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Herpesvirus sylvilagus IN COTTONTAIL RABBITS: ANTIBODY PREVALENCE AND FLEA BURDEN RELATIONSHIPS[1]

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Abstract: A serologic survey of a wild cottontail rabbit (*Sylvilagus floridanus*) population in southern Wisconsin was conducted from November-March, 1971-72 and November-April, 1972-73, to determine prevalence of antibody against *Herpesvirus sylvilagus*. Flea burdens on each live-trapped cottontail were quantified by species. All but six of the 5029 fleas collected were *Cediopsylla simplex*. No correlation was found between flea infestation and viral antibody. Of 101 cottontail rabbits trapped, only six had specific antibody as determined by plaque neutralization in rabbit kidney cell culture. Three of the six developed antibody between January and March of the trapping season. Blood samples from 46 captured rabbits were negative for virus.

INTRODUCTION

In 1968, Hinze[2] isolated and identified *Herpesvirus sylvilagus* from the cottontail rabbit in Wisconsin. *H. sylvilagus* or cottontail herpesvirus (CHV), was discovered to have oncogenic properties in the cottontail. The disease produced by this species-specific virus ranged from benign leukocytosis to generalized lymphoid hyperplasia involving structural changes in major organs.[3]

An understanding of the host-virus relationship and epizootiology of this disease has been sought as a potential model for oncogenic viruses. Attempts to accomplish contact transmission have been negative but the infection has been transmitted by inoculation of blood (Hinze, H. C., personal communication). A CHV serology study of a field population of cottontails in Walworth Co., Wisconsin showed an increase in specific antibody prevalence during cold weather (December-March). A seasonal increase in flea infestation during this same period was noted during other studies.[1,6] Thus, if an insect vector were capable of transmitting CHV, a likely candidate would be the rabbit flea.

This study was designed to 1) confirm CHV transmission during the colder months in another geographic location and 2) investigate possible relationships between prevalence of specific antibody and numbers of fleas infesting cottontails in a field population.

MATERIALS AND METHODS

Trapping

An accessible and concentrated field population of cottontails at the Nevin Fish Hatchery grounds, Fitchburg, Wisconsin, was selected for study. The area consisted of approximately 4 ha of mixed habitat type including woodlots predominated by oaks and aspen, pine and white cedar plantations, stands of brush and multiflora rose and maintained lawns.

Intensive trapping began in November of 1971 and 1972 and continued until the respective March and April of the following year. Cottontails were processed in the field and were released, with a few exceptions, immediately after processing. The population was trapped daily until no new rabbits (or relatively few) were captured, after which the traps were closed for 10-14 days.

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The trap line consisted of 60-80 single-door, wire mesh live traps,[2] both skunk (18 x 18 x 51 cm) and rabbit (23 x 23 x 66 cm) size. Dry cob corn was a convenient bait. Each cottontail was put in a white cotton laundry bag to aid flea collection. Excess handling of the rabbits was avoided to reduce flea escape. During cold weather, bagged animals were placed in a warm automobile to stimulate mobility of fleas.

Sampling and Processing

Animals were held by grasping the pelvic girdle. If inspection indicated flea infestation, they were vacuumed with an automobile battery-operated vacuum cleaner.[3] The vacuum had a removable cloth dirt bag and was used with the cleaning head removed. The rabbit was vacuumed for 2 min or until no fleas were observed. Breathing into the pelage invariably stimulated movement of the fleas to the surface of the fur. Special attention was given to the head, neck and hindquarters of the rabbits.

Cottontails were sexed, and weighed to the nearest 25 g. The left hind foot was measured to the nearest 0.1 cm. Blood was withdrawn by cardiac puncture using 5 ml Vacutainers®[4] and 20 gauge needles.[4] Ordinarily, only cottontails captured at intervals greater than 10 days were bled a second time.

Prior to release, the rabbits were tattooed with a serial number in the right ear.[5] Their tails were stained yellow with an alcoholic solution of picric acid to identify them in the field as processed animals.

In the laboratory, fleas from each sample were immobilized with chloroform-soaked cotton wads and then placed in a Petri dish. Fleas were counted, identified and sexed.[4,6]

Virus Isolation and Serology

Kidney cells from domestic and cottontail rabbits were maintained, with minor modifications, according to Hinze[2] who also provided seed cells and CHV. Trisene[6] was used in the maintenance medium at 0.01 M. final concentration when long term buffering was required.

Clotted blood samples from the field were tested for CHV, provided they were not more than 5 hr old. After serum harvest, the clot was macerated with a pipette or by swirling sterile glass beads with the clot using a Vortex®[7] mixer. Further processing was performed as described by Hinze.[2]

Stocks of CHV were produced in 10 x 22 cm roller bottle[8] cultures of rabbit kidney cells using standard techniques. Virus titration utilized monolayers grown in six or 24-well Linbro® plastic plates[4] according to Hinze[2] with minor modifications. Virus was adsorbed for 4 hr. Due to the highly cell-associated nature of CHV, a solid or semi-solid nutrient overlay such as agar or gum tragacanth was not necessary for plaque formation or to prevent spread of the virus from the primary locus of replication. The inoculated monolayers were therefore overlaid with maintenance medium (Medium 199[9] with three percent fetal bovine serum),[9] incubated at 37 C, and observed daily for plaques. Plaques became clearly visible by 3 to 6 days. Monolayers were fixed and stained in a formalin solution of one per cent crystal violet according to Issel,[5] before plaques were counted.

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[2] Tomahawk Live Trap Co., Tomahawk, Wisconsin 54487, USA.
[3] Sears, Roebuck and Co., Chicago, Illinois 60607, USA.
[4] Becton-Dickenson and Co., Rutherford, New Jersey 07070, USA.
[5] Nasco, Fort Atkinson, Wisconsin 53538, USA.
[6] Nutritional Biochemicals Corp., Cleveland, Ohio 44128, USA.
[7] American Hospital Supply Corp., Evanston, Illinois 60204, USA.
[8] Belco Glass, Inc., Vineland, New Jersey 08360, USA.
[9] International Scientific Industries, Inc., Cary, Illinois 60013, USA.

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Testing of cottontail sera for CHV antibody was done in the above rabbit cell system using six-well Linbro plates. Sera were diluted 1:10 in maintenance medium and mixed with equal volumes of CHV diluted so that 0.5 ml of the suspension yielded 60-100 plaques in each of three monolayers. The serum-virus mixtures were incubated for 1 hr at 37 C before inoculating. A greater than 50% reduction in plaque count was regarded as positive for specific antibody.

RESULTS

During both trapping periods, only six of 101 captured cottontails had CHV-neutralizing antibody (Table 1). Two of these developed antibody during January-February, 1972, one during February-March, 1973 and another between February, 1972 and March, 1973. The remaining two rabbits were trapped and sampled only once so it was impossible to ascertain when the antibody was acquired. Of the six rabbits having neutralizing antibody, those captured during the winter season of 1971-72 were females and those from the 1972-73 trapping were males. Cottontail no. 54, a male, had become infected and developed antibody sometime between 20 February, 1972 and 8 March, 1973. Virus was not recovered from 46 blood clot samples obtained from trapped cottontails tested by inoculation into cell culture.

Flea burdens from the six cottontails becoming infected with CHV were highly variable (Tables 2 and 3). Rabbits not infected with the virus had equally variable flea numbers. Considering the entire cottontail population, the difference between the mean fleas per sample during the 1972-73 trapping ($\bar{x} = 39$) compared to 1971-72 ($\bar{x} = 25$) approached significance ($P = .06$), with no corresponding change in CHV antibody prevalence. All but six of the 5029 fleas collected were the common eastern rabbit flea (*Cediopsylla simplex*). Four were the giant eastern rabbit fleas (*Odontopsylla multispinosus*) and two were mouse fleas *Leptopsylla segnis*).

TABLE 1. Prevalence of cottontail herpesvirus antibody in a cottontail population at Fitchburg, Wisconsin, winters 1971-72, 1972-73.

	1971-72	1972-73	Total
No. rabbits with antibody	3	3	6
No. rabbits trapped	63	38	101
Antibody prevalence (per cent)	5	8	6
No. sera tested	118	58	176

TABLE 2. Results of a flea infestation survey of a cottontail population trapped at Fitchburg, Wisconsin, winters 1971-72, 1972-73.

	1971-72	1972-73	Total
No. cottontails trapped	63	42[a]	101
No. times trapped	234	73	307
Total fleas collected	2923	2100	5023
Mean no. fleas/sample	25 ± 60	39 ± 59	22
Mean no. fleas/rabbit	46 ± 181	50 ± 207	50

[a] Four of these were individuals sampled over both seasons.

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TABLE 3. Rabbit flea **(Cediopsylla simplex)** counts and timing of cottontail herpesvirus acquisition for four cottontail rabbits surveyed at Fitchburg, Wisconsin.

1971 - 1972				1972 - 1973			
Rabbit No.	Date Trapped	No. Fleas	Antibody Status	Rabbit No.	Date Trapped	No. Fleas	Antibody Status
8	11-22-71	30	Neg.	88	01-31-73	10	Neg.
female	12-02-71	0	Neg.	male	02-02-73	0	Neg.
	12-05-71	7	Neg.		03-08-73	124	Pos.
	12-19-71	4	Neg.		03-10-73	0	Pos.
	12-22-71	0	Neg.				
	12-30-71	0	Neg.	54	02-18-74	2	Neg.
	01-06-72	3	Neg.	male	02-20-72	0	Neg.
	01-26-72	0	Pos.		03-08-72	88	Pos.
	02-19-72	0	Pos.				
	02-23-72	0	Pos.				
44	01-03-72	NS[a]	Neg.				
female	01-05-72	0	Neg.				
	02-25-72	7	Pos.				

[a] Not sampled

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DISCUSSION

The purpose of this field investigation was to test the hypothesis that CHV is transmissible by the fleas commonly infesting wild cottontail rabbits. No relationship between flea abundance and acquisition of neutralizing antibody was detected, although only six percent of 101 cottontails sampled were serologically positive. Virus isolation was not attempted from fleas collected from the field because they were used either in laboratory transmission trials or were preserved.

The results of this study support the contention that CHV transmission occurs during the colder months and documents presence of CHV in a cottontail population 100 km. from the original population studied by Lewis and Hinze in Walworth Co., Wisconsin from January, 1970 to January, 1971 (personal communication). Similar serologic assay systems have been used in both studies; however, they detected a considerably higher CHV prevalence of antibody; 22% among 136 cottontails as opposed to 6% for the present study. Of the 10 Walworth Co. rabbits developing antibody, half did so between 22 January and 4 April. This roughly corresponded chronologically to the four Fitchburg cottontails producing antibody between January and March. Thus, our study shows that CHV can be maintained in a relatively dense cottontail population at very low levels.

The mechanism of CHV transmission in the wild is unknown. Despite the lack of correlation between flea infestation and prevalence of CHV, laboratory tranmission trials testing vector capabilities of fleas may aid in assessing the likelihood of their transmitting the virus.

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