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RAPID AND SIMPLE METHOD FOR SCREENING WILD RODENTS FOR ANTIBODIES TO SIN NOMBRE HANTAVIRUS

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ABSTRACT: Sin Nombre hantavirus (SNV) is the primary etiologic agent of hantavirus cardiopulmonary syndrome (HCPS) in the United States and Canada. Hantavirus cardiopulmonary syndrome is a zoonotic disease. The most common reservoir is the deer mouse (*Peromyscus maniculatus),* although numerous other species of wild rodent can carry the viruses that cause HCPS throughout the Americas. Infected rodents show no signs of clinical disease but they develop persistent infection. Sin Nombre virus can be contracted by exposure to feces, urine, or saliva of its rodent reservoirs. Detection of infection in rodents is most often based upon detection of specific antibodies; many laboratories use enzyme linked immunosorbent assays (ELISAs), which require a specialized electrical ELISA reader. Enzyme linked immunosorbent assay readers are not readily amenable to field usage. We describe a portable test, the strip immunoblot assay (SIA), which we have utilized in field diagnosis. The test can be conducted in approximately 6 hr during the day or can be conducted overnight. The test can be used to detect rodents positive for SNV antibody while they are in traps. We show that results with the SIA have excellent concordance with western blot and reverse transcriptase polymerase chain reaction tests.

Key words: Antibodies, deer mice, diagnosis, hantavirus, Sin Nombre virus, zoonotic diseases.

INTRODUCTION

In North America, the prevalence of Sin Nombre virus (SNV) infection in rodent populations varies widely by geography, rodent species, and season. Rodent hosts develop chronic infections with little, if any, pathologic effects and no apparent effects on fertility or longevity (Hjelle and Yates, 2001). The risk of human infection is thought to be associated with high viral seroprevalence and/or high rodent population densities, although this seemingly obvious association has been easier to demonstrate in Europe than in North America (Childs et al., 1994; Niklasson et al., 1995). Hantaviruses are transmitted to humans through inhalation of aerosols or ingestion of virus contaminated rodent excreta, which seems to occur almost exclusively in closed spaces with poor ventilation (Schmaljohn and Hjelle, 1997; Hjelle and Glass, 2000). Human infection, which is fatal in about 40% of cases, progresses very quickly and early diagnosis facilitates effective treatment. A simple and reliable test that could be performed either in the laboratory or in the field would help laboratory workers, field crews, and others who must work with or otherwise encounter wild rodents. It would be particularly valuable for workers to be able to quickly determine whether a significant fraction of a particular wild rodent population is infected with SNV.

Rodents infected with SNV mount a strong humoral immune response to nucleocapsid antigen by about 14 days after exposure (Botten et al., 2000). In the past, we have used western blot analysis extensively to detect antibodies to SNV nucleocapsid antigen in rodent blood, and have found it to be highly concordant with reverse transcriptase polymerase chain reaction (RT-PCR) tests for viral RNA in tissues (Hjelle et al., 1994, 1996). However, the production of western blots consumes a considerable quantity of purified recom-

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binant antigen and requires several days to produce large numbers of strips. For that reason, we developed a strip immunoblot assay (SIA), which differs from the western blot in that the purified recombinant N antigen is directly vacuumed onto the nitrocellulose membrane rather than using an electrophoretic transfer from a polyacrylamide gel. The SIA strips described herein require 25-fold less antigen per test than that needed for western blots. Furthermore, given the availability of purified antigen, several hundred strips can be produced and quality control tested in 1 day.

MATERIALS AND METHODS

Protein purification

Sin Nombre virus nucleocapsid antigen (SNV-N) was expressed and purified using the pET His·Bind metal chelation column affinity system from Novagen (Novagen, Inc., Madison, Wisconsin, USA). We cloned the full-length SNV-N antigen (strain 3H226) into the pET23b vector as we described previously (Rawlings et al., 1996). For expression, we transformed the pSNV-N/pET-1 construct into Novagen BL21(DE3) cells by heat shock, as recommended by Novagen. Expression was conducted in liquid Luria-Bertani (LB) medium containing 50 μg/ml ampicillin at 37 C. When the turbidity of the culture reached 0.25 at 600 nm, expression of the fusion protein was induced with addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 400 μM. After an additional 4 hr at 37 C, the cells were centrifuged and the proteins were solubilized from bacterial pellet in 6M urea. We subjected the SNV-N antigen to affinity column purification, over a metal chelation column as described (Rawlings et al., 1996; Bharadwaj et al., 1997). Proteins were checked for purity by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitated by the method of Bradford (1976; Bio-Rad Laboratories, Hercules, California, USA).

RT-PCR assays

Nested RT-PCR was performed using primers that recognize the middle (M) genomic segment of SN virus (Hjelle et al., 1994). The RNA was extracted from approximately 30 mg of *P. maniculatus* lung. The RNA extracted from 2 mg of lung was loaded in each PCR reaction. The RT and "outer" PCR reactions were conducted in a single tube, using 20 pmol of each outer PCR primer. The nesting reactions were performed with internal primers and 3 μl of outer product in a total volume of 100 μl, as described (Hjelle et al., 1994). Outer primer sequences were as follows: (Har M1+) TAG TAG TAG ACT CCG CA(AC) GAA GA and (Har M 403−) GGA GGA ATA TTA CAT GTG CCT TT, whereas inner primers were (Har M 49+) AGA ATG GTA GGG TGG GTT TGC AT and (Har M 369−) TCT TTT GTT TTA GCC TCA AAT GT. Thermal cycling was performed in a Perkin Elmer model 480 cycler (Perkin Elmer, Boston, Massachusetts, USA) using a 100 μl reaction volume. Thermal cycling conditions were as follows: For the outer reaction, an initial RT step of 39 C for 20 min and 42 C for 40 min was followed by thermal cycling at 94 C-42 C-72 C (1, 1, and 1.5 min each) for eight cycles, then 94 C-47 C-72 C (1, 1, and 1.5 min) for 28 cycles. For the nesting reactions temperatures were 94 C-42 C-72 C (1, 1, and 1.5 min) for eight cycles, then 94 C-47 C-72 C for 1, 1, and 1.5 min for 28 cycles.

Western blot assays

Western blot strips were produced from a standard 12.5% SDS-PAGE tris-glycine stacking gel (Mini-Protean II, Bio-Rad Laboratories). Approximately 15 μg of purified recombinant SNV-N fusion protein was loaded evenly across an 8 cm×1 mm preparative comb. The marker lane was loaded with Bio-Rad low molecular weight pre-stained marker. The gel was run at 200 V for 1 hr. The proteins then were electrophoretically transferred to nitrocellulose membrane (BA83, Schleicher & Schuell, Inc., Keene, New Hampshire, USA) at 4 C at 100 V for 1 hr using 1x tris-glycine buffer containing 20% methanol. The pre-stained molecular weight marker served as a visual indicator of successful transfer. After transfer, a line was penciled across the top of the nitrocellulose for future orientation. The marker lane and excess nitrocellulose were trimmed from the outer aspects of the preparative protein lane. The remaining western blot was cut lengthwise into 1.6 mm strips using a hand held paper shredder. Approximately 40 strips are obtained from each membrane.

Western blot testing was conducted using 5 μl of whole rodent blood or serum in a total volume of 1 ml per test strip. All incubations were performed in mini-incubation trays (Bio-Rad Laboratories) on a lab rocker. To decrease binding of serum antibodies to *E. coli* antigens, we preincubated blood samples for 30 minutes in blotto buffer (5% non-fat powdered milk, 0.9% sodium chloride, 0.1% antifoam A, 0.1% sodium azide; Jenison et al., 1994; Bharadwaj

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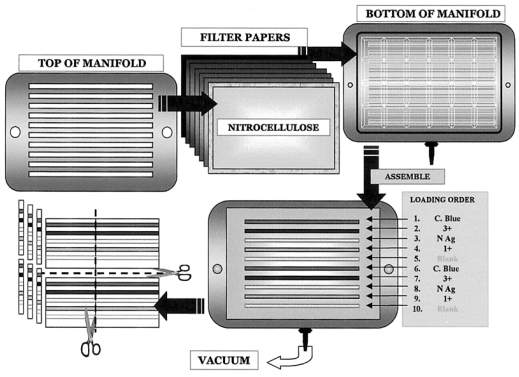


FIGURE 1. Protocol for the manufacture of strip immunoblot assays (SIA). We sandwiched a pre-sized nitrocellulose membrane between the lower (vacuum) and upper panels of an Immunetics SB-10 mini slotter, and then applied Coomassie brilliant blue (C. Blue), diluted deer mouse serum (3+ and 1+), and recombinant nucleocapsid antigen (N Ag) in separate wells in a total volume of 1 ml each. After applying the vacuum, we removed the membrane and cut it lengthwise with a paper shredder to produce the SIA strips.

et al., 2000) containing 0.1% Triton X-100, 0.1% deoxycholic acid, and 5% *E. coli* lysate blocking reagent. Strips were added to the wells and incubated at room temperature for 4 hr to overnight with rocking at a frequency of ~10/min. We then washed the strips three times in wash buffer (10 mM sodium phosphate pH 7.4, 0.1 M NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid) for 1–5 min per wash, with rocking. We then added alkaline phosphatase-conjugated secondary anti-*Peromyscus leucopus* IgG at a 1:1,000 dilution in 1 ml of blotto buffer per well for 1 hr at room temperature followed by washing as above. We visualized the bound alkaline phosphatase with a freshly-prepared color developer solution (100 mM Tris-HCL pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg/ml nitroblue tetrazolium [NBT], 0.165 mg/ml 5-bromo-4-chloro-3-indoyl phosphate [BCIP]). We stopped the development with two or three washes in water after 15 min.

Strip immunoblot assays

We prepared SIA strips using a model SB-10 mini slot blot apparatus (Immunetics, Inc., Cambridge, Massachusetts). Each SIA strip had from the top: an orientation band of Coomassie blue dye, a 3+ intensity control band, an SNV-N antigen band, and a 1+ (faint) intensity control band (Fig. 1). All components were vacuum blotted onto nitrocellulose membrane (BA83; Schleicher & Schuell, Inc.).

When we assembled the mini slot blot apparatus we created a cushion layer consisting of six pieces of 3mm chromatography paper cut to a rectangle of 7.5×10 cm and wetted in 1x tris-glycine buffer (200 mM glycine, 25 mM tris base, 0.1% sodium dodecyl sulfate) containing 20% methanol. This layer is necessary to create a seal tight enough to prevent leaking from the wells during vacuum blotting. We cut the nitrocellulose membrane to the same size and wet it in the same buffer, then layered it on top of the chromatography paper. The apparatus was clamped tight using the hand screws. We applied Coomassie brilliant blue stain, normal *P. maniculatus* serum for the 3+/1+ control bands, and approximately 1–2 μg of purified SNV-N antigen evenly across the length of the wells in the order depicted in Figure 1. The vacuum was applied for 2 min at 0.5 m Hg. We trimmed the excess nitrocellulose from the outer aspects of the vacuum blotting area and cut the membrane perpendicularly with a hand held paper shredder, creating strips 1.6 mm wide. Approximately 45 strips were obtained from each membrane. The strips were stored at 4 C in blotto buffer for up to 1 yr. The milk buffer was replaced every 2–3 mo with fresh buffer.

The orientation band consisted of Coomassie brilliant blue diluted 1:200 from a 5 mg/ml stock into phosphate buffered saline (Sigma – Aldrich Chemicals, St. Louis, Missouri) containing 5% acetic acid and 22.5% methanol. One milliliter of this solution was loaded into the vacuum blotter well. To produce the intensity control bands we prepared dilutions of *P. maniculatus* serum of sufficient concentration to produce 3+ and 1+ band intensity after exposure to 1:1,000 anti- *P. leucopus* IgG conjugate. The SNV-N band consisted of purified nucleocapsid antigen protein. The dilutions of serum and SNV-N antigen were suspended in 1 ml of phosphate buffered saline to facilitate even loading across the well.

To determine the amounts of *P. maniculatus* serum and SNV-N antigen to be loaded per well, we prepared "titration" strips containing varying amounts of serum or antigen. Each lot of pooled normal serum or purified recombinant protein will vary in reactivity and must be titered to determine optimal loading. We used a range of 0.5–15 μl/well of *P. maniculatus* serum or 0.1–5 μg/well of purified SN virus-N antigen to determine the optimal loading. The SNV-N positive and negative control sera were used to optimize the loading of SNV-N antigen. We loaded enough antigen to detect weak positive deer mouse sera without detecting any re-

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activity from negative control sera. We loaded dilutions of deer mouse serum that produced approximately 50% and 10% gray intensities in the 3+ and 1+ control lanes, respectively.

For standard SIA testing we used 5 μl of whole blood or serum for a 1:200 dilution of the primary antibody. The samples were preincubated in mini-incubation trays and mixed gently on a benchtop rocker for 30 min in blotto buffer. The SIA strips were then added and incubated at room temperature for 4 hr to overnight while rocking at 5–10/min. The strips were washed three times in wash buffer (10 mM sodium phosphate pH 7.4, 0.1 M NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid). We added alkaline phosphatase-conjugated secondary anti-*P. leucopus* IgG at 1:1,000 dilution in blotto buffer and incubated it for 1 hr at room temperature and then washed the membranes as above. We visualized bound alkaline phosphatase conjugates with the same NBT/BCIP color developer solution described above for western blots and allowed color to develop for 15 min, whereupon we washed the membranes twice with deionized water.

Use of the SIA test in the field

The reagents used for SIA testing are stable in refrigeration and can be stored on ice for short periods (1–4 days). The most heat-labile reagents are the NBT/BCIP, the strips, the milk buffer, the antibody conjugates, and the positive and negative control sera. Strip immunoblot assay field testing requires portage of heat labile reagents on ice in an insulated cooler. Other supplies include: western blot trays, heat-stable buffers, pipets, and a platform rocker powered by a 500W gas generator. The assay procedure was the same as described above. We applied the same biological safety standards to the laboratory component of the field study as we did to trapping and handling mice (Mills et al., 1995).

SIA and western blot comparisons

We conducted side-by-side comparisons of SIA strips and western blots strips in the laboratory using serum collected from 75 wild caught rodents collected in Adams County, Colorado (USA; 39.49N,104.52W). The rodents were live-captured in Sherman traps and killed with an overdose of ketamine hydrochloride. Blood samples were obtained from the heart and stored on ice for transport to the laboratory for serum separation and stored at −70 C until they were tested.

SIA and RT-PCR tests comparison using deer mouse lung RNA

A side-by side comparison of the SIA on rodent serum and nested RT-PCR assay on rodent lung was conducted in the laboratory on a separate set of samples collected from 22 wild caught rodents from Orange County, California (33.35.34–33.43.09N, 117.44.01–117.50.17W). The rodents were captured in Sherman traps and killed with CO2 from dry ice. Blood samples were obtained from the heart and stored on ice for transport to the laboratory for serum separation and stored at −70 C until tested. Samples of lung for RT-PCR were collected from these same animals.

Use of SIA in the field

We compared results of field and laboratory SIA tests using whole blood collected from rodents collected over consecutive nights. Rodents were anesthetized using methoxyflurane or halothane and blood samples obtained from the retro-orbital sinus. We selected sites for field-testing (Elmore County, Idaho, USA, 43.04N, 115.86W; Socorro County, New Mexico, USA, 33.56N, 107.49W) using available resources such as tents or buildings for weather protection. The primary antibody incubation was carried out overnight, and strips developed the following day. Temperatures varied from 6.7–33.3 C during testing. We repeated the SIA in the laboratory using frozen aliquots of the rodent sera.

RESULTS

In all cases, there was concordance between the SIA and western blot methods for detection of rodent antibody (Fig. 2). We found 100% correlation between the results of SIA and nested RT-PCR assays using the lung tissues as the source of template RNA (data not shown). We found 100% correlation of positive and negative results between field and laboratory testing of the SIA (Fig. 3, Table 1).

DISCUSSION

We have been using the SIA for mass screening of rodents in the laboratory for more than 4 yr and have adapted this technique for routine testing of human sera (Hjelle et al., 1997; Bharadwaj et al., 2000). The SIA strips used to detect human IgG are loaded with a slightly more concentrated purified N antigen stock and

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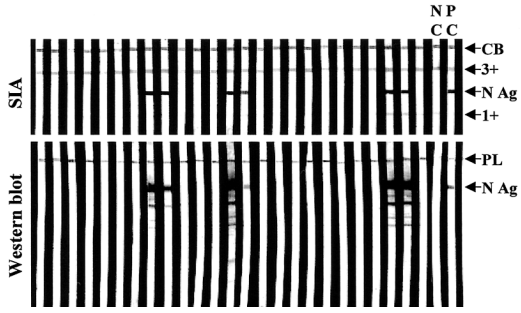


FIGURE 2. A representative series of SIA strips used to test deer mice sera in the laboratory are aligned with the strips resulting from a repeat laboratory analysis of the same samples by western blot. The order of samples loaded, from top to bottom, are Coomassie blue (CB), diluted deer mouse serum (3+), recombinant nucleocapsid antigen (N Ag), and more dilute deer mouse serum (1+). The positive control (PC), negative control (NC), and a pencil line (PL, for orientation) are shown.

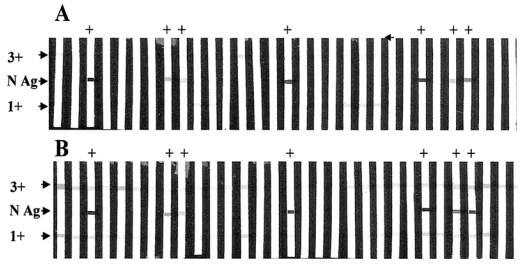


FIGURE 3. A representative series of SIA strips used to test deer mice in the field at the Idaho sampling site (A) and a repeat analysis of the same samples in the laboratory conducted the following week (B). While the same specimens were positive in both series, the reactivities for some samples appeared slightly diminished in the field assay. Note that the SIAs used in this figure were made in an atypical manner in that they lacked a Coomassie blue marker and the 3+ and 1+ control bands were made at blood concentrations that produced approximately equal intensities. The plus signs at top and bottom reflect positive SIA interpretations for both analyses of these samples.

SNV G1 antigen is added in a separate well. G1 antigen is not used in the SIA for rodent antibodies because rodent sera do not demonstrate reactivity to G1 antigen in this format (Hjelle et al., 1994). We have found the SIA to be more economical and easier than methods such as western blot assays for both human and rodent testing. The SIA includes controls to provide assurance that the antigen is present and conjugate reactivity can be standardized, capabilities not available with a western blot assay. The SIA also produces a permanent record that may be stored and reviewed months or years later.

There is no universally accepted "gold standard" diagnostic test for detection of antibodies to SNV in animals, nor is there an FDA-approved product for human diagnosis. Thus, we are unable to determine a meaningful sensitivity and specificity except through our limited cross-comparison between the SIA and the RT-PCR assay, a test that detects the genome of the virus itself. This comparison showed no apparent discrepancy between these two tests, which measure completely different analytes. In addition to the SIA, a number of laboratories have used enzyme linked immunosorbent assays (ELISA) such as those prepared by the Centers for Disease Control and Prevention (CDC) for the detection of serum antibodies (Mills et al., 1997). This test was not available to us, and we do not have direct comparisons between the two tests.

Both the CDC ELISA and the SNV SIA measure the same analytes, IgG-class antibodies directed against the SNV-N anti-

TABLE 1. Results of strip immunoblot assays for IgG antibodies to Sin Nombre virus in Idaho and New Mexico.

Species	Total number tested	Positive	Correlation with laboratory results
Idaho[a]			
Peromyscus maniculatus	104	17	100%
Mus musculus	5	0	100%
New Mexico[b]			
P. maniculatus	40	5	100%

[a] Testing conducted in unheated barracks, outdoor temperature range 6.7–33.3 C, 7–14 June 2000. Temperature recordings taken from regional climate center of similar elevation and latitude.

[b] Testing conducted outdoors without heat, outdoor temperature range 6.7–27.2 C, 4–5 September 1998. Temperature recordings taken from regional climate center of similar elevation and latitude.

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gen. The SIA can, however, be adapted for field settings in places where ELISA readers are unavailable or impractical. We have shown the SIA to be reliable when used with a generator at trapping locations. Rodents can be briefly quarantined and tested, then handled as little as 6 hr later with knowledge of their antibody status.

The SNV-N antigen is recognized by cross-reacting antibodies directed against a wide variety of hantaviruses in either human or rodent infection (Hjelle et al., 1997). Thus, rodents that are infected with other hantaviruses such as Black Creek Canal, Bayou, and El Moro Canyon viruses will frequently score positive in a test that uses SNV-N antigen as target (Hjelle et al., 1995).

Antibodies to SNV are not invariably indicative of active infection. Prevalence of anti-SNV IgG in wild deer mice is highest in those <10.9 g mass, declines in mice ≥10.9–≤14.3 g, and increases in those >14.3 g. The group of small mice probably acquired anti-SNV antibodies passively through the placenta and the decline in seroprevalence between birth and 14.3 g is thought to be due to attrition of maternal antibodies (Mills et al., 1997). Additional experimental evidence has continued to accrue demonstrating that specific anti-hantavirus IgG antibodies can be passed through the placenta and may be protective from challenge by SNV (Borucki et al., 2000; Camaioni, 2001; Botten et al., 2002).

Although the SIA is intended for qualitative screening, it has also been used for assessing endpoint antibody reactivity titers against viral antigens (Bharadwaj et al., 2000). Therefore, the SIA may be useful for the assessment of increasing antibody titers such as during an ongoing or new infection. A fourfold change in specific IgG antibody titer over time has been used as evidence of a recent infection. Anti-IgM tests are not currently available for deer mouse infections because, unlike anti-human IgM, an anti-*Peromyscus* IgM reagent is not commercially available. Thus, we are not able to apply the same technologies to diagnose recent infections in deer mice (Bharadwaj et al., 2000).

The SIA has been highly predictive of PCR-positivity in routine use. However, a negative test for antibodies does not assure that the animal is not infectious. Antibodies to SNV develop 14–21 days after acute infection in experimental infections (Botten et al., 2000). Thus, for establishing new rodent colonies, seronegative animals must be quarantined from one another for several weeks and then retested (Camaioni et al., 2001).

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