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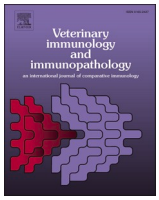

CD4 bovine gene: Differential polymorphisms among cattle breeds and a new tool for rapid identification

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ABSTRACT

Two mutations in the CD4 bovine gene (G>T/Q306H; A>C/K310N) were identified as causative for altered staining with anti-CD4 mAb #CC8. We developed a HRM qPCR for genotyping these mutations and compare with immunophenotyping in different cattle breeds. The assay distinguished five genotypes, B (homozygous, G/A) and C (heterozygous, G/A and T/C), found in taurine, A (homozygous, G/C) and D (heterozygous, T/C and G/C), found in zebu. The E genotype (homozygous, T/C) was not observed in tested animals. As expected, B and C presented high/very high and intermediate CD4 staining, respectively. The lack/low CD4 staining was mainly related to the A, while the intermediate staining was mainly related to D genotype. The developed HRM qPCR assay accurately identified the altered phenotypes associated with CC8 staining in taurine. However, the assay cannot be applicable in zebu or hybrid breeds, probably due to additional mutations in the CD4 gene from zebu descendant animals.

1. Introduction

The cell-surface CD4 receptor expressed by T helper cells is constituted by four immunoglobulin-like domains (D1 to D4) as part of the extracellular domain, a transmembrane domain, and a cytoplasmic tail (Kwong et al., 1990). The extracellular domain binds to the monomorphic region of MHC class II on antigen-presenting cells to increase the affinity of the T cell receptor to the antigen peptide MHC class II complex (Kwong et al., 1990). Three allelic forms of bovine CD4 associated with altered reactivity with anti-CD4 monoclonal antibody (mAb) CC26 clones were described in both *Bos taurus* and *Bos indicus* cattle, indicating that it had arisen prior to the divergence of these subspecies (Morrison et al., 1991). Similarly, allelic variation was also observed for anti-CD4 mAb CC8 clone in pure taurine breeds (Grandoni et al., 2020; Kato-Mori et al., 2021) and taurine/zebu hybrid (Okino et al., 2020), these altered phenotypes were associated to differential susceptibility to *Babesia bovis*, as well as altered immune cells kinetics during primary infection in calves (Okino et al., 2020).

Two missense mutations in the exon 5 (Q306H – G>T and K310N - A>C), pointed out as causative for these altered phenotypic profiles related to anti-CD4 CC8 clone, were identified in Italian dairy cattle (Grandoni et al., 2020), while in Japanese black cattle, an additional mutation in the D4 domain of CD4 gene was also observed (A324T) (Kato-Mori et al., 2021). Thus, our study aimed to evaluate the missense mutations in exon 5 of CD4 gene from different cattle breeds and develop a HRM qPCR assay for genotyping these mutations.

2. Material and methods

2.1. Experimental animals

One hundred-sixty two animals were used in this study (healthy adult, females and males). Forty-eight of Canchim breed (5/8 Charolais + 3/8 Zebu), thirty-four of Holstein breed, and fifty-two of Nelore breed were from Embrapa Pecuária Sudeste experimental farm (located in São Carlos – SP – Brazil), while fourteen Angus, thirteen Ultrablack and one

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¾ Angus x ¼ Nelore were from a farm located at José Bonifácio city, São Paulo state, Brazil. The ¾ Angus x ¼ Nelore animal was here included in the Ultrablack group. The blood was collected from jugular vein using vacutainer tubes and subjected to immunophenotyping and DNA extraction assays. All procedures were approved by the Embrapa Pecuária Sudeste Ethical Committee for Animal Experimentation (CEUA/CPPSE) and Ethics Committee on Animal use of the Instituto de Zootecnia (CEUA/IZ), following the ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation (process number PRT 02/2017 and PRT 328–2021).

2.2. Immunophenotyping of bovine mononuclear cells

Immunophenotyping assays were carried out as previously optimized and described (Okino et al., 2019, 2020). Briefly, twenty-five microliters of whole blood cells were placed into polystyrene tubes with 50 μL of purified mouse IgG (1.25 μg) and incubated at 4 °C for 15 min. The panel consisted of 2 μL anti-CD21 FITC (Cat. MCA1424F, Biorad), 2 μL CD8β RPE (Cat. MCA1654PE, Biorad), and 2 μL CD4 AlexaFluor647 (Cat. MCA1653A647, Biorad). The mAbs were diluted in Stain buffer containing 2% fetal bovine serum to a final volume of 50 μL. The samples were incubated at 4 °C in the dark for 30 min. Then, the erythrocytes were lysed by adding 1 mL of 1 × lysing solution and incubating for 15 min at room temperature. The samples were centrifuged at room temperature for 5 min at 300g. The total leukocytes were washed with 500 μL of Stain buffer and centrifuged at room temperature for 5 min at 300g. The pelleted stained cells were resuspended in 100 μL of Stain buffer, and one drop of cell viability marker 7AAD (Cat. 1351102, Biorad) was added to each sample. All the samples were analyzed by flow cytometry using Accuri C6 Plus (BD Biosciences, Franklin Lakes, USA) on the same day. The cytometer settings were calibrated and adjusted each day after running the cytometer setup and tracking using CS&T RUO beads. At least 30,000 events were acquired in the cell gate. The data were collected using BD Accuri C6 Plus software and analyzed in the Flowjo software version 10.8.1. All data were manually compensated, and a hierarchical gating strategy was applied by gating (in the following sequence: singlet cells, live cells (not stained with 7AAD), and mononuclear cells. The median fluorescence intensity (MFI) of the CD4 + gate was scaled and divided into four groups through cluster analysis using kmeans option of the rattle package of RStudio 2021.09.2 version (R Core Team, 2020).

2.3. DNA extraction

The total DNA extractions were carried out from 30 μL of whole blood using the Easy-DNA™ kit (Invitrogen, Carlsbad, USA, Cat. n. K1800–01—Protocol #1—Small Blood Samples and Hair Follicles). The DNA extracted quantity and purity were estimated by 260 nm ultraviolet absorbance and readings at 260/280 nm. Negative control (PBS instead of blood samples) was added in each extraction lot.

2.4. Primer design

Our strategy was to develop a qPCR assay based on DNA sequence rather than RNA, as the first option is cheaper, making the technique more available to most laboratories. Therefore, a pair of primers was designed for the qPCR assay (CD4_CC8F: 5´- TGACCCTGGATCTCACCAA – 3´and CD4_CC8R: 5′ – ATCTCAGCCTAGCTCCTCAC – 3). The estimated amplicon size was 80 base pairs (bp). The oligonucleotides were designed using PrimerQuest software [http:// https://www.idtdna.com/Primerquest/Home/Index], aiming to get primers flanking the two described missense mutations in the exon 5 of the CD4 gene (Q306H and K310N) (Grandoni et al., 2020; Kato-Mori et al., 2021) and annealing to a region presenting 100% of homology between *Bos taurus* and *Bos indicus* sequences (Supplementary information 1).

2.5. qPCR assay followed by High Resolution Melting analysis

The qPCR was optimized by varying the concentration of oligonucleotides, DMSO addition and annealing temperatures.The final assay was performed in a 10 μL of volume, using 5 μL of Ssofast Evagreen Supermix (Biorad – Cat. 172–5200), 0.3 μL of each 10 μM primer (CD4_CC8F and CD4_CC8R), 2.4 μL of molecular biology grade water and 5 ng of DNA or plasmidial DNA from cloned samples. The thermal profile was 98 °C for 2 min, followed by 40 cycles of 98 °C for 10 s and 62 °C for 10 s (reading) and a melting dissociation curve from 65 °C to 95 °C with 0.2 °C/5 s increments in a CFX96 thermocycler (Biorad). The high-resolution melting (HRM) analysis was performed using Precision Melt Analysis software (Cat 1845015, Biorad). Aiming to evaluate the reproducibility of developed qPCR, two animals from each genotype per breed (A genotype: 2 Ultrablack, 2 Canchim and 2 Nelore; B genotype: 2 Holstein, 2 Angus and 2 Canchim); C genotype (2 Canchim and 2 Holstein); D genotype (2 Canchim, 2 Ultrablack and 2 Nelore) and plasmidial DNA from 8 different clones (a4 and a5 from animal genotyped as A – G/C SNPs; d4 and d5 from animal genotyped as B – G/A SNPs; e1 and e2 from animal genotyped as C – T/C SNPs; i3 and i4 from animal genotyped as D – G/C SNPs – Supplementary information 3) were tested using technical duplicates in five independent runs. Since the E genotype, the homozygous for both mutated SNPs (T/C), was not detected among the experimental animals, the plasmidial DNA from two clones (e1 and e2) of the animal genotyped as C (heterozygous) were used for testing E genotype (Fig. 2 and 3). Mixtures of plasmidial DNA were also tested: d4 + e1 clones and i4 + e1 clones.

2.6. Nucleotide sequencing of qPCR products

Since we have found four genotypes by developed HRM qPCR assay in all tested animals, one animal from each genotype was selected for cloning. Briefly, the assay was performed in a final volume of 50 μL, using Platinum ™ Taq DNA Polymerase High Fidelity kit (Thermofisher – Carlsbad, CA, USA), and the final concentrations of reagents were: 2 mM MgSO4, 1 U of Taq DNA Polymerase PCR Buffer, 0.2 mM dNTP mix (Sigma–Aldrich, St. Louis, USA), 0.6 μM of CD4_CC8F and CD4_CC8R primers and ≅ 60 ng of undiluted DNA. The thermal profile was 95 ∘C for 3 min, followed by 40 cycles at 95 ∘C for 30 s, 60 ∘C for 30 s, and 68 ∘C for 30 s, and a final extension of 68° for 2 min. The amplification products were submitted to electrophoresis in 1.0 % agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Cat. A9282 - Promega). After purifying, the samples were submitted to the A-tailing, ligation, and transformation procedures, according to instructions from pGEM®-T Easy Vector Systems (Cat. A1360 - Promega). Finally, white colonies were picked and submitted to insert verification by colony PCR, with the amplicon-specific primers (Green and Sambrook, 2019). The amplicons from colony PCR were visualized in 1.5% agarose gel. Plasmid minipreps were performed from recombinant colonies according to PureYieldTM Plasmid Miniprep System (Cat. A1222 - Promega). The plasmids were quantified in Qubit 3.0 fluorometer (Life technologies) with the Qubit™ dsDNA HS assay kit (Cat.Q32851 - Life technologies). The sequencing reaction was performed using M13 primers and BigDye Terminator v3.1 cycle sequencing kit (Thermofisher) and then analyzed by an ABI Prism 3730XL DNA analyzer (Thermofisher). The resulting DNA sequences were aligned and assembled using Bioedit version 7.2.5. software and compared to those already deposited in GenBank.

3. Results

3.1. CD4 staining using anti-CD4 CC8 clone

The median fluorescence intensity of CD4 cell staining with mAb of CC8 clone was clustered into four groups (Fig. 1A). The low (*l*) group

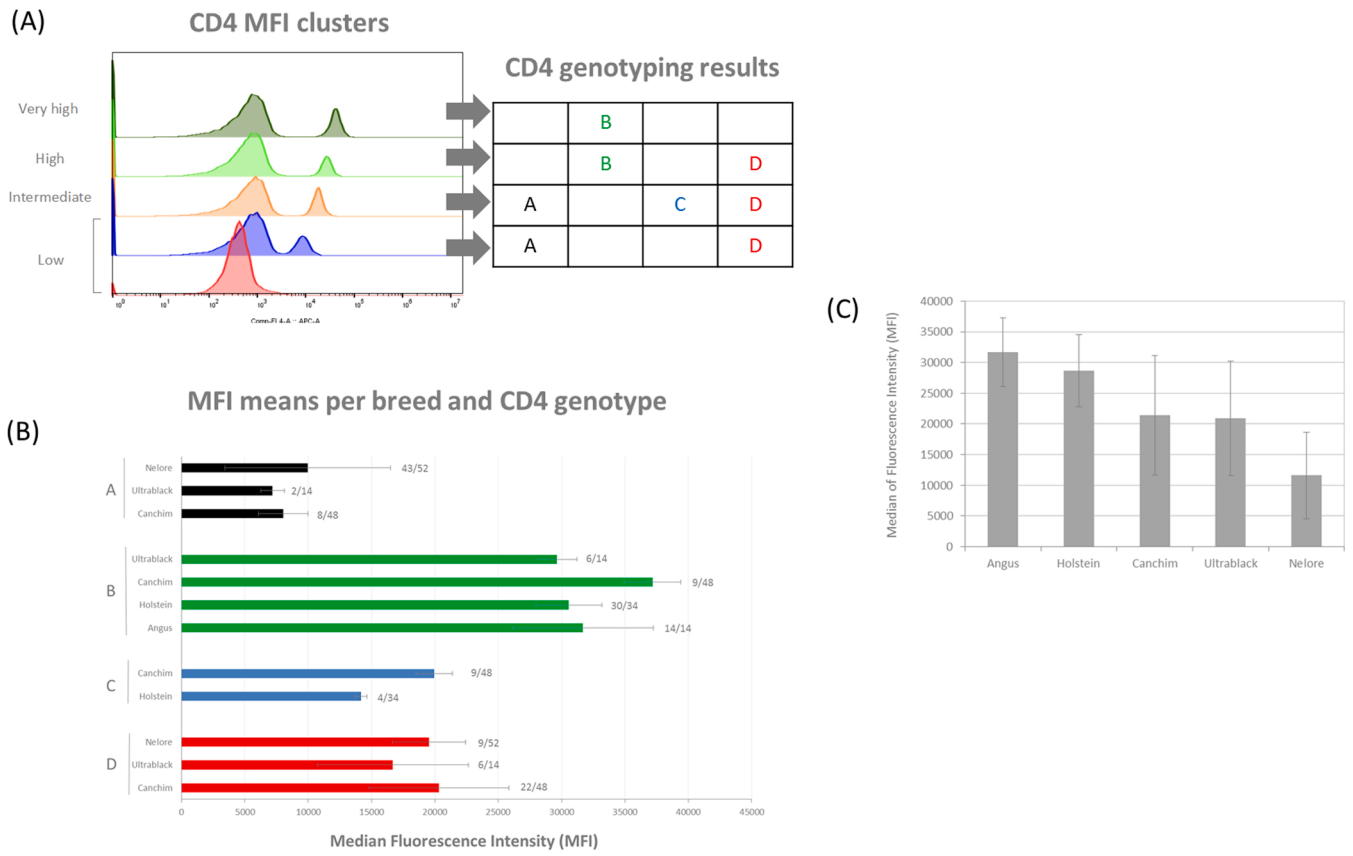

Fig. 1. Median fluorescence intensity (MFI) of CD4 staining using anti-CD4 CC8 clone compared to CD4 genotyping results by the developed HRM qPCR assay. (A) Histogram of animals presenting very high, high, intermediate, and low CD4 staining and respective CD4 genotyping results (A – homozygous, G/C; B – homozygous, G/A; C – heterozygous G/A and T/C; D - heterozygous, T/C and G/C). (B) MFI means per CD4 genotype per breed, including the number of animals per total number of animals evaluated for each breed. (C) Means of MFI per cattle breed.

included animals presenting a lack or very low staining (MFI ranging from 2888 to 12363), which was composed of hybrid (3 Ultrablack and 10 Canchim) and zebu (32 Nelore) breeds. The intermediate (*i*) group included animals presenting moderate staining (MFI ranging from 13574 to 23871), which was composed of taurine (4 Holstein), hybrid (5 Ultrablack and 25 Canchim), and zebu (20 Nelore) breeds. The high (*h*) group included animals presenting high staining (MFI ranging from 24962 to 32503), which was composed of taurine (10 Angus and 22 Holstein), hybrid (6 Ultrablack and 4 Canchim), and zebu (1 Nelore) breeds. Finally, the very high (*v*) group included animals presenting very high staining (MFI ranging from 32768 to 43016), which was composed of taurine (4 Angus and 8 Holstein) and hybrid (9 Canchim) breeds.

All the evaluated samples presented normal staining for anti-CD8 and anti-CD21 mAbs. Since the main purpose of this study was to compare the levels of CD4 staining, these results were not analyzed and were used as controls for the immunophenotyping procedure.

3.2. HRM qPCR assay for CD4 genotyping

The developed HRM qPCR assay for CD4 genotyping presented 100% of reproducibility, since all the five independent run presented same results (Supplementary information 2). Four different genotypes (A, B, C and D) were found in the experimental animals (Figs. 1 and 2), while the E genotype, the homozygous for both mutated SNPs (T/C), was not detected As expected, the mixtures of plasmidial DNA: d4 + e1 and i4 + e1 resulted in C and D genotypes, respectively.

The Holstein breed showed B and C genotypes, which included animals clustered into *h/v* and *i* phenotypes (CD4 staining). Angus breed presented only B genotype harboring animals from *h* and *v* phenotypes. Canchim breed animals were classified in A, B, C, and D genotypes, wherein *v*, *i* and *l* phenotypes were expressed by all the B animals, C and A animals, respectively, while D animals presented *h*, *i*, or *l* phenotypes. Ultrablack breed presented A, B, and D genotypes, and wherein all the B animals were from *h* phenotype, all the A animals showed *l* phenotype and D animals presented *l* or *i* phenotypes. Finally, the Nelore breed revealed the presence of A and D genotypes, wherein A animals were associated to *l* or *i* phenotypes and D animals to *i* and *h* phenotypes. Comparisons between MFI and CD4 genotyping results per breed are shown in the Fig. 1.

3.3. Nucleotide sequencing of qPCR products of CD4 gene

One animal from each CD4 genotype (A, B, C, and D) was selected for cloning and nucleotide sequencing for the genic region flanked by qPCR primers, and compared to sequences previously deposited in the Genbank or Ensembl database (Table 2 and Supplementary information 3). The A and B genotypes were homozygous for G/C (non-mutated) and G/A target SNPs, respectively, wherein the last one presented an altered second SNP (C>A). The C genotype was heterozygous for G/A and T/C target SNPs, while the D was heterozygous for G/C and T/C target SNPs. The homozygous genotype for T/C SNPs, defined as the E genotype in the HRM qPCR assay, was not found in the experimental animals of this study.

4. Discussion

A new rapid and specific HRM qPCR assay for identification of CD4 polymorphisms in cattle related to altered staining by CC8 clone of anti-

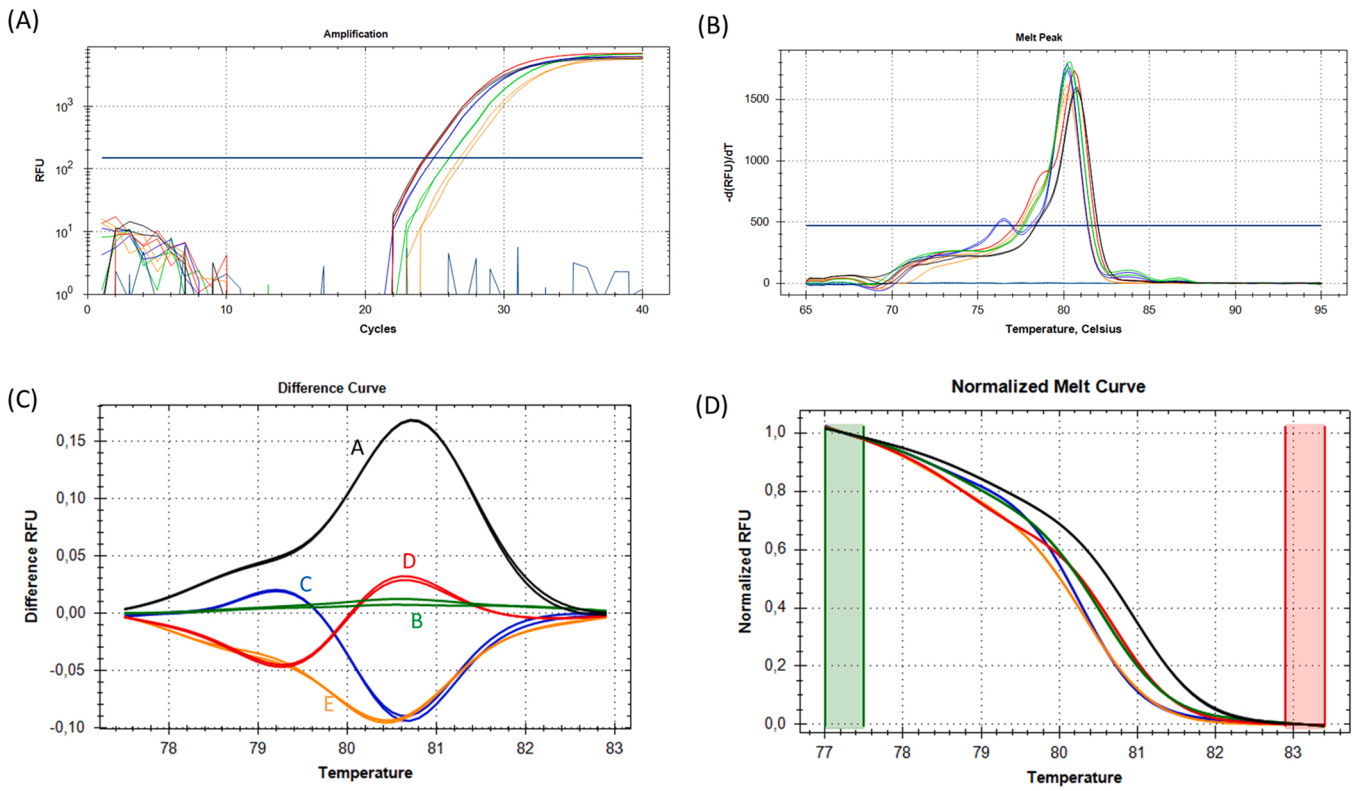

Fig. 2. Results of HRM qPCR assay for CD4 genotyping in cattle samples, including amplification curves (A), melt peaks (B), difference curves (C), and normalized melt curves (D). Black lines – A genotype, green lines – B genotype, blue lines- C genotype, red lines – D genotype, and orange lines- E genotype.

CD4 mAb was developed in this study. In addition, the assay was characterized by high reproducibility since all the samples presented the same results in the five qPCR runs (Supplementary information 2). To our knowledge, this is the first report of a rapid assay able to identify bovine CD4 polymorphisms, since the previous studies were performed by nucleotide sequencing (Grandoni et al., 2020; Kato-Mori et al., 2021). Lower MFI general mean and high frequency of animals presenting low or absent CD4 staining (27.77%) was observed in Nelore breed compared to pure taurine breeds (Angus and Holstein) (Fig. 1C), which may be explained by the second non-polymorphic altered SNP (A>C) in pure zebu animals, also corroborated by the two sequences from zebu breeds (Nelore and Brahman) previously deposited in the Genbank and Ensembl databases (Table 1). Therefore, since the hybrid breeds (Canchim and Ultrablack) also contain partial zebu blood origin, as expected, animals of this breed presented intermediate MFI mean. In cattle, different allelic forms of bovine CD4 were previously described by phenotyping blood mononuclear cells through staining using a CC26 clone of anti-CD4 bovine mAb (Morrison et al., 1991). In this last study, the identical CD4 phenotypes (++, + and -) and the allelic difference were described in both *Bos taurus* and *Bos indicus* cattle, indicating that it had arisen prior to the divergence of these subspecies. However, we hypothesized that for the mAb of CC8 clone, the difference may have arisen after to the divergence of the two subspecies of cattle since the CD4 staining with this clone was found to be lower in zebu compared to pure taurine animals, which led us to conclude that these findings are due to the non-polymorphic altered second target SNP. All the Angus animals were included in the B genotype presenting high or very high CD4 staining. In addition, almost 90% of Holstein animals (30/34) were

Table 1
Target SNPs alignment of nucleotide sequences previously deposited on the Genbank or Ensembl and from our animals.

	Breed	Access number	CD4 staining	Target SNPs
Bos taurus	Japanese Black	AB674569.1	+	G/A
	Japanese Black	AB674570.1	–	T/C
	Holstein	AB674571.1	+	G/A
	Hereford	NM_001103225.1	unknown	G/A
	Hereford	BC151321.1	unknown	G/A
	Hereford	XM_024991418.1	unknown	G/A
	Hereford	XM_024991416.1_	unknown	G/A
	Hereford	XM_024991417.1	unknown	G/A
	Hereford	ENSBTAG00000003255		G/A
Bos indicus	Nelore	XM_019960265.1	unknown	G/C
	Brahman	ENSBTAT00000080175.1	unknown	G/C
Hybrid	Angus x Brahman	XM_027541170.1	unknown	G/C
	Angus x Brahman	XM_027541169.1	unknown	G/C
	Canchim	Animal 10622 (Genotype A)	–	G/C
	Canchim	Animal 10585 (Genotype B)	+ (high)	G/A
	Canchim	Animal 10586 (Genotype C)	+ (intermediate)	G/A and T/C
	Canchim	Animal 10625 (Genotype D)	–	G/C and T/C

also included in the B genotype, homozygous for non-altered target SNPs (G/A), presenting high or very high CD4 staining. In contrast, the four animals genotyped as C, heterozygous for one allele unaltered (G/A) and second allele double altered for the target SNPs (T/C), showed intermediate CD4 staining. These results are in agreement with those observed in Italian dairy cattle (Grandoni et al., 2020) and Japanese black cattle (Kato-Mori et al., 2021), which are both pure taurine breeds, but in our experimental animals, there were no animals homozygous for both SNPs mutated (T/C). Therefore, one of the clones (E1 – Supplementary information 2) obtained from qPCR products of animals genotyped as C, presenting the T/C SNPs, was also included in the reproducibility test, aiming to simulate the assay for this genotype, which was also presented high reproducibility (Supplementary information 2). The Nelore animals were genotyped as A (43/52), which was homozygous with only the second SNP altered (G/C), or D (9/52), which was heterozygous for alleles containing G/C and T/C target SNPs. All the Nelore animals presenting low or lack of CD4 staining were genotyped as A, while the animals showing intermediate CD4 staining were genotyped as A or D. The Canchim breed had animals classified in A, B, C, and D genotypes. Nine animals genotyped as B presented very high CD4 staining, while the eight animals genotyped as A presented low or no CD4 staining. The C animals presented intermediate CD4 staining, while the 22 animals genotyped as D presented low, intermediate, or high CD4 staining. Regarding the Ultrablack animals, A, B and D genotypes were found, wherein the two A animals presented low CD4 staining, the six B animals presented high CD4 staining, while D animals presented low, intermediate, or high CD4 staining. In brief, for pure taurine breeds, the HRM qPCR assay results were accurately associated with CD4 altered staining related to CC8 clone anti-CD4 mAb, while, for hybrid breeds, the D genotype was not associated with any of those phenotypic groups of CD4 staining. However, for pure zebu animals (Nelore), the A animals presented both intermediate and low or absence of CD4 staining.

Our research group had previously associated different CD4 staining profiles with altered resistance and differential immune cell kinetics during primary infection with *Babesia bovis* in Canchim cattle (Okino et al., 2020). In addition, increased somatic cell scores and/or milk protein yields, associated with mastitis indicators in dairy production, were also associated with CD4 gene polymorphisms (He at al., 2011; Usman et al., 2016; Napolitano et al., 2021). Besides, several other studies have also reported an association between CD4 gene polymorphisms and altered disease progression, as observed for human and simian immunodeficiency viruses (HIV/SIV) (He et al., 2019; Bibollet-Ruche et al., 2019).

5. Conclusion

Our developed HRM qPCR assay might accurately identify the altered phenotypes associated with anti-CD4 mAb CC8 clone staining in taurine breeds. However, the assay cannot be applicable in zebu or hybrid breeds, probably due to additional mutations in the CD4 gene from zebu descendant animals, which may hinder the antibody binding to the target epitope. Therefore, further studies must be performed to elucidate better the CD4 gene polymorphisms associated with anti-CD4 CC8 clone altered staining in zebu breeds.

Declarations of interest

None.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110462.

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