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Short Communication

Determination of CD Markers Profile of the Cell Line Infected by S15 Vaccine Strain of *Theileria annulata* Schizont Using RT-PCR Analysis

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ABSTRACT

The aim of this study was to identify the cell surface cluster of differentiation (CD) markers of the cell lines infected by *Theileria annulata* schizont. The CD molecules are very useful for the characterization of cells and different subpopulations of leukocytes. They are usually recognized by specific antibodies using flow cytometry and immunohistochemistry. In the current study, we applied reverse transcriptase-polymerase chain reaction (RT-PCR) to define the profile of cell surface markers in a cell line infected by an attenuated S15 vaccine strain of *T. annulata* schizont and a new laboratory-established cell line infected by a non-attenuated form. In order to determine the specific markers that can be used for excluding the non-attenuated cell lines, the characterization of the surface proteins profile of the S15 vaccine cell line is important. The RT-PCR was carried out by specifically designed primers using a panel of seven bovine CD markers, as well as beta-actin as an internal control house-keeping gene. We showed that both of the examined cell lines had a consistent expression of CD4, CD5, CD11a, CD14, CD43, and CD45 markers. However, the specific finding in this study was the expression of B-cell markers CD79a and CD5 by the newly-transformed cell line. On the other hand, CD5 as a marker for B-cell subset was expressed by S15 vaccine strain. In conclusion, we consider CD79a surface protein as a new marker for the cell lines infected by non-attenuated *T. annulata* schizont, while the cell lines infected by the vaccine strain do not express this marker. In addition, the identification of CD marker expression based on the RT-PCR assay might be a suitable and appropriate alternative technique for flow cytometry.

Keywords: CD marker, CD79a, RT-PCR, *Theileria annulata*, Vaccine

Détermination des Profils d'Expression des Marqueurs CD de la Lignée Cellulaire Infectée par la Souche Vaccinale S15 de *Theileria annulata* Schizont à l'Aide d'une Analyse RT-PCR

Résumé: Le but de cette étude était d'identifier les marqueurs de surface cellulaire de "cluster de différenciation" (CD) des lignées cellulaires infectées par le *Theileria annulata* schizont. Les molécules de CD sont très utiles pour la caractérisation des cellules et des différentes sous-populations de leucocytes. Elles sont généralement reconnues par cytométrie en flux et immunohistochimie en utilisant des anticorps spécifiques. Cette étude portait sur l'application de la RT-PCR pour définir les marqueurs de surface cellulaire de la lignée cellulaire infectée par la souche vaccinale S15 de *Theileria annulata* schizont (atténuée) et une nouvelle lignée produite en laboratoire infectée par *T. annulata* schizont (non atténuée). La caractérisation des profils protéiques de surface de la lignée

cellulaire du vaccin S15 est importante pour déterminer les marqueurs spécifiques pouvant être utilisés pour exclure les lignées cellulaires non atténuées. La RT-PCR a été réalisée à l'aide d'amorces spécifiques conçues à l'aide d'un panel de sept marqueurs CD bovins ainsi que de la bêta-actine en tant que contrôle interne d'un gène domestique (housekeeping gene) . Nos résultats ont montré que les deux lignées cellulaires examinées avaient une expression similaire pour les marqueurs CD4, CD5, CD11a, CD14, CD43 et CD45. Cependant, la co-expression des marqueurs de cellules B CD79a et CD5 s'est avérée être spécifique à la lignée cellulaire nouvellement transformée, alors que le marqueur CD5 d'un sous-ensemble de cellules B était exprimé par la souche de vaccin S15. En conclusion, nous considérons la protéine de surface CD79a comme un nouveau marqueur de la lignée cellulaire infectée par *Theileria annulata* schizont non atténuée, non-exprimé par la souche vaccinale. L'évaluation de l'expression du marqueur CD par RT-PCR pourrait donc représenter une alternative appropriée à la cytométrie en flux.

Mots-clés: Marqueur de CD, CD79a, RT-PCR, *Theileria annulata*, Vaccin

INTRODUCTION

Bovine tropical theileriosis caused by the protozoan parasite *Theileria annulata* is a major problem for cattle health in the tropical and sub-tropical regions of the world, including Iran (Hashemi-Fesharki, 1988). Theileria infestation begins when the sporozoite is injected into the skin by an infected *Hyalomma* tick. Theileria sporozoites invade mostly monocytes, macrophages, and to a lesser extent B cells (Glass et al., 1989). The virulent *T. annulata*-infected cell lines can be attenuated by prolonged in vitro cultivation and this process is used to generate effective live vaccines (Hall, 1988; Singh, 1990). The S15 *T. annulata* vaccine strain has been attenuated for producing commercial bovine theileriosis vaccine in Iran since 1973 (Hashemi-Fesharki and Shad-Del, 1973). It is essential to identify and characterize the vaccine cell strain as a vaccine seed for completing the required documents for Good Manufacturing Practice (GMP) process. Therefore, continuing the previous studies we decided to determine the specific markers that can be used to exclude virulent cells from attenuated cell lines. In this study, we analyzed the expression profile of the cell surface cluster of differentiation (CD) markers in two *T. annulata* schizont-infected cell lines using reverse transcriptase-polymerase chain reaction (RT-PCR). The Sa vaccine seed cell line was previously characterized by PCR-restriction fragment length polymorphism (PCR-RFLP) using the *Tams-1* gene. Moreover, the molecular phylogenetic analysis was performed based on *18S rRNA* gene (Habibi, 2012). The CD markers are proteins expressed on the surface of cells that conveniently serve as the markers of specific cell types. However, CD molecules are very useful for the identification and characterization of cells and the different subpopulations of leukocytes. These markers are usually recognized by specific antibodies using flow cytometry and immunohistochemistry (Pruszak et al., 2007). The characterization of surface protein profiles of the S15 Theileriosis vaccine cell line is important for determining the specific markers that can be used to exclude the non-attenuated cell lines. In the current investigation, we emphasized on applying RT-PCR for defining the cell markers of S15 bovine leukocyte vaccine cell line with a high passage number, in comparison with a new laboratory-established *T. annulata* schizont-infected cell line with a low passage number.

MATERIAL AND METHODS

***T. annulata* Schizont-Infected Cell Lines.** Two cell lines of S15 vaccine strain with the passage number of 262 and new laboratory-established *T. annulata*-infected cell lines with 5 and 88 passages were used in this study.

Cell Culture. Both cell lines were cultured in Stoker medium supplemented with 10% inactivated adult normal bovine serum (Razi institute, Iran), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C. the cells were grown in 25-Cm^2 flasks at the concentration of 700,000 cells/ml for 4 days at 37ºC. The clumps of cell lines in the suspension culture were passaged at the intervals of 3-5 days by adding fresh medium to the confluent cell culture.

RNA Extraction. Total RNA isolation was performed by YTzol Pure RNA reagent according to the instructions of the manufacturer (Yekta Tajhiz Azma, Iran).

cDNA Synthesis. The reverse transcriptase master mix reaction consisting of buffer 10×, dNTPs, $Oligo(dT)_{18}$, RNase inhibitor, and M-MLV reverse transcriptase was added to the DNase-treated RNA samples (Yekta Tajhiz Azma, Iran). Finally, the samples were kept at 42 ºC for 60 min for cDNA synthesis and the enzyme was inactivated for 10 min at 70 ºC.

PCR. The PCR was carried out by specific primers for a panel of bovine CD markers, including CD4, CD5, CD11a, CD14, CD43, CD45, and CD79a, as well as beta-actin as an internal control (Table 1) (Bio-Rad, 2018). Each PCR contained cDNA, 10 pmole forward and reverse primers, 2X PCR Super Master Mix (Yekta Tajhiz Azma, Iran). The PCR program was adjusted as denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 15 s, 72 °C for 30 min, and a final extension for 5 min at 72 °C in thermocycler (Corbett system, Australia). The PCR products were electrophoresed for the predicted sizes after electrophoresis on 1.5% agarose gel when compared to a 100-bp DNA size marker.

RESULTS AND DISCUSSION

The two *T. annulata* schizont-infected cell lines were successfully cultured at the required passages. The confluent cultures were used for harvesting cell pellets and the concentrated cells were utilized for RNA isolation. We applied vaccine cell line at passage 262 and a laboratory-established cell line with 5 and 88 passages for RNA extraction and cDNA synthesis. The results of PCR for beta-actin as the housekeeping gene showed that RT-PCR worked correctly without any problem in terms of RNA integrity. The PCR products for the seven cell CD markers were amplified as the expected size by comparing along with the appropriate DNA size marker (Table 1 and Figure 1). Our results revealed the expression of CD4, CD5, CD11a, CD14, CD43, CD45, and CD79a by *T. annulata* schizont-infected bovine cell lines (Figure 1). As demonstrated in Figure 1, the cell line of passage number 5 expressed CD4, CD5, CD11a, CD14, CD43, CD45, and CD79a, while S15 vaccine strain (passage number 262) expressed all of the mentioned CDs except CD79a. The CD markers are cell-surface proteins that are categorized in different classes, namely integrins, adhesion molecules, glycoproteins, and receptors. Consequently, CD markers could be considered as a useful tool for identifying and differentiating the living cells based on their profile of surface markers expression using flow cytometry and RT-PCR (Pruszak et al., 2007; Hertzano et al., 2010; Chang et al., 2014).The CD markers associated with *T. annulata* schizont-infected cell lines are CD2, CD3, CD4, CD5, CD40, CD80, IL-2/Tac antigen, IgG, MHC II, and γ/δ TCR+ (Dobbelaere et al., 1990; Howard et al., 1993; Moreau et al., 1999). In the present study, the expression of seven CD markers was studied in two *T. annulata* schizont-infected cell lines, some of which were previously reported to be associated with *T. annulata*-infected cell lines. We showed that both of the transformed cell lines had a consistent expression of CD4, CD5, CD11a, CD14, CD43, and CD45 markers. These markers were previously claimed to be associated with *T. annulata* schizont infection (Dobbelaere et al., 1990; Moreau et al., 1999). The most remarkable finding in this study was the expression of B cell marker CD79a and CD5 by low-passage transformed cell line. It should be mentioned

that CD5 as a marker for B-cell subset was expressed by both cell lines. On the other hand, the cell line with 88 passages and the cell line of the S15 vaccine strain did not express CD79a. As a result, CD79a cell marker may be useful as a novel exclusive marker of the non-attenuated or virulent cell lines from the attenuated cell populations. Therefore, we propose a CD79a cell marker as a candidate surrogate marker for non-attenuated cell lines to facilitate the identification of virulent cell lines from attenuated before considering more *in vivo* studies and further vaccine production. However, further clinical experiments by the new laboratory-established cell line are required for a better understanding of the outcome of CD79a+ cell line inoculation to a susceptible calf in different passage numbers. Our results indicated that CD4 as a general marker for T lymphocytes was expressed by both low- and high-passages. However, some reports show that the CD4 marker can be expressed by monocyte/macrophage lineage (Dobbelaere et al., 1990; Zhen et al., 2014). Moreover, CD11a and CD14 as two markers for monocyte/macrophage lineage were expressed by both

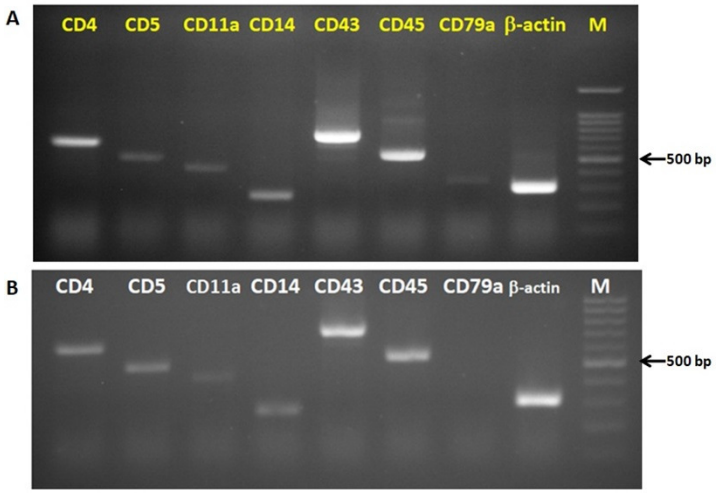

Figure 1. Gel agarose electrophoresis of bovine CD markers; the cDNAs derived from *T. annulata*-infected cell lines were amplified for CD markers; lanes are named from left for the seven cell surface markers of CD4, CD5, CD11a, CD14, CD43, CD45, CD79a, beta-actin as the internal control, and "M" as the 100-bp DNA size marker; A) the cell line with 5 passages was positive for CD4, CD5, CD11a, CD14, CD43, CD45, and CD79a; B) cell line with 262 passages S15 vaccine strain demonstrated the expression of all CDs except CD79a.

Table 1. Oligonucleotide primers used in the RT-PCR analysis of leukocyte cell surface molecules

CD marker	Primer sequences	PCR product length (bp)	Reference
CD4	5'-ACCACTGAACTGAGCCATCG 5'-GTGAGACGCAGTGGTAGCTT	629	NM_001103225
CD5	5'-GGAAGCCCAGACACAAAAGC 5'-GCCAAAATAATGCTCATCACGGC	504	BC113237
CD11a	5'-CGAGGGACCATGTCATTGCT 5'-CTGGGGTGCCCATTGAAGAT	437	BC148897
CD14	5'-AAGCACACTCGCTTGCC 5'-CACATCGGGTAGCACCC	279	Moreau et al. 1999, Pomeroy et al. 2016
CD43	5'-CTGAGCACCAGACCTTACCG 5'-CTTGAGAGAGGTAGCTGGCG	734	NM_001103102
CD45	5'-CCTTCGTGTGAGCCTCCATT 5'-GGGCTTGTAACACCACCGTA	550	AJ400864
CD79a	5'-GGCAGCAACACCAACGTTAC 5'-TCCTGGATATCAGCCCCGAA	362	NM_174266

newly in vitro transformed and S15 vaccine cell lines. The marker CD43 is the cell marker for T and B lymphocyte, monocyte, and macrophage, while CD45 is the general marker for leukocytes. The findings of the current study revealed that both CD43 and CD45 were expressed by the two Theileria-infected cell lines. According to the literature, *T. annulata* has been found to infect monocytes and B lymphocytes, but not *T lymphocytes*. However, *T. parva* infects T cells of both CD4+ and CD8+ lineages (Nene and Morrison, 2016). Moreau et al. (1999) showed that *T. annulata* can infect CD5+ B cells in vitro and B1 cells are common in bovine peripheral blood. Therefore, infection and transformation by Theileria parasites might result in uncontrolled proliferation of CD5+ cells as was reported in the present study. Dobbelaere et al. (1990) revealed the expression of CD markers CD2, CD4, IL-2/Tac antigen, IgG, and MHC II by a *T. annulata*-infected cell line. Although CD4 was expressed by the *T. annulata*-infected cell line, this cell line was negative for CD3 showing that the cells are not necessarily T cells. Howard et al. (1993) demonstrated that *T. annulata*-infected cell lines did not express specific marker for monocyte or B cells and were not derived from T cells. Results for an uncloned cell line generated from an infected calf showed the expression of CD3+ and γ/δ TCR+. These findings indicate that a wider range of host cell types may be infected in vivo than thought previously and some cells may express an abnormal pattern of surface molecules. It has been reported that many cell types other than CD4+ T cells express CD4 marker, particularly human monocytes (Zhen et al., 2014). Considering the previous reports regarding CD4 expression by monocyte/macrophage lineage and the obtained data in this study, the origin of the vaccine cell line seems to be a combination of monocyte/macrophage lineage and B1 cells. To our knowledge, CD79a has not been previously reported to be associated with *T. annulata* infection. Based on our findings, this surface protein might be a novel marker for non-attenuated transformed cell lines. In order to confirm the efficacy of this concept, further experiments are required on a new laboratory-established cell line for in vivo studies. The present investigation is the first study concerning the determination of *T. annulata* schizont-infected cell line markers for cell identity tests by RT-PCR. Our findings showed that this technique might be an alternative assay for flow cytometry.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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