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LONG-TERM DYNAMICS OF SIN NOMBRE VIRAL RNA AND ANTIBODY IN DEER MICE IN MONTANA

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ABSTRACT: Infections with hantaviruses in the natural host rodent may result in persistent, asymptomatic infections involving shedding of virus into the environment. Laboratory studies have partially characterized the acute and persistent infection by Sin Nombre virus (SNV) in its natural host, the deer mouse (*Peromyscus maniculatus*). However, these studies have posed questions that may best be addressed using longitudinal studies involving sequential sampling of individual wild-caught, naturally infected mice. Using enzyme immunoassay and polymerase chain reaction (PCR) analysis of monthly blood samples, we followed the infection status of deer mice in a mark-recapture study in Montana for 2 yr. Only six of 907 samples without IgG antibody to SNV contained detectable SNV RNA, suggesting that there is a very brief period of viremia before the host develops detectable antibody. The simultaneous presence of both antibody and viral RNA in blood was detected in consecutive monthly samples for as long as 3 mo. However, chronic infection was typified by alternating characteristics of PCR positivity and PCR negativity. Two possible interpretations of these results are that 1) viral RNA may be consistently present in the blood of chronically infected deer mouse, but that viral RNA is near the limits of PCR detectability or 2) SNV RNA sporadically appears in blood as a consequence of unknown physiological events. The occurrence of seasonal patterns in the proportion of samples that contains antibody and that also contained SNV RNA demonstrated a temporal association between recent infection (antibody acquisition) and presence of viral RNA in blood.

Key words: Deer mouse, enzyme-linked immunoassay, epizootiology, hantavirus, *Peromyscus maniculatus,* polymerase chain reaction, seasonal, Sin Nombre virus, transmission.

INTRODUCTION

Hantaviruses (family *Bunyaviridae,* genus *Hantavirus*) are rodent-borne pathogens that may produce chronic (persistent) infections in their reservoir hosts. Approximately 40 hantaviruses are recognized throughout the world, with each usually restricted to a particular rodent host species in the family Muridae. In humans, distinct hantaviruses are responsible for hemorrhagic fever with renal syndrome in Asia and Europe, or hantavirus pulmonary syndrome (HPS) in the Americas (Plyusnin and Morzunov, 2001; Plyusnin, 2002). Most cases of HPS in the United States are caused by Sin Nombre virus (SNV), the principal reservoir of which is the deer mouse (*Peromyscus maniculatus*; Nichol et al., 1993; Childs et al., 1994).

Following the first recognized outbreak of HPS in the United States in 1993, studies documented the occurrence of deer mice with antibody throughout the range of that species in the United States, Canada, and Mexico (Kaufman et al., 1994; Weigler et al., 1996; Mills et al., 1997; Langlois et al., 2001; Suzan et al., 2001). To study the epizootiology of SNV within deer mouse populations and to thereby provide data necessary to predict and reduce human risk of SNV infection, long-term mark-recapture studies were initiated in the southwestern United States and Montana (Mills et al., 1999). These studies were designed to monitor changes in rodent population densities, to determine prevalence and incidence of SNV infection in host populations, and to quantify environmental and ecological factors associated with changes in these parameters (Mills et al., 1999).

Laboratory studies have demonstrated a consistent pattern of events following hantavirus infections of their natural rodent hosts (Lee et al., 1981; Yanagihara et al., 1985; Gavrilovskaya et al., 1990; Hutchinson et al., 1998; Botten et al., 2000, 2003). First, there is a brief viremia, followed by the development of circulating antibody, and then consequent clearance of virus from the blood. At the same time, these viruses establish chronic infections in certain cells and virus is shed in urine, feces, and saliva.

Because of the simplicity of antibody assays, most investigators use these techniques to determine past or current infections of deer mice or other hosts of hantaviruses. It has been generally accepted that once a host is infected it will remain infected and infectious (capable of shedding infectious virus into the environment) for the remainder of its life. Although this may be correct, it is likely that the period of occurrence of the highest rate of shedding of SNV from the infected host is during the first several weeks after infection, when virus is shed at the highest titer (Lee et al., 1981; Gavrilovskaya et al., 1990; Hutchinson et al., 1998). This period of infectivity may be quenched by the appearance of neutralizing antibody and the consequent disappearance of infectious virus from the blood. Several authors recently have addressed virus and antibody dynamics in the deer mouse-SNV system. Netski et al. (1999) divided wild-caught deer mice into four hypothetical groups based on serology (for viral antibody) and polymerase chain reaction (PCR; for viral RNA) results in blood samples. These authors labeled these groups as naive (antibody negative and RNA negative), recent infection (antibody negative and RNA positive), acute infection (antibody positive and RNA positive), and chronic infection (antibody positive and RNA negative). The appropriateness of this classification, which assumes that deer mice in the chronic stage of infection do not have viral RNA in their blood, has not been tested. More recent studies have suggested that, for the SNV-deer mouse system, there may be periodic episodes of viral recrudescence during persistent infection. Botten et al. (2003) found that some laboratory-inoculated deer mice had detectable viral RNA in blood for as much as 217 days postinfection, while others did not. Because individual mice were not followed through time, it was unclear whether this finding represented periodic recrudescence or genetically determined differences in responses to infection among individual mice.

In this article, we use PCR and enzyme-linked immunosorbent assay (ELISA) data from a longitudinal mark-recapture study, following individual rodents through time to improve our understanding of SNV infection in its natural host and to test interpretations of previous cross-sectional laboratory studies. We also present data regarding 1) the proportion of deer mice in both peridomestic and sylvan populations with antibody to SNV and with SNV RNA in their blood, 2) seasonal patterns of SNV RNA prevalence, and 3) temporal relationships between antibody acquisition and presence of circulating SNV RNA.

MATERIALS AND METHODS

Study sites

Peridomestic populations were sampled at three study sites in western Montana and sylvan populations were sampled at six sites in western and central Montana. Detailed descriptions of the peridomestic sites and populations can be found in Kuenzi et al. (2001), while details of the sylvan sites and populations are published in Douglass et al. (2001).

Trapping rodents and collecting blood samples

Peridomestic deer mice were live trapped at three sites for three consecutive nights each month from November 1996 to August 1999. Sylvan deer mice were live trapped at five sites for three consecutive nights each month from May through October in 1996 and 1997. The sixth sylvan site was trapped monthly from January 1996 through December 1997. All live trapping was conducted according to standardized protocols (Douglass et al., 2001; Kuenzi et al., 2001). Briefly, captured deer mice in both

peridomestic and sylvan habitats were transported to a central site for processing, where pertinent data (species, gender, mass, age, and reproductive condition) were recorded and blood samples were collected. Blood samples were collected from the retro-orbital sinus of each animal using a heparinized capillary tube and then stored in plastic cryovials on dry ice until transferred to a −70°C mechanical freezer. Rodents were identified with sequentially numbered metal ear tags and returned to the appropriate field or peridomestic setting.

Enzyme-linked immunosorbent assays and reverse transcriptase polymerase chain reaction

Serologic testing was conducted at Montana State University (Bozeman, Montana, USA). All samples of whole blood were diluted 1:100 and tested for IgG antibody reactive with SNV recombinant nucleocapsid protein (supplied by the US Centers for Disease Control and Prevention, Atlanta, Georgia, USA) by ELISA, according to a standardized protocol (Feldmann et al., 1993). Samples were tested at four-fold dilutions from 1:100 to 1:6400.

All samples of whole blood from the sylvan populations of deer mice were tested for the presence of SNV RNA using PCR. Because of logistical constraints, only antibody-positive deer mice from peridomestic populations were tested by PCR. The extraction method for the PCR was modified from Chomczynski (1993). Fifty microliters of blood were diluted in 200 μl of phosphate-buffered saline containing 0.2% Tween 20 (Sigma Chemical Co., St. Louis, Missouri, USA). Twenty-five microliters of microcarrier gel BD and 750 μl of TriBD were added and the RNA extraction was completed as recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, Ohio, USA). Reverse transcription of the RNA was done using PCR with a nested set protocol using the Access PCR kit, as recommended by the manufacturer (Promega, Madison, Wisconsin, UDA). External primers were SNM2581P (5′-CTCCAGCCAGGTGATACCTT) and SNM3330M (5′-CCAGCACTTGATTGTACAGG); internal primers were SNM2755P (5′-GCTACGACACCAACATGTGA) and SNM3094M (5′-TGTCACTGTGGCTCCATAAC). The PCR amplicons were identified by agarose gel electrophoresis (1% agarose [Sigma] in Tris acetate-EDTA buffer) (Voytas, 1999).

We used chi-square tests (Zar, 1984) to determine whether the proportions of antibody-positive samples and of individuals that were PCR positive were similar among subgroups of deer mice. All statistical tests were run using JMP (SAS Institute Inc., 1996) and an alpha of 0.05.

RESULTS

Because only a subset of the blood samples without antibody were tested by PCR (only those individuals from sylvan habitats), we present results separately for samples with and samples without antibody. For brevity and simplicity, we refer to the mice or samples in which we detected IgG antibody reactive with SNV as Ab+ and those without detectable antibody as Ab−. We refer to samples or mice that were found to have SNV viral RNA in blood by PCR as RNA+ and those without PCR-detectable RNA as RNA−.

Deer mice with antibody

PCR assays were completed with 883 blood samples (from 532 deer mice) from those with antibody to SNV. Of these, 572 samples (352 deer mice) were from those captured in a peridomestic habitat and 311 samples (180 deer mice) were from those captured in a sylvan habitat (Table 1). SNV RNA was detected in 19% of the blood samples with antibody (26% of the mice). The proportions of samples with antibody and individuals that were PCR positive were similar when peridomestic and sylvan habitats were compared (for samples: $\chi^2=0.44$, $P=0.50$; for individuals: $\chi^2=1.5$, $P=0.22$). The proportions of samples that were Ab+ and individuals that were RNA+ were similar when males and females were compared, in both peridomestic habitats (for samples: χ^2 with Yates' correction=0.57, $P=0.45$; for individuals: $\chi^2=0.28$, $P=0.60$) and sylvan habitats (for samples: $\chi^2=0.87$, $P=0.85$; for individuals: $\chi^2=0.19$, $P=0.67$).

Deer mice without antibody

We completed PCR assays of 907 blood samples (546 deer mice) from those without antibody in sylvan populations (Table 1). SNV RNA was found in less than 1% of these samples (i.e., approximately 1% of individual mice—Table 1). These RNA− samples were from six Ab− individuals that were captured only once. All had

TABLE 1. Results of reverse transcription polymerase chain reaction (PCR) assays on 883 blood samples from antibody-positive deer mice captured in sylvan and peridomestic habitats and 907 antibody-negative blood samples from deer mice captured in sylvan habitats in western Montana.

		Antibody-positive blood samples		Antibody-negative blood samples	
Type of population	Sex	% blood samples PCR positive (no. positive/total)	% individuals PCR positive (no. positive/total)	% blood samples PCR positive (no. positive/total)	% individuals PCR positive (no. positive/total)
Peridomestic	Both	20 (115/572)	28 (97/352)	None tested	None tested
	Male	21 (73/343)	29 (63/219)	None tested	None tested
	Female	18 (42/229)	26 (34/133)	None tested	None tested
Sylvan	Both	18 (56/311)	22 (40/180)	0.66 (6/907)	1 (6/546)
	Male	16 (31/192)	21 (23/111)	0.43 (2/465)	0.69 (2/289)
	Female	21 (25/119)	25 (17/69)	0.90 (4/442)	2 (4/257)
Total	Both	19 (171/883)	26 (137/532)		
	Male	19 (104/535)	26 (86/330)		
	Female	19 (67/348)	25 (51/202)		

been captured in the spring: one in March, four in April, and one in May.

Analysis of the samples with antibody allowed us to track certain individual mice through time. A total of 194 deer mice were captured two or more times, were tested by both IgG ELISA and PCR, and eventually were shown to have acquired antibody to SNV; that is, to have become infected. None of 23 mice that had at first been captured prior to infection (Ab−/RNA−) were subsequently captured as Ab−/RNA+. This is not surprising, given that only 1% of the deer mice without antibody were in this presumably very brief recent-infection stage (Table 1). However, seven of the 23 deer mice that were initially captured prior to infection were later captured as Ab+/RNA+; the remaining 16 later were captured as Ab+/RNA−. Thirty-one mice were Ab+/RNA+ when first captured and Ab+/RNA− one or more times subsequently. A large number (58) of mice were found Ab+/RNA− after being found Ab+/RNA−, or alternated between Ab+/RNA+ and Ab+/RNA− on several subsequent captures after they were found Ab+/RNA−.

The most consecutive months that an individual mouse was RNA+ was three; the most consecutive months that an Ab+ mouse was RNA− was five. When it occurred, the alternating condition lasted as long as 13 mo; six mice had detectable SNV RNA and then did not (alternated between RNA+ and RNA−) three times over periods of 5–13 mo.

We differentiated antibody titers as high (≥6,400) or low (≤1,600) and found a statistically significant positive association between antibody titer and PCR positivity (χ^2=8.32, P=.004). Of 73 samples with antibody titers ≥6,400, 26% were PCR positive; of the 93 samples with titers ≤1,600, only 17% were PCR positive.

Seasonal patterns

Monthly SNV RNA prevalence in blood (percentage blood samples with antibody that also contained SNV RNA) ranged from 0% to 31% in sylvan populations and 0% to 49% in peridomestic populations (Fig. 1). The SNV RNA was detected in at least some blood samples collected in all months of the year, but the highest prevalences were observed during the early part of the breeding season (January–April) in peridomestic populations. We were able to collect few samples from mice in sylvan habitats during the early part of the breeding season, but the highest prevalences of SNV RNA in those we did capture were found during the mid-part of the breeding season (April–July). In both sylvan and peridomestic populations, SNV RNA prevalence decreased as

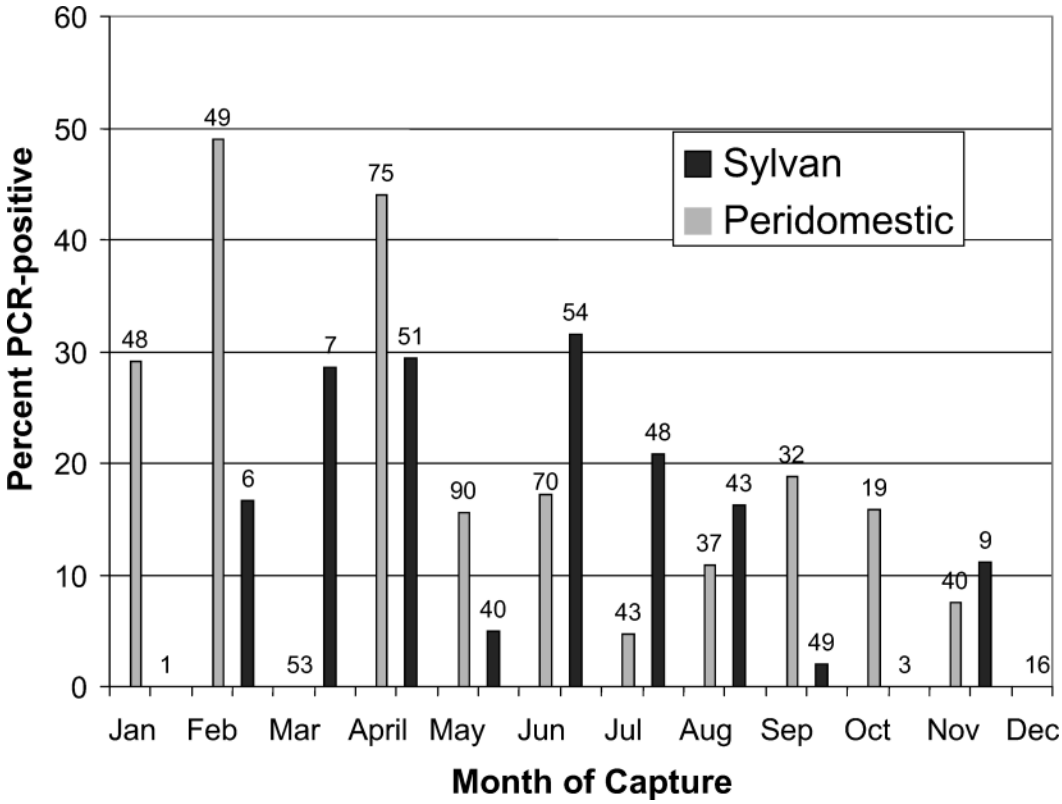

FIGURE 1. Percentage of deer mice with antibody to Sin Nombre virus that also had circulating SNV RNA, by month (1996 and 1997 combined). Total numbers of samples with antibody are shown above bars.

the breeding season came to an end in October and November (Fig. 1).

Peridomestic deer mouse population sizes were sufficient to determine seasonal acquisition of SNV antibody, which we take as an indicator of infection. Of 2,185 individuals captured in peridomestic settings (Kuenzi et al., 2001), we documented 131 that serocoverted. Most of these (88/131, 67%) were males in a population comprising essentially 50% males. Ninety-nine mice seroconverted between consecutive trapping sessions. The greatest proportion of these seroconversions occurred in spring, between April and May, with another peak in the late summer to early fall, between August and November (Fig. 2).

DISCUSSION

This is the first study to present data concerning the presence of SNV RNA in samples from natural populations of deer mice collected over time. We found that the presence of SNV RNA in the blood of infected deer mice was variable and mice were not consistently PCR negative over time. Thus, the notion (Netski et al., 1999) that four stages of infection can be distinguished using serology and PCR results is not supported by our data.

The apparent chronic stage of infection was characterized by alternating PCR-positive and PCR-negative results, suggesting that viral RNA was present intermittently in blood or was consistently present at low levels, near the threshold of PCR detectability. Other studies suggest that hantaviral replication in tissues is variable during chronic infection; intervals of virus shedding in urine, feces, and saliva and intermittent presence of viral RNA or even infectious virus in blood can be expected

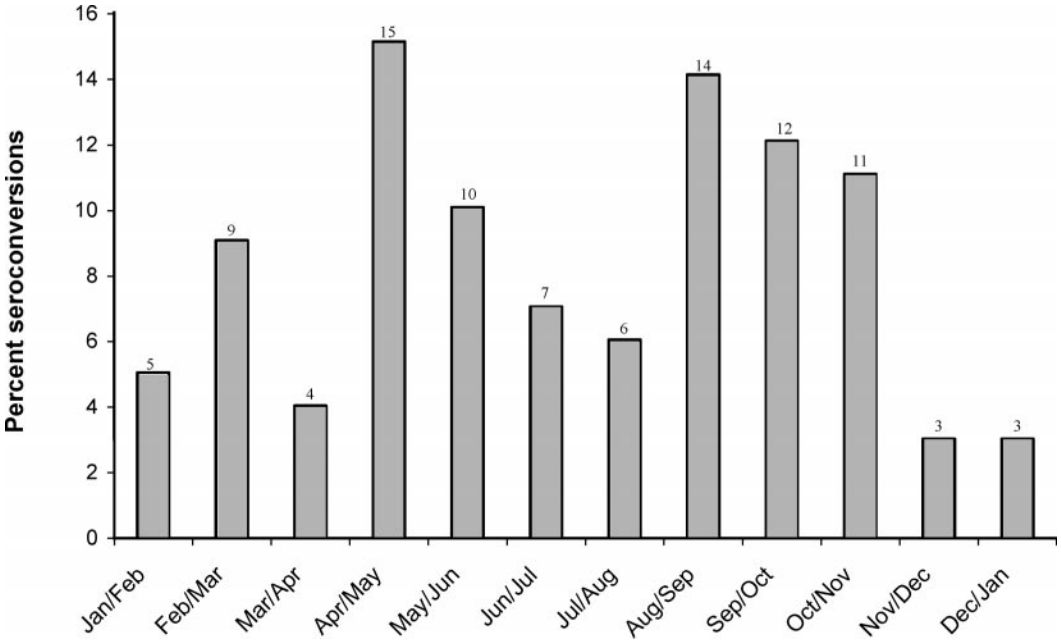

FIGURE 2. Seasonal distribution of acquisition of antibody to Sin Nombre virus in 99 deer mice seroconverting between consecutive monthly captures in Montana, 1996–97. Numbers above bars represent numbers of mice seroconverting during each period.

(Hutchinson et al., 1998; Botten et al., 2002). A second possible explanation for the alternating nature of viral RNA is viral recrudescence. Under various forms of stress, including immunosuppression, immunologic dyscrasias, malignancies, heat, cold, shock, pregnancy, etc., herpesviruses, *Hepatitis B virus, Hepatitis C virus, B19 virus,* and other viruses may be reactivated (Halford et al., 1996; Mehta et al., 2004). The SNV reactivation in deer mice due to seasonal influences or immunosuppression has been suggested (Botten et al., 2002) and replication of Seoul virus appears to be much faster in the immunosuppressed nude rat than in normal rats (Dohme et al., 1994). The exact initiating causes of recrudescence in these cases are unclear.

Based on laboratory infections, Botten et al. (2003) described two broad categories of SNV infection in deer mice: a) a widespread infection characterized by the presence of viral RNA in blood, abundant antigen expression, and abundant replicative form RNA in tissues and b) a more restricted infection pattern with no RNA in blood, only rare antigen expression, and little or no detectable replicative form RNA in tissues. Because experimental animals were sacrificed, these authors could not determine whether these two categories reflected temporal changes in individual mice or whether they represented differences (perhaps genetically fixed) in response to infection by different mice. Our only indicator of virus replication was the presence of SNV RNA in blood. However, it is likely that the condition of Ab+/RNA+ corresponds to the widespread-infection condition described by Botten et al. (2003). Thus, our data support the existence of these two broad patterns and suggest that the two patterns are stages that occur periodically in individual mice during the course of persistent infection.

The positive association between antibody titer and PCR positivity was surprising. This contrasts with what was predicted in the four-stage pattern suggested by Netski et al. (1999) and may indicate that

a large percentage of the samples with antibody and SNV RNA were from deer mice that were in the later chronic stage of infection and not the early acute stage, when IgG antibody titers would be expected to be still relatively low.

Our study does not address the temporal pattern of infectiousness or shedding of virus by infected deer mice. In laboratory studies, recently and acutely infected hispid cotton rats (*Sigmodon hispidus*) clearly had more *Black Creek Canal virus* in their tissues, blood, urine, and saliva (Hutchinson et al., 1998) than they did later in their infections. These studies also demonstrated that rodents infected with hantaviruses may lack detectable viral RNA in their blood and still have high titer viruria (Hutchinson et al., 1998). Given this array of results, it is reasonable to assume that an accurate field assessment of the relative infectiousness of vertebrate carriers of hantaviruses can only be made using an assay for infectious virus.

Of 907 samples without antibody that were tested by PCR, only 6 (about 0.7%) had SNV RNA; these likely represented recent infections. These results are concordant with findings by Botten et al. (2000), who also found this stage of infection to be transient or rarely detected. Netski et al. (1999) found that SNV antigen concentrations were highest in the kidneys of wild-caught deer mice that had no antibody to SNV but had SNV RNA in the blood (those that they presumed to have been infected very recently). That we rarely found deer mice in this early stage of infection may partially explain the infrequence of human infections with SNV, even in the presence of many chronically infected deer mice. All the mice we identified as having SNV RNA but no antibody had been captured in the spring (March–May), suggesting that this may be the period of highest risk of SNV transmission to humans in Montana. Indeed, eight of the 22 confirmed HPS cases in Montana had onset dates in spring (March–May); seven had onset in summer, three in autumn, and four in winter (J. Murphy 2004, Montana Public Health and Human Services, pers. comm.). Even if all the RNA+ deer mice were shedding infectious virus, their overall numbers were small relative to the entire deer mouse population. In Montana, where an average of 13% of sylvan deer mice had antibody to SNV (1994–98) (Douglass et al., 2001), one could expect about 3% ($0.22 \times 13\%$) of individuals in the population to have SNV RNA in their blood at any particular time. In peridomestic populations in Montana, in which an average antibody prevalence of 25% has been observed (Kuenzi et al., 2001), one might expect about 7% ($0.28 \times 25\%$) of individuals in the population to have SNV RNA in their blood at any particular time.

Despite our findings that PCR positivity may not necessarily indicate recent infection, we did find a clear seasonal pattern to the occurrence of SNV RNA in blood. The greatest proportion of samples with antibody also contained SNV RNA early in the breeding season, with a second and lower peak detected late in the breeding season.

In Montana, breeding in deer mice, as indicated by an increase in the proportion of males with testes in the scrotal position, begins during the apparently most physiologically stressful time of year, January and February, in both sylvan and peridomestic settings (Kuenzi et al., 2001). Weather conditions during these months are characterized by cold temperatures (as low as −38 C) and persistent snow cover. These weather conditions occur at the same time that deer mice are depending on the dwindling supply of food produced the previous summer.

The temporal pattern of the proportion of peridomestically captured deer mice that had circulating SNV RNA (Fig. 1) had characteristics in common with the pattern of seroconversions (Fig. 2). In both sylvan and peridomestic samples, the prevalence of deer mice that had SNV RNA was high early in the year, when breeding began

(Douglass et al., 2001; Kuenzi et al., 2001), and low at year end, perhaps showing a secondary peak in fall, at least in the peridomestic population. This pattern may indicate an infection cycle that begins in overwintered adults, with a decline in infection in the middle of the breeding season, and then an increase in infection when young of the year begin to breed. However, in sylvan populations (Douglass et al., 2001), incidence was not observed to occur bimodally through the breeding season. It may be that sylvan deer mice do not begin breeding as early as do peridomestic deer mice and that the April–May seroconversion peak associated with spring breeding in peridomestic populations did not occur (Douglass et al., 2001). Thus, for humans in contact with peridomestic rodent populations, there may be a second period of increased disease risk in the summer to fall period. Because most human cases derive from peridomestic exposure, this may be an important observation.

The resolution of questions concerning the exact intervals during which SNV-infected deer mice are capable of transmitting infectious virus will require the development and application of a reliable assay for infectious virus. Meanwhile, the very low proportion of presumably infectious deer mice, compared with the high proportion of deer mice with antibody at high population densities, may, at least in part, explain the low incidence of human infections with SNV and of HPS.

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