Egyptian Journal Of

GENETICS AND CYTOLOGY

INTERNATIONAL JOURNAL DEVOTED TO GENETICAL AND CYTOLOGICAL SCIENCES

Published by

THE EGYPTIAN SOCIETY OF GENETICS

Volume 37 **July 2008** **No. 2**

DETECTION OF SPLICE FORMS IN TWO ß- DEFENSINS IN EGYPTIAN NATIVE AND FRISIAN CROSSBRED CATTLE cDNA SEQUENCES

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Defensins are cationic and cysteine-rich peptides that play a crucial role in many organisms defense against microorganisms by their capability to permeabilize bacterial membranes (Bauer *et al.,* 2001). Defensins are one of the two most common types of antimicrobial peptides (Defensins & Cathelicidins). Ganz *et al*. (1985) and Selsted *et al*. (1985) were the first to use the term 'defensin' to refer to three short peptides with antimicrobial activity isolated from human neutrophil granules. Since then, a large number of defensins have been identified in many organisms. Over 360 entries had been recorded in a defensin database (http://defensins.bii.a-star.edu.sg/). Among vertebrates, numerous defensin genes have been detected, but their evolutionary background is under discussion (Luenser *et al.,* 2005). β-defensins coding sequences consist of two exons. The first exon includes the 5′-untranslated region and encodes the leader domain of the preproprotein; the second exon encodes the mature peptide with the six-cysteine domain (Brian *et al*., 2002). The low sequence similarity among the members of the large mammalian ß-defensin family suggests that their antimicrobial activity is largely independent of their primary structure

(Bauer *et al.,* 2001). β-defensins in bovine are encoded by a large gene family expressed in a wide variety of tissues (Gallagher *et al*., 1995). The cystine motif in bovine neutrophil β-defensin 12 (BNBD-12) differs from that of classical defensins indicating that the beta-defensins and defensins must have different folded chains, though they share several functional properties (Tang and Selsted, 1993).In eukaryotic genes, the introns have to be spliced out in order to form a continuous coding sequence (mRNA) that can be recognized by the translation machinery. In principle, an intron contains all the necessary information to be spliced out, which enables it to function independently from the exon sequence (de Roos, 2005)**.** Analysis of alternative splicing (AS) using bioinformatics has emerged as an important field, and has significantly changed opinions regarding genome function (Lee *et al*., 2003). AS plays a major role in genome evolution allowing new exons to evolve with less constraint (Boue *et al.,* 2003). Approximately 70-88% of AS events result in changes in the encoded protein (Modrek *et al.,* 2001; Kan *et al*., 2002). Processes such as alternative use of promoters, splice sites, translational start sites and translational termination codons can serve as mechanisms for regulating AS (Kurlender *et al*., 2005). AS may generate variable segments of mRNA that can insert or remove amino acids, shift the reading frame, or introduce a termination codon (Faustino and Cooper, 2003). A large fraction of AS events are regulated in a cell- or tissue-specific manner in which the splicing pathways are modulated according to cell-type, developmental stage, gender, or in response to external stimuli (Savkur *et al***.,** 2003). The enormous amount of phenotypic diversity among different phyla or classes of organisms is a product of accumulation of novel mutations and their conservation that have facilitated adaptation to different environments. It appears that the driving force of phenotypic evolution is mutation, and natural selection is of secondary importance (Nei, 2007). Based on the current knowledge of defensin structure, it is expected that extensive mutational studies will help to establish a structure-function relationship and to provide a molecular basis for the development of potential new drug therapies for the treatment of infectious diseases based on defensins (Bauer *et al.,* 2001). This study aims at studying bovine neutrophil β-defensin 11 and 12 (BNBD-11 & BNBD-12) in both Egyptian native and Frisian crossbred cattle.

MATERIALS AND METHODS

Materials

Tissue samples were obtained from healthy Egyptian native and Frisian crossbred cattle at the slaughter house.

RNA isolation of the recovery of the first strand cDNA

Different tissues of native and crossbred cattle were tested for expression of BNBD-11 & BNBD-12. Total RNAs

were isolated from native and crossbred cattle frozen tissues using Trifast reagent (Peqlab, biotechnologie GmbH) according to the manufacturer's specifications. First strand cDNA synthesis has been conducted using READY TO GO YOU Prime-First Strand Beads kit (Amersham). The first-strand cDNA synthesis was done on 2 µg of total RNA using reverse transcriptase (FPLC pure) and the oligo (dT) primer, pd (T) 12-18 (Amersham Biosciences).

PCR

Primers specific for BNBD-11 & BNBD-12 genes were designed using known cDNA sequences of *Bos Taurus* BNBD-11 & BNBD-12 cDNA published sequences. The sequence of the forward and reverse primers was determined using the software Primer 3 (http://www.genome.wi.mit.edu). PCR primers were selected on the basis that the 5' and 3' ends span exons I and II, so that the amplification product obtained from the cDNA would be of different length from that obtained from genomic DNA which comprises intronic sequence. The primers were synthesized by Amersham Pharmacia Biotech.

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 $MgCl_2$, 0.01% gelatin (W/V), 1.25 units *Taq* polymerase and 1 µM upper and lower primers. Cycling parameters were set as follows: one cycle of preheating at 94°C for 3 min and 30 cycles, each of denaturation at 94°C for 1 min., annealing at the temperature specific for each primer for 2 min and extension at 72°C for 2 min. A final extension step of 10 min at 72°C was performed in order to complete the PCR reaction. Primers sequences, annealing temperatures and accession numbers, are shown in Table (1). PCR products were resolved on a 1.5% agarose gel. The gels were examined with a UV lamp at a wave length 312 nm and photographed using $MP4^+$ Polaroid Camera. No PCR products were detected in the absence of reverse transcriptase, which indicates the lack of contaminating genomic DNA.

The PCR trachea-products were purified and sequenced at the Center of Genetic Engineering; Ain Shams University; Cairo; Egypt. Nucleotide sequencing has been performed using ABI PRISM version 3.7. Alignment analysis of the nucleotide sequences were carried out using CLUSTAL-W (Gasteiger *et al.*, 2003). Six frame translation of nucleotide sequence was carried out using http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html. The protein alignment was carried out using CLUSTAL-W analysis (http://www.ebi.ac.uk/Tools/clustalw/index.html).

RESULTS

In the present study, cDNA of different tissues of native and crossbred cattle were tested for expression of BNBD-11 & BNBD-12 genes. Primers were determined so that the 5' & 3' ends

span exon-I and exon-II. Amplified products were 164 &163 bp for BNBD-11 & BNBD-12, respectively, in all tested cDNAs (Figs. 1-4).

Trachea-amplified fragments were chosen for sequencing. The reverse complement nucleotide sequence of the downstream strand of BNBD-11 (146bp & 135 bp) and BNBD-12 (146 bp & 161 bp) amplicons for native and crossbred cattle, respectively, are presented in Figs. (5 & 6).

CLUSTAL-W analysis revealed that native and crossbred cattle trachea BNBD-11 cDNA sequences have 85% identity. Native cattle BNBD-11 cDNA sequence was aligned with *Bos taurus* BNBD-11 (183 nucleotides, exon I-II) PA: |AJ567992.1|. The results showed 66% identity and the presence of 12 nucleotides (from 41-52) in native cattle which were absent in *Bos taurus***.** In order to identify the nature of these 12 nucleotides, we examined *Bos taurus* BNBD-11 exon-I (gi|55416133|emb| AJ567992.1|) & exon-II (gi|55415755| emb|AJ567993.1|), where both have partial sequence of intron-I. We found that the 12 nucleotides of intron-I which precede exon-II in *Bos taurus* correspond to those reported in native cattle (Fig. 5).

The same results were found when crossbred cattle BNBD-11 cDNA sequence was aligned with *Bos taurus* BNBD-11 (183 nucleotides, exon I-II) PA: |AJ567992.1|. The results showed 72% identity and the presence of the 12 nucleotides reported earlier in native cattle. However the crossbred cattle nucleotide segment showed one deletion and one substitution compared to native cattle (Fig. 5). Few deletions and several substitutions were detected in both native and crossbred cattle BNBD-11 cDNA sequences compared to *Bos taurus* BNBD-11 (183 nucleotides, exon I-II) PA: |AJ567992.1|.

CLUSTAL-W analysis between native and crossbred cattle BNBD-12 cDNA showed 65% identities. Crossbred cattle has 15 nt insertion from 50-64 nt compared to native cattle (Fig. 6). In order to study the differences between native and crossbred cattle sequences, which both include partial exon-I and partial exon-II, and the corresponding mRNA sequence of *Bos taurus* BNBD-12, we combined *Bos taurus* BNBD-12 sequences of exon-I and exon-II deducted from gi|5410321|emb| AF105370.1| and gi|5410322|emb| AF105371.1|, respectively, since there was no published segment that has both exon-I and exon-II as in case of BNBD-11. The combined sequence is heretofore called "*Bos taurus* BNBD-12 exon-I&II".

Native and crossbred cattle BNBD-12 cDNA sequences were aligned with "*Bos taurus* BNBD-12 exon-I&II". Alignment of native BNBD-12 cDNA sequence with *"Bos taurus* BNBD-12 exon-I&II" revealed 64% identity and the presence of 12 nt in native sequence not present in *"Bos taurus* BNBD-12 exon-I&II". When *Bos taurus* BNBD-12 exon-I

(gi|5410321|emb| AF105370.1|) and exon-II (gi|5410322|emb| AF105371.1|) {which both have partial intron-I} were investigated, these 12 nt were found to be part of intron-I immediately preceding exon-II with some mutations (Fig. 6). The above mentioned extra 12 nt present in native BNBD-12 cDNA were also found to be present in crossbred cattle with some mutations. The 15nt insertion detected in crossbred cattle resides within the 12 nt (Fig. 6). A 62% identity was found between crossbred (excluding the 15nt) cattle and *"Bos taurus* BNBD-12 exon-I&II". Several deletions and substitutions were also found in both native and crossbred BNBD-12 cDNA cattle sequences compared to *"Bos taurus* BNBD-12 exon-I&II ".

The six frame translation of amino acid (aa) sequences of both native and crossbred cattle BNBD-11& 12 cDNAs were carried out. The results showed that "aa Frame +1" should be used for both native and crossbred cattle BNBD-11 cDNA, whereas "aa Frame +2" should be used for both native and crossbred cattle BNBD-12 cDNA. This was detected by aligning both native and crossbred cattle BNBD-11 & 12 cDNAs with the corresponding *Bos taurus* BNBD-11 exon-I (gi|55416133|emb|AJ567992.1|) & exon-II (gi|55415755|emb|AJ567993.1|) and BNBD-12 exon-I (gi|5410321| emb|AF105370.1|) & exon-II (gi|5410322| emb|AF105371.1|).

Native and crossbred cattle trachea BNBD-11 cDNA translated aa sequences showed 36% & 25% identities with *Bos taurus* BNBD-11 exon-I & exon-II amino acid sequences, respectively. Whereas for BNBD-12 they showed 33% and 42% identities with *Bos taurus* exon-I & exon-II, respectively.

Six internal stop codons (4 TAA, TAG and TGA) were detected in BNBD-11 cDNA (Fig. 5) and three internal stop codons (TAA, 2 TGA one of them inside the 15 bp) in BNBD-12 cDNA translated aa sequences in crossbred cattle (Fig. 6). No stop codons were detected in BNBD-11 or BNBD-12 cDNA translated amino acid sequence in native cattle (Figs 5 & 6).

DISCUSSION

In the present study, BNBD-11 and BNBD-12 genes have been expressed in several tissues in native and crossbred cattle. The presence of β-defensin in different tissues has been reported earlier (Ogata *et al*., 1992; Gallagher *et al*., 1995; Ganz, 2003; Das *et al*., 2007).

Native and crossbred cattle trachea BNBD-12 cDNAs showed 65% identities. The analysis showed insertion of 15 bp (from 50 to 64nt) in the sequence of crossbred cattle compared to native cattle sequences. This insertion may be of little consequence, since inserts that are evenly divisible by three does not cause frame shift (Taylor *et al.,* 2004) or disrupt the grouping of the codons, however it will result in insertion of five aa in the encoded protein.

CLUSTAL-W analysis revealed that the sequence of native and crossbred cattle trachea BNBD-11 cDNA sequences showed identities of 66% for native and 72% for crossbred cattle compared to *Bos taurus* BNBD-11 (183nt, exon I-II) PA: |AJ567992.1|. Whereas native and crossbred cattle trachea BNBD-12 cDNA sequences showed identities of 64% for native and 62% for crossbred (excluding the 15nt) compared to *" Bos taurus* BNBD-12 exon-I&II *"*. The low percent identities may indicate that native and crossbred cattle trachea BNBD-11& 12 cDNA sequences are alternatively spliced, since 60% identity account for lower conservation of alternatively spliced regions, whereas for constitutive exons it is 70% (Nurtdinov *et al.*, 2003). Insertions, specially at the intron-exon border reveal AS events (Hanke *et al*., 1999; Brett *et al*., 2000; Kan *et al*., 2002; Zavolan *et al.,* 2003). The presence of 12 nt insertions immediately preceding the 5' end of exon-II as well as the presence of some mutations at the same end in native and crossbred cattle BNBD-11 and BNBD-12 cDNAs compared to *Bos taurus* corresponding sequences, confirm the presence of alternative splicing. It is worth mentioning that exon-II in β-definsins encodes the mature peptide with the six cysteine domain (Brian *et al*., 2002).

The mutation reported in native and crossbred cattle, inside the exon or along the flanking intron, results in the creation of a new splice site that competes with the original one, leading to alternative splice site selection (Koren *et al*., 2007). Intron-retention and alternative splicing have been reported earlier in native and Frisian crossbred cattle Cathelicidin-4 (CATHL-4) (Abou Mossallam *et al.*, 2007) and in native river buffalo (*Bubalus bubalis*) (Abou Mossallam, unpublished data).

The translated amino acid sequences of both native and crossbred cattle trachea BNBD-11 and BNBD-12 cDNAs was found to be different in the coding region than the translated amino acid sequences reported in *Bos taurus* BNBD-11 & 12, which is due to alternative splicing and mutations. It has been reported that about 80% of AS results in changes in the encoded protein of human genes (Modrek and Lee, 2002) and up to 59% of the human genes generate multiple mRNAs by alternative splicing (Lander *et al,*. 2001). AS often creates novel isoforms by the insertion of new, functional protein sequences that probably originated from noncoding sequences of introns (Kondrashov and Koonin, 2003). AS is predicted to affect diverse functions including: gene expression, signal transduction, cell death, immune defense, and susceptibility to diseases (Calarco *et al.,* 2007).

Numerous mutations were observed in both native and crossbred cattle trachea BNBD-11 & 12 cDNAs. Nucleotide substitutions can have a relatively minor effect on the sequence of amino acids since only one codon is altered in the mRNA. However deletions

and insertions can have deleterious effects. Insertions and deletions (indels), together with substitutions, provide the raw material for evolutionary change in gene sequences (Soding and Lupas, 2003; Taylor *et al*., 2004). In crossbred cattle BNBD-11 cDNA, the three detected single deletions caused frame shifts and formed six internal stop codons (termination codons). Three internal stop codons were also formed in crossbred cattle BNBD-12 cDNA, whereas no stop codons were detected in BNBD-11 and BNBD-12 cDNAs of native cattle. Stop codons may act on the amino acid chain to stop growing prematurely, resulting in a truncated protein which is typically nonfunctional, leading to insufficiency and in some cases to disease (Carnes *et al.,* 2003). The absence of stop codons within the coding region in native cattle may be the cause of their higher resistance to diseases when compared to crossbred ones.

SUMMARY

Bovine neutrophil β-defensin-11 (BNBD-11) and β-defensin-12 (BNBD-12) were investigated in Egyptian native and Frisian crossbred cattle. BNBD-11 and BNBD-12 genes have been expressed in several tissues of both breeds. CLUSTAL-W analysis revealed that native and crossbred cattle trachea BNBD-11 and BNBD-12 cDNA sequences have 85% and 65% identity, respectively. Crossbred cattle BNBD-12 showed an insertion of 15 nt compared to native cattle. Sequence alignment of native and crossbred cattle trachea BNBD-11 cDNA showed 66% identities in native and 72% in crossbred cattle compared to *Bos taurus* BNBD-11 (183 nt, exon I-II), PA: |AJ567992.1|. Whereas native and crossbred cattle trachea BNBD-12 cDNA sequences showed 64% identities in native and 62% in crossbred (excluding 15nt) cattle compared to "*Bos taurus* BNBD-12 exon I & II". Alternative splicing of native and crossbred cattle trachea BNBD-11 & 12 cDNA sequences is confirmed by the presence of the 12nt (which are absent in *Bos taurus*) at 3' end of intron-I sequence immediately preceding the 5' end of exon-II. The translated amino acid sequences of both native and crossbred cattle trachea BNBD-11 and BNBD-12 cDNAs were found to be different from those reported in *Bos taurus* BNBD-11& 12. Several internal stop codons were found in crossbred cattle BNBD-11& 12 cDNA, whereas no stop codons were noticed in native cattle BNBD-11 or BNBD-12. These stop codons may act on the amino acid chain to stop growing prematurely, resulting in the production of a defective protein. The absence of stop codons within the coding region in native cattle may be the cause of their higher resistance to diseases when compared to crossbred ones.

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Table (1): Sequences, accession numbers and annealing temperatures of primers tested.

Name	Sequence	Accession no.	Exon no.	Ann. Temp.
Bovine neutrophil β-defensin 11 (BNBD-11)	F: CTCCTCGCGCTCCTCTTCC R:TACCACGACCTGCAGCATTTTAC	AJ567992.1 AJ567993.1	Exon-I Exon-II	61.5°C
Bovine neutrophil β-defensin 12 (BNBD-12)	F:GCTCCTCGCGCTCCTCTTC R:CCACGACCTGCAGCATTTTAC	AF105370.1 AF105371.1	Exon-I Exon-II	61.5°C

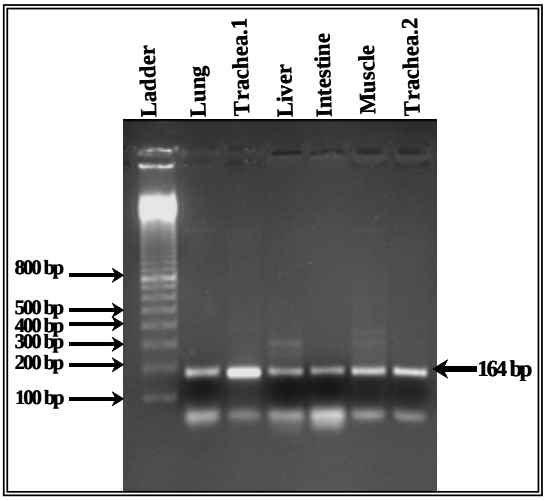

Fig. (1): Amplified PCR products of BNBD-11 in different tissues of native cattle cDNA. Ladder: 100 bp (Amersham).

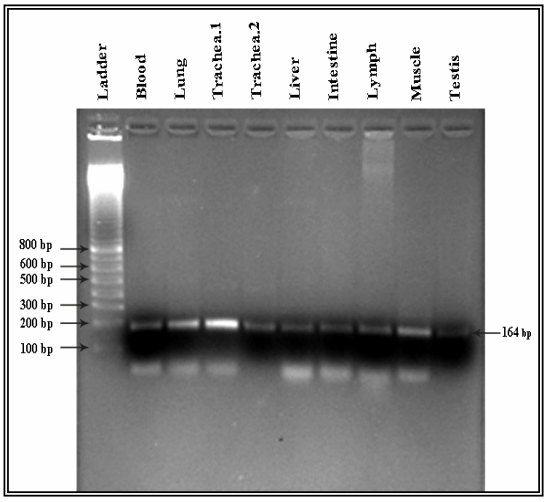

Fig. (2): Amplified PCR products of BNBD-11 in different tissues of Frisian crossbred cattle cDNA. Ladder: 100 bp (Amersham).

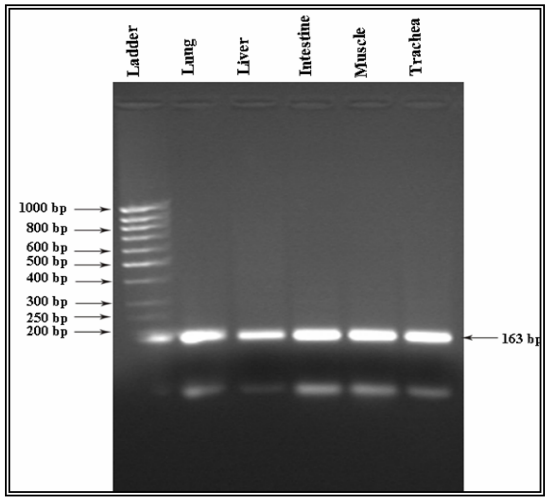

Fig. (3): Amplified PCR products of BNBD-12 in different tissues of native cattle cDNA. Ladder: 50 bp (Amersham).

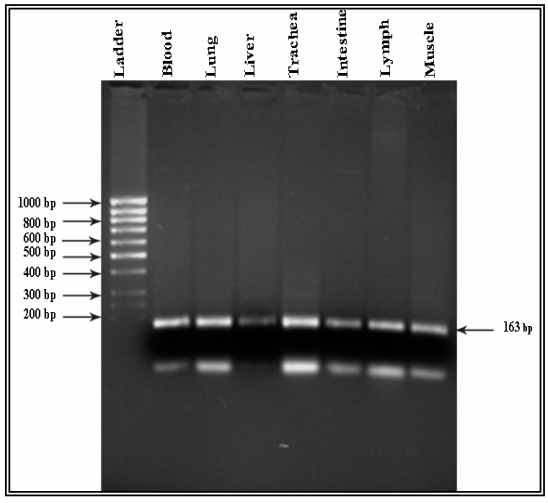

Fig. (4): Amplified PCR products of BNBD-12 in different tissues of Frisian crossbred cattle cDNA. Ladder: 50 bp (Amersham).

Native cattle BNBD-11

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  1  cccctcgcgcttctcttcctggtcctgtttgcttggtcagGATTTACTCAAGgagtaaga
     P  L  A  L  L  F  L  V  L  F  A  W  S  G  F  T  Q  G  V  R
 61  aatcctctaagctgtggtaggaataaaggcatctgtgtgccgatcaagtgctctgtaaac
     N  P  L  S  C  G  R  N  K  G  I  C  V  P  I  K  C  S  V  N
121  atgagtcagagcgtagccaaccctcc
146  M  S  Q  S  V  A  N  P
```

Frisian crossbred cattle BNBD-11

```
  1  ctcctcctcctggtcctgtctcctgggtcagGACTACTCAAGgagTAAgaaatcctcTAA
     L  L  L  L  V  L  S  P  G  S  G  L  L  K  E  *  E  I  L  *
 61  cctccgTAGgaaTAAaggcatctgtgtgccgatcaggtgcccTGAcgcatgagtcagatc
     P  P  *  E  *  R  H  L  C  A  D  Q  V  P  *  R  M  S  Q  I
121  gtagccTAAccaacc
135  V  A  *  P  T
```

Bos taurus BNBD-11 exon-II (gi|55415755|emb|AJ567993.1|)

```
  1 tgactcctgg cggaaggtgg atgtagcaga gcttcccagt ctttgcctca tggtggagct
 61 gacctgcaca caaccggggt ctcacacccc agtcctcagc ctgtggttct gaaaacgtga
121 gggtccacca gagcctgggc acggtcagtg tctgtctgga agctggtcta cggggtcctg
181 gactcatatc ttgttgtcat gaggccgtgt gcgcatctca gcacagaaag ccccagggcc
241 tcgctcagag gggacacagt gggccttctc gagatgccgc tttcctgctg acacgttttt
301 ccctttttgt tctcttttta tGATTTACTC AAGgaataag tggtcctcta agctgccgta
361 ggaatggagg cgtctgtatt ccgatcaggt gccctggacc catgagacag attggcacct
421 gtttcgggcg cccagtaaaa tgctgcaggt cgtggtagaa gaaggcgaag atgcggccgg
481 gaccgatgcg gagacagaaa ctgtgcccct cgacagaacg tctaaaattt aaaccagaat
541 aaattttgtt caaagtt
```

Fig. (5): The reverse complement nucleotide sequences of the downstream strand of BNBD-11 amplified fragment of native and Frisian crossbred cattle trachea cDNA & *Bos taurus* BNBD-11 exon-II (from 334 to 543). The 12nt-insertion is underlined and in bold typeface. The translated amino acids are indicated in capital letters. Asterisks denote the stop codons (in Bold).

Native cattle BNBD12

```
  2 ccccccccgctccttctcccgggcccgtttggttggtcaggaatttctcaaagagttggg
    P  P  P  L  L  L  P  G  P  F  G  W  S  G  I  S  Q  R  V  G
 62 aatcctctaagctgtggtaggaatagaggcatctgtgtgccgatcaagtgttctgaaatg
    N  P  L  S  C  G  R  N  R  G  I  C  V  P  I  K  C  S  E  M
122 cgagacatgagtgtcccacttgccc 146
    R  D  M  S  V  P  L  A
```

Frisian crossbred cattle BNBD12

```
  2 ctcctcgtgctcctgctcctggccctgcttttgaggcaggatttgctggatccggatga
    L  L  V  L  L  L  L  A  L  L  F  E  A  G  F  A  G  S  G  *
 62 atcctaggagctagagttggtatatccgcaggtaggaatagaggcatctgtgtgccgacc
    I  L  G  A  R  V  G  I  S  A  G  R  N  R  G  I  C  V  P  T
122 aagtgcacataaccacgagacagattgtcatgacctctcc
161 K  C  T  *  P  R  D  R  L  S  *  P  L
```

Bos taurus BNBD12 exon-II (gi|5410322|emb| AF105371.1|).

```
  1 gggtcagtgt ctgtctggaa gctggtccta cggggtcctg gactcatatc ttgttgtcat
 61 gagtctgtgt gtgcatctca gcacagaagc cccagggcct cgctcagagg ggacacagtg
121 ggccttctcg agatgccgct ttcctgctga cacgtttttc cctctttgtt ctcttttat
181 gatttactca aggaataagt ggtcctctaa gctgtggtag gaatggaggc gtctgtattc
241 cgatcaggtg ccctgtaccc atgagacaga ttggcacctg tttcgggcgc ccagtaaaat
301 gctgcaggtc gtggtagaag aaggcgaaga tgcggccggg acctacgcgg agacagaaac
361 tgtgcccttc gacagaacgt ctaaaattta aaccagaata aattttgttc aaagttaaag
421 aatcttgccc actggtcatt gaggttgttg tgtggtgtct gatcccaggc gaattcg
```

Fig. (6): The reverse complement nucleotide sequences of the downstream strand of BNBD-12 amplified fragment of native and Frisian crossbred cattle trachea cDNA & *Bos taurus* BNBD-12 exon-II (from 193 to 314). The 12nt- insertion is underlined and in bold typeface. 15nt in Frisian crossbred cattle are indicated in underlined italics. The translated amino acids are indicated in capital letters. Asterisks denote the stop codons (in Bold).