First isolation and establishment of in vitro culture of *Theileria lestoquardi* from a naturally infected cow

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Abstract *Theileria* infected cell line was isolated from the prescapular lymph node of an adult crossbred cow. Molecular study confirmed this cell line of bovine lymphocyte has been transformed by the *Theileria lestoquardi*. This strain of *T. lestoquardi* designated Ka-6 and sheep were inoculated with this strain didn't show any clinical signs of theileriosis which shows the significance of this cell line to develop a tissue-culture vaccine against malignant ovine theileriosis. Contrary to accepted belief that the *T. lestoquardi* not capable of causing disease in cattle, the present study describes the first isolation and establishment of in vitro culture of *T. lestoquardi*-infected cell line from a naturally infected cow with typical signs of acute theileriosis.

Keywords *Theileria lestoquardi* · Cell line · Cow

Introduction

Tick-borne protozoan parasites of the genus *Theileria* infect the wild and domestic ruminants. *Theileria annulata*, commonly a parasite of cattle, leads to tropical theileriosis,

whereas *Theileria lestoquardi* causes severe theileriosis in small ruminants (Uilenberg 1981). *Theileria lestoquardi* has some common features with *T. annulata* of cattle. For example, they have antigenic similarities in the indirect fluorescent antibody test (IFAT) (Leemans et al. 1997). However, a polymerase chain reaction (PCR) and isoenzyme studied showed distinguishes between these two parasites (Kirvar et al. 1998; Leemans et al. 1999a). Nevertheless, based on previous reports infection of cattle by *T. lestoquardi* could not cause disease, while sheep can become infected with *T. annulata* (Leemans et al. 1999a, b). The present study is the first report of infectivity of *T. lestoquardi* in cow and production of a new cell line derived from lymphocyte of this cow.

Materials and method

A 3-year-old female Iranian Holstein dairy cow was referred for clinical evaluation. The animal exhibited typical clinical signs of acute theileriosis including high fever, salivation, nasal discharge, lacrimation with significant peripheral lymphadenopathy and the prescapular lymph nodes were remarkably enlarged. Despite the treatment, the disease led to death of the cow. The needle biopsy of lymph node specimen was directly smeared on glass slide and subjected to Giemsa stain followed by analyzed for the presence of schizont in possible infected cells. The remaining specimen was inoculated to in vitro culture of DMEM supplemented with 10 % fetal calf serum and antibiotic/antimycotic (penicillin, 10,000 U/ml; streptomycin, 100 µg/ml; and amphotericin B, 25 µg/ml (Invitrogen, USA)). The culture was incubated in an incubator at 37 °C facilitated with 5 % CO₂ in a humidified atmosphere. The culture was then examined daily until

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lymphocytic clusters were seen. The suspension cells was subcultured every 3 or 4 days cultivation.

The DNA was extracted from the samples by using a commercially available kit (DNGTM-PLUS, CinnaGen Company, Iran) according to the manufacture's protocol. The DNA sample was stored at -20°C before use. Primers RLB-F2 and RLB-R2 genus-specific for *Theileria* and *Babesia*, respectively were used to amplify the V4 hyper-variable region of the 18S rRNA genes of the parasites present in the sample, as described previously (Gubbels et al. 1999). Sequencing of PCR products was carried out by automated sequencer. The species-specific primers SP1 and SP2 were used to amplify bases between 90–651 of the *T. annulata* and bases 271–710 of the *T. lestoquardi* SP gene sequences (Habibi et al. 2008). The primer pairs VAC1 and VAC2 were also used to identify the cells originating from the cattle host (Santacarla et al. 2007).

Eight of 6 to 12-month-old Karakul breed sheep were used for safety and skin tests. These sheep were purchased from Ab-Barik sheep breeding farm under tick-free conditions in Fars province, Iran. Prior to the experiment, sheep blood samples were taken and molecular test was done to ensure the absence of previous contamination of *Theileria* and *Babesia*. Four sheep were inoculated subcutaneously with 5×10^6 cells of *T. lestoquardi* isolate designated as Ka-6, from the 10th in vitro passage. This dose was tenfold higher than recommended sheep vaccine dose. The other four sheep were used as control group which received healthy normal bovine lymphocytes. Clinical symptoms and temperature changes were evaluated during the 30 days after injection. Intradermal skin test to evaluate cell-mediated immunity was performed 2 months post infection as described by Hashemi-Fesharki et al. (1998) and Namavari et al. (2004). Briefly the freeze-dried *Theileria* antigen was obtained from Department of Protozoology, Razi Vaccine and Serum Research Institute, Karaj, Iran, and then the reconstituted antigen was injected intradermally on shaved side of the animal neck with a dose of 0.1 ml per each sheep of two groups. Skin thickness at the site of inoculation was evaluated 72 h later.

Results

Giemsa stain revealed that the schizonts were present in the cytoplasm of the lymph node cells (Fig. 1). The organism also seen grow in in vitro culture (Fig. 2). Subsequently, the PCR with genus-specific primers RLB-F2 test revealed *Theileria* positive against culture and blood samples (Fig. 3). The amplicon sequences were blast with 18S rRNA gene sequence of the reference retrieved from the Genbank revealed that this cow was infected by *T. lestoquardi*, in which showed 100 % identity with

T. lestoquardi JF309152. The 18S rRNA gene sequence has been submitted to Genbank with accession no. GU233774.1. Beside, the result of SP gene amplification reconfirmed that Ka-6 isolate is *T. lestoquardi* (Fig. 4). While the PCR product yield from primer pairs VAC1 and VAC2 was showed that Ka-6 infected-cells culture were origin from the bovine host (Fig. 5). On the other hand, there was no evidence of clinical symptom in the inoculated animals. Price measurement after 72 h skin test showed that the immunized sheep revealed an increase of skin thickening of 1 mm or more at the inoculation site, whereas skin thickening was not seen in the control animals.

Discussion

Theileria parasites have the ability to infect and transform their host cells of immune system. Infection with this parasite leads to reversible transformed phenotype with uncontrolled proliferation of host cells that remain persistently infected (Dobbelaere and Kuenzi 2004; Gardner et al. 2005). Cell line containing *T. lestoquardi* derived from ovine lymphocyte has previously been introduced (Hooshmand-Rad and Hawa 1975) whereas in this report Ka-6 cell line has established from transformed bovine lymphocyte with *T. lestoquardi*. This finding is interesting because it is inconsistent with previous reports that stated while *T. lestoquardi* readily infected sheep and caused severe disease, it did not infect cattle (Leemans et al. 1999a, b).

Fig. 1 Giemsa stain smear of a bovine lymph node showing the schizont of *Theileria* (arrow)

Fig. 2 Observation of bovine transformed lymphocytes on cell culture

Fig. 3 Gel agarose electrophoresis of blood sample and cell line with RLB primers to show the presence of *Theileria* in Ka-6. *M* marker, *Lane 1* positive control (blood sample), *Lane 2* negative control, *Lane 3* Ka-6

The results of our study demonstrated that the Ka-6 cell line could use to create suitable cell immunity response in immunized sheep with this isolate without producing any considerable side effects. The previous studies showed that the skin test provided reliable index for quality assessment of cellular immunity in immunized animal against *T. annulata* and *T. lestoquardi* (Hashemi-Fesharki et al. 1998; Namavari et al. 2004).

Ka-6 is a sample of lymphocytic suspended cells whit bovine origin that has been transformed by *T. lestoquardi*,

Fig. 4 Gel agarose electrophoresis of three samples with SP primers for differentiation of *T. annulata* and *T. lestoquardi*. *Lane 1* Ka-6, *Lane 2* positive control (vaccinal strain of *T. lestoquardi*), *Lane 3* nonvaccinal strain of *T. annulata*, *Lane 4* positive control (vaccinal strain of *T. annulata*)

Fig. 5 Gel agarose electrophoresis of cell line with VAC primers to show the bovine origin of Ka-6. *M* marker, *Lane 1* negative control, *Lane 2* positive control (blood sample), *Lane 3* Ka-6

this cell line can use in security against theileriosis and also well as other cell lines in biological research and even it is used for propagation of vaccine production. Also there is the possibility of its mass production in cell culture bioreactors due to suspension culture of this cell line.

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