Puumala Virus Antibody and Immunoglobulin G Avidity Assays Based on a Recombinant Nucleocapsid Antigen

HANNIMARI KALLIO-KOKKO,* OLLI VAPALAHTI, KLAUS HEDMAN, MARKUS BRUMMER-KORVENKONTIO, AND ANTTI VAHERI

Department of Virology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

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Puumala virus is one of the three human-pathogenic hantaviruses which cause hemorrhagic fevers with renal syndrome. The severity of the disease differs among these viruses, Hantaan virus being the most pathogenic, causing Korean hemorrhagic fever (KHF) with a mortality rate of 3 to 5%, and Puumala virus being the mildest, causing nephropathia epidemica (NE) with a mortality rate of ~0.2%. Puumala virus has been found to be endemic in northern and central Europe (5, 11). Typical symptoms of NE include fever, headache, nausea, back pain, vomiting, oliguria, stomach pain, and diarrhea (4).

Hantaviruses belong to the Bunyaviridae family, and they all possess a three-segmented negative-stranded RNA genome (7). The L (large) segment codes for the polymerase enzyme (8), the M (medium) segment codes for the two glycoproteins G1 and G2 (10), and the S (small) segment codes for the nucleocapsid (N) protein (9). The most antigenic protein has been shown to be the nucleocapsid protein (13).

There were more than 1,300 verified cases of NE in Finland during the years 1989 to 1991 (3). Diagnosis is made by an immunofluorescence (IF) method that measures the antigen-binding avidity (2) of Puumala virus-specific immunoglobulin G (IgG) antibodies (3). Although the method is fast, easy, and sensitive, IF suffers from the need to propagate the pathogenic and slowly growing virus in cell cultures in a biosafety level 3 laboratory and from subjectivity in interpretation of the results.

The aim of the present study was to develop an alternative way to produce Puumala virus antigen for diagnostic purposes and to develop fast and reliable diagnostic enzyme immunoassays (EIAs) with a recombinant protein as the antigen.

MATERIALS AND METHODS

Expression. The S segment of Puumala virus strain Sokkamo was cloned, and the nucleocapsid protein was expressed as a fusion protein as described earlier (12). The nucleocapsid-coding S segment cDNA construct, created by polymerase chain reaction, was ligated into the pEX2 expression vector. The recombinant protein was purified by cutting the protein band from an agarose gel, melting the agarose, and removing the protein by freezing, incubation on ice, and centrifugation. The recovery was about 1 to 5 mg/200 ml of bacterial suspension, sufficient for coating 100 to 500 enzyme immunoassay microtiter plates. In a study of 312 IF-positive and 233 IF-negative serum samples from NE patients, the recombinant-N-protein enzyme immunoassay detected immunoglobulin G antibodies to Puumala virus with 97.8% sensitivity and 98.5% specificity compared with the IF test results. In addition, an immunoglobulin G avidity enzyme immunoassay was developed and used successfully to diagnose acute NE from a single serum sample. The results demonstrate that the bioengineered antigen is suitable for use in routine diagnostic assays for Puumala virus immunity and recent infection.
the patients was 44 years (43 years for males and 46 years for females; range, 7 to 84 years).

For the IgG EIA, 312 IF-positive and 233 IF-negative sera were tested; for the IgM EIA, 158 IF-positive and 76 IF-negative sera were tested; and for the IgG avidity EIA, 79 IF-positive sera were tested. Twenty-four serum samples from KHF patients (kindly provided by H. W. Lee, Institute for Viral Diseases, University of Korea, Seoul) were also tested in the IgG EIA, including old-immunity samples and 10 pairs of acute-phase samples (each pair taken 7 and 27 days after the onset of illness) (4a).

The approximate time criteria for the different phases are less than 1 month after onset of illness for the acute phase, from 1 to 2 months after onset for the borderline phase, and more than 2 months after onset for old immunity.

**IF assay.** The IgG avidity IF assay (3) used in routine diagnosis was considered the reference method when evaluating the EIA results. The antigen used in IF was the Puumala virus Sotkamo strain-infected Vero E6 cell line. In this test, binding of human IgG antibodies to acetone-fixed Puumala virus-infected cell layers is detected by using fluorescein-conjugated anti-human IgG antibodies.

**Coating of the EIA plates.** The EIA microtiter plates (Nunc, Roskilde, Denmark) were coated with 1 μg of nucleocapsid-β-galactosidase fusion protein per ml in phosphate-buffered saline (PBS) overnight at room temperature. The plates were washed three times for five min each with 8 M urea in PBS and then three times for 10 min each with PBS containing 0.05% Tween 20 (PBST); after these washes, they were ready for use in the EIA. The plates were stored at −20°C.

**IgG EIA.** For the IgG EIA, the serum samples were diluted 1:50 in PBST. A 100-μl amount of each serum dilution was pipetted (in duplicate) per microtiter well, and the plates were incubated for 30 min at 37°C. After three washes with PBST, 100 μl of alkaline phosphatase-conjugated anti-human IgG (γ-chain specific; Orion Diagnostica, Espoo, Finland) was applied at a dilution of 1:100 for 30 min. The plates were washed three times with PBST before the substrate (1 mg of p-nitrophenol phosphate disodium [Sigma, St. Louis, Mo.] per ml of diethanolamine-MgCl₂ buffer [Orion Diagnostica]) was added. The reaction was stopped after 30 min of incubation at room temperature by adding 100 μl of 1 M NaOH. An A₄₀₅ of 0.25 was considered the cutoff value for a positive result. This value was equal to the mean absorbance of the IF-negative samples (n = 233) plus 3 standard deviations.

**IgM EIA.** The IgM EIA was performed like the IgG EIA except that the conjugate was μ-chain specific (Orion Diagnostica). The cutoff A₄₀₅ value for a positive result was 0.2, calculated as the mean absorbance of the IF-negative samples (n = 76) plus 3 standard deviations.

**IgG avidity EIA.** For the IgG avidity EIA, the serum samples were diluted serially in PBST in fourfold steps (1:12.5 to 1:3,200) and incubated in the EIA plates for 60 min at 37°C. After the incubation period, the dilutions from 1:12.5 to 1:800 were washed three times with 6 M urea-PBST and the dilutions from 1:50 to 1:3,200 were washed with PBST. Alkaline phosphatase-conjugated anti-human IgG (Orion Diagnostica) was applied for 60 min at 37°C, and the substrate (see above; 1 mg/ml) was added and incubated for 30 min at 37°C. The reaction was stopped by adding 1 M NaOH, and the A₄₀₅ was measured. The IgG avidity result was calculated by comparing the absorbances in the urea-washed wells with those in the PBST-washed wells and expressing the ratio as a percentage (3). We considered 20% the cutoff value for low avidity (acute phase) after comparison with the results obtained by the reference IF avidity method (3). The cutoff point was selected as the percentage value that best differentiated the known old-immunity cases from the acute-phase cases.

**RESULTS**

**Preparation of EIA antigen.** Immunoblot analysis of the Puumala virus N protein-β-galactosidase fusion proteins expressed in pEX2 showed that the whole nucleocapsid protein and its amino-terminal two-thirds were immunogenic, whereas neither the last third of the nucleocapsid protein nor the β-galactosidase control protein showed any antigenic reactivity with NE-positive sera (12). Antibodies against the fusion protein produced in a rabbit could specifically recognize the nucleocapsid protein, whereas control antibodies against β-galactosidase gave no reaction with Puumala virus components (data not shown). The entire nucleocapsid-β-galactosidase fusion protein was purified by preparative agarose gel electrophoresis, with a yield of 1 to 5 mg of protein per 200 ml of E. coli suspension.

**IgG EIA with NE-positive and NE-negative sera.** The IgG EIA detected IgG antibodies to Puumala virus in serum samples...
from patients with NE with 97.8% sensitivity (calculated as the ratio of positive EIA results to positive results in the reference IF test) and 98.5% specificity (calculated as the ratio of positive and negative EIA results to positive and negative results in the reference IF test) (Fig. 2). No false-positive IgG EIA results were obtained (n = 233) in comparison with IF test results (Fig. 2).

**IgG EIA with KHF sera.** Of the 24 serum samples from KHF patients that were positive in the Hantaan virus-specific IF (4a), 8 were positive in the EIA for IgG antibodies to Puumala virus.

**IgM EIA.** The IgM test gave a positive result for a few very early phase serum samples that could not be diagnosed by the IgG EIA or IF test (Table 1). However, of 28 old-immunity samples (classified by the reference IF test), 5 gave a positive result in the IgM EIA. No positive results were obtained for 76 IF-negative sera.

**IgG avidity EIA.** With a cutoff value of 20%, all of the old-immunity serum samples gave IgG avidity EIA results (>20%) that indicated old immunity. Likewise, all of the acute-phase samples gave results below 20% (Fig. 3).

**DISCUSSION**

Because hemorrhagic fevers with renal syndrome are widespread diseases and difficult to diagnose on clinical grounds alone, specific diagnostic procedures are necessary. The aim of this work was to establish an easy and safe way of producing hantavirus antigen for diagnostic purposes.

As judged from immunoblotting analyses with patient sera and rabbit antisera raised against the Puumala virus nucleocapsid-β-galactosidase fusion protein and against β-galactosidase, the Puumala virus nucleocapsid fusion protein was suitable for use as a diagnostic antigen. The results of the IgG EIA with the recombinant nucleocapsid antigen correlated well with those of the conventional IF test (Fig. 2). Thus, the present IgG EIA provides a good alternative method for routine diagnostic purposes. The recombinant protein-based IgM EIA showed promising results for a few very early phase serum samples in which the IgG EIA and the IF test could not detect any antibodies but the IgM EIA could (Table 1). The indirect IgM EIA seemed, however, to suffer especially from false-positive results for some of the old-immunity serum samples. Other investigators have shown the suitability of μ-capture IgM EIA with conventional viral antigen for rapid diagnosis from a single acute-phase serum specimen (6). Thus, it will be of interest to

**FIG. 2.** $A_{405}$ values in EIA for IgG antibodies to Puumala virus versus time after onset of illness in testing of 312 NE-IF-positive serum samples. The $A_{405}$ cutoff value was 0.25 (dotted line).

**FIG. 3.** Correlation between IgG avidity EIA (AVI-EIA) and IgG avidity IF (AVI-IF) in testing of 79 old-immunity (AVI-IF titer of 1 to 4), borderline-immunity (AVI-IF titer of 8 to 16), and acute-immunity (AVI-IF titer of 32 to 256) serum samples. The cutoff value for acute immunity was 20% (dotted line).

**TABLE 1.** Usefulness of IgM EIA for early-phase NE, when IF gives unclear results and IgG EIA has not yet become positive

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Serum sample (day postonset)</th>
<th>Test result</th>
<th>IgG EIA</th>
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a Results are expressed as no detectable reaction (ND), acute phase, or old immunity.
evaluate the suitability of the present recombinant nucleocapsid protein as an antigen in μ-capture IgM EIA.

At present, the most reliable test to distinguish acute illness from old immunity is the IgG avidity EIA, which showed a good correlation with the IF avidity assay (3) (Fig. 3). An advantage of IgG avidity tests is that they give information about the phase of immunity from single serum samples, as do tests that measure IgM class antibodies.

Our results showed that about a third of the serum samples from KHF patients showed some reactivity in EIA tests and some also reacted in the immunoblotting assay (12) with the recombinant Puumala virus nucleocapsid protein as the antigen. Such cross-reactivity was especially seen in some of the old-immunity cases of KHF, yet a few of the acute-phase serum samples were also positive (data not shown). Previously, similar partial serological cross-reactivity between Puumala virus and Hantaan virus has been demonstrated in the hemagglutination inhibition and indirect IF tests (1) but not in the plaque reduction neutralization test (8). Clearly, even if some positive reactions are observed, the EIA with the Puumala virus nucleocapsid fusion protein as the antigen cannot be regarded as useful for the diagnosis of KHF, for which tests employing Hantaan virus-specific antigens seem to be needed.

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REFERENCES