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Isolation of Montana Snowshoe Hare Serotype of California Encephalitis Virus Group from a Snowshoe Hare and Aedes Mosquitoes [1]

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Abstract

Two isolations of the Montana snowshoe hare serotype of the California encephalitis virus group were made in the summer of 1968 from materials collected at Rochester, Alberta, Canada. One isolation was made from the blood of a snowshoe hare approximately 24-days old while the other was from a pool of unengorged *Aedes communis* group mosquitoes. The isolation of the agent from mosquitoes demonstrates the continued endemicity of the virus in the area. The recovery of the virus from the hare represents the second time a California encephalitis virus has been recovered from a snowshoe hare and relates the virus recovered from the mosquito to the known serology of the virus in hares.

Introduction

Throughout the investigation of a well documented snowshoe hare population in north central Alberta, serologic surveillance has shown members of the California encephalitis (CE) group of viruses to be infecting the hares and possibly producing arbovirus - associated mortality.[5,19] While prior to 1968, no CE virus group members were recovered from the hares, six isolations of two serotypes were made from *Aedes* mosquitoes in 1964 and 1965.[7] This paper reports the isolation and characterization in 1968 of the snowshoe hare serotype of CE virus from a snowshoe hare and from a pool of *Aedes* mosquitoes.

Materials and Methods

Field investigations were conducted near Rochester, Alberta (54 N, 113 W), Canada. Blood samples collected from wildlife and domestic animals were obtained from the area surrounding Rochester. Mosquito collections were made only on a one square mile study area just east of Rochester. A black spruce

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bordered tamarack - sphagnum bog runs north and south through the eastern edge of the study area. Aspen forest predominates to the east and in a few areas on the western side of the bog. Although the western half of the square mile area suffered repeated forest fires in 1961, 1965 and 1968,[4] much of the area had only a ground fire which left the canopy intact. Some of the aspen and spruce suffered a crown fire and in these areas all vegetation was destroyed. Most of the unburned section was in the bog and its spruce border.

Snowshoe hares, as well as all other wild vertebrates sampled with the exception of red-tailed hawks, were live trapped,[8] subjected to various observations and measurements,[9] and subsequently bled by cardiac puncture. Juvenile red-tailed hawks, that had been tethered on the forest floor for several weeks,[10] were bled from the wing vein prior to release. Blood samples were also obtained from domestic rabbits and poultry on local farms. All blood samples were allowed to clot for 24 hours at 4 C and then centrifuged. Sera and the clots were separated, placed in sterile, screw capped, glass vials sealed with adhesive tape. They were then stored on dry ice.

Continental rabbit ticks, *Haemaphysalis leporis - palustris,* were removed from snowshoe hares, field mice, grouse and red-tailed hawks. No attempt was made to secure all of the ticks feeding on a given animal. The ticks were placed in sterile vials sealed with adhesive tape and frozen on dry ice.

Unengorged and engorged mosquitoes were collected by aspiration from human bait. All efforts were made to minimize the many factors which influence this sampling method as listed by Muirhead-Thomson.[12] The human serving as the mosquito attractant was examined for HI antibodies to the CE virus group prior to initiation of the field work, immediately after the conclusion of the field work and two months after the field work. The five collecting sites used on the square mile study area have been described previously.[7] However, due to the May fire and spring drought, the sites differed greatly in the amount of vegetation available for mosquito resting places. Therefore, the majority of the mosquitoes collected were from two sites on the western side of the bog, i.e., the black spruce stand along the edge of the muskeg and the aspen-black spruce ecotone. At the collecting sites, mosquitoes were transferred to snap-capped, plastic vials the lids of which were sealed with adhesive tape. The vials were placed on dry ice at the collecting sites.

The identification of the arthropods was conducted in the Department of Entomology, University of Wisconsin and the procedures have been previously described.[5,7] Serological results concerning CE virus group activity in the hare population through 1968 and the preparation of materials for virus isolation attempts have been presented elsewhere.[5]

All steps in the virus isolation attempts were conducted in laboratory facilities where no stocks of identified arboviruses were being used or stored. Two to four day old white Ham/ICR mice were inoculated intracerebrally with 0.02-0.03 ml. of inocula and observed for 14 days. Sterile technique was used to remove the brains from sick or dead mice. Passage of brain material continued until a reproducible pattern of sickness and death occurred in mice inoculated with mouse-brain suspension shown by culture to be free of bacteria. Reisolation was attempted from an aliquot of the original sample. After successful reisolation, each isolate was tested for ether sensitivity and specific hyperimmune mouse ascitic fluid (MAF) was produced against it.[1] Complement - fixation,[16] agar-gel diffusion[13,15] and virus neutralization tests were used to identfy the isolates and to determine the relationships between the isolates and other members of the CE group. Commercially prepared agar-gel double diffusion plates, pattern C, (Hyland Laboratories, Costa Mesa, California) were used.

Additionally, the isolates were inoculated into suckling (2-4 day old), weanling (28 day old) and adult (5 month old) mice and chick embryos (6 and 10 day old). A ten-fold dilution series was inoculated into each system. All the mice were inoculated intracerebrally and with

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the exception of the adults, intraperitoneally, and were observed for 14 days. Six-day old chick embryos were inoculated via the yolk sac and 10-day embryos via the allantoic cavity. After inoculation, the eggs were incubated at both 32 C and 37 C and observed for embryonic mortality daily for eight days.[17]

Results

During the summer of 1968, samples for virus isolation attempts were collected in July and August. Snowshoe hares and other vertebrates were generally abundant throughout the summer. The combined effect of the May fire and spring drought suppressed mosquito numbers until the middle of July, however, even then their distribution was spotty. On the square mile study area, mosquitoes were found only in those areas near the bog which did not suffer appreciable fire damage. During 15 minute collecting periods, mosquito catches ranged from 0 to 476 individuals, with the largest percent of the total catch coming from the black spruce stand bordering the western edge of the bog. Mosquitoes of the *Aedes communis* group represented 77.3% of the total catch while *Aedes* spp. accounted for another 21% (Table 1).

Three isolations were made from a total of 483 blood clots (Table 2) and 2,311 mosquitoes (Table 1). No isolations were made from ticks. On the basis of complement-fixation (Table 3), neutralization of infectivity (Table 4) and agar-gel double diffusion, two of the isolates were shown to be members of the California encephalitis virus group and the other was identified as Silverwater virus. One of the CE virus group isolations was made from the blood of a snowshoe hare approximately 24-days old while the other came from a pool of 50 unengorged *Aedes communis* group mosquitoes. The Silverwater isolate came from the blood of an approximately 35-day old snowshoe hare and is described elsewhere.[6] The human bait at no time possessed HI antibodies to CE virus group agents and may, therefore, be excluded as the source of infection for the *Aedes* mosquitoes. The snowshoe hare from which the CE virus group agent was isolated was captured almost eight miles from where the mosquitoes were collected.

By comparative complement - fixation (Table 3) and neutralization (Table 4) tests, both CE isolates were shown to be most closely related to the Montana snowshoe hare serotype. In the agar-gel double diffusion test, single lines of identity developed between Roch XXVI and Roch XXVII and snowshoe hare virus, while lines of partial identity developed with LaCrosse virus and no lines with Jamestown Canyon virus. Both isolates 1) were ether sensitive, 2) killed suckling and weanling mice via the intracerebral and interaperitoneal routes, 3) killed adult mice via the intracerebral route and 4) killed chick embryos inoculated via the yolk sac and incubated at both 32 C and 37 C (Table 5).

TABLE 1. *Mosquitoes collected for virus isolation and isolations by species*

	# Mosquitoes	% Total	# Pools	Ave. # Pool	Isolations
Aedes communis group	1786	77.3	44	40	1
Aedes stimulans group	23	.9	1	23	0
Aedes cinereus	1	——	1	1	0
Aedes species	487	21.0	18	27	0
Mansonia perturbans	9	.5	1	9	0
Culiseta inornata	5	.3	1	5	0

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TABLE 2. *Number of blood clots by species tested for virus isolation.*

Mammals:	# Tested	# Isolations
Snowshoe hare *(Lepus americanus)*	226	2
Long-tailed weasel *(Mustela frenata)*	7	0
Red squirrel *(Tamiasciures hudsonicus)*	3	0
Richardson's ground sq. *(Citellus richardsonii)*	91	0
Franklin's ground sq. *(Citellus franklinii)*	1	0
White-footed deer mouse *(Peromyscus maniculatis)*	13	0
Red-backed vole *(Clethrionomys gapperi)*	3	0
Porcupine *(Erethizon dorsatum)*	2	0
Domestic rabbit	39	0
Birds:		
Red-tailed hawk *(Buteo jamaicensis)*	8	0
Ruffed grouse *(Bonasa umbellus)*	6	0
Domestic chicken	46	0
Domestic turkey	7	0
Domestic duck	9	0
Domestic goose	5	0
Guinea fowl	1	0
Amphibians:		
Toad *(Bufo cognatus)*	3	0
Frog *(Rana sylvatica)*	3	0
	483	2

TABLE 3. *Complement-fixation test results.*

	Virus antigens							
MAF*	LAC	TVT	SSH	BFS-283	JC	BUN	Roch XXVI	Roch XXVII
LaCrosse	8**	3	5	5	5	<1	4	4
Trivittatus	5	9	6	4	5	<1	4	4
Snowshoe Hare	5	4	7	5	5	<1	6	6
BFS-283	5	5	6	6	6	<1	5	5
Jamestown Canyon	5	3	4	5	7	<1	4	3
Bunyamwera W-523	<1	<1	<1	<1	<1	6	<1	<1
Roch XXVI	7	5	8	6	5	<1	8	7
Roch XXVII	7	6	8	6	5	<1	7	7
Normal	<1	<1	<1	<	<1	<	<1	<1

* MAF = Immune mouse ascitic fluid

** CF Titer: 1 = 1:16, 2 = 1:32, ..., 8 = 1:2048

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TABLE 4. *Cross-comparison by neutralization test* of Rochester viruses with Montana snowshoe hare, LaCrosse and Jamestown Canyon viruses.*

Virus	MAF** Roch XXVI	Roch XXVII	SSH	LAC	JC
Roch XXVII	5.4***	5.1	5.0	3.5	2.0
Roch XXVII	5.0	5.0	5.2	4.0	2.1
Snowshoe hare	4.9	5.3	5.8	4.2	2.0
LaCrosse	4.4	4.4	4.0	6.2	2.7
Jamestown	2.7	1.8	2.9	2.9	4.0

* Suckling mouse, intracerebral neutralization test
** Immune mouse ascitic fluid
*** Neutralization index

TABLE 5. *Characteristics of California encephalitis group isolates.*

	Roch XXVI	Roch XXVII
Date collected:	25 July 1968	8 August 1968
Sources:	Snowshoe hare	*Ae. communis*
Reisolation successful:	Yes	Yes
Ether sensitive:	Yes	Yes
Titer obtained in mice:		
2-4 day (IC)	6.4 (a)	6.7
28 day (IC)	5.7	5.7
28 day (IP)	4.5	1.7
150 day (IC)	6.5	4.3
Titer in chick embryo:		
6 day yolk sac @ 32C	3.2	6.6
6 day yolk sac @ 37C	3.8	2.5
10 day allant. cav. @ 37C	0.0	0.0
Antigenic relationship:		
Neut. of infectivity	SSH (b)	SSH
Gel-diffusion	SSH	SSH
Complement-fixation	SSH	SSH

(a) Titer expressed as a LD_{50} per 0.02 ml. of inoculum
(b) SSH — Snowshoe hare serotype of CE virus group

Discussion

It has been easier to associate California encephalitis group agents with snowshoe hares than to assess what role the hares play in the epizootiology of the viruses. Serological surveys of snowshoe hares over a wide geographical area have shown in general, a high prevalence of CE virus group neutralizing anti-

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bodies.[14,18,11,5] However, members of the CE virus group have been isolated only twice from naturally viremic hares. The first isolation came in 1959 from a hare in Montana[2] and the second was made from a 24-day old hare in the present study. Both isolates are of the same serotype which is also the one for which Yuill and Hanson[18] demonstrated the greatest specificity of hare sera from Alberta.

On the basis of the two isolations from the blood of snowshoe hares, it is apparent that naturally occurring viremias with the Montana snowshoe hare serotype do exist, however, the question of whether or not the titers are high enough to permit transmission to hematophagous arthropods has yet to be resolved. Yuill and Hanson[18] experimentally infected juvenile snowshoe hares with the Montana snowshoe hare serotype and obtained viremias lasting up to 3 days with titers up to $10^{4.0}$ mouse LD_{50} per ml whole blood. They also observed an increase in the length of viremia with increasing age of the hares. These titers are below the $10^{4.5}$ mouse LD_{50} per ml infection threshold reported by Chernesky[3] for the boreal mosquito species, *Aedes vexans,* with the Montana snowshoe hare serotype. However, infection thresholds obtained with one species of mosquito cannot be extrapolated to other species.

At Rochester, the only arthropods from which CE virus group agents have been isolated are *Aedes* mosquitoes. Excluding transovarial transmission, it is evident that snowshoe hares or some other wildlife species in the area are capable of producing viremias high enough to infect these mosquitoes. The Montana snowshoe hare serotype was isolated from a pool of unengorged *Aedes communis* group mosquitoes in the present study, while Iversen et al.[7] isolated this serotype and the Jamestown Canyon serotype from *Aedes* mosquitoes in 1964 and 1965. Of this total of seven isolates, five are of the serotype which has been demonstrated to be infecting the hares. All of the isolations made by Iversen et al. came from mosquitoes collected in the spring and early summer while the isolation in the present study was made from mosquitoes collected in August. These isolations and serology[5] suggest that CE virus group agents are endemic at Rochester and that transmission occurs throughout the entire summer.

No isolation of members of the CE virus group have been made from ticks, *Haemaphysalis leporis-palustris,* removed from snowshoe hares and other species at Rochester. Newhouse et al.[14] have isolated CE group viruses from *H. leporis-palustris* collected from a snowshoe hare and from *Dermacentor andersoni* collected from a ground squirrel and a chipmunk. However, the amount of virus recovered was minimal and preliminary laboratory experiments with both tick species indicated that the ticks lost all traces of the virus within 48 hours after infectious feedings. Therefore, ticks are probably of little if any importance in CE virus group dissemination within the hare population.

Since the snowshoe hare is often one of the most abundant mammals in the boreal forest, it seems likely that it is one of the major reservoir hosts of the CE virus group. Serology of the hare population and the isolation of the Montana snowshoe hare serotype from a hare would support this hypothesis. The repeated isolations of the Montana snowshoe hare serotype from boreal *Aedes* mosquitoes implicates these species as possible vectors. However, without transmission studies a definite vector - host relationship cannot be established.

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