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Infection rate of *Ehrlichia risticii*, the agent of Potomac horse fever, in freshwater stream snails (*Juga yrekaensis*) from northern California

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Abstract

Juga yrekaensis freshwater snails were tested for trematode stages and for *Ehrlichia risticii* DNA using a nested PCR assay. Snails were collected monthly from two Potomac horse fever (PHF) endemic locations in northern California (Montague and Weed).

The trematode infection rate varied between 40 and 93.3% in large snails (shell size >15 mm) and between 0 and 13.3% in small snails (<15 mm). The highest trematode infection rate for large and small snails was recorded in September and the lowest infection rate for large snails was recorded in June (Weed) and October (Montague). The *E. risticii* PCR infection rate among small snails from both sites was similar and varied monthly between 0 and 3.3%. The PCR infection rate for large snails from Weed was high in May (20.0%) and decreased progressively until November (10.0%). The PCR infection rate for large snails from Montague was 5.0% in May, 26.3% in August and 16.7% in October. PCR-positive snails were always related to the microscopic detection of trematode stages (virgulate cercariae).

This study provides evidence that *J. yrekaensis* are infected with trematode cercariae that harbor *E. risticii*. The number of snails harboring trematode stages and the number of PCR positive snails varied with the size of the snails, the month of collection, and the geographic origin.

Keywords: *Ehrlichia risticii*; Potomac horse fever; Freshwater snails; Seasonal prevalence; California

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1. Introduction

Ehrlichia risticii is the causative agent of Potomac horse fever (PHF), a febrile gastrointestinal disorder of horses reported in North America and Europe (Holland et al., 1985; Ristic, 1990). PHF continues to be a significant problem for horses in the United States, while the mode of transmission and the maintenance cycle of *E. risticii* in nature are still unclear. Since the earliest reports on PHF, an association of cases with aquatic habitats has been recognized. Recent work has shown the close phylogenetic relationship between *E. risticii* and the helminth-associated *Neorickettsia helminthoeca*, the agent of 'salmon poisoning' in dogs (Rikihisa, 1991; Pretzman et al., 1995). Reubel et al. (1998) first reported that *E. risticii* DNA is found in virgulate cercariae released from freshwater operculate snails and suggested that the snails are the intermediate host of a trematode carrying the agent of PHF. Recently, our laboratory reported the successful experimental transmission of *E. risticii* to horses through inoculation of virgulate cercariae collected from *Juga yrekaensis* snails (Pusterla et al., 2000).

The purpose of this study was to determine, by nested PCR, the prevalence of *E. risticii* in freshwater snails, collected from May through October 1999 from a PHF enzootic region in northern California.

2. Materials and methods

2.1. Snail collection

Freshwater snails were collected monthly from May through October 1999 from pastures in Weed and Montague, California (Siskiyou County), with a history of PHF in some resident horses. The pastures are located 15 miles from each other. Snails were collected by hand or with a net from the clear, shallow margins of the Shasta River which flows through both endemic pastures and is accessible to horses for drinking. Thirty small (shell size <15 mm) and 30 large (>15 mm) *J. yrekaensis* snails were collected monthly from each location and transported in chilled water (10–12°C) to our laboratory at the University of California, Davis, for examination. In this study, we focused our analyses exclusively on *J. yrekaensis* snails for the following reasons:

1. *J. yrekaensis* is the most abundant freshwater snail at the endemic sites (Burch, 1989);
2. this snail has previously been reported to be infected with virgulate cercariae carrying *E. risticii* (Reubel et al., 1998); and
3. *E. risticii*-infected trematode stages collected from *J. yrekaensis* were previously shown to transmit PHF to horses (Pusterla et al., 2000).

2.2. Processing of snails

Snails (30 small, 30 large) were measured, extracted from their shell using sterile scissors and forceps, and each was placed in a 1.5 ml microcentrifuge tube containing 1 ml of phosphate-buffered saline (PBS). The tubes were vortexed vigorously for 10 s to disrupt the fragile snail tissue, primarily the hepatopancreas or digestive glands, and to release

the trematode stages (sporocysts and cercariae). To determine the presence of trematode stages, 40 μl of the PBS-mixture was placed on a slide and examined by light microscopy (magnification, 20×). An additional 50 μl of each PBS-mixture was processed for DNA extraction using a modified DNA alkaline extraction protocol for genomic DNA (Rudbeck and Dissing, 1998). Briefly, the mixture was centrifuged at 15,000×*g* for 5 min and the supernatant discarded. The pellets were mixed with 20 μl of 0.2 M NaOH, covered with mineral oil to prevent evaporation, and incubated at 75°C for 30 min, followed by denaturation at 95°C for an additional 15 min. The extracts were neutralized with 180 μl of 0.04 M Tris-HCl (pH 7.5). The tubes were kept on ice, centrifuged (15,000×*g*) at 4°C for 5 min and the supernatant used as template DNA for PCR amplification. The templates were tested by a nested PCR that amplifies a 527-bp segment of the 16S rRNA gene of *E. risticii* (Barlough et al., 1997). The primer pairs used were ER-2 (5′-GTTTTAAATGCAGTTCTTGG-3′) and ER-3 (5′-ATTTGAGAGTTTGATCCTGG-3′) in the first round and ER-2a (5′-CACACCTAAC TTACGGG-3′) and ER-3a (5′-CTAGCGGTAGGCTTAAC-3′) in the nested round. The cycling parameters used were as follows: preheating at 94°C for 5 min and then 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1.5 min, followed by a final extension step at 72°C for 7 min. The PCR products were visualized in ethidium bromide-stained 1.5% agarose gels.

2.3. Sequencing of amplified PCR products

Twenty nested PCR products derived from single snails were selected for sequencing to verify their identity. The fragments were purified using a gel extraction Kit (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA). DNA sequencing of both strands of each purified PCR product was performed using a fluorescence-based automated sequencing system (PE Applied Biosystems, Foster City, CA).

3. Results

A total of 720 *J. yrekaensis* snails were collected over the 6-month period. The snails collected from the two locations were similar in size with 12.8±1.8 mm (mean±s.d.) for small snails and 23.6±2.7 mm for large snails. The type of cercaria found was the virgulate cercaria, which was identifiable by the presence of a bilobed or pyriform virgula organ located in the region of the oral sucker, a tail shorter than the body, and the ventral sucker smaller than the oral sucker, according to the taxonomic keys of Schell (1970). The virgulate cercariae were often found within sporocysts characterized by an ovoid form, a cellular body wall, a body cavity, germinal cells, excretory system and a terminal birth pore through which cercariae escape. The trematode infection rate varied with the size of the snails, the collection time and the geographic origin (Tables 1 and 2). Large snails were significantly more often infected with trematodes than small snails (χ^2-test, $p<0.001$). The highest trematode infection rate for large and small snails was recorded in September and the lowest infection rate for large snails was recorded in June (Weed) and October (Montague). In Weed, the trematode infection rates for large snails for May and September were significantly higher than in October ($p<0.05$). In Montague, the trematode infection rates for large snails were

Table 1
Trematode and *E. risticii* infection rates in *J. yrekaensis* small snails from Montague and Weed

Month	Trematode infection rate/total (%)		*E. risticii* infection rate/total (%)	
	Montague	Weed	Montague	Weed
May	1/30 (3.3)	1/30 (3.3)	0/30	1/30 (3.3)
Jun	0/30	1/30 (3.3)	0/30	1/30 (3.3)
Jul	1/30 (3.3)	1/30 (3.3)	0/30	1/30 (3.3)
Aug	1/30 (3.3)	0/30	1/30 (3.3)	0/30
Sep	4/30 (13.3)	2/30 (6.7)	0/30	0/30
Oct	2/30 (6.7)	2/30 (6.7)	0/30	1/30 (3.3)

significantly higher in September than in June and October ($p<0.05$). When comparing the monthly trematode infection rates in large snails between both locations, the infection rates in July, August, September and October were significantly higher in Weed than in Montague ($p<0.05$).

The rate of *E. risticii* infection, based on PCR amplification of the 16S rRNA gene among small snails from both sites, was similar and varied monthly between 0 and 3.3%. The monthly PCR infection rate for large snails was significantly higher than in small snails (χ^2-test, $p<0.001$). The PCR infection rate for large snails followed different patterns for both locations: the infection rate for snails from Weed was higher in late spring and decreased during summer and early fall; whereas the infection rate for snails from Montague was lower in late spring, increased during summer and declined in early fall. However, no significant differences were detected between the monthly infection rate of *E. risticii* in large snails from a similar location ($p>0.05$). When comparing the monthly infection rates of *E. risticii* in large snails between the two locations, only the infection rate in May was significantly higher in Weed than in Montague ($p<0.01$). PCR-positive snails were always found to be infected with virgulate cercariae.

All the nucleotide sequences of PCR products isolated from trematodes were identical and showed a 100% homology to the 16S rRNA gene sequences of *E. risticii* isolated from horses with PHF from northern California, and were similar to the gene sequences of other strains of *E. risticii* reported previously (Barlough et al., 1998; Reubel et al., 1998).

Table 2
Trematode and *E. risticii* infection rates in *J. yrekaensis* large snails from Montague and Weed

Month	Trematode infection rate/total (%)		*E. risticii* infection rate/total (%)		*E. risticii*/trematode (%)	
	Montague	Weed	Montague	Weed	Montague	Weed
May	20/30 (66.7)	26/30 (86.7)	1/30 (3.3)	6/30 (20.0)	1/20 (5.0)	6/26 (23.1)
Jun	16/30 (53.3)	20/30 (66.7)	3/30 (10.0)	5/30 (16.7)	3/16 (18.7)	5/20 (25.0)
Jul	18/30 (60.0)	25/30 (83.3)	4/30 (13.3)	3/30 (10.0)	4/18 (22.2)	3/25 (12.0)
Aug	19/30 (63.3)	26/30 (86.7)	5/30 (16.7)	3/30 (10.0)	5/19 (26.3)	3/26 (11.5)
Sep	22/30 (73.3)	28/30 (93.3)	5/30 (16.7)	4/30 (13.3)	5/22 (22.7)	4/28 (14.3)
Oct	12/30 (40.0)	21/30 (70.0)	2/30 (6.7)	3/30 (10.0)	2/12 (16.7)	3/21 (14.3)

4. Discussion

Juga yrekaensis is a common pleurocerid snail that inhabits fresh or brackish stream water in northwestern USA (Burch, 1989). Pleurocerid snails are known to be first intermediate hosts of several trematode species in the Pacific Northwest (Schell, 1970). Burns (1961) reported that *Oxytrema silicula* from the state of Oregon is the intermediate host of four virgulate cercariae. This snail harbors the *Nanophyetus salmincola* trematode, which is the vector and reservoir of *Neorickettsia helminthoeca* (Bennington and Pratt, 1960; Millemann and Knapp, 1970). In the present study, virgulate cercariae were the observed type of cercariae found in *J. yrekaensis* snails. Snails with a shell of 15 mm or more in length had significantly higher trematode infection rates than did smaller snails. Bennington and Pratt (1960) reported that usually only large snails, 20 mm or more in length, were infected with *N. salmincola*. The higher infection rate in the larger snails, which live several years, is believed to be related to the age of the snails and, therefore, to the increased opportunity for exposure to miracidia (Bennington and Pratt, 1960).

In this study, the *E. risticii* PCR infection rate in small snails was significantly lower than in large snails, which we believe can be explained by the concurrently low trematode infection rate. The *E. risticii* infection rate reported for large snails ranged from 3.3 to 16.7% in Montague and from 10.0 to 20.0% in Weed. Barlough et al. (1998) reported a minimal prevalence rate of 3.5% from pooled *Juga* sp. snails collected in August 1996 from the same location in Weed. The discrepancies in the prevalence rates for the same time period in these studies could result from use of different DNA templates, although ecological factors may also be involved. The highest infection rate of *E. risticii* in Weed and Montague was found during late spring (May) and late summer (August–September), respectively. Clinical cases of PHF are typically seen in spring and summer months along the Shasta River, with the early cases developing closer to the river and later cases occurring progressively farther away (Madigan et al., 1997). However, we should be aware that the *E. risticii* infection rate in snails does not necessarily indicate the exposure risk for horses, but may rather reflect biological, ecological, and environmental pressure in the endemic locations, such as host–parasite interactions, population densities of intermediate and definitive hosts, and flow rates and water temperatures of the creeks, rivers and streams.

The mode of transmission, the epidemiology, and reservoir hosts of *E. risticii* are gradually unfolding. This study provides evidence that *J. yrekaensis*, a pleurocerid snail, is infected with trematode cercariae that harbor *E. risticii*. The *E. risticii* infection rate was directly related to the trematode infection rate in snails from both locations studied. Although the monthly *E. risticii* infection rates were slightly different between the two locations, peak values of both coincided with the seasonal periods when PHF is known to occur (Palmer, 1993). These findings indicate that a complex aquatic ecosystem is involved in the epizootology of *E. risticii* where snails, trematodes, and unidentified intermediate hosts interact.

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