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Bioinformatic characterization of the Transmembrane protein95 gene (*TMEM95*) in Murrah buffalo (*Bubalus bubalis*)

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ABSTRACT

Idiopathic male subfertility is often a neglected phenotype with respect to male fertility in bovines. The gene *TMEM95* plays a crucial role in idiopathic male subfertility in cattle. Using the DNA sequence information from cattle *TMEM95* gene, we characterized the gene in Murrah buffalo. A total of 2.6 kb of a fragment orthologous to cattle was sequenced from Murrah buffalo and Gir cattle. A 2 bp deletion is present in Murrah buffalo, causing missense mutations in three isoforms that are present in cattle. The functional effects of various non-synonymous mutations were predicted using the SNAP2 program, and showed that the non-synonymous SNPs could affect the protein function. Functional motif annotation revealed the presence of a Casein kinase II phosphorylation site that plays an important role in sperm morphology, Leucine zipper pattern, N-myristoylation site, protein kinase C phosphorylation site, CHRD domain profile, N-glycosylation site and HIT zinc finger motifs in cattle. The HIT ZF motif is absent in all of the functional isoforms in buffalo. The results together suggest that the subfertility gene *TMEM95* in cattle and buffalo must have evolved with different functions but plays a role in male fertility as in other mammals.

Key words: characterization; *TMEM95*; murrah buffalo; idiopathic subfertility

Introduction

Infertility or sub-fertility is a major concern in cattle and buffaloes. Despite providing optimum herd management, nutrition and environment, persistence of the problem may be attributed to a male fertility factor which is often ignored. In some cases the semen quality appears apparently normal, but the aetiology of sub-fertility remains unknown. Unascertained infertility is generally referred to as idiopathic infertility. Male reproductive performance in cattle is in general low to moderately heritable (DRUET et al., 2009). A number of genes have been reported to influence bull fertility

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(PENAGARICANO et al., 2012, MISHRA et al., 2013, GAO et al., 2014). A recent study indicated that a non-sense mutation in the gene *Transmembrane protein95* (*TMEM95*) led to idiopathic male subfertility in Flekvieah cattle (PAUSCH et al., 2014). *TMEM95* is a gene that encodes the protein transmembrane protein95, located on the surface of the spermatozoa. The integrity of the TMEM95 protein is essential for undisturbed fertilization. Further, it was established that spermatozoa that lack or have truncated *TMEM95* gene protein failed to interact with oocyte vestments, leading to infertility (FERNANDEZ-FUERTES et al., 2017). The non-sense mutation in the *TMEM95* gene does not affect sperm quality. These findings indicate that the routine procedures followed to assess spermatozoa in artificial insemination programs are insufficient, and it is important to understand the genetic causes of male infertility or subfertility. Moreover, the majority of such mutations exhibit their phenotype under homozygous recessive states, and heterozygous carriers are always a threat in the population (JOLLY and WINDSOR, 2010).

The Indian subcontinent has a large number of buffalo populations that contribute significantly to the national economy, as well as milk protein needs. However, the genome of buffalo has scarcely been explored. The first draft of the complete genome sequence of water buffalo was published three years ago (WILLIAMS et al., 2017). Comparative genetic analysis is often a tool to understand buffalo genes in contrast to cattle genes (ZHAO et al., 2012), owing to the low genetic diversity between the two species (MOAEEN-UD-DIN and BILAL, 2015). There are certain fundamental differences between cattle and buffalo as far as the male reproductive system is concerned. The bulls reach puberty earlier than buffalo bulls (OSWIN PERERA, 1999). Buffalo bulls are known to be relatively poor reproductive performers, and exhibit lower conception rates with artificial insemination (GORDON, 1996).

In the present study, the DNA sequence of the *TMEM95* gene region was obtained from the Murrah buffalo and Gir cattle breeds of Indian origin. Comparative genetic analysis between cattle and buffalo for the *TMEM95* gene region was performed to characterize the gene in buffalo.

Materials and methods

The genome sequence of Herford cattle (Accession No. AC_000176.1) was utilized to obtain information on the *TMEM95* gene. The gene is located on chromosome 19 of cattle, flanked by the *KCTD11* and *TNK1* genes on either side, respectively. Usingm the BLAST program (ALTSCHUL et al., 1990), the orthologous regions of the Ongole (Nelore) cattle (*Bos indicus*) (Accession No. CM003039) and Mediterranean buffalo (*Bubalus bubalis*) (Accession No. GCA_000471725.1) were obtained. The sequences were aligned using ClastalW (LARKIN et al., 2007), and conserved primers were designed. Five pairs of overlapping conserved primers were designed (Table 1) to amplify and sequence the region in Murrah buffalo (*Bubalus bubalis*) and Gir cattle (*Bos indicus*). The overlapping fragments were sequenced using the Sanger method and the chromatograms were verified for quality. The sequences were aligned and assembled using CodonCode™ Aligner-Software (LI-COR, Inc., Lincoln, USA). The Genewise program (BIRNEY et al., 2004) was used to predict the isoforms in buffalo, using cattle protein sequences for different isoforms. The effect of mutation on protein function was predicted using the SNAP (screening for non-acceptable polymorphisms) program (BROMBERG and ROST, 2007).

The transmembrane topology and signal peptide prediction of different isoforms were obtained using the Phobius program (KALL et al., 2004), and motifs were predicted using the Motif Scan program (https://myhits.isb-sib.ch/cgi-bin/motif_scan) to understand the function of the isoforms. The functional implications of amino acid substitutions were predicted using the SNAP program (KORBER, 2000).

Results

Sequence variation between cattle and buffalo. The region of the *TMEM95* gene was sequenced in Murrah buffalo and Gir cattle breeds. A total of 2610 base pairs (bp) corresponding to the orthologous region of 2631 bp in Herford cattle were obtained from the five overlapping sequenced regions from Murrah buffalo.

Table 1. Primers designed for amplification of the *TMEM95* genomic region

Fragment	Primer Sequence	Tm (Melting temperature)	Product (bp)
1	F: CTGTTTTGGTACCAGGTGTG	56.6	617
	R: AGGTTCTAGAGCATTCTAGCAC	57.3	
2	F: TCCATCTCCCACTAGAAGAATC	56.4	696
	R: CTTCCAAGCCCCTAGGTTGT	59.3	
3	F: TCCAGGTCAGTGAGGGAATC	58.4	689
	R: CAACAGTACACCTCGAAGCC	58.6	
4	F: CAGAGAGGAACGGAGGCTTC	59.8	687
	R: GACCATCTGACACTGGGACT	58.7	
5	F: GCCTAGCCTCCAGTTCTAAG	57.1	621
	R: GTTGAGAACCACTGTGCTAC	56.4	

Table 2. The effect of non-synonymous substitutions on the protein function as predicted by SNAP

Isoform	AA position	AA in Buffalo	AA in Cattle	SNAP predicted Effect
X2	33	Q	R	Neutral
	108	A	S	Neutral
	121	H	N	Neutral
	162	S	P	Effect
	179	I	T	Effect
	193	A	V	Neutral
	196	S	P	Effect
X5	52	Q	R	Neutral
	127	A	S	Neutral
X6	33	Q	R	Neutral
	108	A	S	Neutral
	116	Y	S	Effect

Table 3. Comparative isoforms of *TMEM95* between cattle and buffalo

	Cattle			Buffalo		
Isoform	AA length	No. of Exons	No. of Introns	AA length	No. of Exons	No. of Introns
X1	263	5	4	-	-	-
X2	220	6	5	220	6	5
X3	218	4	3	-	-	-
X4	217	6	5	-	-	-
X5	185	6	5	185	6	5
X6	183	7	6	183	7	6

Table 4. The transmembrane topology and signal peptide predictions in proteins from different isoforms of *TMEM95* gene and their corresponding AA positions

Isoform	Peptide length	Species	Signal peptide	Noncytoplasmic	Transmembrane	Cytoplasmic
X1	263	Cattle	1-16	17- 263		
X2	220	Cattle	1-16	17-220		
		Buffalo	1-16	17-220		
X3	218	Cattle	1-16	17-218		
X4	217	Cattle	1-16	17-188	189-213	214-217
X5	185	Cattle		1-19	20- 41; 165-184	42-164; 185-185
		Buffalo		1-19	20- 41; 165-184	42-164; 185-185
X6	183	Cattle	1-16	17-154	155-179	180-183
		Buffalo	1-16	17-154	155-179	180-183

Table 5. Predicted motifs and their number for different isoforms in cattle and buffalo

Isoform	Species	Motif						
		Casein kinase II phosphorylation site	Leucine zipper pattern	N-myristoylation site	Protein kinase C phosphorylation site	CHRD domain profile	N-glycosylation site	HIT zinc finger
X1	Cattle	3	1	5	3	1	x	x
X2	Cattle	2	1	4	5	1	x	x
	Buffalo	1	1	4	4	1	x	x
X3	Cattle	3	x	4	2	1	x	x
X4	Cattle	2	x	3	2	1	1	1
X5	Cattle	1	x	3	4	x	1	1
	Buffalo	1	x	3	3	x	1	x
X6	Cattle	1	x	2	3	1	1	x
	Buffalo	1	x	2	3	1	1	x

x - absence of the motif

The sequences were submitted to the NCBI database (Accession No. MF521483). Two deletions were observed in the buffalo sequence compared to cattle. A 2 bp deletion in the exonic region, and a 19 bp deletion in the intronic region were observed in buffalo. The 2 bp deletion was segregated in the bubaline species, as the same deletion was also observed in the recently sequenced Mediterranean buffalo genome. The second deletion, 19 bp, was observed in the intronic region in both the Murrah buffalo and the Mediterranean buffalo. At three positions, heterozygous alleles were observed in the Murrah breed (1254: T and G, 1430: C and A and 1865: G and A; the positions indicated correspond to the Hereford cattle genomic sequence). Apart from these, few non-synonymous variants were observed

between cattle and buffalo (Table 2). The sequence analysis of the Gir cattle indicated that there were no indels between the Gir and the Herford cattle. Three variant positions were observed between the Gir and the Herford breed at 1527 (A→C), 1581 (C→G) and 2195 (T→G) positions. These positions are located in the non-coding region of the gene.

Annotation of the TMEM95 gene in buffalo. Six exons were predicted in cattle for the *TMEM95* gene using the NCBI gene prediction tool Gnomon (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/gnomon/). Comparative analysis in buffalo indicated that the isoforms X1, X3 and X4 may not exist in buffalo, or may remain as pseudogene due to the 2 bp deletion in the coding region. As a result, only three isoforms are possible in buffalo (Table 3).

Functional predictions of TMEM95 gene in cattle and buffalo. Subcellular localization and transmembrane topology predictions are important to understand the function of a protein. The isoforms XI, X2, X3, X4 and X6 have a signal peptide in the N-terminal region, whereas the isoform X5 is lacking a signal peptide in both cattle and buffalo. A single transmembrane domain is present in isoforms X4 and X6, and two in isoform X5 (Table 4). To understand the function of the protein, the isoform peptides were scanned for known motifs. Casein kinase II phosphorylation site, Leucine zipper pattern, N-myristoylation site, Protein kinase C phosphorylation site, CHRD domain profile, N-glycosylation site, HIT Zinc finger domain are present in different isoforms (Table 5). The HIT Zinc finger domain is completely absent in any of the functional isoforms in buffalo.

Discussion

Comparative analysis at the nucleotide and protein level of the *TMEM95* gene between cattle and buffalo indicated plausible significant functional differences in the locus between the two species. The gene is associated with idiopathic male subfertility in Fleckvieh cattle (PAUSCH et al., 2014) and reproductive traits in pigs (LIU et al., 2017). In cattle, the sperm produced by the mutant *TMEM95* is apparently normal but it fails in the fertilization process. In cattle, the gene exists in 6 isoforms named as X1, X2, X3, X4, X5 and X6. In the present study, the orthologous of the *TMEM95* gene in buffalo showed a 2 bp deletion in the first coding exon of the isoforms X1, X3 and X4, resulting in truncated protein or non-formation of those three isoforms. A non-sense mutation (rs378652941) led to a premature stop codon in place of cysteine. PAUSCH et al. (2014) did not indicate the specific isoform that causes idiopathic male subfertility in cattle. The non-sense mutation described by PAUSCH et al. (2014) could produce a truncated protein product of the isoforms X4 and X6 of cattle, that results in a protein that lacks a transmembrane domain. Alternative splicing of a gene, resulting in different isoforms, is known to diversify the functional characteristics of the gene (YANG et al., 2016). In cows, relatively lower number of genes are alternatively spliced when compared to humans (21% opposed to 60% in humans) (CHACKO and RANGANATHAN, 2009). The genome of buffalo was only recently published (WILLIAMS et al., 2017), and the rate of alternative splicing in buffalo is not yet known. Even though it is not clear the protein product of which isoform is mainly responsible for the idiopathic male sub-fertility in cattle, the isoform orthologous to X6 is the likely candidate causing this phenotype in buffalo as it is conserved between the two species. Further, it appears that the isoform X2 of the gene in cattle and buffalo might serve diverse functions as there are certain non-synonymous substitutions that may affect the function of the protein (Table 2).

On analysis of the signal peptide and transmembrane domains of the isoforms to understand the function of the protein, it appears that alternatively spliced forms serve different functions in cattle and buffalo. For instance, the isoform X5 lacks the N-terminal signal peptide domain which is essential for guiding the protein for the secretory pathway. Isoforms X1, X2 and X3 lack a transmembrane domain and cytoplasmic localization (Table 4). Isoform X6 (names as *TMEM95*-SV1) and a non-coding isoform (*TMEM95*-SV2) are expressed in both the testis and brain (ZHANG et al., 2016). The results together suggest that the gene *TMEM95* has functional diversity within and between cattle and buffaloes, plausibly with different functions.

In order to understand the role of the *TMEM95* transcripts, functional motifs were predicted in various isoforms of the *TMEM95* gene. The casein Kinase II (CKII) phosphorylation site is present in all the isoforms. The domain is known to have a functional role in sperm morphology. In mice, the lack of the CKII phosphorylation site leads to morphological defects in sperm function (XU et al., 1999). Other domains observed in the isoforms are involved in regulatory function. Interestingly, due to the lack of isoforms X1, X3 and X5, none of the functional isoforms possess a HIT Zinc finger domain. The HIT type zinc finger domain binds to two zinc ions (HE et al., 2007). Zinc plays an essential role in general metabolism by activating enzymes to form metalloenzymes, thereby playing a crucial role in male fertility. The precise role of *TMEM95* in zinc metabolism is not known. However, if the gene plays a role in the mobilization of zinc ions, it may perhaps play different roles in cattle and buffalo. The results taken together indicate that the male subfertility gene *TMEM95* plays a conserved role in cattle and buffalo, but exhibits various degrees of functional diversity. Exploring the spatial and temporal expression profiling of these isoforms would provide a better understanding of the function of the gene.

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SAŽETAK

Pri razmatranju plodnosti goveda, idiopatska neplodnost mužjaka često je zanemareno fenotipsko obilježje. S obzirom na gensku osnovu, smatra se da je obilježje povezano s genom *TMEM9*. U radu je, upotrebom DNA sekvencijskih informacija dobivenih od goveđeg *TMEM95*, provedena karakterizaciju tog gena u murah bivola. Uz

bivola, fragment od ukupno 2.6 kb koji je ortologan govedima, sekvenciran je i u gir goveda. Delecija od 2 bp prisutna je u murah bivola što uzrokuje pogrešnu mutaciju u tri izoformna oblika prisutna u goveda. Funkcijski učinci nesinonimnih mutacija predviđani su primjenom SNAP2 programa koji je pokazao da nesinonimni SNP-i mogu utjecati na funkciju proteina. Funkcionalna bilješka motiva pokazala je prisustvo kazein kinaze II fosforilacijskog mjesta koje ima važnu ulogu u morfologiji spermija, zatim prisustvo leucin patentnog-zatvarača, N-miristoilacijskog mjesta, protein kinaze C-fosforilacijskog mjesta, CHRD domene profila, N-glikokosilacijskog mjesta i HIT motiva cinkovog prsta u goveda. U nijednoj od funkcionalnih izoformi u bivola nije prisutan HIT motiv cinkovog prsta. Rezultati skupno ukazuju da se gen subfertilnosti *TMEM95* između goveda i bivola razvijao sa različitim funkcijama ali ima ulogu u muškoj plodnosti kao i u drugih sisavaca.

Ključne riječi: karakterizacija; *TMEM95*; murah bivol; idiopatska neplodnost