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ISSN 1678-4596 PARASITOLOGY

Oral infection of neonate gerbils by *Neospora caninum* tachyzoites

Infecção oral de gerbils neonatos com taquizoítos de *Neospora caninum*

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ABSTRACT

*Neosporosis is a parasitic disease caused by the protozoan **Neospora caninum** which results in major economic losses for cattle breeding due to abortion and other reproductive disorders. Gerbils (**Meriones unguiculatus**) are commonly used as experimental models for neosporosis due to their high susceptibility to **N. caninum** infection, both by oocysts ingestion as by tachyzoites/bradyzoites parenteral inoculation. However, the risk of transmission by tachyzoites ingestion is not fully elucidated. In this study, infection of neonate gerbils by **N. caninum** (NC-1 strain) tachyzoites inoculated by the oral route and the parasite distribution in gerbils' tissues were evaluated by protozoan DNA detection. Seventeen neonate gerbils, aged 4-5 days, were inoculated with $4x10^5$ tachyzoites by the oral route and one gerbil was kept as uninfected control. **N. caninum** DNA was detected in 100% of the inoculated gerbils, showing that the oral route is effective as a potential route of infection of neonates by **N. caninum** tachyzoites. **N. caninum** DNA was reported in all organs evaluated (heart, lungs, kidneys, liver, spleen and brain), with different frequencies. These results showed systemically distributed infection of neonate gerbils after oral inoculation of tachyzoites.*

Key words: *molecular diagnosis, experimental model, bovine neosporosis, rodents.*

RESUMO

*Neosporose é uma doença parasitária causada pelo protozoário **Neospora caninum** e resulta em grandes perdas econômicas para a bovinocultura, o que se deve a abortos e outras desordens reprodutivas. Gerbils (**Meriones unguiculatus**) são comumente utilizados como modelos experimentais para neosporose, devido a sua alta susceptibilidade à infecção por **N. caninum**, seja pela ingestão de oocistos ou pela inoculação parenteral de taquizoítos/bradizoítos. Porém, o risco de transmissão pela ingestão de taquizoítos não está totalmente elucidado. Neste estudo, foram avaliadas a infecção de gerbils neonatos por taquizoítos de **N. caninum** (cepa NC-1) inoculados por via oral e a distribuição do parasita nos tecidos desses gerbils, através da detecção de DNA do protozoário. Dezessete gerbils neonatos, com idade de 4-5 dias, foram inoculados com $4x10^5$ taquizoítos por via oral e um gerbil foi mantido como controle não-infectado. DNA de **N. caninum** foi detectado em 100% dos gerbils inoculados, demonstrando a eficácia da via oral como potencial rota de infecção de neonatos por taquizoítos de **N. caninum**. DNA de **N. caninum** foi encontrado, em diferentes frequências, em todos os órgãos avaliados (coração, pulmões, rins, fígado, baço e cérebro). Esses resultados demonstraram infecção sistêmica dos gerbils neonatos após a inoculação oral de taquizoítos.*

Palavras-chave: *diagnóstico molecular, modelo experimental, neosporose bovina, roedores.*

INTRODUCTION

Protozoan ***Neospora caninum*** (Apicomplexa) is the cosmopolitan etiologic agent of neosporosis (DUBEY et al., 2007). Canids are definitive hosts and cattle, as several mammals, are intermediate hosts of ***N. caninum***. Bovine neosporosis is characterized by embryo mortality, abortion, fetal mummification, stillbirth or birth of weak or healthy persistently infected calves, causing major losses for

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cattle industry, mainly in dairy herds (INNES et al., 2002). As proven by serology and histopathology, the birth of clinically healthy calves, congenitally infected by tachyzoites vertical transmission, allows ***N. caninum*** maintenance in a herd (BARR et al., 1993, DUBEY et al., 2006). Vertical (transplacental) infection of the fetus is considered the main route of ***N. caninum*** transmission in cattle (SCHARES et al., 1998), since horizontal transmission occurs by ingestion of sporulated oocysts from environment (GONDIM et al., 2004).

Natural route of horizontal infection for herbivores is the ingestion of ***N. caninum*** environmental oocysts. However, small amounts of oocysts are excreted by infected dogs, hindering their collection. Thus, tachyzoites are commonly used for experimental purposes, due to their easy obtainment from *in vitro* cell culture (RAMAMOORTHY et al., 2005). Experimental models using rodents are desirable, since studies with cattle or dogs are expensive and demand for more time and infrastructure. Moreover, rodents can be used in studies of vaccines, drugs and strain characterization (QUINN et al., 2002).

Gerbils (***Meriones unguiculatus***) are excellent models for acute neosporosis, since they are highly susceptible to ***N. caninum*** replication without previous immunosuppression (GONDIM et al. 2001; TONIN, et al., 2013; BOTTARI et al., 2014). Neosporosis was already reproduced in gerbils in different experimental conditions (DUBEY & LINDSAY, 2000; GONDIM et al., 2001; RAMAMOORTHY et al., 2005; KANG et al., 2009; TOSCAN et al., 2012; TONIN et al., 2013; BOTTARI et al., 2014). Among these studies, acute infection was commonly performed by intraperitoneal inoculation of tachyzoites (CUDDON et al., 1992; RAMAMOORTHY et al., 2005; KANG et al., 2009; TOSCAN et al., 2012) and, eventually, by oral inoculation of oocysts (DUBEY & LINDSAY 2000). Studies on ***N. caninum*** pathogenesis in gerbils are useful for development of control strategies, as vaccines or drugs, for canine and bovine neosporosis (KANG et al., 2009).

Studies indicated that bovine fetuses and neonate calves can be infected by ingestion of ***N. caninum*** tachyzoites from amniotic fluid or colostrum, but specially soon after birth (DUBEY & SHARMA 2003, ROMERO & FRANKENA 2003, HOBSON et al., 2005). Experiments proved that calves are orally infected by ***caninum*** tachyzoites from colostrums and milk (UGGLA et al., 1998, DAVISON et al., 2001). However, the use of tachyzoites for oral infection of neonate gerbils as experimental model of neosporosis was not evaluated.

The aims of this study were (1) to evaluate potential transmission of ***N. caninum*** by tachyzoites to neonate gerbils by the oral route; (2) to characterize organic distribution and identify preferable tissues used for parasite replication, using ***N. caninum*** DNA detection by PCR; and (3) to establish an useful experimental model for prophylaxis and control studies on neosporosis in neonate animals.

MATERIALS AND METHODS

In vitro culture of ***Neospora caninum*** tachyzoites

N. caninum (NC-1 strain) was cultured in Vero cell monolayers, with RPMI medium (Invitrogen, Brazil), supplemented with 10% bovine fetal serum (Nutricell, Brazil), at 37°C, saturated humidity and 5% CO_2 atmosphere. Tachyzoites for inoculum were obtained after cellular suspension and disruption, and solution was decanted during 30 min., at 4°C, in sterile tube to diminish cell debris. Supernatant was recovered, washed once by centrifugation, resuspended and centrifuged with RPMI for tachyzoites concentration. Tachyzoites were counted using Neubauer's chamber and re-diluted with RPMI at the desired concentration.

Animals

Eighteen neonate gerbils (aged 4-5 days) from three litters of ***N. caninum*** seronegative female gerbils, provided by UFSM animal facilities, were used. During pregnancy and lactation, adult and neonate gerbils were kept in controlled environment, allocated in appropriate boxes, with fresh water and pelletized food *ad libitum*.

Inoculation

Seventeen neonate gerbils were inoculated with $4x10^5$ live tachyzoites per oral (as described by TOSCAN et al., 2012). Briefly, tachyzoites were diluted in 10µL saline solution and administered directly into the gerbils' mouths using 0.5-10µL pipette and sterile tip. One neonate gerbil was kept as uninfected control (Table 1).

Infection monitoring

Gerbils were daily evaluated during the experimental period. Clinical signs as lateral head tilt, rough coat, anorexia, abdominal swelling were monitored as indicative of acute neosporosis (AGUADO-MARTÍNEZ et al., 2009).

Table 1 - ***Neospora caninum*** DNA detection in tissues of neonate gerbils (***Meriones unguiculatus***) after inoculation of $4x10^5$ tachyzoites by oral route.

Animal	Heart	Lungs	Spleen	Kidneys	Liver	CNS
1	+	-	+	+	+	-
2	+	-	+	+	+	+
3	+	-	+	+	+	-
4	+	+	+	+	+	+
5	+	-	+	-	+	+
6	+	+	+	-	+	-
7	+	-	+	+	-	-
8	+	+	+	+	-	-
9	+	+	+	-	-	-
10	+	+	-	-	-	-
11	+	+	+	-	-	-
12	-	-	+	-	-	-
13	+	+	+	+	-	+
14	+	+	+	+	-	+
15	+	+	+	+	-	+
16	+	+	+	+	-	+
17	+	+	+	+	-	+
C(-)*	-	-	-	-	-	-
Total (%)	16/17 (94.1) [a]	11/17 (64.7) [b]	16/17 (94.1) [a]	11/17 (64.7) [b]	6/17 (35.3) [c]	8/17 (47.0) [bc]

C(-): negative (uninfected) control.
Different letters (a, b, c) in each column indicated significant difference among the frequencies ($P<0.05$).

Tissue collection

Central nervous system (brain, cerebellum and brainstem), liver, spleen, kidneys, lungs and heart were collected after spontaneous death or euthanasia from each gerbil. Uninfected control gerbil was euthanized at the end of the experimental period. Tissues were washed with sterile PBS and stored at -20ºC in microtubes up to DNA extraction.

DNA extraction and PCR

Genomic DNA was extracted from each sample using the Wizard® Genomic DNA Purification Kit (Promega™, Madison, USA) according to manufacturer's recommendation. DNA samples were stored at -20ºC up to molecular analysis. Before the PCR, DNA concentration was evaluated using a PicoDrop microliter visible UV spectrophotometer (Picodrop, Cambridge, UK), at the absorbance level of 260nm.

PCR was performed using a primer set for specific amplification of ***N. caninum*** Nc-5 gene: forward (Np21), 5'CCCAGTGCGTCCAATCCTGTAAC-3'; and reverse (Np6), 5'CTCGCCAGTCAACCTACGTCTTCT-3' (YAMAGE et al., 1996). Amplification product (328bp) was evaluated by electrophoretic running in 1% agarosis gel, stained with SYBR® safe DNA gel stain (Invitrogen™, USA) and visualized under ultraviolet light. DNA samples extracted from ***N. caninum***-infected and uninfected Vero cells were included as positive and negative controls, respectively, in each PCR.

Statistical analysis

Data were checked for normality by Shapiro-Wilk test at 95% confidence level. Since normality was not confirmed, data were analyzed by Wilcoxon-Mann-Whitney test (non-parametric) at 95% confidence level, comparing the frequencies of ***N. caninum*** DNA positive samples for each tissue considering all the positive gerbils.

RESULTS AND DISCUSSION

Protozoan DNA was detected in several tissues, showing effective oral infection of neonate gerbils by ***N. caninum*** tachyzoites. In addition, clinical signs of acute neosporosis were observed in all of the gerbils inoculated. These clinical signs were anorexia and decreasing body condition and occurred from five to seventeen days after inoculation. Other clinical signs of acute neosporosis, commonly reported in adult gerbils (RAMAMOORTHY et

al., 2005; YANG et al., 2009; TONIN et al., 2014), were not reported in the neonate gerbils, indicating differences in the clinical course in neonate and adult animals. Mortality (spontaneous death or euthanasia *in extremis*) of neonate gerbils occurred from the eighth to seventeenth days. These results were in accordance to other studies, which showed the high susceptibility of the gerbils to ***N. caninum*** (DUBEY & LINDSAY, 2000; RAMAMOORTHY et al., 2005; TONIN et al., 2013; BOTTARI et al., 2014). Uninfected control gerbil remained clinically healthy up to the end of the experimental period and was euthanized at day seventeen.

Clinical course was related with parasite distribution, since ***N. caninum*** DNA was found with higher frequencies in tissues from gerbils dead earlier. At necropsies, all infected neonate gerbils showed increased volume of kidneys and liver in comparison to uninfected control, as also observed by TOSCAN et al. (2012) in young gerbils. Besides, hemorrhagic lungs were observed in some of the inoculated neonate gerbils.

In this study, the presence of ***N. caninum*** DNA was detected in tissues from 100% of the inoculated animals (Table 1). Frequencies of parasite DNA detected by PCR were 94.1% (16/17) in heart and spleen; 64.7% (11/17) in lungs and kidneys; 47.0% (8/17) in CNS; and 35.3% (6/17) in liver samples. The presence of ***N. caninum*** DNA was used to describe the organic distribution of the parasite. PCR analysis was negative in all tissues from the uninfected neonate gerbil (control).

In a study performed by UGGLA et al. (1998), four calves were inoculated by the oral route with around 3 to 10 x 10^7 ***N. caninum*** tachyzoites diluted in colostrum up to six hours after birth. Colostrum was administered using bottles (for two calves) or gastric tubes (for the other two calves). Both calves which received the inoculums using bottles were infected by ***N. caninum,*** as proven by positive serology and parasite DNA detection in the CNS by PCR. These data showed the potential transmission of ***N. caninum*** to neonate calves due to ingestion of colostrum containing tachyzoites. Nevertheless, gastric micro environmental conditions could inactivate the free tachyzoites that reach the stomach (DUBEY, 1980). However, production of hydrochloric acid and enzymes is limited in neonates' gastrointestinal tract and proteolytic activity is decreased by the action of trypsin inhibitors contained in colostrum (ROY, 1990; TIZARD, 1996).

DAVISON et al. (2001) performed three experiments to evaluate oral transmission of ***N. caninum*** by tachyzoites in cattle. Firstly, six calves received colostrum or milk containing, approximately, 10^7 tachyzoites, administered at four times using bottles (the first time up to 12 hours after birth, and other three times at weekly intervals). In the second experiment, two calves were feed with bovine placental tissue infected by ***N. caninum*** (bradyzoites/tachyzoites), before 12 hours after birth. In the third experiment, seven calves were breastfed by seropositive cows since one to three days up to 12 weeks after birth. Positive serology was observed in all calves in the first experiment and in one calf in the third experiment.

Results from studies with calves and, from the present study with gerbils, showed that per oral transmission by ingestion of ***N. caninum*** tachyzoites can occur in neonate animals. However, the experimental infection was performed with high concentration (around 10^7) of tachyzoites administered for calves. Thus, at the field, calves can probably be episodically infected by the oral route if a high concentration of tachyzoites is present in colostrum or milk from infected cows (DAVISON et al., 2001). In this study, a concentration of $4x10^5$ tachyzoites was enough for infection of 100% of neonate gerbils. However, further investigation is required to determinate the role of tachyzoites ingestion as a source of ***N. caninum*** transmission to neonate animals. Moreover, it is not clear if the concentration of tachyzoites in colostrum or milk of infected cows could be enough high to cause neonatal infection.

The high frequency of ***N. caninum*** DNA detected in gerbils showed that oral inoculation of tachyzoites resulted in effective replication and broad organic distribution of the parasite. The results of the present study with neonate gerbils are in agreement with data from LÓPEZ-PÉREZ et al. (2006) and TOSCAN et al. (2012), which detected ***N. caninum*** DNA in lungs, heart, spleen, liver and kidneys, during acute infection in adult gerbils. These data showed the affinity of the parasite to these organs in the early period of infection. Therefore, these are the preferable tissues for investigation of ***N. caninum*** during acute infection in rodents.

Regarding tissue distribution of the parasite, KANG et al. (2009) showed that the first target-organs in ***N. caninum*** acute infection were liver, spleen and kidneys and that brain tissue was the last reached by the parasite. Thus, parasite distribution frequency was: liver/spleen/kidney > heart/muscle/spinal cord/blood > lung > brain. In the study of TOSCAN et al. (2012), the distribution frequency was: kidney > lung > spleen/heart > liver >

brain > spinal cord. In the present study, ***N. caninum*** tissue distribution was: heart/spleen > lungs/kidneys/brain, whilst the lowest frequency was reported in liver (but with no difference between brain and liver; $P<0.05$) (Table 1). These results were not fully concordant with reports of KANG et al. (2009) and TOSCAN et al. (2012). This fact may be related to the age of the animals. Neonate gerbils were, apparently, more susceptible to broad and systemic ***N. caninum*** infection, including brain invasion.

Gerbils immaturity is a contributive factor to ***N. caninum*** infection, since neonate animals are more susceptible to neosporosis due to their restricted immune competency. Thus, oral inoculation of $4x10^5$ tachyzoites was effective to reproduce acute neosporosis in 100% of the neonate gerbils, as an experimental model. However, there are still doubts on the concentration of tachyzoites in colostrum or milk of infected cows and the actual role of the oral route in natural transmission of neosporosis to neonate cattle, by tachyzoites ingestion. These aspects showed that further investigation is required to clarify the role of colostrum or milk in bovine neosporosis transmission.

CONCLUSION

Inoculation of $4x10^5$ ***N. caninum*** live tachyzoites by the oral route resulted in infection and clinical acute neosporosis in neonate gerbils. ***N. caninum*** DNA was distributed by several tissues, including central nervous system, of these animals. This study showed that neonate gerbils can be used as experimental models to reproduce acute neosporosis by oral infection with live tachyzoites.

BIOETHICHS AND BIOSECURITY COMMITTEE APPROVAL

This study was approved by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal de Santa Maria (UFSM). Assent no. 052/2012.

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