

Molecular investigation of *Neorickettsia risticii* in trematodes and snails in a region with serological evidence of this agent in horses, state of Rio de Janeiro

[*Pesquisa molecular de Neorickettsia risticii em trematódeos e moluscos em região com evidência sorológica do agente em equinos, estado do Rio de Janeiro*]

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ABSTRACT

In Brazil, some studies have indicated that *Neorickettsia risticii* circulates in horses, but it is unclear which are the possible intermediate vectors of this bacterium in the country. The aim of this study was to use molecular techniques in order to analyze the presence of *N. risticii* in snails and larval stages of trematodes in farms in a region with a history of seroreactive horses towards this bacterium, in Rio de Janeiro, Brazil. Convenience sampling was used in the studied region. The collected snails were exposed to incandescent light (60W) for 2-4 hours in order to investigate trematodes in larval forms. Deoxyribonucleic acid (DNA) was extracted from snail tissue and trematode. Real-time PCR (qPCR) technique was used to investigate the presence of a 16S *rRNA* gene fragment of *N. risticii*. Snail specimens (n=410) were collected from 11 horse-breeding farms, and the following species were identified: *Melanoides tuberculata*, *Pomacea* sp., *Biomphalaria tenagophila*, *Physa acuta*, *Drepanotrema anatinum* and *Biomphalaria straminea*. Only 3.17% (n=13/410) of the collected snails were infected by trematodes. The cercariae obtained from these snails were classified as *Megalourous cercariae*, *Pleurolophocercus cercariae* and *Furcocercous cercariae*. There was no amplification of the target DNA of *N. risticii* in the snail and trematode samples tested by qPCR. Based on these data, the transmission of *N. risticii* by trematodes using these snail species in this region does not appear to occur or occurs at very low rates. Thus, further studies are needed in order to clarify which species of invertebrate hosts are infected by this bacterium and potentially participate in the transmission chain of equine neorickettsiosis in the state of Rio de Janeiro, Brazil.

Keywords: equine neorickettsiosis, molecular diagnosis, qPCR, intermediate hosts

RESUMO

No Brasil, estudos apontam a circulação de *Neorickettsia risticii* em equinos, contudo não estão claros quais os possíveis vetores intermediários dessa bactéria no país. O objetivo do presente estudo foi analisar a presença de *N. risticii*, utilizando-se técnicas moleculares, em caramujos e estágios larvais de trematódeos em propriedades rurais de uma região com histórico de equinos sororreativos para essa bactéria, no Rio de Janeiro, Brasil. Uma amostragem por conveniência foi utilizada na região de estudo. Os caramujos coletados foram expostos à luz incandescente (60W) durante duas-quatro horas para a investigação de trematódeos nas formas larvais. A extração de ácido desoxirribonucleico (DNA) foi realizada em tecidos de caramujos e trematódeos. A técnica de PCR em tempo real (qPCR) foi utilizada para investigar a presença de um fragmento do gene 16S *rRNA* de *N. risticii*. Foram coletados 410 espécimes de caramujos de 11 propriedades com criações de equinos, sendo identificadas as seguintes espécies: *Melanoides tuberculata*, *Pomacea* sp., *Biomphalaria tenagophila*, *Physa acuta*, *Drepanotrema*

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anatinum e *Biomphalaria straminea*. Apenas 3,17% (n=13/410) dos caramujos identificados estavam infectados por trematódeos. As cercárias obtidas desses caramujos foram classificadas em *Megalourous cercariae*, *Pleurolophocercus cercariae* e *Furcocercous cercariae*. Não foi observada a amplificação do DNA-alvo de *N. risticii*, por meio da qPCR, em nenhuma das amostras de caramujos e trematódeos testadas. Com base nesses dados, a transmissão de *N. risticii* por trematódeos que utilizam as espécies de caramujos nessa região parece não ocorrer ou ocorre a taxas muito reduzidas. Portanto, novos estudos são necessários para elucidar quais espécies de hospedeiros invertebrados se infectam por essa bactéria e potencialmente participam da cadeia de transmissão da neorickettsiose equina no estado do Rio de Janeiro, Brasil.

Palavras-chave: neorickettsiose equina, diagnóstico molecular, qPCR, hospedeiros intermediários

INTRODUCTION

Neorickettsia risticii (Holland *et al.*, 1985) Dumler *et al.*, 2001 (previously described as *Ehrlichia risticii*), a bacterium of the Anaplasmataceae family, is the etiological agent of equine neorickettsiosis (Pusterla *et al.*, 2003), also known as Potomac horse fever (Rikihisa *et al.*, 1984). This bacterium preferentially invades cells of the mononuclear system, such as monocytes, tissue macrophages and intestinal epithelial cells (Rikihisa *et al.*, 1984).

This disease affects horses at all ages and the clinical signs vary, but include fever, depression, anorexia, laminitis, colic, and acute diarrhea (Holland *et al.*, 1985).

Studies indicate that transmission to horses occurs through oral ingestion of the trematodes infected with *N. risticii* (Madigan *et al.*, 2000; Mott *et al.*, 2002). These helminths might be free-living in the water that horses drink, attached to vegetation or encysted in intermediate hosts (Barlough *et al.*, 1998). Snails are always used by digenetic trematodes as the first intermediate host. Aquatic insects are used as a second intermediate host (Chae *et al.*, 2000). The horse might accidentally ingest these insects containing metacercariae infected with *N. risticii* and acquire the infection (Mott *et al.*, 2002). Insectivorous birds and bats are definitive hosts for trematodes that are vectors for *N. risticii* (Pusterla *et al.*, 2003; Gilbson *et al.*, 2005). Studies have shown that snails of the families Pleuroceridae (genus *Juga*), Planorbidae (genus *Planorbella*) and Hydrobiidae (genus *Heleobia*) have a direct relationship with the transmission of this bacterium (Pusterla *et al.*, 2000a; Coimbra *et al.*, 2005; Pusterla *et al.*, 2013). These snails have been found to be positive for *N. risticii* using nested PCR (nPCR) and real-time PCR

(qPCR) as diagnostic techniques (Barlough *et al.*, 1998; Pusterla *et al.*, 2000b).

Geographical distribution of equine neorickettsiosis in the United States has been well demonstrated (Palmer *et al.*, 1986). Clinical and serological evidence shows that the disease also occurs in Canada, Brazil, Uruguay, and some countries in the Europe (Van Der Kolk *et al.*, 1991; Dutra *et al.*, 2001; Baird and Arroyo, 2013). In Brazil, compatible cases with the clinical disease were firstly described in the state of Rio Grande do Sul (Dutra *et al.*, 2001; Coimbra *et al.*, 2006). Serological evidence has also indicated that the agent circulates in horses in the states of Santa Catarina, São Paulo and Rio de Janeiro (Ferrão *et al.*, 2007; Moreira *et al.*, 2013).

Only snails of the genus *Heleobia* have tested positive for *N. risticii* using the PCR technique in southern Brazil. This genus has been indicated as participant in the transmission chain of the equine neorickettsiosis in Brazil (Coimbra *et al.*, 2005). There is a need for further studies relating the bacterium to other possible intermediate hosts in Brazil, especially in areas with serological evidence of its circulation. The aim of the present study was to detect *N. risticii* DNA in larval trematodes and snails that are present in natural and artificial sources of water, on horse-breeding farms in the Itaguaí microregion, state of Rio de Janeiro, using qPCR as a diagnostic technique.

MATERIAL AND METHODS

This study was conducted between January and May 2013, in the municipalities of Seropédica and Mangaratiba (43° 46' 31" W and 22° 51' 08" S), which are located in the Itaguaí microregion of the state of Rio de Janeiro, Brazil. These

municipalities were selected because of a previous study (Roier, 2011) which showed seroreactive horses to *N. risticii* in this region. Each collection point was georeferenced using the global positioning system (GPS; *eTrex 30*, Garmin®, Kansas, USA) (Fig. 1). The Itaguaí microregion is characterized as tropical, with dry

winters and rainy summers (Aw), with a hot and humid climate in general.

The farms were selected due to the presence of grazing areas and at least one source of water, such as lakes, rivers, streams, ponds and artificial water bodies. Convenience sampling of snails was carried out in these municipalities.

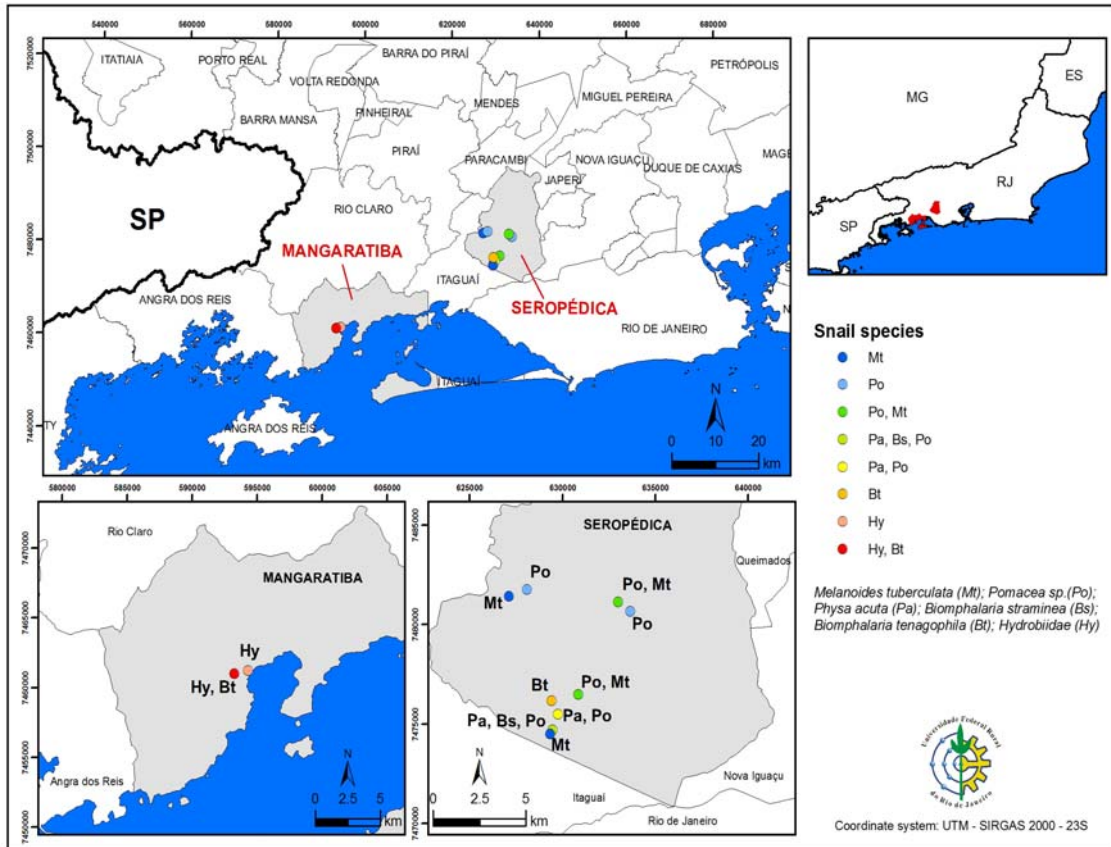


Figure 1. Geographical location of the areas where the snails were collected, according to the species and the municipality in the state of Rio de Janeiro.

The snails were collected using 2mm fine-mesh netting and were transported to the laboratory. They were separated according to the genus and then, the snails were identified in species using conchology and anatomical dissections with the assistance of the Laboratório Nacional de Referência em Malacologia do Instituto Oswaldo Cruz (LNRM/ IOC/FIOCRUZ-RJ).

The snails were removed from their shells and were sectioned into four anatomical divisions: gonads (containing a small portion of the digestive gland), digestive gland, cephalopodal mass, and pallial cavity. These anatomical parts

were grouped into pools containing 10 specimens (divided according to genus, sexual dimorphism and collection site), and were stored at -80°C until DNA extraction.

Larval trematodes in the snails were surveyed as described by Coutinho (1950). The snails in which no cercariae were observed after exposure to light were kept in the dark until the next day and they were subsequently reexamined to ascertain which larval forms presented nocturnal activity.

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The larval forms of trematode were fixed in acetic acid 1:2 (AFA) and stained with Delafield hematoxylin (Merck, Darmstadt, Germany) for morphological identification. To determine the group, the larval forms were classified according to morphometric characteristics using an optical microscope (Olympus BX51[®], Pennsylvania, USA) coupled to the Cell[^]D software (Olympus Europe Software Informer[®], Pennsylvania, USA). The identification of species was based on morphological characteristics (Freitas, 1977).

Different anatomical regions of the snails, such as their digestive glands, gonads, cephalopod mass and pallial cavity, were separated as described previously, in order to perform DNA extraction. Each sample was weighed on a precision balance and 15mg were macerated using a glass rod. Genomic DNA was extracted using a commercial kit, in accordance with the manufacturer's recommendations (Wizard genomic DNA purification kit from tissue, Promega[®], Madison, USA).

Larval trematodes were placed in polypropylene tubes containing 2mL of distilled water. The tubes were centrifuged at 6,000g for 4 minutes and DNA extraction was performed on the sediment, using a commercial kit (Wizard genomic DNA purification kit, Promega[®], Madison, USA).

All of the DNA samples were quantified using a spectrophotometer (NanoDrop ND-2000[®], Thermo Fisher Scientific Inc., Massachusetts, USA). They were diluted to 100ng/ μ L and stored at -20°C until molecular analysis.

Neorickettsia risticii DNA from snails and larval trematodes was detected using the TaqMan real-time PCR system in the StepOnePlus[®] equipment (Applied Biosystems, California, USA), in order to amplify a fragment of 85bp (base pairs) of the 16S *rRNA* gene (Pusterla *et al.*, 2000b). Samples with cycle quantification (Cq) values below or equal to 40 cycles were considered positive. The reactions were performed in duplicate on different plates with a final volume of 20 μ L, containing: 1X TaqMan[®] Universal PCR Master

Mix (Applied Biosystems[®], California, USA), 0.6mM of each primer, 0.2mM of the probe and 100ng of total DNA. The positive control sample was obtained from DH82 cell culture infected with *N. risticii*, removed from glass slides prepared for indirect immunofluorescence (Fuller Laboratories, California, USA). The slide was scraped and the contents were eluted in PBS solution. DNA extraction was performed subsequently using the DNeasy blood and tissue kit (Qiagen, Valencia, California, USA), in accordance with the manufacturer's recommendations.

The distribution of snails by municipalities and the morphometric data of the studied trematodes were submitted to descriptive analysis in the BioEstat 5.0 software (Ayres *et al.*, 2007).

These procedures were approved by Comissão de Ética na Pesquisa da Universidade Federal Rural do Rio de Janeiro (COMEP/UFRRJ), under protocol number 364/2013 and process number 23083.003989/2013-69.

RESULTS

The taxonomy classifications of the collected snails are shown in Tab. 1. A total of 410 snails were collected from the horse-breeding farms in the Itaguaí microregion. Among the species that were identified, *Melanoides tuberculata* (Muller, 1774) was the most frequent (Fig. 2), representing 71.7% (n=294/410) of the collected snails, followed by *Pomacea* sp. (Perry, 1810) with a frequency of 10.97% (n=45/410). *Biomphalaria straminea* (d'Orbigny, 1835) was the less frequent species, with only one specimen collected. These snails were deposited in the snail collection of IOC/Fiocruz-RJ under numbers 8260 to 8276. It was observed that *M. tuberculata* occurred in all of the municipalities analyzed, while *B. straminea* and *P. acuta* (Draparnaud, 1805) were observed only in the municipality of Seropédica. Likewise, *Drepanotrema anatinum* (Orbigny, 1835) and Hydobriidae (Stimpson, 1865) were observed only in the Mangaratiba municipality.

Table 1. Taxonomy classification of the snails collected in the Itaguaí microregion, Seropédica, and Mangaratiba municipalities separately, state of Rio de Janeiro, Brazil

Snails	Total area		Seropédica		Mangaratiba	
	N	(%)	N	(%)	N	(%)
<i>Melanoides tuberculata</i>	294	71.70	260	76.47	34	48.57
<i>Pomacea</i> sp.	45	10.97	45	13.23	-	-
<i>Drepanotrema anatinum</i>	15	3.65	-	-	15	21.43
<i>Biomphalaria tenagophila</i>	27	6.58	11	3.23	16	22.86
<i>Biomphalaria straminea</i>	1	0.24	1	0.29	-	-
<i>Physa acuta</i>	23	5.61	23	6.76	-	-
Hydrobriidae	5	1.25	-	-	5	7.14
Total	410	100.0	340	100.0	70	100.0

Larval trematodes were found in 3.17% (n=13/410) of the snails collected in the studied area. Regarding the snail species that were naturally infected by trematodes, 25.92%

belonged to *B. tenagophila* (n=7/27), 1.70% to *M. tuberculata* (n=5/294), and 20% to Hydrobriidae (n=1/5).



Figure 2. Specimens of *Melanoides tuberculata* collected in the Itaguaí microregion, state of Rio de Janeiro.

Regarding the municipalities, the highest frequency of snails infected by trematodes was observed in Mangaratiba, at a rate of 4.28% (n=3/70), followed by Seropédica, with 2.94% (n=10/340) of frequency. It was possible to detect trematode larvae, rediae, cercariae, and metacercariae in the digestive gland and cephalopodal mass of the parasitized snails. Morphometric data relating to cercariae morphotypes of infected snails are shown in Tab. 2.

Among the thiarids, the presence of several cercariae morphotypes was observed (Fig. 3). Morphotype 1 was characterized as the *Megalourous* cercariae group, infecting 1.02%

(n=3/294) of the collected snails. Curiously, the metacercariae had a piriform shape and were observed only once, since their main feature was rapid encystment, which occurred in about one hour. Morphotype 2 was characterized as the *Pleurolophocercous* cercariae group and was found parasitizing *M. tuberculata* specimens at a frequency of 0.68% (n=2/294). Morphotype 3 was obtained from *B. tenagophila* and its cercariae were characterized as *Furcocercous* cercariae. Although a single specimen of morphotype 4 was also observed infecting Hydrobriidae snails, it was not possible to stain and measure the specimen collected, because of the rapid degradation of the material that occurred through the staining process.

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Table 2. Trematode stage, anatomical structures and morphometric data obtained in relation to the three cercariae morphotypes of infected snails that were collected in the Itaguaí microregion, state of Rio de Janeiro, Brazil

Stage	Anatomical structure	Parameter measured	Type 1	Type 2	Type 3
			M ± SD	M ± SD	M ± SD
Cercariae	BO	L	309.7 ± 71.1	174.3 ± 4.2	154.8 ± 72.3
		W	85.0 ± 48.3	88.0 ± 1.3	37.8 ± 27
	OS	D	52.5 ± 6.5	16.6 ± 1.6	-
	VS	D	43.4 ± 14.4	20.6 ± 3.5	-
	TA	L	46.1 ± 1.0	101.8 ± 7.9	125.7 ± 3.1
		W	21.9 ± 1.8	7.9 ± 3.5	18.8 ± 0.3
	RO	D	-	6.3 ± 1.0	-
	LO	D	-	5.7 ± 0.2	-

L = length, W = width, D = diameter, OS = oral sucker, VS = ventral sucker, RO = right ocellus, LO = left ocellus, BO = body, TA = tail, M = mean, SD = standard deviation.

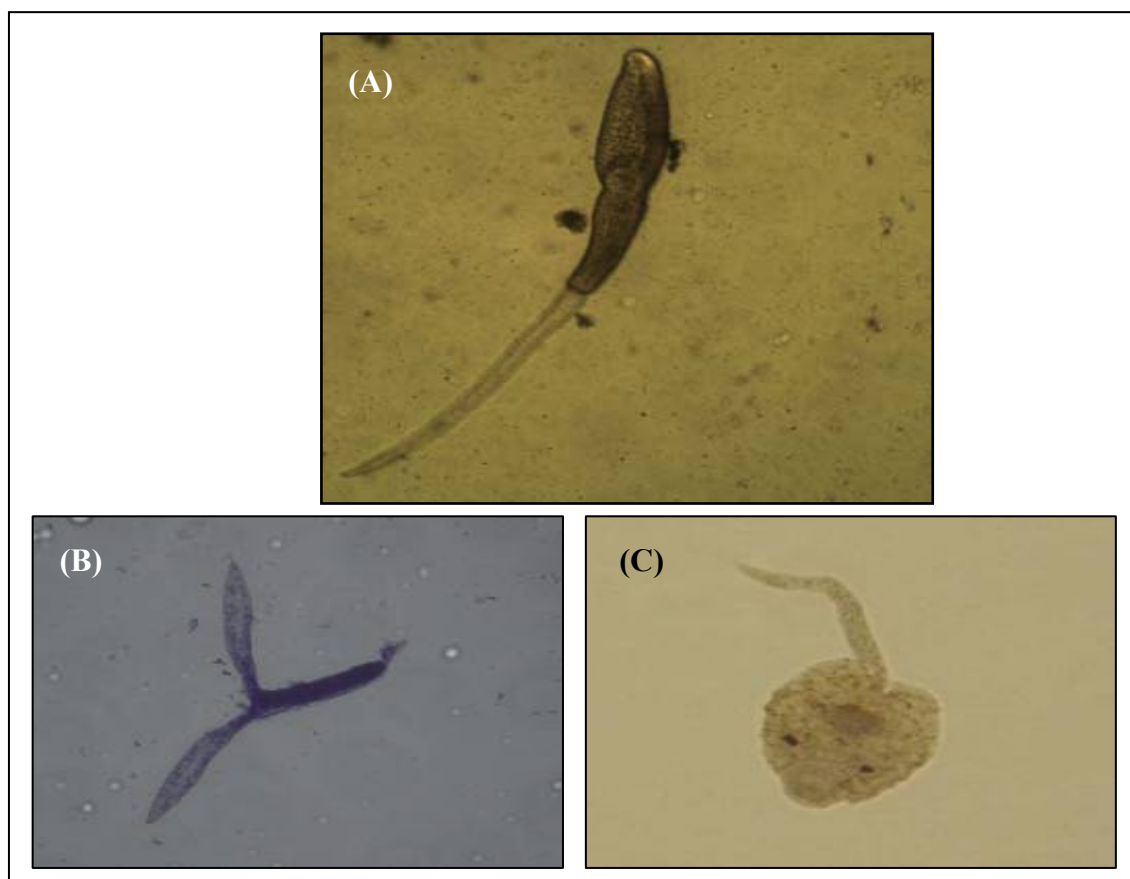


Figure 3. Cercariae morphotypes observed amongst infected *Melanoides tuberculata* collected in the Itaguaí microregion, state of Rio de Janeiro. (A) *Megalourous* cercariae; (B) *Furcocercous* cercariae; (C) *Pleurolophocercous* cercariae (400X magnification under an optical microscope).

Real time PCR analyzes on the snail samples showed that there was no amplification of *N. risticii* DNA. Similarly, no amplification of the 16S *rRNA* gene of *N. risticii* from trematode samples was observed.

DISCUSSION

In the present study, the target DNA of *N. risticii* in snail tissues and larval trematode samples was not detected. Previous studies have shown the

movement of this bacterium between horses in the Itaguaí microregion, Rio de Janeiro state (Roier, 2011; Moreira *et al.*, 2013). Furthermore, clinical evidence of equine neorickettsiosis has been reported in the same state (Ferrão *et al.*, 2007). These results do not rule out the possibility of this bacterium infection in trematodes in the studied region, with very low prevalence.

It is well known that this agent has a relatively low prevalence in snails, even in areas with a history of neorickettsiosis in horses. Studies conducted in the United States have shown that the positivity rates of snails infected by *N. risticii* range from 0.7 to 12.5%, even in endemic areas for the disease (Barlough *et al.*, 1998; Pusterla *et al.*, 2000a; Pusterla *et al.*, 2013). In Brazil, a study conducted by Coimbra *et al.* (2005) showed that the frequency of snails infected by this bacterium was 13.3% in a region with a history of neorickettsiosis in horses. Furthermore, the percentage of infected snails can be influenced by certain biological factors, such as the type and maturation status of snail and trematode vectors, the period of the year and the region of study (Pusterla *et al.*, 2000a).

The biology of the transmitters is associated with seasonality, and this influence affects the distribution of equine neorickettsiosis. In the United States, Palmer *et al.* (1986) observed that this disease occurred in the hottest months of the year (June to September), a period compatible with greater availability of snails. In addition, occurrences of equine neorickettsiosis were associated with wetlands, large river valleys and low areas near large lakes or irrigation ditches (Palmer *et al.*, 1986; Dutra *et al.*, 2001; Coimbra *et al.*, 2006). Although the present study was conducted during the warmer months and in areas with characteristics similar to those already described, the presence of *N. risticii* DNA was not observed in any of the snail samples analyzed. This fact highlights the low prevalence of this bacterium in snails, in areas with a history or serological evidence of equine neorickettsiosis (Barlough *et al.*, 1998; Pusterla *et al.*, 2000a; Chae *et al.*, 2003; Pusterla *et al.*, 2013). It is important to mention that the rate of snail infection by *N. risticii* is related directly to the trematode infection rate. In the present study, the trematodes infection rate in snails was very low (3.17%).

Recent studies have shown that different genera of snails are involved in the equine neorickettsiosis transmission cycle. In the United States, *Juga* sp., *Planorbella* sp., *Stagnicola* sp. and *Elimia* sp. were considered to be the main species of freshwater snails associated with the life cycle of the digenetic trematode vectors of *N. risticii* (Kanter *et al.*, 2000; Pusterla *et al.*, 2000a; Pusterla *et al.*, 2013). In Brazil, so far, only the genus *Heleobia* has been correlated with the equine neorickettsiosis cycle in the state of Rio Grande do Sul (Coimbra *et al.*, 2005). None of these species were observed in the present study, but the mollusk fauna collected corroborate the findings of Thiengo *et al.* (2001). *Melanooides tuberculata* was the most common species of snail in this region. However, this species has not been incriminated as a snail vector of *N. risticii* in other studies. It is possible that other genera or species of snails are related to the transmission of *N. risticii* in the state of Rio de Janeiro.

Interestingly, *B. straminea* and *B. tenagophila* were found in the studied area. Both species are a public health concern given that these are intermediate hosts of the nematode *Schistosoma mansoni*, which is the etiological agent of schistosomiasis in humans.

None of the trematodes were positive for *N. risticii*, although *Furcocercous*-type cercariae were observed, which have already been identified as a vector for this bacterium in other studies (Chae *et al.*, 2003; Park *et al.*, 2003). The absence of the bacterium in these helminths may be related to agent specificity for its hosts, and also to transmission capacity for subsequent generations, when infected (Greiman *et al.*, 2013).

Other intermediate hosts such as aquatic insects may act as an infection source to a trematode hosting the bacterium and to accidental hosts, as horses (Madigan *et al.*, 2000). Chae *et al.* (2000) described 13 species of aquatic insects that might be directly related to the trematode vector cycle in Northern California. In Brazil, few studies have been conducted focusing in other possible intermediate hosts of trematode vectors beside the snails (Coimbra, 2010). Moreover, some studies have shown involvement of birds and bats as wild reservoirs of *N. risticii* (Pusterla *et al.*, 2003; Gibson *et al.*, 2005). Thus, this study

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does not exclude the possibility of different hosts participating in the equine neorickettsiosis transmission chain in Brazil.

In Brazil, the acute phase of this disease was reported just in the southern region, where the climate is comparable with American states (Dutra *et al.*, 2001; Coimbra *et al.*, 2006). In other Brazilian regions, only serological evidence of *N. risticii* exists (Ferrão *et al.*, 2007; Moreira *et al.*, 2013). This serological evidence demonstrates that the animal has been exposed to the bacterium even though the agent has not been directly identified. This constitutes the main critical point in the understanding of the epidemiological chain of equine neorickettsiosis in the studied region. The absence of bacterial DNA might be explained by little or no movement of *N. risticii* in the Itaguaí microregion. Further studies are still needed using a larger sampling of snails, at different seasonal periods to confirm the low occurrence or the absence of *N. risticii* in these hosts in studied region.

CONCLUSION

It was not possible to identify which species of snails and trematodes are participating in the transmission chain of *N. risticii* to horses in the Itaguaí microrregion, state of Rio de Janeiro, Brazil.

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