Suspension culture of *Besnoitia caprae* by murine macrophage

R. Sadoughifar · M. Namavari · A. Oryan

Received: 9 December 2013 / Accepted: 7 February 2014 / Published online: 22 February 2014
© Indian Society for Parasitology 2014

Abstract *Besnoitia caprae* is a tissue cyst-forming protozoan that infects goats and has considerable economic importance in certain regions of Asia and Africa. Murine macrophage J774 cell line was inoculated with tachyzoites of *Besnoitia caprae* (BC-Pars isolate) collected from mice. A significant growth of tachyzoites was observed in J774. Mice were inoculated with tachyzoites harvested from J774 cell culture. Skin samples from the mice infected with tachyzoites of BC-Pars were PCR positive. One mouse showed alopecia and skin lesions on 45 DPI. Dermal lesions started from around right eye and gradually developed more and more. After euthanasia on 60 DPI, histopathological evaluation of skins around the eye showed necrosis of the epidermis and follicular adnexa with chronic inflammatory cell infiltration. Histopathological sections of their skin showed the presence of necrosis and mononuclear cell infiltration. To the authors' knowledge, this is the first report of successful production of *Besnoitia caprae* tachyzoites was achieved in vitro by suspension culture technique. Another interesting finding is the report of the alopecia and skin lesions around the eye in mouse that quite similar to lesions of goats due to infection of *Besnoitia caprae*.

Keywords *Besnoitia caprae* · Cell culture · J774 cell line · Mice

Introduction

Parasites of the genus *Besnoitia* has been formerly reported in ten genres of various animals (Dubey and Yabsley 2010; Leighton and Gajadhar 2001). Besnoitiosis is a severe but usually non-fatal disease with considerable economic impact (Frenkel 1977; Glover et al. 1990; Heerden et al. 1993; Neuman and Nobel 1981). Caprine besnoitiosis due to *Besnoitia caprae* is recognized as a host specific parasite in goats as intermediate hosts; while the definitive host for this protozoa is still mysterious (Bwangamoi 1967, 1968). It is endemic in domestic and wild goats of some of the African and Asian countries (Cheema and Toofanian 1979; Bwangamoi et al. 1989; Bwangamoi and Njenga 1993; Oryan and Sadeghi 1997). Reducing the spermatogenic activity and devaluation of the skin and leather quality are the consequences of this parasitic disease which makes great economic losses in the goat industry (Bwangamoi et al. 1989; Bwangamoi and Njenga 1993; Cheema and Toofanian 1979; Njenga et al. 1999; Oryan and Azizi 2008). Oryan et al. (2010) displayed significant different biological characteristics between the two isolates (BC-1 and BC-2) of *B. caprae* and they revealed that the inbred BALB/c mice infected with a high dose of BC-1 died and those infected with lower doses showed consistent pathological lesions. Since large-scale experiments on goats are costly, laboratory animal models and cultivation in vitro are used in studies on *Besnoitia caprae*. There are several reports available on cell cultivation of *Besnoitia besnoiti*, for instance cultivation of *Besnoitia besnoiti* on five mammalian cell types which showed Vero (green monkey kidney), L₉ 29 (mouse fibroblasts) and BEK (bovine embryo kidney) cells were highly susceptible and were almost destroyed after 5 days of incubation, while MDBK (Madin-darby bovine kidney)

R. Sadoughifar · A. Oryan
Department of Pathobiology, School of Veterinary Medicine,
Shiraz University, Shiraz, Iran

M. Namavari (✉)
Razi Vaccine and Serum Research Institute, Shiraz, Iran
e-mail: namavari@yahoo.com

and BL (*Theileria annulata*-infected bovine lymphoid) cells were less affected (Shkap et al. 1987).

Up to now, there is just one report available on cell cultivation of *Besnoitia caprae*. Namavari et al. (2012) has successfully cultured *Besnoitia caprae* on BHK₂₁ monolayer cell line, but their attempts to replication of parasites in MDCK and Vero cell had no result. According to the limitations of monolayer culture systems, in this study we tried to culture *B. caprae* on J774 cell line to evaluate the capacity of this cell line as a suspension cell culture to support its growth.

Materials and methods

Multiplication of *Besnoitia caprae* tachyzoites

Seven weeks old male healthy BALB/c mice were infected with Pars isolate of *Besnoitia caprae* (Namazi et al. 2011). Tachyzoites at seed count of 2×10^5 were suspended in 0.2 ml RPMI-1640 medium, and intraperitoneally injected into the mice.

Cell cultivation

The tachyzoites obtained from mice were released from the cells by passing them through a 27 G needle and counted in a Neubauer chamber. The J774 cell line which used as host cell was inoculated with 5×10^5 tachyzoites of *B. caprae*. The host cell was maintained in DMEM, 2 % FCS and an antibiotic/antimycotic solution, containing penicillin (10,000 U/ml), streptomycin (100 µg/ml), and amphotericin B (25 µg/ml) (Invitrogen, USA). Cell line and parasites were maintained in an incubator at 37 °C with 5 % CO₂ in a humidified atmosphere. Whole development process of *B. caprae* tachyzoites in J774 cell line was observed daily under an inverted phase-contrast microscope for 7 days. The numbers of tachyzoites from 5 samplings in each well were counted using a Neubauer chamber and the result being expressed as an average.

Inoculation of *B. caprae* tachyzoites into BALB/c mice

Two groups each having eight mice were inoculated intraperitoneally with 1×10^5 and 5×10^4 tachyzoites obtained from J774, while the control group was inoculated with 200 µl DMEM. The animals were monitored daily for overt clinical signs such as changes in demeanour or skin appearance.

Histopathological and PCR analysis

The samples for histopathological examinations were fixed in 10 % neutral buffered formalin, embedded in paraffin

and sections of 5 µm in thickness were stained with haematoxylin and eosin (H&E) and studied with an ordinary light microscope (Olympus, Tokyo, Japan). The samples for DNA extraction and PCR analysis were labeled and kept in −70 °C. The employed protocol for DNA extraction and PCR assay of skin samples was as in the previous description (Oryan et al. 2010; Namazi et al. 2010). Specific primers were designed for the amplification of the whole internal transcribed spacer 1 (ITS1), 5.8S rDNA and ITS2 regions of the ribosomal DNA. Sterile water was used as the negative control.

Results

The mice inoculated with bradyzoites of BC-Pars died and tachyzoites were then harvested from the peritoneal cavity by lavage with 8 ml RPMI-1640 medium. Numbers of BC-Pars tachyzoites, cultured in J774 cells, increased regularly up to day 6 without showing any decline except on day 7, when cells became almost lysed. The mice received 1×10^5 tachyzoites died, however the mice inoculated with 5×10^4 tachyzoites obtained from J774 survived until 60 DPI. One of the survived mice showed alopecia and skin lesions on 45 DPI (Fig. 1a). Dermal lesions started from around the left eye and gradually developed more and more and finally prolonged to mouse.

Gross examination the skin showed hyperkeratosis and thickening of the area

After euthanasia on 60 DPI, histopathological evaluation of skins around the eye showed mild to moderate necrosis, chronic inflammatory cell infiltration with mild hyperkeratosis (Fig. 2). The control animals showed no mortality, clinical signs or histopathological lesions. Skin samples from the mice infected with tachyzoites of BC-Pars showed a single product of 755 bp similar to those of the positive control but negative control was PCR negative.

Fig. 1 a Alopecia together with skin lesions around the eye of an experimentally infected mouse. b Typically alopecia of a goat infected with *B. caprae*

Fig. 2 Histopathological section from the skin around the eye of a mouse infected with 5×10^4 tachyzoites. Presence of moderate necrosis (thick arrow), mononuclear cell infiltration (O). (H&E, scale bar 56 μ m)

Discussion

There are several reports on the in vitro isolation of *B. besnoiti* in cell cultures (Bigalke 1968; Neuman 1974; Gobel et al. 1985; Shkap et al. 1987; Cortes et al. 2006). Vero cells are often used for the in vitro cultivation of *B. besnoiti* (Bigalke et al. 1974; Neuman 1974; Gobel et al. 1985; Shkap et al. 1987; Cortes et al. 2006). Namavari et al. (2012) introduced BHK₂₁ as a suitable cell line to support BC-Pars growth, while there was no report available on cultivation of *B. caprae* yet. They employed Vero and BHK21 cell lines which had successfully been used for the cultivation of *B. besnoiti* before. Their result was in agreement with the previous findings reported by Schares et al. (2009) who observed complete failure to isolate *B. besnoiti* via Vero cells and the best replication of tachyzoites was in BHK21 cells. There are some significant limitation aspects in scaling up the parasite production on monolayer cell lines. They are anchorage-dependent, and in the absence of surface attachment interactions between the integrins and extracellular matrix, these cells do not proliferate (Folkman and Moscona 1978; Assoian 1997; Griffiths 2001). In this regard, suspension culture systems as homogeneous systems independent of any carrier did not return any limitation regarding scaling up that parasite, in vitro and the area where cells are grown could be critically controlled (Assoian 1997; Griffiths 2001). Cell lines that can proliferate in suspension would greatly facilitate the process of *B. caprae* tachyzoite production. In that sense, in the present investigation J774 suspension was used for *B. caprae* cell culture to evaluate if it could be used to promote its growth.

J774 cells inoculated with tachyzoites obtained from the peritoneal cavity of mice supported the growth of the parasite and an acceptable replication rate was observed in

this cell line. Our results also showed that we can induce skin lesions as clinical signs of besnoitiosis in the laboratory animals which these findings are in agreement with the previous findings reported by Namavari et al. (2012). In addition, we observed dermal lesions around the eye prolonged to nose, which had never been reported in mice before. This lesion resembles the lesion which had seen in goat besnoitiosis before (Fig. 1b). Pols (1960) and Bigalke (1981) stated that *Besnoitia* of all species have a high affinity for dermal, mucosal and serosal surfaces in their intermediate hosts. Bovine isolates showed a tendency to involve skin more than viscera, while wildebeest isolates are evidently more viscerotropists (Bigalke 1968). Thickening, alopecia and hyperkeratosis in the skin of the infected goat occurs due to localization of *Besnoitia* cysts in the dermis and subcutaneous tissues that causes tissue necrosis and infiltration of mononuclear and eosinophils in the affected region (Dubey et al. 2004, 2005; Njagi et al. 1998; Oryan and Sadeghi 1997; Oryan et al. 2008). Oryan and Sadeghi (1997) also reported that the best method for confirming a diagnosis of caprine besnoitiosis in its intermediate host is by histopathological examination of the dermis and subcutaneous connective tissue of the tarsal and carpal areas, face, tendons, testis and the sclera of the eyes. Our histopathological evaluation of around the eye skin of mouse showed alopecia, hyperkeratosis and necrosis of the epidermis associated with chronic inflammatory cell infiltration, which were in agreement of similar previous studies in goats (Oryan and Sadeghi 1997).

The mouse is cheap and available in all countries, so based on the present study it could be the selective choice for the isolation of *B. caprae*. This finding could be used in biological study of the parasite and other applicable investigations. The results of the present study showed the first successful isolation and growth of *B. caprae* culture in suspension cell line which could facilitate the future experimental studies on *B. caprae* like chemotherapeutic agents, diagnosis and control of besnoitiosis. In addition, the in vitro cultivation of *B. caprae* can produce a pure parasite for access to surface antigens and future vaccine production.

Acknowledgments This study was financially supported by Razi Vaccine and Serum Research Institute, grant no. 2-84-18-91115. We thank Dr. Omid-Reza Amrabadi for his kind assistance.

References

- Assoian RK (1997) Anchorage-dependent cell cycle progression. *J Cell Biol* 136:1–4
- Bigalke RD (1968) New concepts on the epidemiological feature of bovine besnoitiosis as determined by laboratory and field investigations. *Onderstepoort J Vet Res* 35:3–138

- Bigalke RD (1981) Besnoitiosis. In: Ristic M, McIntyre I (eds) Diseases of cattle in the tropics. Martinus Nijhoff, The Hague, pp 429–442
- Bigalke RD, Schoeman JH, McCully RM (1974) Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. Onderstepoort J Vet Res 41:1–5
- Bwangamoi OA (1967) Preliminary report on the finding of *Besnoitia besnoiti* in goat skins affected with dimple in Kenya. Bull Epizoot Dis Afr 15:263–271
- Bwangamoi OA (1968) A survey of skin diseases of domesticated animals and the defects which down grade hides and skins in east Africa. Bull Epizoot Dis Afr 17:185–195
- Bwangamoi OA, Njenga JM (1993) A review of besnoitiosis with special reference to goats. Zimbabwe Vet J 24:23–27
- Bwangamoi OA, Carles AB, Wandera C (1989) An epidemic of besnoitiosis in goats in Kenya. Vet Rec 125:461
- Cheema AH, Toofanian F (1979) Besnoitiosis in wild and domestic goats in Iran. Cornell Vet 69:159–168
- Cortes HC, Reis Y, Waap H, Vidal R, Soares H, Marques I, Pereira da Fronseca I, Fazendeiro I, Ferreira ML, Caeiro V, Shkap V, Hemphill A, Leitao A (2006) Isolation of *Besnoitia besnoiti* from infected cattle in Portugal. Vet Parasitol 141:226–233
- Dubey JP, Yabsley MJ (2010) *Besnoitia neotomofelis* n. sp. (Protozoa: Apicomplexa) from the southern plains woodrat (*Neotoma micropus*). Parasitology 137:1731–1747
- Dubey JP, Sreekumar C, Rosenthal BM, Vianna MCB, Nylund M, Nikander S, Oksanen A (2004) Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). Int J Parasitol 34:1273–1287
- Dubey JP, Sreekumar C, Donovan T, Rozmanee M, Rosenthal BM, Vianna MCB, Davis WP, Belden JS (2005) Redescription of *Besnoitia bennetti* (Protozoa: Apicomplexa) from the donkey (*Equus acinus*). Int J Parasitol 35:659–672
- Folkman J, Moscona A (1978) Role of cell shape in growth control. Nature 273:345–349
- Frenkel JK (1977) *Besnoitia wallacei* of cats and rodents: with a reclassification of other cyst-forming isosporoid coccidia. J Parasitol 63:611–628
- Glover GJ, Swendrowski M, Cawthorn RJ (1990) An epizootic of besnoitiosis in captive caribou (*Rangifer tarandus caribou*), reindeer (*Rangifer tarandus tarandus*) and mule deer (*Odocoileus hemionus hemionus*). J Wildl Dis 26:186–195
- Gobel E, Widauer R, Reimann M, Munz E (1985) Ultrastructure of the asexual multiplication of 80 *Besnoitia besnoiti* (Marotel, 1912) in Vero and CRFK-cell cultures. Zbl Vet Med B 32:202–212
- Griffiths B (2001) Scale-up of suspension and anchorage-dependent animal cells. Mol Biotechnol 17:225–238
- Heerden JV, Els HJ, Raubenheimer EJ, Williams JH (1993) Besnoitiosis in a horse. J S Afr Vet Assoc 64:92–95
- Leighton FA, Gajadhar AA (2001) *Besnoitia* spp. and besnoitiosis. In: Samuel WM, Pybus MJ, Kocan AA (eds) Parasitic diseases of wild mammals. Iowa State University Press, Ames, pp 478–486
- Namavari M, Namazi F, Oryan A, Mansourian M, Rahimian A, Tahamtan Y (2012) In vitro cultivation of *Besnoitia caprae*. Online J Vet Res 16:72–80
- Namazi F, Oryan A, Namavari MM, Rahimian A (2010) Experimental infection of embryonated eggs of chicken with *Besnoitia caprae*. Trop Biomed 27:417–423
- Namazi F, Namavari MM, Rahimi E, Lotfi M, Tahamtan Y (2011) Standardization of the outbred BALB/c mice as a suitable animal model for *Besnoitia caprae* studies. Vet Res Commun 35:425–431
- Neuman M (1974) Cultivation of *Besnoitia besnoiti* Marotel, 1912, in cell culture. Z Tropenmed Parasitol 25:243–249
- Neuman M, Nobel TA (1981) Observations on the pathology of besnoitiosis in experimental animals. Zbl Vet Med B 28:345–354
- Njagi ON, Ndarathi CM, Nyaga PN, Munga LK (1998) An epidemic of besnoitiosis in cattle in Kenya. Onderst J Vet Res 65:133–136
- Njenga JM, Munyua SJ, Mutiga ER, Gathuma JM, Kang'ethe EK, Bwangamoi O (1999) Semen characteristics of goats with subacute, acute and chronic besnoitiosis. J S Afr Vet Assoc 70:18–20
- Oryan A, Azizi S (2008) Ultrastructure and pathology of *Besnoitia caprae* in the naturally infected goats of Kerman, East of Iran. Parasitol Res 102:1171–1176
- Oryan A, Sadeghi MJ (1997) An epizootic of besnoitiosis in goats in Fars province of Iran. Vet Res Commun 21:559–570
- Oryan A, Nazifi S, Mohebbi H (2008) Pathology and serum biochemical changes in natural caprine besnoitiosis. Revue Med Vet 159:27–32
- Oryan A, Namazi F, Namavari MM, Sharifiyazdi H, Moraveji M (2010) Comparison of the pathogenesis of two isolates of *Besnoitia caprae* in inbred BALB/c mice. Vet Res Commun 34:423–434
- Pols JW (1960) Studies on bovine besnoitiosis with special reference to the aetiology. Onderstepoort J Vet Res 28:265–356
- Schares G, Basso W, Majzoub M, Cortes HCE, Rostaher A, Selmaier J, Hermanns W, Conraths FJ, Gollnick NS (2009) First in vitro isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet Parasitol 163:315–322
- Shkap V, Pipano E, Greenblatt C (1987) Cultivation of *Besnoitia besnoiti* and evaluation of susceptibility of laboratory animals to cultured parasites. Vet Parasitol 23:169–178