Journal of Virological Methods 151 (2008) 204–210

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of an ELISA to detect Sin Nombre virus-specific IgM from deer mice (*Peromyscus maniculatus*)

Mariana G. Bego[a,*], Darcy Bawiec[a], Deepa Dandge[a], Benjamin Martino[c], Denise Dearing[b], Eric Wilson[c], Stephen St. Jeor[a]

[a] *University of Nevada, Reno, School of Medicine, Department of Microbiology, 1664 N Virginia St. MS 200, Reno, NV 89557, USA*
[b] *University of Utah, Biology Department, 257 South 1400 East, Salt Lake City, UT 84112, USA*
[c] *Brigham Young University, Department of Microbiology and Molecular Biology, Provo, UT 84602, USA*

ARTICLE INFO

Article history:
Received 9 November 2007
Received in revised form 11 April 2008
Accepted 12 May 2008
Available online 30 June 2008

Keywords:
Hantavirus
IgM
ELISA
Peromyscus maniculatus
Sin Nombre virus

ABSTRACT

Peromyscus maniculatus (deer mouse) is the primary reservoir for Sin Nombre virus (SNV). Although the presence of IgG antibodies is often used as a marker of infection, it provides little information on active infections in a population but usually is an indicator of past infections. The presence of IgM antibodies is a much better marker for determining whether active infections are present in a population. A μ-capture SNV-specific IgM enzyme linked immunosorbent assay (ELISA) was developed. From live-trap and release studies a total of 68 rodent sera were studied for the presence of Sin Nombre virus-specific IgG and IgM antibodies. In these studies, IgM responses were detected in a number of animals. In some cases early SNV infection was determined through the presence of anti-SNV IgM before IgG antibodies could be detected. From the set of animals analyzed, it was concluded that the IgM response against SNV can persist anywhere from 1 to up to over 2 months, with a median of less than 1 month. Most importantly, it was demonstrated that anti-Sin Nombre virus IgM is an important tool for detection of early infections in rodents and should be considered as a key diagnostic tool.

1. Introduction

Hantaviruses are segmented negative strand viruses belonging to the *Bunyaviridae* family. They are widespread throughout North and South America, Europe and Asia. In the old world, Hantavirus infections are associated with hemorrhagic fever with renal syndrome (HFRS) (Vapalahti et al., 2003) and in the new world they are linked to hantavirus cardiopulmonary syndrome (HCPS, also referred to as hantavirus pulmonary syndrome or HPS) (Khan et al., 1996). Several Hantaviruses that are pathogenic for humans have been identified. One member of the Hantavirus genus of particular medical importance, Sin Nombre virus (SNV), is the primary causative agent of HCPS in the United States. Sin Nombre virus infections in humans are associated with a high mortality rate.

All Hantaviruses establish a persistent infection in their rodent host, without indication of severe disease. Experimental infections of SNV natural reservoir, the deer mouse (*Peromyscus maniculatus*) are restricted to BSL-4 animal facilities. Hence very little is understood of the basic biology, ecology and immune response of SNV and its primary host. Hantavirus RNA has been isolated from various rodent host tissues as well as from blood and saliva (Kuenzi et al., 2005; Safronetz et al., 2005). Since viral RNA is highly unstable and difficult to detect, researchers have relied heavily upon detection of anti-SNV IgG antibodies to determine the infection status of individual animals. IgG antibodies do not arise until weeks after an animal is exposed to virus (Botten et al., 2000).

Antibody-based immune responses begin with the production of polymeric IgM antibodies. In humans, IgM antibodies appear for a short period of time soon after primary infection. IgG antibodies that appear after IgM, persist for many years as part of the memory response. ELISA IgG and ELISA capture for IgM are used routinely to determine Hantavirus infection in humans (Bostik et al., 2000; Meisel et al., 2006). In contrast, ELISA IgG is currently the only diagnosis method for infections in rodents (Childs et al., 1994; Jay et al., 1997; Otteson et al., 1996). The patterns of SNV-specific IgM in

Abbreviations: SNV, Sin Nombre virus; ELISA, enzyme linked immunosorbent assay; HFRS, hemorrhagic fever with renal syndrome; HCPS, hantavirus cardiopulmonary syndrome; HPS, hantavirus pulmonary syndrome; PEMA, *Peromyscus maniculatus*; MUS, *Mus musculus*.

* Corresponding author. Tel.: +1 775 784 4275; fax: +1 775 327 2332.
E-mail addresses: marianabego@gmail.com (M.G. Bego), dbawiec@medicine.nevada.edu (D. Bawiec), dandged@unr.nevada.edu (D. Dandge), bjmartino@medicine.tamhsc.edu (B. Martino), denise.dearing@utah.edu (D. Dearing), ericwilson@byu.edu (E. Wilson), stjeor@gmail.com (S. St. Jeor).

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.05.008

P. maniculatus are not known. The detection of IgM antibodies in *P. maniculatus* has not previously been shown due to the lack of appropriate reagents.

A μ-capture SNV-specific IgM ELISA was developed to be used with *P. maniculatus* samples. *P. maniculatus* sera from a long term sampling study where more than 5000 samples were analyzed. Through the analysis of animals trapped multiple times throughout the study it was shown that in some animals anti-SNV IgM antibodies are present in animals testing negative for anti-SNV IgG antibodies. In subsequent trapping of these animals, antigen specific IgG antibodies were detected as the immune system converted to an IgG type response. In addition to early detection of SNV in rodents the use of virus specific IgM antibodies could be used to determine if increased incidences in human SNV infection correlate with increases in infections acquired recently in wild populations of rodents.

2. Material and methods

2.1. Archive rodent samples

Archive samples from a catch and release field study, which took place between 1995 and 1998 were used (Boone et al., 1998, 2002). Traps were operated from 2 to 5 days of each month, at 48 sites during the spring through fall. Rodents at every site were live-trapped according to a standardized protocol. Weight, sex and species identification were recorded for each animal. A blood sample was collected from each rodent at the trap station by retro-orbital puncture with a heparinized capillary tube or Pasteur pipette. Animals were marked with identifying ear tags or fur clips. Rodents were released at the point of trapping. Blood samples were placed immediately on dry ice until they could be returned to the laboratory for analysis. At the laboratory sera and clots were divided, and after the initial IgG testing, sera were archived (Boone et al., 1998, 2002).

P. maniculatus sera from a 5-year live-trap and release study, where more than 5000 samples were collected, were examined. Of all the animals collected, the only ones used in this study were animals that were negative for SNV IgG antibodies the first time they were trapped but subsequently seroconverted. Only 51 animals seroconverted between trapping. From the initial 51 seroconverted animals' samples, some were used for the development of ELISA. Other samples did not have sufficient volume left after the initial testing. A total of 35 animals were selected for the IgM study. For most animals at least two samples were available, allowing a total of 68 rodent sera to be tested for anti-SNV IgG and IgM presence.

2.2. SNV-specific ELISA IgG

ELISA IgG testing was done using a Center for Diseases Control (CDC) established protocol as described previously (Otteson et al., 1996). Briefly, ELISA plates were coated overnight at 4 °C with recombinant affinity purified SNV N antigen (CDC SPR569, Cat# VA2273, lot#98-0042L, 1:2000 dilution in phosphate buffered saline (PBS)). Plates were rinsed four times with ELISA wash (PBS supplemented with 0.5% Tween-20). Serum samples were diluted 1:100 in ELISA diluent (ELISA wash supplemented with 5% skim milk) and tested in duplicate. After incubation at 37 °C for 60 min, wells were rinsed 4 times with ELISA wash. Samples were then incubated with peroxidase-labeled goat anti-*Peromyscus leucopus* IgG secondary antibody (Kirkegaard and Perry Laboratories) diluted 1:1000 in ELISA diluent, for 60 min at 37 °C. Plates were rinsed four times with ELISA wash; peroxidase substrate (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid, Kirkegaard and Perry Laboratories) was added and the plates were incubated for 30 min at 37 °C. Color development was quantified by reading the optical density at 405 nm (OD405). Results were considered positive if the average OD405 for the samples was higher than 3 times the average OD405 for the negative control samples.

2.3. Reactivity of available anti-IgM reagents

An IgM capture assay was developed to determine if *P. maniculatus* IgM antibodies could be captured using reagents developed against *Mus musculus* IgM. A variety of anti-*Mus musculus* and anti-*Rattus norvegicus* reagents from different vendors, raised in different species were tested for specificity and reactivity to *P. maniculatus*. Of those tested, the goat anti-*Mus musculus* IgM reagent from Southern Biotech (San Diego, CA) exhibited the highest signal to noise ratio. Plates were coated at 4 °C overnight with different dilutions of goat anti-*Mus musculus* IgM antibodies (Southern Biotech, San Diego, CA). The dilutions (1:100, 1:300, and 1:1000) were made using PBS. Different dilutions of either *Mus musculus* control or, *P. maniculatus* sera from a rodent colony never exposed to SNV were made in ELISA diluent. Five serial dilutions ranged from 1:100 to 1:3200. Plates were incubated with the diluted sera at 37 °C for 60 min, and rinsed four times with ELISA wash. A secondary antibody peroxidase labeled goat anti-*Mus musculus* IgM (Southern Biotech, San Diego, CA) diluted 1:1000 in ELISA diluent was added and plates were incubated at 37 °C for 60 min. Plates were rinsed four times with ELISA wash; peroxidase substrate (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the plates were incubated for 30 min at 37 °C. Color development was quantified by reading the optical density at 405 nm (OD405).

2.4. Sin Nombre virus-specific ELISA capture IgM

Several strategies were tested to develop an ELISA IgM capture ELISA protocol that yielded a higher signal/noise ratio. The protocol described below was used. Plates were coated at 4 °C overnight with the capture antibody: goat anti-*Mus musculus* IgM (Southern Biotech) diluted 1:1000 in PBS. Nonspecific binding was blocked by adding the ELISA diluent containing 5% goat serum to the plates, followed by 60 min incubation at 37 °C. Sera to be tested were diluted 1:100 in ELISA diluent, and plated in duplicate. As a negative control, *P. maniculatus* serum from an animal never exposed to SNV diluted 1:100 in ELISA diluent was used in each plate. An additional negative control, where only ELISA diluent and no sera were added, was also included in each plate. Samples were plated in such a way that the wells surrounding each sample were empty to avoid cross-well contamination. After the addition of sera, plates were incubated at 37 °C for 60 min followed by rinsing four times with ELISA wash, and recombinant SNV N antigen (CDC SPR569, Cat# VA2273, lot#98-0042L, diluted 1:1000 in ELISA diluent) was added and plates were incubated at 37 °C for 60 min. Plates were rinsed four times with ELISA wash, and rabbit anti-SNV nucleocapsid (Immunology Consultant Laboratories Inc., Newberg, OR; diluted 1:1000 in ELISA diluent) was added and plates were incubated at 37 °C for 60 min. Plates were rinsed four times with ELISA wash, and the peroxidase labeled goat anti-rabbit IgG antibody (Southern Biotech; diluted 1:1000 in ELISA diluent) was added and plates were incubated at 37 °C for 60 min. Following incubation plates were rinsed again four times with ELISA wash; peroxidase substrate (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and they were incubated for 30 min at 37 °C. Color development was quantified by reading the optical density at 405 nm (OD405). Samples were considered positive if the average of their OD values was higher than the average OD for the negative *Peromyscus* serum plus 3 times the standard devi-

Table 1
Capture of *Peromyscus* IgM

		Sera dilution				
		1:100	1:400	1:800	1:1600	1:3200
Coating 1:100	PEMA	2.98	2.92	2.72	2.24	1.60
	MUS	2.98	2.96	2.92	2.87	2.87
	No serum	0.17	0.15	0.11	0.58	0.09
Coating 1:300	PEMA	3.03	2.87	2.57	1.84	1.24
	MUS	3.07	3.04	2.98	2.93	2.92
	No serum	0.30	0.15	0.13	0.09	0.09
Coating 1:1,000	PEMA	2.99	2.90	2.58	1.76	1.41
	MUS	3.10	3.07	3.04	3.01	2.95

Average of OD405 values shown. *Notes*: PEMA, *Peromyscus maniculatus*; MUS, *Mus musculus*. Each value represents average OD405 values for duplicate reading.

ation of these negative OD values. This protocol was tested with a number of known negative *Peromyscus* samples and demonstrated to yield consistent results.

3. Results

3.1. Capture of P. maniculatus IgM molecules using commercially available reagents

ELISA plates were coated overnight with goat anti-*Mus musculus* IgM unlabeled antibodies as described in Section 2. Different dilutions of anti-*Mus musculus* IgM antibody in PBS were used. *P. maniculatus* (PEMA) serum was diluted serially 5 times, from 1:100 to 1:3200, in an ELISA diluent. As a positive control *Mus musculus* (MUS) serum was used, and as a negative control no rodent sera was added to the plates. To detect if the *Peromyscus* IgM molecules were captured successfully, peroxidase labeled goat anti-*Mus musculus* IgM (capture antibody) was used as described in Section 2. As seen in Table 1, detection of *P. maniculatus* IgM from sera was concentration dependent.

3.2. Development of an ELISA capture IgM for detection of early SNV infections

3.2.1. Experimental setup

Several strategies were examined in order to develop an ELISA IgM capture protocol that yielded an optimal signal/noise ratio. The general ELISA capture IgM outline is shown in Fig. 1. Each step was omitted to determine source of background readings. Alternative reagents were tested for each step. For all steps in Fig. 1, different dilutions were tested and the optimal dilution was used in all subsequent experiments.

Plates were coated using goat anti-*Mus musculus* IgM (Fig. 1—Step 1). The presence or absence of blocking between different steps during the ELISA was tested. Blocking was determined to be more efficient after coating. Samples from rodents that were never exposed to the virus were used as negative controls, while several samples from live-trap and release studies were also tested (Fig. 1—Step 3). Two SNV antigens were tested for the next step in the procedure, affinity purified SNV nucleocapsid (CDC), or SNV infected cell slurry (CDC). Optimal results were obtained when using the purified nucleocapsid antigen (Fig. 1—Step 4). Several detection antibodies were tested including, rabbit anti-N; rabbit anti-G1/G2, or human convalescent sera. Antibodies raised in mouse should be avoided at this stage. Rabbit sera against the SNV nucleocapsid gave the best results (Fig. 1—Step 5). Rabbit polyclonal anti-SNV N (Cat# RSNV-55, Immunology Consultant Laboratories Inc., Newberg, OR) was obtained from animals immunized with recombinant protein, and had a 1:50,000 titer with the conventional ELISA IgG.

3.2.2. Assay sensitivity

The main objective of any assay validation is to provide test results that identify samples as positive or negative with a preset degree of statistical confidence. The cut-off for an assay result is selected at a threshold value at and above which findings are interpreted as positive. Fifteen samples from the live-trap and release study with known IgG titers were used for ELISA IgM testing. The samples were diluted 1:100, and tested in triplicate. Serum samples from a known SNV negative *P. maniculatus* rodent were used as a negative control. Two other groups of results were observed, those whose OD405 average was significantly higher than this initial cut-off value (positive group, OD average higher than three times standard deviations over average of negative controls), and those whose OD405 values were closer than this established value (borderline group, OD average higher than two times standard deviations over average of negative controls, Fig. 2). Although cut-off OD405 values may seem higher when compared to other ELISA set ups, like ELISA IgG, these values are not uncommon for ELISA IgM

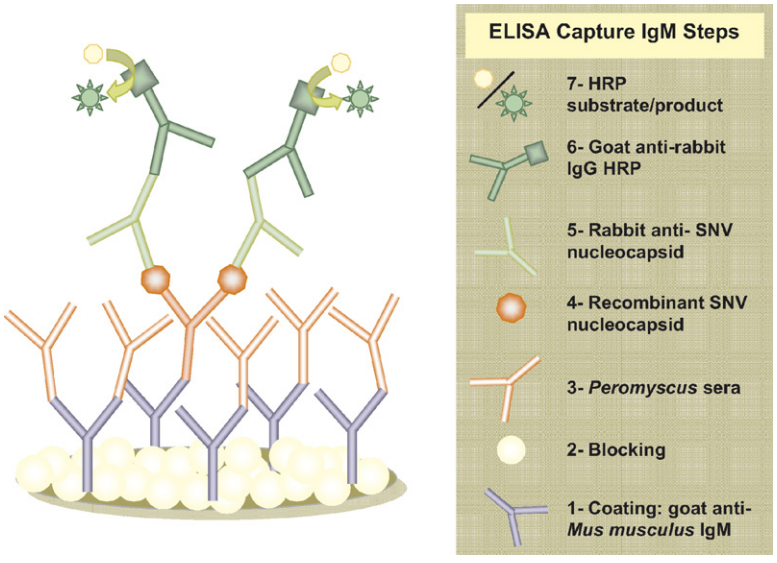

Fig. 1. ELISA capture IgM steps. In order to simplify the graphic IgM is shown as a monomer.

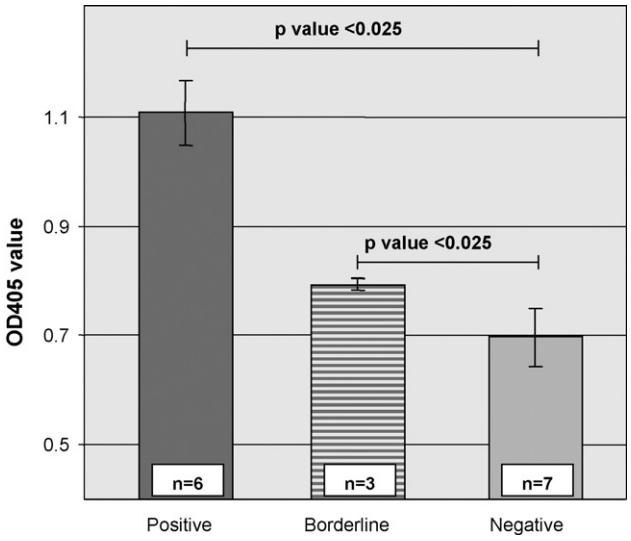

Fig. 2. ELISA IgM results for a negative control and 15 unknown samples. OD405 values for each sample is the average of three independent readings. Three significantly distinct groups were observed, six samples were classified as positive, three as borderline, and six as negative. A known negative control was also included. Comparison between groups was done using unpaired *t*-test.

(Melo Cardoso Almeida et al., 2008; Nielsen and Vestergaard, 2002; Rota et al., 1994; Takahashi et al., 1999).

OD405 values of the three groups were compared using an unpaired *t*-test. Comparison between positive and negative groups was done using unpaired *t*-test for two samples with equal variance (p value = 2.1×10^{-7}) and using unpaired *t*-test for two samples with unequal variance for borderline and negative group comparison (**p value = 0.0012). Since two independent *t*-tests were used to analyze the data set, the Bon Ferroni correction was applied. It was concluded that the samples can be divided in three distinct groups since the p values observed were smaller than 0.025.

3.2.3. Assay reproducibility

To determine assay reproducibly samples were tested several times by independent assays. In each plate the sample was tested in duplicate. Eleven positive samples were tested by at least two independent assays, while four were tested three times. Twenty negative samples were tested at least twice, from those 20, five were tested three times, and two four times (data not shown; see supplementary material tables).

Table 2
Rodents with recorded seroconversion to IgM prior to IgG

Rodent #	Capture dates	Specie	Sex	Age	Previous ELISA IgG	ELISA IgM	ELISA IgG
2520	07-22-98	PEMA	F	A	0	NEG	NEG
	10-14-98	PEMA	F	A	1	POS	NEG
2604	08-15-98	PEMA	M	A	0	POS	NEG
	10-14-98	PEMA	M	A	4	POS	POS
2653	09-09-98	PEMA	F	A	0	BORD	NEG
	10-14-98	PEMA	F	A	0	BORD	NEG
	06-22-99	PEMA	F	A	4	NEG	POS

Notes: PEMA, *Peromyscus maniculatus*; F, female; M, male; J, juvenile; A, adult. Previous ELISA graded in a scale from 0 (negative), to 5. sna, sample not available; NEG, negative; POS, positive; BORD, borderline.

Table 3
Rodents that had IgM and IgG detected at the same time

Rodent #	Capture dates	Specie	Sex	Age	Previous ELISA IgG	ELISA IgM	ELISA IgG
382	09-18-98	PEMA	M	A	0	NEG	NEG
	11-16-98	PEMA	M	A	0	NEG	NEG
	05-12-99	PEMA	M	A	5	POS	POS
	06-08-99	PEMA	M	A	5	NEG	POS
893	03-19-98	PEMA	F	A	0	sna	sna
	05-16-98	PEMA	F	A	5	sna	sna
	06-17-98	PEMA	F	A	5	POS	POS
974	07-09-97	PEMA	M	A	0	sna	sna
	07-22-98	PEMA	M	A	3	BORD	POS
	08-15-98	PEMA	M	A	5	NEG	POS
1414	10-14-98	PEMA	F	A	0	NEG	NEG
	07-26-99	PEMA	F	A	5	POS	POS
1714	06-07-98	PEMA	F	A	0	sna	sna
	07-22-98	PEMA	F	A	4	POS	POS
1969	10-10-97	*PETR*	F	A	0	sna	sna
	04-25-98	*PETR*	F	A	0	sna	sna
	05-18-98	*PETR*	F	A	5	sna	sna
	06-10-98	*PETR*	F	A	4	POS	POS
	07-01-98	*PETR*	F	A	5	POS	POS
2388	06-28-98	PEMA	F	A	0	sna	sna
	07-22-98	PEMA	F	A	0	NEG	NEG
	08-15-98	PEMA	F	A	5	POS	POS
2408	06-28-98	PEMA	M	A	0	NEG	NEG
	09-09-98	PEMA	M	A	5	POS	POS
2409	06-28-98	PEMA	M	A	0	POS	POS
	09-09-98	PEMA	M	A	5	NEG	POS
2412	06-28-98	PEMA	F	J	0	sna	sna
	07-22-98	PEMA	F	A	0	sna	sna
	08-15-98	PEMA	F	A	5	POS	POS
	09-09-98	PEMA	F	A	5	NEG	POS
2530	07-22-98	PEMA	M	J	0	NEG	NEG
	10-14-98	PEMA	M	A	0	NEG	NEG
	09-08-99	PEMA	M	A	5	POS	POS
2616	08-15-98	PEMA	M	A	0	sna	sna
	09-09-98	PEMA	M	A	5	POS	POS
	10-14-98	PEMA	M	A	5	POS	POS
2623	08-15-98	PEMA	F	A	0	sna	sna
	10-14-98	PEMA	F	A	4	POS	POS
2710	09-09-98	PEMA	M	A	0	NEG	NEG
	10-14-98	PEMA	M	A	5	POS	POS

Notes: PEMA, *Peromyscus maniculatus*; PETR, *Peromyscus truei*; F, female; M, male; J, juvenile; A, adult. Previous ELISA graded in a scale from 0 (negative), to 5. sna, sample not available; NEG, negative; POS, positive; BORD, borderline.

3.3. Evaluation of the ELISA capture IgM for the detection of early SNV infections

3.3.1. Rodents with recorded seroconversion to IgM prior to IgG

In three animals (Table 2), it was possible to detected IgM prior to the appearance of IgG, suggesting these animals had recently acquired SNV infections. The response in rodent 2604, demonstrated that the IgM response can persist at a minimum for 2 months, and overlaps with the IgG response. Sample from the first and second times rodent 2653 was trapped were positive for IgM, but not IgG. A sample from the third time rodent 2653 was trapped, 8 months after the second, was IgG positive but IgM negative. These findings indicated that as seen in humans, the IgM response in *P. maniculatus* is short-lived, even during persistent infection.

3.3.2. Rodents that had IgM and IgG detected at the same time

In the present study, as predicted, no instances were found of IgG antibodies arising before IgM antibodies. These results support the specificity of the anti-IgM reagents. Several examples of IgG and IgM antibodies detected simultaneously were observed, Table 3. For example, samples collected from rodent 382 on the first and second time it was live-trapped were negative for SNV-specific IgM and IgG antibodies. The sample collected from this rodent's third live-trap was positive for both SNV-specific immunoglobulins. The sample collected from this rodent last live-trap was SNV-specific IgG positive but IgM negative. From this result, it can be concluded that this animal was infected between its second and third time it was trapped (from November to May; however, the exact date of seroconversion could not be determined). By the following June (<7 months) the animal was SNV-specific IgM negative but IgG positive, indicating that the IgM response persisted less than 7 months.

Rodent 1669 was the first animal examined that was not a *P. maniculatus*, but *Peromyscus truei*. Samples collected from this animal's first two times it was trapped were negative for SNV-specific IgG. Samples collected from this animal's following three times it was trapped were positive for SNV-specific IgG. Only samples from this animal last two times it was trapped were available for further IgM analysis. These last two samples were positive for SNV-specific IgG and IgM antibodies, indicating that this animal was likely infected between April and May, and retained its SNV-specific IgM response at least for 3 months.

3.3.3. Samples with no IgM response

In 18 rodents, an IgM immune response was detected, Table 4. In this group, two other *Peromyscus truei* were present in the study set, rodents 2199 and 2236. Samples collected from rodent 2199 first three times it was trapped were SNV-specific IgG negative, while samples collected from its five following trappings were SNV-specific IgG positive. Since this animal was always IgM negative, it was postulated that these molecules appeared after the last IgG negative sample was collected, and disappeared before the first IgG positive sample was taken, persisting for less than 1 month. The same scenario was seen with rodent 453 (1 month between last taken negative sample and IgG seroconversion). In most of the rodents analyzed, between one and one and a half months passed between trapping for seroconversion to IgG (1694, 1740, 2138 and 2524).

Table 4
Rodents without detected IgM response

Rodent #	Capture dates	Specie	Sex	Age	Recorded ELISA IgG	ELISA IgM	ELISA IgG
339	09-18-98	PEMA	F	J	0	NEG	NEG
	11-16-98	PEMA	F	A	1	NEG	NEG
413	08-11-98	PEMA	M	A	0	sna	sna
	09-18-98	PEMA	M	A	0	NEG	NEG
	11-16-98	PEMA	M	A	3	sna	sna
453	08-29-98	PEMA	M	A	0	NEG	NEG
	10-08-98	PEMA	M	A	5	NEG	POS
	11-21-98	PEMA	M	A	4	NEG	POS
862	04-18-98	PEMA	M	A	0	sna	sna
	06-23-98	PEMA	M	A	4	NEG	POS
1694	06-07-98	PEMA	M	A	0	sna	sna
	06-28-98	PEMA	M	A	0	NEG	NEG
	07-22-98	PEMA	M	A	4	NEG	POS
1697	06-07-98	PEMA	F	A	0	sna	sna
	06-28-98	PEMA	F	A	3	sna	sna
	07-22-98	PEMA	F	A	3	NEG	POS
1740	06-07-98	PEMA	M	A	0	NEG	NEG
	07-22-98	PEMA	M	A	4	NEG	POS
1743	06-07-98	PEMA	M	A	0	sna	sna
	06-28-98	PEMA	M	A	0	sna	sna
	07-22-98	PEMA	M	A	4	NEG	POS
	08-15-98	PEMA	M	A	5	sna	sna
1769	06-10-98	PEMA	M	A	0	NEG	NEG
	09-12-98	PEMA	M	A	4	sna	sna
2121	03-23-98	PEMA	F	A	0	NEG	NEG
	04-18-98	PEMA	F	A	0	NEG	NEG
	05-19-98	PEMA	F		1	NEG	NEG
2132	04-18-98	PEMA	M	A	0	sna	sna
	09-18-98	PEMA	M	A	5	NEG	POS
	11-16-98	PEMA	M	A	5	sna	sna
2138	04-18-98	PEMA	F	A	0	NEG	NEG
	05-19-98	PEMA	F	A	4	NEG	POS
2199	04-25-98	*PETR*	M	A	0	sna	sna
	05-18-98	*PETR*	M	J	0	sna	sna
	06-10-98	*PETR*	M	J	4	NEG	sna
	07-01-98	*PETR*	M	A	5	NEG	POS
	08-11-98	*PETR*	M	A	5	NEG	POS
	09-12-98	*PETR*	M	A	5	NEG	POS
	10-04-98	*PETR*	M	A	4	NEG	POS
	10-27-98	*PETR*	M	A	4	NEG	POS
2236	04-25-98	*PETR*	F	J	0	sna	sna
	05-18-98	*PETR*	F	J	0	sna	sna
	06-10-98	*PETR*	F	J/A	1	NEG	NEG
2377	06-28-98	PEMA	M	A	0	sna	sna
	07-22-98	PEMA	M	A	3	NEG	POS
2575	08-15-98	PEMA	F	A	0	NEG	NEG
	10-14-98	PEMA	F	A	3	NEG	POS
2658	09-09-98	PEMA	M	A	0	NEG	NEG
	10-14-98	PEMA	M	A	5	sna	sna
2768	10-04-98	PEMA	M	A	0	NEG	NEG
	10-27-98	PEMA	M	A	1	sna	sna

Notes: PEMA, *Peromyscus maniculatus*; PETR, *Peromyscus truei*; F, female; M, male; J, juvenile; A, adult. Previous ELISA graded in a scale from 0 (negative), to 5. sna, sample not available; NEG, negative; POS, positive.

4. Discussion

IgM antibodies are the first antibodies produced after an infection and are usually replaced with higher affinity IgG antibodies as the immune response progresses. It had been demonstrated previously that low affinity IgM antibodies do not compete efficiently with higher affinity IgG for antigen binding in ELISA (Kallio-Kokko et al., 1998). When an excess of IgG molecules are present, most of the antigenic epitopes will be bound to IgG, blocking binding of IgM molecules. In order to detect the presence of antigen specific IgM antibodies, the sera need to be cleared of IgG against the antigen being tested. In our procedure, all *P. maniculatus* IgM antibodies were first captured, unbound antibodies of other isotypes were then washed away and finally SNV specific IgM antibodies were detected through the use of recombinant nucleocapsid antigen. This ELISA technique is known as ELISA capture for IgM. This technique thus allows the detection of low affinity IgM antibodies without the interference of high affinity IgG antibodies.

Standard ELISA capture IgM requires specific antibodies to recognize IgM molecules. Currently antibodies against *Mus musculus* IgM are commercially available, but not against *Peromyscus* IgM. In order to determine if *Peromyscus* IgM antibodies could be captured using available reagents, *Peromyscus* sera were tested using anti-IgM antibodies from *Mus musculus.* The results of this study indicated that although there are evolutionary differences between *P. maniculatus* and *Mus musculus* sufficient homology exists in their

immunoglobulin genes that *P. maniculatus* IgM can be detected readily using anti-*Mus* reagents.

In the past, IgG avidity assays were used to try to estimate differences between recent and past Hantavirus infections (Gavrilovskaya et al., 1993; Hedman et al., 1991; Kallio-Kokko et al., 1993, 1998; Safronetz et al., 2006). Although the increase of IgG avidity in a given rodent is a clear indication of recent infection, comparison between samples is not direct. Some rodents can produce a high avidity IgG faster after infection. On the other hand, some other rodents can increase the IgG avidity at a much slower pace, and may never generate high avidity IgG antibody titers. Conversely, the presence of an IgM response, as it demonstrated in this manuscript, is a direct indication of an early/recent infection. As demonstrated by data obtained in this study, 8.5% (3/35) of SNV infected animals would not have been detected by traditional virus specific IgG assays.

SNV-specific IgM antibodies were detected in 17 of 35 animals which seroconverted during the initial study. In three animals it was possible to detect the presence of anti-SNV IgM before they were anti-SNV IgG positive. This indicates that, as seen in humans, IgM antibodies are the first responders after SNV infections. Although in 18 of the animals studied IgG seroconversion was detected, IgM response was negative. This would indicate that the animal was not trapped in the window of time where IgM antibodies were present. Among the ELISA IgM positive rodents, several instances of individual animals seroconverting to IgG positive IgM negative were documented during the study. SNV specific IgG antibodies were never detected prior to IgM antibodies.

For most of the animals in the study, the data collected and samples available were not useful to determine how long IgM can persist. Although from 13 animals data were gathered regarding possible periods of IgM seropositivity. Two animals maintained their IgM antibodies for over 2 months (rodents 1969 and 2604), while two animals (rodents 893 and 2653) were IgM seropositive for at least 1 month. In seven of the thirteen animals tested, IgM antibodies were not detected even when IgG seroconversion was recorded within a month interval. This would indicate that the presence of IgM persisted less than 1 month after infection. For the remaining three animals detectable levels of IgM antibodies were shown to persist less than 2 months. From the set of animals tested, it can be concluded that the IgM response against SNV can persist anywhere from less than 1 to up to over 2 months, with a median of less than 1 month. The dramatically different lengths of time animals remained IgM positive may relate to the size of the infectious inoculum or other environmental factors beyond our control. The IgM response seen in rodents corresponded to previous findings in Hantavirus infections in humans (Bostik et al., 2000). It is highly unlikely that patterns observed in our study are due to random effects.

It should be recognized that because animal studies with the natural reservoir of SNV are limited to a BSL-4 laboratory it is difficult to conduct animal studies where the time of infection can be controlled. BSL-4 laboratory space is extremely difficult to obtain. In the present study, catch and release of wild rodents which represents a natural infection in a natural setting were used, which makes it impossible to determine the exact time of virus exposure. Future experiments in a laboratory setting could not only establish accurately the kinetics of IgM and IgG antibody responses but also control for inoculum size and other factors that are impossible to control in a natural setting.

In conclusion, an ELISA that detects consistently IgM antibodies against SNV in their rodent host was developed. ELISA IgM tests similar to the one presented in this manuscript can be easily adapted to detect newly acquired infection of other pathogens in *Peromycus* rodents. A large set of animals was studied. It was demonstrated that anti-SNV IgM profiles in *P. maniculatus* are similar to the profiles observed in human infections. Most importantly, it was also demonstrated that ELISA IgM is an important tool in order to detect new Sin Nombre virus infections in their natural reservoir. Since these types of antibodies are the first responders, ELISA IgM should be considered as a key diagnostic and epidemiologic tool. The use of an IgM capture assay is much more informative than an IgG assay since it provides information on the presence of active virus in a population of animals.

Acknowledgements

We would like to thank Dr. John Boone and Elmer Otteson for the archive samples. Work supported by NSF EID Program EF 0326999 and NIAD Award number AI-65359.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.jviromet.2008.05.008.

References

Boone, J.D., McGwire, K.C., Otteson, E.W., DeBaca, R.S., Kuhn, E.A., St Jeor, S.C., 2002. Infection dynamics of Sin Nombre virus after a widespread decline in host populations. Am. J. Trop. Med. Hyg. 67, 310–318.

Boone, J.D., Otteson, E.W., McGwire, K.C., Villard, P., Rowe, J.E., St Jeor, S.C., 1998. Ecology and demographics of hantavirus infections in rodent populations in the Walker River Basin of Nevada and California. Am. J. Trop. Med. Hyg. 59, 445–451.

Bostik, P., Winter, J., Ksiazek, T.G., Rollin, P.E., Villinger, F., Zaki, S.R., Peters, C.J., Ansari, A.A., 2000. Sin nombre virus (SNV) Ig isotype antibody response during acute and convalescent phases of hantavirus pulmonary syndrome. Emerg. Infect. Dis. 6, 184–187.

Botten, J., Mirowsky, K., Kusewitt, D., Bharadwaj, M., Yee, J., Ricci, R., Feddersen, R.M., Hjelle, B., 2000. Experimental infection model for Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*). Proc. Natl. Acad. Sci. U.S.A. 97, 10578–10583.

Childs, J.E., Ksiazek, T.G., Spiropoulou, C.F., Krebs, J.W., Morzunov, S., Maupin, G.O., Gage, K.L., Rollin, P.E., Sarisky, J., Enscore, R.E., et al., 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J. Infect. Dis. 169, 1271–1280.

Gavrilovskaya, I., Apekina, N., Okulova, N., Demina, V., Bernshtein, A., Myasnikov, Y., 1993. IgG avidity assay for estimation of the time after onset of hantavirus infection in colonized and wild bank voles. Arch. Virol. 132, 359–367.

Hedman, K., Vaheri, A., Brummer-Korvenkontio, M., 1991. Rapid diagnosis of hantavirus disease with an IgG-avidity assay. Lancet 338, 1353–1356.

Jay, M., Ascher, M.S., Chomel, B.B., Madon, M., Sesline, D., Enge, B.A., Hjelle, B., Ksiazek, T.G., Rollin, P.E., Kass, P.H., Reilly, K., 1997. Seroepidemiologic studies of hantavirus infection among wild rodents in California. Emerg. Infect. Dis. 3, 183–190.

Kallio-Kokko, H., Vapalahti, O., Hedman, K., Brummer-Korvenkontio, M., Vaheri, A., 1993. Puumala virus antibody and immunoglobulin G avidity assays based on a recombinant nucleocapsid antigen. J. Clin. Microbiol. 31, 677–680.

Kallio-Kokko, H., Vapalahti, O., Lundkvist, A., Vaheri, A., 1998. Evaluation of Puumala virus IgG and IgM enzyme immunoassays based on recombinant baculovirus-expressed nucleocapsid protein for early nephropathia epidemica diagnosis. Clin. Diagn. Virol. 10, 83–90.

Khan, A.S., Ksiazek, T.G., Peters, C.J., 1996. Hantavirus pulmonary syndrome. Lancet 347, 739–741.

Kuenzi, A.J., Douglass, R.J., Bond, C.W., Calisher, C.H., Mills, J.N., 2005. Long-term dynamics of Sin Nombre viral RNA and antibody in deer mice in Montana. J. Wildl. Dis. 41, 473–481.

Meisel, H., Wolbert, A., Razanskiene, A., Marg, A., Kazaks, A., Sasnauskas, K., Pauli, G., Ulrich, R., Kruger, D.H., 2006. Development of novel immunoglobulin G (IgG), IgA, and IgM enzyme immunoassays based on recombinant Puumala and Dobrava hantavirus nucleocapsid proteins. Clin. Vaccine Immunol. 13, 1349–1357.

Melo Cardoso Almeida, C., Vasconcelos Jr., A.C., Kipnis, A., Andrade, A.L., Junqueira-Kipnis, A.P., 2008. Humoral immune responses of tuberculosis patients in Brazil indicate recognition of *Mycobacterium tuberculosis* MPT-51 and GlcB. Clin. Vaccine Immunol. 15, 579–581.

Nielsen, L., Vestergaard, B.F., 2002. A mu-capture immunoassay for detection of human herpes virus-6 (HHV-6) IgM antibodies in human serum. J. Clin. Virol. 25, 145–154.

Otteson, E.W., Riolo, J., Rowe, J.E., Nichol, S.T., Ksiazek, T.G., Rollin, P.E., St Jeor, S.C., 1996. Occurrence of hantavirus within the rodent population of northeastern California and Nevada. Am. J. Trop. Med. Hyg. 54, 127–133.

Rota, S., Beyazova, U., Karsligil, T., Cevheroglu, C., 1994. Humoral immune response against antigen 60 in BCG-vaccinated infants. Eur. J. Epidemiol. 10, 713–718.
Safronetz, D., Lindsay, R., Dibernardo, A., Hjelle, B., Xiao, R., Artsob, H., Drebot, M.A., 2005. A preliminary study of the patterns of Sin Nombre viral infection and shedding in naturally infected deer mice (*Peromyscus maniculatus*). Vector Borne Zoonotic Dis. 5, 127–132.
Safronetz, D., Lindsay, R., Hjelle, B., Medina, R.A., Mirowsky-Garcia, K., Drebot, M.A., 2006. Use of IgG avidity to indirectly monitor epizootic transmission of sin nombre virus in deer mice (*Peromyscus maniculatus*). Am. J. Trop. Med. Hyg. 75, 1135–1139.
Takahashi, T., Johnson, T.D., Nishinaka, Y., Morton, D.L., Irie, R.F., 1999. IgM anti-ganglioside antibodies induced by melanoma cell vaccine correlate with survival of melanoma patients. J. Invest. Dermatol. 112, 205–209.
Vapalahti, O., Mustonen, J., Lundkvist, A., Henttonen, H., Plyusnin, A., Vaheri, A., 2003. Hantavirus infections in Europe. Lancet Infect. Dis. 3, 653–661.