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Semi-quantitative Analysis of Expression of Various Genes in relation to Possible Markers for *Theileria annulata* Attenuation

Habibi*[1], G.R., Esmaeil Nia[1], K., Bozorgi[1], S., Hashemi-Fesharki[1], R. Bordbar[2], N.

1. Department of Protozoology & Protozoal Vaccine Production, Razi Vaccine and Serum Research Institute, Karaj, Iran
2. Department of Quality control, Razi Vaccine and Serum Research Institute, Karaj, Iran

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ABSTRACT

The sporozoites of *Theileria annulata* invade bovine MHC II cells, where they differentiate into schizonts. The later can immortalize and induce fundamental changes in their host cells. Live attenuated vaccine is an important way of controlling *T. annulata* infection of cattle. Production is by prolonged cultivation of macroschizont-infected cells. The mechanisms underlying this transformation are not understood. The objects of this work were to analyze the expression levels of MMPs, Pro-inflammatory cytokines, Tams1 and TashHN genes in relation to possible markers for *Theileria annulata* attenuation. Semi-quantitative polymerase chain reaction (RT-PCR) was applied to quantify and compare variations in gene expression level among different passage numbers of three cell lines. The results of this study demonstrated that the infected cells show detectable specific transcripts for MMP9 in low passage cultures, but it decreased in long term passages (S15 vaccine strain and high passage number of C1 and C2 cell lines). The analyses of three available cell lines indicated detectable amount of specific mRNAs for TashHN. Tams1 specific transcripts were detected in low passage number of C1 and C2 cell lines, but not obtained in attenuated S15 vaccine and prolonged culture of C1 and C2 cell lines. Two pro-inflammatory cytokines, IL-1-beta and TNF-alpha, were detected with high fluctuations in all three *T. annulata* infected cell lines, both in low and high passage number. In conclusion, the results of this work clearly showed that the level of MMP9 transcripts is in contrast with the amounts of Tams1 mRNAs in *T.annulata* schizont infected cell lines that might be considered for virulence and attenuation respectively. Understanding the mechanisms of virulence and attenuation of infected cell line by using molecular biology methods and *in vivo* animal experiments could help to increase our knowledge about attenuation mechanisms and preparing and identifying appropriate cell lines in order to develop the new *T. annulata* vaccine cell lines.

Keywords: *Theileria annulata*, Gene Expression, Matrix metalloproteinase, Cell line, Attenuation, Vaccine

INTRODUCTION

Tropical Theileriosis represents a serious threat to cattle health in tropical and sub-tropical regions of the world, with some 240 million animals at risk (Gill *et al* 1980, Glass & Jensen 2007). *Theileria annulata* infects bovine leukocytes and stimulates them to proliferate in a cancer-like manner (Bishop *et al* 2004, Dubbelaere *et al* 1988, Ahmed *et al*

*Author for correspondence. E-mail: g.habibi@rvsri.ir

1984, Spooner *et al* 1989; Glass *et al* 1989). The schizont-infected cells became disseminated rapidly through the lymphoid tissues from the lymph node draining the site of inoculation to distant lymph nodes and to non-lymphoid organs (Forsyth *et al* 1999). The virulence of *T.annulata* infected cells can be attenuated by prolonged *in vitro* cultivation and this process is used to generate effective live vaccines (Hall 1988, Tait & Hall 1990, Singh 1990). As infection progressed, the schizonts differentiated into merozoites, but the parasites within attenuated cell lines have been reported to lose the ability to differentiate to piroplasms *in vivo*, following prolonged *in vitro* culture (Pipano 1974). The mechanisms of infected cell lines transformation and metastasis are not completely understood, but some hypotheses are discussed. Available evidences indicate that the parasite up-regulates a number of signaling pathways promoting cell proliferation and stimulatory down-regulations apoptotic pathways (Dobbelaere *et al* 2000, Hall *et al* 1999 and Ahmed *et al* 1999). Alteration in level of Matrix metalloproteinases gene expression is more studied and demonstrated (Somerville *et al* 1998b, Hall *et al* 1999, Adamson *et al* 2000a & 2000b). Pro-inflammatory cytokines are the other postulated mechanism in both pathology and immune evasion (Ahmed 2002, Graham *et al* 2001). Recent studies with *T. annulata* have identified a family of DNA-binding polypeptides, some members of which are exported from the schizont into host cell nucleus (Swan *et al* 1999, Swan et al. 2001, Dobbelaere *et al* 2000). This family of AT hook proteins and particularly *T. annulata* schizont host nucleoproteins (TashHN) are shown to be involved in cell transformation and proliferation (Swan *et al* 1999). The specific transcripts of TashHNs decreased during differentiation to the merozoite. On the other hand, severely attenuated cell lines showed a substantial increase in TashHN expression (Swan *et al* 2003). In order to answer this question" How we can estimate the level of virulence or attenuation of new isolated *T. annulata* infected cell lines for future preclinical and immunological studies?" we decided to measure, the level of discussed protozoa and host gene expression, involved in virulence attenuation alteration. Thereby, we performed analysis of MMP2, 9, 13, TashHN, IL-1-beta, TNF-alpha and Tams1 genes at transcriptional level in three particular Iranian S15 vaccine strain and two isolated C1 and C2 *T. annulata* infected cell lines.

MATERIALS AND METHODS

***Theileria annulata* infected cell lines.** Three *T. annulata* infected cell lines "S15 vaccine strain", C1 and C2 were used in this study. S15 strain has been used for live attenuated vaccine production in Iran more than three decades (Hashemi-Fesharki 1998). This vaccine strain is a local cell line and has been attenuated after 260 passages (Hashemi-Fesharki 1988). The two *T.annulata* infected cell lines "C1" and "C2" were isolated and established in protozoal vaccine research and production department of Razi institute in 2006 from Boein-Zahra region in Qazvine province of Iran.

Cell culture and passage. C1 and C2 *T. annulata* infected cell line were isolated from two splenectomized Friesian calves experimentally infected and cell culture was performed as previously described (Hashemi-Fesharki 1988) with some modifications. Briefly, lymph node biopsy was washed with 5X Penicillin streptomycin, finely minced and were centrifuged at 400 × g for 10 min and the supernatant was discarded. The pellets were washed twice with Stoker media and resuspended in complete Stoker medium (added 10% bovine serum, Penicillin 100 IU/ml and Streptomycin 100 μg per ml). Cell pellets were resuspended in the same medium and cultured in 25-Cm^2 culture flasks in humidified atmosphere at 37 °C, under 5% CO_2 until they reached confluence. Preparing cell culture passage or subcultures could be made by

simply transferring an adequate number of cells to establish a culture, into a new tissue culture flask and fresh medium was supplied after 4 to 5 days. Three *T. annulata* infected cell lines were grown in different passage numbers (between 3 and 100); cells were harvested at the end of the culture period and were preserved in deep freeze, until total RNA extraction and further gene expression analysis.

***In vivo* assessment of cell line virulency.** Two Friesian calves were injected subcutaneously with ten millions of grown C2 *T.annulata* infected cell line in passage 40. Animals were observed for 8 weeks. Animals were observed for clinical theileriosis in eight weeks. Rectal temperature was daily recorded. Mucosa and conjunctiva were inspected for petechial hemorrhages. The pre-scapular lymph node was examined. Biopsy material and blood smears were taken and examined for schizont and piroplasm forms of *T. annulata*.

***Theileria annulata* infected cell line heat induction.** The S15, C1 and C2 macroschizont-infected cell lines (low, medium and high passage numbers) were grown in stoker medium at 37°C, after that the grown cultures were induced to differentiate at 41°C (Shiels et al. 1994). After 2 days incubation at 41°C, the cultures were centrifuged (300 g) and harvested. The cell pellets were used for RNA isolation and further gene expression analysis.

RNA extraction and Reverse Transcription-PCR. Total RNA was isolated using the TRIzol extraction reagent (Life Technologies). 2.5 ml of TRIzol was added to concentrated macroschizont infected cell cultures from 25 Cm^2 flasks. Following isolation, RNA was dissolved in distilled water and quantified by UV-spectrophotometry. Total RNA was treated by RNase free DNase. All DNase treated RNAs were confirmed using GAPDH primers to make sure this treatment was complete. These steps were done to ensure that no DNA remains in the RNA sample. The quality of RNA samples was confirmed by observation of ribosomal RNA integrity following electrophoresis and ethidium bromide staining (Chomczynski and Sacchi 1987, Sambrook *et al* 1989). Subsequently, RNAs were used for reverse-transcription polymerase chain reaction (RT- PCR). Complementary DNA was prepared from total DNase treated RNA using a reverse transcription system (Fermentas, Germany). Briefly, 2μg of total DNase treated RNA, 5X RT buffer, 20 U RNase Inhibitor were added to 200 Units of RevertAid ^{TM} M-MuLV reverse transcriptase, 500 μM each of dNTP, 160 pm of oligo $(dT)_{15}$ primer, and 5 mM $MgCl_2$ in a total volume of 20 μl. The reaction was incubated at 42 ^oC for 1 hour, followed by 10 min at 70^oC to inactivate the enzyme.

Polymerase Chain Reaction. An aliquot of 2 μl of the reverse transcription reaction solution was amplified by PCR, using 1 U Taq DNA Polymerase (Fermentas) in a 20 μl reaction volume that contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 μM of the primers for the template of interest. Specific primers for target mRNAs are shown in table1 (Balcerzak *et al* 2001). All oligo nucleotide primers were synthesized by Cinnagen^{TM} (Tehran, Iran). Reaction mixtures were amplified in a RG1-96 thermocycler (Corbett Research, Sydney, Australia): The initial denaturation step was 2 min at 95 ^oC, each cycle consisted of a denaturing step of 15 seconds at 95 ^oC, an annealing step of 45 seconds at 57^oC, and an extension step of 45 seconds at 72 ^oC, followed by final extension step of 5 min at 72 ^oC. The number of cycles of amplification required to obtain a detectable signal is dependent on the abundance of the mRNA in the sample material; 28 to 34 cycles were required for the different gene products. Identity of the PCR products was confirmed by restriction digestion and analysis of the fragments on agarose gels.

Quantification of cytokine mRNA expression (analysis of amplified DNA). After the PCR amplification, 8 μl of the samples with 2 μl of a

tracking dye were run on a 2% agarose gel containing 1 µg/ml ethidium bromide. The PCR products were scanned (Uvidoc, Gel Documentation System, Cambridge, UK) and the amount of PCR products present in each lane was determined using UVIdoc Software Version 12.5. The intensity of bands was measured by densitometry and normalized on the basis of the GAPDH expression. Under ideal or theoretical conditions, the amount of PCR products will be double during each cycle of the PCR reaction. The relative initial amounts of a target sequence and the endogenous standard can be determined. A different number of amplification cycles for target and standard sequences are often required (Habibi *et al* 2001).

Statistical Analysis. Data were analyzed using the Microsoft Excel program. Data were pooled and the means were calculated with the Microsoft Excel graphing. Correlation analysis was performed to determine whether a relationship existed between the six measurements of target mRNAs.

RESULTS

The results of *in vivo* study of cell line virulency. No severe symptoms of bovine theileriosis were observed clinically including raise of body temperature, weakness, weight loss, anorexia, petechia on the conjunctival mucosa, swollen lymph nodes and anemia and microscopically in red blood cells, lymph nodes biopsies after injection of C2 *T.annulata* infected cell line at passage 40 in two injected Friesian calves.

The results of PCR. The PCRs were optimized for each gene considering the exponential phase of amplification regarding to the needed cycle numbers for amplification (Figure 1). PCR products were confirmed by expected PCR product size, and validated by restriction enzyme analysis by using appropriate enzymes (Figure 2).

Table 1. PCR Primers for Gene Expression Detection and Quantification, and amplified products of bovine Matrix metalloproteinases, TashHN, TNF-alpha, IL-1-beta, GAPDH and Tams1.

Target mRNA	Primer sequences	GenBank Accession no.	PCR fragments
MMP2	5' CTT CCC CCG CCA GCC CAA GTG GG	AF135231	510
	3' GGT GAA CAG GGC TTC ATG GGG GC		
MMP9	5' ACG TGG ACA TCT TCG ACG C	AF135234	359
	3' CGA ACC TCC AGA AGC TCT GC		
MMP13	5' TCT GGT CTG CTG GCT CAC GC	AF135235	472
	3' TAG GCA GCA TCA ATA CGG TTG G		
GAPDH	5' CCA TCA CCA TCT TCC AGG AGC	AJ000039	309
	3' TCA TAA GTC CCT CCA CGA TGC		
TashHN	5' ACC ACC GAT CCC TTC AAC ACG	AJ564565	252
	3' TCC AGA GGC ACC TCT ACT TGC		
TNF-alpha	5' CCT CTG GTT CAA ACA CTC AGG	Z48808	252
	3' GGT GTG GGT GAG GAA CAA GG		
IL-1-beta	5' GCA AAC TCC AGG ACA GAG AGC	M35589	350
	3' AGT GCT GAT GTA CCA GTT AGG G		
Tams1	5' CGA GAC CTA CTA CGA TGA AG	Z48739	472
	3' GAT AAG TTG TTA CGA ACA TGG		

The results of MMP gene expression. In different passages of C1 *T. annulata* infected cell line the level of expression for MMP9 and 13 was up-regulated in low level passages, but decreased after passages 15 entirely. But MMP2 showed fluctuation in level of expression during low and high passages. In different passages of C2 *T. annulata* infected cell line all three MMPs studied in this research exhibited high level of expression in low passage numbers and showed severe decreasing in expression after passage 20. In different passages of

S15 *T. annulata* infected cell line the cell line showed low level of expression for two MMP9 and 13 but not for MMP2 (Figure 3).

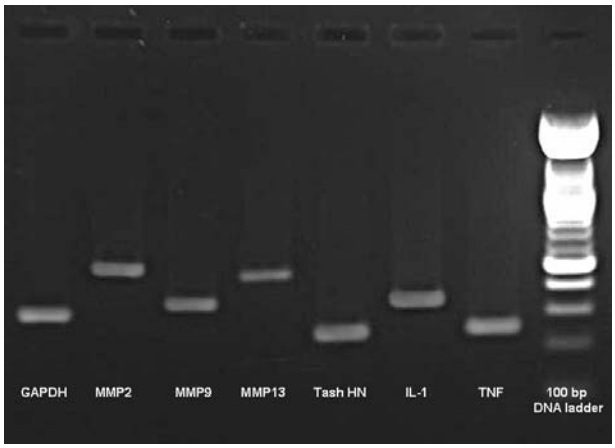

Figure 1. Gel agarose electrophoresis of PCR optimization of MMPs, TashHN, Pro-inflammatory cytokines and bovine GAPDH genes.

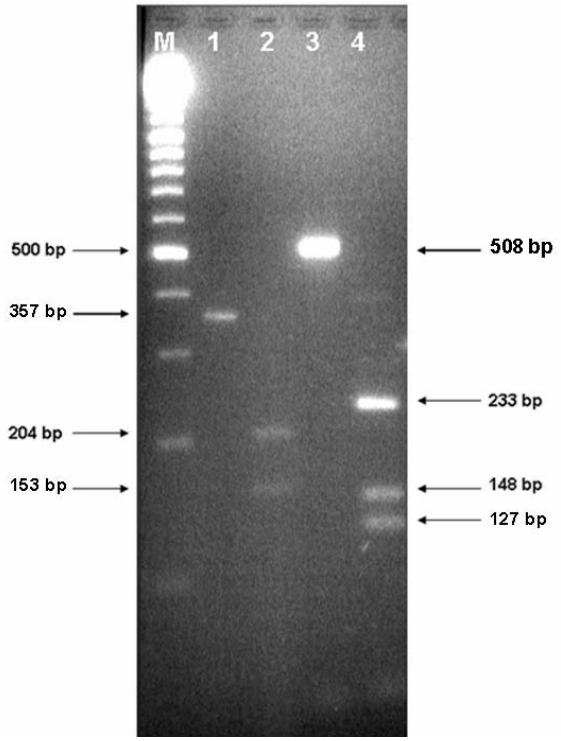

Figure 2. Gel agarose electrophoresis of MMP9 and MMP2 digestion by Hinf I restriction enzyme. Lane M, 100 bp DNA size marker; lane 1, MMP9 before digestion; lane 2, MMP9 after digestion (204 and 153 bp fragments); lane 3, MMP2 before digestion and lane 4, MMP2 after digestion (233, 148 and 127 bp fragments).

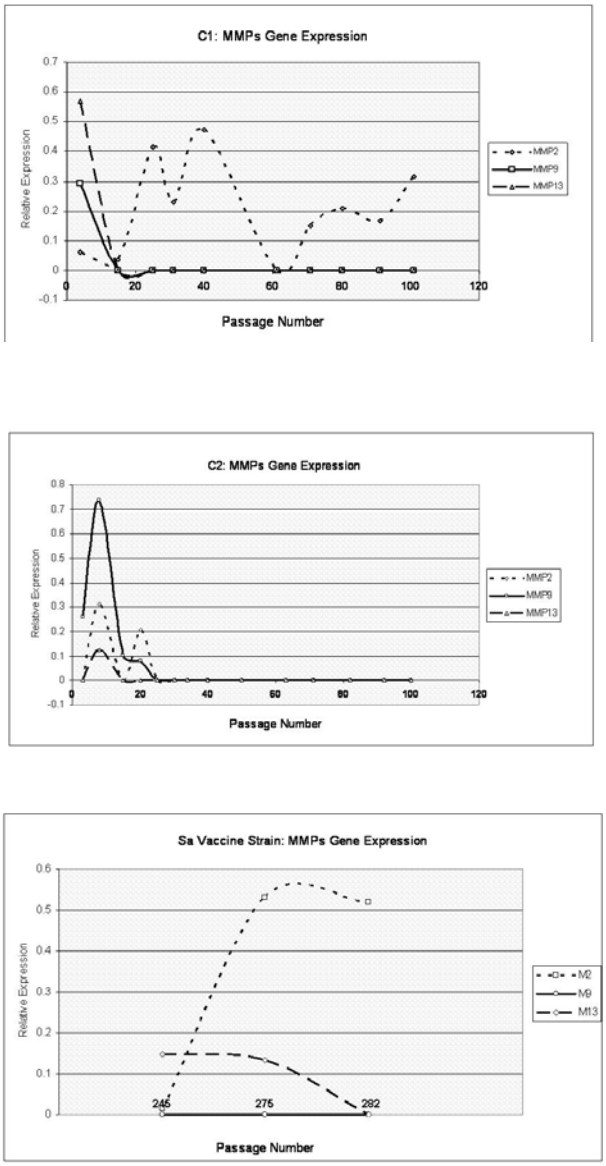

Figure 3. The level of matrix metalloproteinases (MMP2, 9 and 13) relative gene expression in three examined cell lines "C1, C2 and S15 vaccine strain" in different passages

The results of TashHN gene expression in different passages of C1, C2 and S15 *T. annulata* infected cell lines. All three studied Theileria infected cell lines showed different level of expression for TashHN gene expression all through examined passages numbers.

The results of Pro-inflammatory cytokines gene expression in different passages of C1, C2

and S15 *T. annulata* infected cell lines. All three studied Theileria infected cell lines showed fluctuation in expression for both IL-1-beta and TNF-alpha gene transcripts through examined passages numbers.

The results of Tams1 gene expression. In different passages of C1 and C2 *T. annulata* infected cell line both cell lines showed specific transcripts for Tams1 in low passages numbers, but not in medium and high passage cultures. In addition, different passages of S15 vaccine cell line showed no specific Tams1 RNA.

The results of correlation analysis. The correlation coefficient of IL-1 beta and TNF-alpha in each cell line, were 0.85 and 0.77 for C1 and C2 respectively, but the correlation coefficient of TashHN, IL-1 and TNF between two C1 and C2 cell lines ranged between 0.38 and 0.42 and did not reveal statistically significant associations.

DISCUSSION

Prolonged passage of *Theileria annulata* infected cell lines resulted in reduced genetic diversity and this loss in diversity may account for low capacity to provide protection against challenge with heterologous parasite isolates (Morrison & McKeever 2006). Furthermore, there are some evidences of antigenic heterogeneity among isolated of *T.annulata*, by evaluating of immune status of inoculated animals with attenuated infected *T.annulata* cell lines (Pipano 1974, Darghouth *et al* 1996). Despite of limitations, attenuated parasitized cell lines have been used successfully in the number of countries. Therefore, developing the attenuated *T. annulata* vaccine cell line requires to availability of attenuated, potent and heterologous parasitized cell lines. S15 *T. annulata* vaccine strain has been used for commercial bovine theileriosis vaccine production in Iran since 1973 (Hashemi-Fesharki and Shad-Del 1973). Two new *T. annulata* schizont infected cell lines C1 and C2 have been isolated in 2006; subcultures were prepared to more than 100 passages. In this study we have evaluated the role of some involved genes in attenuation, virulence and cell transformation in three Iranian parasitized cell lines at mRNA level by using semi-quantitative RT-PCR. Analysis of virulence and gene expression indicated that prolonged *in vitro* culture of *T. annulata* infected cell lines results in their attenuation and this process is associated with alteration in both host and parasite gene expression (Baylis *et al* 1995, Adamson & Hall 1997, Somerville *et al* 1998a, Ahmed *et al* 1999 and Adamson *et al* 2000a). Whereas, a loss in bovine MMP expression in high passage cultures suggests that these parasite induced MMPs might be virulence factors (Somerville *et al* 1998b, Hall *et al* 1999 & Adamson *et al* 2000b). Two C1 and C2 cell lines were shown virulence in low passage cultures when they were obtained from two infected calves with severe bovine theileriosis, and have been attenuated after 40 passages when they were experimentally injected to two susceptible intact Holstein Friesian calves. Whereas, S15 vaccine strain was demonstrated virulent in low passage culture clinically (Hashemi-Fesharki 1973) and attenuated in high passage culture by inoculating to intact calves (data not shown). However, S15 vaccine strain was available no more than in high passage numbers. Here we demonstrated that gene expression profiles differ between low and high passage numbers of the Iranian *T. annulata* macroschizont infected cell lines. The attenuated S15 cell line was found with a marked reduction in MMP9 expression in the passage numbers of 245 to 282. In parallel with this, two *Theileria* infected C1 and C2 cell lines showed clear reduction of MMP9 in high passages (25 and more) but high level of MMP9 expression in low passage numbers (below 20) (Figure 3).Study on vaccine cell lines showed alteration results in the loss of MMP activity and this may partly explain the loss of virulence (Baylis *et al* 1995 and Somerville *et al* 1998b). An

interesting comparison of MMP gene expression and virulent status between two *T. annulata* parasitized cell lines have been reported, that Uzbek strain showed completely attenuated on passage 80 with low level of MMP activity. In contrast, there was no direct correlation between virulence and proteolytic activity in Israeli strain. However, finding a parasite strain to be used for vaccine production, *in vivo* studies will be remains the most reliable method to assess the degree of attenuation (Shkap *et al* 2003). Up-regulation of the major merozoite/piroplasm surface antigen occurs at merogony and can be induced by culturing schizont-infected lymphocytes at 42 °C (Skilton *et al* 2000). Analysis of Tams1 gene expression in low and high passage cultures of three *T. annulata* infected cell lines demonstrated loss in attenuated S15 vaccine strain and two long term passage parasites C1 and C2, but there were detectable levels of Tams1 RNAs in both C1 and C2 at low passage cultures. This result is in agreement with those found by Pipano (1974), who demonstrated that attenuated *T. annulata* infected cell lines lose their ability to differentiate into merogony stage, therefore, it might be concluded that Tams1 gene as specific piroplasm antigen could not be transcribed in attenuated cell lines. Pro-inflammatory cytokines are the other postulated subject in explanation of attenuation/virulence mechanism in both pathology and immune evasion (Graham *et al* 2001). We have experienced this hypothesis and evaluated macrophage origin cytokines (IL-1-beta and TNF-alpha) gene expression to evaluate virulence of three Iranian infected *T. annulata* cell lines. The results showed all three cell lines expressed different levels of Pro-inflammatory cytokines with no direct association to passage number. However, previous findings suggests both high and low cytokine expressing cell lines protected cattle against heterologous challenge infection, offering no direct correlation of cytokine expression and pathogenicity of cell lines (Graham *et al* 2001). On the other hand, both two Pro-inflammatory cytokine profiles exhibited statistically significant association in each two studied cell lines. The AT hook proteins and particularly *T. annulata* schizont host nucleoproteins (TashHN) are shown to be involved in cell transformation and proliferation (Swan et al. 1999 and 2001). In this study, we demonstrated the detectable levels of TashHN transcripts in all *T.annulata* infected cell lines in both low and high passage cultures; those could be exhibited by proliferative growth phenotype. This relationship of cell lines proliferative behavior and detectable amount of TashHN mRNAs shows the role of this protein as a growth factor in three examined cell lines. In addition, it seems the considerable amount of TashHN transcripts could be detected throughout *in vitro* growing *T. annulata* schizont infected cell lines. The same data were previously reported that analysis of attenuated cell lines indicated a substantial increase in TashHN expression, with host nuclear reactivity (Swan *et al* 2003). All three parasitized cell lines transform and proliferate continuously, with high expression of TashHN, whereas MMP9 gene expression is not activated in all three high passage cell lines. In addition, Tams1 gene expression down regulated in vaccine strain and high passage culture of C1 and C2 cell lines. In conclusion, based on our results it seems that the parasite-dependent continuous proliferation of infected cell lines is associated with gene expression of TashHN, but attenuation showed a relationship with exclusion of MMP9 and Tams1 special transcripts. Therefore, these findings as well as *in vivo* studies may be of support to choose a reliable *T. annulata* schizont infected cell line for attenuated live bovine theileriosis vaccine.

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