CHARACTERIZATION OF TWO B-DEFENSINS (BNBD5 AND BNBD10) IN EGYPTIAN NATIVE AND FRISIAN CROSSBRED CATTLE

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The emergence of antibiotic-resistant microorganisms has heightened interest in the study of endogenous antimicrobial substances and their role in host defense. Recently, numerous antimicrobial peptides have been isolated from mammals (Hancock and Chapple, 1999), amphibians (Cunha Filho *et al*., 2005) and insects (Vizioli and Salzet, 2002), representing a broad and previously unrecognized component of animal host defense.

Production of antimicrobial peptides and proteins is an important means of host defense in eukaryotes. The large antimicrobial proteins contain more than 100 amino acids. They are often lytic enzymes, nutrient-binding proteins or contain sites that target specific microbial macromolecules, whereas, the small antimicrobial peptides act largely by disrupting the structure or function of microbial cell membranes. Hundreds of antimicrobial peptides have been found in the epithelial layers, phagocytic cells and body fluids of multicellular animals, from mollusks to humans. Some antimicrobial peptides are produced constitutively; others are induced in response to infection or inflammation (Ganz, 2003).

Defensins are cationic antimicrobial peptides that are characterized by the presence of a conserved cysteine-rich defensin motif. α-, β-, and θ-defensins are mostly found in mammalian species. β-Defensin genes code for multifunctional peptides with a broad-range of antimicrobial activity (Roosen *et al*., 2004). They are microbicidal peptides implicated in host defense functions of phagocytic leukocytes and certain surface epithelial cells (Yount *et al.,* 1999). Bovine β-defensin gene family is represented by 13 cationic, tridisulfide-containing peptides which are characterized by a highly cationic 38-42-residue chain that includes six invariantly spaced cysteines which form the three disulfides (Selsted *et al*., 1993).

Neutrophil β-defensins have potent antibacterial activities against *Staphylococcus aureus* and *Escherichia coli in vitro* (Selsted *et al*., 1993). In addition to their abundant expression in neutrophils, related β-defensins are expressed in the epithelium of bovine trachea (tracheal antimicrobial peptide, or TAP; Diamond *et al*., 1991), tongue (lingual antimicrobial peptide, or LAP; Schonwetter *et al.,*

1995) and intestine (enteric β-defensin, or EBD; Tarver *et al*., 1998). Several homologous peptides were also purified from chicken and turkey leukocytes (Harwig *et al.,* 1994; Evans *et al.,* 1994). Members of the β-defensin (BNBD) gene family have been characterized at the DNA level in sheep (Mahoney *et al*., 1995), pig (Zhang *et al.,* 1998), mouse (Huttner *et al.,* 1997; Morrison *et al*., 1998), cattle (Diamond *et al.,* 1991; Selsted *et al.,* 1993) and human (Schutte *et al*., 2002; Bensch *et al.,* 1995; Goldman *et al*., 1997).

Because of the importance of the neutrophil β-defensins in the innate immune response, the neutrophil β-defensin genes of farm animals have attracted considerable attention. Neutrophil β-defensin genes have been characterized in many domestic animals such as: cattle, sheep, goat and pig (Mahoney *et al*., 1995; Zhang *et al.,* 1998; Yount *et al.,* 1999; Zhao *et al*., 1999). On the other hand, comparative analysis of the defensin clusters among chicken, mouse and human suggested that vertebrate defensins have evolved from a single β-defensin-like gene, which has undergone rapid duplication, diversification and translocation in various vertebrate lineages during evolution (Xiao *et al*., 2004). Analysis of α, β and θ-defensins in different vertebrate lineages, is therefore required to shed light on the mechanisms of host defense and evolution of innate immunity.

The β-defensins are encoded by genes consisting of two exons. The primary translation product is an inactive precursor (prepropeptide) constructed of an N-terminal signal sequence, a short propiece and a C-terminal mature peptide, which is cleaved from the propiece. The first exon encodes the signal sequence; the second exon encodes the pro- and the mature peptide (Ganz and Lehrer, 1994).

Mutations are the source of new variation important for evolution. Mutations create variations in the gene pool, and the less favorable (or deleterious) mutations are reduced in frequency in the gene pool by natural selection, while more favorable (beneficial or advantageous) mutations tend to accumulate, resulting in evolutionary change (Knight *et al*., 2006).

We aim to study the bovine neutrophil β-defensin genes 5 and 10 (BNBD5 and BNBD10), focusing on characterization and detection of mutations in native (also known as Baladi) and Frisian-native crossbred cattle.

MATERIALS AND METHODS

RNA isolation and first-strand cDNA synthesis

RNA was extracted from samples taken from lung, trachea, liver and muscle of both native and Frisian crossbred cattle, obtained at the slaughter house. In addition, blood, testis and lymph samples were taken from Frisian crossbred cattle, whereas intestine was taken from Native cattle. RNA was extracted from the above samples according to Grubor *et al.* (2004).

cDNAs were synthesized using RT-PCR READY TO GO YOU prime-First Strand Beads kit (Amersham Biosciences) according to manufacturer instructions.

Primer design

Primers specific for bovine BNBD5 and BNBD10 genes (Table 1) were designed using *Bos taurus* cDNA sequences [accession numbers: 11125504 (for BNBD5) and 55416131-55415754 (for BNBD10)] and the software Primer 3 [http://www.genome.wi.mit.edu] (Marone *et al*., 2001). PCR primers were selected on the basis that the 5' and 3' ends span exons I and II. The primers were synthesized by Amersham Pharmacia Biotech.

Polymerase chain reaction (PCR)

Amplification reactions (100 µl) contained 5 µl of first-strand cattle cDNAs, 0.2 mM dNTPs, 10 mM Tris, 50 mM Kcl, 1.5 mM MgCl2, 0.01% gelatin (W/V), 1.25 units Taq polymerase and 1 µM forward and reverse primers. The reaction mixture was overlaid with sterile mineral oil. PCR was performed using MJ research PTC-100 thermocycler using 1 cycle (3 min.) at 94°C, followed by 30 cycles for (1 min. at 94°C, 2 min. at 60°C for both BNBD5 & 10 and 2 min. at 72°C) and finally 1 cycle (10 min.) at 72°C. The reaction products were electrophoresed on 1.5% agarose in 1X- Tris acetate buffer (TAE) containing 0.8 µl of 10 mg/ml ethidium bromide (Ausubel *et al*., 1990).

Sequence analysis

The PCR products were purified and sequenced at the Center of Genetic Engineering; Ain Shams University; Cairo; Egypt. Sequence analysis and alignments were carried out using CLUSTAL W (1.83) analysis (Gasteiger *et al.,* 2003).

RESULTS AND DISCUSSION

Defensins were the first antimicrobial peptides isolated from leukocytes. Search for the classical defensins in bovine neutrophils led to the discovery of a new class of distinct but related peptide antibiotics, β-defensin. β-defensin constitutes a highly conserved family of at least 13 neutrophil peptides, which are characterized by a disulphide motif different from that of the other defensin family (Selsted *et al*., 1993).

The present study focused on the use of primer pairs specific for two β-defensin antimicrobial peptides, BNBD5 and BNBD10, to test their expression in different tissues of Frisian crossbred and native cattle. Primers specific for BNBD5 amplified a fragment of 192 bp in lung, trachea and liver cDNAs in Frisian crossbred (Fig. 1) but only in lung and trachea of native cattle (Fig. 2).On the other hand primers specific for BNBD10 amplified a fragment of 165bp. This was only present in lung and trachea cDNAs of Frisian crossbred cattle (Fig. 3), whereas in native cattle it was expressed only in lung, trachea, liver and intestine (Fig. 4).

BNBD5 and BNBD10 trachea cDNA amplified segments of Frisian crossbred and native cattle were sequenced. The reverse complement nucleotide sequences and the translated amino acids of the downstream strand of BNBD5 and BNBD10 amplicons are presented in Figs (5 and 6), respectively.

CLUSTAL W (1.83) multiple nucleotide sequence alignment between BNBD5 amplicons of Frisian crossbred and native cattle showed 64% identity. *Bos taurus* (AF014108) showed 77% identity with Frisian crossbred cattle and 67% identity with native cattle (Table 2). CLUSTAL analysis for BNBD10 sequences showed 93% alignment between Frisian crossbred and native, 93% between Frisian crossbred and *Bos taurus* Exon 1 and 2 (AJ56799), and 90% between native and *Bos taurus* (AJ56799) (Table 3).

Point mutations were detected in native and crossbred BNBD5 and BNBD10; however they were higher in BNBD5. Three deletions were noticed in both Frisian crossbred and native cattle BNBD5. They were detected at the sites corresponding to *Bos taurus* nucleotides (nts) number 86, 165 and 166 in native cattle and 86, 123 and 158 in Frisian crossbred cattle (Table 2). On the other hand, only one deletion was detected in BNBD10 Native cattle at the site corresponding to *Bos taurus* nt number 131 bp, in addition to two insertions in both native and crossbred cattle at the sites corresponding to *Bos taurus* nts number 77 and 126 bp. Nucleotide deletion and insertion cause frame shift mutations which lead to a dramatic change of amino acid sequence (Galvani and Slatkin, 2003). Many substitutions were detected in BNBD5 and BNBD10 of native and crossbred cattle (Tables 2 and 3). Substitution mutation has a relatively minor effect on the sequence of amino acids because only one codon in the mRNA is altered. Evolutionary events depend on mutations because this is the only way that new alleles are created (Galvani and Slatkin, 2003).

In order to detect differences in gene translation between native and crossbred cattle and *Bos taurus*, NCBI open reading frame (ORF) finder: [http://www.ncbi.nlm.nih.gov/gorf/gorf.htmlURL] was used for amino acid (aa) translations. This was done by comparing the six frame amino acid sequences of the two breeds with *Bos taurus* BNBD5 (AF014108) and BNBD10 (AJ567990 & AJ567991). Amino acid F+3 and aa F+1 were selected for BNBD5 and BNBD10, respectively. Native cattle BNBD5 aa translation showed four stop codons at nts number 69, 87, 123 and 132; whereas, two stop codons were detected in crossbred at nts number 87 and 153 (Fig. 5). On the other hand, no stop codons were detected in aa translation of native and crossbred cattle BNBD10 (Fig. 6). Nonsense mutation, the one that changes an amino acid codon into a stop codon, has been reported by Galvani and Slatkin, (2003). The presence of a stop codon causes the amino acid chain to stop growing, prematurely, which results in a

truncated protein. A very small percentage of all mutations actually have a beneficial effect. These mutations lead to new versions of proteins that help an organism and its future generations better adapt to changes in their environment (Knight *et al.,* 2006).

Nucleotide insertions, deletions, substitutions observed in this study denote the greater tendency of BNBD5 to mutations compared to BNBD10 which is reflected at the level of translated aa sequences. Homology percentage of BNBD5 aa sequences of native and Frisian crossbred cattle and that of *Bos taurus* (26% and 27%, respectively) (Table 4) are lower than the corresponding values for BNBD10 aa sequences (82% and 73%, respectively) (Table 5).

In conclusion, the characterization of BNBD5 and BNBD10 antimicrobial peptides in native and Frisian crossbred cattle reared in Egypt revealed more tendencies to variations or mutations in BNBD5, rather than BNBD10. Further studies, especially at the protein level, are required to study the effects of the aa changes on the protein production and its relation to cattle health, performance and disease resistance which may direct breeders to better selections. On the other hand, mutations detection may lead to new versions of proteins that help an organism and its descendents for better adaptation to changes in their environment, in addition to a better understanding of host defense mechanisms and evolution of innate immunity.

SUMMARY

Two β-Defensin antimicrobial peptides namely bovine neutrophil β-defensin 5 and 10 (BNBD5 and BNBD10) were investigated in Native and Frisian crossbred cattle reared in Egypt. The two antimicrobial peptides were tested for reaction with cDNA of various cattle tissues such as lung, trachea, liver, intestine, blood, muscle, testis and lymph node using PCR. BNBD5 was positive in lung, trachea and liver of Frisian crossbred but only in lung and trachea of native cattle. BNBD10 was positive in lung and trachea of Frisian crossbred, whereas in native cattle it was expressed in lung, trachea, liver and intestine.

CLUSTAL W (1.83) multiple nucleotide sequence alignment between BNBD5 amplicons of Frisian crossbred, native cattle and of *Bos taurus* (AF014108) showed 64% alignment between Frisian crossbred and native, 77% between Frisian crossbred and *Bos taurus* and 67% between native and *Bos taurus*. CLUSTAL analysis for BNBD10 sequences showed 93% alignment between Frisian crossbred and native, 93% between Frisian crossbred and *Bos taurus* Exon 1 and 2 (AJ56799), and 90% between native and *Bos taurus* (AJ56799).

Some point mutations were observed in BNBD5 rather than BNBD10 in native and Frisian crossbred cattle. BNBD5 alignments showed three deletions and two stop codons in Frisian crossbred cattle, whereas in native cattle three deletions and four stop codons were

detected. No stop codons were detected in BNBD10; only two insertions were detected in crossbred and native cattle in addition to one deletion in the latter. The above findings were reflected on the amino acid translated sequences, where lower homologies and higher tendency of BNBD5 to mutations than BNBD10 were detected.

REFERENCES

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Seidman and K. Struhl (1990). Current Protocols in Molecular Biology. Chapter 2. Green Publishing and Wiley-Interscience, New York.

Bensch, K. W., M. Raida, H. J. Magert, P. Schulz-Knappe and W. G. Forssmann (1995). hBD-1: a novel beta-defensin from human plasma. FEBS Lett., 368: 331-335.

Cunha Filho, G. A., C. A. Schwartz, I. S. Resck, M. M. Murta, S. S. Lemos, M. S. Castro, C. Kyaw, Jr. O. R. Pires, J. R. S. Leite, Jr. Carlos Bloch and E. F. Schwartz (2005). Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the toad *Bufo rubescens*. Toxicon**,** 45: 777-782.

Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W. L. Maloy and C. L. Bevins (1991). Tracheal Antimicrobial Peptide, A Cysteine-Rich Peptide from Mammalian Tracheal Mucosa: Peptide Isolation and Cloning of a cDNA. Proc. Natl. Acad. Sci. USA, 88: 3952-3956.

Evans, E. W., G. G. Beach, J. Wunderlich and B. G. Harmon (1994). Isolation of antimicrobial peptides from avian heterophils J. Leukocyte Biol., 56: 661-665.

Galvani, A. and M. Slatkin (2003). Evaluating plague and smallpox as historical selective pressures for the CCR5-Δ32 HIV-resistance allele. Proc. Natl. Acad. Sci., USA, 100: 15276-15279.

Ganz, T. (2003). The Role of Antimicrobial Peptides in Innate Immunity. Integrative and Comparative Biology, 43: 300-304.

Ganz, T. and R. I. Lehrer (1994). Defensins. Curr Opin Immunol, 6: 584-589.

Gasteiger E., A. Gattiker, C. Hoogland, I. Ivanyi, R. D. Appel and A. Bairoch (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res., 31: 3784-3788.

Goldman, M. J., G. M. Anderson, E. D. Stolzenberg, U. P. Kari, M. Zasloffand J. M. Wilson (1997). Human beta-defensin-1 is a salt sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell, 88: 553-560.

Grubor, B., J. M. Gallup, D. K. Meyerholz, E. C. Crouch, R. B. Evans, K. A. Brogden, H. D. Lehmkuhl and M. R. Ackermann (2004). Enhanced surfactant protein and defensin mRNA level and reduced viral replication during parainfluenza virus Type 3 pneumonia in Neonatal lamb. Clin. Diagn. Lab. Immunol., 11: 599-607.

Hancock, R. E. W. and D. S. Chapple (1999). Peptide Antibiotics. Antimicrob Agents Chemother, 43: 1317-1323.

Harwig, S. S. L., K. M. Swiderek, V. N. Kokryakov, L. Tan, T. D. Lee, E. A. Panyutich, G. M. Aleshina, O. V. Shamova and R. I. Lehrer (1994). Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett., 342: 281-285.

[http://www.genome.wi.mit.edu]

[http://www.ncbi.nlm.nih.gov/gorf/gorf.htmlURL]

Huttner, K. M., C. A. Kozak, and C. L. Bevins (1997). The mouse genome encodes a single homolog of the antimicrobial peptide human beta-defensin 1. FEBS Lett., 413: 45-49.

Knight, C. G., N. Zitzmann, S. Prabhakar, R. Antrobus, R. Dwek, H. Hebestreit and P. B. Rainey (2006). Unraveling adaptive evolution: how a single point mutation affects the protein coregulation network. Nat. Genet., 38: 979-980.

Mahoney, M. M., A. Y. Lee, D. J. Brezinski-Caliguriand and K. M. Huttner (1995). Molecular analysis of the sheep cathelin family reveals a novel antimicrobial peptide. FEBS Lett., 377: 519-522.

Marone M., S. Mozzetti, D. De Ritis, I. Pierelli and G. Scambia (2001). Semiqualitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biol. Proceed., 3: 19-25.

Morrison, G. M., D. J. Davidson, F. M. Kilanowski, D. W. Borthwick, K. Crook, A. I. Maxwell, J. R. W. Govan and J. R. Dorin (1998). Mouse beta defensin-1 is a functional homolog of human beta defensin-1. Mamm. Genome, 9: 453-457.

Roosen, S., K. Exner, S. Paul, J. Michael Schröder, E. Kalm and C. Looft. (2004). Bovine ß-defensins: Identification and characterization of novel bovine ß-defensin genes and their expression in mammary gland tissue. Mamm. Genome, 15: 834-842.

Schonwetter, B. S., E. D. Stolzenberg and M. A. Zasloff (1995). Epithelial antibiotics induced at sites of inflammation. Science, 267: 1645-1648.

Schutte, B. C., J. P. Mitros, J. A. Bartlett, J. D. Walters, H. P. Jia, M. J. Welsh, T. L. Casavant and P. B. Jr. McCray (2002). Discovery of five conserved beta-defensin gene clusters using a computational search strategy. Proc. Natl. Acad. Sci., USA, 99: 2129-2133.

Selsted, M. E., Y. Q. Tang, W. L. Morris, P. A. McGuire, M. J. Novotny,W. Smith, A. H. Henschen and J. S. Cullor (1993). Purification, primary structures, and antibacterial activities of beta- defensins, a new family of antimicrobial peptides from bovine neutrophils. J. Biol. Chem., 268: 6641-6648.

Tarver, A. P., D. P. Clark, G. Diamond, J. P. Russell, H. Erdjument-Bromage, P. Tempst, K. S. Cohen, D. E. Jones, R. W. Sweeney, M. Wines, S. Hwang and C. L. Bevins (1998). Enteric -defensin: molecular cloning and characterization of a gene with inducible intestinal epithelial cell expression associated with *Cryptosporidium parvum* Infection Infect. Immun., 66: 1045-1056.

Vizioli, J. and M. Salzet (2002). Antimicrobial peptides from animals: focus on invertebrates. Trends in Pharmacological Sciences, 23: 494-496.

Xiao, Y., A. L. Hughes, J. Ando, Y. Matsuda, J. Cheng, D. Noble and G. Zhang (2004). A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics, 5: 56.

Yount, N. Y., J. Yuan, A. Tarver, T. Castro, G. Diamond, P. A. Tran, J. N. Levy, McCullough, J. S. Cullor, C. L. Bevins and M. E. Selsted (1999). Cloning and expression of bovine neutrophil-defensins. J. Biol. Chem., 274: 26249-26258.

Zhang, G., H. Wu, J. Shi, T. Ganz, C. R. Ross and F. Blecha (1998). Molecular cloning and tissue expression of porcine beta-defensin-1. FEBS Lett., 424: 37-40.

Zhao, C., T. Nguyen, L. Liu, O. Shamova, K. Brogden and R. I. Lehrer (1999). Differential expression of caprine β-defensins in digestive and respiratory tissues. Infection and Immunity, 67: 6221.

Table (1): Primers and PCR amplification conditions for BNBD5 and BNBD10.

Name	5'-3' Sequences	Accession no	Size (bp)
BNBD5	F: TGCTCCTCGTGCTCCTCTTC R:TGTTTGCCTTCTTTTACCACCT	AJ278799	192
BNBD10	F: CTCCTGCTCCTCTTGGTGG R: CCTGCAGCATTTTACTCGG	AJ567990 AJ567991	165

Table (2): CLUSTAL W (1.83) multiple sequence alignment of Frisian crossbred (F) and native cattle (N) and of *Bos taurus* (AF014108) BNBD5.

Seq A	Name	Len (nt)	Seq B	Name	Len (nt)	Score
1	BNDB5 (F)	161	2	BNDB5 (N)	161	64
1	BNDB5 (F)	161	3	AF014108	167	77
2	BNDB5 (N)	161	3	AF014108	167	67

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BNDB5Frisian      ----------------------TTCCCCTCGTGCTCCTCTTCCCAGGCCCGTCTGCTGGG 38
AF014108Bos       GCCAGCATGAGGCTCCATCACCTGCTCCTCGTGCTCCTCTTCCTAGTCCTGTCTGCTGGG 60
BNDB5Native       ----------------------TGCTCCTCGTGCTCCTCCTCCTGGCCCTGTTTGTTGAG 38
                                        * * ************ ***  * ** ** ** ** * *


BNDB5Frisian      TCAGGATTTACTCAAATACTAAGGA-TCCTCAAAGCTGCCGTAGGAATATAGGCATCTGT 97
AF014108Bos       TCAGGATTTACTCAAGTAGTAAGAAATCCTCAAAGCTGCCGTTGGAATATGGGTGTCTGT 120
BNDB5Native       GCAGGATTTACTCAATGCGTAAGAA-TCCTCTGAGCTGCTGTAGGAATATAGGCATGTAT 97
                  **************    **** * *****  ****** ** ******* **  * * *

BNDB5Frisian      GT-CCGATCAAGTGCTCTGGCAACATGAGACAGATCG-CACCGTTCCTGATGCTCGATGA 155
AF014108Bos       ATTCCGATCTCGTGCCCTGGCAACATGAGACAGATTGGCACCTGTTC------------- 167
BNDB5Native       GAATAGAGCAAGTGCTCTGGAGACATGAGACAGATAACCACTGA--CAGATTGT-AATAC 154
                       ** *  **** ****  *************   ***     *

BNDB5Frisian      TGCACC- 161
AF014108Bos       ------- 
BNDB5Native       CTCACCC 161
```

Primer sequences are indicated in underlined Bold typeface.

Table (3): CLUSTAL W (1.83) multiple sequence alignment of Frisian crossbred (F) and native cattle (N) and of *Bos taurus* Exon 1 and 2 (AJ567990) BNBD10.

Seq A	Name	Len (nt)	Seq B	Name	Len (nt)	Score
1	BNDB10 (F)	136	2	BNDB5 (N)	135	93
1	BNDB10 (F)	136	3	AF014108	287	93
2	BNDB10 (N)	135	3	AF014108	287	90

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F               -------------------------CTCCTGCTCCTCTTGGTGGTCCTGTCTTCTGGGTC  35
Ex1&2BOS        CAGCATGAGGCTCCATCACCTGCTCCTCCTGCTCCTCTTGGTGGTCCTGTCTTCTGGGTC  60
N               -------------------------CTCCTGCTCCTCTTGGTGGTCCTGTCTTCTGGGTC  35
                                         ************************************

F               AGGATTTACTCAAGGAGTAAGAAGTTATCTAAGCTGCTGGGGGAATAGAGGCATCTGTCT  95
Ex1&2BOS        AGGATTTACTCAAGGA-TAAGAAGTTATCTAAGCTGCTGGGGGAATAGAGGCATCTGTTT 119
N               AGGATTTACTCAAGGAGTAAGAAGTTATCTAAGCTGCTGGGGGAATAGAGGCATCTGTCA  95
                *************** ****************************************

F               GCTGAAGCAGGTGCCCTGTACGCATGAGACAGATGGCGTCC------------------- 136
Ex1&2BOS        GCTTAA-CAGGTGCCCTGGACGCATGAGACAGATGGCACCTGTTTAGCGCCCCGAGTAAA 178
N               GCTGAAGCAGGT-TCCTGTACGCATGAGTCAGATAGAAGGG------------------- 135
                *** ** *****  **** ********* ***** *

F               ------------------------------------------------------------
Ex1&2BOS        ATGCTGCAGGTAAAAGAAGGTGAAGATGCGGCCGGACCGATGCGGAGAGAAACTGGACCC 238
N               ------------------------------------------------------------

F               -------------------------------------------------
Ex1&2BOS        TTTGACAGAGCGTCTAAAATTTAAACCAGAATAAATTTTGTTCAAAGTT 287
N               -------------------------------------------------
```

Primer sequences are indicated in underlined Bold typeface.

Table (4): CLUSTAL W (1.83) multiple sequence alignment of Frisian crossbred (F) and native cattle (N) and of *Bos taurus* BNBD5 amino acid sequences.

Seq A	Name	Len (aa)	Seq B	Name	Len (aa)	Score
1	BNDB5 (N)	49	2	BNDB5 (F)	51	24
1	BNDB5 (N)	49	3	BNDB5 *Bos taurus*	54	26
2	BNDB5 (F)	51	3	BNDB5 *Bos taurus*	54	27

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BNDB5(F)          ------PLVLLFPGPSAGSGFTQILRILKAAVGIASVSDQVLWQHETDRTVPDARCT--- 51
BNDB5 Bos taurus  MRLHHLLLVLLFLVLSAGSGFTQVVRNPQSCRWNMGVCIPISCPGNMRQIG---TCS--- 54
BNDB5(N)          ------LLVLLLLALFVEAGFTQCVRIL-AAVGIACMNRASALETDRPLTD----CNTSP 49
                        ****        ****  *                                *
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Table (5): CLUSTAL W (1.83) multiple sequence alignment of Frisian crossbred (F) and native cattle (N) and of *Bos taurus* BNBD10 amino acid sequences.

Seq A	Name	Len (aa)	Seq B	Name	Len (aa)	Score
1	BNDB10 (F)	46	2	BNDB10 (F)	45	80
1	BNDB10 (F)	46	3	BNDB5 *Bos taurus*	62	73
2	BNDB10 (N)	45	3	BNDB5 *Bos taurus*	62	82

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BNBD10(N)         -------LLLLLVVLSSGSGFTQGVRSYLSCWGNRGICQLKQVPVRMSQIEG-------- 45
BNBD10 Bos taurus MRLHHLLLLLLLVVLSSGSGFTQGVRSYLSCWGNRGICLLNRCPGRMRQIGTCLAPRVKC 60
BNBD10(F)         -------LLLLLVVLSSGSGFTQGVRSYLSCWGNRGICLLKQVPCTHETDGVX------- 46
                         ***************************** *   *

BNBD10(N)         --
BNBD10 Bos taurus CR 62
BNBD10(F)         --
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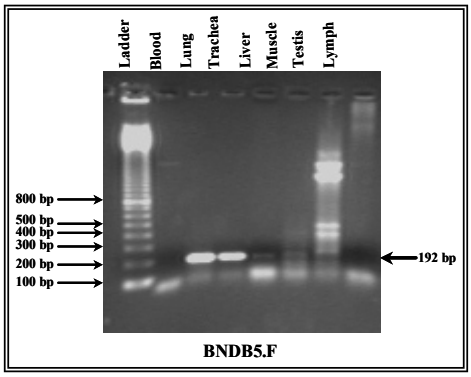

Fig. (1): Amplified PCR products of BNBD5 in different tissues of Frisian crossbred cattle (F) cDNA. L-100 bp: Ladder.

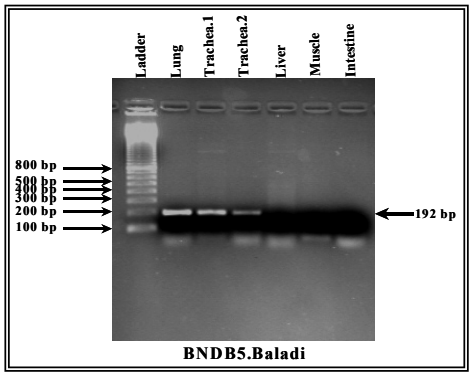

Fig. (2): Amplified PCR products of BNBD5 in different tissues of native (Baladi) cattle cDNA. L-100 bp: Ladder.

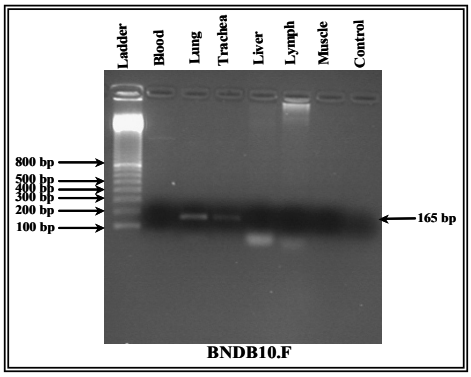

Fig. (3): Amplified PCR products of BNBD10 in different tissues of Frisian cross-bred cattle (F) cDNA. L-100 bp: Ladder.

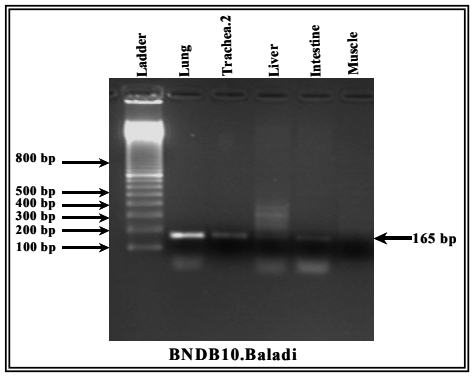

Fig. (4): Amplified PCR products of BNBD10 in different tissues of native (Baladi) cattle cDNA. L-100 bp: Ladder.

BNBD5 (Crossbred Frisian)

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     3 cccctcgtgctcctcttcccaggcccgtctgctgggtcaggatttactcaaatactaagg
       P  L  V  L  L  F  P  G  P  S  A  G  S  G  F  T  Q  I  L  R
    63 atcctcaaagctgccgtaggaata**tag**gcatctgtgtccgatcaagtgctctggcaacat
       I  L  K  A  A  V  G  I  *  A  S  V  S  D  Q  V  L  W  Q  H
   123 gagacagatcgcaccgttcctgatgctcga**tga**tgcacc 161
       E  T  D  R  T  V  P  D  A  R  *  C  T
```

BNBD5 (Native)

```
     3 ctcctcgtgctcctcctcctggccctgtttgttgaggcaggatttactcaatgcgtaaga
       L  L  V  L  L  L  L  A  L  F  V  E  A  G  F  T  Q  C  V  R
    63 atcctc**tga**gctgctgtaggaata**tag**gcatgtatgaatagagcaagtgctctggagaca
       I  L  *  A  A  V  G  I  *  A  C  M  N  R  A  S  A  L  E  T
   123 **tga**gacaga**taa**ccactgacagattgtaatacctcaccc 161
       *  D  R  *  P  L  T  D  C  N  T  S  P
```

Fig. (5): The reverse complement nucleotide sequences of the downstream strand of BNBD5 amplified fragment of Frisian crossbred and native cattle cDNA and their translated aa F+3 sequence. Asterisks denote the stop codons and are in bold typeface.

```
BNBD10  (Crossbred Frisian)
      1 ctcctgctcctcttggtggtcctgtcttctgggtcaggatttactcaaggagtaagaagt
        L  L  L  L  L  V  V  L  S  S  G  S  G  F  T  Q  G  V  R  S
     61 tatctaagctgctgggggaatagaggcatctgtctgctgaagcaggtgccctgtacgcat
        Y  L  S  C  W  G  N  R  G  I  C  L  L  K  Q  V  P  C  T  H
    121 gagacagatggcgtcc 136
        E  T  D  G  V

BNBD10  (Native)
      1 ctcctgctcctcttggtggtcctgtcttctgggtcaggatttactcaaggagtaagaagt
        L  L  L  L  L  V  V  L  S  S  G  S  G  F  T  Q  G  V  R  S
     61 tatctaagctgctgggggaatagaggcatctgtcagctgaagcaggttcctgtacgcatg
        Y  L  S  C  W  G  N  R  G  I  C  Q  L  K  Q  V  P  V  R  M
    121 agtcagatagaaggg 135
        S  Q  I  E  G
```

Fig. (6): The reverse complement nucleotide sequence of the downstream strand of BNBD10 amplified fragment of Frisian crossbred and native cattle cDNA and its translated aa F+1 sequence.