Veterinary Parasitology 249 (2018) 70–73

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Neospora caninum bioassay in gerbils using placental tissues from naturally infected goats

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ARTICLE INFO

Keywords:
Meriones unguiculatus
Capra hircus
Apicomplexan parasite
Encephalitis
Immunohistochemistry
PCR

ABSTRACT

Neospora caninum is one of the main agents that causes abortions in cattle worldwide. However, little is known about the pathogenesis of neosporosis in small ruminants, especially goats. Gerbils (*Meriones unguiculatus*) have been used as a model for neosporosis, and this species is highly susceptible to infection by bovine *N. caninum* strains. The present study aimed to evaluate the susceptibility of gerbils to a *N. caninum* isolate from goats. The placentas were obtained from naturally infected goats, that presented with mild to severe lymphoplasmacytic and histiocytic infiltrate, foci of necrosis, calcification and protozoan-like structures. Immunosuppressed gerbils bioassayed with *N. caninum*-infected placental tissues showed severe neurological signs. Microscopic lesions in these gerbils were characterized by encephalitis, myocarditis, myositis and pancreatitis. These lesions were often associated with a small to moderate number of *N. caninum* tachyzoites, confirmed by immunohistochemistry and PCR. This is the first report showing that goat *N. caninum* strains could infect immunocompetent gerbils and cause severe lesions and clinical signs in immunosuppressed gerbils.

1. Introduction

Neospora caninum is an apicomplexan parasite that is known worldwide as an important cause of abortion, especially in dairy cattle (Dubey and Schares, 2011). There are few reports about the pathogenicity of *N. caninum* strains in goats, and to date, there are no reports of *N. caninum* isolated from goats (Porto et al., 2016). Few reports show that *N. caninum* can cause reproductive disorders and encephalitis in goats (Mesquita et al., 2013; Costa et al., 2014). Gerbils (*Meriones unguiculatus*) have been widely used as a model for neosporosis because this species is considered to be more susceptible to infection compared to inbred and outbred mouse strains (Ramamoorthy et al., 2005). To date, no studies have evaluated the pathogenicity of *N. caninum* from naturally infected goats in gerbil models. Therefore, the aim of the present study was to evaluate the susceptibility of gerbils to infection by *N. caninum* goat strains.

2. Materials and methods

The *N. caninum* samples used in the present study were obtained from the placenta of three goats who had a history of previous abortions and congenital transmission of *N. caninum* to goat kids (Mesquita et al., 2013; Costa et al., 2014). These goats tested negative for agents that may cause abortions, such as *Toxoplasma gondii*, *Brucella* spp., *Coxiella burnetti* and *Chlamydophila abortus*. All animals were located in an experimental paddock, free of contact with dogs, and with periodic *N. caninum* IFAT testing in negative animals, to guarantee the absence of the possibility to a new horizontal infection by *N. caninum* oocysts from other routes as water and food. The placentas of these goats were collected immediately after parturition, and samples were collected for DNA extraction, histology and immunohistochemistry (IHC) to confirm the presence of *N. caninum*. Fresh placental samples were processed for inoculation of gerbils. Briefly, 100 g of cotyledons were randomly collected from different areas of the placenta, and the cotyledonary epithelium was aseptically debrided with a scalpel blade and digested with a 0.25% trypsin-EDTA solution for 45 min at 37 °C. Then, the samples were filtered in a 70 μm cell strainer, washed twice with phosphate buffered saline, centrifuged at 1200*g* for 10 min and suspended in RPMI 1640 medium to a final volume of 10 ml. The placental suspensions were intraperitoneally inoculated in six gerbils (0,5 ml for each gerbil) for each goat (case Nos. 1–18). Three gerbils from each group were immunosuppressed with three subcutaneous doses of 0.4 mg methylprednisolone at an interval of 7 days (case Nos. 4–6, 10–12 and 16–18). As controls, six gerbils were inoculated with the same volume of RPMI

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https://doi.org/10.1016/j.vetpar.2017.11.009
Received 13 February 2017; Received in revised form 25 October 2017; Accepted 20 November 2017

used for inoculation of experimental animals. Three control gerbils were immunosuppressed as previously described. The gerbils were evaluated daily and were euthanatized with an overdose of isoflurane on the 30th day post inoculation. All procedures in this work were approved by the Ethics Committee for animal use of the Universidade Federal de Lavras, under the protocol number 018/17.

Tissue samples of brain, hearth, skeletal muscle, pancreas, kidney, liver, lung, spleen, eye, tongue, testicles, and mesenteric lymph nodes were collected in 10% buffered formalin for histology and IHC for *N. caninum*. IHC was performed following a standard protocol with a peroxidase method as previously described (Costa et al., 2014). In tissues from gerbils, a semi-quantitative analysis of IHC was performed, in which IHC was classified as mild, moderate or severe. Mild IHC labeling was determined when rare small parasite structures ressembling tachyzoites were observed within histological tissues. A moderate IHC labeling was used when numerous structures ressembling tachyzoites and up to 5 aggregated parasites structures ressembling cysts were observed in 5 high power fields (HPF, 400X), and severe IHC labeling was used when more than 5 cysts were observed in 5 HPFs. DNA from the placentas and gerbil tissues (brain, pancreas, liver, spleen, heart and skeletal muscle), were extracted with a commercial kit (Reliaprep gDNA Tissue Miniprep System − Promega), according to the manufacturer's instructions. Detection of *N. caninum* DNA was performed using primers for a specific region of the Nc5 gene as previously described (Collantes-Fernández et al., 2002). PCR was performed for detection of presence or absence of *N. caninum* NC5 gene. Serum samples from gerbils were collected one day before inoculation and at day of euthanasia, and from the goats at parturition, for the indirect fluorescent antibody test (IFAT) (Bandini et al., 2011; Mesquita et al., 2013). An initial serum dilution of 1:50 was used. Then, the slides were labeled with FITC conjugated Rabbit anti Gerbil IgG (ICCL) or caprine fluorescein-conjugated anti-IgG antibody (Sigma-Aldrich) both at 1:100 dilution. PCR for amplification of the MS10 microsatellite was performed according to Regidor-Cerrillo et al. (2006), and the product was sequenced by Sanger sequencing method.

3. Results and discussion

Goat 1 had an IFAT titer of 1:3200 at parturition, and both goats 2 and 3 had titers of 1:1600. Grossly, there was no lesions within the placentas. Microscopically, the placenta of goat 1 had a moderate to severe placentitis with multifocal infiltrates of a large number of macrophages and lymphocytes, and a lesser number of plasma cells within the stroma (Fig. 1), with multifocal areas of calcification and focal necrotic areas within the chorionic villi involving the trophoblastic cells. Protozoan-like structures were observed within the chorionic epithelium (Fig. 2) and stroma associated or not with inflammation. In the placentas of goats 2 and 3, a mild and multifocal lymphohistiocytic placentitis with few foci of calcification were observed. Structures resembling cysts and tachyzoites of *N. caninum* located mainly on the chorionic epithelium but also on the stroma of goat 1 and 2 were immunolabeled for *N. caninum*. Immunolabeled protozoan-like structures were not detected within the placenta of goat 3. The placentas from all goats (goats 1, 2 and 3) were positive for *N. caninum* NC5 gene.

The results about gerbils IFAT titer, clinical signs, PCR and IHC are summarized in Table 1.

All gerbils inoculated with placental suspension from goat 1 (case Nos. 1–6) seroconverted at 30 days post inoculation (dpi), while 4 gerbils inoculated with placental suspension from goat 2 (case Nos. 7, 10–12), and two gerbils from goat 3 (case Nos. 16–17) seroconverted at 30 dpi. Two gerbils (4, 16) presented with significant clinical signs, such as a severe head tilt at 16 and 28 dpi, respectively, and there was apparently no loss of consciousness or coordination. Grossly, the pancreatic surface was slightly shrunken (case Nos. 4–6, 16). In all gerbils that seroconverted, *N. caninum* DNA was detected within the brain (case Nos. 1–7, 10–12, 16–17). *N. caninum* DNA was also detected within the pancreas, liver (case Nos. 4–7, 10–12, 16), heart and skeletal muscle (case Nos. 4–6) of *N. caninum*-infected gerbils. Histologically, the main affected organs of *N. caninum*-infected gerbils included the pancreas, brain, skeletal muscle and heart. The pancreatic architecture was partially effaced by a large number of macrophages and neutrophils as well as a small number of lymphocytes and plasma cells. There was also a multifocal necrosis and mild to moderate pancreatic fibrosis (Fig. 3) (case Nos. 4–7, 10–12 and 16–17). In the brains of *N. caninum*-infected gerbils (case Nos. 4, 5, 6, 10, 11, 12 and 16), there was a multifocal, mild to severe gliosis that was mainly in the telencephalon, thalamus, and brainstem (Fig. 4). Central nervous system (CNS) lesions were more prominent in gerbils that exhibited severe clinical signs (case Nos. 4 and 16). A few groups of tachyzoites-like structures (Fig. 4), as well as intraneuronal cysts-like structures labeled by *N. caninum* in IHC (Fig. 5), were observed associated or not with areas of gliosis. Only gerbils inoculated with placental suspension from goat 1 had myocardial and skeletal muscle lesions. Myocardial and skeletal muscle fibers were mildly to moderately expanded by a large number of macrophages with multifocal areas of muscle fiber degeneration and necrosis with dystrophic calcification (Fig. 6). Within the myocardium and skeletal muscle, there were multifocal to coalescing areas of fibrosis. Rare tachyzoite-like structures were immunolabeled for *N. caninum* within the heart and skeletal muscle.

The samples from three goats in this study presented the MS10 microsatellite allele $(ACT)_6(AGA)_{15}(TGA)_8$. The microsatellite MS10 alone have been used to differentiate *N. caninum* strains (Reis et al., 2016; Peters et al., 2017). This allele, which was found in the *N. caninum* genome from the goats in the present study, was also found in a frequency between 20% and 30% in German and Scotland, and with a low frequency in Argentina and Spain (Regidor-Cerrillo et al., 2016). However, this allele was not previously described in *N. caninum* strains from Brazil.

In the present study, gerbils were successfully infected by *N. caninum* using placental tissue from naturally infected goats. These placental tissues had lesions, which were characterized by mild necrosis and calcification with moderate to severe lymphohistiocytic inflammatory infiltrate. In addition, goat 1 had a large number of parasite-like structures observed in histology and IHC. These placental lesions are similar to those described in experimental studies when the parasite was inoculated in cows (Regidor-Cerrillo et al., 2014) and goats (Porto et al., 2016), which consisted of inflammatory infiltrates mainly composed by mononuclear cells and necrotic foci. Cattle inoculated at early gestational period with two *N. caninum* strains with different pathogenicity, NC-Spain7 (more pathogenic) and NC-Spain8 (less pathogenic), showed similar severe inflammatory infiltrate of mononuclear cells and rare neutrophils and presented a high rate of fetal loss (Regidor-Cerrillo et al., 2014). In contrast, goats experimentally infected at early and mid-gestational periods with the NC-Spain7 strain showed more severe lesions consisting by moderate to extensive necrotic placentitis with mild non-purulent inflammatory infiltrate, and at a late gestational period, the necrotic lesions and inflammatory infiltrate were mild (Porto et al., 2016). Similarly, cattle inoculated at early and mid-gestational period with NC-1 strain showed severe placental necrosis and nonpurulent inflammatory infiltrate (Maley et al., 2006; Macaldowie et al., 2004).

The seroconversion of immunocompetent gerbils inoculated with goat *N. caninum* strains shows that the parasite burden inoculated through the placental samples was enough to cause infection. However, the initial parasite load inoculated in gerbils and the parasite stage could not be evaluated in this study, as fresh placental tissue was used. In contrast, other studies show that gerbils are a highly susceptible to *N. caninum* infection, using tachyzoites from NC-Kr2 (Kang et al., 2009) and Nc-1 (Ramamoorthy et al., 2005) strains, and oocysts from NC-Liv strain (Dubey and Lindsay, 2000). In contrast, Oliveira et al. (2017), using the same number of oocysts as Dubey and Lindsay (2000), but from a different strain, reported that gerbils did not develop clinical