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Short Communications

Cloning of actin, beta (ACTB) cDNA and assessment of its suitability as a reference gene in uterine endometrial tissue of buffalo *(Bubalus bubalis*)

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Actin is a highly conserved protein found in abundance in all the eukaryotic cells. In mammals, 6 distinct isoforms of actin are identified which can be classified into 3 major categories: non-muscle or cytoplasmic types (b and g in both brain and thymus), smooth muscle types (vascular and nonvascular) and striated muscle types *(a* skeletal and *a* cardiac). Both cytoplasmic b and g actin isoforms are coexpressed in dividing non-muscle cells and undifferentiated myoblasts (Vandekerckhove and Weber 1978, Vandekerckhove *et al*. 1980, Vandekerckhove and Weber 1979, Zakut *et al.* 1982). Amongst these, "actin, beta" (ACTB) is one of the most studied and gene encoding ACTB has been characterized in a wide range of species from protozoan like *Dictyostelium* (McKeown and Firtel 1981) to human (Engel *et al.* 1981). The constitutive expression of ACTB mRNA in cells has made it as one of the most widely used reference gene for gene expression studies. However, use of ACTB as a universal reference gene has been contested and criticized. A recent study reported the least stable expression of ACTB mRNA in the bovine mammary tissue during lactation as compared to other reference genes (Bionaz and Loor 2007). In another study in fish, estrogen treatment significantly down regulated ACTB mRNA expression in gonad and liver (Filby and Tyler 2007). Consequently, it is proposed that the suitability of ACTB as a reference gene in gene expression studies need to be validated for each organism and each tissue type (Glare *et al.* 2002, Bionaz and Loor 2007, Tramontana *et al.* 2008). Ribosomal protein L19 (RPL19) encodes a protein that belongs to group of proteins organized into large and small ribosomal subunits. RPL19 is predicted to function as RNA chaperones during translation process (Maguire and Zimmermann 2001, Ramakrishnan 2002). RPL19 is widely used in different gene expression studies as reference genes (Lee *et al*. 2002, Garcia-CrespoJuste and Hurtado 2005) and it has been reported that the RPL19 is reliable reference gene for gene expression studies (Al-Bader and Al-Sarraf 2005). In the present study we cloned and characterized the cDNA of water buffalo *(Bubalus bubalis*). Besides, we have also assessed the suitability of ACTB as a reference gene in comparison to RPL19 in studying the differential expression of target genes in the uterine endometrium during different reproductive stages.

Sample collection: Uteri of apparently healthy buffaloes were collected from local abattoir and transported immediately (within 1 h) to the laboratory on ice. On the basis of corpus luteum morphology, patency of cervical *os* and vascularity of uterus (Arosh *et al.* 2002, Yadav *et al.* 2002), uteri were classified into 3 stages of estrous cycle: stage I (days 3 to 5), stage II (days 6 to 15) and stage III (days 16 to 21). Days of pregnancy were approximately determined on the basis of crown rump length and weight of the foetus.

Amplification of ACTB cDNA: Total RNA was isolated from 100 mg of uterine endometrial tissue using TRI reagent following the manufacturer's instructions. One µg of total RNA was reverse transcribed with suitable negative (reverse transcriptase enzyme omitted) controls using the Reverse Transcription System as per the manufacturer's instructions. The second strand of ACTB cDNA was PCR amplified using degenerate primers (BACTIN/30F forward: 5' CCAGYTCRCCATGGATGATGATATYGCYGCGC 3' and BACTIN/1178R reverse: 5' TAACAGTCCGCCTAGAAGCATTTGCGGTGG 3') designed on the basis of available ACTB sequences of cattle (GenBank Acc. No. AY141970) and sheep (GenBank Acc. No. NM_001009784 and U39357). The PCR reaction contained 0.2 unit of *Taq* DNA polymerase and its 1X buffer, first strand of cDNA (5µl), 0.2 mM of each dNTPs, 2.5 mM $MgCl_2$, and 5 pmoles each primer. The PCR protocol involved an initial denaturation at 95°C for 3 min followed by 30 cycles

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of denaturation (95°C for 30 sec), annealing (56°C for 30 sec) and extension (72°C for 45 sec) proceeded by a final extension at 72°C for 10 min. The PCR product was examined by agarose gel (1%) electrophoresis in 1X TAE buffer after staining with ethidium bromide. Ready-to-use 1 kb DNA ladder was used as a molecular weight marker for electrophoresis. After electrophoresis, the stained gels were recorded with a digital fluorescent image recorder.

Cloning and sequencing of ACTB cDNA: The amplicon was cloned into the pTZ57R/T vector following the manufacturer's instruction. Positive recombinant clones were identified using blue and white screening. Further, the presence of the insert was confirmed by restriction digestion with *Eco*RI and *Bam*HI and using plasmid PCR. The positive clones were sequenced using an ABI PRISM automatic sequencer (Version 2.0) using standard cycle conditions by Sanger's dideoxy chain termination method with the standard M13F and M13R sequencing primers. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotide as well as deduced amino acid sequence was aligned with that of available species in the GenBank database using the ClustalW method of Megalign Programme of Lasergene Software. Phylogram was constructed using neighbourhood joining method in MEGA, version 4 (Tamura *et al.* 2007). Secondary structure and domains were predicted by homology modeling using Expasy's CPH (model 2.0) tool.

Expression analysis of buffalo ACTB and RPL19 mRNA: The expression of ACTB and RP L19 mRNA in the uterine endometrium during different stages of estrous cycle and pregnancy was quantified by real-time PCR (qPCR). Primers for ACTB (BACTINFP forward 5'-AGCTCGCCATGGATGATGA –3'; BACTINRP reverse 5'-TGCCGGAGCCGTTGTC –3') were designed on the basis of the buffalo ACTB cDNA sequence characterized in this study. Primers for RPL19 (forward 5'-GGGCAGGCATATGGGTATAGG –3'; reverse 5'-CTCGGGCATTCGAGCATT –3') were designed based on the bovine RPL19 gene (Acc. No. NM_001040516.1). All of the above primers were designed using Primer Express 3.0 software. All qPCR reactions were performed in duplicates. The amplification was carried out in 5µl volume containing 1X Fast SYBR® Green Master Mix, 5 pmols of each gene-specific primer and 2.5 µl of cDNA template. PCR cycling conditions were: initial denaturation at 95 °C for 20s, followed by 40 cycles of denaturation at 95 °C for 1 s; annealing/extension at 60 °C for 20 s. In order to check the DNA contamination, a control reaction without reverse transcriptase enzyme was kept during cDNA synthesis. A dissociation curve was generated using inbuilt software in 7900HT Fast Real-Time PCR System after completion of amplification and analyzed in comparison to negative (cDNA omitted reaction) and positive controls (recombinant plasmids as template), to determine the specificity of the PCR reaction. The level of mRNA expression was expressed as threshold cycle (Ct). The Ct values for each sample were determined by using the inbuilt software in 7900HT Fast Real-Time PCR System. The efficiency of the selected primer pairs was calculated as follows: a serial dilution of template was run with each primer pair and the log of the dilution was plotted against the Ct value of each dilution. The slope of the resulting regression equation was used to calculate the efficiency (E) of the PCR reaction using $E = -1+10^{(-1/slope)}$ equation (Rutledge and Cote 2003).

Statistical analysis: All numerical data were expressed as a mean±SE of the biological replicates (n=3) from each stage of the estrous cycle and pregnancy. Shapiro-Wilk test was used to check the normality of the components of experiment and the equality of variance was tested by Levene's test. Effect of different stages of estrous cycle and early pregnancy on ACTB and RPL19 gene expression was analyzed by one-way ANOVA.

Amplification, cloning, sequencing and characterization of ACTB cDNA: Agarose gel electrophoresis of the PCR product revealed an amplification of a fragment of approximately 1143 bp (Fig. 1). Nucleotide sequencing of the amplicon also confirmed the size. The BLAST analysis of the obtained nucleotide sequence retrieved the sequence of ACTB cDNA of different domestic animal species exhibiting more than 90% identity. Accordingly, the sequence has been submitted to GenBank under Acc. No. DQ661647. The buffalo ACTB sequence (Fig. 2) has an open reading frame (ORF) of 1128 nucleotides (reads through nucleotide 11 to 1138 bp) that encodes a peptide of 375 amino acids. The size of ORF of buffalo ACTB cDNA is conserved across the species including mammals and birds (Groenen *et al.* 1993).

The nucleotide sequence of buffalo ACTB showed 98, 97, 94.6, 94, 93, 90, 89, 87 and 86% identity with that of cattle, sheep, yak, horse, dog, human, rat, mouse, chicken, turkey and zebrafish, respectively. However, the deduced amino acid sequence exhibited more than 97% identity with that of above-mentioned species indicating its high degree

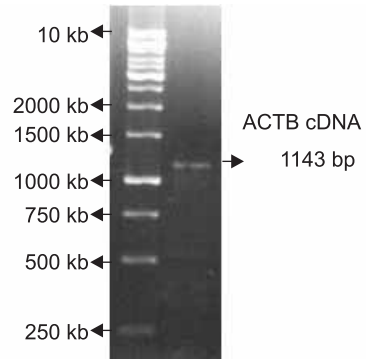

Fig. 1. Reverse transcription-PCR amplified product of bubaline ACTB from total RNA isolated from uterine endometrium of buffalo.

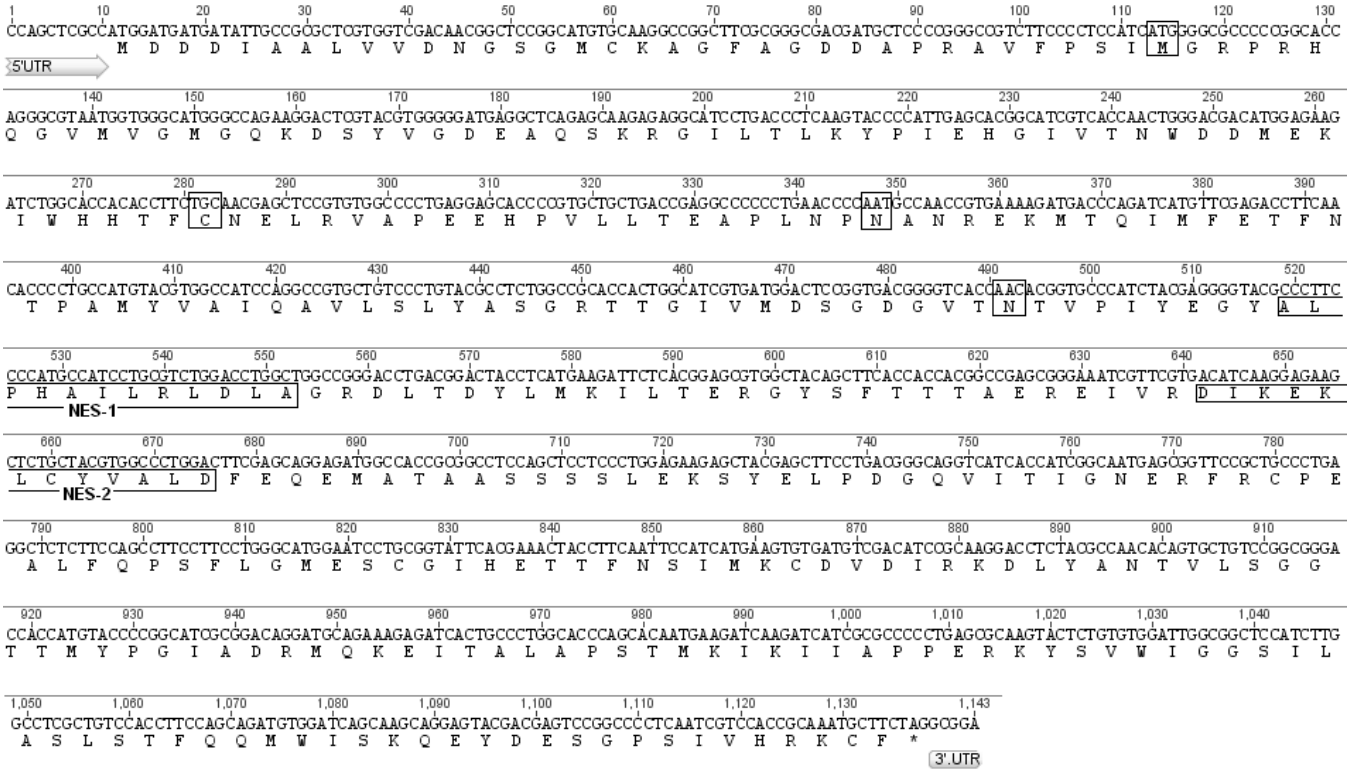

Fig. 2. Nucleotide sequence with deduced amino acid sequence of buffalo ACTB. Non synonymous nucleotide substitution (based on orthologue sequences) and the amino acid changes are indicated by boxes. Predicted 2 nuclear export signals (NES-1. NES–2) are also conserved in buffalo ACTB.

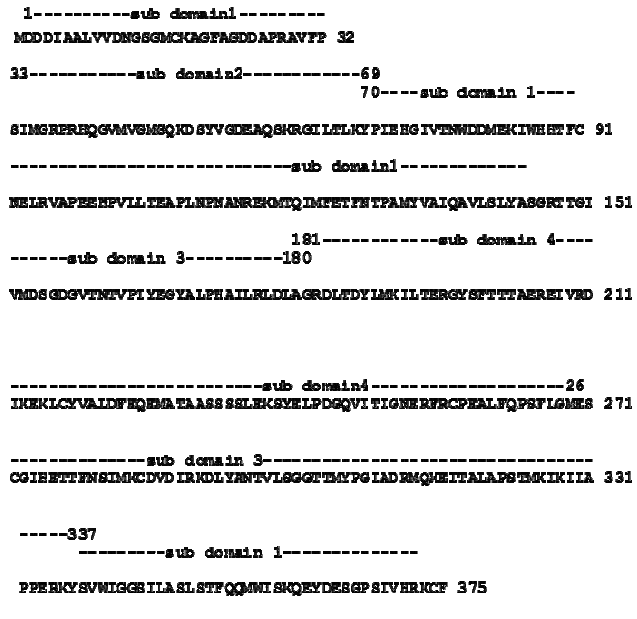

Fig. 3. Predicted subdomains of buffalo ACTB protein

of conservation. When compared with cattle, 19 nucleotide changes are observed in buffalo ACTB. Among these, only 4 nucleotide changes are non-synonymous causing amino acid substitution at codons 35 (Val to Met), 91 (Tyr to Cys), 113 (Lys to Asn), and 161 (His to Asn) (Fig. 2).

ACTB consists of 2 functional domains, referred as small and large domains. Each domain is further divided into two sub-domains (Kabsch *et al*. 1990).The large and small domains are connected by helices Gln 137-Ala 144 and Ser 338-Ser348 which are usually a part of the common structural motif as reported in other actin molecules (Kabsch and Holmes 1995). The Ser 14 and Asp 157 residues of ACTB are predicted to be involved in inter-domain communication (Schuler *et al*. 1999). The sub-domains of buffalo ACTB, as predicted based on rabbit skeletal actin, (Kabsch *et al*. 1990) are conserved across the species (Fig 3). Buffalo ACTB protein also has two conserved nucleotide export signal

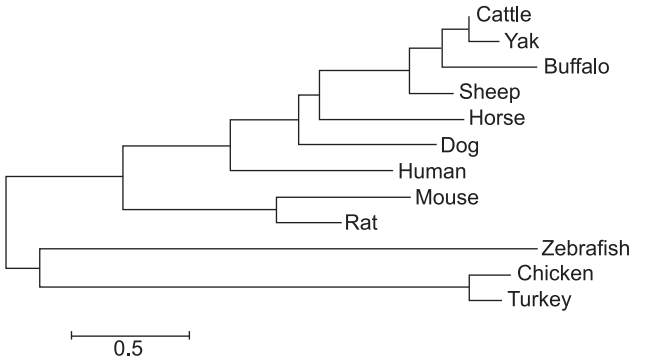

Fig. 4. Phylogram based on deduced amino acids sequences of ACTB of different species including buffalo.

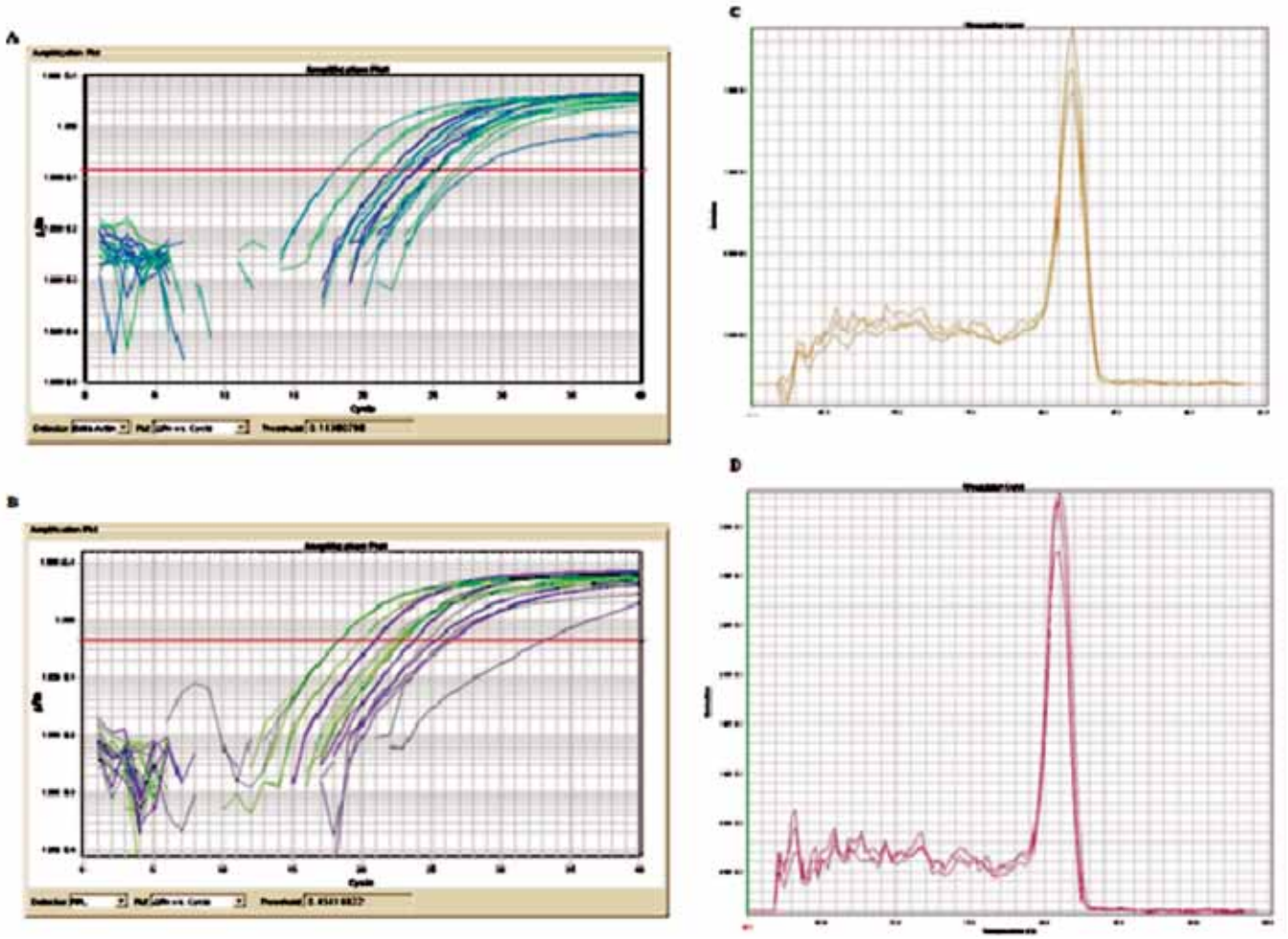

Fig. 5A. Amplification plots of ACTB (A) and RPL19 (B) mRNA expression were shown. Dissociation curve analysis was performed for the amplified products of ACTB (C) and RPL19 (D) and single peak in the amplified product indicates the presence of one specific product of a respective gene.

(NES) sequences (aa 170–181: NES-1 and aa 211–222: NES 2). These NES sequences are proved to be involved in the shuttling of actin molecules between nucleus and cytoplasm (Wada *et al.* 1998).

Phylogram constructed based on the deduced amino acid sequences of ACTB of various species revealed that ACTB of mammals, avian and fishes fall into 3 distinct clads. In the clad which include the mammalian species, all the ruminants were in close proximity followed by dog, horse, human, rat and mice (Fig. 4).

Endometrial mRNA expression profile of ACTB and RPL: The PCR efficiency (E) for ACTB and RPL19 were 104 and 115 respectively. Expression of ACTB (Fig. 5A.) and RPL mRNA in the uterine endometrial tissues was observed and presence of single peak in the dissociation curve for the amplified products of the gene indicates the specificity of the qPCR reaction (Fig. 5A. C, D). Expression of ACTB and RPL mRNA in the uterine endometrial tissues did not vary significantly (P≥0.05) during the different stages of estrous

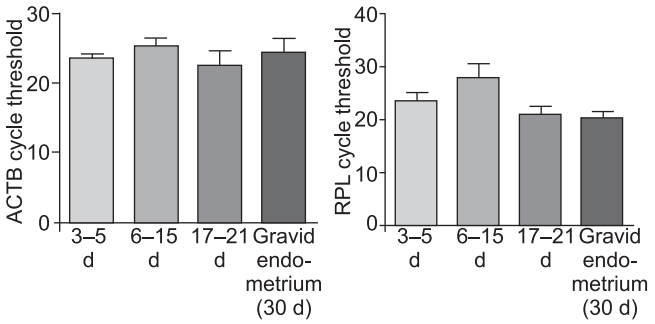

Fig. 5B. SYBR green quantitative PCR analysis of buffalo ACTB (A) and RPL19 (B) mRNAs in uterine endometrial tissue of buffalo during different days of estrous cycle and early pregnancy (~d30). Threshold cycle (Ct) values were calculated for both ACTB and RPL19 and values are presented as the mean±SE of threshold cycle. Threshold cycle values of the selected reproductive stages were analyzed by one-way ANOVA. There was no significant difference in Ct values of ACTB (p=0.6568) and RPL (0.0661) found among different stages of estrous cycle and early pregnancy.

cycle as well as during early pregnancy (Fig. 5B). The R square values (estimated in one way ANOVA) of ACTB and RPL genes were 0.1733 and 0.5734 respectively. Lesser R^2 value for ACTB indicates comparatively less variability in ACTB mRNA expression than that of RPL during different stages of the estrous cycle. In gene expression studies, using quantitative real time PCR (RTqPCR) of any tissue or cell types, the variation at the level of RNA quantity and reverse transcription process is usually controlled using reference gene (Karge *et al*. 1998). Therefore, selection of appropriate reference gene is very important to avoid any errors in the interpretation of changes in gene expression and subsequently to determine the functionality of the tissue or cells (Thellin *et al*. 1999). Accordingly, ACTB gene is turned out to be as a reliable reference gene in studying gene expression in the uteine endometrium of buffalo.

The entire coding sequence of buffalo ACTB, deduced amino acid sequence as highly conserved. Further, the suitability of ACTB as a reliable reference gene for studying the gene expression in the uterine endometrium is validated.

SUMMARY

In the present study, we have cloned 1143 bp of β-actin (ACTB) cDNA (GenBank Acc. No. DQ661647) having an open reading frame of 1128 bp that encodes a peptide of 375 amino acids. The nucleotide as well as deduced amino acid sequence of buffalo ACTB showed > 86 and 97% identity with that of other mammalian species indicating high degree of conservation across the species. Expression of ACTB mRNA in the bubaline endometrial tissues did not vary significantly during different phases of estrous cycle and early pregnancy. This result validated the suitability of ACTB as a reliable reference gene in gene-expression studies in uterine endometrial tissues of buffalo.

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