Tropical Biomedicine 27(3): 417–423 (2010)

Experimental infection of embryonated eggs of chicken with *Besnoitia caprae*

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Received 17 March 2010; received in revised form 9 June 2010; accepted 12 June 2010

Abstract. Knowledge on parasites of the genus *Besnoitia*, especially *Besnoitia caprae*, is sparse. *Besnoitia caprae*, an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa, is the causative agent of caprine besnoitiosis. This experiment was conducted to determine the infectivity of the bradyzoites and the resultant histopathological lesions after inoculation of *B. caprae* bradyzoites in the embryonated egg. Eight groups, each having six embryonated eggs, were assigned in this experiment. Seven groups were inoculated with different doses of *B. caprae* bradyzoite inoculums ($1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $5x10^6$, $1x10^7$ and $2x10^7$) via the allantoic cavity route. The 8^{th} group was considered as control. The embryos inoculated with higher doses showed mortality between 14 and 21 days of incubation (5–12 days post-infection). Those embryos that received lower doses were hatched on day 21 of incubation; however, they presented loss of feathers and paralysis and showed hyperemia in the skin of the feet regions. Histopathological sections of the skin revealed the presence of hemorrhages, hyperemia and inflammatory responses. Some of the chickens were euthanized after 50 days post-infection (DPI) and histopathological examination of their tissues revealed haemorrhages and coagulative necrosis with the presence of mononuclear cells infiltration in the liver and heart with interstitial pneumonia. It seems that the embryonated eggs could be a useful model to study the parasite's biology.

INTRODUCTION

Parasites of the genus *Besnoitia* are classified in the subfamily Toxoplasmatinae of the phylum Apicomplexa (Ellis *et al.*, 2000). Besnoitiosis is an economically important parasitic disease (Heerden & Els, 1993) in a wide range of domestic and wild animals that has previously been reported in cattle, goats, equids, reindeer/caribou, opossums, rabbits, rodents, and lizards (Cheema & Toofanian, 1979; Bwangamoi *et al.*, 1989; Ng'ang'a *et al.*, 1994; Oryan & Sadeghi, 1997; Dubey *et al.*, 2002, 2003a, 2004, 2005; Cortes *et al.*, 2005). To date, nine species in the genus *Besnoitia* have been named (Dubey *et al.*, 2003a, 2003b); however, considerable uncertainty exists regarding the identity of some of these species because the life cycles of only *Besnoitia darlingi*, *Besnoitia wallacei*, and *Besnoitia oryctofelisi* are known, and morphological differences among the remaining species are poorly defined (Dubey *et al.*, 2003a). *Besnoitia* infections in goats have been reported in wild and domestic goats in Iran (Cheema & Toofanian, 1979; Oryan & Sadeghi, 1997) and Kenya (Bwangamoi *et al.*, 1989; Bwangamoi & Njenga, 1993). The life cycle of this *Besnoitia* species has not been fully elucidated and the intermediate host range and the definitive host for the *Besnoitia* species infecting goats are unknown. Clinical diagnosis of caprine besnoitiosis relies on the demonstration of the characteristic grayish-white cysts in the scleral conjunctiva. Confirmation may

be made by histological demonstration of the cysts in skin biopsies or postmortem samples from the skin of the tarsal and carpal areas, skin covering the frontal bone, scrotum together with samples from the testicles, epididymis and tunica albugina and tunica vaginalis (Bwangamoi & Njenga, 1993; Oryan *et al*., 2008a, 2008b). Thickening, alopecia, hyperkeratosis, parakeratosis, acanthosis and other skin lesions in the infected goats occur due to development of the *Besnoitia* cysts in the dermis and subcutaneous tissues and further tissue necrosis and inflammatory cells infiltration in the affected areas (Oryan & Sadeghi, 1997; Njagi *et al*., 1998; Dubey *et al*., 2004). The presence of the cysts in the parenchyma of testis and epididymis and their adverse effect on spermatogenesis, animal breeding and reproduction in male goats have previously been reported (Njenga *et al*., 1999b; Oryan *et al*., 2008a, 2008b). Overall, this parasite causes heavy economical loss by reduction of male fertility together with skin and leather quality (Bwangamoi *et al*., 1989; Bwangamoi & Njenga, 1993; Njenga *et al*., 1999a; Ellis *et al*., 2000**;** Oryan & Azizi, 2008). Hence, it is essential to have a better understanding of the biology and life cycle of this parasite, including the intermediate host range and the definitive host(s) to formulate adequate control measures. Therefore, the present experiment was conducted to determine the infectivity of the bradyzoites of *Besnoitia caprae* on the embryonated eggs.

MATERIALS AND METHODS

Isolation of *Besnoitia caprae*

A naturally infected goat, presenting chronic manifestation of skin disease, was painlessly euthanized according to the Iranian Veterinary Organization ethic rules, and subcutaneous tissues from this animal were collected and stored at 4°C. To remove surface contamination, tissue pieces were separately washed in a petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal calf serum (FCS), 1% antibiotic solution (10,000 IU Penicillin and 10,000 mg Streptomycin/ml solution) and 1% amphotericin B (250 mg/ml). Subsequently, the tissue sections were cut into 1-2 mm^3 pieces. The DMEM with liberated bradyzoites was collected and centrifuged at 770x g for 15 min at 4°C. The pellets were resuspended in DMEM and the *B. caprae* bradyzoites were counted using a neubauer chamber. Cryopreservation of the bradyzoites was conducted by resuspending the bradyzoites in FCS containing 10% DMSO, and freezing and storage in liquid nitrogen.

Culture of *B. caprae* on Vero cells

The parasites were cultured in Vero cell line with fresh RPMI medium (Sigma Co., USA) and 2% fetal calf serum. RPMI medium was supplemented with an antibiotic/antimycotic solution, containing penicillin (10,000 U), streptomycin (100 µg), and amphotericin B (25 µg) (Invitrogen, USA). Confluent monolayers were passaged routinely every 6 days.

Animals and experimental infections

Eight groups, each having six embryonated eggs, were assigned in this experiment. Seven groups were inoculated with different doses of *B. caprae* bradyzoite inoculums ($1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $5x10^6$, $1x10^7$ and $2x10^7$). Inoculation was performed via the allantoic cavity route (Warren & Russ, 1948). The 8^{th} group was considered as the control and was inoculated with the supernatant of the parasite culture fluid after centrifuge. Eggs were presented on day 9 of incubation at the time of experimental infection and were then maintained in an incubator at a controlled temperature, humidity and rotation. The embryos were monitored until hatching; however, prior mortalities were recorded.

Necropsy and histopathological examination

Samples of brain, lungs, heart, liver, spleen, limb muscles and skin were fixed in 10% neutral buffered formalin. Paraffin-

embedded sections were cut in 5 µm thickness, and examined after staining with haematoxylin and eosin (H&E) and studied by an ordinary light microscope.

RESULTS

Although after 40 days in Vero cell no replication of bradyzoites or CPE were seen. As shown in Table 1, the embryos inoculated with different doses of *B. caprae* bradyzoite inoculums showed mortality between 14 and 21 days of incubation (5–12 days post-infection) and there was a significant association between the embryo death and the inoculum's dose. While no embryo died up to 4 DPI in any of the experimental or control groups, all the embryos that received $2x10^7$, $1x10^7$ and $5x10^6$, five chickens of the group inoculated with $1x10^6$, and two animals from the group infected with $1x10^5$ died from 5-8 days post inoculation. The remaining chickens of the $1x10^6$ and $1x10^5$ groups and 4 and 3 animals from $1x10^4$ and $1x10^3$ respectively, died on 9-12 days post-inoculation. Gross examination of most of the embryos showed haemorrhages and thickening of the chorioallantoic membranes.

Table 1. Mortality rates in 10-day-old embryonated eggs experimentally infected with different doses of *Besnoitia caprae* bradyzoites inoculums ($1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $5x10^6$, $1x10^7$, $2x10^7$ and control)

Doses	Days post-infection		
	1–4	5–8	9–12
$1x10^3$	–	–	3
$1x10^4$	–	–	4
$1x10^5$	–	2	4
$1x10^6$	–	5	1
$5x10^6$	–	6	–
$10x10^6$	–	6	–
$20x10^6$	–	6	–
Control	–	–	–

Histopathologically, the chorioallantoic membranes were congested and showed focal inflammation with the presence of mononuclear and polymorphonuclear cell infiltration.

The surviving embryos that received lower doses and hatched after 21 days of incubation presented loss of feather and paralysis and showed hyperemia in the skin of the feet regions. Histopathological sections of the skin revealed the presence of haemorrhages, hyperemia and inflammatory responses. The surviving chickens were euthanized after 50 days post-infection and their macroscopic and microscopic lesions were monitored. The hearts revealed necrosis and infiltration of lymphocytes, macrophages and heterophils among the cardiac muscle fibers (Fig. 1) and the livers showed haemorrhages, moderate to severe cell necrosis and mononuclear cells infiltration (Fig. 2). Interstitial pneumonia with haemorrhages, together with macrophages and lymphocytes infiltration in the lumen of the alveoli and bronchioles were seen in the tissue sections of these chickens. The brain showed gliosis.

DISCUSSION

Embryonated eggs have been used for decades as a model for protozoan isolation (Buttitta, 1951), propagation (Wunderlin *et al.*, 1997) and parasite biology studies (Mello & Deane, 1976; Que *et al.*, 2004; Furuta *et al.*, 2007). The use of chicken embryos, due to its low cost and sterile nature, was shown to be a very feasible method to produce *Toxoplasma gondii* antigens from the late 1940's until the early 1970's. This experimental model also has the advantage of avoiding animal euthanasia (Wunderlin *et al.*, 1997). Moreover, embryonated eggs may serve as a valuable tool to study different parasite stages, as seen with Trypanosoma cruzi, which displayed vertebrate and invertebrate specific stages inside egg yolk and corio-allantoic membranes (Mello &

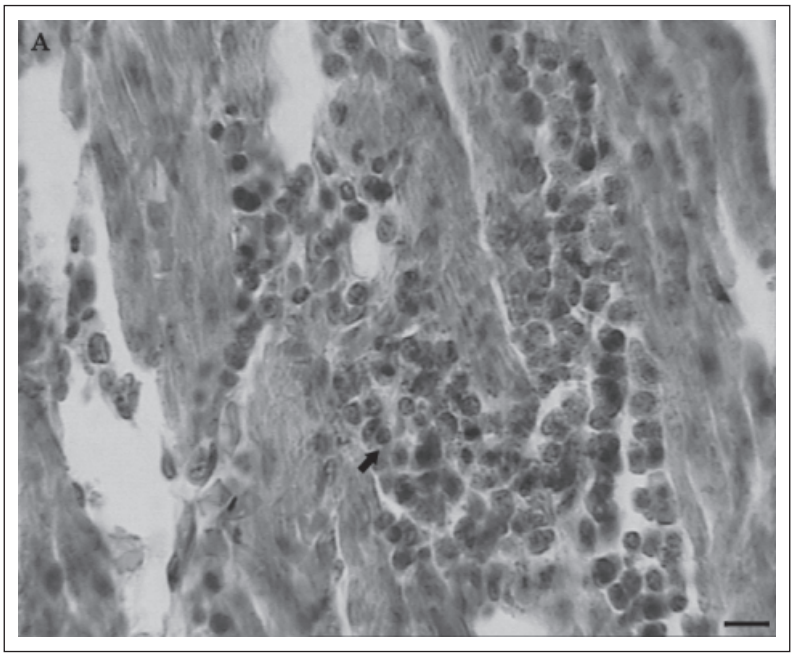

Figure 1. Histopathologic section from the heart of a chicken was experimentally infected with $1x10^3$ bradyzoites of *Besnoitia caprae*. Lymphocytes, plasma cells, macrophages and heterophils are infiltrated among myocardial muscle fibers (arrow) (H & E, scale bar=38 µm)

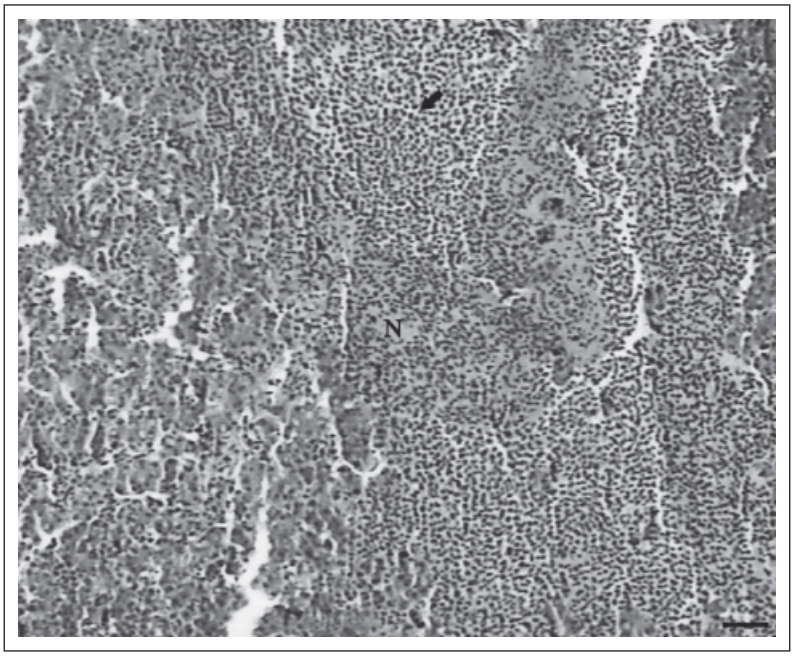

Figure 2. Liver section from an infected chicken with $1x10^4$ bradyzoites of *Besnoitia caprae*. Massive necrosis of hepatocytes (N) with hemorrhages and severe mononuclear cell infiltration is evident (arrow) in this histopathologic section (H & E, scale bar=150 µm)

Deane, 1976). Mineo *et al.* (2009) reported that pigeons are good experimental models for avian *Neospora caninum* infection.

When analyzing eggs inoculated at earlier incubation dates in the present study, the parasite infection effects observed for 10 day old embryos were potentially increased, probably due to prolonged exposure of the parasite to embryos with immature immune responses, since immunological competence is reached only in the second half of the incubation period (Davidson, 2003). In this case, it is speculated that parasites divide themselves freely without any barrier imposed by the host, which would explain the higher mortality and lesion rates found in these groups. In addition, it seems an incubation period of at least five days is needed for localization of the bradyzoites in the vital organs and initiating tissue injuries. In the present study, the mortality rate was completely dependent on the inoculums dose as well. In the groups inoculated with $1x10^3$ and $1x10^4$ bradyzoites, there were 3 and 2 hatches respectively, but there were no hatchings in groups inoculated with $1x10^5$, $1x10^6$, 5×10^6, 1×10^7 and 2×10^7 bradyzoites.

No *Besnoitia* cyst was detected in the skin, subcutaneous fascia, liver, heart, kidneys, lungs and other examined tissues, however, moderate to severe cell necrosis and mononuclear cells infiltration were present in the liver parenchyma, and the muscle fibers of the myocardium were severely infiltrated with lymphocytes, plasma cells, macrophages and heterophils. The tissue cysts of *B. caprae* have not yet been produced in laboratory animals by other investigators either (Dubey *et al*., 2004). The results of the present study are comparable with those of Kharole *et al*. (1979). They infected the chickens experimentally with bradyzoites of *B. besnoiti* liberated from ruptured cysts and found severe haemorrhages with mononuclear cell infiltration in the myocardium of the infected chickens.

Developing adequate laboratory models would promote the study of the biology and epidemiology of ungulate besnoitiosis. From these findings, it could be concluded that the embryo of the chicken could be a suitable model for biological studies and estimating the pathogenesis of this species of *Besnoitia*. Further studies should be performed in order to have a better understanding of the immunization, treatment, route of transmission and other criteria of this parasite.

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