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Detection of larvae of *Toxocara canis* in milk: an experimental study in rabbits

Detecção de larvas de *Toxocara canis* em leite: um estudo experimental em coelhas

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Abstract

Toxocariasis, caused most commonly by *Toxocara canis*, is an important cosmopolitan zoonosis. Paratenic hosts have been employed to provide knowledge regard to the transmission of toxocariasis. Transmammary transmission in murine experimentally infected was observed based on the recovery of larvae from the tissue. The aim of this study was to evaluate the possibility of transmammary transmission of *Toxocara canis* in rabbits by detecting larvae directly in milk. Seventeen sexually mature virgin white New Zealand female rabbits were divided into two groups. Twelve animals were orally inoculated with 1,000 *T. canis* embryonated eggs (infected group), and five animals remained uninfected (control group). One month following the infection, the females were mated. Manual collection of 500 µL of milk from each rabbit was performed on days +7, +14 and +21 of lactation for three consecutive lactations. The recovery of larvae was determined via a centrifuge-sedimentation technique using ether and formalin solutions. ELISA test was run to confirm the production of anti-*T. canis* antibodies (IgG) by infected rabbits. The presence of larvae was observed in milk samples from 5 (41.7%) of the 12 infected rabbits. The total number of recovered larvae was 20, ranging from 1 to 4 larvae per lactation/rabbit. Larvae were recovered exclusively on days 7 and 14 of lactation. Recovery was verified in different lactations. No significant difference was observed with respect to the number of larvae either in the same lactation period or in different lactation periods. Anti-*T. canis* antibodies were detected in all infected rabbits. In conclusion, the presence of larvae in rabbit milk samples suggests the possibility of galactogenic transmission of *T. canis* in paratenic hosts. Moreover, the technique employed in this study allows for the recovery of larvae directly from milk.

Key words: Toxocariasis, lactation, diagnosis

Resumo

Toxocaríase, causada geralmente pelo *Toxocara canis*, é uma importante zoonose de distribuição mundial. Hospedeiros paratênicos têm sido utilizados para obtenção de informações sobre a transmissão de *T. canis*. A transmissão transmamária em murinos infectados experimentalmente foi observada com

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a recuperação de larvas. O objetivo do presente estudo foi de avaliar a possibilidade de transmissão transmamária de *Toxocara canis* em coelhos, pela detecção direta de larvas no leite. Dezesete coelhas (Nova Zelândia branca) púberes e virgens foram distribuídas em dois grupos. Doze fêmeas foram infectadas com 1000 ovos embrionados de *T. canis* (Grupo Infectado), por via oral, enquanto outras cinco coelhas foram mantidas sem infecção (Grupo Controle). Um mês após a inoculação, as coelhas foram acasaladas. Nos dias +7, +14 e +21 após o nascimento dos filhotes, foram coletados, por ordenha manual, 500µL de leite, em três lactações consecutivas. A recuperação de larvas foi determinada pelo uso da técnica de centrífugo-sedimentação com formol-éter. A técnica de ELISA foi empregada para confirmar a produção de anticorpos (IgG) anti-*T. canis* pelas fêmeas infectadas. Observou-se a presença de larvas em cinco das doze (41,7%) coelhas por amostra. As larvas foram recuperadas exclusivamente nos dias +7, +14 de lactação. A detecção foi observada em diferentes lactações. Não houve diferença significativa entre o número de larvas na mesma lactação ou entre as diferentes lactações. Anticorpos anti-*T. canis* foram detectados em todas as coelhas infectadas. Em conclusão, a presença de larvas no leite de coelhas sugere a possibilidade de transmissão lactogênica em hospedeiros paratênicos. Ademais, a técnica empregada no estudo permite a recuperação de larvas diretamente do leite.

Palavras-chave: Toxocaríase, lactação, diagnóstico

Introduction

The causal agents of human toxocariasis are the ascarid nematodes (roundworms) *Toxocara canis* and *T. cati*, whose hosts are dogs and cats, respectively. However, *T. canis* is the most commonly studied agent in experimental infections using animal models (SANTARÉM; RUBINSKY-ELEFANT; FERREIRA, 2011).

In dogs, *T. canis* is transmitted most commonly via the placenta; however, other routes of transmission also occur, including the ingestion of embryonated eggs in the soil, the ingestion of paratenic hosts, and the ingestion by puppies of milk infected with larvae from nursing bitches (RUBINSKY-ELEFANT et al., 2010).

Humans represent an accidental host of *T. canis*, whereby transmission occurs via the ingestion of eggs present either in soil or on dog hair or via the ingestion of undercooked or raw meat from paratenic hosts, including ruminants (ESPAÑA et al., 1993; ALDAWEK et al., 2002; YOSHIKAWA et al., 2011; PARK et al., 2012) and birds (MORIMATSU et al., 2006). In Argentina, a case report describing an infected premature child presenting with an ophthalmic disturbance caused by *T. canis* larvae suggests that toxocariasis can be transmitted congenitally to humans (MAFFRAND et al., 2006).

The galactogenic transmission of *T. canis* to man has not been reported in the literature. However, studies have demonstrated that via milk maternal–neonatal transmission of larvae can occur in experimentally infected mice, based on the recovery of larvae from the tissue of the newborn animals (REITEROVÁ; TOMASOVICOVÁ; DUBINSKÝ, 2003; JIN; AKAO; OHTA, 2008; DIAS et al., 2010). Nevertheless, information regard to recovery of larvae directly from milk in paratenic hosts is scarce in literature.

Based on these statements, the aim of the current study was to recover *T. canis* larvae directly from milk samples collected from experimentally infected rabbits.

Material and Methods

Animals

Seventeen sexually mature virgin white New Zealand female rabbits (two months of age) were included in this study. All animals were confirmed to be free of helminths and protozoa by examination of a sample of their faeces using the Willis-Molay and Hoffman et al. techniques (HOFFMANN, 1987).

This experiment involved two experimental groups of rabbits. The first group included 12 females experimentally infected with *T. canis* eggs,

and the second group, the control group, included five rabbits that were not infected.

Fourteen days preceding the experiment, the animals were kept in individual cages and were handled by researchers each day for 15 minutes, for a period of seven days, to make the animal handling easier during the experimental period (HÖRAK; TUMMELEHT; TALVIK, 2006). The animals were nourished with a commercial feed for rabbits according to the manufacturer's recommendations.

Experimental infection

The eggs of *T. canis* that were used for animal infection were extracted from adult female worms. The eggs were incubated in 2% formalin (1.0 mL of PBS containing eggs:5.0 mL of formalin) for approximately one month at 28°C. Following this period, the eggs were washed three times and collected by centrifugation (3 minutes at 2,000 rpm). After the washes, 1,000 eggs containing larva were counted in a Neubauer chamber and resuspended in 5.0 mL of distilled water.

After being mildly anesthetised via intramuscular injection with a 30 mg/kg tiletamine/zolazepam solution (Zoletil 5%, Virbac) diluted in saline solution (KANASHIRO; CASSU, 2008), each rabbit was orally infected with eggs via a plastic tube. The control group was inoculated with 5.0 mL of saline solution following the same anaesthetic procedure.

One month following the infection, 17 pairs of 4-month-old rabbits were mated in separate cages during a three-day period. The rabbits were kept in individual cages during pregnancy and throughout the lactational period. The offspring were allowed to nurse for three weeks, at which time the offspring were separate from their mothers. The mating process was then repeated twice for a total of three consecutive lactations throughout the duration of the study. The pairs (male and female rabbits) remained the same for each mating.

Collect of milk

After parturition (day 0), milk samples were collected once per day on days 7, 14 and 21. The milk samples (approximately 0.5 mL) were obtained by manual milking the teats of the rabbits. To promote the production of a sufficient amount of milk for analysis, the offspring were separated from their mothers for six hours before the milking process.

To obtain serum, blood samples were taken from the experimentally infected and control animals via central ear artery puncture in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Iowa (research.uiowa.edu/animal/?get=rbt_tech). Blood samples were collected on days 0 (pre-infection), 7, 14, 21 and 28 post-infection (DPI) and on the first day following the weaning.

Recovery of larvae

The recovery of *T. canis* larvae was performed by the centrifuge-sedimentation technique described by Ritchie (1948) with slight modifications (HOFFMANN, 1987). Briefly, 500 µL of milk was transferred to an Eppendorf tube. An aliquot of 200 µL of buffered formalin 7.5% (v/v) solution was then added to the milk. The tube was then allowed to stand for 20 minutes. Next, 200 µL of sulphuric ether solution was added to the milk solution, and the tube was vigorously shaken for 3 minutes and centrifuged at 2,000 rpm (697 g/cm³) for 10 minutes (Excelsa Baby II 206 BL – Fanem). After centrifugation, the top layer of ether and the fat plug were separated. Three aliquots of 20 µL of the sediment layer from the bottom of the tube were transferred to a glass microscope slide, which was then covered with a glass coverslip (22 X 22 mm) and examined microscopically (10X and 40X objectives). Third stage larvae were identified according to the morphological characteristics described by Nichols (1956) and Schacher (1957). The specimens recovered from the milk were

washed in distilled water and preserved at 4°C until identification.

ELISA (Enzyme-linked Immunosorbent Assay)

An indirect ELISA test was performed to evaluate the production of IgG anti-*T. canis* antibodies according to the protocol described elsewhere (DE SAVIGNY; VOLLER; WOODRUFF, 1979) with slight modifications (ELEFANT et al., 2006).

To perform the test, polystyrene 96-well microplates (Corning, Costar, New York, NY) were coated (1.9 µg/mL antigen/well) with excretory-secretory antigens produced by *T. canis* larvae (TES) for 2 h at 37°C, followed by 18 hours at 4°C, and then washed three times for 5 minutes with 0.01 M phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-T). The microplates were blocked with 2.5% skim milk (Molico, Nestlé) in PBS-T (200 µg/well) for 1 hour at 37°C and then washed three times with PBS-T.

Serum samples (100 µL/well) diluted 1:200 were incubated for 40 minutes at 37°C. After three wash cycles, the plates were incubated with 1:40,000 horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in PBS-T (100 µL/well; Sigma-Aldrich A0545, USA) for 40 minutes at 37°C. After a new cycle of washing, the plates were incubated with chromogen solution (100 µL/well; OPD Fast- Sigma, St Louis, USA) composed of ortho-phenylenediamine (0.4 mg/mL) and H₂O₂-urea peroxide (0.4 mg/mL) in 0.05 M phosphate-citrate buffer for 15 minutes at 37°C. The reaction was stopped with 2N H₂SO₄ (50 µL/well), and the optical density was measured at 492 nm (Titertek Multiskan MCC/340, Lab-System, Finland).

The cut-off value was calculated by adding two standard deviations and the mean absorbance of 17 negative control sera (5 controls and 12 pre-infected

animals). Antibody levels are expressed as reactivity indices (RIs), which were calculated as the ratio of the absorbance value of each tested sample to the cut-off value (0.292). Samples with RIs greater than 1 were considered positive.

Statistical analysis

Statistical analysis was performed using the Bioestat 5.0 computational package. The comparison of the number of larvae in different lactations was performed by ANOVA in conjunction with Tukey's method, and the Mann-Whitney test was employed to analyse the averages of the larvae counts at different evaluation time points from the same lactation event. A significance of 5% was set for all analyses (AYRES et al., 2007).

Ethical considerations

This study was approved by the Ethical Research Committee of the University of Oeste Paulista, Unoeste, Presidente Prudente, São Paulo, Brazil (132/09).

Results

In this study, five out of the 12 (41.7%) infected rabbits released *T. canis* larvae into the milk. All the larvae recovered were immobile and most of them were in a spiral form. The size of the larva was in the range of 0,387 to 0,412 mm length by 0,015-0,018 mm width.

A total of twenty larvae were recovered, equivalent to 0.17% of the inoculated material (12,000 embryonated eggs). Five larvae were recovered in the first lactation, 10 larvae in the second, and five in the third lactation (Table 1).

Table 1. The total number of larvae recovered from milk produced by New Zealand female rabbits that were experimentally infected with *Toxocara canis* embryonated eggs. The larvae were collected via a centrifuge-sedimentation technique at various lactation time points (7, 14 and 21 days) during three different lactation periods.

Lactation	Time points of milk sample collection			Total
	+7	+14	+21	
1	0	2	3	5
2	0	4	6	10
3	0	3	2	5
Total	0	9	11	20

Source: Elaboration of the authors.

The presence of larvae in the milk was observed exclusively on the days 14 (nine larvae; 45%) and 21 (11 larvae; 55%) of lactation.

The number of recovered larvae ranged from 1 to 4 per lactation/female, and a maximum number of two larvae were observed in a single sample (Table

2). It was observed that with the exception of one animal whose sample milk was considered positive, all rabbits released larvae through their milk at different time points during the same lactation and during two consecutive lactations.

Table 2. The individual numbers of larvae recovered from the milk produced by five of twelve New Zealand female rabbits that were experimentally infected with *Toxocara canis* embryonated eggs. The eggs were collected via a centrifuge-sedimentation technique at three lactation time points (7, 14 and 21 days) during three different lactation periods.

Animal	Larvae recovered at three time points during different lactations								
	Lactation 1			Lactation 2			Lactation 3		
	+7	+14	+21	+7	+14	+21	+7	+14	+21
03	0	0	0	0	1	2	0	2	1
05	0	0	1	0	0	0	0	0	0
06	0	0	0	0	1	1	0	1	1
07	0	2	2	0	1	2	0	0	0
11	0	0	0	0	1	1	0	0	0

Source: Elaboration of the authors.

No difference was observed with respect to the number of larvae during the same lactation ($p=0.7386$) or with respect to the average number of larvae recovered during different lactations ($p=0.5882$). Regarding the detection of serum antibodies, 11 infected animals were considered positive based on the ELISA on 28 DPI (IR: 1.383-4.639). However, at the time of weaning, antibodies were detected in all of the infected females (IR: 4.557-5.315).

Neither behavioural nor clinical changes were observed in the animals throughout the experimental period.

Discussion

Milk is an important route of transmission of *T. canis* in dogs (BURKE; ROBERSON, 1985). Some studies in mice have found that *T. canis* larvae are able to migrate from the mother to neonates via suckling, based on the recovery of larvae

using a tissue digestion technique (REITEROVÁ; TOMASOVICOVÁ; DUBINSKÝ, 2003; JIN; AKAO; OHTA, 2008; DIAS et al., 2010). In our study, the *T. canis* larvae were recovered directly from the milk of experimentally infected rabbits.

Although the murine model has been considered the ideal model for the study of human toxocariasis (BARDÓN; CUÉLLAR; GUILLÉN, 1994), rabbits were utilised in this study because the amount of milk secreted by nursing female rabbits was considered sufficient for the recovery of larvae using the Ritchie technique. This technique is based on centrifuge-sedimentation and is employed for the detection of helminths and protozoan structures in stool samples as part of routine laboratory and epidemiological studies (VALVERDE et al., 2011). The technique involves the use of ether and formalin solutions to dissolve fat elements present in faeces. In our study, these solutions served to promote the lysis of the fat present in milk and the analysis of the sediment. The authors have considered that the larvae may be retained in the fatty layer, thereby making the analysis difficult.

The larvae recovered in our experiment were in an immobile spiral form, probably due to the formalin-ether action. The characteristics of the specimens were similar to the described by Nichols (1956) and Schacher (1957), and the size was in accordance to the observed by these authors and by Rim (1963), who verified a wide variation in size of third stage larvae recovered from tissues.

Further studies are necessary to optimise the technique employed in this experiment. Nevertheless, the technique employed in this study does not require the evaluation of the animal tissues, thereby avoiding the need for euthanasia. Additionally, it was possible to assess the number of larvae released in milk in different lactations.

The number of larvae recovered from the milk, equivalent to 0.17% of the inoculated material, was considered low. In dogs, Burke and Roberson (1985) observed that the transmission of larvae

from nursing females to offspring occurs almost exclusively via the placenta (98.5%), whereas only 1.5% of transmissions occur via milk. In contrast, Tomašovicová et al. (1993) verified in mice that *T. canis* is primarily transmitted via milk and that uterine migration is sporadic. These conflicting findings suggest that the migrating behaviour of *T. canis* may vary between the usual and paratenic hosts. In addition, the estimated number of larvae may be influenced by the technique employed for recovery.

Hall (1971) observed that the amount of fat present in the milk of New Zealand rabbits increases gradually throughout the lactation. Thus, a gradual reduction in the number of larvae in milk during the lactation would be expected. However, larvae were not recovered in the first week. Larvae were detected only in the second and the third weeks of nursing with no statistically significant difference. Conversely, Jin, Akao and Ota (2008) recovered larvae from the tissue of mice on days 7 and 14 after birth. Moreover, these authors observed that the number of larvae recovered during the second week was significantly greater than during the first week. After administering prolactin to mice, these authors concluded that this hormone is a promoting factor that contributes to the lactational transmission of *T. canis* larvae in mice. In our study, no substance was administered to the animals to promote the secretion of milk.

Other methodological criteria adopted in experimental studies may influence the evaluation of the migration of *T. canis* larvae, including the number of infective eggs and the gestational and timelines of evaluation. Dias et al. (2010) observed that the infective dosage was directly proportional to the level of transmammary transmission of larvae via milk in mice. These authors infected female adult mice two months before mating, observing that larvae were identified 60 days after birth in 15.2% and 85.7% of the mice that nursed from animals infected with 1,200 and 2,500 eggs, respectively. Different from our study, in which

the larvae were recovered directly from milk, these authors recovered the larvae from the tissues of mice. Regarding the number of larvated eggs, we used 1,000 eggs for infection, that has been widely employed for experimental infection in mice (ABO-SHEHADA; HERBERT, 1984; SAMANTA; ANSARI, 1990; BARDÓN; CUÉLLAR; GUILLÉN, 1994; XI; JIN, 1998; REITEROVÁ; TOMASOVICOVÁ; DUBINSKÝ, 2003; CHO et al., 2007).

In the study of Jin, Akao and Ota (2008), two Balb/c female mice were infected with 300 *T. canis* eggs 12 hours following the post-puerperal period. A recovery of 18% of the larvae from the tissue of the offspring was due to mammary transmission. Nevertheless, Reiterová, Tomasovicová and Dubinský (2003) verified that the number of larvae transmitted by milk is independent of the period in which the females are infected (pre or post puerperium).

In the present study, an ELISA test was performed to confirm the infection of the animals, and samples presenting RI values greater than 1 were considered positive. During weaning, antibodies were detected in all infected rabbits (IR: 4.557-5.315), thereby confirming infection.

Our data suggest that the release of *T. canis* larvae via milk may represent an important route of transmission in paratenic hosts. In addition, the technique employed in this study is a feasible tool to recover *Toxocara* spp. larvae directly from the milk.

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