

Serological evidence that *Mus musculus* is the natural host of Theiler's murine encephalomyelitis virus

Howard L. Lipton ^{a,b,c,*}, Byung S. Kim ^c, Hiroyuki Yahikozawa ^c,
Charles F. Nadler ^d

^a Division of Neurology, Evanston Hospital, 2650 Ridge Avenue, Evanston, IL 60201, USA

^b Department of Neurology, Northwestern University Medical School, Chicago, IL 60611, USA

^c Department of Microbiology–Immunology, Northwestern University Medical School, Chicago, IL 60611, USA

^d Department of Medicine, Northwestern University Medical School, Chicago, IL 60611, USA

Received 14 November 2000; received in revised form 13 March 2001; accepted 13 March 2001

Abstract

Theiler's murine encephalomyelitis virus (TMEV) infection is maintained in mouse colonies by fecal-oral spread (with no apparent role for persistent central nervous system infection) from an acutely infected animal to another. Therefore, serological methods offer the principal way to assess infection in mice and related rodent populations. Infection of mouse colonies with TMEV appears to be worldwide, yet no systematic serologic studies have been reported. In this study, enzyme-linked immunoassay and neutralization analysis of sera from feral *Mus musculus* obtained from four locations in the United States and one in Russia revealed antibodies to purified TMEV and two linear viral peptide epitopes in more than 50% of the sera derived from the five different locations. A similar analysis of sera from 26 species of related rodents trapped at multiple locations in North America and Europe indicated the presence of anti-TMEV antibodies only in a small proportion of water and bank voles that belong to a different subfamily. These results indicate that *Mus musculus* is the natural host of TMEV. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Theiler's murine encephalomyelitis virus; Serology; *Mus musculus*; Voles

1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) is an enteric pathogen of mice that be-

longs to the *Cardiovirus* genus in the family *Picornaviridae*. TMEV is a common cause of asymptomatic enteric infections in mice housed in non-barrier animal colonies. On occasion, TMEV spreads to the central nervous system (CNS) to cause poliomyelitis (flaccid paralysis) and more rarely, encephalitis (Thompson et al., 1951). There is no evidence of persistent infection in extraneu-

* Corresponding author. Tel.: +1-847-5702168; fax: +1-847-5701568.

E-mail address: hllipton@merle.acns.nwu.edu (H.L. Lipton).

ral organs, e.g. intestine, or the CNS; therefore, TMEV is only maintained by fecal-oral transmission from acutely infected to non-immune mice. Only one TMEV strain, MHG virus, has been recovered from another species, i.e. laboratory rats (Hemelt et al., 1974), and no isolations have been reported from rodents other than mice or from other animals in nature. Thus, TMEV appears to have a very narrow host range. In contrast, the closely related encephalomyocarditis group of cardiociruses (EMCV), which consists of EMCV, Mengo virus, Columbia-SK virus, and Maus-Elberfeld virus, has a wide host range and has been isolated from a variety of mammals (including man), birds and insects (reviewed in Tesh and Wallace, (1978)). Although the prevalence of neutralizing antibodies to EMCV in *Rattus rattus* was found to be among the highest in several animal populations examined in Hawaii (Tesh and Wallace, 1978) the wide host range of EMCV makes it difficult to conclude that rats are the principal natural reservoir for this infection.

Although serologic evidence has been reported indicating that the meadow vole, *Microtus pennsylvanicus* is periodically subject to epizootics of TMEV infection (Descoteaux and Mihok, 1986), no serologic studies of the most likely natural host, wild mice, *Mus musculus* have been done despite the apparent worldwide infection of mouse colonies. In the present study, we analyzed sera of feral *Mus musculus* (family Muridae, subfamily Murinae) from four locations in the United States and one in Russia, for the presence of anti-TMEV antibodies; more than 50% of the sera of *Mus musculus* from the five different locations were enzyme-linked immunoassay (ELISA) positive. Similar analysis of 26 species of related rodents trapped at multiple locations in North American and Europe revealed TMEV antibodies in only a small proportion of water and bank voles which together with meadow voles belong to a different subfamily (Microtinae). Thus, *Mus musculus* appears to be the natural host for TMEV infection.

2. Materials and methods

2.1. Animal sera

Blood was obtained by cardiac puncture under Metafane anesthesia from feral mice trapped live at the Lincoln Park Zoo in Chicago and bled the same day. Sera were obtained by standard procedures, heat-inactivated at 56°C for 30 min. and stored at -80°C. Feral mouse sera from several other locations were kindly provided by Ann Baker (Colorado State University, Fort Collins, CO), Jamie Childs (Centers for Disease Control, Atlanta, GA), and Vadim Agol (Poliomyelitis Institute, Moscow, Russia). Sera from the other rodent species were collected by one of the authors (C.F.N.).

2.2. Cells and viruses

BHK-21 cells were grown in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 100 µg streptomycin and 100 units penicillin/ml, 10% tryptose phosphate broth, and 8% fetal bovine serum. The origin and passage history of wild-type BeAn virus has been described (Rozhon et al., 1983). Wild-type EMCV and an attenuated Mengo virus with a shortened poly(C) tract were kindly provided by Ann Palmenberg, University of Wisconsin, Madison (Duke et al., 1990). The viruses were titered by standard plaque assay in BHK-21 cell monolayers as described (Rozhon et al., 1983).

2.3. Immune mouse sera

Groups of 5–8 outbred CD-1 mice injected intraperitoneal (i.p.) with $\cong 10^5$ plaque-forming units (PFU) of either BeAn virus or attenuated Mengo virus were killed on day 30 and blood was obtained cardiac puncture. Immune sera for each set of virus-inoculated mice were pooled.

2.4. Neutralizing antibody

Two-fold dilutions of heat-inactivated (56°C for 30 min) sera were incubated with 100–150 PFU of BeAn virus or EMCV at 24°C for 1 h.

Each mixture was then assayed in duplicate on BHK-21 cell monolayers in 6-multi-well plates according to standard plaque procedure. Neutralizing antibody was considered present if PFU were reduced by 50% compared with a normal mouse serum control.

2.5. Enzyme-linked immunoassay

An indirect ELISA was used to detect and quantify antibodies against TMEV and EMCV. Briefly, 50 μ l of partially purified virus (0.1 μ g) or synthetic peptides, A1Cb (VP1 262–276) or A2B (VP2 165–179), conjugated to bovine serum albumin (0.3 μ g) in 0.05 M NaHCO₃/Na₂CO₃ buffer, pH 9.6, were adsorbed onto 96-well polyvinyl chloride microtiter plates overnight at 20°C (Inoue et al., 1994). Fifty microliter of serial 2-fold dilutions (starting from 1:100) of sera, diluted in phosphate buffer saline, pH 7.4, containing 0.05% Tween 20 and 1% bovine serum albumin, were added to washed wells. After incubation for 2 h at 37°C, 50 μ l of anti-mouse/rat IgG conjugated with peroxidase (Type VI, Sigma) or alkaline phosphatase (Promega) was added to the wells. Plates were incubated for 3 h at 37°C, and 50 μ l of orthophenylenediamine for peroxidase and *p*-nitrophenyl phosphate for alkaline phosphatase was added. After incubation for \approx 10 min at 20°C, color development was stopped by addition of 50 μ l of 1.2 M H₂SO₄, and the reaction was assessed in a spectrophotometer at 492 nm for peroxidase and at 405 nm for alkaline phosphatase.

Preliminary experiments established that the OD values of serum dilutions \geq 1:200 from BeAn virus-infected CD-1 mice were $>$ 3 standard deviations higher than the values for uninfected mice. Thus, a serum titer \geq 1:200 was considered antibody-positive.

3. Results

3.1. Serological cross-reactivity between TMEV and EMCV

Based on the reported cross-reactivity between

TMEV and EMCV in complement fixation but not neutralization assay (Brown et al., 1994; Dick, 1949; Kerr, 1952; Smithburn, 1953), (Casals, 1963) we evaluated the extent of serologic cross-reactivity between these two related virus groups by ELISA, before testing sera from wild mice and other rodents for TMEV antibodies. Immune sera were pooled from 5 to 8 mice killed one month after i.p. injection of either BeAn virus (TMEV) or attenuated Mengo virus (EMCV group). Attenuated Mengo virus (Duke et al., 1990) was used since wild-type Mengo virus and EMCV are highly neurovirulent for mice. As shown in Fig. 1(A), the TMEV immune sera showed minimal if any reactivity with Mengo virus, while the Mengo virus immune sera showed substantial reactivity with TMEV. Therefore, infection with EMCV may result in cross-reactivity with TMEV, especially at lower serum dilutions. Recently, two major linear antibody epitopes, designated A1C and A2B in the C-terminus of VP1 and in the VP2 puff B region, respectively, have been identified in the TMEV capsid (Yahikozawa et al., 1997). Analysis of the reactivity of the immune sera with peptides representing these two linear epitopes revealed reactivity of the Mengo immune sera with TMEV peptide A2B but not A1C (Fig. 1(B)), which is surprising since there is relatively little amino acid sequence identity between the two viruses in these epitopes (Table 1). Thus, the conformation of the region may allow cross-reactivity of TMEV and Mengo virus, despite the differences in the amino acid residues.

3.2. Serological evidence for prevalence of TMEV infection in wild mice

Sera from a large number of feral mice trapped at the Lincoln Park Zoo in Chicago and from feral mice from other locations in the United States and Russia were tested by for ELISA for IgG antibodies to TMEV (Table 2). There was a high prevalence of antibodies to TMEV in mice from all locations, with an overall 59% of adult mice (range 40–100%) positive for TMEV antibody. As expected juvenile mice showed a somewhat lower incidence of TMEV antibodies than

the adult animals in some locations, although fewer juvenile sera were tested.

To exclude the possibility that the positive sera contained antibodies to EMCV, 16 ELISA-positive sera collected from the first location (Chicago, IL) were examined for neutralizing anti-

bodies to EMCV and BeAn. While all of the sera showed high neutralizing antibody titers to BeAn virus ($>1:4096$), none of these sera neutralized EMCV at a titer of 1:64. Selected ELISA-positive sera from the other locations were also negative by neutralization assay (data not shown). Thus it

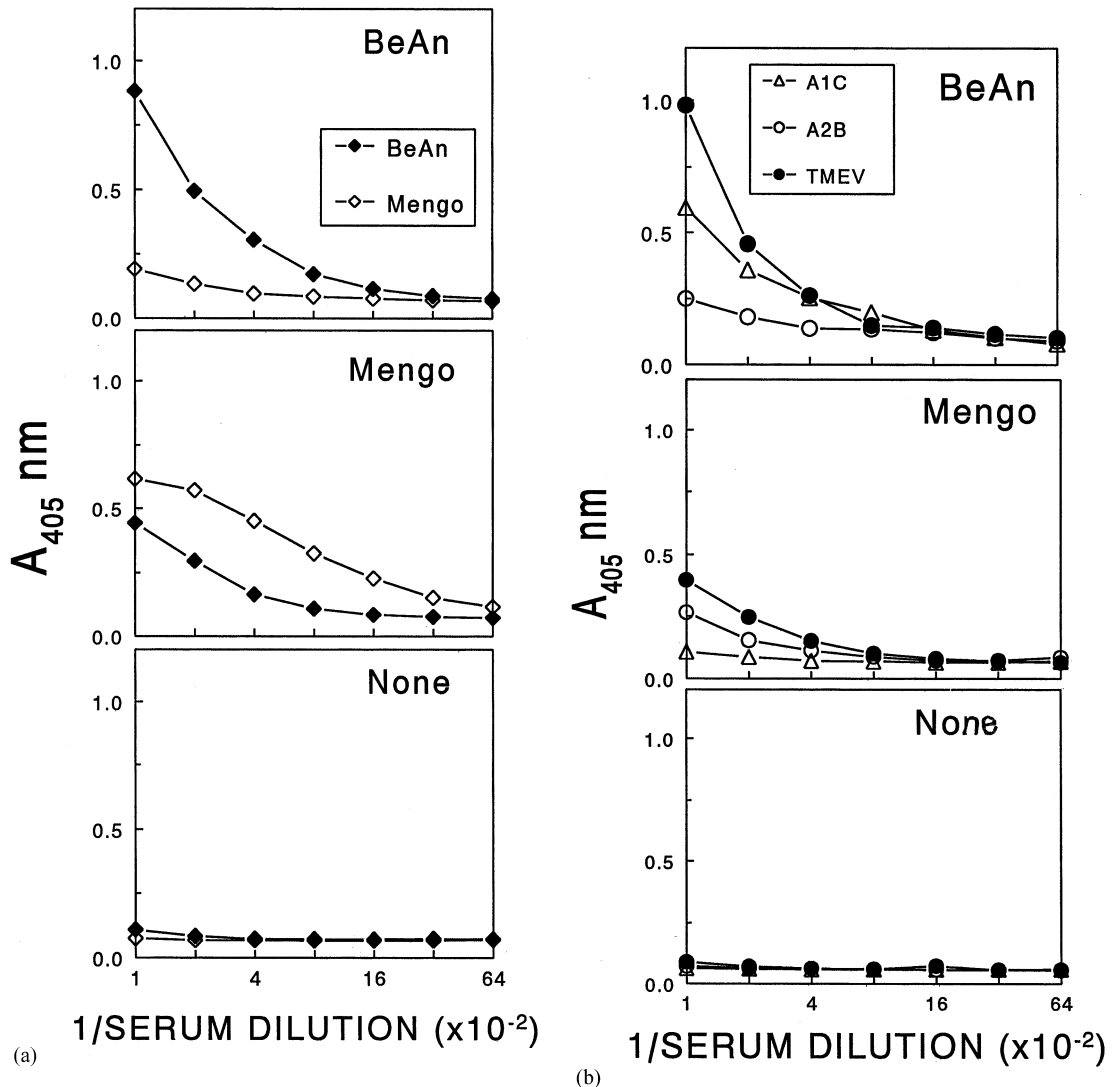


Fig. 1. Assessment of potential cross-reactivity between TMEV and EMCV. (A) Reactivity of sera from mice immunized with either TMEV (BeAn strain) or Mengo virus (EMCV group) and from unimmunized control mice. Label in right upper corner indicates the immunogen used to raise antisera, BeAn, Mengo or None. The antigens, BeAn or Mengo viruses, are shown in the box at the top. (B) Reactivity of sera from mice immunized with TMEV (BeAn strain) or Mengo virus and from unimmunized control mice. Label in right upper corner indicates the immunogen used to raise antisera, BeAn, Mengo or None. The antigens, TMEV linear antibody epitopes A1Cb (VP1) and A2B (VP2), and purified TMEV, are shown in the box at the top.

Table 1

Comparison of amino acids in linear antibody epitopes A2B and A1Cb of TMEV and EMCV

<i>Epitope A2B (VP2 residues 165–179 in puff B)</i>															
BeAn ^a	T	G	Y	R	Y	D	S	R	T	G	F	F	A	T	N
GDVII, FA ^b	R	Q	A	M	.
DA ^c , TO4, WW, Yale ^d	M	.
EMCV ^e	G	T	R	T	Q	T	N	.	K	.	P	.	.	M	D
<i>Epitope A1C (VP1 residues 262–276 at the C-terminus)</i>															
BeAn, Yale	T	K	I	N	A	D	N	P	V	P	I	L	E	L	E
GDVII, FA
DA, TO4, WW, Yale	S
EMCV	D	.	.	D	M	T	P	R	A	G	V	.	M	.	.

^a Pevear et al. (1987).^b Pevear et al. (1988).^c Ohara et al. (1988).^d Michiels et al. (1995).^e Palmenberg et al. (1984).

is highly unlikely that a positive ELISA in this study reflects antibody cross-reactivity with EMCV.

3.3. TMEV infection in other rodent species

A report by Descoteaux and Mihok (1986) suggests that TMEV infection is epidemic in wild meadow voles, *M. pennsylvanicus*. To determine whether rodent species other than feral mice are infected with TMEV, sera were obtained from 26 species of rodents trapped in North American and Europe; and tested by ELISA (Table 3). No sera from *M. pennsylvanicus* were available. Only water voles (*Arvicola terrestris*) and bank voles (*Clethrionomys glareolus*) were positive for TMEV antibodies; 27% of water voles from England, 4% from Switzerland and none from Russia, whereas only 3% of bank voles from Switzerland were TMEV-positive and none of the bank voles from three other locations had TMEV antibodies. These species and *M. pennsylvanicus* are members of the subfamily Microtinae. Thus, TMEV appears to infect water and possibly bank voles (Table 3) in addition to meadow voles (Descoteaux and Mihok, 1986). Although the prevalence of the infection is much lower than in wild mice. Of only two sera available from a single species of rat (*Rattus turkistanicus*), both were negative.

3.4. Antibody responses in wild mice to the A2B and A1C epitopes compared to virions

To determine whether sera of feral mice naturally infected with TMEV show reactivity with the

Table 2
TMEV ELISA Antibodies in Wild Mice, *Mus musculus*

Location	Age ^a	No. positive ^b / no. tested	Percent
<i>Baltimore, MD</i>			
City Parks	Juvenile	10/11	91
	Adults	20/20	100
Residential	Juvenile	6/7	86
	Adult	43/43	100
<i>Chicago, IL</i>			
Lincoln Park Zoo	Juvenile	18/79	23
	Adult	158/314	50
Ft Collins, CO	Juvenile	3/3	100
	Adult	19/20	95
<i>South Florida</i>			
Ft Myers		5/9	56
Everglades		0/2	0
Moscow, Russia	Juvenile	1/7	14
	Adult	8/20	40

^a Mice weighting <20 gm were designated juveniles; mice ≥20 gm were considered adults.

^b Serum titers ≥1:200 in ELISA.

Table 3
TMEV ELISA Antibodies in other rodent species

Rodent	Country	No. positive/No. tested	Percent
Water vole, <i>Arvicola terrestris</i>	England	3/11	27
	Switzerland	1/24	4
	Russia	0/3	0
Bank vole, <i>Clethrionomys glareolus</i>	Switzerland	2/59	3
	Finland, England and Russia	0/32	0
Subtotal positive ^a		6/129	

^a A total of 490 sera were tested, 361 of which were seronegative: *Peromyscus leucopus* (USA) 0/4; *Peromyscus maniculatus* (USA) 0/6; *Arvicola richardsoni* (USA) 0/2; *Clethrionomys ruficanus* (Finland) 0/13; *Clethrionomys rutilus* (Finland, USA & Yukon) 0/47; *Clethrionomys sikotanensis* (Russia) 0/3; *Lagurus curtatus* (USA) 0/20; *Microtus agrestis* (England & Switzerland) 0/19; *Microtus arvalis* (Switzerland and Russia) 0/22; *Microtus longicaudus* (USA & Yukon) 0/13; *Microtus mirus* (Yukon) 0/19; *Microtus nivalis* (Switzerland) 0/13; *Microtus ochrogaster* (USA) 0/24; *Microtus oeconomus* (Finland, USA & Yukon) 0/29; *Microtus pennsylvanicus* (Yukon) 0/15; *Microtus townsendii* (USA) 0/15; *Phaiomys carruthersi* (Russia) 0/14; *Pitymys multiplex* (Switzerland) 0/22; *Pitymys savii* (Switzerland) 0/7; *Pitymys subterrannius* (Switzerland) 0/1; *Pitymys pinetorum* (USA) 0/5; *Synaptomys cooperi* (USA) 0/10; *Apodemus sylvaticus* (Switzerland and Russia) 0/18; *Rattus turkistanicus* (Russia) 0/2; *Zapus princeps* (USA) 0/1.

A2B and A1C linear epitopes, the ELISA response to these peptides was compared to that of purified virus in all of the sera from Moscow (see Table 2). While 9 of the 27 feral mouse sera exhibited antibody titers of $\geq 1:200$ to the virus, only sera from four mice (# 4, # 6, # 8, # 23) showed responses to the A2B epitope and none to A1C (Fig. 2). In two of the sera (# 6, # 8) the ELISA response to A2B was greater than that to the purified virus, and in one serum sample (# 8), there was no detectable antibody to the virus. The variable levels of responses to A2B may reflect differences in the genes involved in antibody production to this epitope. Alternatively the differences may represent cross-reactivity of antibodies with epitopes related to TMEV A2B, as seen in Fig. 1(B). The ELISA antibody responses to a mixture of the peptides were no higher than that to A2B alone (not shown). The low level of antibodies to A1C is not surprising since antibodies to this epitope are relatively low in mice without overt disease after i.c. inoculation compared to mice undergoing TMEV-induced demyelination (Yahikozawa et al., 1997). Thus, neither of the two linear peptide epitopes or a mixture of the two peptides was as sensitive as purified virus for the detection of TMEV antibodies by ELISA.

4. Discussion

Although TMEV persists in the CNS of intracerebrally inoculated mice with the development of demyelinating disease, spread of this virus to the CNS after enteric (per os) infection is a rare event. Any persistently infected mouse would be selected against in nature. Therefore, TMEV infection is maintained by fecal-oral spread under natural conditions. For this reason we did not attempt to isolate or amplify by reverse transcriptase-polymerase chain reaction virus from the CNS in this study. Rather, serology was used to assess the prevalence of TMEV infection in feral mice and other related rodents species. Overall, we detected TMEV antibodies by ELISA in more than half of the sera of *Mus musculus* trapped in four locations in the United States and one site in Russia. Neutralization tests of selected ELISA-positive sera revealed high TMEV neutralizing titers ($\geq 1:4096$) and the lack of a response to EMCV, confirming the specificity of the ELISA for TMEV in this study. These data suggest that TMEV infection in *Mus musculus* is worldwide as it is in colony-bred mice, and that *Mus musculus* is the natural host for TMEV.

Unlike EMCV, which has been isolated from many species of rodents and other animals in nature (Tesh and Wallace, 1978), TMEV has only been isolated from mice, mainly colony-bred mice, and on one occasion, from colony-bred rats (Hemelt et al., 1974); however, TMEV infection has been transmitted to cotton rats and hamsters (Jungeblut, 1944). Because no sera from feral *Rattus rattus* were available for our study, we cannot exclude the possibility that rats are also infected with TMEV infection in the wild.

Serological analysis also indicated that TMEV infects the water vole, *Arvicola terrestris*. Interest-

ingly, Vilyuisk virus, which is serologically related to TMEV, has been isolated from an *Arvicola* species in Siberia (reviewed in Petrov, 1970). Vilyuisk virus was originally isolated from cases of acute and chronic encephalitis that occurs each summer in the Yakut indigenous people of the Sakha Republic in Siberia; however an etiologic link between Vilyuisk virus and the encephalitis remains to be established (Casals, 1963; Goldfarb and Gajdusek, 1992; Petrov, 1970; Sarmonova and Chumachenko, 1960). Both viruses infect water voles in nature, showing a further link between strains of TMEV and Vilyuisk virus.

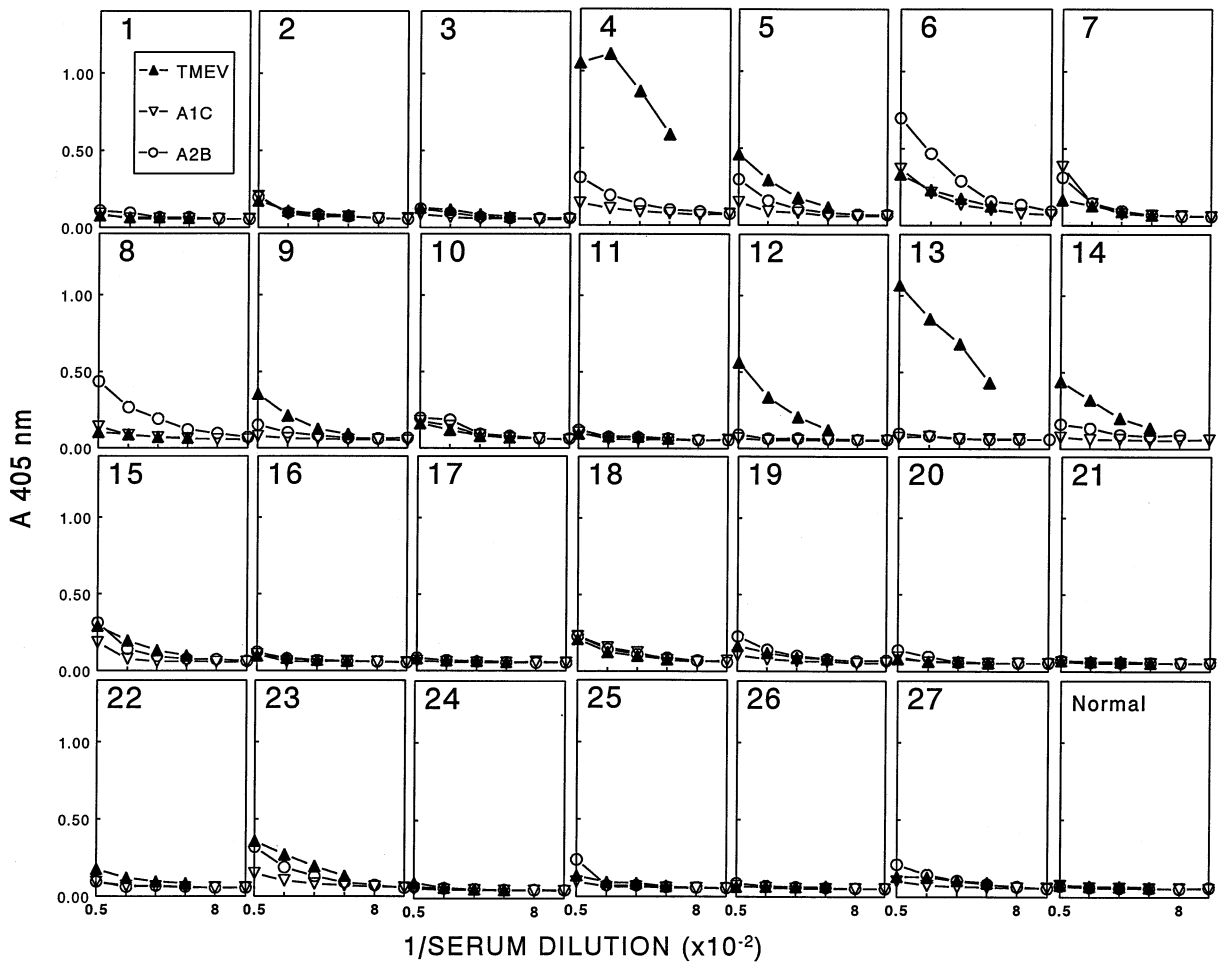


Fig. 2. Reactivity of sera from feral mice from Moscow with TMEV and two TMEV linear epitopes. Sera from individual mice were analyzed for reactivity with purified TMEV and peptides representing the A1Cb and A2B epitopes. A normal, uninfected laboratory mouse was included as a negative control.

Finally, in screening a subset of the *Mus musculus* sera for ELISA antibody responses to two linear peptide epitopes within the capsid (A1C and A2B) (Yahikozawa et al., 1997), we had hoped to find peptides that could substitute for virus in an ELISA. Routine TMEV antibody screening is needed in all mouse colonies used for research, and peptide antigens would provide a more convenient source of antigen than virus lysates. However as described above, neither of the two linear peptide epitopes or a mixture of the two peptides was as sensitive as purified virus for the detection of TMEV antibodies by ELISA, probably because most picornavirus antibody epitopes are conformational in nature.

Acknowledgements

We thank Beth Ostrowski and Steve Whittler for technical assistance. This research was supported by U.S. Public Health Service grants NS 23349 and NS 28752 from the National Institutes of Health and The Leiper Trust.

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