Cloning, characterization and identification of polymorphism in TCR Zeta Gene in deoni cattle

K. Swathi*, M. Gnana Prakash, D. Sakaram, T. Raghunandan, A. Sarat Chandra and P. Kalyani

Department of Animal Genetics and Breeding, College of Veterinary Science, P. V. Narsimha Rao Telangana Veterinary University, Hyderabad- 500 030, Telangana, India.

Received: 16-02-2017 Accepted: 10-01-2018 DOI: 10.18805/ijar.B-3389

ABSTRACT

The cDNA encoding, T-cell receptor zeta (TCR ζ; CD247) molecule of Deoni cattle (Bos indicus), was isolated, cloned and sequenced in the present study. The CD247 cDNA comprised 1078 nucleotides including a 30 nucleotide 5¹-untranslated region (UTR), 495 nucleotide single open reading frame (ORF) and 553 nucleotide 3¹-UTR. Deduced amino acid of cattle CD247 sequence was two residues shorter than the corresponding sheep sequences. However, ruminant-specific insertions and substitutions in transmembrane (TM) and intra-cytoplasmic (IC) domain were present in cattle. Immunoreceptor tyrosine-based activation motifs (ITAMs), the important motifs for TCR signalling, were totally conserved among ruminants including cattle. The 3¹ - UTR region of the cattle CD247 was highly homologous to the corresponding region in the buffalo sequence and showed lack of polymorphism after polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using Hae III and Mse I restriction enzymes in cattle population. Phylogenetically, cattle sequence was closer to buffalo sequence under the ruminant's lineage. The conserved nature of this gene ensures TCR integrity which is vital for induction of optimal and efficient immune response.

Key words: Cattle, CD247, Immunoreceptor tyrosine-based activation motif (ITAM), Phylogeny, T cell receptor zeta.

INTRODUCTION

Thymic derived lymphocytes are typically involved in protecting the host from pathogenic organisms. T-cells typically recognize and respond to a diverse array of antigens presented through the mediation of the T cell receptor (TCR) complex. The T cell receptor (TCR) complex comprises of two subunits: the variable TCR subunit composed of α, β, γ, δ polypeptide chains and invariant signal-transducing CD3 subunit (Weiss, 1991).

The CD3 subunit, a dimer of different γ, δ, ε and ζ chains, has an important role in signal transduction. Among different chains, ζ chain homodimer is most essential for efficient transport of assembled TCR complexes to the cell surface under signal transduction (Sussman et al., 1988). The TCR α chain plays an important role in T-cell activation. This is supported by absence of lymphokine release, calcium mobilization and the generation of inositol phosphates in T-cell mutants which are either deficient in TCR ζ-chain expression (Weissman et al., 1989) or have impaired association of the TCR ζ-chain with other components of TCR/CD3 complex (Sancho et al., 1989) the TCR ζ-chain plays an important role in linking TCR-triggering to several protein tyrosine kinases such as ZAP-70, Syk, Lck and Fyn, which in turn control signal transduction pathways that regulate lymphokine and lymphokine receptor gene expression. Failure of the α chain to associate with the pre-TCR leads to its lysosomal degradation (Klausner et al., 1989).

The ζ chain is encoded by CD247 (CD3Z) gene. This gene is located in the distal regions of human and mouse chromosomes 1 (Modi et al., 1989; Seldin et al., 1989), whereas it has been localised to chromosome 3q11-q14 in cattle (Amarante et al., 1996).

Deoni is considered as an important dual-purpose breed of cattle in India. These animals are quite popular in the tracts of Telangana and adjoining districts of Karnataka and Maharashtra. Deoni cattle are hardy and well adapted to their breeding tract and constitute an important cattle genetic resource of India. However, the genetic basis of these important traits is yet to be explored extensively in cattle. In this study, we cloned and characterised Deoni cattle TCR-zeta (TCR-z) cDNA, which will further help to understand disease resistance in animals.

MATERIALS AND METHODS

This research project was approved by institutional animal ethics committee. For cDNA construction, blood sample was collected from a healthy Deoni cattle maintained at Instructional Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Telangana. Total RNA was isolated from lymphocytes using purelink total RNA blood purification kit (Invitrogen) and the first strand cDNA

*Corresponding author’s e-mail: swathi.koppula014@gmail.com
synthesis was carried out using Verso cDNA synthesis kit (Thermo Scientific). Using the first strands, cDNA of BoLA-TCR Zeta gene was amplified by using specific primers. Primers for PCR amplification were (Forward: 5'-GCTCCGGGACCATCCTGG-3', Reverse: 5'-CTACCTACCCCCATCTCCTG-3') designed on the basis of available cattle sequence (GenBank Acc. no. U25688) using primer blast software of NCBI.

PCR amplification was in a total volume of 25 µL with 1x PCR buffer, 1.5 mM of MgCl2, 200 µM of each dNTPs, 60 pmol of each primer (forward and reverse) and 1 U of Taq DNA polymerase. The polymerase chain reaction (PCR) was carried out in a thermal cycler under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The PCR product of desired length was purified from the gel by using QIA quick gel extraction kit (Qiagen). The purified PCR product was ligated to a pGEM-T easy vector (Promega) and cloned in Escherichia coli DH5α cells. Positive clones with proper inserts were identified by blue-white colony method and colony PCR. These positive colonies were grown in LB broth and the isolated plasmid was sequenced from both directions by an automated sequencing method. The sequence thus obtained was annotated and blasted using a public database (www.ncbi.nlm.nih.gov) and submitted to NCBI GenBank (Accession No.KX388149). The nucleotide and deduced amino acid sequences were compared with orthologous cDNA sequences of cattle and other species. Phylogenetic tree was derived for cDNA sequences from buffalo and other species by using Neighbor-joining method (Nucleotide: Kimura 2 parameter model) of Molecular Evolutionary Genetics Analysis (MEGA) 7 programme (Kumar et al. 2016) based on CD247 cDNA sequences of cattle and other species.

For polymorphism study of the cattle CD247 3-UTR region, venous blood was collected from 60 unrelated Deoni cattle. Genomic DNA was isolated by phenol-chloroform extraction method (Sambrook and Russell, 2001). Primers (Forward: 5'-ACCTATGACGCACCCTCCA 3', Reverse: 5'-ACATGCGCGGCTTACAGT-3') were designed using the obtained cattle sequence encompassing 42 bases of the coding region and remaining bases of 3'-UTR. A 298 bp long product was amplified using 35 amplification cycles (94°C/45 sec, 61.5°C/40 sec and 72°C/45 sec). RFLP analysis of the amplified PCR products was carried out using Hae III and Mse I restriction enzymes.

RESULTS AND DISCUSSION

A 1078 nucleotide long cDNA molecule corresponding to CD247 gene was amplified from Deoni cattle. The annotated CD247 sequence revealed high similarity with homologous sequences of other species. The analysis indicated that the cattle TCR CD3Z gene has the highest homology (83.03%) with sheep CD3Z gene followed by 81.84% with buffalo while the mouse CD3Z gene (Acc. No. BC052824) demonstrated least homology (68.06%). The deduced amino acid sequences of cattle CD247 cDNA showed highest homology with buffalo (82.93%) followed by sheep, pig and human and the least homology with mouse and rat (66.46%) sequences (Table 1).

Predicted topologies for exon and domain boundaries were found to be similar with other ruminant species (Hagens et al., 1996) (Figure 1). However, the transmembrane (TM) domain was shorter by one amino acid residue in cattle than other ruminants and the intracytoplasmic (IC) domain was longer by one amino acid residue in comparison to buffalo. As expected, signal peptide (SP) domain showed highest variability among different domains, whereas extra cellular (EC) domain which is responsible for setting the structural framework for surface expression of assembled receptor was found to be completely conserved across all species.

Some of the important amino acid residues in the hydrophobic trans-membrane (TM) domain are found to be conserved. Residue Cysteine at 32, fully conserved across species, if mutated abolishes signalling (Hagens et al., 1996). Similarly, residue aspartic acid at position 36 (only acidic amino acid in hydrophobic TM domain) mediates disulphide homo dimerization and association to CD16 or TCR complex. Tyrosine at position 42 in TM domain which interact with TCR ζ chain (Johansson et al., 1999) was found to be conserved. Similarly, glycine (Gln) residue at position 43 of dimerization motif, G4x3xxxT (Mingarro et al., 1997) was found to be invariant in cattle similar to other species. Noticeably, mutations at these positions disrupt the key functions of TCR ζ chain. Substitution of residue 46 (Leu to Val/Ile) in consensus motif L31 CY x LD x ILF x YG xx LT x LF x51 (x represents any residue) of TM domain, which is essential for dimer/tetramer formation (Torres et al., 2002), was also found in cattle similar to other ruminant species.

The IC domain, functionally independent for signalling was found to be conserved. However, some changes especially in the antigen recognition activation motifs (ARAMs), ITAMs and guanosine di-/tri-phosphate (GDP/GTP) binding site were observed in ruminants.

There was deletion of two amino acid residues at positions 51 (TM domain) and 52 (IC domain), which was unexpected. The IC domain is responsible for setting the structural framework for surface expression of assembled receptor was found to be completely conserved across all species.

Some of the important amino acid residues in the hydrophobic trans-membrane (TM) domain are found to be conserved. Residue Cysteine at 32, fully conserved across species, if mutated abolishes signalling (Hagens et al., 1996). Similarly, residue aspartic acid at position 36 (only acidic amino acid in hydrophobic TM domain) mediates disulphide homo dimerization and association to CD16 or TCR complex. Tyrosine at position 42 in TM domain which interact with TCR ζ chain (Johansson et al., 1999) was found to be conserved. Similarly, glycine (Gln) residue at position 43 of dimerization motif, G4x3xxxT (Mingarro et al., 1997) was found to be invariant in cattle similar to other species. Noticeably, mutations at these positions disrupt the key functions of TCR ζ chain. Substitution of residue 46 (Leu to Val/Ile) in consensus motif L31 CY x LD x ILF x YG xx LT x LF x51 (x represents any residue) of TM domain, which is essential for dimer/tetramer formation (Torres et al., 2002), was also found in cattle similar to other ruminant species.

The IC domain, functionally independent for signalling was found to be conserved. However, some changes especially in the antigen recognition activation motifs (ARAMs), ITAMs and guanosine di-/tri-phosphate (GDP/GTP) binding site were observed in ruminants.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession number</th>
<th>Nucleotide similarity (%)</th>
<th>Amino acid similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>DQ057984</td>
<td>81.84</td>
<td>82.93</td>
</tr>
<tr>
<td>Sheep</td>
<td>NM_001009417</td>
<td>83.03</td>
<td>82.32</td>
</tr>
<tr>
<td>Pig</td>
<td>NM_214155</td>
<td>71.46</td>
<td>74.39</td>
</tr>
<tr>
<td>Mouse</td>
<td>BC052824</td>
<td>68.06</td>
<td>66.46</td>
</tr>
<tr>
<td>Rat</td>
<td>NM_170789</td>
<td>69.46</td>
<td>66.46</td>
</tr>
<tr>
<td>Human</td>
<td>J04132</td>
<td>70.85</td>
<td>71.34</td>
</tr>
</tbody>
</table>

Table 1: Nucleotide and amino acid similarity of cattle CD247 cDNA sequence with other species.
observed in cattle especially. Amino acid substitutions at positions 70 and 71 of ARAM1 and 103, 107 and 109 of ARAM2 (Tsuzaka et al., 2005) in cattle CD247 were also found in buffalo and sheep sequences, which supports the fact that these substitutions are specific to ruminant species. The GDP/GTP binding domain, ARAM3 (Hagens et al., 1996) and SNID2 (Schaefer et al., 2000) with the sequence G<sub>130</sub>** **RRRGGHDGLYQYG in the loop of helix-loop-helix was increased in length by ruminant-specific insertions at 132, 133 (Asn/Gln) along with ruminant-specific substitutions at 130 (Gly-Ser) and 131 (Gln-Asp).

The heart of TCR signalling, ITAMs (Kersh et al., 1998) presented some surprising substitutions. In ITAM1, at position 77 (Leu-Val) and 84 (Asp-Ala), ruminants differed from non-ruminants. Similarly, in ITAM2 at position 115 (Gln-Arg) ruminant-specific substitution was found. ITAM3 was conserved across species. Rest aside, the Tyr/Ile/Leu of consensus motif YxxL/I/L<sub>Q6</sub>G YxxL/I/L (Schaefer et al., 2000) for TCR signalling along with YxxL for CD<sub>26</sub> mediated signalling remained unchanged across species indicating their enormous role. Mutation of Tyr/Ile/Leu of this consensus motif, especially Tyr would prove disastrous.

Similarly, residues like lysine at position 129 (GTP oxi-modification site) and Gln at position 137 in the GDP/GTP binding domain motif including putative nucleotide binding were also found to be conserved in cattle as in other species. Highly conserved GDP/GTP binding domain, ARAM3 and ITAM3 across the species were also notable features. Substitutions at position 77 (Leu to Val) of SNID1 (72-77), 130 (Gly to Ser) and 131 (Glu to Asp) along with insertions at 132, 133 (Asn/Gln) in SNID2 (123-136, Schaefer et al., 2000) were found to be ruminant specific. There was remarkable interspecies conservation between cattle and buffalo at 3'-UTR of the CD247 sequences (Figure 2).

The phylogenetic tree (Figure 3) based on cDNA sequences of CD247 gene from different species revealed higher resemblance among different ruminant species. Cattle, buffalo and sheep were from a different lineage than the rest of the species compared. Separation of sheep TCR Zeta occurred earlier than cattle and buffalo TCR Zeta from the same lineage. Cattle along with buffalo formed a sub-group while the sheep in other. Of the other lineage, mouse and rat differentiated together whereas pig and human formed a close sub-group.

### Table 1

<table>
<thead>
<tr>
<th>SP domain</th>
<th>EC domain</th>
<th>TM domain</th>
<th>IC domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ex2</td>
<td>Ex3</td>
<td>Ex4</td>
</tr>
<tr>
<td>MKWALVTAILOAQPFTTEA</td>
<td>QSFGLLDVPK</td>
<td>LCYYLIGILFYGVILALTALFL</td>
<td>RAKFSRSDAPAYQQQGONLGRREYVVDLRKRR</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

---

**Fig 1:** A comparison between the predicted amino acid sequences of the cattle CD247 cDNA and corresponding sequences from other species. Accession numbers for the sequences are given in Table 1. Identity is denoted by dot (.) and non-identical amino acids are indicated. Gap is denoted by asterisk (*) and introduced to optimise similarity. ITAM residues of consensus sequence are underlined. Residues highlighted grey are predicted ARAMs of cattle sequence (Hagens et al. 1996). Cysteine for dimer formation is indicated by #. Plus (+) signs indicate the ruminant specific insertions in GDP/GTP binding domain. Exons were ascertained on the basis of human and mice CD3Z sequences (Banijash et al. 1989).
Fig 2: A comparison between 3'-UTR nucleotide sequences of cattle CD247 cDNA and corresponding region of buffalo (Acc. no. DQ057984) and sheep (Acc. no. NM_001009417). Identical nucleotide residues of buffalo and sheep sequences to the cattle are denoted by dot (.), whereas non-identical residues are indicated. Gap is denoted by asterisk (*) and introduced to optimise similarity.

Fig 3: Phylogenetic tree based on amino acid sequence of cDNA of TCR Zeta gene

Fig 4: Hae III digestion of 298bp fragment of TCR zeta gene
Lane 7 and 14: Undigested PCR product (298bp)
Lane 1-13: Hae III digested fragments of TCR zeta gene
Lane M: 100bp ladder used as marker

Fig 5: Mse I digestion of 298bp fragment of TCR zeta gene
Lane 6: Undigested PCR product (298bp)
Lane 1-12: Mse I digested fragments of TCR zeta gene
Lane M: 100bp ladder used as marker
CONCLUSION

Cattle TCR Zeta (CD3Z) gene, which plays a major role in coupling cell surface receptors to intracellular signalling pathways was invariant in critical residues notably in dimerization motif, GTP-oxi-modification site and the very heart of signalling tyrosine residues for phosphorylation in immune receptor tyrosine based activation motif 1, 2 and 3 (ITAM 1, 2 and 3). The conserved nature of this gene ensures the T cell receptor integrity which is vital for induction of optimal and efficient immune response, including the elimination of routine pathogens and elimination of modified cells and molecules. Large number of ruminant-specific substitutions, in important motifs of TCR Zeta molecules, along with their separate lineage, may partly answer as to why not a single autoimmune disease has been reported in them.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Dean, Sri P. V. Narasimha Rao Veterinary University, Hyderabad and Department of Biotechnology (DBT), Government of India, New Delhi for providing all the facilities and funds for this study.

REFERENCES


