Optimization of murine model for *Besnoitia caprae*

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Abstract It has been shown that mice, particularly the BALB/c ones, are susceptible to infection by some of the apicomplexan parasites. To compare the susceptibility of the inbred BALB/c, outbred BALB/c and C57 BL/6 to *Besnoitia caprae* inoculation and to determine LD50, 30 male inbred BALB/c, 30 outbred BALB/c and 30 C57 BL/6 mice were assigned into 18 groups of 5 mice. Each group was inoculated intraperitoneally with 12.5×10^3 , 25×10^3 , 5×10^4 , 1×10^5 , 2×10^5 tachyzoites and a control inoculum of DMEM, respectively. The inbred BALB/c was found the most susceptible strain among the experienced mice strains so the LD50 per inbred BALB/c mouse was calculated as $12.5 \times 10^{3.6}$ tachyzoites while the LD50 for the outbred BALB/c and C57 BL/6 was $25 \times 10^{3.4}$ and 5×10^4 tachyzoites per mouse, respectively. To investigate the impact of different routes of inoculation in the most susceptible mice strain, another seventy five male inbred BALB/c mice were inoculated with 2×10^5 tachyzoites of *B. caprae* via various inoculation routes including: subcutaneous, intramuscular, intraperitoneal, infraorbital and oral. All the mice in the oral and infraorbital groups survived for 60 days, whereas the IM group showed quicker death and more severe pathologic lesions, which was then followed by SC and IP

groups. Therefore, BALB/c mouse is a proper laboratory model and IM inoculation is an ideal method in besnoitiosis induction and a candidate in treatment, prevention and testing the efficacy of vaccines for besnoitiosis.

Keywords *Besnoitia caprae* · Murine model · Susceptibility

Introduction

Parasites of the genus *Besnoitia* are classified in the subfamily toxoplasmatinae of the phylum apicomplexa (Ellis et al. 2000). Besnoitiosis has been formerly reported in ten genres of various animals including cattle, goats, sheep, equids, reindeer, caribou, opossums, rabbits, rodents, wood rat and lizards (Oryan et al. 2008a, b; 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 and Els 1993; Kafi et al. 2007). The life cycle of *Besnoitia* species has not been clarified completely yet but some species can be transmitted mechanically from one intermediated host to other susceptible intermediate hosts through tachyzoites or bradyzoites (Bigalke 1968; Ng'ang'a et al. 1994). One of the species of these protozoa is *Besnoitia caprae* which is recognized as a host specific parasite in goats as intermediate hosts, while the definitive host for this protozoon is still unknown. The first evidence of caprine besnoitiosis was observed in dried skin sections in Kenya in 1967, but the disease existed as early as 1955 (Bwangamoi 1967, 1968). Nevertheless, it has been shown that the *Besnoitia* of cattle and goats differ in several biological features (Ng'ang'a and Kasigazi 1994; Njenga et al. 1993; Oryan and Azizi 2008). Caprine besnoitiosis is

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endemic in domestic and wild goats of some of the African and Asian countries including Iran and Kenya (Cheema and Toofanian 1979; Bwangamoi et al. 1989; Bwangamoi and Njenga 1993; Oryan and Sadeghi 1997; Oryan et al. 2008a, b). The prevalence of caprine besnoitiosis in Fars Province, southern Iran has been reported in the range of 12.0–18.9 % (Oryan and Sadeghi 1997; Oryan et al. 2008a, b).

The skin of the infected goats is thickened, folded and/or wrinkled, and in some instances is associated with alopecia, hyperkeratosis and hyperpigmentation. The typical ‘elephant skin’ which is a feature of scleroderma is sometimes noted with an eventual shedding of the epidermis (Oryan and Sadeghi 1997). In addition, caprine besnoitiosis halts spermatogenesis in male goats which indirectly affects goat production. This adverse effect is not only because of the direct unknown effects of the localized *Besnoitia* cysts in the parenchyma of the testis and epididymis, but also because of further complications such as tubular cell degeneration and necrosis, inflammatory reactions, tissue infarction, mineralization, fibrosis, epididymal duct obstruction, spermatocele, and sperm granuloma formation (Kafi et al. 2007; Oryan et al. 2010). These effects subsequently result in retardation or complete cessation of spermatogenesis and may lead to temporary or permanent infertility (Kafi et al. 2007; Oryan et al. 2008a, b). Therefore, reducing the spermatogenic activity and devaluation of the skin and leather quality are the consequences of this parasitic disease which is responsible for considerable economic losses in the goat industry (Oryan and Azizi 2008).

Oryan et al. (2010) isolated two separate isolates of *B. caprae* (BC-1 and BC-2) (Namazi et al. 2010; Oryan et al. 2010) and introduced the inbred BALB/c mouse as a suitable laboratory animal model for BC-1 (Table 1). They also showed different biological performance between these two isolates in the inbred BALB/c mice. In addition, Namazi et al. (2011) calculated the LD₅₀ of BC-Pars for the outbred BALB/c as $25 \times 10^{3.2}$ tachyzoites/mouse. Genetic susceptibility has been proved in some other apicomplexan protozoa such as *Neospora caninum* and it has been shown that the inbred mouse strains vary in their susceptibility to neosporosis. Most mouse strains are resistant to *N. caninum* but the BALB/c and C57BL/6 mice develop clinical neosporosis and meningoencephalitis after inoculation with the parasite (Lindsay et al. 1995; Long et al. 1998; Namazi et al. 2010). Genetic susceptibility has also been seen in bovine species (Pan et al. 2004). In another study, there are indications from several countries that *N. caninum* seroprevalences varied according to the cattle breed (Bartels et al. 2006). Therefore, in accordance with the previous investigations with other protozoa, the present study was designed to evaluate the presence of

genetic susceptibility of different mice strains to *B. caprae* inoculation and to investigate different routes of inoculation in the most susceptible mice strain.

Due to lack of knowledge on the transmission of this organism to laboratory animals and in accordance with the former investigations about other protozoa, the present study was carried out to compare the genetic susceptibility of three strains of mice including inbred BALB/c, outbred BALB/c and C57 BL/6 to *B. caprae* inoculation. To estimate the impact of inoculation of different parasite dose combinations and routes of inoculation (SC, IM, IP, infraorbital and oral) to evaluate the development of the disease in more susceptible mice strain after infection with *B. caprae* the LD₅₀ was measured. The findings of this study might be helpful to raise awareness about the animal models of this protozoan inoculation and promote further research in this regard to clarify the non-defined aspects of this disease such as treatment and immunization.

Materials and methods

Parasites

All experiments were carried out using the *B. caprae* (Pars) strain isolated by Namazi et al. (2011). For the tachyzoite multiplication, twenty inbred BALB/c mice each were inoculated with 3×10^5 tachyzoites of BC-Pars isolate intraperitoneally. After the mice died their peritoneal washing was centrifuged at 2,500 rpm for 15 min at 4 °C. The sediment was resuspended in DMEM. Tachyzoites of BC-Pars obtained from peritoneal washing of BALB/c mice were counted (≥ 95 % viable) using a Neubauer chamber. These processes were done under biosafety Class II cabins and all safety measures were taken into consideration to avoid external bacterial or fungal contamination. Cryopreservation of the tachyzoites was undertaken at the following passage, by resuspending the tachyzoites in FCS containing 10 % DMSO, and freezing and storage in liquid nitrogen.

Infection of the outbred BALB/c, inbred BALB/c and C57 BL/6 mice

Thirty approximately 10 week-old male mice were used for each of the inbred BALB/c, outbred BALB/c and C57 BL/6 groups. The mice of each group were then randomly assigned into 6 groups of 5 mice. The mice were kept in individual steel cages, at 22 ± 2 °C and 50 ± 10 % humidity and fed standard rodent proprietary mix food and fresh water. The tachyzoites were tested for viability with trypan blue. The animals of each group were intraperitoneally inoculated with different doses of the cryo-preserved

Table 1 Experimental design of 3 mice strain infection with different doses of *Besnoitia caprae*

Mice strain	Group	N	Inoculums (Tachyzoites)
Inbred BALB/c	1	5	12.5×10^3
	2	5	25×10^3
	3	5	5×10^4
	4	5	1×10^5
	5	5	2×10^5
	6	5	1×10^3 DMEM
Outbred BALB/c	7	5	12.5×10^3
	8	5	25×10^3
	9	5	5×10^4
	10	5	1×10^5
	11	5	2×10^5
	12	5	1×10^3 DMEM
C57 BL/6	13	5	12.5×10^3
	14	5	25×10^3
	15	5	5×10^4
	16	5	1×10^5
	17	5	2×10^5
	18	5	1×10^3 DMEM

tachyzoites of BC-Pars or the control inoculum of DMEM as indicated in Table 2. The animals were monitored daily for changes in demeanor, appetite or skin appearance for 60 days. The LD50 was calculated by the Reed and Muench method (Pizzi 1950) for the three mice strains used in the present study.

Infection of the inbred BALB/c mice by different inoculation routes *B. caprae*

Fifty male inbred BALB/c mice, 10 weeks of age, were randomly divided into 5 experimental and 5 control groups, each group having 5 mice. After viability testing of the tachyzoites with trypan blue, the mice in each of the experimental groups were infected with 2×10^5 cryopreserved tachyzoites of BC-Pars and each of the control animals received an inoculum of DMEM with different

Table 2 Experimental design of the inbred BALB/c infection to *Besnoitia caprae* via different inoculation routes

Group	N	Inoculums (Tachyzoites)	Inoculated routes
1	9	2×10^5	IP
2	9	2×10^5	IM
3	9	2×10^5	SC
4	9	2×10^5	Oral
5	9	2×10^5	IO
6	45	1×10^3 DMEM	All routes

IP intraperitoneal, IM intramuscular, SC subcutaneous, IO infra orbital

inoculation routes as indicated in Table 3. The animals were monitored daily for changes in demeanor, appetite or skin appearance.

Sample collection

After necropsy and careful gross examination, the tissue samples were collected from the brain, lungs, heart, liver, spleen, and kidneys for histopathological and PCR evaluations. For histopathological examinations, the samples were fixed in 10 % neutral buffered formalin, embedded in paraffin, and Sections 5 μ m in thickness were stained with haematoxylin and eosin (H & E) and blindly studied by an ordinary light microscope (Olympus, Tokyo, Japan).

Identification of *B. caprae* by PCR

The employed protocol for DNA extraction and PCR assay of the tissue samples was carried out as described by (Oryan et al. 2010). The specificity of the primers was checked on all available sequences from the GenBank database using the Blast program (www.ncbi.nlm.nih.gov/BLAST/). The negative control DNAs from the genetically related organisms (*Neospora*, *Sarcocystis* and *Theileria*) and genomic DNA from a healthy mouse with no previous injection of tachyzoites were also similarly and concomitantly tested.

The study was approved by the local ethics committee of our faculty, in accordance with the ethics standards of “Principles of Laboratory Animal Care”.

Results

Clinical signs and mortality rate in the infected outbred BALB/c, inbred BALB/c and C57 BL/6 mice

All the mice of both outbred BALB/c and C57 BL/6 mice strains infected with 12.5×10^3 tachyzoites survived without showing any clinical signs during 60 DPI. Mortality occurred in all the remaining outbred BALB/c groups (groups 7–12) infected with tachyzoites within 5–10 DPI (Table 3). Two, 3, 3 and 4 mice of the C57 BL/6 groups (groups 13–18) with 25×10^3 , 5×10^4 , 1×10^5 and 2×10^5 tachyzoites respectively died within 5–8 DPI. However, in the inbred BALB/c groups (groups 1–6), the number of died mice with these doses were 2, 3, 4, 5 and 5, respectively within 5–8 DPI. The LD50 was $12.5 \times 10^{3.6}$ tachyzoites per inbred BALB/c mouse while this amount for the outbred BALB/c and C57 BL/6 mice was $25 \times 10^{3.4}$ and 5×10^4 tachyzoites per mouse, respectively. Therefore, the inbred BALB/c mouse was the most susceptible strain among the experienced mice strains. In

Table 3 Mortality rate in 3 mice strains infected with different doses of *Besnoitia caprae*

Type of rodent	Doses (Tachyzoites)	Days post infection			
		1–3	4–6	7–9	10–12
Outbred BALB/c	12.5×10^3	–	–	–	–
	25×10^3	–	–	–	2
	5×10^4	–	–	2	2
	1×10^5	–	–	3	1
	2×10^5	–	4	–	–
	Control	–	–	–	–
Inbred BALB/c	12.5×10^3	–	–	2	–
	25×10^3	–	1	2	–
	5×10^4	–	4	–	–
	1×10^5	3	2	–	–
	2×10^5	5	–	–	–
	Control	–	–	–	–
C 57 BL/6	12.5×10^3	–	–	–	–
	25×10^3	–	–	2	–
	5×10^4	–	–	3	–
	1×10^5	–	3	–	–
	2×10^5	–	4	–	–
	Control	–	–	–	–

estimation of LD₅₀ for BC-Pars tachyzoites in the three mice strains, 12.5×10^3 , 25×10^3 , 5×10^4 , 1×10^5 and 2×10^5 tachyzoites were intraperitoneally administered in 200 µl volumes of DMEM to each mouse. The infected mice presented clinical signs including ruffled coat, trembling, inactivity, leukocoria and anorexia before death and the tachyzoites were observed in their peritoneal washing.

Infection of the inbred BALB/c mice using different inoculation routes of parasite

All the mice in the groups 4 and 5 (oral and infra orbital inoculation) survived for 60 days and did not exhibit any signs of the disease. All the mice administered tachyzoites by both intra muscular and subcutaneous routes, died within 3–5 DPI (Table 4). The mice inoculated intraperitoneally with tachyzoites died within 4–7 DPI. The infected mice in groups 1–3 (IM, SC and IP) presented clinical signs including ruffled coat, trembling, depression, inactivity, leukocoria and reduction in the food uptake before death (Table 5).

Histopathological findings

Histopathological examination of the tissue sections from the kidneys revealed congestion, tubular degeneration and necrosis in association with infiltration of heterophils (Fig. 1). The heart sections showed areas of hemorrhages,

Table 4 Mortality rate in the inbred BALB/c mice strain infected with different inoculation routes by *Besnoitia caprae*

N	Inoculum's dose (Tachyzoites)	Group number				
		1	2	3	4	5
Inoculated route		IM	SC	IP	Oral	Infra orbital
5	1×10^5	D3	D4	D4	K60	K60
5	1×10^5	D3	D5	D5	K60	K60
5	1×10^5	D4	D5	D6	K60	K60
5	1×10^5	D5	D5	D7	K60	K60
5	1×10^5	D5	D5	D7	K60	K60

Table 5 Clinico-pathological changes in experimentally infected mice

Group number	Inoculum's route	Ruffled coat	Hepatitis	Nephritis
1	IM	++	+++	++
2	SC	++	++	+
3	IP	+	++	+
4	Oral	–	–	–
5	Infra orbital	–	–	–

+ Mild, ++ moderate, +++ severe, – no significant lesion

mild to moderate necrosis of the muscle fibers with heterophils and lymphocytes infiltration. Splenitis with hemorrhages and mononuclear and polymorphonuclear cell infiltration were also seen in these animals. The brain showed gliosis. The liver showed severe fatty change in association with congestion, infiltration of macrophages and disarrangement of the hierarchical organization of the liver in the mice inoculated with IM and SC routes (Fig. 2). The vitreous of the eyes displayed infiltration of histiocytes, cistern organization and severe neovascularization. The lungs of the inbred BALB/c mice inoculated showed hyperemia, hemorrhages, severe thickening of the alveolar septa and interstitial pneumonia associated with infiltration of heterophils, macrophages and lymphocytes. Infiltration of macrophages and lymphocytes in the lumen of the alveoli, peribronchiolitis, development of bronchiolar associated lymphatic tissues (BALT) and congestion was evident in the tissue sections of these animals (Fig. 3). No lesions were found in the tissue sections of the brain, heart, lungs, liver, spleen, skin and skeletal muscles of the control animals.

PCR findings

All the peritoneal washings obtained from the animals of all groups, except those of the infraorbital and oral ones, produced a single PCR product of 755 bp similar to those of the positive control. The genomic DNA from a healthy

Fig. 1 Histopathology section from the kidneys of a mouse infected with 1×10^5 tachyzoites. **a.** severe tubular degeneration (H & E, $\times 240$) and **b.** Presence of congestion (*thin arrow*), and mononuclear cells infiltration (*thick arrow*) (H & E, $\times 200$)

Fig. 2 Histopathology section from the liver of a mouse infected with 5×10^4 tachyzoites intramuscularly. Severe fatty change (*thin arrows*) in association with congestion (*thick arrows*) (H & E, $\times 350$)

mouse with no previous injection of tachyzoites which was used as the template did not show this PCR product. Additionally, the PCR testing of the extracted DNA samples from the inoculated animals for *B. caprae*, including: skin, brain, lungs, liver, spleen, heart, kidneys and skeletal

Fig. 3 Histopathology section from the lungs of a mouse infected with 5×10^4 tachyzoites. **a.** congestion (H & E, $\times 110$), **b.** Thickening of alveolar septa and infiltration of inflammatory cells (H & E, $\times 200$)

muscles revealed positive results. However, control DNAs from the genetically related organisms (*Neospora*, *Sarcocystis* and *Theileria*) which were used as the template did not show this PCR product.

Discussion

In the present study the susceptibility of three mice strains to *B. caprae* inoculation was compared in order to find the best laboratory animal model for future studies.

Generally the mice models have previously been widely utilized in evaluation of the vaccine candidates for cerebral neosporosis (Bartley et al. 2006; Ramamoorthy et al. 2007). Mouse is susceptible to infection with some members of the apicomplexan parasites. It has been stated that BALB/c and C57BL/6 mice developed clinical neosporosis and meningoencephalitis after *N. caninum* inoculation (Lindsay et al. 1995). *Besnoitia caprae* has been assumed to be host specific for goats and has not been reported in any other animal. Cattle, sheep, horses and donkeys that co-pastured with the infected goats have not been infected with this parasite (Ng'ang'a and Kasigazi 1994; Oryan and Sadeghi 1997). Therefore, *B. caprae* was identified as the specific etiological agent for the domestic and wild goats (Cheema and Toofanian 1979; Ng'ang'a and Kasigazi 1994; Oryan and Sadeghi 1997).

The LD50 is an extensively accepted tool in vaccine and toxicity investigations. It is of special use to manufacturers

as it is prescribed in most international pharmacopeias as a test to measure the efficacy of vaccines and drugs (Ramamoorthy et al. 2007). Whenever a lethal or sublethal dose of *Besnoitia* inoculation is going to be used, the advantage of LD50 measurement is to determine the exact infective dose which is effective on the immune response of mice.

In this study the comparison of LD50 among different mice strains revealed that the inbred BALB/c was the most susceptible murine strain among the experienced mice strains with the LD50 of $12.5 \times 10^{3.6}$ tachyzoites/mouse while the LD50 for the outbred BALB/c and C57 BL/6 was $25 \times 10^{3.4}$ and 5×10^4 tachyzoites per mouse, respectively. Namazi et al. (2011) determined the LD50 of the outbred BALB/c as $25 \times 10^{3.2}$ tachyzoites per mouse and introduced this mouse strain as a suitable laboratory animal model for *B. caprae*. However, our findings showed that the inbred BALB/c and C57 BL/6 are both more susceptible than the outbred BALB/c. Ramamoorthy et al. (2007) and Kargar et al. (2012) calculated the LD50 for *N. caninum* as 1.5×10^7 tachyzoites/mouse and 2.5×10^7 tachyzoites of C57BL/6 per mouse, respectively, both of which show higher virulence compared to the LD50 of *B. caprae* that was calculated in different mice strains in the present study.

The infectivity of some of the *Besnoitia* species for rodents has previously been reported (Frenkel 1977). For instance, tissue cysts of *B. neotomofelis* have been shown to be infective in mice after the cysts were orally administered and all the inoculated animals died of acute besnoitiosis (Dubey and Yabsley 2010). Persistence of the parasite in the experimental infection of voles with *B. besnoiti* has also been reported (Basso et al. 2011). Shkap and Pipano (1993) reported that gerbils were susceptible to intraperitoneal inoculation of *B. besnoiti* too. In another experiment, oral administration of the bradyzoites of *B. oryctofelisi* was infective for gerbils and cats (Dubey et al. 2003). However, Njenga et al. (1993) and Ng'ang'a and Kasigazi's (1994) attempt in experimental transmission of *Besnoitia caprae* to rabbits and mice was not successful while Oryan et al. (2010) isolated two distinct isolates of *B. caprae* (BC-1 and BC-2) and introduced the inbred BALB/c mice as a suitable laboratory animal model for BC-1. They also showed different biological performance between these two isolates in the inbred BALB/c mice. Namazi et al. (2011) also calculated the LD50 of BC-Pars for the outbred BALB/c as $25 \times 10^{3.2}$ tachyzoites/mouse.

Our results are in agreement with those reported by Oryan et al. (2010) which showed that the inbred BALB/c is not just an appropriate animal model for *B. caprae* but it is more susceptible than the outbred BALB/c and C57 BL/6. Collantes-Fernández et al. (2004) compared pathology and parasite load in outbred and inbred mouse models in

chronic *N. caninum* infection and indicated more severe cerebral lesions and higher brain parasite burdens in the inbred than the outbred mice.

The present study also showed that when the inbred BALB/c mice were intramuscularly administered with BC-Pars this apicomplexan parasite was able to induce a quicker death and more severe pathologic lesions compared to other routes. On the other hand, the intraperitoneal route of infection was the slowest route in inducing infection in the BALB/c mice.

The most significant lesions found in histopathological examination were seen in the kidneys of the IM group and they revealed congestion, moderate/severe tubular degeneration and necrosis in association with infiltration of heterophils. The liver showed severe fatty change in association with congestion, infiltration of macrophages and disarrangement of the hierarchical organization of the liver in the mice inoculated with both IM and SC routes. The new finding in this study, which has not been previously reported was the vitreous of the eyes which displayed infiltration of histiocytes, cistern organization and severe neovascularization. These histopathological changes were presented in the mice which had leukocoria several days after *B. caprae* inoculation.

Savva et al. (1990) suggested that it is possible to detect *T. gondii* in body tissues and fluids by the PCR with specific oligonucleotide primers. In this study all the peritoneal washings and tissue samples obtained from the animals of all groups, except those of the infraorbital and oral ones, produced a single PCR product of 755 bp, similar to those of the positive control.

In the present study no mortality, pathological lesions or positive PCR results were seen in the mice that were inoculated orally or infraorbitally. In contrast, Yaniz et al. (2007) demonstrated that the conjunctival instillation of *N. caninum* tachyzoites in pregnant heifers induces specific systemic antibodies, the immunological and pathological effects of which after conjunctival inoculation remain to be determined not only in the foetus but also in the dam. It was previously reported that the tachyzoites of *T. gondii* invaded epithelial cells, goblet cells and macrophages in the conjunctiva (Skorich et al. 1988), but such events were not studied in the present work.

The data in this investigation confirmed that the inbred BALB/c mice, due to its relatively easy maintenance, handling and high susceptibility to *B. caprae* infection should be selected over other models as the first choice in experimental caprine besnoitiosis. Concerning the route of inoculation, the intramuscular route induced a quicker infection and more severe pathologic lesions, which was then followed by subcutaneous and intraperitoneal routes, respectively, while intraorbital and oral inoculation of *B. caprae* resulted in no mortality or clinical signs in BALB/c

mice. Finally, the findings of the present study indicating that the inbred BALB/c mice is the most susceptible animal model in comparison to other experienced laboratory animal models is extremely helpful in evaluating the future investigations on goat besnoitiosis, and in promoting the study of the biology and epidemiology of the ungulate besnoitiosis.

This study demonstrated that the intramuscularly inoculation of *B. caprae* tachyzoites induced quicker death and more severe pathologic lesions compared to other routes in the inbred BALB/c mice. Further researches should be performed in order to obtain a better understanding of the immunization, treatment, route of transmission and other criteria of this parasite.

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