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A Preliminary Study of the Patterns of Sin Nombre Viral Infection and Shedding in Naturally Infected Deer Mice (*Peromyscus maniculatus*)

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Abstract

Deer mice (*Peromyscus maniculatus*) were trapped in southern Manitoba, Canada and tested for evidence of Sin Nombre virus infection. Viral genome was amplified from tissues as well as saliva/oropharyngeal fluid, and urine samples were collected from seropositive animals. Detection of viral RNA in tissue samples and excreta/secreta from mice suggest that differences may exist between naturally infected rodents with respect to viral shedding.

Keywords

Hantavirus; Deer mice; Infection

INTRODUCTION

In North America, several hantaviruses are associated with hantavirus pulmonary syndrome (HPS); however, only Sin Nombre virus (SNV) has been implicated as an etiological agent in Canada. As of 7 December 2004, 53 laboratory-confirmed cases of HPS have been reported in Canada (Drebot et al. 2000, H. Art-sob, unpublished data). Hantaviruses have been detected in rodent populations from across Canada (Drebot et al. 2000). Within Manitoba, at least two hantaviruses, SNV and Prospect Hill virus, are known to exist in deer mice (*Peromyscus maniculatus*) and meadow voles (*Microtus pennsylvanicus*), respectively (Drebot et al. 2001, D. Safronetz, unpublished data). Although transmission of SNV among rodents and to humans is believed, to date, to occur through contact with virus-contaminated secreta or excreta (i.e., saliva, feces, or urine), few studies have been able to demonstrate SNV in these specimens (Botten et al. 2002).

MATERIALS AND METHODS

This study was conducted under strict safety guidelines to prevent accidental exposures to hantaviruses and other zoonotic agents potentially carried by captured animals. In the field, traps containing animals were placed in a sealed plastic container for transport from the site of

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capture to an on-site field station for processing. While handling animals and collecting samples, personnel wore protective clothing (i.e., disposal gowns or coveralls and nitrile gloves) and N100 respirators or HEPA filtered positive pressure respirators. All additional work with infected rodent tissues was carried out in a biocontainment level 3 facility at the National Microbiology Laboratory.

From August 11th to 15th, 2003, deer mice (*Peromyscus maniculatus*) were captured using Sherman® live traps from an area in Manitoba, Canada where seropositive mice had previously been collected (R. Lindsay, unpublished data). Captured animals were anaesthetized by inhalation of isoflurane and bled via the infra-orbital sinus using Natelson™ blood collection tubes or by cardiac puncture. Whole blood samples were placed into Microtainer™ serum separator tubes for serology and 1.5-mL Sarstedt™ cryovials for preparation of viral RNA. Oropharyngeal fluid was obtained by swabbing the oral cavity of each mouse with Dacron-tipped applicators (VWR®, Mississauga, On-tario) and placing the applicator into lysis buffer RLT (Qiagen®, Mississauga, Ontario) for subsequent RNA extraction. The weight, sex, and estimated relative age (adults or sub-adults) of each animal were recorded. Following sample collection, animals were euthanized with an overdose of isoflurane and the carcasses were stored in individual whirlpak bags. In the field, carcasses and whole blood samples were frozen on dry ice, whereas oral swabs and whole blood samples for serum separation were stored at approximately 4°C. In the laboratory, RNA was extracted from oral swabs within 36 hr. These RNA extracts, the carcasses and whole blood samples were stored at −85°C for later testing.

Serum samples from mice were screened for the presence of immunoglobulin G (IgG) reactive to hantavirus using an indirect enzyme linked immunosorbant assay as previously described (Lindsay et al. 2001). Samples were considered positive if they were reactive at a dilution of 1:400 or greater, and equivocal if positive only at a 1:100 screening dilution.

Total RNA was isolated from 122 oral swabs and 28 whole blood samples (from those mice with serological evidence of SNV infection) using Qiagen® RNeasy kits. Fifteen mice with serological evidence of SNV infection were dissected and approximately 15 mg of salivary gland, skeletal (masseter) muscle, bladder, spleen, kidney, liver, heart, lung, and fat samples were collected. Insofar as possible, urine (approximately 20–60 μL) was collected from the bladder with a sterile syringe and needle and then mixed with 200 μL of lysis buffer. In addition, spleen, kidney, heart, and lung samples were collected from 15 seronegative mice. Tissue samples were placed in 1 mL of BA-1 diluent (Nasci et al. 2002) and mechanically homogenized using a Qiagen® mixer mill. RNA was extracted from 200 μL of the tissue homogenates using Qiagen® RNA extraction kits.

The RNA samples were tested for hantavirus-specific RNA using a real-time RT-PCR assay on an ABI® 7700 sequence detector using previously published protocols (Botten et al. 2002). In addition, quantitative RT-PCR (qRT-PCR) was carried out on six whole blood, five oral swab extracts, two urine, two lung, four heart, and one kidney sample using previously established methods (Botten et al. 2003). Fifty of 116 RT-PCR positive samples (including all positive blood, oral swabs, and urine samples along with one or more representative tissue samples from each SNV infected mouse dissected) were confirmed with nested RT-PCR, using M-segment primers (Johnson et al. 1997) as described by Drebot et al. (2001). To verify the presence of amplified SNV genome, selected M-segment amplicons were sequenced on an ABI 3100 sequencer using BigDye™ Terminator version 3.1 cycle sequencing kits.

Samples of solid tissues (salivary gland, bladder, heart, and lungs) from 10 seropositive mice were tested for infectious virus by cultivation in monolayer cultures of Vero E6 cells. Briefly, 500 μL of the tissue homogenate was passed through a 0.45-μM filter and used to inoculate Vero E6 cells for 1 h. Cells were passaged three times for 7–10 days per passage after

inoculation. The presence of replicating SNV was detected by RT-PCR assays conducted on supernatant extracts and strand-specific RT-PCR assays on cell pellet extracts (Botten et al. 2003). The sampling procedures used to collect oral secretions and urine (i.e., placing samples directly in lysis buffer) did not permit us to determine whether infectious SNV was present in those specimens.

RESULTS

In total, 122 (81 male/41 female; 78 adult/44 sub-adult) deer mice were collected, and antibodies (IgG) to hantavirus were detected in 21 (17.2%) of these animals (titers ranging from 400 to greater than 6400). An additional seven (5.7%) mice had equivocal SNV antibody titers (reactive only at the 1:100 screening dilution). Seropositive mice were predominantly adult (23.1% versus 6.8%, $\chi^2 = 4.34$, df = 1, $p < 0.05$); however, significant sex related differences in seropositive deer mice were not observed (males = 18.5% versus 14.6% females, $\chi^2 = 0.23$, df = 1, $p > 0.05$). Samples collected from seronegative deer mice (94 oral swabs and spleen, kidney, heart, and lung samples from 15 animals) were uniformly negative for the presence of SNV RNA, with the exception of lung samples from one mouse (which may have represented a recent infection; Netski et al. 1999, Botten et al. 2000).

SNV RNA was detected in six (21.4%) of 28 oropharyngeal fluid samples, two (18.2%) of 11 urine samples, and nine (32.1%) of 28 blood samples from seroreactive mice. Viral genome was also amplified from nine (81.8%) of 11 fat samples; 12 (80%) of 15 salivary gland, masseter muscle, spleen, kidney, heart and lung samples; 10 (66.7%) of 15 liver samples; and six (40%) of 15 bladder samples (Table 1). SNV RNA was detected in mice with antibody titers ranging from equivocal (100) to greater than 6400. Only two seropositive mice (Table 1, DM 53 and 78) lacked viral genome in any sample, while another seropositive mouse (Table 1, DM 45) had detectable viral RNA in only the blood sample. Two serologically equivocal mice (DM 37 and DM 57) had SNV RNA in a variety of tissues. A subset of samples tested by nested M-segment RT-PCR demonstrated 100% correlation with the real-time PCR results. The nucleotide sequence of M-segment amplicons generated from the lung samples of five seropositive deer mice displayed significant identity with SNV genotypes previously reported in Manitoba (Drebot et al. 2001), and confirmed the presence of the virus within these mice.

One of five oropharyngeal fluid samples (DM 2) tested by qRT-PCR was quantifiable with 27,000 S-segment copies/swab. Overall concentrations of SNV S-segment in three of four heart samples (DM 2, DM 26 and DM 51) tested were determined to be 500,000, 37,000, and 324,000 copies/mg, respectively. The two lung samples (DM 2 and DM 26) tested had 504,000 and 57,600 copies/mg, respectively. SNV RNA was below the quantifiable limit of detection in the one kidney, two urine and six blood samples tested by qRT-PCR.

Positive and negative strand SNV RNA was detected in Vero E6 cell pellets from five (DM 2 salivary gland, DM 2 heart and DM 2, DM 51, DM 57 lung samples) of 40 samples tested by viral isolation. The supernatant from the same isolations were positive for SNV RNA after two to three passages.

DISCUSSION

The overall dynamics of SNV infection in deer mice collected in Manitoba is similar to those reported elsewhere with respect to sero-prevalence, age bias of infected animals, and the systemic nature of SNV infection (Douglass et al. 2001, Botten et al. 2003, Netski et al. 1999). No significant sexual bias was observed in seropositive deer mice.

To our knowledge, this is the first detection of SNV in urine from either naturally or experimentally infected deer mice and only the second study demonstrating its presence in oral

secretions (Botten et al. 2002). While it is widely assumed that SNV transmission occurs through infectious secreta or excreta, as has been suggested for other hantaviruses (Kariwa et al. 1998, Hutchinson et al. 2000), infectious SNV in saliva/oropharyngeal fluid, urine, or feces has not been well documented. Although the presence of viral RNA does not necessarily predict the presence of infectious virus, the isolation of infectious SNV from the salivary gland of one animal lends support to the hypothesis that SNV replication occurs in this tissue and that SNV virions are shed in the saliva. Alternatively, it is possible that the SNV RNA detected in oropharyngeal fluids, may have originated as respiratory secretions. Both the observation that every deer mouse with detectable SNV RNA in the oropharyngeal fluid also had SNV-positive lungs and the isolation of SNV from three lung samples support the potential involvement of respiratory secretions in SNV transmission. The presence of SNV RNA in the urine from a small number of seropositive mice suggests that virus transmission may occur via excreta. However, further studies are required since our results do not rule out the possibility that the SNV RNA was released from the cells of the luminal surface of the bladder during the freeze-thaw process.

It is still unclear why only a very small fraction of persons with presumed exposure to SNV-infected deer mice actually contract HPS. Perhaps seropositive deer mice differ markedly in their abilities to transmit SNV to humans. Thus, it is of interest to determine whether individual deer mice and their secreta or excreta differ substantially in the degree to which viral RNA can be detected and to further differentiate deer mice as infected (presence of genome in tissues/blood, but no virus/viral RNA detected in excreta or secreta) or potentially infectious (mice with detectable virus/viral RNA in excreta or secreta). The proportion of infected mice which are infectious will be in constant flux as the duration and magnitude of viral shedding in naturally infected deer mice is likely influenced by several factors including the age when infection occurred, route of exposure, infectious dose, host genetics, and perhaps most importantly length of time post-infection. Such factors are impossible to control in a natural setting, thus studies to clarify how these and other parameters impact the dynamics of SNV shedding must be carried out in the laboratory, as has been done with other hantaviruses (Hutchinson et al. 2000, Fulhorst et al. 2002).

Botten et al. (2003), recently described two discrete patterns of persistent SNV infection in experimentally infected deer mice (disseminated and restricted), based on the detection of viral RNA in blood, replicative (positive strand) RNA in the heart, lungs, or brown fat, and the number of antigen-positive tissues. Diverse patterns of SNV persistence in organs of naturally infected deer mice may also exist; however, such patterns could also be explained by the length of time post-infection. Although SNV infection in most naturally infected deer mice appears to be systemic, some animals (Table 1, DM-53 and 78) may be able to clear SNV before infection of multiple organs is established. The lack of detectable SNV RNA in these seropositive mice may alternatively be explained by the detection of maternal antibodies within these rodents, as opposed to antibodies generated post-infection.

The findings of this study provide the foundation for more intensive studies to determine whether detection of SNV in oropharyngeal fluids and/or urine can be used to better understand the transmission dynamics of this virus in naturally infected rodents.

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Table 1

Summary of Serology and RT-PCR Results from 15 Deer Mice

Rodent I.D. no.	*Sex (weight [g])*	*Antibody titer*	*Whole blood*	*OP sec.*	*Salivary gland*	*Masseter muscle*	*Urine*	*Bladder*	*Fat*	*Spleen*	*Kidney*	*Liver*	*Heart*	*Lung*
DM-2	F (22.5)	≥6400	+	+	+	+	n.a.[a]	+	+	+	+	+	+	+
DM-4	M (24.5)	≥6400	−	−	+	+	−	−	n.a.	+	+	+	+	+
DM-41	M (21.5)	≥6400	+	−	+	+	−	−	+	+	+	+	+	+
DM-51	M (28)	≥6400	+	+	+	+	−	+	+	+	+	+	+	+
DM-84	M (23)	≥6400	−	+	+	+	−	−	+	+	+	+	+	+
DM-87	M (23.5)	≥6400	−	−	+	+	+	+	+	+	+	−	+	+
DM-91	F (17)	≥6400	+	+	+	+	−	−	n.a.	+	+	+	+	+
DM-110	M (20)	≥6400	−	−	+	+	−	−	+	+	+	−	+	+
DM-26	M (22)	1600	+	−	+	+	−	−	+	+	+	+	+	+
DM-53	M (18.5)	1600	−	−	−	−	−	−	−	−	−	−	−	−
DM-54	M (21)	1600	+	+	+	+	+	+	+	+	+	+	+	+
DM-78	M (14)	400	−	−	−	−	n.a.	−	n.a.	−	−	−	−	−
DM-45	M (14)	400	+	−	−	−	−	−	−	−	−	−	−	−
DM-37	M (18)	100[b]	+	−	+	+	n.a.	+	+	+	+	+	+	+
DM-57	M (16)	100	+	+	+	+	n.a.	+	n.a.	+	+	+	+	+

[a] Not available.

[b] A titer of 100 is considered equivocal.

OP sec., oropharyngeal secretions.