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.
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. Using 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: CTGTTTTGTACCAGGTGTG  R: AGGTTCTAGACATTCTAGCAC | 56.6, 57.3 | 617, 696 |
| 2        | F: TCCATCTCCCACTAGAAGAATC  R: CTTCCAAGCCCTAGGTTGT | 56.4, 59.3 | 689 |
| 3        | F: TCCAGTGTCAGTGAGGAATC  R: CAACAGTACACCTCAAGGCC | 58.4, 58.6 | 689 |
| 4        | F: CAGAGAGGAACGGAGGCTTC  R: GACCATCTGACACTGGAAGCC | 59.8, 58.7 | 689 |
| 5        | F: GCCTAGGCCTCCAGTTCTAAG  R: GTTGAGAACCCACTGCTACT | 57.1, 56.4 | 621 |

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

| Isoform | Cattle | Buffalo |
|---------|--------|---------|
|         | 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 \textit{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     |
|         |                | Buffalo |               |               | 189-213       |             |
| 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 |
|---------|---------|-------|
|         |         |       |
| X1      | Cattle  | 3     |
| X2      | Cattle  | 2     |
|         | Buffalo | x     |
| X3      | Cattle  | 3     |
| X4      | Cattle  | 3     |
|         |          | x     |
| X5      | Cattle  | 3     |
|         | Buffalo | 3     |
| X6      | Cattle  | 2     |
|         | Buffalo | 2     |

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 involve 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 govedeg 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