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Original Article

Immunological Analysis of Aerobic Bioreactor Bovine Theileriosis Vaccine

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Abstract

Background: In this study, the pilot production of aerobic bioreactor tropical theileriosis vaccine was optimized with the aim of immunological assays for further mass production.

Methods: We have shown earlier the delayed type hypersensitivity (DTH) assay could be used for evaluating the immunity and memory cells against specific *Theileria* antigen in vaccinated animals. In addition, TNF- α is the principle cytokine in modulating the cytotoxic activity of cytotoxic T-lymphocytes (CTL). Immunological analysis of the vaccine was performed by using two cell mediated immunity (CMI) *in vitro* and *in vivo* DTH test (Theilerin) and TNF- α assay.

Results: The results of immune responses of susceptible immunized cattle by bio-reactor vaccine in comparison with conventional flask vaccine revealed a significant stimulation of immune cells by transcription of high level of TNF- α and positive reaction against *Theileria* antigen in Theilerin skin test (DTH).

Conclusion: The equal immunological results achieved in both above mentioned vaccines verified the satisfactory immunity for aerobic bioreactor theileriosis vaccine for advance mass vaccination in the field on a large scale.

Introduction

Tropical theileriosis, caused by the apicomplexan parasite *T. annulata* extends from Southern Europe, North Africa expanding down through Egypt

to Northern Sudan, and the Near and Middle East including Iran, and India, Central Asia, and China (1, 2). *Theileria* infection starts when the sporozoite stage of the parasite is injected

into the skin by an infected *Hyalomma* tick. The sporozoite tends to target cells, mostly mononuclear macrophages and to a lesser extent B cells (3). In infected cells, the protozoan parasite differentiates to multinucleated schizont form that is coincided with symptoms of the disease. At present, parvaquone and buparvaquone are expensive drugs for treatment of both *T. parva* and *T. annulata* infections (4, 5).

Tropical theileriosis could be managed by tick vector control and vaccination by live attenuated macroschizont-infected leukocytes (4). The vaccination against Tropical theileriosis is used by injection of attenuated macroschizont infected cell lines. These attenuated schizonts shift from the vaccine cell lines to the intact recipient host cells and finally results to the establishment of the infection (6, 7). The successful cell line vaccines have been developed in Israel (8), Iran (9), Turkey, India (10), China, and in some countries of the former Soviet Union (11).

In principal, the immunity against *T. annulata* infection includes both humoral and cell mediated immunity. Development of protective cytotoxicity effect of T lymphocytes (CTLs) is generated by live attenuated schizont-infected cell lines vaccination against *T. annulata* (12, 13). However, humoral immunity has demonstrated a small and limited role against free *T. annulata* schizonts/ merozoites, and the most protozoa able to invade the healthy leucocytes and erythrocytes (14). Therefore, activated CD8+ cytotoxic T lymphocytes can be considered as a critical component of the immune system against *T. annulata* infection (15).

The activated *T. annulata* infected macrophages release cytokines and presenting antigen to CD4+ T cells to begin protective immune responses. It has been shown that the CD4+ T lymphocytes produce interferon-gamma (IFN- γ), which activates non-infected macrophages to synthesis TNF- α and nitric oxide (NO), to kill intracellular schizont and piroplasms (16). Cytotoxic T lymphocytes have been shown to recognize and destroy

infected cells with the MHC-antigen complex (16).

In general, two major functional T helper subsets (Th1 and Th2) are explained by their cytokine profiles (17). Th1 cells are characterized by producing IFN- γ , IL-2, TNF- α , and lymphotoxin. Th1 lymphocytes cause cell-mediated immunity, and develop the cellular immunity responses against some protozoa like *Leishmania* and *Trypanosoma*, while CD8+ naive T lymphocytes develop into effectors (killers) CTL after interaction with an antigen-presenting cell (APC) (18).

Likewise, TNF- α is an important cytokine in immune regulation and resistance to various micro-organisms including obligate intracellular parasites (19). While the cytokines such as TNF- α are released from the CTL or nearby macrophages, thus, the estimation of TNF- α gene expression might demonstrate the activation of CTL response. Moreover, the evaluation of DTH response against *Theileria* antigen could prove the presence of memory T cells and potential activation of CMI system against *Theileria* infection.

DTH is a cell-mediated response that a small amount of protein extracted from the micro-organism, is injected into the skin. The test is positive when the case is infected now, or has been infected previously. The maximum skin reactions are seen at 24-72 hours after the antigen injection. The constructed nodule (induration) is densely crowded with mononuclear cells (lymphocytes and macrophages). Basically, DTH response is only observed after induction of a T memory response by prior exposure (sensitization) to particular antigen (20).

The *in vitro* culture of *T. annulata* schizont infected bovine monocytes/ macrophages cell line has been used for production of live attenuated bovine theileriosis vaccine for a long time in many involved countries. The method of vaccine production has been carried out by using the conventional tissue culture flasks for more than four decades in Iran (9). But the technique has some limitations and disadvantages, risk of contamination dur-

ing cell passage and culture manipulating, requires to more space for culture incubation, vessel sterilization facilities and cell harvesting as well. Thus, the new proposed cell culture system has been defined and developed in a pilot scale for culturing the *Theileria* infected cells S15 vaccine strain in an aerobic bioreactor process.

The purpose of this study was to analyze the efficacy of new produced bioreactor theileriosis vaccine. The vaccine efficiency was studied by using *in vitro* cell stimulation and TNF- α cytokine assay in transcription level as a key factor cytokine in CTL response, and delayed-type hypersensitivity responses have been used to assess CMI *in vivo*.

Materials and Methods

Production of bovine theileriosis vaccine

T. annulata schizont infected cells S15 vaccine strain was cultured by two different methods.

1. Production in Aerobic bioreactor. At first, the culture of *T. annulata* infected cell lines were carried out in simple tissue culture bottles, BioMixer as semi-automatic vessel, and then in 10 liter automated aerobic bioreactor. Precise control of temperature, aeration (soluble oxygen), agitation (round per minute), adding antifoam and daily sampling in sterile condition were performed for documenting the growth requirements.

2. Production in tissue culture flasks (the conventional technique). *T. annulata* infected cell line was grown as previously described (9). Briefly, the working seed was cultured in complete Stoker medium (added 10% bovine serum, Penicillin 100 IU/ml and Streptomycin 100 μ g per ml) and cultivated in cell culture flasks at 37 °C, 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 (9, 21).

The cell concentrating and harvesting the propagated cells for formulation and producing the final product were carried out based on the routine methods of the production of Bovine Theileriosis vaccine in Razi institute (9). The best time for cell harvesting during vaccine production is at the stationary phase of cell growth because it was proved that the produced such a vaccine engender a better immunological response than the early harvested cells (9, 22). The quality control of the produced vaccines was performed based on the approved routine methods in Razi institute (22).

The Animals

It was important to choose animals that have no previous exposure to *Theileria* and *Babesia*. This has been determined by history taking from the animal owner and the microscopic and molecular assays on the provided samples for *Theileria* infections. The number of 34 pure breed cattle was selected for immunological assay in this study. The animals were kept in two farms in enzootic regions for bovine theileriosis in Alborz province.

The immunization

The selected cattle were inoculated by the produced vaccines randomly in two chosen farms as the usual method by injecting one milliliter of thawed cell suspension (one dose) through subcutaneous in autumn season earlier to the tick activation period. The sixteen calves and cattle were inoculated with flask produced vaccine as positive control and eighteen individuals were done by bioreactor produced vaccine as the test group.

Sampling

The blood samples were provided to make sure there was no previous *Theileria* infection in selected animals. The sampling was carried out by taking the peripheral blood from the animals two months post immunization. The provided blood samples in cold conditions were brought to the laboratory for peripheral

blood mononuclear cell (PBMC) isolation and further cell culture and specific antigen stimulation.

Microscopic inspection

The prepared blood smears were taken and examined microscopically for the presence of schizont and trophozoite forms of *T. annulata*.

Molecular detection of *Theileria* infection

The specific PCR was performed to prove there is no earlier infection to *Theileria* in selected animals. Briefly, proteinase K and further phenol chloroform purification were performed for DNA extraction (23). The primers SP1 (5' GCG AAT GTG GTC CAT TTC TTC C) and SP2 (5' GAA GAA TGA TCC ACA ACA TTG CG) were used to amplify bases between 90 to 651 of the *T.annulata* (AJ316248) SP gene sequence (24).

The selected immunological assays

As it was mentioned earlier in the introduction, the immunity against *Theileria* is mediated by CMI and specifically the CTL response is the main efficacious response against the *Theileria*-infected cells, therefore the TNF- α assay and specific DTH test by the Theilerin antigen were intended to perform. TNF- α was measured in level of transcription by the semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR assay) as an *in vitro* test (25, 26).

PBMC isolation

PBMCs can be isolated from heparinized whole blood by using Ficoll density gradient (27).

Cell culture and specific stimulation

The isolated PBMCs were cultured in 24 wells plates and stimulated by phytohaemagglutinin (PHA) as a general stimulant (positive control), Theilerin as a specific stimuli and a group of cells were considered without stimuli as non-stimulated or negative control. The incubation conditions were at 37°C in humidified atmosphere in 5% CO₂, after a period of

72 hours the cultured cells were harvested and homogenized in RNA extraction solution and were preserved in deep freeze (-70°C), until total RNA extraction and further gene expression analysis (28).

RNA extraction and Reverse Transcription-PCR

Total RNA was isolated using the TRIzol extraction reagent (Life Technologies) according to the instruction. 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers to make sure the DNase worked well. The quality of RNA samples was confirmed by observation of ribosomal RNA integrity following electrophoresis and ethidium bromide staining (23, 29). 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) according to the instructions.

Polymerase Chain Reaction

An aliquot of 2 μ l of the reverse transcription reaction solution was amplified by PCR, using 1 U Taq DNA Polymerase (Fermentas) in a 20 μ l reaction volume that contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 μ M of the primers for the template of interest. Specific primers for TNF- α are forward "5' CCT CTG GTT CAA ACA CTC AGG" and reverse "5' GGT GTG GGT GAG GAA CAA GG", and for GAPDH are forward "5' CCA TCA CCA TCT TCC AGG AGC" and reverse "5' TCA TAA GTC CCT CCA CGA TGC" the amplicons are 252 bp for TNF- α and 309 bp for GAPDH (30). All oligo nucleotide primers were synthesized by CinnagenTM (Tehran, Iran). Reaction mixtures were amplified in a RG1-96 thermocycler (Corbett Research, Syd-

ney, Australia): The initial denaturation step was 2 min at 95°C, each cycle consisted of a denaturing step of 15 seconds at 95°C, an annealing step of 45 seconds at 57°C, and an extension step of 45 seconds at 72°C, followed by final extension step of 5 min at 72°C. The number of cycles of amplification required to obtain a detectable signal is dependent on the abundance of the mRNA in the sample material; 28-32 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)

The PCR products were photographed and the intensity of bands was measured by densitometry and normalized based on the GAPDH band. The relative TNF- α gene expression was estimated based on the relation of TNF density to the internal housekeeping gene density (25).

The specific DTH Theilerin test as an *in vivo* test (31). The DTH was performed in two times, first two months post-vaccination and the second in summer the season of tick vector activation.

Statistical Analysis

Data were analyzed using the Microsoft Excel program. The paired t-test was used to compare the values of the means from two samples. The *P*-value less than 0.05 were considered significant.

Results

T. annulata infected cell line S15 vaccine strain has successfully grown by using aerobic bioreactor and flask methods. The propagated cells were harvested and formulated in appropriate cryo-preservative medium and then were stored in deep freeze (-70 °C or -196 °C). The final products released for further immunological assay, until their quality has been

verified satisfactory by quality assurance system.

The number of 34 susceptible high-grade exotic breed calves and cattle with no history of vaccination against bovine theileriosis was randomly inoculated by the flask and bioreactor prepared vaccines.

There was no evidence for previous *Theileria* infection by microscopic inspection and PCR assay in provided blood samples.

The results of relative level of TNF- α gene expression assay

The average relative level of TNF- α gene expression in vaccinated cattle with bioreactor theileriosis vaccine were determined for PHA stimulated cells, *Theileria* specific antigen stimulated PBMCs and non-stimulated cells; 6.755, 1.619 and 0.242 respectively (Fig. 1).

Statistical analysis of average relative level of TNF- α gene expression in immunized cattle with bioreactor theileriosis vaccine demonstrated that there is a significant difference between the relative level of TNF- α gene expression for PHA stimulated cells and non-stimulated cells (*P*=0.05). The same results were exhibited for *Theileria* specific antigen stimulated cells and non-stimulated cells (*P*=0.025).

The average relative level of TNF- α gene expression in vaccinated cattle with flask theileriosis vaccine were determined in PHA stimulated cells, *Theileria* specific antigen stimulated PBMCs and non-stimulated cells; 4.264, 1.169 and 0.633 respectively (Fig. 1).

Statistical analysis of average relative level of TNF- α gene expression in immunized cattle with flask theileriosis vaccine revealed there was a significant difference between the average relative level of TNF- α gene expression for PHA stimulated cells and non-stimulated cells (*P*=0.011). Similar consequences were found between the average relative level of TNF- α gene expression for *Theileria* specific antigen stimulated cells and non-stimulated cells (*P*=0.013).

Statistical analysis of average relative level of TNF- α gene expression showed there is no significant difference between the *Theileria* specific antigen stimulated cells for bioreactor and flask ($P=0.409$).

The results of Theilerin skin test (DTH) post-vaccination

The average skin thickness (induration) was 1.81 millimeter (1.81 ± 0.70) in group of ani-

mals received bioreactor theileriosis vaccine (Fig. 2).

The average skin thickness was 1.57 millimeter (1.57 ± 0.76) in group of animals received flask theileriosis vaccine (Fig. 2).

The statistical analysis showed there is a significant difference for skin thickness before and after the DTH test ($P<0.05$) for both bioreactor and flask vaccines.

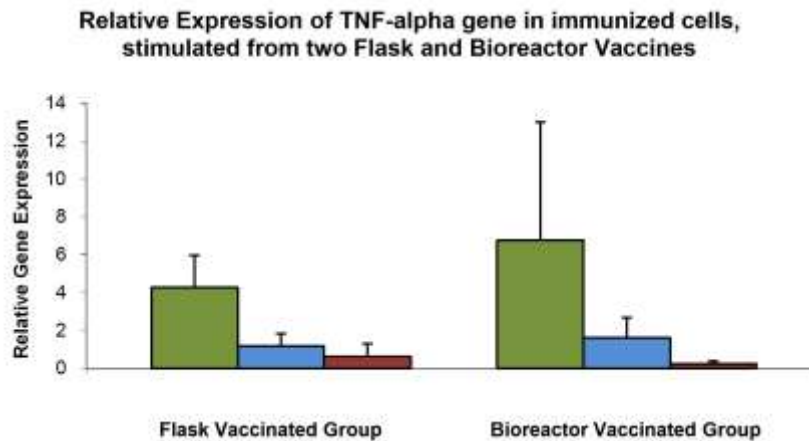


Fig. 1: The mean value of relative level of TNF- α gene expression in PHA stimulated cells (green), specific *Theileria* antigen stimulated cells (blue), and non-stimulated cells (red) in *in vitro* assay. The value of standard deviation bars have been shown on each column. The TNF- α gene expression was presented as relative level. Two groups of susceptible calves and cattle were vaccinated by two aerobic bioreactor and flask theileriosis vaccines

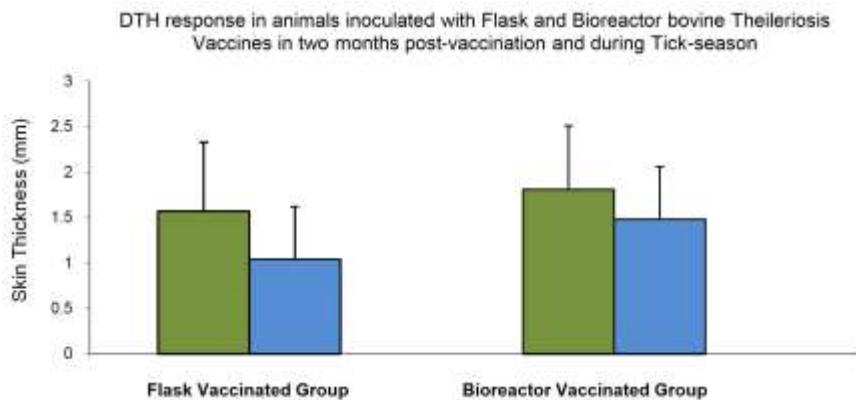


Fig. 2: The mean value of skin thickness in response to *Theileria* antigen in DTH assay was determined in calves inoculated by two bioreactor and flask produced theileriosis vaccine two months post-vaccination (green) and during tick season (high incidence and activity of vector ticks) (blue). The bars on the columns represent the standard deviation of the mean value of skin thickness

The results of skin test (DTH) in summer (high activity and incidence of ticks)

The average skin thickness was 1.48 millimeter (1.48 ± 0.58) in group of animals received bioreactor theileriosis vaccine (Fig. 2).

The average skin thickness was 1.04 millimeter (1.04 ± 0.58) in group of animals received flask theileriosis vaccine (Fig. 2).

The statistical analysis showed there is a significant difference for skin thickness before and after the DTH test ($P < 0.05$) for both bioreactor and flask vaccines.

Discussion

T. annulata infected cell line S15 vaccine strain was successfully propagated in a newly established aerobic bioreactor system in Razi institute. Growth requirements in bioreactor were set up and optimized by preliminary culture in simple cell culture vessels then in Bio-MixerTM and finally in fully controlled aerobic bioreactor was equipped with aeration system and probes for measuring the important cell culture factors e.g. oxygen pressure, pH, and temperature. The process of cell harvesting, formulation and advance quality control tests were carried out for providing the final product for further immunological assays. Additional characterization of the final product by means of immunological techniques may provide valuable information.

Basically, the immunogenicity and effectiveness of new vaccine must be evaluated in clinical trials before the vaccine become available to the market. Therefore, a field trial of a bioreactor vaccine has been performed in enzootic area for tropical theileriosis. The immunological analysis of the bioreactor vaccine was studied on highly susceptible Holstein-Friesians exotic breeds of cattle.

Protection and recovery from tropical theileriosis have been shown to be associated with cell-mediated immune responses; in addition,

CTL and Th1 cytokines particularly IFN- γ and TNF- α activates macrophages to destroy schizont-infected cells (16). Therefore, CMI-based assays have been useful for studying the immunogenicity of newly developed vaccine prior to evaluating the efficacy in large scale field trials.

In the present study, a total of 34 cattle of high-pure exotic breeds were immunized by inoculation of 2.6×10^6 homologous *T. annulata*-infected cells at various ages (between 9-36 months) from different enzootic zones for tropical theileriosis in Karaj and Nazar-Abad areas (Alborz province of Iran) in the early winter when tick activity is low. Two months later, these animals were sampled for *in vitro* analysis of cell mediated immunity specifically TNF- α assay and *in vivo* DTH test for evaluation of memory T cell and macrophage reactivity against specific *Theileria* antigen.

The analysis of TNF- α gene expression in PHA stimulated cells as control positive showed there are significant differences between the mitogen stimulated cells and both antigen-stimulated and non-stimulated cells in all groups of examined (Fig. 1). The achieved results provide reasonable assurance that procedures are carried out properly.

The statistical analysis showed there is a significant difference between antigen-stimulated and non-stimulated cells in both groups of received two kinds of theileriosis vaccine for TNF- α gene transcripts. This statistically significant difference reveals the specific response against employed *Theileria* antigen in PBMCs of those immunized animals.

The comparison of average relative level of TNF- α expression in both two groups of immunized animals received two theileriosis vaccines determined there is no significant difference for TNF- α gene transcripts in both cell stimulated by mitogen (PHA) in two immunized groups. Likewise, same results were obtained for *Theileria* specific antigen that showed no significant difference for relative

level of TNF- α expression in both two groups of immunized animals that received two theileriosis vaccines. Moreover, our results indicate that positive and comparable cell responses are against the theilerin antigen (DTH assay) for both vaccines.

In this study, DTH skin test has been performed twice, two months post-vaccination and the second was during tick season. The first assay was accomplished to have an estimate for induction of immunity, but the purpose was to evaluate the extent of the immune response in tick season.

The results indicate that there is a statistically significant difference between the mean skin thicknesses in animals received two vaccines before and after Theilerin skin test. This diversity demonstrates a positive response against *Theileria* antigen throughout two months post-vaccination and tick season as well. The same results was obtained in period of high activity of tick vectors (tick season) in both two groups of animals, received bioreactor and flask theileriosis vaccines (Fig. 2). But the comparison of average skin thickness for two groups of animals received two vaccines showed no significant difference between the bioreactor and flask theileriosis vaccines. These results were obtained either in two months post-vaccination and tick season. In other words, the vaccines' effectiveness has been documented increase in relative level of TNF- α gene expression and positive DTH responses.

DTH assay is accepted as an *in vivo* cell mediated immunity test for investigating the specific immunity against many pathogens including intracellular parasites (32-38). Hashemi-Fesharki et al. have demonstrated Theilerin test as a specific DTH assay for evaluating the cellular immune response in vaccinated cattle (31).

There are many studies in the field of cell-mediated immunity to *T. annulata* that demonstrated the development of cytotoxic T cells during primary and secondary infections (39). Additionally, Preston & Brown have deter-

mined the role of excreted cytokines from activated macrophages in control of the parasites in the blood by cytostatic effects on schizont-infected cell lines (13). Recent studies on cell mediated immunity confirm the role of cytotoxic T cells and macrophages in induction of protective immunity against *Theileria* infection (13, 40).

In an *in vitro* mixed lymphocyte culture, the PBMCs of immunized animals inhibited the growth of the parasitized cells in an MHC-independent manner, but the naïve PBMC did not. This inhibition effect suggesting that, there is a cytostatic substance in the PBMCs of immune animals on the parasitized cells growth inhibition (41).

A number of cytokines were expressed by *T. annulata* infected cell lines including IL-1 α , IL-1 β , IL-6 and TNF- α (42). Two critical cytokines including TNF- α and IFN- γ may be playing an essential role in controlling the *Theileria* infection. These cytokines interfere with the differentiation of the trophozoites to schizonts, and inhibiting the formation of new infections (42).

TNF- α can be produced by infected cells and/or activated non-infected macrophages. The activated macrophages can produce nitric oxide besides of TNF- α , which inhibits invasion of sporozoites to host cells, and preventing the proliferation of infected cells and finally causes schizont infected cell apoptosis (43).

The protective immune responses against obligatory intracellular protozoan parasites such as *Leishmania* and *Toxoplasma* have been identified by Th1 cell responses (44). The protective immunological responses in bovine theileriosis looks like as those intracellular parasites such as *Leishmania* and *Toxoplasma* by production of cytokines such as IFN- γ and TNF- α for activation of infected macrophages (44, 45).

Therefore, the control of tropical theileriosis depends on the application of tick control measures as well as on vaccination by live attenuated schizont infected cell line, the later

might be enough for fully control of the disease (45-49).

However many published immunization trials are indicating that attenuated vaccines against tropical theileriosis are the only way for disease control and not a means for infection eradication since piroplasms are still abundantly produced in challenged vaccinated calves (50-53).

There is a concern for the possibility of continuing the life cycle of protozoa in the field via live attenuated vaccination. This is probably not a problem where fully attenuated cell lines are used as these do not produce the infected erythrocyte stage and so transmission cannot occur. Hence, the disease cannot be spread via engorged tick vectors on healthy vaccinated cattle to unvaccinated animal (15, 54).

Furthermore, the vaccinated cattle are engendering robust protection against wild *T. annulata* strains due to desirable immunity. Since, there is a considerable cross-protection among different strains of *T. annulata* in vaccinated and recovered animals, thus the vaccine might theoretically create enough protection against homologous parasite strains in the field (16, 55).

In recent years, subunit vaccines have been focused on surface antigens (recombinant SPAG1 and Tams1 surface antigens of *T. annulata* sporozoites and merozoites, respectively). The sporozoite antigen (SPAG1) has been identified as a costimulating recombinant protein with live attenuated vaccine for improving the level of protection against *Theileria* infection (53).

Here in this study, while the challenge strain was not available for evaluation of vaccine efficacy, the study was designed based on immunological parameters by using CMI specific assays to measure the relevant CMI cytokines specifically TNF- α that is important in provoking the CTL response and DTH as specific *in vivo* CMI assay.

In addition, one must also consider the possibility of transferring other diseases by the

use of such vaccine, although this could be eliminated by appropriate screening (if available). However, the good manufacturing practice (GMP) rules remove this important point by seed lot system and master seed examining for important bovine viral infectious particles by supplementary tests as the Razi quality assurance system does.

The achieved results in the present study, clearly demonstrated there are comparable immunological responses in susceptible cattle that vaccinated with two aerobic bioreactor and flask tropical theileriosis vaccines. Here we have used two important immune assays for better understanding the cell mediated immunity in examined animals by *in vivo* Theilerin (DTH) and TNF- α assay as an *in vitro* test. The comparable immunological results obtained in both two vaccines have determined the satisfactory evidences of immunity to theileriosis.

Conclusion

Iranian S15 vaccine strain cell line was successfully cultivated in aerobic bioreactor system. The comparable immune responses achieved by *in vitro* and *in vivo* immunological assays from both bioreactor and flask vaccines verified the satisfactory immunity for advance mass vaccination in the field.

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