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Full Length Research Paper

Identification and isolation of gene differentially expressed on scrotal circumference in crossbreed bulls

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Differential display reverse transcription polymerase chain reaction (DDRT-PCR), reverse northern blot analysis and real-time polymerase chain reaction (RT-PCR) were used to identify tissue-specific expression genes in scrotum of differential scrotal circumference bulls and the function of these specific genes on the development of the bull's scrotum was analyzed in this study. The results indicate that 19 positive fragments were screened out by DDRT-PCR and reverse northern blot analysis. Results of BLAST with GenBank show that three genes or expressed sequence tag (ESTs) were unknown, and there were eight sequences highly identified to be *Bos taurus* mRNA for proline-rich protein P-B and other sequences were *B. taurus* ebd-P2 pseudogene, *B. taurus* similar to F-box only protein 21 isoform 2, *B. taurus* similar to Kinesin heavy chain isoform 5C, *B. taurus* similar to ankyrin repeat domain protein 15 isoform and *B. taurus* similar to galactosidase, beta 1-like. The result of real-time PCR analysis testified more that *B. taurus* was similar to galactosidase, beta 1-like, kinesin heavy chain isoform 5C and ankyrin repeat domain protein 15 isoform. *B. taurus* ebd-P2 pseudogene was highly expressed in bulls which had bigger scrotal circumference. They may be involved with sperm maturation in the epididymis, sperm protection and prevention of the ascent of microorganisms into the adjacent testes and also responsible for converting immature sperm into competent functional cells, as well as movement of spermatozoa.

Key words: Bulls, scrotal circumference, differential display reverse transcription polymerase chain reaction (DDRT-PCR), real time polymerase chain reaction (RT-PCR).

INTRODUCTION

Scrotal circumference(SC) is moderately to highly heritable, at a range of 0.32 to 0.71 (Evans et al., 1999; Gipson et al., 1987; Kealey et al., 2006; Smith et al., 1981). Koots et al. (1994) reported that the average heritability estimate for SC was 0.45. SC was useful for predicting the quality and amount of spermatozoa-producing tissue (Coe, 1999; Elmore et al., 1976) and age at puberty (Brinks et al., 1978). Smith et al. (1989) suggested that for each 1 cm increase in sire's scrotal circumference, there is 0.31 cm increase in the son's scrotal circumference. It has a favorable association with seminal traits. As SC increases, the volume, color, concentration and motility of the semen also increases (Kealey et al 2006). Scrotal circumference positively influences the percentages of normal spermatozoa (Devkota et al., 2008). The incidence of sperm abnormalities decreases as SC increases. Seminal

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characteristics remained poor in bulls with SC less than or equal to 32 cm (Madrid et al., 1988). This means that, when selection pressure was applied to increase SC, all of the phenotypes evaluated would be expected to improve. Predicted correlated responses in semen characteristics per genetic standard deviation (SD) of selection applied to SC were 0.87 genetic SD or less (Kealey et al 2006).

In addition, bulls with larger scrotum at an earlier age result in reducing age at puberty and increasing pregnancy rates in their heifer offspring (Moser et al., 1996). This means that, bulls selection with early puberty, along with others traits, would allow early breeding heifers and improve cow's reproductive performances (Smith et al., 1989). A 0.796 day change in the age at puberty and 0.826 day change in the age at first calving of female offspring per cm of SC in the sire was found (Eler et al., 2004). Shamsuddin et al. (2006) documented that the farmers' average income would increase up to US$ 265 to 561 per year if the age at first calving of heifers could be reduced from 35 to 44 months to 33 to 40 months in zebu and crossbred cows, depending on the dairy production system in Bangladesh. Accordingly, selection of stud bulls at the earliest possible age will not only improve reproduction, but also provide economic advantages by decreasing feeding and management costs and early return of money to the producers (Devkota et al., 2008).

In this study, differential display reverse transcription polymerase chain reaction (DDRT-PCR), reverse northern blot analysis and real-time-PCR were used to identify tissue-specific gene expression in larger scrotal circumference size bulls, so as to analyze the function of the specific expression gene and effects on the development of the bull's scrotum. The results could provide new information and evidence regarding the tissue-specific expression genes in scrotum of differential scrotal circumference bulls and the function of these specific genes on the development of the bull's scrotum, which could be useful in cattle breeding programs.

MATERIALS AND METHODS

Animals and experimental design

The experiment was designed using six-months-old crossbreeds (Charolais with indigenous Fuzhou female). These animals were raised in Beef Cattle Research Centre, Chinese Academy of Agricultural Sciences under the same age, cross generation, raising condition and management in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC) and with relevant legislation. When the feeding was over after six months, the scrotal circumferences of bulls were examined. Six bulls were selected and classified into two groups. A group consisted of three bulls with larger scrotal circumference (26 ± 2.5 cm). The control group was three crossbreed bulls with smaller scrotal circumference (17 ± 2.2 cm) ($P<0.01$). The bulls were castrated by surgical operations, while the scrotal circumferences were measured. A piece of tissue (2 cm^3) was removed from the deeper area of the testis and preserved in liquid nitrogen. A small section (0.5 cm^3) was used for total RNA extraction by using the TRIZOL reagent kit (GIBCO/BRL, Bethesda, MA, USA). The RNA was prepared for DDRT-PCR experiments and quantitative real-time PCR. The total RNA of liver, kidney, lung, fat, muscle, small intestine, rumen and testicle of bulls were extracted and prepared for tissues expression analysis.

The first-strand cDNA synthesis

The cDNA synthesis reaction was designed for four bulls using 3 anchored oligo-dT primers, which were H2T11A (5′-AAGCTTTTTTTTTTTA-3′), H2T11G (5′-AAGCTTTTTTTTTTG-3′) and H2T11C (5′-AAGCTTTTTTTTTTC-3′). From each individual, there were three cDNA pools, which were incubated with 7 arbitrary random primers. The random primers are described as H-AP1(5′-TGCCGAAGCTTTGGTACC-3′), H-AP2 (5′-TGCCGAAGCTTTGGTAGC-3′), H-AP3 (5′-TGCCGAAGCTTTGGTATG-3′), H-AP4 (5′-TGCCGAAGCTTTGGTCAC-3′), H-AP5 (5′-TGCCGAAGCT TTGGTCAG-3′), H-AP6 (5′-TGCCGAAGCTTTGGTCAT-3′) and H-AP7(5′-TG CCGAAGCTTTGGTGAC-3′).The first-strand cDNA used for RT-PCR was synthesized as follows: the first step, 1 µg/µL of DNaseI-treated total RNA (2 µL), 10 µmol/µL of anchored primers (5 µL) and DEPC-treated water (3 µL) (Promega, Madison, WI, USA) were added, heated for 5 min at 70°C and placed on ice immediately. For the next step, 5×RT buffer (5 µL), 10 mmol/L dNTPs (6.25 µL), 40 U/µL RNase inhibitor (1 µL), 200 U M-MLV reverse transcriptase (Promega) (1 µL) and DEPC-treated water (26.75 µL) were added in a total volume of 50 µL at 37°C for 1 h and 95°C for 5 min.

DDRT-PCR and gel electrophoresis of DNA fragments

The PCR protocol was designed to allow the analysis of two cDNA subfractions from two groups with three anchored primers and seven random primers. The PCR reactions were carried out in a volume of 20 µL, which contained 2 µL of the first-strand cDNA product, 2 µL 10×buffer (Tris-HCl 100 mmol/L, KCl 500 mmol/ L, pH 8.3), 10 µmol/L random primers (0.8 µL), 10 µmol/L anchored primers (0.4 µL), 0.75 mmol/L dNTPs (2 µL) and 0.2 µL Taq DNA polymerase (TaKaRa, Dalian, China). The optimum condition of the PCR was 94°C for 5 min, 40°C for 5 min and 72°C for 1 min, followed by 40 cycles of 94°C for 45 s, annealing for 2 min at 60°C, extension for 1 min and a final extension at 72°C for 5 min. We concentrated half of the DDRT-PCR incubation mixture (10 µL) by vacuum and heat (10 min) to 3 µL and adjusted with glycerol to 5%, 0.05% xylene cyanol FF and 0.05% bromophenol blue. Then, we loaded 3 µL onto a prerun 4% polyacrylamide gel with urea and run in TAE buffer at 100 V for 7 h. The patterns of DNA bands were observed and photographed (Figure 1).

Re-amplification reverse northern blot analysis

Cut out the bands (e.g., those differing between the patterns from control and the test individual) from the gel and transfer to Eppendorf tubes. Add 40 µL of DEPC-treated water and boil for 15 min. The re-amplification PCR was carried out in a volume of 40 µL containing eluted cDNA (4 µL), 10×PCR buffer (as aforementioned) (4 µL), 10 µmol/L arbitrary primers (1.6 µL), 10 µmol/L anchored primers (0.8 µL), 0.75 mmol/L dNTPs (4 µL) and 10 U Taq DNA polymerase (0.4 µL) (as earlier mentioned). The anchored, arbitrary primers and the optimum condition of the PCR were the same as the ones given for DDRT-PCR.

The cDNA probes were prepared from 10 to 50 µg of each of the two RNA samples by reverse transcription in a 50 µL reaction which

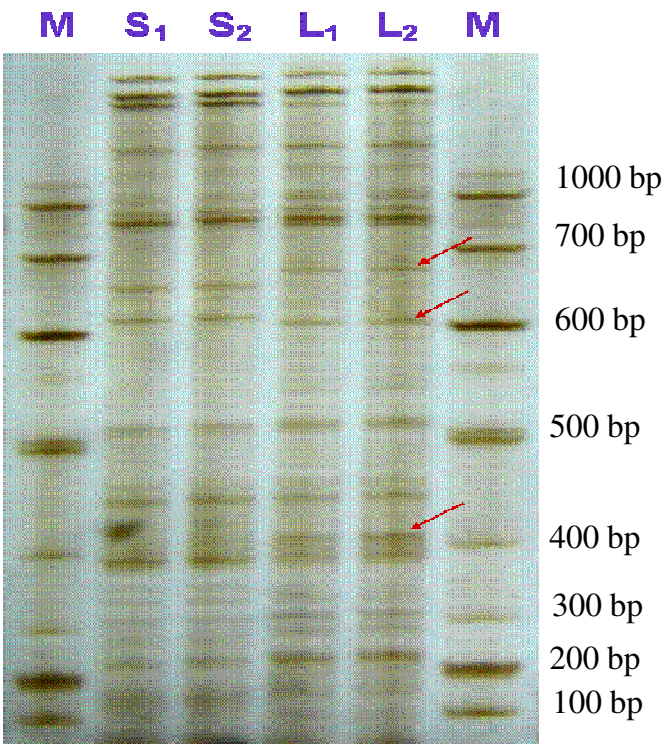

Figure 1. Different display of four bulls using the H2T11G primer combined with H-AP6 and H-AP7. S_1 and S_2 indicate two samples for small SC; L_1 and L_2 indicate two samples for large SC; M is the molecular ladder marker.

consisted of three steps. The first step was the adding of 1 µg/µL of DNaseI-treated total RNA 2 µL, 10 µmol/L oligo-dT18 (5 µL) and DEPC-treated water (3 µL) , then heated for 5 min at 70°C andplaced on ice immediately. The next step included the addition of 5×RT buffer (5 µL), 10 mmol/L dNTPs (6.25 µL), 40 U/µL RNase inhibitor (1 µL), 200 U M-MLV reverse transcriptase (Promega) (1 µL) and DEPC-treated water (26.75 µL) in a total volume of 50 µL at 37°C for 1 h and 95°C for 5 min. Furthermore, purified cDNA probes were labeled by DIG (Roche, SWISS). The re-amplification PCR product was transferred to a nylon membrane (Hybond N+, Amersham) by capillary blotting followed by ultraviolet cross linking. The membranes were hybridized using DIG-labeled specific probes. Hybridization was carried out at 65°C for 4 h. After washing, membranes were exposed to Kodak XAR-5 film at -80°C.

Cloning and sequencing DNA fragments of interest

The DNA fragments of interest were verified by reverse northern blot, purified with wizard PCR Preps DNA purification system (Promega) and cloned into the pGEM T-easy vector (Promega) and then sequenced using an ABI 3730 automatic sequencer (Applied Biosystems, Forster City, CA, USA). All sequences were assembled with SeqMan™ II (Lasergene version 6; DNAStar, Inc., Madison, WI, USA).

Quantitative real time PCR analysis

The gene fragments identified as differentially expressed in DDRT-PCR were confirmed and quantified by comparative qRT-PCR. mRNAs of the two different scrotal circumference were used for qRT-PCR experiments. The measurements were performed on Bio-RAD qPCR system using the SYBR Green qPCR Master mix. qRT-PCR was performed in triple technical replicates for each mRNA sample/ primer combination. The amounts of RNA in each qRT-PCR reaction were normalized using primers specific for β-actin. Primer pairs (Table 2) for qRT-PCR analyses were designed using Primer3 software. Threshold (Ct) values and gene transcript numbers were used to determine the gene expression levels from different cDNA samples.

Tissues expression analysis

The mRNA from liver, kidney, lung, fat, muscle, small intestine, rumen and testicle were subjected to tissues expression analysis and the primers used in this part of the study were shown in Table 3.

RESULTS

In this study, DDRT-PCR was applied to identify differentially expressed genes in the bovine testis between smaller and larger SC and the purified sample RNA of six bulls were amplified using three anchored primers and seven arbitrary random primers. The gel electrophoresis was analyzed for differences between smaller and larger SC, which revealed 77 cDNA

Table 1. Results from sequence blast of differential gene expression in bovine testis.

Number	Primer	Gene bank	Similarity	Gene name
A54	H2T11A, H-AP5	gi55415674	142/145 (97%)	*Bos taurus* ebd-P2 pseudogene
A55	H2T11A, H-AP5			Unknown
A56	H2T11A, H-AP5			Unknown
A57	H2T11A, H-AP5	gi55415674	142/145 (97%)	*Bos taurus* ebd-P2 pseudogene
A58	H2T11A, H-AP5			Unknown
A59	H2T11A, H-AP5	gi76639092	223/224 (99%)	*Bos taurus* similar to F-box only protein 21 isoform2
A62	H2T11A, H-AP6	gi54013468	238/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
A63	H2T11A, H-AP6	gi54013468	239/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
A71	H2T11A,H-AP7	gi76681130	292/293 (99%)	*Bos taurus* similar to septin 10 isoform 1
G31	H2T11G, H-AP3	gi54013468	239/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
G53	H2T11G, H-AP5	gi54013468	215/216 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
G63	H2T11G, H-AP6	gi54013468	239/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
C13	H2T11C, H-AP1	gi54013468	234/240 (97%)	*Bos taurus* mRNA for proline-rich protein P-B
C16	H2T11C, H-AP1	gi76609893	584/585 (99%)	*Bos taurus* similar to Kinesin heavy chain isoform 5C (Kinesin heavy chain neuron-specific 2), transcript variant 1
C18	H2T11C, H-AP1	gi54013468	239/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
C42	H2T11C, H-AP4	gi54013468	239/240 (99%)	*Bos taurus* mRNA for proline-rich protein P-B
C44	H2T11C, H-AP4	gi76671770	480/485 (98%)	*Bos taurus* similar to ankyrin repeat domain protein 15 isoform a
C62	H2T11C, H-AP6	gi76610658	392/395 (99%)	*Bos taurus* similar to galactosidase, beta 1-like

Table 2. The gene-specific primer pairs for quantitative real-time PCR.

Number	Gene name	Primer	Product size (bp)
	β-Actin	FW: GAAGTGTGACGTTGACATCCG RV: GCCTAGAAGCATTTGCGGTG	282
C62	*Bos taurus* similar to galactosidase, beta 1-like	FW: ACCAGGACCTTACATCTGTG RV: TCATGTAGCTCACGTCGCAG	239
C16	*Bos taurus* similar to Kinesin heavy chain isoform 5C	FW: CGACTGTACATCAGCAAGATG RV: CTGCTCCATGTTCTGCATG	195
C44	*Bos taurus* similar to ankyrin repeat domain protein 15 isoform a	FW: TGTGACAGAAGCCATGCTTG RV: AAGTGGCTGGGCCATCATG	214
A59	*Bos taurus* similar to F-box only protein 21 isoform2	FW: AGGGCTGAAGCTCTAAGTG RV:GCATAAATCCGAGAGATGAATCT	191
A62	*Bos taurus* mRNA for proline-rich protein P-B	FW: ATTCGTAATCATGTCATAGCTG RV: AGCGGAAGAGCGCCCAAT	241
A54	*Bos taurus* ebd-P2 pseudogene	FW: CTGCCGTACGTATAGGCTG	274

differential fragments. Figure 1 shows the differential bands of cDNA amplified by the anchored primer H2T11G combined with H-AP6 and H-AP7 in 4 bulls as an example.

Following the 77 bands of interest cut from the gel, the re-amplification PCR was carried out, and then the

Table 3. The gene-specific primer pairs for tissues expression.

Number	Gene name	Primer	Product size (bp)
	β-Actin	FW: GAAGTGTGACGTTGACATCCG RV: GCCTAGAAGCATTTGCGGTG	282
C62	*Bos taurus* similar to galactosidase, beta 1-like	FW: GGGCAACATCATTAGCATTC RV: GAGCCACATTTGAGTCCTTC	159
C16	*Bos taurus* similar to Kinesin heavy chain isoform 5C	FW: CGACTGTACATCAGCAAGATG RV: CGTGAAGACTTTGGCAGATGT	219
C44	*Bos taurus* similar to ankyrin repeat domain protein 15 isoform a	FW: CAGCCACTTGTTTTCAGCAA RV: GGCCCCACCTCTTTATTCTC	164
A59	*Bos taurus* similar to F-box only protein 21 isoform2	FW: ACTAAAAAGGGAAGGGGAGGC RV: TGTCATGGCAACGGTGACTCT	169
A62	*Bos taurus* mRNA for proline-rich protein P-B	FW: ATTCGTAATCATGTCATAGCTG RV: AGCGGAAGAGCGCCCAAT	241
A54	*Bos taurus* ebd-P2 pseudogene	FW: CTGCTCCACACACTTCTCTTCC RV: CAGCATTTTACTGAGGGCGTG	167

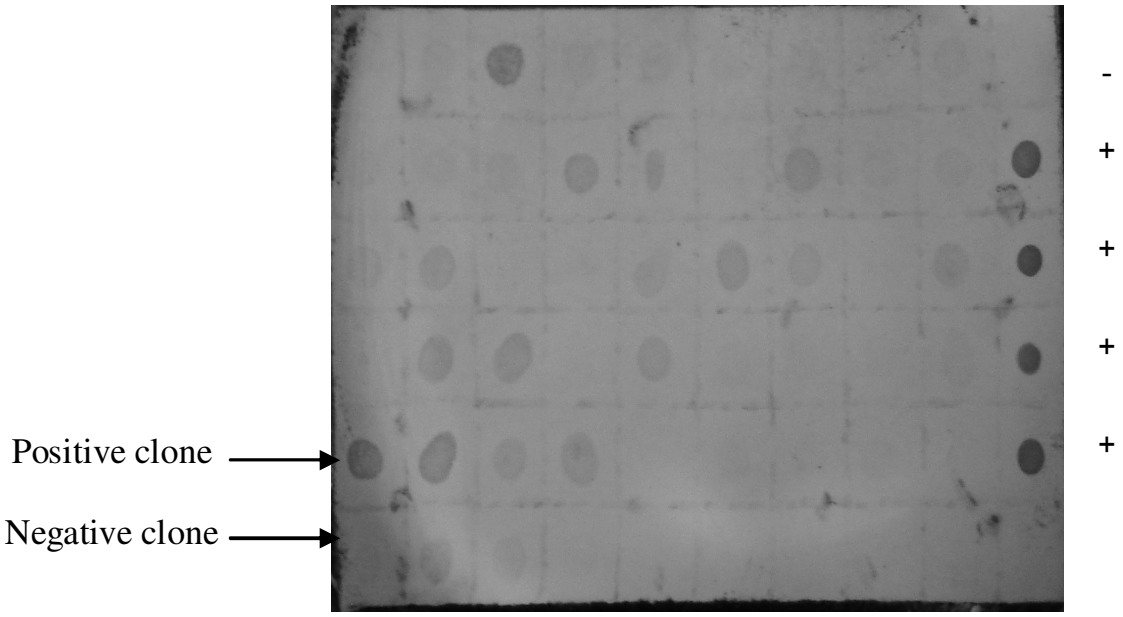

Figure 2. Result of reverse northern dot blot of probe3. +, Positive control; -, negative control.

products of re-amplification of cDNA were hybridized using DIG (Roche, SWISS). Figure 2 shows the results of reverse northern dot blot of probe 3 as an example. By reverse northern high-density blots screening, 19 positive clones were randomly picked up from 77 differential fragments. These 19 positive clones were sequenced and blasted with the sequences of Genbank. The analysis of the cDNA fragments revealed nine different genes or mRNAs with higher expression levels at testis (Table 1). Six genes corresponded to genes of known or inferred function; either the bovine gene or the likely human ortholog and three genes or ESTs were unknown. These were *B. taurus* ebd-P2 pseudogene, *B. taurus* similar to F-box only protein 21 isoform2, *B. taurus*

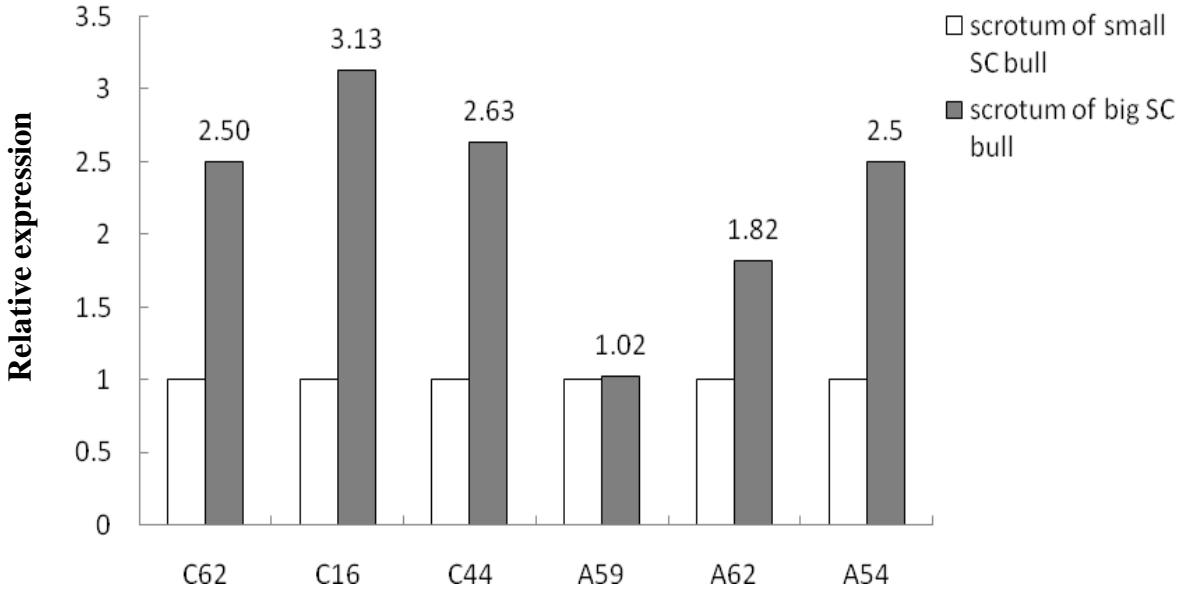

Figure 3. The results of qPCR. C62:*Bos taurus* is similar to galactosidase, beta 1-like; C16:*Bos Taurus* is similar to Kinesin heavy chain isoform 5C; C44:*Bos taurus* is similar to ankyrin repeat domain protein 15 isoform a; A59:*Bos taurus* is similar to F-box only protein 21 isoform2; A62:*Bos taurus* mRNA for proline-rich protein P-B; A54: *Bos taurus* ebd-P2 pseudogene.

mRNA for proline-rich protein P-B, *B. taurus* similar to Kinesin heavy chain isoform 5C, *B. taurus* similar to ankyrin repeat domain protein 15 isoform and *B. taurus* similar to galactosidase, beta 1-like.

An initial research of quantitative real-time PCR showed that these six genes were more highly expressed in scrotum of big SC bull than the small one. As shown in Figure 3, the expression of *B. taurus* was similar to Kinesin heavy chain isoform 5C; was more than three times higher in big bull than in the small SC bull and the expression of *B. taurus* was similar to galactosidase, beta 1-like, and *B. taurus* was similar to ankyrin repeat domain protein 15 isoform a. *B. taurus* ebd-P2 pseudogene also reached two times in the big SC bull than in the small one. As shown in Figure 4, the tissues expression of six genes were detected in all the nine tissues such as liver, kidney, lung, fat, muscle, small intestine, rumen and testicle.

DISCUSSION

B. taurus ebd-P2 pseudogene, a novel member of β-defensins, was first identified in bovine mammary gland tissue (Roosen et al., 2004). In this paper, it was found to be expressed in the epithelia of bovine epididymis. Li et al. (2001) reported that a cloned 385-base pair complementary DNA and its genomic DNA named Bin1b is exclusively expressed in the caput region of the rat epididymis, which suggest that *B. taurus* ebd-P2 pseudogene may have the same function as eBin1b. Bin1b exhibits structural characteristics and antimicrobial activity similar to that of cationic antimicrobial peptides, β-defensins. Unlike other beta-defensins that are expressed in a wide array of organs such as salivary glands, airways and the urogenital tract, Bin1b is exclusively expressed in the caput region of the rat epididymis. Its expression starts in the first 30 days, reaches a maximum during the sexually mature period and decreases in old age. Bin1b is maximally expressed when the rats are sexually mature and can be up-regulated by inflammation. It appears to be a natural epididymis-specific antimicrobial peptide that plays a role in reproductive tract host defense and male fertility that is responsible for sperm maturation, storage, protection and movement. In this study, the *B. taurus* ebd-P2 pseudogene was detected in epithelia of bovine epididymis and highly expressed in bulls which had bigger SC. We deduce that ebd-P2 pseudogene may also be involved with sperm maturation in the epididymis, as well as sperm protection and prevention of the ascent of microorganisms into the adjacent testes where sperm are produced and responsible for converting immature sperm into competent functional cells.

In this work, *B. taurus* was similar to galactosidase and beta 1-like was identified in epithelia of bovine epididymis. Its similarities with galactosidase, beta 1-like of *Homo sapiens* and mouse were 95 and 87%.(Tulsiani. 2003) reported that the bovine testicular beta-galactosidase may be involved with sperm maturation and modification. *B. taurus* was also similar to Kinesin heavy chain isoform 5C (Kinesin heavy chain neuron-specific 2) and to septin 10 isoform 1 too (Tulsiani 2003). Kinesins are microtubule-based motor proteins that

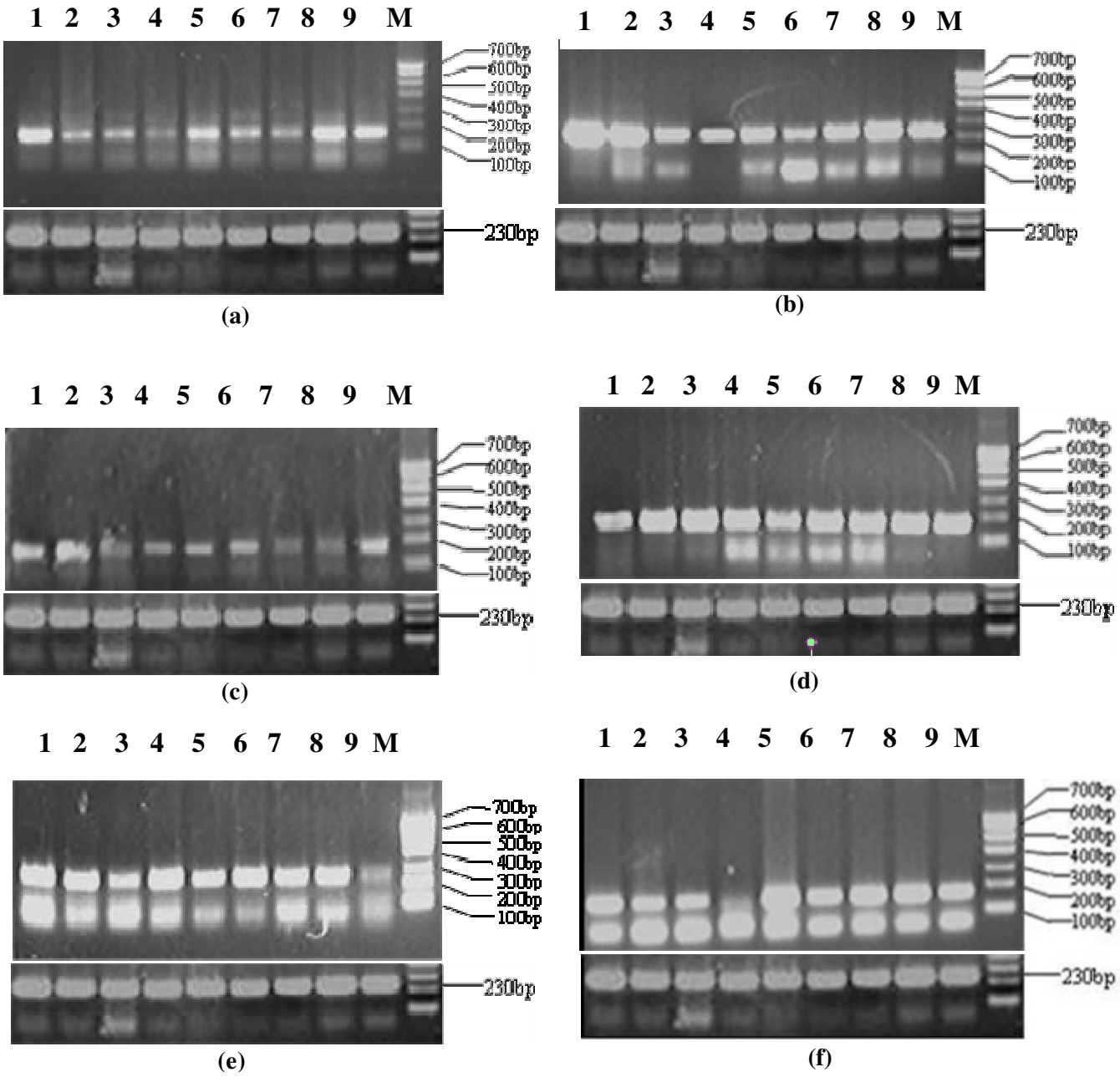

Figure 4. The results of tissues expression. (a) The tissues expression of *Bos taurus* similar to galactosidase, beta 1-like; (b) the tissues expression of *Bos taurus* similar to Kinesin heavy chain isoform 5C; (c) the tissues expression of *Bos taurus* similar to ankyrin repeat domain protein 15 isoform a; (d) the tissues expression of *Bos taurus* similar to F-box only protein 21 isoform2; (e) the tissues expression of *Bos taurus* praline-rich protein P-B; (f) the tissues expression of *Bos taurus* ebd-P2 pseudogene. Top panel: PCR products from cDNAs of various bovine tissues. 1, testicle; 2, kidney; 3, liver; 4, muscle; 5, lung; 6, fat; 7, womb; 8, small intestine; 9, rumen; M, marker(100 bp ladder); Bottom panel: β-actin PCR products from cDNAs of all samples.

perform diverse functions, including the translocation of vesicles, organelles, chromosomes, protein complexes, RNA-binding proteins (RNPs) (Brendza et al., 2000; Cleveland et al., 2003; Guzik and Goldstein, 2004; Hirokawa and Takemura, 2005). They also help to orchestrate microtubule dynamics and determine the morphology of cells (Abrieu et al., 2000; Hirokawa and Takemura, 2004; McIntosh et al., 2002; Yao et al., 2000). Septins are a family of conserved cytoskeletal GTPases that participate in various cell functions such as cytokinesis, vesicle trafficking, cell cycle regulation and so on. Ihara et al. (2005) reported that the conformation of sperm flagellum and movement in rat without the Septin4 gene was affected and develop sterility (Ihara et

al., 2005). These suggested that *B. taurus* was similar to Kinesin heavy chain isoform 5C (Kinesin heavy chain neuron-specific 2) and to septin 10 isoform 1 involved in development and maturity of spermatozoa, the movement especially.

Conclusion

Results from this study, six genes corresponded to genes of known or inferred function, either the bovine gene or the likely human ortholog, while three genes or ESTs were unknown. Those known were *B. taurus* ebd-P2 pseudogene, *B. taurus* similar to F-box only protein 21 isoform2, *B. taurus* mRNA for proline-rich protein P-B, *B. taurus* similar to Kinesin heavy chain isoform 5C, *B. taurus* similar to ankyrin repeat domain protein 15 isoform and *B. taurus* similar to galactosidase, beta 1-like. The *B. taurus* ebd-P2 pseudogene, *B. taurus* similar to galactosidase, beta 1-like, *B. taurus* similar to Kinesin heavy chain isoform 5C and *B. taurus* similar to ankyrin repeat domain protein 15 isoform were found both highly expressed in bulls that have bigger SC. Their functions may be involved with sperm maturation in the epididymis, sperm protection and prevention of the ascent of microorganisms into the adjacent testes, and also responsible for converting immature sperm into competent functional cells, as well as movement of spermatozoa.

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