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Molecular Analysis of *Neorickettsia risticii* in Adult Aquatic Insects in Pennsylvania, in Horses Infected by Ingestion of Insects, and Isolated in Cell Culture

Jason Mott,[1] Yasukazu Muramatsu,[1] Elizabeth Seaton,[1] Carol Martin,[2] Stephen Reed,[3] and Yasuko Rikihisa[1]*

Department of Veterinary Biosciences[1] and Department of Veterinary Clinical Sciences,[3] College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210–1092, and Sunbury, Pennsylvania[2]

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Upon ingestion of adult aquatic insects, horses developed clinical signs of Potomac horse fever, and *Neorickettsia risticii* was isolated from the blood. 16S rRNA and 51-kDa antigen gene sequences from blood, isolates, and caddis flies fed to the horses were identical, proving oral transmission of *N. risticii* from caddis flies to horses.

Potomac horse fever (PHF) is caused by *Neorickettsia risticii* (8). *N. risticii* DNA has been found in virgulate cercariae from freshwater snails (family *Pleuroceridae*: *Juga yrekaensis*) from Northern California (1, 6) and in virgulate xiphidiocercariae isolated from *Elimia livescens* snails from Ohio (3). *N. risticii* in trematodes from snails can infect mice by intraperitoneal inoculation (3) and horses by subcutaneous inoculation (5). *N. risticii* DNA has also been detected in metacercariae from various aquatic insects (2), and recently in Northern California, one horse fed adult caddis flies (family *Limnephilidae*: *Dicosmoecus gilvipes*) developed PHF (4). Since, in that study, the *N. risticii* sequence in the caddis flies fed to the horse was not determined, whether the *N. risticii* strain in the caddis flies actually infected the horse is unknown. In the present study, we determined (i) a species of snails and adult insects infested with trematodes harboring *N. risticii* and the *N. risticii* PCR-positive rates of snail trematodes and larval and adult aquatic insects collected in Pennsylvania, (ii) whether immunocompetent mice can become infected with *N. risticii* by ingesting adult aquatic insects, (iii) the characteristics of prepatent and ehrlichemia periods and time course of disease development in horses after ingestion of insects, and (iv) the sequences of nearly full-length 51-kDa-antigen genes (12) and 16S rRNA genes of *N. risticii* from horses and insects fed to the horses.

E. virginica snails, caddis flies, mayflies, and aquatic insect larvae were collected in an area where PHF is endemic, near the Susquehanna River in central Pennsylvania on 14 to 16 August 2000. The nested-PCR (3)-amplified (382-bp) product of the *N. risticii* 16S rRNA gene was found in 12% (5 of 42 pools from each snail) of sporocysts and cercariae isolated from snails, 11% (4 of 35 pools of two each) of aquatic insect larvae, 20% (2 of 10 pools of two each) of mayflies, and 38% (3 of 8 pools of two each) of caddis flies.

Nine CF-1 mice (4 weeks old) were divided into three groups. The first group was fed aquatic insect larvae, the second was fed adult mayflies (family *Polymitarcyidae*: *Ephoron leukon*), and the third was fed adult caddis flies (family *Leptoceridae*: *Oecetis inconspicua*) freshly collected on 25 August 2000. By PCR, 0% (0 of 14 pools of two each) of larvae, 6.0% (1 of 15 pools of two each) of mayflies, and 86.7% (13 of 15 pools of two each) caddis flies were *N. risticii* positive. No mouse showed any signs of illness throughout the 16- to 24-day period. Blood collected at day 6 was positive for one mayfly-fed mouse. One mouse from each group was sacrificed on day 16, 23, or 24, and blood, spleen, and liver DNA samples were collected. One mouse (C3) fed caddis flies and killed on day 24 postfeeding was positive for both the 16S rRNA and 51-kDa-antigen genes by nested PCR. No trematode was found in the mouse intestines. We concluded that immunocompetent mice would serve as a convenient oral-infection model for PHF.

Horse 1 (a 3-year-old gelding) was fed adult mayflies (family *Heptageniidae*: *Leucrocuta minerva*) mixed with caddis flies (family *Hydropsychidae*: *Cheumatopsyche campyla* and *Hydropsyche hageni*) on 7 September 2000 (day 0 of feeding) and mayflies alone on 11 September 2000. Horse 2 (a 10-year-old mare) was fed caddis flies (*C. campyla* and *H. hageni*) only on 11 September 2000 (day 0 of feeding). Before feeding, both horses were *N. risticii* negative by PCR and by indirect fluorescence antibody testing. The same batch was used for all feedings, and 0% (0 of 20 pools of two each) of mayflies and 80% of caddis flies (12 of 15 pools of two each) were *N. risticii* 16S rRNA PCR positive. Horse 1 developed slightly digital pulses and warm feet at day 13, followed by slight depression, anorexia, and decreased borborygmus on day 18, which immediately preceded development of fever and diarrhea on days 19 through 25. Horse 2 developed anorexia, depression, high fever, and decreased borborygmus on day 15 followed by warm feet and digital pulses on day 19. Horse 1 developed leukopenia from days 19 through 22, and horse 2 developed neutropenia from days 11 to 18 and leukopenia from days 11 to 15. Platelet counts decreased starting on day 14 and remained low throughout

* Corresponding author. Mailing address: Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210-1092. Phone: (614) 292-5661. Fax. (614) 292-6473. E-mail: Rikihisa.1@osu.edu.

TABLE 1. Culture isolation of *N. risticii*, immunofluorescence assay (IFA) titers, and PCR results for horses 1 and 2 following ingestion of aquatic insects

Horse	Days postingestion	*N. risticii* isolation	Days in culture	IFA titer	PCR result
1	6	+	29	0	−
	11	+	20	40	−
	15	+	16	40	+
	19	+	12	320	+
	22	+	10	320	+
	26	+	14	2,560	+
	28	+	18	2,560	+
2	2	−	34	0	−
	7	−	29	20	−
	11	+	16	20	−
	15	+	12	160	+
	18	+	8	320	+
	22	+	14	1,280	+
	24	+	11	2,560	+

the study period, but other clinical signs disappeared from horses 1 and 2 by days 27 and 23, respectively, without antibiotic treatment. Horses 1 and 2 seroconverted on days 11 and 7, respectively (Table 1). Since mayflies were PCR negative and time courses of development of clinical signs, PCR, and culture isolation were similar between horses 1 and 2, both horses appear to have acquired *N. risticii* from caddis flies. Ehrlichemia in horses began 6 to 11 days after ingestion of insects, similar to that in dogs after ingestion of *Neorickettsia helminthoeca* in *Nanophyetus salmincola* metacercariae in fish (11). Fewer culture days were required for *N. risticii* isolation after days 15 and 11 for horses 1 and 2, respectively, suggesting that an increase in organism numbers in the blood preceded the onset of clinical signs (Table 1). Transmission electron microscopy of culture-isolated *N. risticii* from blood collected on days 15 and 11 postingestion from horses 1 and 2, respectively, revealed ultrastructures clearly resembling that of *N. risticii* Virginia strain found in experimentally infected horse intestines (10) and in $P388D_1$ cells after isolation from naturally infected horses in Maryland, Ohio, and Kentucky (7, 9).

The nearly complete (1,452-bp) 16S rRNA gene sequences found in isolates from horses 1 and 2 and caddis flies fed to the horses and the partial (333-bp) hypervariable region of the 16S rRNA gene sequences obtained from buffy coat DNA of both horses from the earliest PCR-positive dates, from mouse C3 spleen tissue, and from adult insects (both mayflies and caddis flies) fed to mice were identical (Table 2). Since the 16S rRNA gene sequence is highly conserved, we also sequenced the nearly complete 51-kDa-antigen gene (1,449 bp). The almost complete amino acid sequences of a 51-kDa protein (483 of 558 amino acids [aa] [87.0% of the total aa]) in culture isolates from horses 1 and 2 and the caddis flies fed to the horses and the partial sequence (171 aa) in blood from horses 1 and 2 and in mouse C3 spleen tissue were identical, proving the transmission of *N. risticii* from adult caddis flies to the horses and a mouse (Table 3). Comparison of amino acid sequences of the 51-kDa antigen suggested a geographic segregation of *N. risticii* strains. Sequences from *N. risticii* DNA originating in the eastern United States (Ohio, Maryland, Kentucky, and Pennsylvania) were least identical to sequences originating from California (Table 4) (3). The 16S

TABLE 2. Nucleotide differences between 16S rRNA genes of *N. risticii* from selected sources

Source	Nucleotide at position[a]:																
	92	131	168	186	242	309	382	395	436	619	956	971	1043	1221	1226	1231	1246
Type strain	T	G	A	A	G	A	C	G	A	G	T	G	G	C	G	T	G
Horse 1 isolate[b]	●	●	●	●	●	●	●	●	●	●	●	●	—	●	●	●	●
Horse 2 isolate[c]	●	●	●	●	●	●	●	●	●	●	●	●	—	●	●	●	●
Caddis fly[d]	●	●	●	●	●	●	●	●	●	●	●	●	—	●	●	●	●
Caddis fly[e]	●	●	●	●	●	●	●	●	●	/	/	/	/	/	/	/	/
Horse 1 blood[f]	●	●	●	●	●	●	●	●	●	/	/	/	/	/	/	/	/
Horse 2 blood[g]	●	●	●	●	●	●	●	●	●	/	/	/	/	/	/	/	/
Mouse C3[h]	●	●	●	●	●	●	●	●	●	/	/	/	/	/	/	/	/
Mayfly[i]	●	●	●	●	●	●	●	●	●	/	/	/	/	/	/	/	/
Eclipse[j]	●	●	●	●	●	●	T	●	●	●	●	●	●	●	A	●	●
S22[k]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
S6[k]	●	A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
SHSN-1[l]	●	●	●	●	●	G	●	●	●	●	C	●	●	T	●	T	A
Ohio 081[m]	C	●	●	●	●	●	●	●	●	T	C	A	●	T	●	T	A

[a] Bullets represent conserved nucleotides (relative to the sequence of the type strain; GenBank no. M21290). Slashes indicate that the 16S rRNA sequence was not completely determined. Dashes indicate gaps in the sequence.
[b] Culture isolate from horse 1, 15 days after feeding with caddis flies (*C. campyla* and *H. hageni*) (GenBank no. AF380257).
[c] Culture isolate from horse 2, 11 days after feeding with caddis flies (GenBank no. AF380258).
[d] Caddis flies (*C. campyla* and *H. hageni*) collected in central Pennsylvania and fed to horses (GenBank no. AF380264).
[e] Caddis flies (*O. inconspicua*) collected in central Pennsylvania and fed to mice (GenBank no. AF380262).
[f] Buffy coat from horse 1, 11 days after caddis fly feeding (GenBank no. AF380259).
[g] Buffy coat from horse 2, 15 days after caddis fly feeding (GenBank no. AF380260).
[h] Spleen of mouse fed infected caddis flies (GenBank no. AF380261).
[i] Mayflies (*E. leukon*) collected in central Pennsylvania and fed to mice (GenBank no. AF380263).
[j] Horse in Elizabethville, Pa. (GenBank no. AF036653).
[k] Virgulate trematode pool isolated from *E. livescens* in Warsaw, Ohio (GenBank no. AY005439).
[l] Juga sp. in Weed, Calif. (GenBank no. AF037210).
[m] Isolate from a horse in Findlay, Ohio (13).

TABLE 3. Amino acid differences of 51-kDa antigens among *N. risticii* sources

Source	Amino acid at indicated position[a]														
	95	108	114	128	135	141	142	143	144	145	146	147	148	149	150
90-12 strain[b]	R	S	K	A	S	T	S	E	A	N	S	N	S	V	N
Horse 1[c]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Horse 2[d]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Caddis fly[e]	/	/	/	/	/	/	/	/	/	/	/	/	●	●	●
Mouse C3[f]	/	/	/	/	/	/	/	/	/	/	/	/	●	●	●
S21[g]	/	/	/	/	/	/	/	/	●	●	●	●	●	●	●
Eclipse[h]	/	/	/	/	/	●	●	●	●	●	●	●	●	●	●
Juga[i]	/	/	/	/	/	●	●	G	S	G	●	●	T	A	S
SHSN-1[j]	/	/	/	/	/	●	●	G	S	G	●	●	T	A	S
Ohio 081[k]	K	R	E	T	A	A	N	—	—	—	—	—	●	T	N

Source	151	154	163	185	187	199	204	226	230	232	236	239	247	250	262
90-12 strain[b]	N	A	D	G	F	R	A	D	Q	I	V	I	N	S	L
Horse 1[c]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Horse 2[d]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Caddis fly[e]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Mouse C3[f]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
S21[g]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Eclipse[h]	●	●	●	●	L	●	●	●	●	●	●	●	●	●	●
Juga[i]	S	T	●	●	●	●	●	●	E	●	●	●	●	●	●
SHSN-1[j]	S	T	●	●	●	●	●	●	E	●	●	●	●	●	●
Ohio 081[k]	●	●	N	S	●	K	S	E	K	L	A	V	E	N	I

Source	263	266	267	269	270	271	272	273	274	274[l]	275	276	277	282	284
90-12 strain[b]	G	H	S	G	T	G	S	S	S	—	Q	S	K	V	P
Horse 1[c]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
Horse 2[d]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
Caddis fly[e]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
Mouse C3[f]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
S21[g]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
Eclipse[h]	●	●	●	●	●	●	●	●	●	—	●	●	●	●	R
Juga[i]	●	●	N	●	●	S	G	N	N	—	●	●	●	●	H
SHSN-1[j]	●	●	N	●	●	S	G	N	N	—	●	●	●	●	H
Ohio 081[k]	N	R	T	N	—	—	G	●	T	T	T	C	G	A	H

Source	286	290	291	294	295	312	319	331	333	336	340	347	358
90-12 strain[b]	S	A	D	S	P	F	S	I	V	G	K	P	P
Horse 1[c]	F	●	●	T	●	L	T	●	●	●	●	A	A
Horse 2[d]	F	●	●	T	●	L	T	●	●	●	●	A	A
Caddis fly[e]	F	●	●	T	●	L	T	/	/	/	/	/	/
Mouse C3[f]	F	●	●	T	●	L	T	/	/	/	/	/	/
S21[g]	F	●	●	T	●	L	/	/	/	/	/	/	/
Eclipse[h]	F	●	●	T	●	L	T	/	/	/	/	/	/
Juga[i]	F	●	N	T	●	L	●	/	/	/	/	/	/
SHSN-1[j]	F	●	N	T	●	L	●	/	/	/	/	/	/
Ohio 081[k]	F	T	N	T	Q	L	T	V	L	A	Q	G	/

[a] Bullets represent conserved amino acids (relative to the sequence of strain 90- 12). Slashes indicate that the amino acid sequence was not determined. Dashes indicate gaps in the sequence. Sequences available from GenBank were used for comparison.
[b] Isolate from a horse in Maryland (GenBank no. U85784).
[c] Culture isolate from horse 1 (GenBank no. AF380265).
[d] Culture isolate from horse 2 (GenBank no. AF380266).
[e] Caddis flies collected in western Pennsylvania and fed to horses (GenBank no. AF380270).
[f] Spleen of mouse fed infected caddis flies (GenBank no. AF380269).
[g] Virgulate trematode pool isolated from *E. livescens* in Warsaw, Ohio (GenBank no. AY005440).
[h] Horse in Elizabethville, Pa. (GenBank no. AF036673).
[i] Secretions from *Juga* spp. in Weed, Calif. (GenBank no. AF036674).
[j] *Juga* sp. in Weed, Calif. (GenBank no. AF037215).
[k] Isolate from a horse in Findlay, Ohio (GenBank no. AY005443).
[l] This position is absent in strain 90-12.

rRNA gene sequence and 51-kDa-antigen amino acid sequences of the Ohio 081 isolate remain quite divergent from both those in eastern and western *N. risticii* groups (Tables 3 and 4).

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TABLE 4. Amino acid sequence identities and evolutionary distances between 51-kDa antigen sequences from selected *N. risticii* sources

Source	% Amino acid sequence identity or evolutionary distance[a]:									
	90-12	Horse 1	Horse 2	Caddis fly	Mouse C3	S21	Eclipse	*Juga*	SHSN-1	Ohio 081
90-12[b]		0.008	0.008	0.008	0.008	0.008	0.010	0.089	0.089	0.217
Horse 1[c]	99.2		0.000	0.000	0.000	0.000	0.002	0.082	0.082	0.209
Horse 2[d]	99.2	100.0		0.000	0.000	0.000	0.002	0.082	0.082	0.209
Caddis fly[e]	99.2	100.0	100.0		0.000	0.000	0.002	0.082	0.082	0.209
Mouse C3[f]	99.2	100.0	100.0	100.0		0.000	0.002	0.082	0.082	0.209
S21[g]	99.2	100.0	100.0	100.0	100.0		0.002	0.082	0.082	0.209
Eclipse[h]	99.0	99.8	99.8	99.8	99.8	99.8		0.084	0.084	0.212
Juga[i]	91.0	91.6	91.6	91.6	91.6	91.6	91.4		0.000	0.224
SHSN-1[j]	91.0	91.6	91.6	91.6	91.6	91.6	91.4	100.0		0.224
Ohio 081[k]	79.1	79.7	79.7	79.7	79.7	79.7	77.5	79.3	77.3	

[a] Values in the upper right portion are evolutionary distances; values in the lower left portion are percent amino acid sequence identities. The values were calculated by comparison of a total of 169 or 170 amino acids of the smallest sequence from the alignment.
[b] Isolate from a horse in Maryland (GenBank no. U85784).
[c] Culture isolate from horse 1 (GenBank no. AF380265).
[d] Culture isolate from horse 2 (GenBank no. AF380266).
[e] Caddis flies collected in central Pennsylvania and fed to horses (GenBank no. AF380270).
[f] Spleen of mouse fed infected caddis flies (GenBank no. AF380269).
[g] Virgulate trematode pool isolated from *E. livescens* in Warsaw, Ohio (GenBank no. AY005440).
[h] Horse in Elizabethville, Pa. (GenBank no. AF036673).
[i] Secretions from *Juga* spp. in Weed, Calif. (GenBank no. AF036674).
[j] *Juga* sp. in Weed, Calif. (GenBank no. AF037215).
[k] Isolate from a horse in Findlay, Ohio (GenBank no. AY005443).

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