pathogen-associated molecular patterns and endogenous damage-associated molecular patterns and initiate intracellular signalling pathways that regulate inflammatory and antimicrobial responses (Akira, 2001; Akira *et al.*, 2006; Kawai and Akira, 2010) [12, 13, 14]. Among the functional Toll-like receptors identified in cattle, Toll-like receptor 4 (*TLR4*) plays a central role in recognizing bacterial lipopolysaccharide and several endogenous danger signals (Miller *et al.*, 2005; Kawai and Akira, 2010) [15, 14].

The bovine *TLR4* gene has been mapped to chromosome 8 and is expressed in several immune-cell populations, including macrophages, dendritic cells and neutrophils (McGuire *et al.*, 2006) [16]. Activation of *TLR4* initiates MyD88-dependent and TRIF-dependent signalling pathways. Through its effects on dendritic-cell maturation, macrophage activation, antigen presentation and cytokine production, *TLR4* provides an important functional link between innate and adaptive immunity (Akira *et al.*, 2006; Vlasova and Saif, 2021) [13, 17]. Enhanced activation of antigen-presenting cells promotes T-lymphocyte stimulation, B-cell differentiation and antibody production indicating that *TLR4*-mediated signalling may influence the magnitude and quality of vaccine induced immunity (Glass *et al.*, 2012 [9]; Vlasova and Saif, 2021 [17]) [9, 17].

Polymorphisms in the bovine *TLR4* gene have been associated with variation in mastitis resistance, paratuberculosis, inflammatory responses, reproductive traits and vaccine responsiveness (Wang *et al.*, 2007; Glass *et al.*, 2012; Girish *et al.*, 2016; Gopi *et al.*, 2020; Chen *et al.*, 2022) [18, 9, 19, 20]. Despite the recognized role of *TLR4* in innate immune activation, information regarding its genetic variation in relation to antibody response following LSD vaccination is limited. Hallikar cattle represent an indigenous *Bos indicus* breed adapted to tropical environmental conditions whereas Crossbred Holstein Friesian cattle possess both exotic and indigenous genetic contributions. These contrasting genetic backgrounds may contribute to breed wise differences in immune-gene polymorphisms and vaccine responses. Therefore, the present study was designed to identify single nucleotide polymorphisms (SNPs) in exon 1 of the *TLR4* gene in Hallikar and Crossbred Holstein Friesian cattle and to evaluate their association with variation in vaccine induced antibody responses following LSD vaccination (Bayyappa MR *et al.*, 2025) [23].

Materials and Methods

Experimental animals and pre-vaccination management

A total of 154 cattle, comprising 86 Hallikar and 68 Crossbred Holstein Friesian (CB-HF) cattle were identified from different villages of Bagepalli and Shidlaghatta taluks

of Chikkaballapur District, Karnataka. Animals were selected based on dentition, and only cattle possessing a minimum of one pair of permanent incisor teeth were considered for screening. The age of each animal was recorded in years based on the incisor eruption pattern. Before vaccination, all the identified animals were dewormed with fenbendazole bolus 1.5g and were supplemented with a trace mineral bolus to minimize the possible confounding effects of parasitic infestation and mineral deficiency on vaccine-induced immune responses.

Animal selection and vaccination

Before enrolment, all identified animals were screened for pre-existing antibodies against LSDV using indirect enzyme-linked immunosorbent assay (iELISA) and Serum Neutralization Test (SNT). Animals exhibiting an iELISA percentage positivity value of less than 35 PPV and an SNT antibody titre of less than 1:8 were considered seronegative and included in the study. Based on these criteria, 50 Hallikar and 50 CB-HF cattle were enrolled in the study and vaccinated subcutaneously with 1 ml of live attenuated Goatpox vaccine (Uttarkashi strain; Hester Biosciences Ltd., India) containing not less than 1×10^3 TCID₅₀ per dose, as per the manufacturer's recommendations and the Government of India guidelines for heterologous vaccination against LSD.

Classification of antibody responder groups

Post-vaccination antibody responses were assessed at 28 days using iELISA and SNT. Since SNT is the reference assay for evaluating functional neutralizing antibodies, SNT titres were used for fold-change calculations and responder classification. Animals were categorized into higher and lower antibody responder groups based on the fold change in log₂-transformed SNT titres by using quartile based distribution. Animals with fold-change values $\leq$ Q1 (25th percentile) were designated as lower responders, those with values between Q1 and Q3 (25th-75th percentile; including Q2, the median) as average responders, and those with values $\geq$ Q3 (75th percentile) as higher responders. Only the higher and lower responder groups were selected for sequencing and subsequent SNP association analysis.

DNA isolation and quality assessment

Genomic DNA was isolated from EDTA-anticoagulated whole blood samples using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed using a UV spectrophotometer, and DNA integrity was confirmed by electrophoresis on 1.5 percent agarose gel. DNA samples of adequate quality and concentration were used for amplification of *TLR4* exon 1.

Selection of the target region and primer design

TLR4 exon 1 was selected as one of the candidate immune response gene regions for molecular characterization. The *Bos taurus* reference sequence was obtained from the Ensembl Genome Browser (*TLR4* gene/transcript ID: ENSBTAT00000008190, *TLR4* exon ID: ENSBTAE00000066213). Primers were designed using PerlPrimer and synthesized by Eurofins Genomics India, Bengaluru, the details of the primers are represented in Table 1.

Table 1: Details of primer sequences used for amplification of bovine *TLR4* exon 1

Gene	Exon	Primer	Sequence (5'-3')	Expected product size (bp)
<i>TLR4</i>	Exon 1	Forward	TTTGTTATGGCTGGAACATAGG	620
		Reverse	CACAGGGAAACTGAGCAGAG	

Primer reconstitution

Lyophilized primers were reconstituted in nuclease free water to obtain a stock concentration of 100 pmol/μl or 100 μM. A working concentration of 10 pmol/μl or 10 μM was prepared by mixing 10 μl of primer stock with 90 μl of nuclease-free water. Stock and working primer solutions were stored at -20 °C and 4 °C, respectively.

PCR reaction mixture

PCR amplification was carried out in a final volume of 25 μl using the reaction composition as mentioned in the Table 2.

Table 2: Composition of PCR reaction mixture

Sl. No.	Component	Volume (μl)
1.	PCR Master Mix (2X)	12.5
2.	Template DNA	2.0
3.	Forward primer	1.0
4.	Reverse primer	1.0
5.	Nuclease-free water	8.5
	Total volume	25.0

PCR thermal cycling conditions

PCR amplification of *TLR4* exon 1 was performed using the standardized thermal profile, the details of the PCR thermal cycling conditions are mentioned in Table 3.

Table 3: PCR thermal cycling conditions used for amplification of bovine *TLR* exon 1

Sl. No.	Step	Temperature	Duration
1.	Initial denaturation	94 °C	5 min
2.	Denaturation	94 °C	40 sec
3.	Annealing	58.0 °C	30 sec
4.	Extension	72 °C	30 sec
5.	Step 2 to 4 were repeated 35 times		
6.	Final extension	72 °C	7 min
7.	Hold	4 °C	Until removal

Agarose gel electrophoresis

PCR products were evaluated using 1.5 percent agarose gel electrophoresis in 1× TBE buffer. Electrophoresis was performed with a 100 bp DNA ladder at 80 V for 60 minutes. Amplified products were visualized under ultraviolet illumination and documented using a gel documentation system.

Purification and Sanger sequencing

PCR amplified products selected from the higher and lower responder groups were purified and subjected to bidirectional sequencing using the Sanger sequencing method.

Sequence editing and consensus sequence generation

Forward and reverse Sanger sequencing reads were quality checked and low quality bases at the 5' and 3' ends were trimmed using CLC Genomics Workbench (version 9.5.2). The reverse reads were reverse-complemented and assembled with the corresponding forward reads to generate consensus sequences.

Sequence alignment and SNP identification

The consensus sequences were aligned with the *Bos taurus* reference sequence using the CLUSTAL W algorithm implemented in BioEdit (version 7.2.5) to identify SNPs. Chromatograms corresponding to the identified polymorphic positions were manually inspected to confirm single peaks and overlapping heterozygous peaks using Chromas Lite (version 2.6.6).

Statistical analysis

The association between identified *TLR4* SNPs and antibody response category was evaluated using the two-tailed Fisher's exact test. Contingency tables were constructed by comparing the distribution of reference and variant nucleotides between higher and lower antibody responder groups. The statistical analysis was performed using IBM SPSS Statistics. A P-value below 0.05 was considered statistically significant.

Results

Post vaccination humoral immune response

A marked increase in antibody titres was observed in both breeds at 28 days post-vaccination (dpv). The post-vaccination indirect ELISA PPV values, SNT titres, and geometric mean titres (GMT) are presented in Table 4.

Table 4: Post-vaccination humoral immune response in Hallikar and CB-HF cattle

Parameter	Hallikar (Mean ± SE)	CB-HF (Mean ± SE)
Indirect ELISA PPV (0 dpv)	20.67 ± 1.48	24.51 ± 1.43
Indirect ELISA PPV (28 dpv)	91.29 ± 3.08	85.75 ± 1.90
SNT titre (log ₂) (0 dpv)	1.98 ± 0.07	2.19 ± 0.07
SNT titre (log ₂) (28 dpv)	6.19 ± 0.08	5.91 ± 0.07
GMT (0 dpv)	3.94	4.56
GMT (28 dpv)	72.93	60.13

Values for indirect ELISA PPV and log₂-transformed SNT titres are presented as mean ± SE. Geometric mean titres are presented as absolute values.

Classification of antibody responder groups

The fold change in log₂-transformed SNT titres was calculated separately for Hallikar and Crossbred Holstein Friesian (CB-HF) cattle and classified using a quartile based distribution. In Hallikar cattle, the first quartile (Q1), median (Q2) and third quartile (Q3) values were 4.03, 4.04 and 5.03, respectively. Accordingly, animals with fold-change values ≤ 4.03 were classified as lower responders, those with values > 4.03 and < 5.03 were classified as average responders, and animals with fold-change values ≥ 5.03 were classified as higher responders.

In CB-HF cattle, the Q1, Q2 and Q3 values were 3.04, 4.03 and 4.04, respectively. Animals with fold-change values ≤ 3.04 were categorized as lower responders, those with values > 3.04 and < 4.04 were categorized as average responders, and animals with fold-change values ≥ 4.04 were categorized as higher responders. Based on this classification, 12 higher and 12 lower antibody responder animals from each breed were selected for bidirectional

Sanger sequencing and subsequent SNP association analysis.

Genomic DNA isolation and quality assessment

High quality genomic DNA was successfully isolated from all selected animals. The DNA concentration ranged from 60 to 110 ng/μl, with a mean concentration of 85 ± 12 ng/μl. The A260/A280 nm ratio ranged from 1.75 to 2.00, indicating good purity. Agarose gel electrophoresis showed

intact high-molecular-weight genomic DNA suitable for PCR amplification.

PCR amplification of *TLR4* exon 1

PCR amplification of *TLR4* exon 1 was successful in all selected animals. Agarose gel (1.5%) electrophoresis revealed distinct bands of the expected size without non-specific amplification or primer-dimer formation (Figure 1).

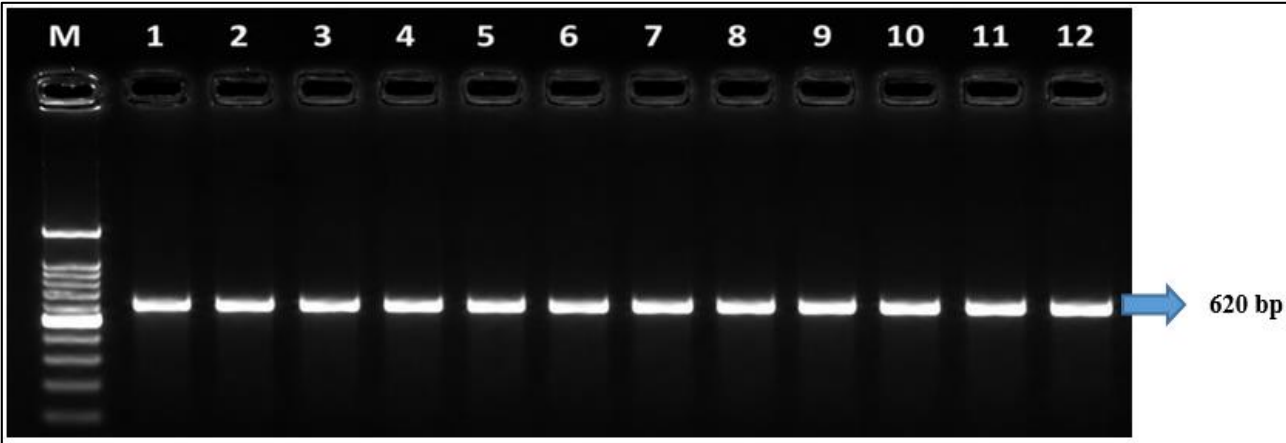


Fig 1: Agarose gel (1.5%) showing PCR amplified product of *TLR4* (exon 1) gene. Lane M: Molecular marker (100 bp DNA ladder), Lanes 1, 2, 3, 4, 5, 6: PCR amplified product 620 bp (Hallikar) and Lane 7, 8, 9, 10, 11, 12 : PCR amplified product 620 bp (CB-HF cattle)

Identification of *TLR4* exon 1 polymorphisms

Sequence alignment of *TLR4* exon 1 identified breed specific SNPs in both Hallikar and Crossbred Holstein Friesian cattle. Two polymorphic nucleotide positions were detected in Hallikar cattle whereas, one polymorphic nucleotide position was identified in CB-HF cattle. The identified nucleotide substitutions comprised both transition and transversion.

analysis of Hallikar cattle identified two nucleotide polymorphisms at positions 413 and 534 in the studied population. At nucleotide position 413, the reference nucleotide G was replaced by A, representing a transition substitution. At nucleotide position 534, the reference nucleotide A was substituted by C, representing a transversion. Representative multiple sequence alignment and chromatograms illustrating the detected polymorphisms are presented in Figures 2, 3 and 4, respectively.

***TLR4* exon 1 polymorphisms in Hallikar cattle: Sequence**

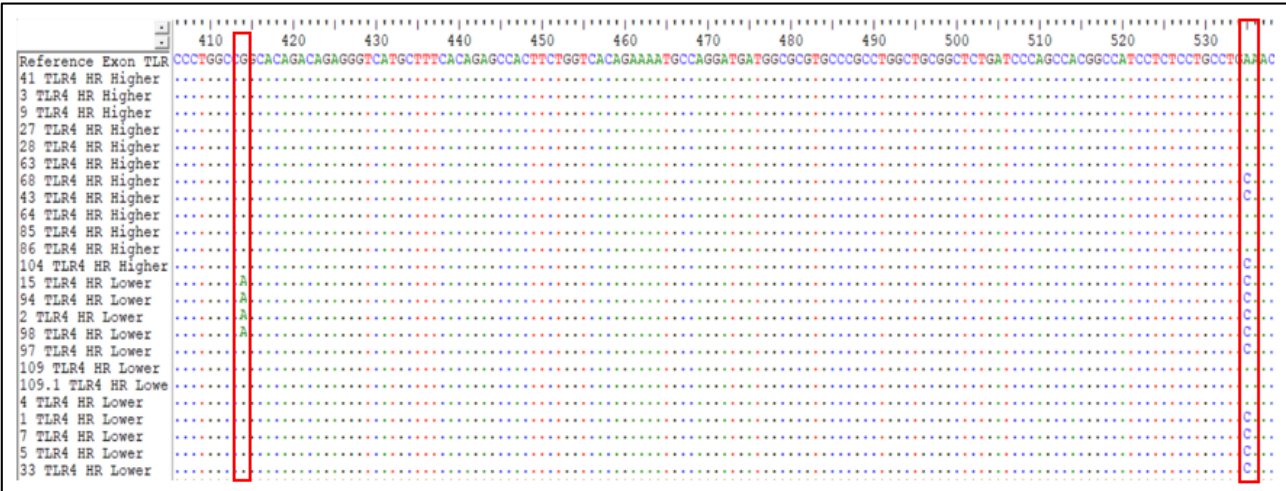


Fig 2: Multiple sequence alignment of *TLR4* exon 1 sequences from Hallikar cattle showing nucleotide variations at positions 413 and 534

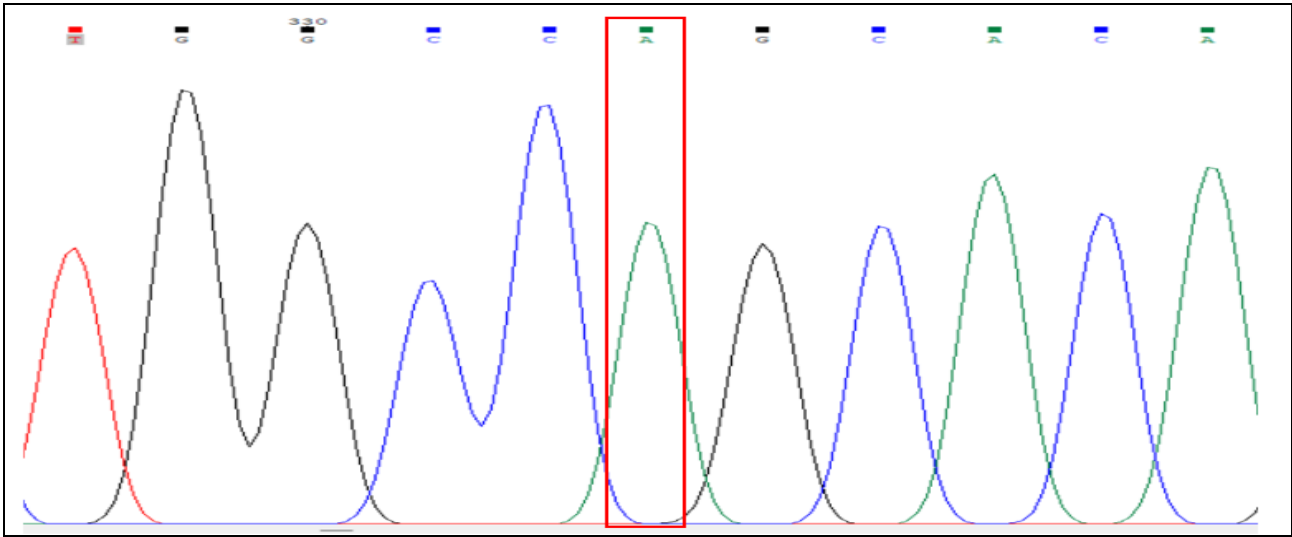


Fig 3: A representative chromatogram c.413G>A polymorphism in the *TLR4* exon 1 in Hallikar Cattle at position 413

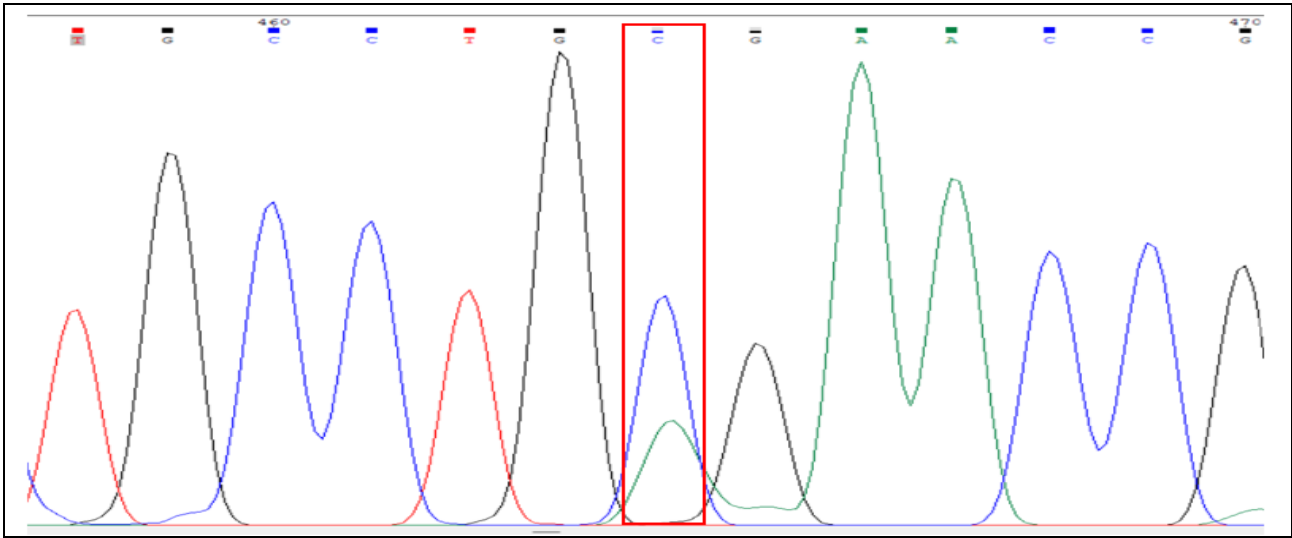


Fig 4: A representative chromatogram c.534 A>C polymorphism in the *TLR4* exon 1 in Hallikar Cattle at position 534

***TLR4* exon 1 polymorphism in Crossbred Holstein Friesian cattle**
Sequence alignment of CB-HF cattle identified a single polymorphic nucleotide position at 245. The reference

nucleotide G was replaced by C, representing a transversion mutation. Representative multiple sequence alignment and chromatograms illustrating the detected polymorphisms are presented in Figures 5 and 6, respectively.

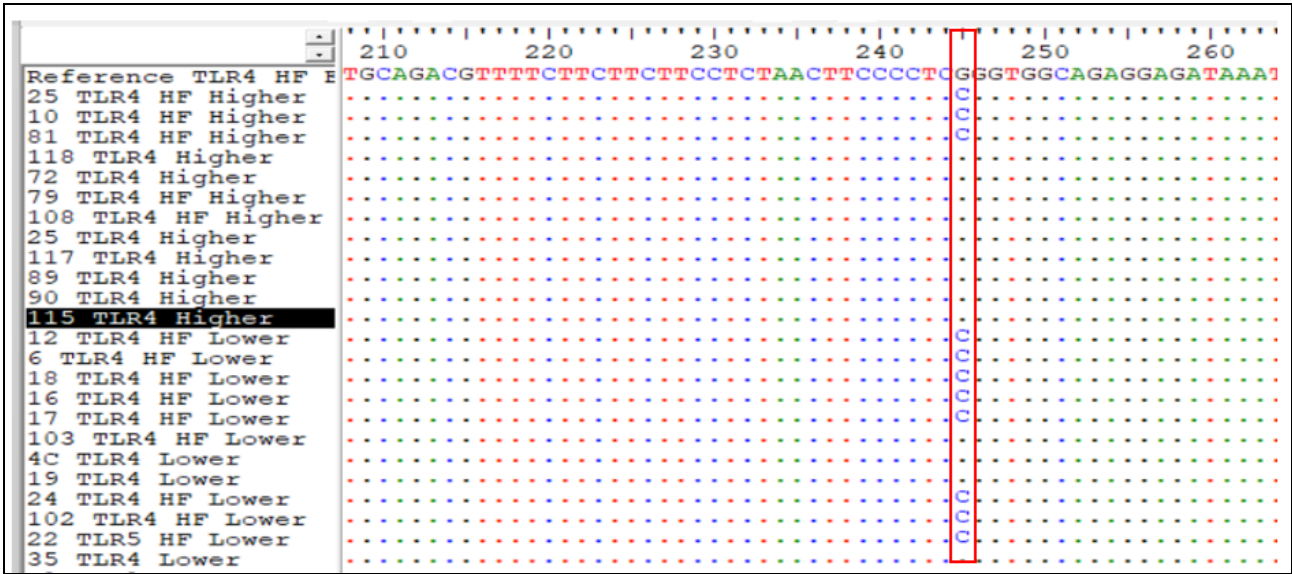


Fig 5: Multiple sequence alignment of *TLR4* exon 1 sequences from CB-HF cattle showing nucleotide variations at position 245

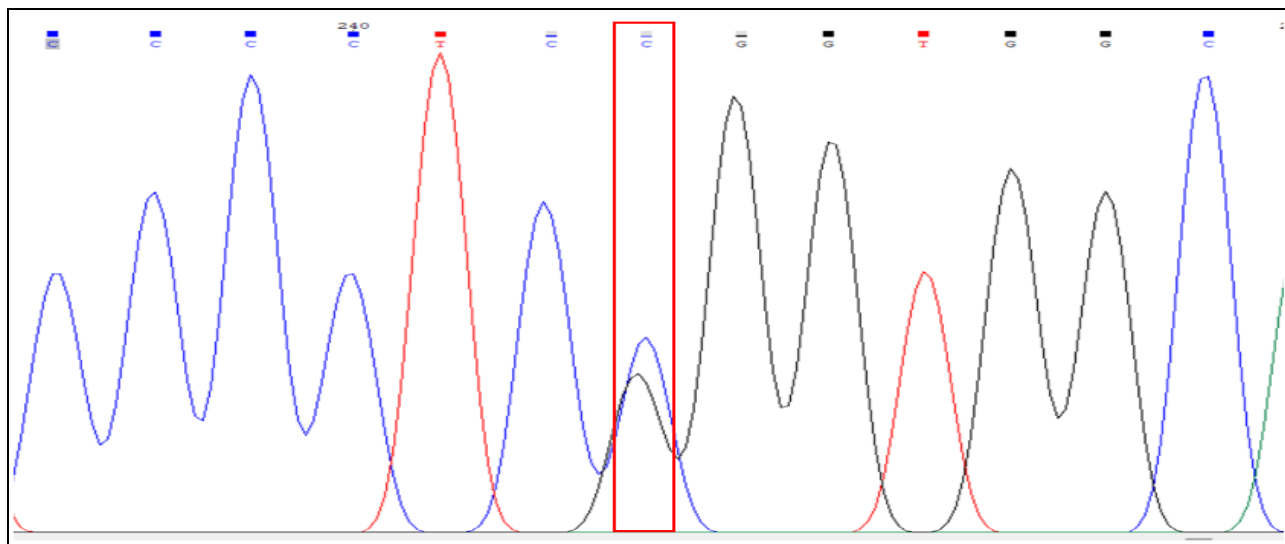


Fig 6: A representative chromatogram c.245G>C polymorphism in the *TLR4* exon 1 in CB-HF cattle at position 245

Association of *TLR4* exon 1 polymorphisms with antibody response

The distribution of *TLR4* exon 1 SNPs between higher and lower antibody responder groups was evaluated using the two-tailed Fisher's exact test. Among the three polymorphic positions identified, only the A>C substitution at nucleotide position 534 in Hallikar cattle showed a significant ($P = 0.0084$) association with antibody response following

vaccination. In contrast, the G>A substitution at position 413 in Hallikar cattle and the G>C substitution at position 245 in CB-HF cattle were not significantly associated with antibody responder status. The identified *TLR4* exon 1 polymorphisms and their association with antibody response in Hallikar and Crossbred Holstein Friesian (CB-HF) cattle are summarized in Table 5.

Table 5: Association of *TLR4* exon 1 polymorphisms with antibody response in Hallikar and Crossbred Holstein Friesian (CB-HF) cattle

Breed	Nucleotide position	Reference nucleotide	Variant nucleotide	Higher responders (2n = 24)	Lower responders (2n = 24)	Fisher's exact test (P-value)	Significance
Hallikar	413	G/G	A	G = 22, A = 2	G = 16, A = 8	0.0723	Non-significant
	534	A/A	C	A = 3, C = 9	A = 9, C = 3	0.0084*	Significant
CB-HF	245	G/G	C	C = 8, G = 16	C = 14, G = 10	0.1468	Non-significant

Note: Values for higher and lower responder groups represent allele counts (2n = 24 alleles) obtained from 12 animals in each responder group. Statistical significance was evaluated using the two-tailed Fisher's exact test.

Discussion

In the present study, sequence analysis of *TLR4* exon 1 identified two polymorphic nucleotide positions (413 and 534) in Hallikar cattle and one polymorphic position (245) in Crossbred Holstein Friesian (CB-HF) cattle demonstrates the existence of breed specific genetic variation within the *TLR4* gene. Similar genetic diversity in the bovine *TLR4* gene has been reported previously in different indigenous and crossbred cattle populations (Wang *et al.*, 2007; Girish *et al.*, 2016; Chen *et al.*, 2022) [18, 19, 21], but the polymorphic positions identified in the present study differed from those reported earlier. The differences may be attributed to variation in cattle breeds, population structure, genomic regions analysed and evolutionary selection pressures. At nucleotide position 413 in Hallikar cattle, G>A transition was identified. Although the variant A nucleotide occurred more frequently among lower antibody responders than higher responders, the association was not statistically significant. Similarly, in CB-HF cattle, G>C transversion was identified at nucleotide position 245, where the C nucleotide occurred more frequently among lower responders, the difference was also not statistically significant. These findings suggest that the identified polymorphisms may not have a measurable influence on

vaccine-induced antibody response in the studied population. Comparable observations have been reported by Girish *et al.* (2016) [19] who identified several *TLR4* polymorphisms in the entire gene in indigenous and crossbred cattle but did not observe significant associations between all variants and mastitis resistance. Likewise, Sonwane *et al.* (2023) [22] reported *TLR4* polymorphisms in exon 3 in HF × Gir crossbred cattle without detecting significant association with somatic cell count among 40 subclinical mastitis affected and 20 healthy cows. In contrast, nucleotide position 534 in Hallikar cattle showed a significant association ($P = 0.0084$) with antibody response. The variant 'C' nucleotide was more frequently observed among higher antibody responders whereas the reference 'A' nucleotide predominated among lower responders. This observation suggests that the polymorphism at position 534, or another functional variant in linkage disequilibrium with it, may influence vaccine induced humoral immunity. Although the exact biological effect of this nucleotide substitution could not be determined in the present study, the observed association indicates that this locus may serve as a potential genetic marker for variation in antibody response following LSD vaccination.

The present findings are consistent with previous studies demonstrating the involvement of *TLR4* polymorphisms in bovine immune responses. Glass *et al.* (2012) [9] reported that genetic variation in *TLR4* influenced IgG1 and IgG2 responses following vaccination against bovine respiratory syncytial virus and foot-and-mouth disease virus, indicating that innate immune receptor polymorphisms contribute to variation in adaptive immune responses. Similarly, Chen *et al.* (2022) [21] demonstrated that the missense mutation rs8193069 within the *TLR4* gene was significantly associated ($p < 0.05$) with mastitis resistance and somatic cell count in Holstein cattle. Functional analysis suggested that the mutation altered the receptor structure and downstream signalling, thereby influencing inflammatory responses. Wang *et al.* (2007) [18] also reported associations between *TLR4* polymorphisms at nucleotide 4525 in intron 1, and a C→T substitution at nucleotide 1397 in exon 3 and mastitis resistance traits in dairy cattle, supporting the importance of *TLR4* gene in disease resistance.

However, the present investigation provides the first evidence to our knowledge, of an association between a *TLR4* exon 1 polymorphism and antibody response following LSD vaccination in Hallikar cattle. Future studies integrating genome wide association analyses, transcriptomics, functional assays and protein modelling would help determine the biological significance of this polymorphism and evaluate its potential application in marker-assisted selection for improved vaccine responsiveness and disease resistance.

Conclusions

The present study identified two polymorphic nucleotide positions (413 and 534) in Hallikar cattle and one polymorphic position (245) in CB-HF cattle. Among these, the A>C substitution at nucleotide position 534 in Hallikar cattle showed a significant association ($P = 0.0084$) with the vaccine-induced antibody response. These findings suggest that the *TLR4* polymorphism at position 534 may represent a potential candidate marker for variation in vaccine responsiveness in Hallikar cattle. However, validation in larger and genetically diverse cattle populations, together with functional characterization of the identified variants is required before their application in marker assisted selection programmes aimed at improving vaccine responsiveness and disease resistance.

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