Short Communication

Detection and quantification of bovine signal joint T-cell receptor excision circles

Rinnosuke Hisazumi [a,b], Miya Kayumi [c], Ryuji Kikukawa [d], Tetsuo Nasu [a,c], Masahiro Yasuda [a,c*]

[a] *Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, Miyazaki 889-2192, Japan*
[b] *Forensic Science Laboratory, Miyazaki Prefectural Police H.Q., Miyazaki 880-8509, Japan*
[c] *Department of Veterinary Anatomy, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan*
[d] *NOSAI Miyakonojyo, Miyazaki 885-0012, Japan*

[*]Corresponding author: Masahiro Yasuda
Tel: +81-985-58-7264
E-mail: yasudaja@cc.miyazaki-u.ac.jp

Abstract

A signal joint (sj) T-cell receptor excision circle (TREC) is produced by T-cell receptor (TCR) gene rearrangements during αβ T-cell maturation in the thymus. sjTREC have been studied as a marker of thymus function in several spices. We designed specific primers for δrec-ψJα sj region to identify the location of the bovine sjTREC region and determined the nucleotide sequence of the PCR product. The obtained sequences were subjected to a BLAST search, which identified a matching region. This matching region contained TCR δ genes and was identified on bovine chromosome 10. We also confirmed the polymorphism of the sj region by sequencing of ten PCR products, and observed irregular insertion of bases in the δrec-ψJα recombination signal sequence. We then developed a quantitative PCR (QPCR) assay for evaluation of sjTRECs level in order to evaluate bovine thymus function for application in the veterinary clinic. This QPCR assay specifically amplified the sj region of bovine sjTREC and could detected 10^1 - 10^7 copy numbers of sjTRECs. Using this assay we found that the number of sjTRECs in peripheral blood mononuclear cells was less than 10% that of the thymus.

Key words: calf, peripheral blood mononuclear cells, quantitative PCR, sjTREC, thymus

1. Introduction

The thymus is a primary lymphoid organ, where the primary T-cell repertoire is generated (Tonegawa, 1983). The T-cells that mature in the thymus and migrate to the periphery as recent thymic emigrants (RTE) play an important role in cellular immunity. In the thymus, the TCR is produced by rearrangements of the TCR gene cluster. These TCR gene rearrangements are regulated by the enzyme recombinase, which recognizes the recombination signal sequence (RSS) of the variable (V), diversity (D), and joining (J) gene segments, based on the 12/23 spacer rule (Sawchuk et al., 1997). These gene rearrangements generate circular DNAs called T-cell receptor excision circles (TRECs). During the precursor T-cell differentiate into the αβ T-cell, a signal joint TREC (sjTREC) is generated by δrec-ψJα rearrangement, which including the TCR δ gene deletion from chromosome. The δrec-ψJα signal joint (sj) region and the TCR δ gene cluster are contained in the sjTREC, and the δrec-ψJα coding joint region and TCR α gene cluster are retained in the chromosome. Therefore, the number of αβ T-cell emigrated from thymus match the number of sjTREC.

Thymus function has been evaluated by the analysis of naïve T-cell phenotype in the periphery and the measurement of thymus size by computed tomography (Hazenberg et al., 2001). Since sjTREC are included only in the αβ T-cells that emigrate from the thymus, they have been used as a marker to evaluate the thymus function after the hematopoietic stem cell transplantation for the infants with serve combined immunodeficiency or the high active antiretroviral therapy for the HIV infected patients (Douek et al., 1998; Patel et al., 2000). sjTREC have some unique characteristics: they are stable in peripheral T-cells, they are not replicated by mitosis, and they are diluted-out with each cell division (Livak ey al., 1996; Kong et al., 1999; Sempowski et al., 2001). Moreover, since the level of sjTRECs in PBMC has been reported to decrease with age, sjTREC level is a promising indicator of age estimation

in criminal investigations (Zubakov et al., 2010; Ou et al., 2011; Ou et al., 2012). Although sjTRECs of humans, rhesus monkeys, sooty mangabeys, baboons, mice, pigs, and sheep have been reported (Sodora et al., 2000; Broers et al., 2002; Vallabhajosyula et al., 2011; Tena et al., 2011; Ou et al., 2011), bovine sjTREC have not yet been studied. To evaluate the bovine thymus function under the chronic diseases such as pneumonia and diarrhea, we planned to use the measurement of bovine sjTREC. In this study, we identified the bovine sjTREC region by PCR amplification of the bovine δrec-ψJα sj region. In addition, we established a method for quantification of bovine sjTRECs by using a TaqMan PCR assay.

2. Materials and Methods

2.1. Animals and samples

Japanese black calves (1-13 months old, n=5) were obtained from local farms in Miyazaki Prefecture. The animals were killed by electric shock following intravenous injection of a combination of xylazine (0.2 mg/kg) and pentobarbital (15 mg/kg). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki. The thymus was collected, frozen on dry ice, and then stored at -80 °C until use. Whole blood cells were collected and PBMCs were separated using Ficoll-Paque (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) and then stored at -80 °C until use.

2.2 DNA preparation

Genomic DNA was extracted and purified from the thymus and PBMCs using the EZ1® DNA Investigator kit (Qiagen, Hilden, Germany). The concentration of genomic DNA was then determined using NanoVue plus (GE Healthcare UK Limited).

2.3 Sequencing of the sj region of bovine sjTREC

The ψJα primer: 5'-GAAGTCTCTGCATCGTGTGATAAC-3' and the δrec primer: 5'- CTTACCCTCTGCTGCCATCTAGTG-3' were designed based on sheep sjTREC sequencing primers (Vallabhajosyula et al., 2011). The PCR mixtures (25 μl) contained 10 pmol of the ψJα primer, 10 pmol of the δ-rec primer, 50 ng of genomic DNA, 1.25 U of Ampli Taq Gold® DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 μl of 10×PCR Gold buffer, 2.5 μl of 25 mM $MgCl_2$ solution, and 2.5 μl of a 10 mM GeneAmp® dNTP Mix. Amplification proceeded using the GeneAmp® PCR System 9700 (Applied Biosystems) at 95 °C for 15 min followed by 35 cycles of 94 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 1 min. The PCR product was purified using the QuickStep™ 2 PCR Purification Kit (Edge Biosystems, Gaithersburg, MD, USA). The nucleotide sequences of the PCR products were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI 3130*xl* DNA analyzer.

2.4. Location of the sjTREC region on bovine chromosomes

A search for homology between the nucleotide sequences of the sj region and the sequences of bovine chromosomes was performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Development of a QPCR assay for bovine sjTREC

The bovine sjTREC QPCR primers Q-ψJα primer: 5'-TGCCACATCCCTTTCAACCAC-3' and Q-δrec primer: 5'-GAGCAGACAGAGCACAGCGAC-3' and the TaqMan probe 5'-CACAGGAGTGAACACCTTTACA-MGB-3' were designed based on the sequence of the sj region.

For the QPCR assay, we used the PCR product (also 1004 bp) as the sjTREC standard. The sjTREC copy numbers of the standard were calculated as follows: copy numbers = [concentration (μg / μl) × 6.023 × 10^{23}] / [the number of nucleotides × 660]

The PCR mixtures (25 μl) contained 1 × TaqMan Gene Expression Master Mix (Applied Biosystems), 900 nM each of the Q-ψJα primer and the Q-δrec primer, 250 nM of the TaqMan Probe, and 100 ng of genomic template DNA. Amplification proceeded using a 7500 Real Time PCR system (Applied Biosystems) at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 65 °C for 30 sec.

The specificity of the QPCR assay was checked by electrophoresis (Reliant® Gel System 4% NuSieve® 3:1, LONZA, Rockland, ME, USA) and direct sequencing of the QPCR product.

2.6. QPCR assay for bovine sjTREC

The genomic DNA served as templates for QPCR assay in triplicate as described in section 2.5., and sjTREC copy numbers per genomic DNA (100 ng) was calculated using the software provided with the Applied Biosystems 7500 (Applied Biosystems).

3. Results and Discussion

The thymus function that ability to produce the T-cells is closely related to the cellular immunity in the periphery. The measurement of sjTREC in PBMC is able to detect RTE such as naïve T-cells in the periphery. Therefore, sjTREC have been studied as a marker of thymus function (Hazenberg et al., 2001). In this study, to identify the location of bovine sjTREC, the sj region of sjTREC was amplified using primers that were designed based on sheep sjTREC sequencing primers. A single PCR

product was obtained and the nucleotide sequence of the PCR product was determined (Fig. 1A). The sequence was divided into two domains, and the sequences of these domains matched with an approximately 185 kb region at the TRA/D locus on bovine chromosome 10 by NCBI-BLAST (Fig. 1B). In addition, an homology search for the putative bovine sjTREC region was performed (Fig. 1C). The putative bovine sjTREC region showed homology with the TCR δ gene (TRDV, TRDD, TRDJ, and TRDC segments). Two putative TRDV segments (V1 and V2), nine putative TRDD segments (D1 to D9), four putative TRDJ segments (J1 to J4), and one putative TRDC segment (C1) were found in this region (Fig. 1C). Furthermore, these putative TCR δ gene segments are lined up in the expected order (TRDV, TRDD, TRDJ, and TRDC segments). We determined the localization of the bovine sjTREC region in the TCR α chain C-like region on the bovine chromosome 10 (NM_003104268.1; 12192074-12377457).

To develop bovine sjTREC quantitative primers, we first confirmed the polymorphism of the sj region in the sjTREC. Some of polymorphic sites (data not shown) were observed by sequencing of ten of the PCR products (Fig. 2A). Primers and a TaqMan probe that amplify only the sj region in sjTREC were designed. This primer set does not amplify the germline DNA (Fig. 2B).

The sjTRECs in the range of 1×10^{1} copies to 1×10^{7} copies were detected, and amplification efficiency was 98%. The specificity of the PCR product was checked and non-specific amplification was not observed (data not shown).

Quantification of sjTRECs in thymus DNA and in PBMCs DNA was then performed using this assay. The sjTREC was detected from both samples. The number of sjTRECs in PBMCs was less than 10% that of the thymus (Fig. 3). This finding indicated that the number of RTEs in the PBMCs is less than 10% the number of α gene rearranged T-cells in the thymus. Therefore, the difference in these values

between the thymus and PBMCs might indicate the occurrence of T-cell selection in the thymus and/or proliferation of T-cell in the periphery. sjTRECs quantification has been reported for several species. In human, quantification of sjTRECs in autoimmune disease patients and in Down's syndrome patients has been performed, which revealed changes and differences in thymus function in these patients compared to normal controls (Passerini et al., 2003; Prada et al., 2005). In clinics, quantification of sjTREC is being used for diagnosis of severe combined immunodeficiency in newborn infants (Vogel et al., 2014). In other animals, in a study of a thymic xenograft between a baboon and a pig, baboon thymopoiesis in a porcine thymic xenograft was confirmed by quantitation of sjTRECs (Tena et al., 2011).

In conclusion, we determined the localization of the bovine sjTREC region in the TCR α chain C-like region on the bovine chromosome 10 (NW_003104268.1; 12192074-12377457) and developed the QPCR assay for bovine sjTREC. We are investigating the relationships between bovine thymus function and aging, gender differences and seasonal variations, by quantification of bovine sjTRECs in PBMC. It has been found that the bovine thymus function is decreased with age and changed with the seasonal variations. In addition, we speculate that this method of measurement of sjTRECs in PBMCs will be useful for evaluation of calf thymus function and for checking the medical condition of chronic infectious diseases.

References

Broers, A.E., Meijerink, J.P., van Dongen, J.J., Posthumus, S.J., Lowenberg, B., Braakman, E., Cornelissen, J.J., 2002. Quantification of newly developed T cells in mice by real-time quantitative PCR of T-cell receptor rearrangement excision circles. Exp. Hematol. 30, 745-750.

Douek, D.C., McFarland, R.D., Keiser, P.H., Gage, E.A., Massey, J.M., Haynes, B.F., Polis, M.A., Haase, A.T., Feinberg, M.B., Sullivan, J.L., Jamieson, B.D., Zack, J.A., Picker, L.J., Koup, R.A., 1998. Changes in thymic function with age and during the treatment of HIV infection. Nature 396, 690-695.

Hazenberg, M.D., Verschuren, M.C., Hamann, D., Miedema, F., van Dongen, J.J., 2001. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J. Mol. Med. 79, 631-640.

Kong, F.K., Chen, C.L., Six, A., Hockett, R.D., Cooper, M.D., 1999. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. Proc. Natl. Acad. Sci. U.S.A. 96, 1536-1540.

Livak, F., Schatz, D.G., 1996. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. Mol. Cell. Biol. 16, 609-618.

Ou, X., Zhao, H., Sun, H., Yang, Z., Xie, B., Shi, Y., Wu, X., 2011. Detection and quantification of the age-related sjTREC decline in human peripheral blood. Int. J. Legal Med. 125, 603-608.

Ou, X.L., Gao, J., Wang, H., Wang, H.S., Lu, H.L., Sun, H.Y., 2012. Predicting human age with bloodstains by sjTREC quantification. PLoS ONE 7, e42412.

Passerini, L., Bernasconi, P., Baggi, F., Cornelio, F., Mantegazza, R., 2003. Analysis of SjTREC levels in thymus from MG patients and normal children. Ann. N. Y. Acad. Sci. 998, 270-274.

Patel, D.D., Gooding, M.E., Parrott, R.E., Curtis, K.M., Haynes, B.F., Buckley, R.H., 2000. Thymic function after hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. The New England journal of medicine 342, 1325-1332.

Prada, N., Nasi, M., Troiano, L., Roat, E., Pinti, M., Nemes, E., Lugli, E., Ferraresi, R., Ciacci, L., Bertoni, D., Biagioni, O., Gibertoni, M., Cornia, C., Meschiari, L., Gramazio, E., Mariotti, M., Consolo, U., Balli, F., Cossarizza, A., 2005. Direct analysis of thymic function in children with Down's syndrome. Immun Ageing 2, 4.

Prieto-Hinojosa, A., Knight, A., Compton, C., Gleeson, M., Travers, P.J., 2014. Reduced thymic output in elite athletes. Brain Behav. Immun. 39, 75-79.

Sawchuk, D.J., Weis-Garcia, F., Malik, S., Besmer, E., Bustin, M., Nussenzweig, M.C.,

Cortes, P., 1997. V(D)J recombination: modulation of RAG1 and RAG2 cleavage activity on 12/23 substrates by whole cell extract and DNA-bending proteins. J. Exp. Med. 185, 2025-2032.

Sempowski, G., Thomasch, J., Gooding, M., Hale, L., Edwards, L., Ciafaloni, E., Sanders, D., Massey, J., Douek, D., Koup, R., Haynes, B., 2001. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. J. Immunol. 166, 2808-2817.

Sempowski, G.D., Gooding, M.E., Liao, H.X., Le, P.T., Haynes, B.F., 2002. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol. Immunol. 38, 841-848.

Sodora, D.L., Douek, D.C., Silvestri, G., Montgomery, L., Rosenzweig, M., Igarashi, T., Bernacky, B., Johnson, R.P., Feinberg, M.B., Martin, M.A., Koup, R.A., 2000. Quantification of thymic function by measuring T cell receptor excision circles within peripheral blood and lymphoid tissues in monkeys. Eur. J. Immunol. 30, 1145-1153.

Tena, A., Vallabhajosyula, P., Hawley, R.J., Griesemer, A., Yamada, K., Sachs, D.H., 2011. Quantification of baboon thymopoiesis in porcine thymokidney xenografts by the signal-joining T-cell receptor excision circle assay. Transplantation 91, 639-644.

Tonegawa, S., 1983. Somatic generation of antibody diversity. Nature 302, 575-581.

Vallabhajosyula, P., Tena, A., Yamada, K., Sachs, D.H., 2011. Signal joint T-cell receptor excision circle assay in miniature swine. Transplantation 92, 634-640.

Vogel, B.H., Bonagura, V., Weinberg, G.A., Ballow, M., Isabelle, J., DiAntonio, L., Parker, A., Young, A., Cunningham-Rundles, C., Fong, C.T., Celestin, J., Lehman, H., Rubinstein, A., Siegel, S., Weiner, L., Saavedra-Matiz, C., Kay, D.M., Caggana, M., 2014. Newborn screening for SCID in New York State: experience from the first two years. J. Clin. Immunol. 34, 289-303.

Zubakov, D., Liu, F., van Zelm, M.C., Vermeulen, J., Oostra, B.A., van Duijn, C.M., Driessen, G.J., van Dongen, J.J., Kayser, M., Langerak, A.W., 2010. Estimating human age from T-cell DNA rearrangements. Curr. Biol. 20, R970-971.

Figure captions

Fig. 1. Localization of the sjTREC region in bovine chromosomes. (A) The sequence of the 1004 bp PCR amplification product for determination of the location of the bovine sjTREC region is shown. The sequences of the sequencing primers (squares), the δrec and ψJα recombination signal sequences (RSSs) (underlines), and the location of the δrec RSSs and ψJα RSSs separated site (double slashes) are shown. The division into domains a and b, which was based on the results of a BLAST search, is indicated. (B) Identification of a putative bovine sjTREC. The sequences of the domains a and b shown in (A) match with an approximately 185 kb region at the T-cell receptor alpha chain C-region-like region on the bovine chromosome 10. The data suggest that this region forms a circle. (C) The segments of the putative bovine sjTREC region (NW_003104268.1). This region has two TRDV (squares), nine TRDD (triangles), four TRDJ (vertical lines) and one TRDC (circle) gene segments.

Fig. 2. Design of the QPCR assay for bovine sjTRECs. (A) The primers (double underlines) and the TaqMan probe (bold underline) used for the QPCR assay of bovine sjTRECs are shown. Using this assay, polymorphism of the sj region was confirmed and random insertion of bases in the recombination site was revealed. (B) The QPCR assay detects only the sj region of sjTREC and this primer set did not amplify the germline DNA.

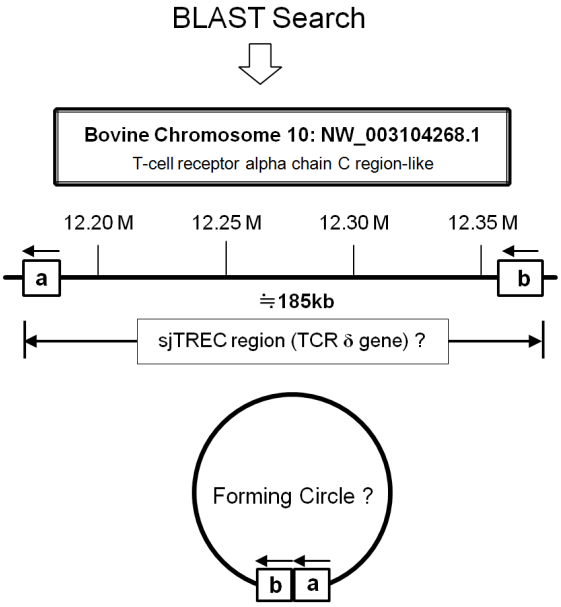
Fig. 3. Quantification of bovine sjTRECs in the thymus and PBMCs. sjTREC levels, as measured using the QPCR assay, were higher in the thymus than in PBMCs. The data are shown as means ± SD.

Figure 1

A


1 GAAGTCTCTGCATCGTGTGATAAGATTTTGATATCTTATTTATCATCTTCATCCCTGAAATCTA
66 CTCAGCCACCTACTATCTCTGTCCTAACAGAGGGAAGAGGGCAGACCTTTCCCAGGAAAGAG
128 GGGGCCCCTGGGGAGGACAGACAGCTCTCTCTGTCTAGTGAGGAAGGGGAACAGAGGCGA
188 TGCCACATCCCTTTCAACCACGCTGACGCTTCTAGTTTTTGTAAAGGTGTTCACTCCTGTG //
248 CACAGCAGTACGCAGGCACCTGCAGCCCCATCCTAAACCCTGCAGTCGCTGTGCTCTGTCT
310 GCTCTGCTTTCACTGCTCTCGTTAGCTCTAAGGACAAGCTGGCTTGTGTTGCATTAGGTCTTC
373 CATCCTGGGAAGTGTTTCACAGCTTTGCACACATCCCCAGGCCCCAGGCTAATGAGTAAGGG
435 TTGAAAATACATTCAGGTGTCATCATGGGCAATGGAGCAGGTGTCAAACACGGTCTGCAGGT
497 CAGGCTGGAGCAAGCTTCCACTTGCTTGCTCTGTACGGAAATAGGTGGGCGGTTGGCTGAA
558 GACACCAACGAATTTCCCTTTGGCTCTGTGCTTTCTCCATCTCTGCCTCTGGGGATTCTTGAA
621 GATACAGTGGGAAATGTCAGCATGAGATGCAGGACTCTATCTCTCTCCAGGATTCACCTGGG
683 ATTTTGGCAAGACATGCCAGGTCTTCACGGTTTACTTTTAGAGTCTGAAAGGTGGATAGATTT
746 GTAAGAAAGTGGACAGACAATCTCTCTCCAGAAGAGAAAGGGCCCTCTCGGGCTCCAAGAT
808 GCAGTCCCAGCGTTGCTCAGGCAGATGTCAGGAGATGGGTCCTGACCCTCCGTTGCGACAA
869 GGACTCTGAGGAAAGCCATGACTGTGTGTTTCTGTACCCGTCTTCCTCCTGCTTCACGGGTG
931 CTGAGACTTCAGCCCTGTTTAGGAAAAGGACAACGCAGAGGAGGAAAGTGGCCACTAGATG
992 GCAGCAGAGGGTAAG
a
b
:Primer, __ :RSS, // :separation site

B


BLAST Search
Bovine Chromosome 10: NW_003104268.1
T-cell receptor alpha chain C region-like
12.20 M
12.25 M
12.30 M
12.35 M
a
b
≒185kb
sjTREC region (TCR δ gene) ?
Forming Circle ?
b
a

C

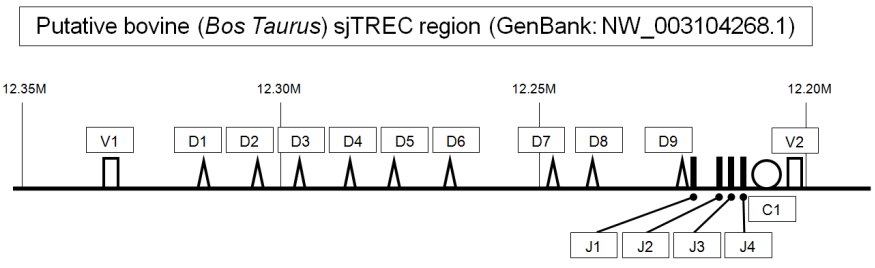
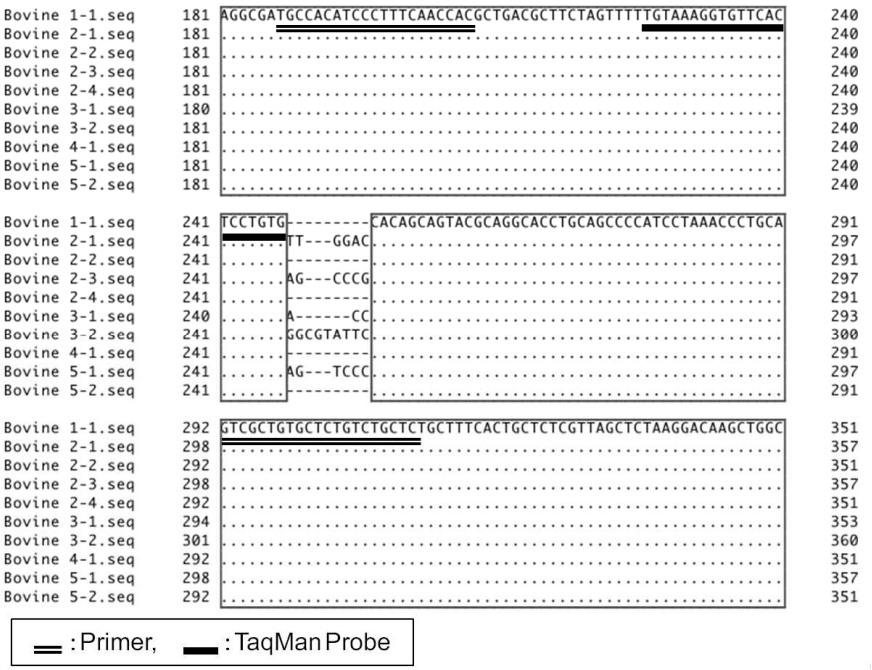

Putative bovine (Bos Taurus) sjTREC region (GenBank: NW_003104268.1)
12.35M
12.30M
12.25M
12.20M
V1
D1
D2
D3
D4
D5
D6
D7
D8
D9
V2
C1
J1
J2
J3
J4

Figure 2

A


Bovine 1-1.seq 181 AGGCGATGCCACATCCCTTTCAACCACGCTGACGCTTCTAGTTTTTGTAAAGGTGTTCAC 240
Bovine 2-1.seq 181 240
Bovine 2-2.seq 181 240
Bovine 2-3.seq 181 240
Bovine 2-4.seq 181 240
Bovine 3-1.seq 180 239
Bovine 3-2.seq 181 240
Bovine 4-1.seq 181 240
Bovine 5-1.seq 181 240
Bovine 5-2.seq 181 240
Bovine 1-1.seq 241 TCCTGTG---------CACAGCAGTACGCAGGCACCTGCAGCCCCATCCTAAACCCTGCA 291
Bovine 2-1.seq 241 .....TT---GGAC..... 297
Bovine 2-2.seq 241 .....--------..... 291
Bovine 2-3.seq 241 .....AG---CCCG..... 297
Bovine 2-4.seq 241 .....--------..... 291
Bovine 3-1.seq 240 .....A------CC..... 293
Bovine 3-2.seq 241 .....GGCGTATTC..... 300
Bovine 4-1.seq 241 .....--------..... 291
Bovine 5-1.seq 241 .....AG---TCCC..... 297
Bovine 5-2.seq 241 .....--------..... 291
Bovine 1-1.seq 292 GTCGCTGTGCTCTGTCTGCTCTGCTTTCACTGCTCTCGTTAGCTCTAAGGACAAGCTGGC 351
Bovine 2-1.seq 298 357
Bovine 2-2.seq 292 351
Bovine 2-3.seq 298 357
Bovine 2-4.seq 292 351
Bovine 3-1.seq 294 353
Bovine 3-2.seq 301 360
Bovine 4-1.seq 292 351
Bovine 5-1.seq 298 357
Bovine 5-2.seq 292 351
: Primer, : TaqMan Probe

B

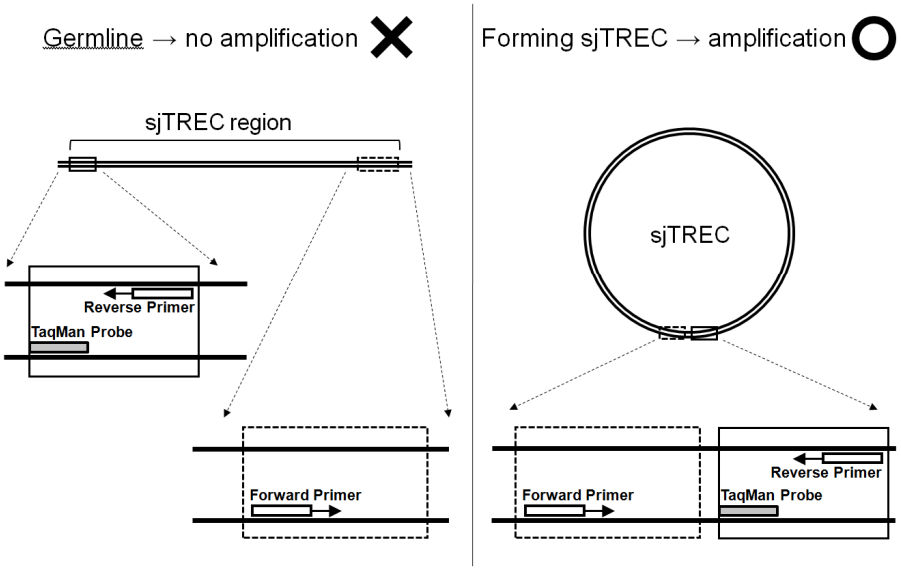
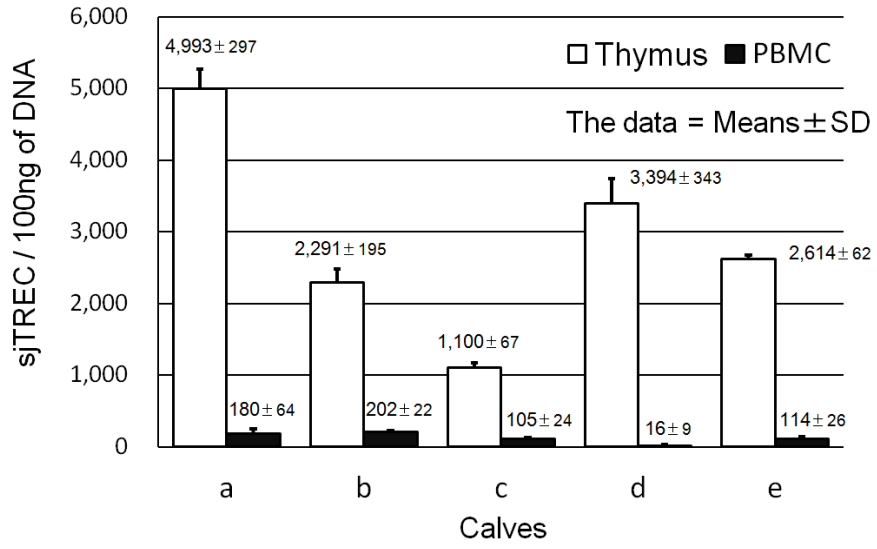

Germline → no amplification
Forming sjTREC → amplification
sjTREC region
Reverse Primer
TaqMan Probe
Forward Primer
sjTREC
Forward Primer
Reverse Primer
TaqMan Probe

Figure 3


6,000
5,000
4,000
3,000
2,000
1,000
0
sjTREC / 100ng of DNA
Thymus
PBMC
The data = Means ± SD
4,993 ± 297
180 ± 64
2,291 ± 195
202 ± 22
1,100 ± 67
105 ± 24
3,394 ± 343
16 ± 9
2,614 ± 62
114 ± 26
a
b
c
d
e
Calves