Utilization of Hantavirus Antibody Results Generated Over a Five-Year Period to Develop an Improved Serologic Algorithm for Detecting Acute Sin Nombre Hantavirus Infection

Harry E. Prince,* Xin Su, and Wayne R. Hogrefe
Focus Diagnostics Inc., Cypress, California

In the United States most cases of hantavirus pulmonary syndrome (HPS) are caused by the Sin Nombre virus (SNV) and are typically identified by serology. The goal of this study was to assess the performance of our hantavirus serologic testing algorithm by reviewing results generated over five years. Sera were screened for pan-hantavirus immunoglobulin (Ig)G and IgM by enzyme-linked immunosorbent assay (ELISA). Screen IgG+ sera were then tested by immunoblot for SNV glycoprotein-specific IgG, and screen IgM+ sera were tested for SNV-specific IgM using an ELISA that measured differential reactivity to SNV and Seoul nucleocapsid proteins. Although only 13% of sera were positive in

one or both screening assays, 85% of screen+ sera lacked SNV antibodies. Nearly all (97%) screen IgM–IgG+ samples lacked SNV IgG, and 90% of screen IgM+IgG– samples lacked SNV IgM. However, SNV IgM testing of screen IgM+IgG– samples appears to be necessary, since this test identified nine of 37 patients with acute HPS (based on clinical feedback). A screen IgM+IgG+ result was a good predictor of SNV antibody detection and acute HPS. These findings were used to design a modified algorithm that identified all 37 patients with acute HPS, but reduced the number of specimens that required SNV antibody testing by 42%. J. Clin. Lab. Anal. 21:7–13, 2007. © 2007 Wiley-Liss, Inc.

Key words: Sin Nombre virus; hantavirus; serology; hantavirus pulmonary syndrome

INTRODUCTION

Hantaviruses are RNA viruses that may be transmitted to humans via inhalation of virus-laden aerosols arising from rodent excreta (1–3). Human hantavirus infections are associated with two distinct illnesses that reflect the geographic distribution of the causative agents. In Europe and Asia the primary illness is hemorrhagic fever with renal syndrome (HFRS), which is associated with Dobrava/Puumala hantaviruses and Hantaan/Seoul hantaviruses, respectively (2,4). In North America the primary illness is hantavirus pulmonary syndrome (HPS), and most cases are caused by the Sin Nombre virus (SNV) (1,3,5–8).

HPS is characterized by fever, pulmonary infiltrates, thrombocytopenia, and hemoconcentration (2,3,8). Since it was first described in 1993 in the southwestern United States, over 400 cases of HPS have been reported to the U.S. Centers for Disease Control and Prevention, with an overall mortality rate of 36% (9–11). Nearly all cases have occurred in states west of the Mississippi River, consistent with the geographic range of the SNV

reservoir, the deer mouse (*Peromyscus maniculatus*) (12,13).

SNV infection in humans is characterized by short-lived viremia (8); however, assays to detect SNV RNA are not routinely available. Serology has thus emerged as the most common laboratory tool for diagnosing HPS (3). Detection of immunoglobulin (Ig)M to SNV nucleocapsid protein (NP) at the time of disease onset effectively identifies patients with acute SNV infection (3,8,13,14). The acute IgG response is also directed toward NP, but is followed during early convalescence by an even stronger response to the envelope glycoproteins, particularly G1 (3,13,14).

Due to the presence of conserved NP epitopes (1), NP antibodies produced by infection with one hantavirus exhibit varying levels of cross-reactivity with NP from

*Correspondence to: Harry E. Prince, 5785 Corporate Ave., Cypress, CA 90630. E-mail: hprince@focusdx.com

Received 7 July 2006; Accepted 26 October 2006

DOI 10.1002/jcla.20145

Published online in Wiley InterScience (www.interscience.wiley.com).

other hantaviruses (15). NP antibodies induced by SNV infection exhibit greater cross-reactivity with the more closely related Puumala hantavirus than with the more distant Seoul hantavirus (5,14,16). In contrast, the antibody response to G1 is specific for the infecting hantavirus, and thus G1 IgG antibodies induced by infection with hantaviruses other than SNV do not react with SNV glycoprotein G1 (5,14).

Based on this information, we developed a two-tiered algorithm for identifying SNV-specific antibodies. In the first tier (applicable to the detection of antibodies induced by any hantavirus infection), sera are screened by enzyme-linked immunosorbent assay (ELISA) for hantavirus IgG and IgM antibodies using a mixture of recombinant hantavirus NP (17). In the second tier, sera that tested positive in the screening assays are tested with assays designed to detect SNV-specific antibodies. A similar algorithm was previously evaluated by other investigators using selected panels of sera from blood donors and patients with hantavirus infections, and was shown to be effective for identifying SNV infection (8,13). We assessed the algorithm's performance by retrospectively analyzing the results obtained over a five-year period.

MATERIALS AND METHODS

Specimens

Serum samples were submitted to the Focus Diagnostics Reference Laboratory for hantavirus antibody testing between 1 January 2001 and 31 December 2005.

Hantavirus Screening ELISAs

Sera were screened for pan-hantavirus IgG and IgM using ELISA kits manufactured by Focus Diagnostics (Products Division), according to the manufacturer's instructions (4,18). Both assays utilized microtiter wells coated with a mixture of SNV and Seoul recombinant NP produced using a Baculovirus system. The results were expressed as an index, which we calculated by dividing the absorbance value of the patient serum by the absorbance value of a calibrator serum. Per the package inserts, index values <0.90 were considered negative, 0.90 – 1.10 equivocal, and >1.10 positive. All equivocal and positive samples were further tested for SNV IgG and/or SNV IgM as appropriate; thus, per the current algorithm, samples with index values ≥ 0.90 were defined as positive, as opposed to the >1.10 index value given in the package insert.

SNV-Specific IgG Immunoblot

Screen IgG+ samples were tested for SNV-specific IgG with the use of a homebrew immunoblot system. This

assay was validated in 1998 in sera from healthy controls and patients with HPS or HFRS, and exhibited 100% sensitivity and specificity. Antigen strips were prepared by the application of three protein preparations to nitrocellulose paper: anti-human IgG (serum addition control band), a mixture of SNV and Seoul NP, and a synthetic peptide representing a type-specific epitope of the SNV G1 glycoprotein complexed with bovine serum albumin (14). Strips were added to individual wells of an incubation tray, followed by the addition of a 1:100 dilution of control or patient serum specimens. After incubation for one hour at room temperature (RT), the strips were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), and the wells then received alkaline phosphatase-conjugated goat anti-human IgG (Fc fragment-specific; Jackson ImmunoResearch, West Grove, PA). After 30 minutes at RT and another series of washes with PBST, the wells received 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium (Moss, Pasadena, MD). To stop color development after five to seven minutes, the substrate was removed and the strips were washed with deionized water. The strips were transferred to absorbent paper and allowed to dry at RT. Band staining intensity was assessed visually using the G1 peptide band of the positive control as the reading band. A positive result was defined as detection of all three bands.

SNV-Specific IgM ELISA

Screen IgM+ samples were tested for SNV-specific IgM with the use of a homebrew ELISA system based on differential reactivity to SNV and Seoul NP (5,14,16). The assay was validated in 2000 using screen IgM+ sera that were shown to be positive or negative for SNV IgM at another laboratory (University of New Mexico, Albuquerque, NM). The results from the homebrew assay showed 100% agreement with those from the other laboratory. Serum was diluted 1:100 in the sample diluent used for the hantavirus screening IgM ELISA, and then added to a microtiter well coated with a mixture of SNV and Seoul NP (12 ng of SNV NP and 12 ng Seoul NP per well) and to an adjacent microtiter well coated with only Seoul NP (64 ng/well). After an hour at RT and washing, the wells were processed in the same manner used for the screening IgM ELISA. The absorbance values of the mixture well and the Seoul well for a given sample were then compared. An SNV IgM+ result was defined as a higher absorbance value for the mixture well compared to the Seoul well, whereas a SNV IgM– result was defined as a lower absorbance value for the mixture well compared to the Seoul well. This differential reactivity approach for identifying SNV-specific IgM was necessary because

all the recombinant SNV NP, which was produced for the purpose of making pan-hantavirus IgG and IgM ELISA kits, had been mixed with Seoul NP, and thus no material was available to coat microtiter wells with SNV NP only.

Postanalytical Activities

Statistical analysis of differences among proportions was performed with the use of the Chi-square test. Because of the patient management and public health implications of SNV infection, one of the authors (H.E.P.) attempted to contact (via telephone) all ordering physicians of patients who tested positive for SNV-specific antibodies. Upon contact, some physicians voluntarily supplied relevant clinical information.

RESULTS

Over the five-year period of this study (2001–2005), 5,831 serum specimens were submitted for Hantavirus antibody testing. The results for these sera are summarized in Fig. 1. Overall, 13% of sera were positive in one or both screening assays. Of 143 screen IgM–IgG+ sera further tested for SNV-specific IgG, only four samples were positive for SNV IgG. Available clinical information indicated that two of these four SNV IgG+ sera were from patients with a documented history of HPS. Of 555 screen IgM+IgG– samples further tested for

SNV-specific IgM, 55 (10%) were SNV IgM+. Clinical information was available for 20 of these 55 samples, and 12 samples were from nine patients with acute HPS. In contrast, eight samples were from seven patients with non-HPS illnesses; three patients had acute Epstein Barr virus (EBV) infection, one patient was presumed to have Rocky Mountain spotted fever based on serology, one patient was presumed to have Dengue virus infection based on serology, and two patients (who contributed three samples) had undefined illnesses without acute respiratory distress or thrombocytopenia (see Fig. 2). Of 88 screen IgM+IgG+ sera, 42 were both SNV IgG+ and SNV IgM+. Clinical information was available for 30 of these samples (from 27 patients), and all 27 patients were identified as having acute HPS. An additional 12 screen IgM+IgG+ samples were SNV IgM+ but SNV IgG–. Clinical information available for three of these samples indicated acute HPS (one of these HPS patients was among the group of 27 patients with acute HPS). Lastly, six screen IgM+IgG+ samples were SNV IgG+ but SNV IgM–. Clinical information was available only for two samples from a single patient, and indicated hay-induced allergic alveolitis rather than HPS.

Because of the unexpectedly large number of screen IgM+IgG– samples and the low proportion of these samples that were positive for SNV-specific IgM, we investigated the relationship among screen IgM index values, SNV IgM assay results, and clinical findings

Fig. 1. Summary of results generated using the current hantavirus serology testing algorithm over a five-year period. Sera with positive results in one or both pan-hantavirus antibody screening ELISAs were further tested in the relevant SNV-specific antibody assay(s). IgM and IgG are abbreviated M and G, respectively. Percentage values in parentheses were calculated using the number in the linked box above as the denominator. Available clinical information is summarized in the bottom row of boxes. *One sample represented a later sample from an HPS patient in the box to the left; that sample was the acute sample, whereas this sample (showing IgG seroconversion) is the convalescent sample. **One sample represented an earlier sample from an HPS patient in the box to the left.

Fig. 2. Relationship among screen IgM index, SNV IgM reactivity, and clinical findings for the 555 screen IgM+IgG– specimens indicated in Fig. 1. The format is the same as that described in Fig. 1. *An earlier sample from an HPS patient in the box to the left.

(Fig. 2). The proportion of samples that yielded an SNV IgM+ result was significantly higher in samples with screen IgM index values >3.0 compared to samples with lower screen IgM index values ($P<0.0001$). A similar trend was observed when we compared samples with screen IgM index values of 1.11–3.0 and samples with screen IgM index values of 0.90–1.10 ($P=0.0105$). Nearly all samples linked to HPS patients, as well as non-HPS patients, exhibited screen IgM index values >1.10 .

Similarly, we examined the relationship among screening assay index values, SNV antibody assay results, and clinical findings for screen IgM+IgG+ samples (Fig. 3). Over 80% of these samples exhibited index values >1.10 in both screening assays. Over 90% of the sera that tested positive for SNV antibodies, including sera from all 27 patients with acute HPS, were found in this sample group with index values >1.10 in both screening assays.

These findings were used to design a modified algorithm. Under the new algorithm, the definition of “positive” for the screening assays was changed from an index ≥ 0.90 to an index >1.10 , and sera with screen IgM–IgG+ results were no longer tested in the SNV IgG immunoblot assay. The modified algorithm was applied to the existing data set, and the findings were compared with those obtained using the current algorithm (Table 1). The new algorithm forwarded 8% of all samples for SNV antibody testing vs. 13% using the current algorithm, and this change represented a 42%

reduction in the number of samples that required SNV antibody testing (457 by the new algorithm vs. 786 by the current algorithm). However, the new algorithm still detected 90% of the SNV antibody+ samples detected using the current algorithm, and identified all 37 patients with clinically defined acute HPS.

DISCUSSION

A retrospective review of results obtained using our current algorithm for detecting SNV IgG and IgM showed that the pan-hantavirus ELISAs worked fairly well as a screening system, and reduced the number of specimens to be tested for SNV-specific antibodies by 87%. Further, symptoms consistent with HPS characterized 79% of the SNV antibody-positive patients for whom clinical information was available, demonstrating the clinical relevance of a positive SNV antibody result, particularly SNV IgM.

The data review also identified two major issues with the current algorithm. First was the lack of clinical utility of SNV IgG measurements in screen IgM–IgG+ samples for identifying cases of acute HPS. Only four of 143 screen IgM–IgG+ samples were SNV IgG+, and two of these four samples were from individuals with past, rather than acute, HPS. Second was the surprisingly large number of sera that were screen IgM+IgG– but SNV IgM–. This group of sera accounted for nearly 9% of all specimens tested using the current algorithm. False-positive reactivity in the hantavirus screen IgM

Fig. 3. Relationship among screen IgM and IgG index values, SNV IgM and IgG reactivity, and clinical findings for the 88 screen IgM+IgG+ specimens indicated in Fig. 1. The format is the same as that described in Fig. 1. *An earlier sample from one of the 27 HPS patients indicated in the lower right box. **One sample represented an earlier sample from one of the 27 HPS patients indicated in the lower right box.

TABLE 1. Comparison of the Current Algorithm and a Modified Algorithm*

Parameter	Current algorithm	Modified algorithm	% change, modified vs. current
Screen M–G+	143	111	–22%
SNV G+	4	Not measured	Not applicable
Screen M+G–	555	385	–31%
SNV M+	55	53	–4%
Screen M+G+	88	72	–18%
SNV M+G+	42	41	–2%
SNV M+G–	12	10	–17%
SNV M–G+	6	4	–33%
Total tested for SNV antibodies	786 ^a	457 ^b	–42%
Total tested for SNV IgG	231	72	–69%
Total tested for SNV IgM	643	457	–29%
Total positive for SNV antibodies	119	108	–9%
Total positive for SNV IgG	52	45	–13%
Total positive for SNV IgM	109	104	–5%
Acute HPS patients identified	37	37	0%

*Modified algorithm: positive in screening assays defined as >1.10 rather than ≥ 0.90 , and screen IgM–IgG+ samples no longer tested for SNV IgG.

^a88 screen M+G+ samples were tested for both SNV IgG and SNV IgM; thus, this value of 786 samples tested for SNV antibodies is 88 less than the total of the two boxes below ($231+643=874$).

^b72 screen M+G+ samples were tested for both SNV IgG and SNV IgM; thus, this value of 457 samples tested for SNV antibodies is 72 less than the total of the two boxes below ($72+457=529$).

assay due to acute EBV and cytomegalovirus infections has been reported previously (4,18), but it is unlikely that herpesvirus infections explain the large number of samples with this reaction pattern. The environmental triggers for this hantavirus screen IgM reactivity remain unknown, and further studies are needed to determine 1) what proportion are due to acute herpesvirus infections, 2) whether other non-hantavirus infections induce cross-reactive antibodies, 3) whether heretofore unrecognized hantavirus infections account for some of this reactivity, and 4) whether other conditions are responsible for this reaction pattern. However, it appears that SNV IgM testing of screen IgM+IgG– samples is necessary, since nine of the 37 patients with acute HPS exhibited this screen reaction pattern.

These issues led us to explore options for improving the algorithm. Discontinuing SNV IgG testing on screen IgM–IgG+ samples was an obvious change that would reduce the number of samples reflexed for additional testing without jeopardizing detection of acute HPS cases. We also targeted the screen IgM+IgG– subset of samples and found that raising the index for defining positive had a minimal impact on the number of SNV IgM+ samples detected while it markedly reduced the number of screened samples that required SNV IgM testing. Further analysis showed that this higher index for defining positive could be applied to both the IgG and IgM screening assays without adversely affecting the clinical utility of a screen IgM+IgG+ reaction pattern. When the modified algorithm incorporating all these changes was applied to the current data set, all clinically defined HPS patients were still identified, but 42% fewer samples were targeted for SNV-specific antibody testing.

An unexpected finding from this study was that some patients who exhibited a screen IgM+IgG– SNV IgM+ reactivity pattern had clinical conditions other than HPS. Seven of the 16 patients who exhibited this reaction pattern, and for whom available clinical information was available, had conditions other than HPS, including infections due to EBV, dengue virus, and Rocky Mountain spotted fever. Clinicians thus need to be educated concerning possible false-positive SNV IgM reactivity, particularly in conjunction with failure to detect hantavirus IgG.

Hantaviruses other than SNV have been shown to cause a few cases of HCPS-like illness in geographic areas of the United States outside the West/Southwest. Cases caused by Bayou hantavirus have been identified in Louisiana and east Texas (19,20), and Black Creek Canal hantavirus infection has been documented in Florida (21). It is unclear what reaction pattern(s) such sera would show in our hantavirus serology assays. In a previous study Hjelle et al. (14) used a polyvalent conjugate (specific for human immunoglobulin heavy

and light chains) and found that sera from patients infected with Bayou or Black Creek Canal hantaviruses showed extensive cross-reactivity with SNV NP, but no cross-reactivity with SNV G1. We thus predict that such sera would exhibit a screen IgM+IgG+SNV IgM+SNV IgG– reaction pattern in our algorithm; however, further studies are needed to characterize the serologic response of North American patients with non-SNV hantavirus infections.

Limitations of this study include 1) the lack of clinical information for 50% (58/116) of the samples that tested positive for SNV-specific IgG and/or IgM, and 2) our dependence on physician assessment of clinical findings consistent with HPS, rather than defined clinical criteria for HPS. However, the available data support the use of a serologic testing algorithm to identify HPS patients. Additional studies with improved patient follow-up and systematic assessment of clinical signs and symptoms are needed to fully assess the efficacy of these serologic assays for hantavirus antibodies.

ACKNOWLEDGMENTS

We thank Dr. James Carlson for a critical review of the manuscript, and the staff of the Focus Diagnostics Immunology Laboratory for performing the described assays.

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