Identification and sequence analysis of two antimicrobial peptides in Egyptian native and crossbred frisian cattle

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ABSTRACT

Two antimicrobial peptides namely bovine tracheal antimicrobial peptide (TAP) and bovine neutrophil β-defensin 4 (BNBD4) were investigated in crossbred Frisian and Egyptian native cattle. TAP and BNBD4 were tested for reaction with cDNA of various cattle tissues such as liver, lung, trachea, intestine, mammary gland, colon and blood using PCR. TAP and BNBD4 were positive in all tissues of the native cattle whereas in crossbred Frisian only the trachea PCR- product sequence was positive with both. NCBI-Blast analysis of TAP and BNBD4 sequences showed 89% and 92% alignment between crossbred Frisian and native cattle, respectively. Blast analysis of TAP PCR-product sequences of both native and crossbred Frisian cattle with GenBank published sequences revealed sequence homology between both native and crossbred Frisian cattle and Bos taurus TAP mRNA. Analysis also revealed homology (90%) between crossbred Frisian TAP and Bos taurus lingual antimicrobial peptide (LAP), whereas native cattle TAP was homologous (86%) to Bubalus bubalis LAP. Blast analysis of BNBD4 PCR-products sequence revealed sequence homology between both native and crossbred Frisian cattle and Bos taurus BNBD4 mRNA. Analysis also revealed homology with Bubalus bubalis enteric beta-defensin preproprotein mRNA, however the native cattle showed higher homology (128 nucleotide shared) than the crossbred Frisian where only 62 nucleotides were shared. The presence of TAP and BNBD4 in all tested tissues of native cattle may reflect a higher tendency towards disease resistance in native cattle compared to the crossbred Frisian ones. Further studies on the expression of these genes in different organs of the native cattle will shed more light on their disease resistance.

Key words: *Antimicrobial peptides- TAP- BNBD4- cattle breed.*

INTRODUCTION

Disease is one of the major factors contributing to poor livestock productivity in developing countries. The innate immune system is the first line of defense against pathogenic insult, which is followed by acquired immune responses associated with the activation of T and B cells aimed against specific antigens. Essential to innate immunity are receptors that recognize colonizing or invading microorganisms and initiate a host defense reaction (Fearon and Locksley, 1996; Medzhitov and Janeway, 2000).

Antimicrobial peptides are effector molecules of innate immunity with direct antimicrobial and mediator function. They are an endogenous part of the innate immune

system of vertebrates, insects and plants. Antimicrobial peptides of various families differ in size, amino acid sequence and certain structural motifs. Defensins, a class of antimicrobial peptides, includes three subfamilies: α, β and ɵ defensins.

β-defensins are a component of the first line of defense against micro-organisms in the mucosal surfaces of tissues where the body interfaces with its environment. Bovine neutrophil granules contain β-defensins, a family represented by 13 cationic, tridisulfide-containing peptides containing 38-42 residues (Selsted *et al.*, 1993). They are found in nearly all epithelial tissues of vertebrates and invertebrates as well as neutrophil granulocytes macrophages (Conejo Garcia *et al.*, 2001). β-defensins have potent antibacterial activities against *Staphylococcus aureus* and *Escherichia coli in vitro*. In addition to their abundant expression in neutrophils, related β-defensins such as (tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP), enteric β-defensin (EBd) are present in the epithelium of bovine trachea (Diamond *et al.*, 1991), tongue (Schonwetter *et al.*, 1995), and intestine (Tarver *et al.*, 1998), respectively.

The genomic structure of most defensins consists of 2 exons and one intron (Lehrer and Ganz, 2002). Several homologous peptides were also purified from chicken and turkey leukocytes (Harwig *et al.*, 1994; Evans *et al.*, 1994). Members of the β-defensin gene family from sheep (Mahoney *et al.*, 1995), pig (Zhang *et al.*, 1998), and mouse and human (Huttner *et al.*, 1997; Morrison *et al.*, 1998; Yamaguchi *et al.*, 2002) and equine (Looft *et al.*, 2006) have been characterized at the DNA level.

In Egypt, as in other developing countries, an increase in supplies of livestock products is necessary to meet the growing demand from burgeoning populations and rapid urbanization. Several projects were conducted in order to increase cattle productivity. Few decades ago Frisian cattle were imported to Egypt; they were crossed with native cattle in an attempt to improve native cattle productivity.

This investigation aimed at studying bovine tracheal antimicrobial peptide (TAP) and bovine neutrophil β-defensin 4 (BNBD4) in both crossbred Frisian and Egyptian native cattle.

MATERIALS AND METHODS

Tissue and RNA preparation

Different tissue samples were obtained from healthy crossbred Frisian and native cattle at the slaughter house. They include blood, lung, trachea, intestine, mammary gland, colon and liver. RNA was extracted using Trifast isolation kit (Grubor *et al.*, 2004). cDNA was synthesized from RNA with the RT- PCR (Ready- to- go kit, GE healthcare UK) and then stored frozen.

Primer design

Primers specific for the antimicrobial peptide genes TAP and BNBD4 were designed using known cDNA sequences of *Bos taurus* published in database. The sequence of the primers was determined using the software Primer 3 at http://www.genome.wi.mit.edu (Marone *et al.*, 2001). PCR primers were selected on the basis that the 5′ and 3′ ends span the two exons, so that the amplification product obtained from the cDNA would be of different length from that obtained from any contaminant genomic DNA comprising intronic sequences. The primers were synthesized by Amersham Pharmacia Biotech.

Polymerase chain reaction (PCR)

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 TAP and BNDB4, and 2 min. at 72°C) and finally 1 cycle (10 min.) at 72°C. The reaction products were electrophoresed on 1.5% agarose gel. Primer sequences, annealing temperatures, product sizes and accession numbers are shown in Table (1).

Table (1): Sequences of TAP and BNBD4 primers.

Name	Sequence 5/ 3/	Accession No. (gi)	Ann. Temp	Size (bp)
Tracheal Antimicrobial Peptide (TAP)	ATTGATCCCGGCTGTGTCTTG CGCGCTCCTCTTCCTGGTC	2226433	60	201
Bovine Neutrophil β-Defensin 4 (BNBD4)	CCTCTTCCTGGTCCTGTCTG GTTTCTGACTCCGCATTGG	4102641	60	209

Sequence analysis

The PCR products were purified and sequenced at the Center of Genetic Engineering, Ain-Shams University, Cairo, Egypt. Sequence analysis and alignment were carried out using NCBI-BLASTN 2.2.14 version (Altschul *et al.*, 1997).

RESULTS

The presence or absence of TAP and BNBD4 in crossbred Frisian and native cattle were tested in blood, lung, trachea, intestine, mammary gland, colon and liver cDNAs prepared from total RNA extracted from the different tissues.TAP and BNBD4 cDNAs gave bands at 201 and 209 bp in crossbred Frisian and native cattle. In crossbred Frisian they were found only in trachea (Fig.1 and 2) whereas in native cattle they were present in all examined tissues (Fig. 3 and 4).

A one way sequence of TAP and BNBD4 amplified segments of crossbred Frisian and native cattle which have been submitted to DDBJ/ EMBL/ GenBank nucleotide sequences database (accession numbers AB299971 & AB299980 and AB297660 & AB297970) are presented in Figures 5 and 6 and Figures 7and 8, respectively. NCBI-Blast analysis showed 89% alignment between TAP crossbred Frisian amplicon, (from 39 to 166 bp) and native cattle TAP sequence (from 30 to 158 bp). BNBD4 crossbred Frisian amplicon (from 84 to 174 bp) showed a 92% alignment with native cattle BNBD4 (from 96 to 186 bp).

The obtained TAP and BNBD4 amplicon sequences for crossbred Frisian and native cattle were compared with published sequences in GenBank. The amplified segment of crossbred Frisian TAP cDNA revealed a 93% alignment with *Bos taurus* TAP mRNA sequences of the GenBank database (gene bank accession numbers gi: 28849942, gi|4102639|; gi|2226433| and gi|289395|). Whereas the native cattle TAP cDNA revealed 94% alignment with mRNA of *Bos taurus* TAP gi: 28849942, gi|4102639|. TAP cDNA sequence of crossbred Frisian (from 47 to 109 bp) showed 90% alignment with *Bos taurus* lingual antimicrobial peptide (LAP) mRNA (from 119 to 182 bp), accession number gi| 44681481. No alignment was found between native cattle TAP cDNA and *Bos taurus* LAP however a sequence homology of 86% (from 30 to 151 bp) was found with the *ubalus bubalis* LAP mRNA (from 101 to 222 bp); gi| 91807132 .

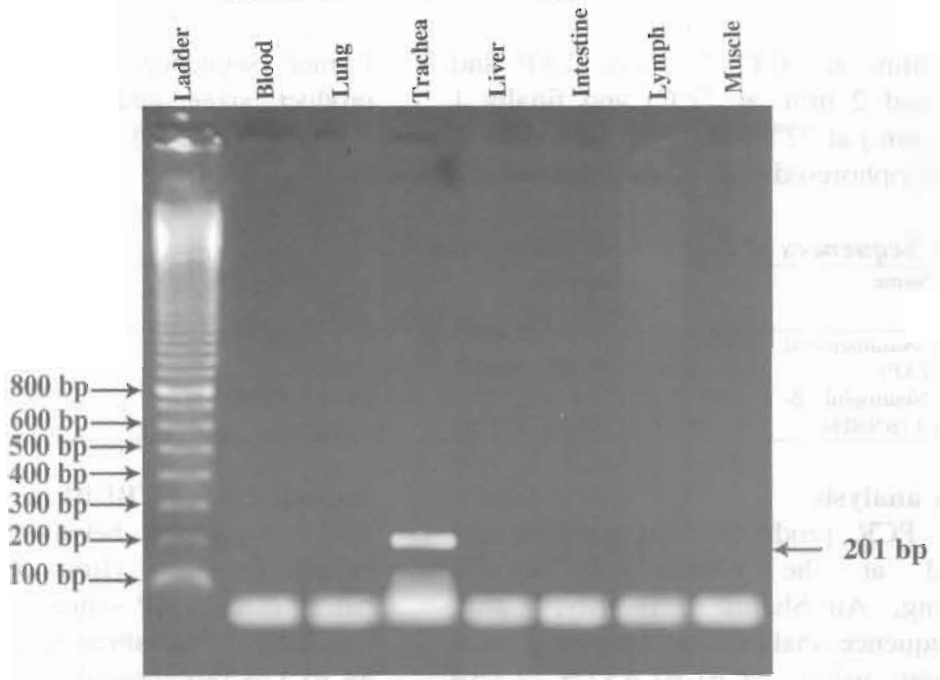

Fig.(1): Amplified PCR products of Tracheal Antimicrobial Peptide (TAP) in different tissues of crossbred Frisian cattle cDNA. Ladder (100 bp, Pharmacia).

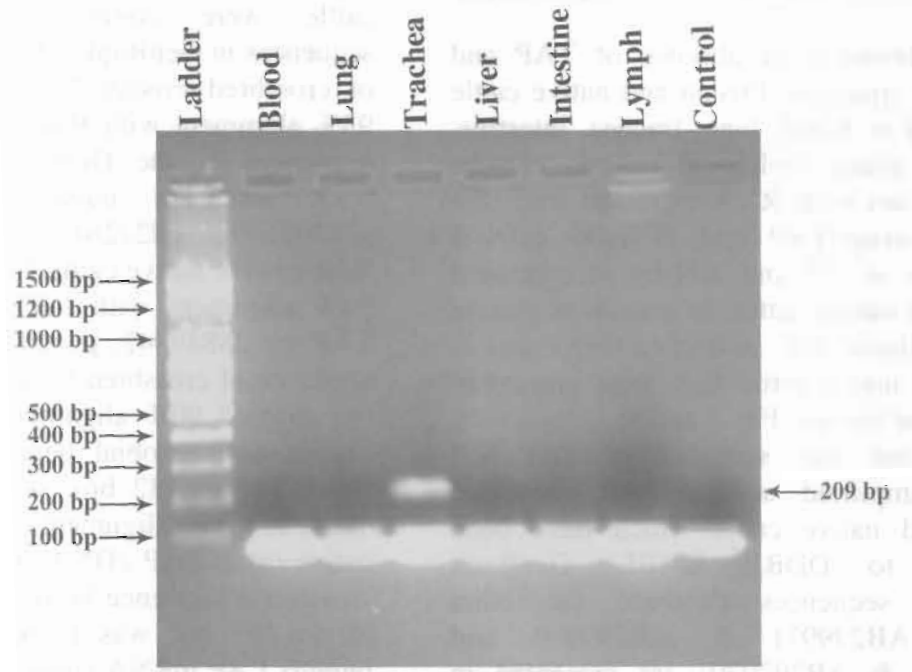

Fig. (2): Amplified PCR products of bovine Neutrophil β-Defensin 4 (BNBD4) in different issues of crossbred Frisian cattle cDNA. Ladder (100 bp, Pharmacia).

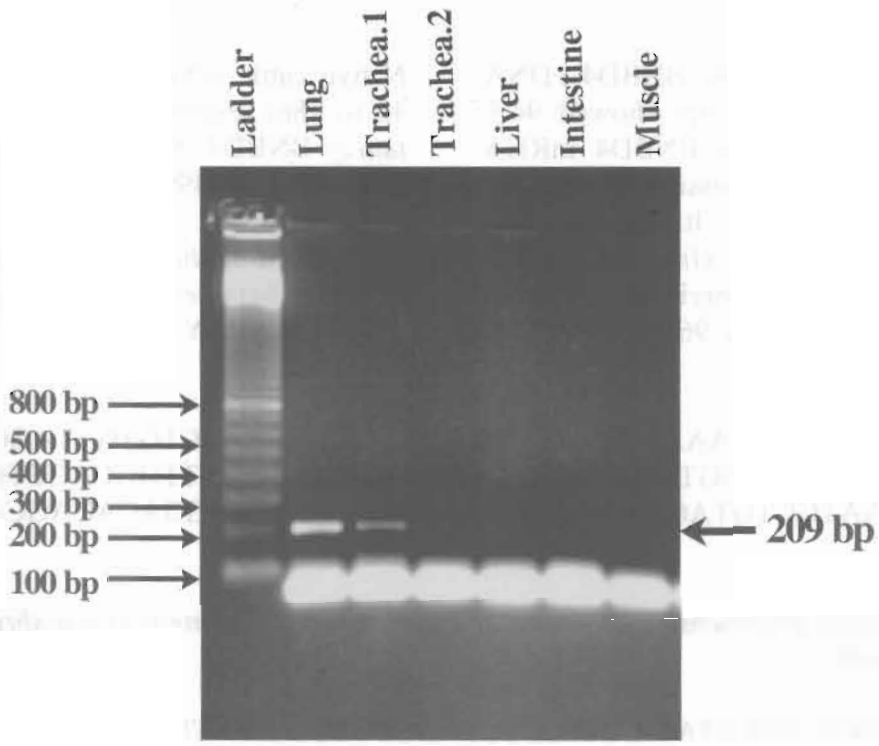

Fig.(3): Amplified PCR products of Tracheal Antimicrobial Peptide (TAP) in different tissues of native cattle cDNA. Ladder (100 bp, Pharmacia).

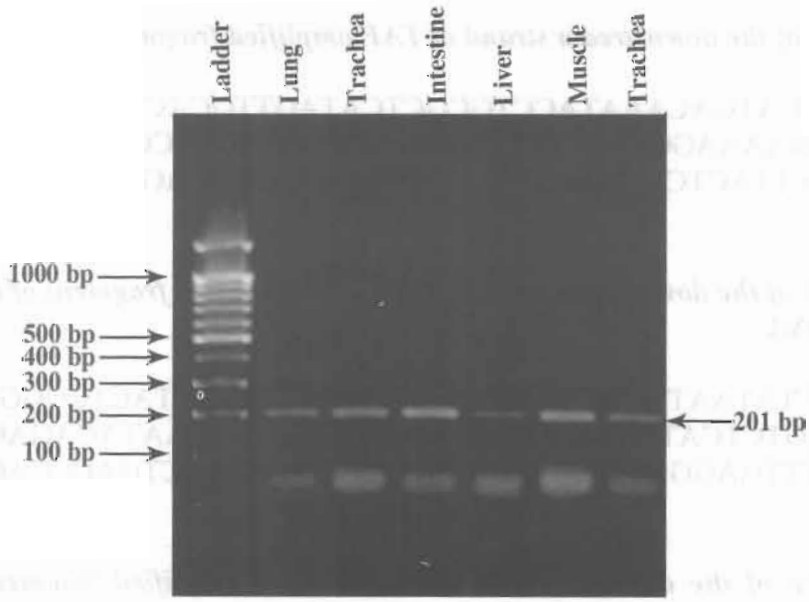

Fig.(4): Amplified PCR products of Bovine Neutrophil β-Defensin 4 (BNBD4) in different tissues of native cattle cDNA. Ladder (100 bp, Pharmacia).

Crossbred Frisian cattle BNBD4 cDNA sequence (from 83 to 175 bp) showed 94% alignment with *Bos taurus* BNBD4 mRNA (from 256 to 164 bp), accession number gi: 28849940/ref/NM 174775.1. It also showed a sequence homology of 98% (from 113 to 175 bp) with *Bubalus bubalis* enteric beta-defensin preproprotein mRNA (from 96 to 34 bp); gi: 31747506 / gb/AY301005.1.

Native cattle BNBD4 cDNA sequence (from 42 to 186) revealed 95% alignment with *Bos taurus* BNBD4 mRNA sequences (from 165-309); gi: 28849940/ref/NM 174775.1, and 88% alignment (from 58 to 186bp) with mRNA of *Bubalus bubalis* (from 161 to 35bp) enteric beta-defensin preproprotein; gi: 31747506/gb/AY 301005.1.

AAAAAACGGAATTAATAAAGGTATCCTGAAATGCTGTAAGCTGTGTTAGGAATAAAGG
CATCTGTGTGCCGATAGGTGAACAGGAAACATGAAACAGATTGGCACCTGTGTTGGCC
GGGCCGAAAAATGCTGTAGAAAGAAGTAAAAGAAGGCCAGGACACAGCCGGGATCGA
TAATTA

Fig.(5): Sequence of the downstream strand of TAP amplified fragment of crossbred Frisian cattle cDNA.

CGTCCGGATAATCAGTATATAAATCCTCTAGCTGTGGTAGGTATAAAGGCAT
CTGTGTGCCGATCTGGTGGGCTGGAAACATGAAACAGATTGGCACCTGTGTCGGGCAG
GCAGTAAAATGCTGCAGAAAGAAGTAAAAGAAGGCCAAGACACAGCCGAGGATCAAT
AA

Fig (6): Sequence of the downstream strand of TAP amplified fragment of native cattle cDNA.

GGCAATTAAGGCATGACAAATACCTGCGCTCATAGTTGCACTGGGCCCGAACAGGTGC
CATCTGTCTAAAAAAAGGGAACGGACGGAATACAGACACCCATATTAAACGAAAAGC
TTTGAGGATTTCTTACTCTTTGAGTAAATCCTGACCCAGCAGACAGGACCAGGAAGAG
GATTCT

Fig. (7): Sequence of the downstream strand of BNBD4 amplified fragment of crossbred Frisian cattle cDNA.

GACTTTTACTTTTATAATTCGCTCTGATCATGAAGCTTGTCTACTCGGGGCCCGAAACA
GGTGCCAATCTGTCTCATGTTAACAAGGAACGAGATCGGAATACAGACACCCATATTC
CAACGGCAGCTTTGAGGATTTCTTACTCTTTGAGTAAATCCTGACCCAGCAGACAGGAC
CAGGAAGAGG

Fig. (8): Sequence of the downstream strand of BNBD4 amplified fragment of native cattle cDNA.

DISCUSSION

Several studies were conducted to better understand innate host defense mechanisms with a principal focus on defining the role of antimicrobial peptides of epithelial origin because of their major importance in defending respiratory diseases. Bovine Respir-atory Disease (BRD) was found to be the most common cause of illness and death in beef cattle. In addition to the costs associated with treatment, wasted feed and cattle deaths, BRD also results in performance losses due to decreased weight gain and feed conversion efficiency (Controlling BRD, 2001).

This investigation was an attempt to find out the differences in innate immunity between the native cattle and the crossbred Frisian cattle at the molecular level. Two β-defensin genes were investigated; TAP and BNBD4 in both native and crossbred Frisian cattle by comparing their sequences. Beta-defensins are bactericidal peptides serving as effector molecules of the innate immune system. They are synthesized in neutrophile granulocytes but also in a variety of epithelial tissues. The encoding genes form a multigene family in cattle comprising at least 13 members (Selsted *et al.*, 1993). A phylogenetic analysis placed the gene encoding the tracheal antibacterial peptide (TAP) into a separate basal position of β-Defensin gene evolution while the other genes formed distinct paired groups indicating recent events of gene duplication (Looft *et al.*, 2006 and Diamond *et al.*, 1991).TAP has a potent antimicrobial activity and was first isolated (38-amino acid peptide) from the bovine tracheal mucosa by Diamond *et al.* (1991). The mRNA encoding this peptide was found to be more abundant in the respiratory mucosa than in whole lung tissue.

BNBD-4 is a prototypic bovine neutrophil β-defensin. Yount *et al.* (1999) characterized the corresponding cDNA and indicated that BNBD4 (41 residues) derives from a 63-amino acid prepropeptide. BNBD4 and BNBD-12/13 mRNAs were most abundant in bone marrow, but were expressed differentially in certain non-myeloid tissues. BNBD4, a β–defensin, is synthesized in neutrophile granulocytes but also found in a variety of epithelial tissues (Yount *et al.*, 1999).

The results showed that TAP and BNBD4 are expressed in epithelial cells of all examined tissues of the native cattle whereas in crossbred Frisian cattle it is expressed only in the trachea. Sequence homology between crossbred Frisian and native cattle TAP cDNAs is 89%. They showed 93% and 94% alignment with *Bos taurus* TAP mRNA, respectively. Despite the high alignment between crossbred Frisian and native cattle TAP cDNA, the native cattle TAP cDNA showed 86% alignment with *Bubalus bubalis* lingual antimicrobial peptide (LAP) mRNA. The similarity with buffaloes (*Bubalus bubalis*) which are known for having high disease resistance (Report of the advisory committee on Technology Innovation, 1981) indicates the tendency of native cattle for disease resistance. Whereas, the crossbred Frisian TAP cDNA showed 90% alignment with *Bos taurus* LAP mRNA. TAP and LAP belong to the β-defensins (Diamond *et al.*, 1991 and Schonwetter *et al.*, 1995).

Crossbred Frisian and native cattle BNBD4 cDNAs showed 94% and 95% alignments with *Bos taurus* BNBD4 mRNA, respectively. They also showed 98% and 88% alignment with *Bubalus bubalis* enteric beta-defensin preproprotein mRNA, respectively. However the number of nucleotides shared between crossbred Frisian BNBD4 cDNAs and buffalo enteric beta-defensin preproprotein

mRNA were only 62 compared to 128 between native cattle and buffalo.

Certain facts note the stronger immune tolerance of buffalo compared to cattle. In Egypt, Italy, Bulgaria and other Balkan states, certain herds of buffaloes have surpassed the local cattle in growth, environmental tolerance and health. They were also found to be notably resistant to various diseases like ticks, contagious pleuropneumonia, anaplasmosis and babesiosis (Report of the advisory comm.-ittee on Technology Innovation, 1981).

The presence of TAP and BNBD4 in all investigated tissues of native cattle, while they are only found in the trachea of crossbred Frisian cattle, and the closer sequence similarity between native cattle and buffalo may give an indication of the advantage of native cattle for disease resistance compared to the crossbred Frisian.

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الملخص العربي

تعريف و تحليل التتابع النيوكلوتيدى لأثنتين من الببتيدات المضادة للميكروبات في الأبقار المحلية المصرية و الفريزيان المهجنة

أحلام أحمد ابو مسلم , منى أحمد بيبرس , ايمان رشدى محفوظ , سهير محمد النحاس
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تم اختبار إثنتين من الببتيدات المضادة للميكروبات فى الأبقار الفريزيان المهجنة و المحلية فى مصر و هم bovine tracheal antimicrobial peptide (TAP) and bovine neutrophil β-defensin-4 (BNBD4) . تم اختبار تواجد الجين الخاص بكل منهما فى cDNA نسجة الأبقار المختلفة مثل الكبد و الرئة و القصبة الهوائية و الأمعاء و الغدد اللبنية و القولون و الدم باستخدام تفاعل البلمرة المتسلسل (PCR). ثبت تواجد كل من TAP & BNBD4 فى كل الأنسجة الخاصة بالأبقار المحلية، بينما فى الأبقار الفريزيان المهجنة أعطى تفاعل PCR نتيجة موجبة لكل من الجينين فى القصبة الهوائية فقط. عن طريق البحث باستخدام NCBI- Blast وجد أن كلا من TAP & BNBD4 تطابق بنسبة ٨٩ % و ٩٢ % بين الفريزيان الهجين و الأبقار المحلية على التوالى. على صعيد آخر، فإن مقارنة تتابعات TAP فى كل من الأبقار المحلية و الفريزيان المهجنة مع التتابعات المنشورة فى GenBank أوضحت وجود تشابه بين كل من النوعين من الأبقار و *Bos taurus* TAP mRNA. كما أوضح التحليل البحثى وجود تشابه بنسبة ٩٠% بين TAP الخاص بالأبقار الفريزيان المهجنة و *Bos taurus* (LAP) ، بينما الأبقار المحلية تشابهت بنسبة ٨٦% مع الجاموس (*Bubalus bubalis* LAP) أيضا أوضح البحث باستخدام Blast analysis لناتج PCR ل BNBD4 ان التتابع تشابه بين كل من الأبقار الفريزيان المهجنة و المحلية و بين . *Bos taurus* BNBD4 mRNA كما أوضح التحليل أيضا وجود تشابه مع الجاموس Bubalus bubalis enteric beta-defensin preproprotein mRNA مع الأخذ فى الاعتبارتشابه أكبر (١٢٨ نيوكلوتيدة) مع الأبقارالمحلية عنه فى الفريزيان المهجنة (٦٢ نيوكلوتيدة فقط). إن وجود كل من TAP & BNBD4 فى كل الأنسجة المختبرة فى الأبقار المحلية قد يعكس استعداداً أكبر للمناعة ضد الأمراض مقارنة بالأبقار الفريزيان المهجنة. إلا أن مزيداً من الدراسات على التعبير الجينى فى الأعضاء المختلفة للأبقار المحلية سوف يلقى المزيد من الضوء على مدى استعدادها لمقاومة الأمراض.