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CHARACTERIZATION OF WHITNEY'S *Clethrionomys gapperi* VIRUS ISOLATES FROM MASSACHUSETTS[1]

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Abstract: Six strains of virus were recovered from the blood and/or liver of five *Clethrionomys gapperi ochraceus* trapped in southeastern Massachusetts during 1969. Biological, antigenic and physiochemical properties of these isolates are reported. USA M-2268a was selected as the reference strain. This strain was identical by complement-fixation and neutralization tests to Whitney's *C. gapperie* virus (USA 64-7855) from New York State and was related to, but distinct from, an unpublished agent (Johnson's *Microtus montanus* enterovirus USA M-1146) isolated in June, 1962 from voles trapped in Klamath County, Oregon. USA M-2268a was resistant to lipid solvents and acid pH and was stable at temperatures of 4 C, 22 C, and 37 C. Virus was detected over a 10-day observation period in four species of mosquitoes inoculated with USA M-2268a, although there was no evidence of infection or replication, and transmission attemps by bite failed. Neutralizing antibody was detected in *C.g. gapperi* and *C. g. ochraceus* in various habitats throughout the state.

INTRODUCTION

Whitney and co-workers[11] first reported the isolation of a small (<50 mu), solvent resistant, heat stable agent from spleen, liver, and pooled blood, liver, and spleen suspensions from three boreal red-backed voles, *Clethrionomys gapperi*, captured in St. Lawrence Co., New York. The virus was referred to as *C. gapperi* virus, and USA 64-7855 was selected as the prototype strain.

Strains of this virus were subsequently recovered from the blood and liver of *C. gapperi* trapped in Massachusetts. The isolation and characterization of these strains are the subject of this paper.

MATERIALS AND METHODS

The *C. gapperi ochraceus* included in this study were live-trapped in Massachusetts as part of an arbovirus surveillance program by the Encephalitis Field Station (EFS) of the State Department of Public Health. Most of these studies were concentrated in the Hockomock, a 2500 ha white cedar (*Chamaecyparis thyoides*) — red maple (*Acer rubrum*) swamp located in the southeastern part of the state.

Voles were exsanguinated by cardiac puncture and the blood diluted 1:5 with 0.025 mg/ml heparin. Organ samples were triturated in mortars with Alundum and 10% suspensions were prepared in

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tris buffered (pH 7.6) Hanks' balanced salt solution with 5% fetal calf serum plus penicillin, streptomycin, neomycin, and amphotericin B. Suspensions were spun at 2500 (1600 x g) rpm for 20 min in a refrigerated centrifuge and the supernatant stored at —60 C.

Blood and organ samples were screened for the presence of virus by intracerebral (ic) inoculation of a 1- to 4-day old Swiss mice; mice were observed daily for two weeks for signs of illness. Early serial passages (1-3) of 10% brain suspensions in 5% fetal calf serum of sick mice were attempted at the EFS until uniform death patterns were attained. Later passages were done in 0.75% bovine albumin in phosphate buffered saline (pH 7.2) (BAP) or 0.85% NaCl at the Yale Arbovirus Research Unit (YARU). Hemagglutination (HA) and complement-fixation (CF) tests were attempted with sucrose-acetone extracted suckling mouse brain antigens in microtiter by standard techniques.[3,4] Neutralization (NT) tests were done by ic inoculation of suckling mice with serial tenfold virus dilutions incubated with an equal volume of undiluted ascitic fluid for one hr at 37 C.

Immune ascitic fluids were tapped by paracentesis from adult female Swiss mice following four weekly intraperitoneal (ip) injections with 0.1 ml of 10% infected mouse brain in 0.85% NaCl plus 0.1 ml of Freund's complete adjuvant; an additional inoculation with adjuvant in saline was given during the third week. Sarcoma-180 was used to induce ascites in some immunized mice. Reference virus stocks, antigens, and immune ascitic fluids were supplied by the WHO International Arbovirus Reference Centre at YARU and by the Research Resources Branch at the National Institutes of Health in Bethesda, Maryland.

Virus stability was determined by incubating tenfold serial dilutions of infected mouse brain in 0.75% BAP in stoppered test tubes at temperatures of 4 C, 22 C and 37 C; these suspensions were inoculated into suckling mice after selected intervals over a six-week period. Virus sensitivity to ether, sodium deoxycholate (SDC), and acidic pH was tested by the techniques of Andrewes and Horstmann,[1] Theiler,[10] and Borden and co-workers,[2] respectively.

The susceptibility of suckling (1- to 4-day old), weanling (3- to 4-week old), and adult (8-week old) Swiss mice, baby chicks (3-day old sex-linked cockerels), and bats (adult *Myotis lucifugus* and *Myotis keenii*) was examined by inoculating 10% infected mouse brain suspensions in 0.75% BAP by ic, ip, or subcutaneous (sc) routes. Mosquito susceptibility was determined by intrathoracic inoculation of adult female *Aedes aegypti, Culex pipiens quinquefasciatus, Anopheles quadrimaculatus,* and *Culiseta melanura* with approximately 0.0006 ml of 1.0% infected mouse brain suspensions. These mosquitoes were maintained in pint cartons at 27 C and 75% relative humidity and fed 10% dextrose. Lots of five inoculated mosquitoes were removed from the cartons at selected intervals, triturated in 1.0 ml of 0.75% BAP with antibiotics and titrated in suckling mice. At 10 days post-inoculation (pi), 3-day old mice were exposed to the bite of the remaining mosquitoes; these mice were observed for evidence of infections, including CF antibody determinations at seven weeks pi.

Samples from experimental animals were screened and titrated in suckling mice; virus identifications were confirmed by CF tests performed with crude mouse brain suspensions in veronal buffer.

NT antibody was detected by incubating approximately 100 $SMicLD_{50}$ of virus with an equal volume of serum for 1 hr at 37 C and inoculating this ic into suckling mice.

RESULTS

Six strains of virus were recovered from the blood and/or liver of five of 53 *C. gapperi ochraceus* trapped in the Hockomock Swamp during November, 1969 (Table 1). Virus was not isolated from the blood of 116 small mammals of other species collected at the same time; liver samples from 37 of these animals were also negative.

TABLE 1. Virus isolations from **Clethrionomys gapperi ochraceus** collected in the Hockomock Swamp in southern Massachusetts during November, 1969.

Specimen Number	Age	Sex	Collected Date	Virus Isolations			
				Blood		Liver	
				Primary	Reisolation	Primary	Reisolation
USA M-2268*	adult	male	11/15/69	5/6**	6/6	1/4	2/6
USA M-2233	adult	female	11/13/69	0/6	nd	2/4	6/6
USA M-2309	adult	male	11/19/69	0/6	nd	3/4	2/6
USA M-2313	adult	female	11/20/69	0/6	nd	4/4	2/6
USA M-2537	adult	male	11/23/69	0/6	nd	1/4	0/6

* The brain, salivary glands, heart, lung, pancreas, kidney and spleen from this vole were negative in suckling mice.

** Number of sick mice/number of suckling mice inoculated.

nd = not done.

TABLE 2. Susceptibility of suckling mice, weanling mice and baby chicks to infection with approximately 7.0 dex* of Whitney's **Clethrionomys gapperi** virus from a vole (USA M-2268a from the blood; USA M-2268g from the liver) from Massachusetts.

Strain	Route	Mouse Passage History	Evidence of Infection	No. Inoculated / No. Dead	Incubation Period (days)	Titer in Brain (dex)
A. Suckling Mice (1- to 4-day old)						
USA M-2268a	ic	2 - 5	Pa, D	>200/>200	3 - 6	5.9 - 7.5
USA M-2268a	ip	3 - 4	Pa, D	12/14	6 - 9	7.2
USA M-2268g	ic	2 - 4	Pa, D	65/65	3 - 5	
USA M-2268g	ip	2	Pa, D	6/6	5 - 6	
B. Weanling Mice (3- to 4-week old)						
USA M-2268a	ic	2 - 4	Occ. D	3/16	8	
USA M-2268a	ip	2 - 3	none	0/15		
USA M-2268g	ic	4	Occ. D	1/4	6	
USA M-2268g	ip	3	none	0/4		
C. Baby chicks (3-day old cockerels)						
USA M-2268a	ic	4	none	0/2		
USA M-2268a	sc	4	Occ. D	1/14	5	negative

Pa = paralysis
D = Death
Occ.D. = Occasional Deaths
*Dex = decimal exponent [5]

ic = intracerebral inoculation
ip = intraperitoneal inoculation
sc = subcutaneous inoculation

Suckling mice became sick on days 8 through 12 after inoculation with the original samples. After a single passage, all mice were paralyzed or moribund on pi days 5 and 6; by the second passage, the average survival time was 4 days by ic inoculation. The incubation period was slightly longer (5 to 9) days in suckling mice inoculated ip with passage material. Occasional deaths were observed in weanling mice following ic, but not ip, inoculation (Table 2).

USA M-2268a, the isolate from the blood of a vole, was selected as the reference strain. This strain passed through a 220 mu Millipore filter and no evidence of bacterial growth was detected in inoculated thioglycollate cultures under aerobic or anaerobic conditions.

Sonicated sucrose-acetone extracted antigens prepared from each of the six strains failed to hemagglutinate goose erythrocytes at temperatures of 4 C, 22 C and 37 C over a pH range of 5.8 to 7.2.

These antigens did fix complement in the presence of the homologous and the five heterologous antisera. No antigenic differences were detected among the six strains by CF. Strains USA M-2268a and/or USA M-2309b were compared in CF tests with 461 antigens and 344 sera available at the YARU (Table 3). These included reovirus type 3 and several agents serologically similar or identical to encephalomyocarditis virus. Dilutions of antigens (homologous titers of 1:64 to 1:128) and antisera (titering 1:128 to 1:512) were used at 1:4 in these tests. Serological relationships were detected with only two viruses: USA 64-7855, an isolate from *C. gapperi* trapped in St. Lawrence Co., New York in 1964[11] and Johnson's *Microtus montanus* enterovirus M-1146, an agent recovered in June, 1962 from mice trapped in Klamath County, Oregon (Johnson, H. N., personal communication). This virus, together with Klamath virus,[9] was referred to by Johnson[8] as two unnamed viruses from meadow mice. USA M-2268a was identical to USA 64-7855, but distinct from USA M-1146, by CF and NT (Table 4).

TABLE 3. Reference antigens and antisera compared with USA M-2268a and/or USA M-2309b in complement-fixation tests.

Virus Group	Number antigens tested	Number antisera tested	
		Specific	Grouping
Herpesvirus	4	4	
Poxvirus	4	4	
Picornavirus	5	4	
Togavirus	81	56	2
Reovirus	1	1	
Orbivirus	43	25	2
Arenavirus	7	5	1
Myxovirus	1	1	
Paramyxovirus	1	1	
Rhabdovirus	20	21	3
Bunyavirus	163	105	19
Coronavirus	1	1	
Unknown or Assigned	130	83	6
Total	461	311	33

Strain USA M-2268a was not neutralized by any of Lim Beynesh-Melnick pools (A-H)[7,8] or specific enterovirus sera indicating that the virus is not any of the following 42 human enteroviruses: echovirus types 1-7, 9, 11-27, 29-33; coxsackievirus A types 7, 9, 16; coxsackievirus B types 1-6; or Poliovirus types 1-3.

USA M-2309b, and/or USA M-2268a were resistant to SDC, ether, and acidic pH (Table 5). There was little or no loss in titer after 35 days at 4 C or 22 C; titers dropped significantly in samples maintained at 37C, although infectious virus was detected after five weeks (Table 6).

Virus was recovered from the liver, but not the blood, of adult mice inoculated sc with USA M-2268a (Table 7). Three-day old cockerels were resistant to infection by sc and ic routes; virus was

TABLE 4. Serological comparison of virus strains from **Clethrionomys gapperi** in Massachusetts (USA M-2268a) and New York (USA 64-7855) and from **Microtus montanus** in Oregon (USA M-1146).

A. Complement-fixation tests

	Sera			
Antigen	USA M-2268a	USA 64-7855	USA M-1146	Normal
USA M-2268a	256/64*	256/64	8/16	0
USA 64-7855	256/64	256/64	0	0
USA M-1146	64/32	64/32	64/32	0
Normal	0	0	0	0

*Reciprocal of serum titer/reciprocal of antigen titer.

B. Neutralization tests

	Sera						
	USA M-2268a		USA 64-7855		USA M-1146		Normal
Virus	dex+	LNI*	dex	LNI	dex	LNI	dex
USA M-2268a	1.6	5.8	2.1	5.3	7.1	0.3	7.4
USA 64-7855	3.3	4.8	2.8	5.3	6.7	1.4	8.1
USA M-1146	4.0	2.4	4.5	1.9	2.9	3.5	6.4

*LNI = Log of Neutralization Index

+ dex = decimal exponent [5]

TABLE 5. Sensitivity of two strains of Whitney's **Clethrionomys gapperi** virus to sodium deoxycholate, ether and acidic pH.

	USA M-2268a (passages 4 & 5)		USA M-2309b (passages 3)	
	treated	control*	treated	control*
Sodium deoxycholate (10%)	5.7**	5.9	7.1	7.2
Ether (1:2)	6.8	6.9		
pH 3.0	8.1	8.3		

* controls: 0.75% bovine albumin or pH 7.2

** dex: decimal exponent [5]

TABLE 6. Stability of USA M-2268a (passage 6) in 0.75% bovine albumin at 4 C, 22 C and 37 C.

Day	4C	22C	37C
0	7.5*	7.5	7.5
1	7.3	7.9	7.1
2	7.6	7.2	6.3
3	6.8	6.8	6.2
4	7.3	6.7	5.8
5	≧6.5	≧6.5	6.4
6	≧6.5	6.3	6.3
7	6.4	7.0	6.1
14	7.3	7.0	5.4
21	6.4	6.4	3.7
28	7.1	6.7	3.5
35	7.0	6.3	1.9
42	≧3.5	≧2.5	nd

*dex = decimal exponent [5]
nd = not done

isolated from the blood (on day 1) but not the brain of a chick that died on day 5 pi (Table 7). Virus was recovered from 11 of 17 bats (*M. lucifugus* and *M. keenii*) inoculated sc and maintained at various temperature schedules (Table 8). Infected organs included the liver (7 bats), intrascapular brown fat (7), spleen (2), blood (2), and salivary glands (1). Virus was recovered from the liver and spleen of hibernating bats as long as 35 days pi.

There was little or no evidence of virus replication in *A. aegypti, C. p. quinquefasciatus, A. quadrimaculatus,* and *C. melanura* following intrathoracic inoculation with USA M-2268a, although virus was recovered from all four species over a 10-day observation period (Table 9). This probably represents residual virus from the inoculum rather than multiplication in the arthropod, considering the lack of any significant increase in virus titers during incubation and the stability of the virus outside of living tissues. There was no evidence of virus transmission to suckling mice by the bite of inoculated mosquitoes at 10 days pi (Table 9).

Sera from 12 of 54 (22.2%) red-backed voles trapped in Massachusetts neutralized USA M-2268a virus. Neutralization was demonstrated in *C. gapperi gapperi* collected in an upland coniferous forest in Chester (3 of 5 voles tested) and a lowland sphagnum bog in Belchertown (8 of 41) both in western Massachusetts and in *C. gapperi ochraceus* from mixed woodlands along a brackish river in Berkley (1 of 2) in the eastern part of the state. None of 57 bat sera (10 *M. keenii,* 46 *M. lucifugus,* 1 *Eptesicus fuscus*) collected in Massachusetts neutralized this virus.

DISCUSSION

Whitney's *Clethrionomys gapperi* virus was isolated from the blood and/or liver of boreal red-backed voles, *C. gapperi ochraceus,* trapped in southeastern Massachusetts during 1969. Serosurveys indicated that the virus was prevalent among both subspecies of *C. gapperi* found in Massachusetts wherever significant numbers of voles were tested. The virus isolation rate among *C. gapperi* in the Hockomock Swamp during November,

TABLE 7. Susceptibility of adult mice (8-weeks old) and baby chicks (3-days old) to infection with Whitney's **Clethrionomys gapperi** virus (USA M-2268a).

Route	Tissue sampled	Virus Isolation Attempts days post-inoculation 1	2	3	4	5	6	7	8	14	21	28	CF Antibody 28
A. Mice													
Virus	blood	0/2*	0/2	0/2	0/2	0/2	0/2	0/2		0/2			7/8
(sc)	liver	0/2	2/2	0/2	0/2	1/2	0/2	1/2		1/2		0/8	
controls	blood	0/1	0/1	0/1	0/1	0/1	0/1	0/1		0/1			0/4
(sc)	liver	0/1	0/1	0/1	0/1	0/1	0/1	0/1		0/1		0/4	
B. Chicks													
Virus	blood	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		
(sc)	liver									0/2	0/2		
	brain					0/1				0/2	0/2		
Virus	blood									0/1	0/1		
(ic)	liver									0/1	0/1		
controls	blood	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		
(sc)	liver									0/1	0/1		
	brain									0/1	0/1		

* number positive/number tested

TABLE 8. Virus isolation attempts from **Myotis lucifugus** (l) and **Myotis keenii** (k) following subcutaneous inoculation with 3.0 dex* of USA M-2268a.

A. bats maintained at 37 C for days 0 to 7 and 4 C for days 7 to 14

days:	1		2		3		4		5		7	14
species:	k	k	k	k	k	k	k	k	k	l	l	k
sex:	♂	♂	♂	♂	♂	♀	♂	♂	♂	♂	♀	♀
blood**	—	—	+	—	—	—	—	—	—	—	+	—
liver	+	+	+	—	—	—	—	—	—	—	—	—
salivary glands	—	—	—	—	—	—	—	—	—	—	—	—
brown fat	—	—	+	—	+	—	+	+	—	—	—	—
spleen	+	—	—	—	—	—	—	—	—	—	—	—
lung	—	—	—	—	—	—	—	—	—	—	—	—
kidney	—	—	—	—	—	—	—	—	—	—	—	—
heart	—	—	—	—	—	—	—	—	—	—	—	—
pancreas	—	—	—	—	—	—	—	—	—	—	—	—
brain	—	—	—	—	—	—	—	—	—	—	—	—

B. bats maintained at 4 C for days 0 to 35

days:	7	14	21	28	35	Control 35
species:	l	l	l	l	k	l
sex:	♂	♂	♂	♂	♀	♂
blood**	—	—	—	—	—	—
liver	—	+	+	+	+	—
salivary glands	—	+	—	—	—	—
brain fat	—	+	+	+	—	—
spleen	—	—	—	—	+	—
lung	—	—	—	—	—	—
kidney	—	—	—	—	—	—
heart	—	—	—	—	—	—
pancreas	—	—	—	—	—	—
brain	—	—	—	—	—	—

* dex-decimal exponent [5]

** sera all negative for complement-fixing antibody.

1969 was 10.6%, while the prevalence rate, determined by neutralizing antibody, varied from 20 to 60%. Because bats were being used in the laboratory to determine the effects of hibernation on arboviral infections, USA M-2268a was inoculated into *M. lucifugus* and *M. keenii* held at temperatures of 4 C and 37 C (Table 8). Virus was detected in 7 of 11 bats held at 37 C over a 7 day observation period and 4 of 5 bats held at 4 C for as long as 5 weeks after inoculation. CF antibody was not detected in sera from any of the inoculated bats; none of the samples were tested for neutralizing antibody.

TABLE 9. Susceptibility of four species of mosquitoes to infections with Whitney's **Clethrionomys gapperi** virus following intrathoracic inoculation with approximately 4.5 dex* of USA M-2268a.

Day	*Aedes aegypti*	*Culex pipiens quinqefasciatus*	*Anopheles quadrimaculatus*	*Culiseta melanura*
0	2.8 dex**	2.8	2.8	2.6
1	3.4	2.7	3.5	2.4
2	3.2	2.7	2.2	2.8
3	>4.5	3.6		
4	2.5	3.6	3.7	3.4
5	2.9	4.0		
6	3.6	3.8	3.6	
8	2.9	3.7	3.4	
10	3.0	3.2	2.5	3.3

* dex = decimal exponent [5]

** total of five inoculated mosquitoes triturated in 1.0 ml of diluent.

Although the virus was recovered from the blood of one vole, there is no field or experimental evidence to suggest that it is an arthropod-borne agent. Attempts to transmit the virus to suckling mice by the bite of inoculated mosquitoes of four species failed. In addition, this virus has not been recovered from several thousand mosquitoes collected in the Hockomock Swamp (EFS, unpublished data).

The biological, physical, and chemical properties of this agent suggest that it is a rodent picornavirus or possibly a reovirus or parvovirus, depending upon the nucleic acid, which has not yet been characterized. Whitney[11] originally determined that the virus was not MM, Theiler's TO, lymphocytic choriomeningitis, reovirus type 3, Theiler's GD VII, or K virus. In the present study no serological relationships were detected by CF with several strains of encephalomyocarditis, Theiler's mouse encephalomyelitis virus (GD VII), and reovirus type 3, nor was this virus neutralized by antisera to 42 human enteroviruses. This virus did share CF antigens with an ungrouped, unpublished isolate from the blood of a montane vole, *M. montanus,* collected in Oregon in 1962; no other serologic affinities were detected.

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