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Molecular characterization of *β-Defensin123* gene in river buffalo.

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ABSTRACT

The water buffalo (*Bubalus bubalis*) with a population exceeding 195 million heads is an economically important livestock. Water buffalo (*Bubalus bubalis*) is a good source for high quality milk and meat. Defensins play a prominent role in the protection of various epithelial surfaces. *β-Defensin123* is a novel defensin, detected in cattle with potent inhibitory properties against pathogenic microbes like Escherichia coli and Staphylococcus. The present study characterize β- Defensin123 gene in river buffalo for the first time. DNA was extracted from uterus tissue samples obtained from Egyptian buffalo. PCR products of *β-Defensin123* flanking the 2 exons were purified and sequenced. Sequence analysis revealed the presence of nine noncoding SNPs and one signal peptide polymorphism. The latter was a non-synonymous substitution T>G in the 2nd position of the initiating triplet resulting in 2 variants. Sequence with ATG initiation codon (variant1) results in full precursor peptide, whereas the non-ATG sequence (variant2), results in 5 amino acid shorter precursor peptide. The mature proteins of the two sequences had the same length since the 5 amino acids short occurred in the signal peptide region. The two variants had intrinsically disordered protein (5 amino acids) at the mature peptide. In addition, vatiant1 had a disordered region in the signal peptide that is missed in variant2**.**

Keywords: River buffalo, *β-Defensin123*, Intrinsically disordered proteins, Polymorphic sites

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INTRODUCTION

Water buffalo *Bubalus bubalis* includes two subspecies: swamp (2n=48) and river buffaloes (2n=50) [1]. Egyptian buffaloes are of the river type and belong to one breed [2-3] with a population exceeds 4 million heads; it ranks the third after Indian (115 million) and Pakistani (31.7 million) buffalo [4]. Water buffalo is a good source for high quality milk and meat [5]. Unlike domestic animals, buffaloes are more resistant to most of the infectious diseases, although they live in hot humid condition leading to disease development [6].

Antimicrobial peptides (AMPs) are new generation antibiotics with a vital role in both innate and adaptive immune systems of animals and humans [7]. Defensins and cathelicidins can be considered the most important antimicrobial peptides with broad spectrum activity against enveloped viruses, bacteria, and fungi [8-11].

Mammalian defensins are segregated into three major types, α- defensins, β-defensins, and Θ-defensins, according to cellular origin, cysteine position [12], specific intermolecular disulfide-bond pattern and length of amino acids sequence [13-14].

β-defensins are characterized by having six cysteines and three disulfide bonds between cysteines residues (Cys1–Cys5, Cys2–Cys4, Cys3–Cys6) [15]. β-defensin have been identified in epithelial cells of buffalo [16]. A comprehensive bioinformatics search of the sequenced bovine genome identified 57 open reading frames of the β-Defensins genes [17]. β-Defensin genes exist in a single cluster in birds, but 4 clusters exist in dog, rat, mouse, and cow [18]. The four clusters: A, B, C and D are located on bovine chromosome 8, 13, 23, and 27 respectively [18]. β-Defensin cluster B is a relatively stable cluster in higher mammals with few gene gain or loss events disrupting the syntenic order and gene orientation [19].

β-Defensin123 gene is among a group of nineteen bovine genes in cluster B that spans 320 kb on chromosome 13 which are predominantly expressed in the reproductive tract of cattle [17]. It has shown to be more effective against methicillin-resistant Staphylococcus aureus (MRSA) [20] and E.coli [17] that can invade the reproductive tract. Peptides of β-defensins genes in cluster D such as Tracheal antimicrobial peptide (TAP) [21], Enteric β- defensin [22], and Neutrophil β-defensins (β-defensins 1-13) [23] are tissue specific.

Molecular analysis of β-defensins is of great importance as a first step to study its evolutionary relationship and biological role [24]. So, the aim of the present study was to characterize *β-Defensin123* gene in buffalo.

MATERIAL AND METHODS

Samples collection and DNA extraction

Uterine tissue samples of twenty five Egyptian buffalo were collected from the slaughter house for DNA extraction. Genomic DNA was extracted from Uterine tissue of healthy buffalo by salting out method according to Miller *et al.* [25]. The DNA quality and purity were determined by NanoDrop 1000 (Thermo Scientific) and used as a template in Polymerase chain reaction (PCR).

Primers design

Two primers pairs were designed to flank the two exons of *β-Defensin123* using known DNA sequences of Bos taurus # NC_007311 via Primer3 [26]. To ensure primers specificity, primers were tested by Oligo Analyzer program (ver. 1.0.3) and were synthesized by Amersham Pharmacia Biotech. The first primer pairs [F: GTGACATCTGTTCCGGTTGG and R: TCCTAGCTTCCCAACACAGG] was designed to amplify 574bp including 5′ UTR (114bp), exon 1 (58bp) and a part of the intron (402bp).The second primer pairs [F: CAGAGCACAGAAGCAGAT and R: TCAGCCAGAGATGTTTAT] was designed to amplify 709bp including part of the intron (532bp), exon 2(146bp) and 3′ UTR (62bp).

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Polymerase chain reaction and sequencing

Each PCR reaction was performed using 2 µl of 50 ng/µl DNA template, 12.75 µl of RNAse, DNAse free water, 2.5µl of 2mM dNTPs, 2.5 µl of 10X Taq polymerase buffer, and 2.5 µl forward and reverse primers (10 µM), as well as 0.25µl (5U) of Taq polymerase in 25 µl reaction final volume. The following cycling conditions were used: 5 min. at 94ºC; 35 cycles for 1 min at 94ºC; 1 min at 62 ºC and 52 ºC for first and second primer pairs respectively; 2 min at 72ºC and a final extension for 10 min at 72ºC. The PCR products were electrophoresed on 1.5% agarose gel stained with ethidium bromide (Applichem).The gels were examined under UV and photographed using Gel documentation system (In Genius, Syngene Bio-Imaging). The PCR products were purified using MEGAquick-spinTM Total Fragment DNA Purification Kit (iNtRON biotechnology) according to the manufacturer's instructions. Each PCR product was 2 ways sequenced using Sanger method by Macrogen.

Sequence analysis and SNPs identification.

Multiple sequence alignment of the investigated buffalo was performed using CLUSTAL Omega program (**http://www.ebi.ac.uk/Tools/msa/clustalo**) in order to identify nucleotide differences between samples. Single nucleotide polymorphic sites (SNPs) were determined by visual examination of sequence chromatogram. The protein sequence was identified using Open Reading Frame (ORF) (**https://www.ncbi.nlm.nih.gov/orffinder/**) and the Conserved Domain Database (**http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi**) was used to predict the protein domains of the investigated gene. Prediction of intrinsically disordered protein was accomplished by using PONDR software (**http://www.pondr.com/**) whereas the SignalP 4.1 software was used to predict the signal peptide and the cleavage sites (**http://www.cbs.dtu.dk/services/ SignalP/**).

RESULTS

The two primers pairs used in this study for *β-Defensin123* resulted in two non-overlapped amplified amplicons 574 bp and 709 bp and the full gene (1383bp) was deduced and submitted to GenBank acc**#** MF069183. It included 5′ UTR (114bp), exon1 (58 bp: c.1-c.58) and 402 bp of 5'intron1, unknown segment of intron1 (100bp), 501 bp of 3' intron1, exon 2 (146 bp: c.59-c.204) and 62bp of 3'UTR. Analysis of *β-Defensin123* sequence revealed the presence of 10 SNPs: A/G in 5' UTR at c.1-g.29, T/G in exon1 at c.2 (signal peptide polymorphism) and 8 intronic SNPs C/G at c.58+g.47; A/G at c.58+g.70; C/T at c.58+g.334; A/G at c.58+g.370; A/G at c.59-g.485; A/C at c.59-g.251; A/G at c.59-g.21 and C/T at c.59-g.19. A full coding region of buffalo *β-Defensin123* was deduced from the genomic sequence. ATG and alternatives initiation codons predictor of the Open Reading Frame software (ORF) was used resulting in two sequences. Table 1 showed the different *β-Defensin123*: SNP positions, allele frequency, and genotype frequency.

Table 1: SNP positions, allele frequency and genotype frequency of buffalo *β-Defensin123* gene.

SNPs position			Allele frequency (%)		Genotype frequency (%)		
c.1-g.29	5′ UTR	R(A/G)	A	G	AA	GG	AG
			34	66	11	44	45
c.2	exon	K(G/T)	G	T	TT	GG	TG
			24	76	58	6	36
c.58+g.47	Intron	S(C/G)	C	G	CC	GG	CG
			26	74	7	55	38
c.58+g.70	Intron	R(A/G)	A	G	AA	GG	AG
			20	80	4	64	32
c.58+g.334	Intron	Y(C/T)	C	T	TT	CC	TC
			68	32	10	46	44
c.58+g.370	Intron	R(A/G)	A	G	AA	GG	AG

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			32	68		10	46	44
c.59-g.485	Intron	R(A/G)	A	G		AA	GG	AG
			66	34		44	11	45
c.59-g.251	Intron	M(A/C)	A	C		AA	CC	AC
			34	66		11	44	45
c.59-g.21	Intron	R(A/G)	A	G		AA	GG	AG
			38	62		39	14	47
c.59-g.19	Intron	Y(C/T)	C	T		CC	TT	TC
			62	38		39	14	47

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ATG Seq.       MKLLSLILAGLLLLSQLTPGGTRKcWNFHGKcRHKcFKKEKVYVYcTNNKLccVKSRYLP   60
Non-ATG Seq.   -----MILAGLLLLSQLTPGGTRKcWNFHGKcRHKcFKKEKVYVYcTNNKLccVKSRYLP   55
                    :**************************************************

ATG Seq.       KNLPWQI    67
Non-ATG Seq.   KNLPWQI    62
               *******
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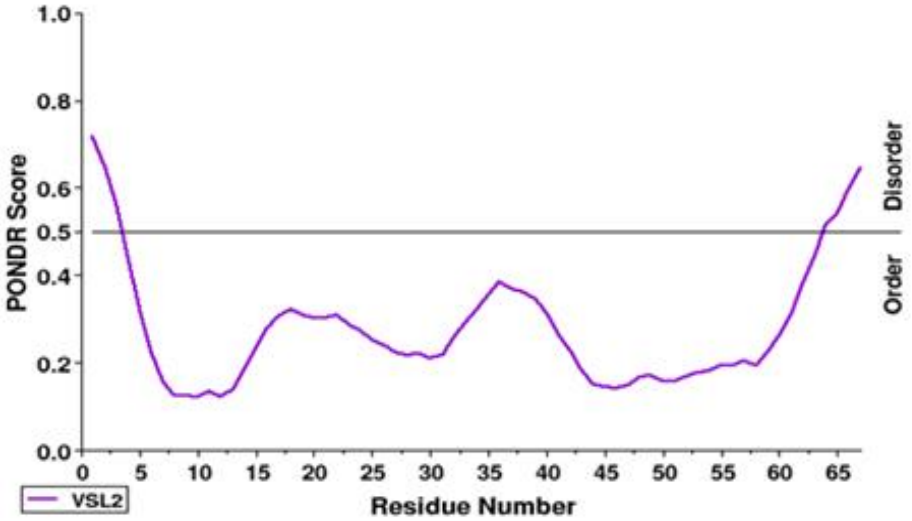
Figure 1: Alignment between ATG and non-ATG proteins of buffalo β-Defensin123. Signal peptides are underlined; '⁎' indicates identical amino acids; '⁚' conserved amino acids; conserved domains are in bold; the cysteine residues are in small letter.

Variant2 is characterized by the missing of five amino acid that are present at variant2. Variant1 and variant2 have different length of signal peptide 22 and 17 amino acid ,respectively. The mature proteins in both variants had the same length where The six cysteine residues are conserved at positions 3,10,14, 24, 30 and 31.

a




```
1       MKLLSLILAG LLLLSQLTPG GTRKCWNFHG KCRHKCFKKE KVYVYCTNNK
        DDD

51      LCCVKSRYLP KNLPWQI
                   DDDD
```

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b

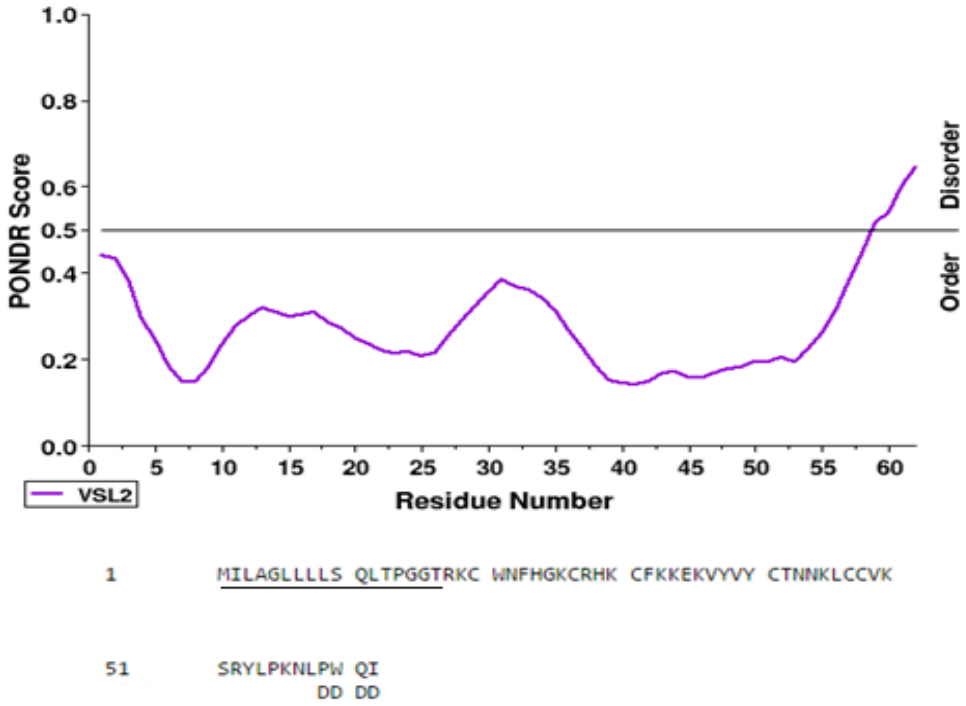

Figure 2: Graphical representation and amino acids sequences of PONDR for buffalo *β Defensin123* ATG (a) and non-ATG (b) sequences."D" indicates disordered amino acids; Signal peptide is underlined.

Both variants had a disordered region that consists of 4 amino acids of the mature peptide. In addition, variant1 had a disordered region in the signal peptide that are missed in variant2.

The exonic SNP (T/G) detected in the second position of the initiating triplet was nonsynonymous resulting in two different codons: ATG (Methionine) and AGG (arginine). Sequence starting with the initiating codon ATG resulted in full 67 amino acids precursor peptide (variant1). However, since AGG is less efficient than CTG as an initiating codon, a substitute non-ATG initiating-codon (CTG) resulted in a 62 amino acids precursor peptide sequence (variant2). Buffalo *β-Defensin123* gene was submitted to GenBank*:* variant 1 acc# MF069183, and variant 2 acc# MF150116.

Signal peptide prediction

Signal peptide analysis of the two different precursor peptides showed that buffalo *β-Defensin123* is a secretory protein (Y-score: 0.594, 0.476) with a signal peptide present from amino acids 1 to 22 in ATG-sequence and from 1 to17 in non-ATG sequence since their S-scores were high (0.977, 0.877 and S-mean 0.881, 0.732, respectively). High S-scores indicate that the corresponding amino acid is a part of a signal peptide whereas low scores indicate that the amino acid is a part of mature protein. The cleavage sites were between codon 22/23 in ATG-sequence and 17/18 in non-ATG sequence. The mature proteins in both sequences were not different (45 amino acid) since the 5 amino acids difference between the two sequences were in the signal peptide region**.**

Analysis of buffalo *β-Defensin123* coding regions also showed the presence of conserved domains with the six cysteine residues characteristic for defensin genes. The six cysteine residues in ATG and non-ATG sequences are conserved at positions 25, 32, 36, 46, 52, 53 and 20, 27, 31, 41, 47, 48 of the precursor protein, respectively and at 3,10,14, 24, 30 and 31 of the mature protein in both variants (Fig 1).

Prediction of intrinsically disordered protein

Prediction of intrinsically disordered protein in buffalo *β-Defensin123* two variants was accomplished using the most accurate predictor in the PONDR family (VSL2 Predictor) **(http://www.pondr.com/)** [27]. Both

variants had a disordered region that consists of 4 amino acids of the mature peptide. In addition, variant1 had a disordered region in the signal peptide that consists of the first 3 amino acids that are present in the 5 missing amino acids in variant2 (Fig. 2 a, b).

DISCUSSION

In the present study, we investigated *β-Defensin123* gene in river buffalo for the first time. Defensins and cathelicidins can be considered the most important antimicrobial peptides. Characterizations of beta-defensin genes are important to evaluate the innate immune system. Bovine *β-Defensin123* is characterized by potent inhibitory properties against pathogenic microbes like Escherichia coli, Listeriamonocyto genes, Klebsiellapneumoniae, Streptococcus pneumoniae, Pseudomonas aeroginosa, and Staphylococcus species in both cattle [17] and human [28].

In this study, we have reported ten polymorphic sites, one in 5'UTR eight in the intronic region and one in the coding region. The latter was a non-synonymous substitution T>G (a purine substitution) in the 2nd position of the initiating triplet resulting in 2 variants with different precursor peptides that differed by 5 amino acids in length. The second position of the initiator triplet was reported to be more sensitive to substitution and cannot tolerate the presence of a purine residue [29] this allowed for a non-ATG initiating codon (CTG) to be used resulting in a precursor peptide five amino acids shorter. CTG is more efficient than AAG and AGG in mammals and plants [29].The presence of non-initiation codon has been reported by Tikole and Sankararamakrishnan [30] who accessed at least 38 mRNAs from 23 genes in GenBank databases with non-AUG initiation codons and by Ivanov *et al.* [31] who identified about 42 human genes with non-AUG-initiated codon.

The difference between the two precursor peptides resulting from SNP T/G was in the signal peptide. Signal peptide polymorphism has an integral role in the translocation of secretory proteins across the membrane of the endoplasmic reticulum. The presence of two signal peptides alleles that differ in size has been reported by Visvikis *et al.* [32] in Apolipoprotein B gene (APO B). The shorter signal peptide was reported to have less effective translocation across the endoplasmic reticulum membrane which may influence the production of triacylglycerol [33]. The polymorphic signal peptide of APO B was found to have an impact on protein secretion, cholesterol, and low density lipoprotein levels [34], cholesterol transport [35], and the prevalence of atherosclerosis [36]. The polymorphic signal peptide of acid sphingomyelinase (ASM) was reported to regulate ASM secretion without affecting its cellular activity [37].

Analysis of *β-Defensin123* sequences in buffalo revealed the presence of six cysteine residues and eight intronic SNPs. The six cysteine residues play an essential role for the resistance of defensins to bacterial proteolysis [38]. In buffalo *β-Defensin123* the six cysteines residues are at positions 3, 10, 14, 24, 30 and 31 of the mature protein. Their positions are different from *β-Defensin* 4, 5, Tap, and enteric *β*-defensin which have conserved positions at 9, 16, 21, 31, 38, and 39 [39].

In buffalo *β-Defensin123* the cationic residues (3 arginine and 9 lysine) are clustered within c-terminus of mature peptide. The antimicrobial activities of defensins are related to the clustering of cationic arginine and lysine residues [40-41].Clustering of cationic residues are characteristics of defensin [13].

The mature peptide of buffalo *β-Defensin123* has a consensus amino acid sequence of $x_2Cx_5(G)xCx_3Cx_9Cx_5CCx_{14}$which falls in the consensus amino acid sequence ($x_{2\text{-}10}\,Cx_{5\text{-}6}(G/A)xCx_{3\text{-}4}Cx_{9\text{-}13}Cx_{4\text{-}7}CCx_n$) reported in primates by Yang *et al.* [9].
Three out of the eight identified intronic SNPs (C/G at c.58+g.47; A/G at c.59-g.21; and C/T at c.59-g.19) in buffalo were located within the extended consensus sequences of exon -intron splice junctions. Intronic SNPs in these regulatory regions can regulate gene expression and influence either the transcriptional activity or the splicing efficiency of genes [42].

Buffalo *β-Defensin123* ATG translated precursor peptide was found to have two regions of intrinsically disordered protein one in the signal peptide and the other in mature peptide. Whereas the non-ATG translated precursor peptide has one intrinsically disordered protein in mature peptide. It has been reported that disordered regions in many proteins are more susceptible to amino acid substitutions, insertions, and deletions than the ordered regions of the same proteins [43-44]. Intrinsically disorder proteins in defensins are

critical in the maturation process of these antibacterial peptides [45]. Placing of disordered regions within proteolytic cleavage sites of pro-defensins make them easily accessible for proteases [46]. The interactions of cationic defensins with anionic membranes of invading pathogens are also defined by intrinsic disorder protein. Furthermore, Intrinsic disorder is also needed for post-translational modifications of defensins [15]. Intrinsic disorder proteins are associated with conformational plasticity [47] this gives an evidence that the biological activity of β-defensins does not require a rigid β-sheet structure or the presence of three properly connected disulfide bonds [48].

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