The mRNA expression study on small amount of *Theileria annulata* lymph node biopsy sample using SMART-cDNA technology

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Abstract:

BACKGROUND: A major issue in many gene expression studies utilizing small amount of biological materials is the limited quantity of RNA purified from clinical samples, which is often used for RT-PCR or standard Northern blot analysis. **OBJECTIVES:** The SMART cDNA synthesis method and subsequent SMART-cDNA-PCR technique was used to analyse 3 genes in macroschizonts of *Theileria annulata* in small lymph node biopsy material. **METHODS:** The SMART-cDNA of *TaSp* gene was cloned in pTZ57R/T-vector and sequenced. We focused on genes encoding surface proteins *TaSp*, *TaD* and *HSP70*. **RESULTS:** Our results showed that SMART cDNA dependably reproduces the expression profile found in messenger RNA. The RT-SMART-PCR showed the amplification of the processed mRNAs. The sequencing analysis showed that the amplified cDNA was coded for *TaSp* protein in *Theileria annulata*. **CONCLUSIONS:** It was concluded that the SMART PCR technique is practical for amplification of complete sequence of mRNAs in the form of cDNAs, and therefore for gene expression studies if only small amounts of starting material are available.

Introduction

Tropical theileriosis caused by the protozoan parasite *T. annulata* is a disease by cattle with high economic losses worldwide (Dolan, 1989). Diagnosis of theileriosis is performed in Iran normally with the traditional Gimsa-staining method with the blood smears, which is accompanied by some problems such as low accuracy in detection of parasitic agent (Shayan et al, 2008). The PCR method is one of the gold standard methods for identification of *Theileria annulata* carrier animals, but for acute phase of disease is not recommended. Diagnosis of acute phase of theileriosis is mostly dependent on the detection of schizonts in the peripheral blood or lymphnode nuclear cells and PCR cannot differentiate between piroplasm and schizont DNA sources. The serological methods such as IFAT and immunoperoxidase (Shayan et al., 2007) are also used for diagnosis of theileriosis. Due to the cross reactivity between different *Theileria* species, the diagnosis methods based on immunogenic antigens need species specific antigens such as species specific recombinant proteins. Cloning of the species specific genes requires enough biological materials. Analysis of differential gene expression in very small quantities of RNA is in increasing demand. A standard Northern blot requires 10 to 15 µg of total RNA or 1 to 4 µg of mRNA. At least 1 µg of total RNA is required to confirm a differential gene expression in an RNAse protection assay. Reverse transcription (RT) and successive cDNA amplification overcomes the problem of limited starting material but does not always generate full length transcripts. Therefore, it is questionable as to whether the resulting DNA patterns are representative of the original RNA.

SMART™ cDNA synthesis is a recently developed method ensuring full length mRNA transcription (Chenchik, 1996). In this method, a customized oligo (dT) primer is used to selectively prime mRNAs in total RNA. Moloney murine leukemia virus reverse transcriptase (MMLV-RT) is employed for the RT reaction. When reaching the 5' -end of the template, the enzyme adds a short oligo (dC) tail to the first strand of the newly synthesized cDNA because of its intrinsic terminal transferase activity. A special oligonucleotide with an oligo (G) tail now hybridizes to the oligo (dC) end. MMLV-RT then switches templates, thereby extending the synthesized cDNA and appending the sequence of the oligonucleotide. Consequently, the resulting cDNA has two defined ends encompassing the full mRNA sequence. These ends serve as primer sites for subsequent PCR amplification. DNA generated by this technique can be regarded as an mRNA equivalent and can be analyzed using standard Southern hybridization (Edery, 1995 and Endege, 1999). Therefore, the method is also called 'virtual Northern' blot. We were keen to employ the technology described above for gene expression analyses in biopsy samples from infected lymphonods with *Theileria annulata*.

Materials and Methods

DNA Extraction and PCR: Total DNA was extracted from a jugular lymph node biopsy of a *Theileria* infected female dairy cow using DNA Extraction kit (MBST, Iran), according to the manufacturer's protocol. Two pair of primers were designed for amplifying the bovine ß-actin (P1/P2) and *T. annulata* 18s rRNA (P3/P4) encoding genes. In two separate PCR programs, approximately 10 ng DNA solution was used for the PCR analysis performed in 100 µL total volume including 10x PCR buffer, 2.5 U Taq polymerase (Cinnagen, Iran), 2 µL of each primer (20 µM, Cinnagen, Iran), 2 µL of each dATP, dTTP, dCTP and dGTP, (200 µM, Fermentas), 1.5 mM MgCl2, in automated Thermo cycler (MWG Biotech Primus, Germany) with the following program: 5 min incubation at 95°C to denature double strand DNA, followed by 38 cycles of 45 s at 94°C (denaturation), 45 s at 55 or 60°C (annealing) and 45 s at 72°C (extension) and an additional extension step at 72°C for 5 min. Annealing temperature used for ß-actin and T.annulata primer sets were 60°C and 55°C, respectively. All of the used primers were listed in Table 1. The expected PCR product length for ß-actin and T.annulata were 639 and 430 bp, respectively. 10 µL of each PCR product was subjected to electrophoresis on a 1.5% agarose gel in TBE buffer and visualized under UV light by ethidium bromide staining.

SMART-Double Strand (DS)-cDNA synthesis: Total RNA was extracted immediately from the second biopsy from the same lymph node described above using TriPure Reagent (Roche, Germany) according to the manufacturer's instruction. One microgram of total RNA was subjected to SMART cDNA synthesis following manufacturer's instruction (Clontech, USA). Ten micro liter of SMART cDNA was amplified with the primer supplied by Kit (Clontech, USA) under the following conditions: Denaturation 1 min at 95°C followed by 27 amplification cycles of 15 s at 95°C (denaturation), 30 s at 65°C (annealing), 6 min at 68°C (extension). Finally, the PCR products were analyzed by agarose gel electrophoresis.

SMART-cDNA-RT-PCR: One µL of SMART-DS-cDNA was amplified using specific primers (P5/P6, P11/P12 and P9/P10, respectively) derived from gene coding for *TaSp*, *TaD* and *HSP70* proteins under the following conditions: Initial denaturing temperature at 95°C for 5 min, 38 cycles of 95°C for 45 sec (denaturation), at 68°C for 45 sec (annealing), at 72°C for 45 sec (elongation) and an additional elongation for 10 min at 72°C. Expected PCR product of amplified *TaSp* , *TAD* and *HSP70* encoding genes should be 941 bp, 533 and 687 bp in length, respectively. 100 µL of *TaSp* PCR product was purified with PCR Product Purification Kit (MBST, Iran) according to the manufacturer's protocol. The purified PCR product was used for nested PCR analysis. The SMART-double cDNA was evaluated with seven primers (p15-P21) derived from complete mRNA of the *HSP70*.

Nested-PCR of *TaSp* encoding gene: In order to confirm that the *TaSp* PCR product was *TaSp* specific, two inner primer sets (P7/P8) were designed (Table 1). The sense primer had at the 5`-end the nucleotide sequences GGATCC which is the recognition site for restriction endonucleasis Bam H I and the antisense primer had at the 5`-end the nucleotide sequences

AAGCTT which is the recognition site for restriction of endonucleasis Hind III. The Nested PCR was runned on 10 ng of purified *TaSp* PCR product as nearly the same program for *TaSp* PCR with the distinct annealing temperature at 62°C. The expected nested PCR product was 704 bp in length. The nested PCR product was subjected to electrophoresis on 1.5% agarose gel and visualized under UV light by ethidium bromide staining.

Cloning in pTZ57R/T vector: 100 µL of *TaSp* nested PCR product was purified using PCR product kit (MBST, Iran) according to the manufacturer's protocol. Four µL of the purified nested PCR product was added to the solution containing 3 µL of plasmid vector pTZ57R/T DNA (0.18 pmol ends), 3 µL of 5X ligation buffer, 1 µL T4 DNA Ligase and 19 µL de ionized water, then incubated at 22°C for 1 hour.

Preparation of competent cells and Transfection: For preparation of *E. coli* competent cells, 300 µL of overnight grown *E .coli* DH5-a culture were added to 20 mL LB-medium and incubated for about 3 hours in 37°C with shaking condition until the OD650 of 0.6-0.8 was achieved. The bacteria-culture was then transferred in small portions into 1.5 mL micro tubes and centrifuged at 5000 rpm for 1 minute. Two hundred micro liter of 0.1 M MgCl2 were added to each pellet and incubated on ice for 15 minutes. After centrifugation for 1 min at 5000 rpm, 600 µL of 0.1 M CaCl2 was added to each pellet, mixed well and incubated again for 20 min on ice. After centrifugation for 1 min at 5000 rpm, the pellet was re-suspended in 60 µL of 0.5 M CaCl2. For transfection, 5 µL of ligation mixture containing the recombinant plasmid was mixed with 20 µL of competent cells and incubated on ice for 15 min. The mixture was then incubated first at 42°C for 90 seconds in water bath and transferred immediately into a dish containing ice. After 90 seconds, 500 µL of LB medium was added to the reaction and incubated for 1.5 hours at 37°C. Finally, 10 µL of the reaction was cultured overnight at 37°C on LB-Ampicillin-agar medium.

Amplification of Insert DNA: One colony transfected with recombinant plasmid was transferred into 10 mL LB-Ampicillin medium and incubated overnight at 37°C under shaking condition. One micro liter of liquid grown culture used in a PCR program using specific multi cloning site flanking primers (P13/P14) for amplification of the 704 bp *TaSp* inserted DNA inserted in multi cloning site of pTZ57R/T vector. PCR was performed as nearly the same program for *TaSp* PCR with the distinct annealing temperature at 54°C. Ten µL of PCR product was subjected to electrophoresis on 1.5 % agarose gel and visualized using ethidium bromide under UV light. Expected PCR product was 821 bp in length. The recombinant plasmid containing *TaSp* was extracted in large quantity using the Plasmid Extraction Kit (MBST, Iran) according to the manufacturer's protocol.

Restriction endonucleasis Treatment: Fifteen microliter solution containing 10 µg of extracted recombinant plasmid used for restriction endonucleasis treatment in 20 microliter reaction including 1 µL restriction endonucleasis BamH I (10 U/µL, Fermentas), 1 µL restriction endonucleasis Hind III (10 U/µL, Fermentas), 2 µL restriction endonuclease buffer and 1 µL de ionized water and incubated at 37°C for one hour. Five micro liter of the reaction was subjected to the gel electrophoresis on 1.5 % agarose gel and visualized using ethidium bromide under UV light.

Nucleotide sequence analysis: The extracted recombinant plasmid was first analyzed on agarose gel under UV condition and sequenced according to the Sanger Method by Kowsar Company (Iran).

Results

After DNA extraction from a jugular lymph node biopsy of a *Theileria* infected female dairy cow, two separate PCR reactions with two pair of primers designed for amplifying the bovine ß-actin and *T. annulata* 18s rRNA encoding genes were done. As expected , analysis of 10 µL of each PCR product with electrophoresis, showed PCR product length of ß-actin and T.annulata was 639 and 430 bp, respectively (Data not shown). After the confirmation of *Theileria* infection with PCR, Total RNA was extracted from the second biopsy and used for SMART-DS-cDNA synthesis as mentiond above. Ten micro liter of total SMART-DScDNAs was subjected for electrophoresis on 1.5 % agarose gel and visualized under UV light by ethidium bromide staining. The smear pattern showed the presence of total Double strand SMART-cDNAs (Figure 1, Left). *HSP70* encoding

Table 1. The nucleotide sequence of used primers for PCR applications.

No	Name of primer	Accession no.	Nucleotide sequences
P1	Bovine β-actin sense	NM_173979	5' atcactgccctggcacccag 3'
P2	Bovine β-actin antisense	NM_173979	5' cttagagagaagcggggtggc3'
P3	*T. annulata* sense	AY150056.3	5' cacagggaggtagtgacaag 3'
P4	*T. annulata* antisense	AY150056.3	5' ctaagaatttcacctctgacag 3'
P5	*TaSp* sense	AJ316249.1	5'atgaaattcttctacctttttgttctatttcca 3'
P6	*TaSp* antisense	AJ316249.1	5'acaacaatcttcgttaatgcg 3'
P7	*Tasp* sense	AJ316249.1	5' atcggatcccctatcgattttgatcccaatgatg 3'
P8	*Tasp* antisense	AJ316249.1	5' atcaagctttcagtccaatgcataagcacag 3'
P9	*Hsp70*-sense	J04653.1	5 caagctgtg acgaacccagag 3
P10	*Hsp70*-anti-sense	J04653.1	5 ctggtctgctgttataaacggcaagttaa 3
P11	*TaD*-sense	AJ784421	5 atgaaattga ccgctggatt 3
P12	*TaD*-anti-sense	AJ784421	5 gaaccgcccgaggcttcaccttt 3
P13	Vector pTZ57R/T- sequencing sense	pBR322	5` gaattcgagctcggtacctc 3`
P14	Vector pTZ57R/T- sequencing antisense	pBR322	5` accatgattacgccaagctc 3`
P15	Sal sense	J04653.1	5` actaaatatgttaattgttac 3`
P16	Sal antisense	J04653.1	5` ggtaaataaattatttattagg 3`
P17	Sam sense	J04653.1	5` cacaaaacaagcaatactcacc 3`
P18	Sam antisense	J04653.1	5` ctttgctaaaatatacgctct 3`
P19	Sau sense	J04653.1	5` cctcgtgggaggaatgacaag 3`
P20	Sau antisense	J04653.1	5` aggtgactgaagtagtcaaaa 3`
P21	Sagu antisense	J04653.1	5` gcgtcgaaaagcaactcactga 3`

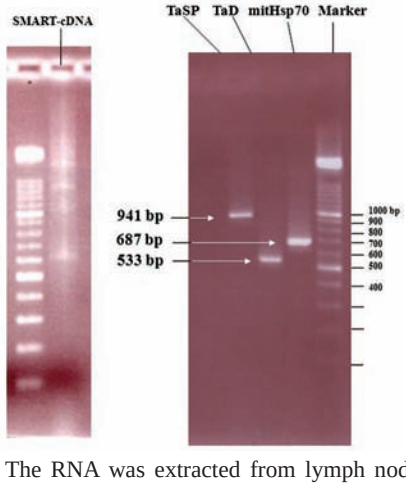

Figure 1. The RNA was extracted from lymph node biopsy material and used for preparing of SMART-double strand-cDNA (at the left side). The prepared SMART-double strand cDNA was amplified using primers (P5/P6 for TaSP), (P9/P10 for HSP70) and (P11/P12 for TaD).

gene was selected for accuracy of SMART-DS-cDNA synthesis procedure. For this aim seven primer pairs (P15-P21, Table 1) were designed for amplifying different regions of *HSP70* SMART-cDNA with overlapping regions between the corresponding PCR products (Figure 2). Six separate SMART-PCR reactions resulted in PCR products with 1401 bp, 734 bp, 722 bp, 641 bp, 54 bp and 50 bp in length (Figure 3). These expected PCR products confirmed that the complete sequence of *HSP70* mRNAs was transcripted to the SMART-DS-cDNA. To prove whether the SMARTcDNA probe was representative for all mRNAs, the SMART-cDNA was amplified with the primer pairs derived from the genes coding for *TaSP*, *TAD* and *HSP70* proteins (Table 1) resulting in PCR products of 941 bp, 533 bp and 687 bp in length, respectively (Figure 1, Right). The PCR product of *TaSp* gene with the 704 bp in length was separately purified and cloned in pTZ57R/T vector. The plasmid DNA was isolated and was digested with the restriction endonucleasis Hind III and BamH I and analysed on the agarose gel (Figure 4, at the right sides). The recombinant plasmid was amplified with the primer pair derived from the flanking region of multicloning side of the vector resulting in a PCR product of 821 bp in length (Figure 4, at the left side, lanes 2, 3, 6 and 8). The PCR results showed that the *TaSp* DNA of 704 bp in length was successfully cloned in the vector. The PCR amplification of

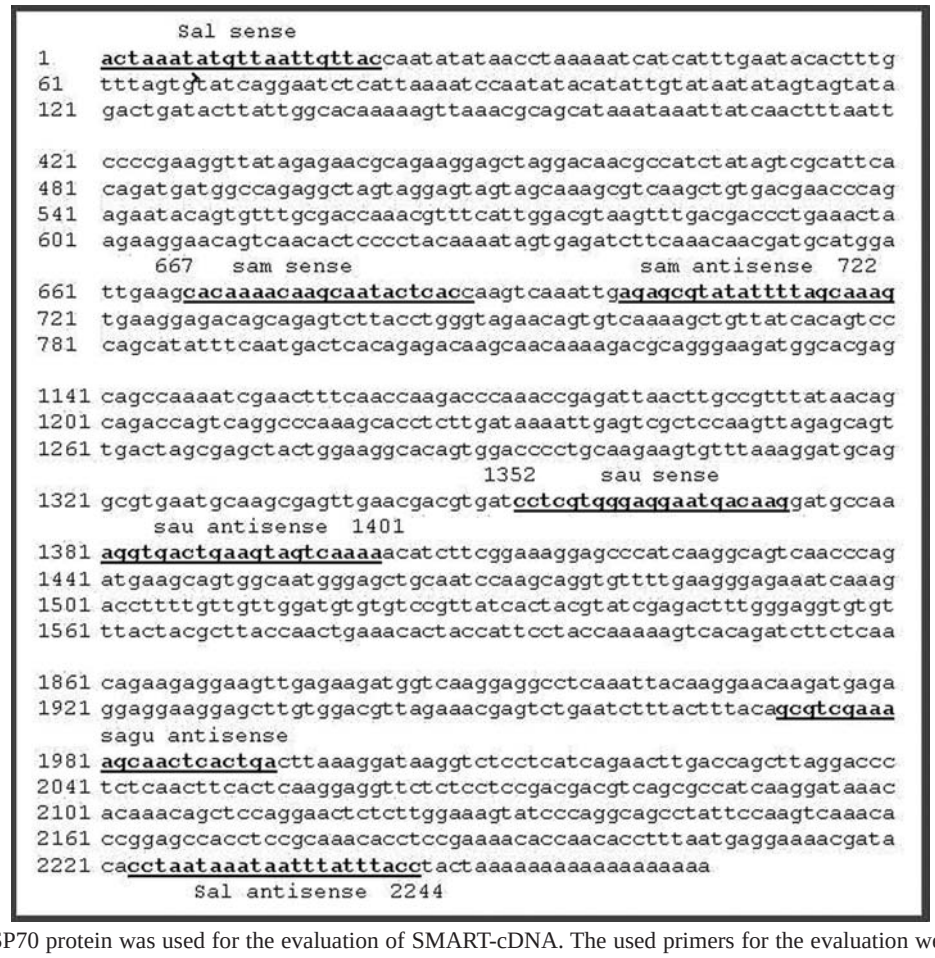

Figure 2. The HSP70 protein was used for the evaluation of SMART-cDNA. The used primers for the evaluation were marked on the nucleotide sequence as boiled and underlined regions.

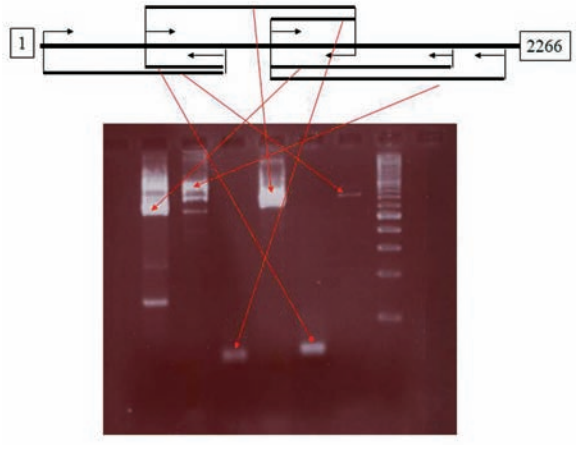

Figure 3. The SMART-double strand cDNA was amplified with different primers derived from the complete mRNA of HSP70 with 2266 b in length. The primers used were given on the top of the figure in schematic form.

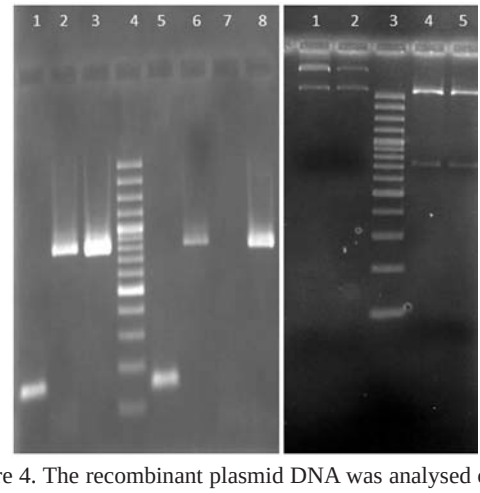

Figure 4. The recombinant plasmid DNA was analysed on the agarose gel (figure at the right side, lane 1 and 2). The recombinant TaSp-pTZ57R/T-vector was cut with the restriction endonucleasis BamH I and Hind III and analysed on agarose gel (figure at the at right side, lanes 4 and 5). Figure at the left side: The recombinant TaSp-pTZ57R/T-vector was amplified with the primers flanking the multi cloning site (13/14), lanes 2, 3, 6 and 8 showed the TaSp-insert-DNA. The lanes 1 and 5 showed the amplificates from the pTZ57R/T-vector without insert DNA.

plasmid DNA without insert resulted in the expected PCR product of 117 bp in length (Figure 4, at the left side, lanes 1 and 5). The recombinant plasmid DNA

was sequenced and the sequenced data was registered under accession number JQ003240 in GenBank. The nucleotide sequence analysis showed that the nucleotide sequence of insert DNA had 94% homology to the *Theileria annulata TaSp* gene registered under accession number AJ316249.1 in GenBank.

Discussion

Tropical theileriosis caused by *Theileria annulata* is a severe disease for cattle with high economic losses in animal husbandry worldwide (Dolan, 1989). Control of this disease is of great importance in animal health management. *Theileria annulata* is transmitted predominantly by Hyalomma ticks, which were most abundant in each zone, especially in the mountainous area of Iran (Nabian et al., 2009 and Rahbari et al., 2009). One of the interesting aspects of parasite-host interaction by *Theileria annulata* is that the shizont-infected mononuclear cells transform to the immortal proliferating cells (Brown, 1995). The molecular mechanisms involved in the signal transduction causing immortal status of the infected cells are unknown. In many cases there is only a small amount of biological material for the molecular studies based on the expression status of the certain gene available. For such cases, it is desirable to prepare the mRNAs in the form of amplificable cDNAs which are representative of the original mRNAs. In the case of lymph node biopsy from *Theileria annulata* infected cattle, we used SMART-cDNA technology to prepare the amplificable cDNAs from the whole mRNAs isolated from the biopsy. There are methods like Oligocapping (Kato et al., 1994 and Maruyama et al., 1994), CAPture (Edery, 1995) and CAP-trapper (Carninci, 1996) to produce full-length cDNA sequences but because of some difficulties like multi-step manipulations of the cap structure and mRNA and cDNA intermediates, or degradation of mRNA or cDNA-RNA duplexes, these methods are not suitable enough, especially for cases in which starting material is limited (Hung et al., 1999 and Zhu et al., 2001). One of the disadvantages of the current methods for construction of cDNA libraries, especially when poly (dT) primer is used, is that they may lead to the under-representation of the 5' -end of genes during the first strand cDNA synthesis (Schmidt et al., 1999). The SMART-double strand cDNA prepared from biopsy material was evaluated with the gene coding for *HSP70*. All primers derived from different parts of this gene could amplify the expected PCR product, revealing the presence of representative cDNA for complete *HSP70* mRNA. For further evaluation the SMART-double strand cDNAs representative for genes encoding *TaSp* and *TaD* proteins could be shown within the prepared SMART-cDNA. *HSP70*, *TaSp* and *TaD* belong to the proteins expressed by *Theileria* schizonts (Schnittger et al., 2000, Schnittger et al., 2002 and Schneider et al., 2004). Production of recombinant proteins in recent years has led to the development of new enzyme-linked immunosorbant assays (ELISA) for detection of several important *Theileria* species such as T. parva and *T. annulata* (Katende et al., 1998 and Gubbles et al., 1999). The characterization of *T. annulata* surface protein *TaSp* and its followed application in indirect ELISA makes *TaSp* protein as a serious candidate molecule for the development of detecting and/or controlling tools (Bakheit, 2004 and ,Seitzer et al., 2004). Application of *T. annulata* surface protein in an indirect *TaSp*-ELISA in Sudan showed a sensitivity of 99.1% and specificity of 90.47% compared to the IFAT test which was used as a reference test there (Salih et al., 2005). The SMART-double strand cDNA was cloned in the pTZ57R/T vector and its nucleotide sequence was determined. The nucleotide sequence analysis showed 94% homology to the *Theileria annulata TaSp* gene registered under accession number AJ316249.1 in GenBank. Translation of the presented *Tasp* sequence to amino acid level using Expasy Protein Translation tool revealed that the translated protein was also comparable to the abovementioned *TaSp* protein from GenBank. Our results showed that the SMART-cDNA technology can be used as a suitable method for the generation of amplificable cDNAs representative of the original mRNAs if a small amount of biological material is available.

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مجله طب دامی ایران، ۱۳۹۲، دوره ۷، شماره ۱، ۲۲-۱۵

مطالعه بیان mRNA در مقدار کم نمونه اخذ شده از عقده لنفاوی آلوده به تیلریا آنولاتا با استفاده از تکنیک SMART-cDNA

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چکیده

زمینه مطالعه: یکی از مشکلات در تحقیقات مرتبط با بیان ژنی که با روش های RT-PCR و یا Northern blot آنالیز می گردند، مقادیر کم مواد بیولوژیک می باشد. **هدف:** در مطالعه حاضر از روش سنتز SMART-cDNA و تکنیک SMART-cDNA-PCR برای بررسی بیان سه ژن مرحله ماکروشیزونتی انگل تیلریا آنولاتا در مقدار کم نمونه اخذ شده از عقده لنفاوی استفاده شد. **روش کار:** سه ژن مورد بررسی در این مطالعه بیان کننده سه پروتئین TaSp, TaD و HSP70 بودند. با روش RT-SMART-PCR، cDNA های کامل از mRNA های پردازش شده با موفقیت تکثیر شدند. **نتایج:** نتایج نشان داد که روش SMART-cDNA می تواند الگوهای mRNA را بصورت cDNA بطور کامل رونوشت برداری کند. SMART-cDNA مشتق شده از ژن TaSp در وکتور pTZ57R/T کلون و تعیین توالی شد. توالی نوکلئوتیدی قطعه DNA کلون شده نشان داد که این توالی با توالی نوکلئوتیدی کدکننده پروتئین TaSp همخوانی دارد. **نتیجه گیری نهایی:** نتایج نشان داد که تکنیک SMART-PCR روشی کاربردی جهت تکثیر توالی کامل mRNAs بصورت cDNAs می باشد و می تواند برای تحقیقات بیان ژن از مقادیر کم مواد بیولوژیک بخوبی مورد استفاده قرار گیرد.

واژه های کلیدی: تیلریا آنولاتا، SMART-cDNA, TaSp, TaD, HSP70

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