Diagnosis of hantavirus infection in humans


Salim Mattar*1, Camilo Guzmán1 and Luis Tadeu Figueiredo2

1Instituto de Investigaciones Biológicas del Trópico, Universidad de Córdoba, Montería, Colombia
2School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil
*Author for correspondence: mattarsalim@hotmail.com

Rodent-borne hantaviruses (family Bunyaviridae, genus Hantavirus) cause hantavirus pulmonary syndrome in the Americas and hemorrhagic fever with renal syndrome in Europe and Asia. The viruses are transmitted to humans mainly by inhalation of virus-contaminated aerosols of rodent excreta and secreta. Classic clinical hemorrhagic fever with renal syndrome occurs in five phases: fever, hypotension, oliguria, polyuria, and convalescence. Hantavirus pulmonary syndrome is a severe acute disease that is associated with respiratory failure, pulmonary edema and cardiogenic shock. The diagnosis of hantavirus infections in humans is based on clinical and epidemiological information as well as laboratory tests. We review diagnosis for hantavirus infections based on serology, PCR, immunochemistry and virus culture.

KEYWORDS: differential diagnosis • epidemiology • hantavirus • reservoir • symptoms

Hantaviruses (family Bunyaviridae, genus Hantavirus) are rodent-borne zoonotic viruses that produce two major clinical syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and hantavirus pulmonary syndrome (HPS) in the Americas. Diseases compatible with HFRS were recognized as early as 1930 in China and Russia, but the first isolation of a causative agent did not occur until Hantaan virus was isolated in Korea [1]. Since HPS was initially characterized in the USA in 1993, and since the associated hantavirus (Sin Nombre virus [SNV]) was identified, increasing numbers of human cases and SNV-related viruses have been identified in most countries of North and South America [2]. There have been more than 640 HPS cases in the USA with a fatality rate of 36% (http://www.cdc.gov/hantavirus/surveillance/index.html). HPS is caused by any of at least 25 hantavirus genotypes distributed throughout the Americas. All hantaviruses that cause HPS are hosted by rodents of the family Cricetidae. In general, each hantavirus is associated with a different rodent species or subspecies [3,4] (Table 1). Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) are among the most important viruses causing HFRS in Europe. In Asia, Hantaviruses (HTNV) and Seoul virus (SEOV) are important. In North America SNV is the most prevalent virus. In South America, hantaviruses have been described from Argentina, Bolivia, Brazil, Chile, Colombia, Paraguay, Peru, Uruguay and Venezuela [3-7]; in sum, all South American countries except for Ecuador, Surinam and Guyana. In Central America, hantaviruses are described from Panama, Honduras and Costa Rica [2,8-10]. Recently, antigenically and genetically distinct hantaviruses have been described from shrews, moles and bats [11-15]. Nevertheless, only the rodent-borne hantaviruses are known to cause human disease.

Laboratory diagnosis of hantavirus infection

The diagnosis of hantavirus infection in humans is based on clinical and epidemiological information as well as laboratory tests. A definitive diagnosis cannot be based solely on clinical findings, especially in cases where disease is mild to moderate [16,17]. Laboratory testing should be performed on samples from patients with fever of unknown origin, severe myalgia, thrombocytopenia, renal failure or respiratory distress, and patients living in hantavirus disease-endemic regions, or persons with recent outdoor activities during which there was possible exposure to rodents or their excreta [18,19]. Because hantaviruses differ in their geographic distribution, course of infection and likely outcome, specific and accurate laboratory diagnostic tests are important [20]. Laboratory diagnosis of hantavirus
infection is based on four primary categories of tests: serology, reverse transcription (RT)-PCR, immunochrometry and virus culture.

**Serologic diagnosis**

The most practical approach for the laboratory diagnosis of hantavirus infection is based on serologic tests [21-23]. The three structural proteins of hantaviruses (Gn, Gc and N) can induce a high level of IgM antibodies, which are detectable at the onset of symptoms [24].

**Enzyme-linked immunosorbent assay**

The ELISA is the most commonly used serologic test to identify antibodies of the IgM class (acute phase) and IgG (convalescent stage). Antibodies of the IgG class against hantaviruses persist for the life of the individual and can be used in retro-spective studies [22,25]. Thus, while seroconversion to a hantavirus can be determined by using IgG or IgM ELISA, only IgM detection indicates recent exposure [26]. IgM antibody appears relatively early after infection; timing of appearance varies depending on incubation period, dosage, immune status and multiple additional factors. After several weeks, IgM begins to decline over 3–6 months. IgG is detectable slightly later (sometime concurrently), but titers may remain elevated for many years [4]. These dynamics appear to be similar for most hantaviruses.

Hantavirus antigens in most current serologic tests use protein antigens obtained by recombinant DNA techniques (Table 2). The use of recombinant proteins avoids risk to laboratory personnel who otherwise would need to handle large quantities of virus in the preparation of conventional antigens. These antigens are mostly N proteins, but Gn and Gc proteins have also been produced. The N protein has been expressed and purified from several recombinant expression systems, primarily bacterial [27].

An ELISA method based on a recombinant viral antigen was successfully used to investigate hantavirus infections in rodents and human clinical samples. For the development of ELISAs in the Americas, recombinant antigens of SNV (CDC, USA) and ANDV (Instituto Carlos Malbrán, Argentina), have been produced by cloning and expression of genes encoding viral proteins [4,28]. In Brazil, Figueiredo et al. [24,27] produced a recombinant N protein from Araraquara virus, which is being used as an ELISA antigen (Table 2). In Argentina, Maciel virus, which is not known to be pathogenic, has been cultured for use in serodiagnosis; Maciel virus is cross-reactive with all recognized New World hantaviruses [2].

In 2004, we detected IgG antibodies reactive with SNV by ELISA in 14% of serum samples from 88 rural volunteers in northwestern Colombia [29]. In 2013, we detected IgG antibodies to Maciel or Araraquara hantaviruses in 8.4% of bodela-Be1grade hantavirus strains (mild form of HFRS), which are much higher than those found in serosurveys in North America. For example, even when high-risk occupational groups were chosen (e.g., mammalogists), only 0.5% of North Americans had antibody reactive with SNV 30. Other than being rural workers, the Colombian volunteers did not have specific occupational risks. This suggests that rural workers in northwestern Colombia are frequently exposed to one or more hantaviruses [2].
Table 2. Immuno-enzymatic assays and antigens for the diagnosis of hantavirus infection.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Antigen type</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Commercial name</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Sin Nombre Seoul Hantaan Dobrava Puumala</td>
<td>95.1%</td>
<td>94.1%</td>
<td>Hantavirus IgG and IgMDxSelect ELISA Kits Focus Diagnostics</td>
<td>[33]</td>
</tr>
<tr>
<td>ELISA</td>
<td>Hantaan, Dobrava, Seoul Puumala</td>
<td>96.7%</td>
<td>98.7%</td>
<td>Hantavirus IgG/IgM Combo Test (Colloidal gold immunochromatographic assay, Xiamen Biotech, China)</td>
<td>[31]</td>
</tr>
<tr>
<td>ELISA</td>
<td>Araraquara (ARAVrN) Puumala</td>
<td>97.2%</td>
<td>100%</td>
<td>In-house ELISA</td>
<td>[27]</td>
</tr>
<tr>
<td>IFA</td>
<td>Puumala/Hantaan</td>
<td>98%</td>
<td>91%</td>
<td>Progen® IFA</td>
<td>[20]</td>
</tr>
<tr>
<td>Immunoblot assay</td>
<td>Puumala, Hantaan, Dobrava-Belgrade, Seoul</td>
<td>96%</td>
<td>100%</td>
<td>kit Mikrogen®, Neuried, Germany</td>
<td>[32]</td>
</tr>
</tbody>
</table>

IFA: Immunofluorescence assay.

Immunofluorescence assay
The initial test used for serodiagnoses of HFRS in Europe and Asia was IFA smearing hantavirus fixed as an antigen on microscope slides. The use of virus-infected cells for serologic tests is not widely used because cell culture infections require biosafety 3 laboratories (BSL-3) [30]. The IFA is often used in Europe (e.g., Euroimmun Anti-Hantavirus-IIFT Mosaic II Test, Lübeck, Germany) (Table 2). Diluted serum samples are added to reaction fields on biochips containing uninfected cells or cells infected with one or more hantaviruses that are being tested for.

Immunoblot assay
Immunoblot tests (Table 2) have also been tested in Europe (e.g., recomLine® Bunyavirus IgG/IgM test kit; Mikrogen, Neuried, Germany). Diluted sera are incubated on recomLine test strips enclosing six antigens with complete nucleocapsid proteins from HTNV and PUUV, or a recombinant N-terminus of the nucleocapsid antigen from PUUV, HTNV, DOBV and SEOV. A control band is integrated for the antibody class (IgG or IgM).

Focus reduction neutralization test
Although the assays described above are used to determine whether a patient has been infected with a hantavirus, none of these tests can determine which hantavirus was responsible for the infection because significant humoral cross-reaction occurs among hantavirus antibodies [30,31]. The specific infecting hantavirus can be identified by focus reduction neutralization test (FRNT), which is the gold standard for hantavirus testing [30,32]. FRNT can detect and measure neutralizing antibodies by comparing titers with the relevant hantavirus strains [31,32] and although it distinguishes hantaviruses with serum from experimentally infected rodents, it was less specific when serum from acute phase patients was tested [31]. Because FRNT requires cell culture, it has to be conducted under BSL-3. The assay is also time consuming and labor intensive [30,32].

Molecular detection of hantaviruses
Molecular diagnostic methods for hantavirus infections also are widely used. The methods are based on sensitive, practical RT-PCR. Often the diagnosis is by post-mortem, using blood and tissue from fatal cases [5,33–35]. A distinct advantage of PCR is that the PCR product may be sequenced to identify the virus and conduct phylogenetic analysis. Using PCR, hantavirus RNA is detectable 7–10 days after onset of symptoms. RT-PCR also has been used in the diagnosis of hantavirus infections, using primers specific for the hantavirus S and M segments (Table 3).

PCR provides results within 24 h, allowing rapid detection of hantavirus infection and is especially useful in cases where the disease is rapidly evolving and cases may progress from a febrile prodrome to severe respiratory disease in < 24 h [31,36]. Nevertheless, PCR using blood samples is only useful in the early stages of infection when patients are viremic [30,37,38]. Real-time RT-PCR is a sensitive tool for early detection of hantavirus RNA even prior to the appearance of IgM antibodies. Hantavirus real-time RT-PCR, when combined with immunologic techniques, can contribute to pathogenesis studies and understanding antiviral treatments [39].

Virus isolation in cell culture
Because virus isolation from human samples is rare, it is not an option in the diagnosis of human hantavirus infection [30]. However, the isolation of hantaviruses for research purposes may be achieved using clinical materials that are inoculated onto Vero E-6 cell cultures. One to 2 weeks after inoculation, hantavirus-infected cells may be identified using immunofluorescence. The isolation is laborious and time consuming, involves high risk of viral contamination in the laboratory and requires biosafety measures to protect the handler from exposure to aerosols [40–43]. In Brazil, for example, there are no reports of the isolation of hantaviruses; only the detection of their genetic material by RT-PCR.
Differential diagnosis

The differential diagnosis of hantavirus infection depends on the phase of infection of the patient. In the prodromal phase, diagnosis basically poses differential viral states with non-specific or febrile syndromes of undetermined origin. For the cardiopulmonary phase, in which pulmonary and cardiac involvement are established, diagnosis must be made considering differentiation from pathologies involving acute respiratory failure. These pathologies are presented as differential diagnoses both in the prodromal and cardiopulmonary phases of HPS in immunocompetent patients (Table 4).

Severe atypical pneumonia

In the early phase of infection, the complete blood count is the laboratory test most useful for distinguishing hantavirus infection (especially HPS) from atypical pneumonias caused by *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella* spp. Eighty-seven percent of HPS patients have hemoconcentration, leukocytosis and thrombocytopenia.

Pneumonia influenza

Pneumonia influenza often occurs in outbreaks during the winter and has more upper respiratory airway symptoms and signs, including cough, sore throat, runny nose and conjunctivitis. Diagnostic confirmation is accomplished by technical immunoassay.

Heart failure

Heart failure should be accompanied by a history of hypertension, rheumatic disease or valvular or coronary arteriosclerosis.

The clinical evaluation includes dyspnea (which can be mild, moderate or severe), cough, edema of limbs or venous congestion. Radiology may show cardiomegaly, pulmonary edema distribution butterfly wings or pleural effusion.

Leptospirosis

The pulmonary hemorrhagic phase of leptospirosis sparsely courses with jaundice or renal disease or hemorrhagic manifestations in other tissues. Leptospirosis is generally confirmed by serologic diagnosis.

Rickettsiosis

Rickettsiosis are typified by maculopapular rash, which may evolve into hemorrhagic papules that can mimic other hemorrhagic fevers. In the laboratory, the leukocyte count is usually normal. The diagnosis relies on the presence of an inoculation eschar (tick bite). Signs may be multisystemic and may include pulmonary edema.

Conclusion

Three important methods exist for the etiologic diagnosis of hantavirus infections: i) serology: This is the method of choice for the diagnosis of hantavirus infections. The detection of IgG or IgM antibodies to hantavirus using ELISA is the method most commonly used in the diagnosis given that the majority of HPS cases show IgM antibodies during the acute period of the illness and they remain detectable for 6–8 months. The appearance of specific IgG can occur during or shortly after the appearance of IgM antibodies, they can remain at high levels for several years.
The sensitivity and specificity depend on the antigen; in South America there appears to be antigenic similarity between different variants or circulating strains (Andes virus, Laguna Negra, Juquitiba, etc.). In North America, the antigen of SNV is the most used. However, cross-reactivity has been reported with antigens in South America and PUUV, the most widely used antigen in Europe. The antigenic cross-reactivity between hantaviruses makes ELISA the most appropriate test not only for diagnosis, but also for the initial search for exposure to hantavirus in geographic areas without previous reports of infection. Immunofluorescence assays detect antibodies in the acute period of the disease and persist for years. The indirect immunofluorescence assay is useful for researching new hantaviruses because of the high cross-reactivity observed between hantaviruses by this technique.

- **ii) Detection of viral antigen:** Immunohistochemistry is a method of great utility in the demonstration of viral antigen in tissues, particularly in fatal cases in which there is no other type of sample.
- **iii) Molecular detection:** The use of RT-PCR allows the detection of viral RNA in whole blood, clot or tissues obtained in the first 10 days of the period of the disease and persist for years. The method is also useful for detecting viral RNA in tissues of infected rodents.

For the diagnosis, it is desirable to select primers that are complementary to gene sequences that are highly conserved between the different hantaviruses (e.g., the S segment that encodes the viral nucleoprotein). Post-mortem molecular diagnosis of hantavirus can be performed on samples of blood and organs, mainly lung and kidney. The isolation of the hantavirus is difficult due to its poor growth in Vero cells; for this reason, isolation is not used for diagnostic purposes.

**Expert commentary**

Hantaviruses are rodent-borne zoonotic viruses that produce two major clinical syndromes in humans: HFRS in Asia and Europe, and HPS in the Americas. Case fatality rates for HFRS can reach as high as 15% and for HPS can exceed 40%. Very recently, the occurrence of hantaviruses and HFRS was also shown in Africa.

In endemic areas, there is a set of clinical or laboratory signs that are pathognomonic of hantavirus infection, so acknowledgement...
of the local epidemiology and high clinical suspicion is necessary. Since initial clinical signs of the hantavirus and the symptoms are similar to those of many other tropical febrile illnesses, it is necessary to search for strategies of detection more sensitive and appropriate for the presence of these infections that could threaten the health of human populations.

Viral isolation of hantavirus is very difficult and therefore rarely used in current practice. Furthermore, this technique involves direct contact with the virus that is highly infectious, so it should be done only in laboratory biosafety level category four. RT-PCR specific to hantavirus can be used to diagnose the virus in the beginning of acute phase of the disease. However, the sensitivity of this test decreases in the recovery phase. In current practice, serology is the most common method for diagnosing the disease. Nearly all patients with SPH have a positive IgM titer at the time of evaluation. In a patient with a compatible clinical picture, the presence of an established ELISA or IFA-positive IgM allows a presumptive diagnosis, which should be confirmed with the increase in IgG or IgM titers in 2–4 weeks. On the other hand, immunological tests can also follow the evolution of the patient, detect undiagnosed prior infection and contribute to programs of epidemiologic surveillance in human beings and rodents for evaluation and control of this zoonosis.

Five-year view
Outbreaks of tropical diseases such as the hantavirus will still appear in environments where public health surveillance is lacking or non-existent. In order to better protect these communities of future outbreaks, surveillance and rapid diagnostic tests are needed to ensure a timely public health response. Immunological methods play an important role in epidemiological surveillance, however, the major disadvantage of these immunological tests is that production of the antigens used is infecting cell cultures, this means the restriction of BSL-3/4 and the variability that may exist between the antigens produced by different laboratories. For this reason, it would be of great value having a diagnostic method that does not involve the manipulation of the pathogenic virus in its development of DNA recombinant antigens. In the future, immunological methods based on recombinant antigens developed to diagnose hantavirus will be used in combination with different classes of real-time RT-PCR.

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Key issues

- Hantaviruses are transmitted by rodents, humans become infected by inhaling aerosols of excreta and urine.
- Hantaviruses cause hemorrhagic fever with renal syndrome in Europe and Asia and with cardiopulmonary syndrome in the Americas.
- The clinical course of HPS can be basically divided into three periods: a febrile prodrome, a cardiopulmonary stage and the convalescence. There is 14–17 days incubation period after exposure, which is followed by the prodrome phase, which usually lasts for 3–6 days with myalgia, malaise and fever of abrupt onset in the absence of cough and coryza. Additional symptoms seen at early stages could include mainly gastrointestinal manifestations, headache and chills.
- Laboratory abnormalities include increased hematocrit, thrombocytopenia with neutrophilia and relative lymphopenia. The first to appear is thrombocytopenia, which can anticipate the respiratory failure of 1 or 2 days. Leukocytosis is later and more specific for progression to severe cases. There are changes in blood chemistry, increased lactate dehydrogenase and transaminases.
- Detection of antigens or virus RNA is essential for early diagnosis in patients with hantavirus. Hantaviruses N-protein is the most abundant and conserved structural protein in infected cells and virions and it is the target commonly selected for the detection of IgG or IgM antibodies. Therefore, hantaviruses N-protein is more suitable for the development of monoclonal antibodies in acute or convalescent patients.
- Detection of RNA viral by RT-PCR is required to confirm clinical or post-mortem cases.
- Viral isolation is difficult and requires biosafety 3 and 4 laboratories (BSL-3/4).
- The differential diagnosis of this syndrome in tropical countries include several atypical pneumonia, influenza, heart failure, malaria, dengue, arenavirus, leptospirosis and rickettsia.
References

Papers of special note have been highlighted as:
- of considerable interest

- This is a review of hantavirus, which discussed the basic properties of molecular and cell biology of hantavirus and offers an overview of virus-induced pathology, it describes signs and symptoms of the disease, emphasizing on immunopathology and vascular problems.
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- This paper is important because it is the first serological evidence of the movement of hantavirus in Colombia in rural workers exposed to the virus and in rodents.
- This paper provides baseline data for future monitoring of hantavirus seroprevalence in Switzerland and it shows highlights of changes in the estimating of seroprevalence of these viruses in a country with very low endemicity.
37. Lundkvist A, Hörlich J, Niklasson B. In Europe, the humoral response to Puumula (nephropathia epidemica) and Dobrava virus infection is investigated by viral protein specific immunosassays. Arch Virol 1993. 130(1-2):121-30. Available from: http://dx.doi.org/10.1007/BF01319001PMid:8099274
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- This review summarizes clinical features and current epidemiological and clinical findings, as well as concepts about immunology, pathogenesis and intervention strategies of human hantaviral diseases.


- This article emphasizes critical patients with FFRS and highlights the importance of managing the potentially fatal complications and biochemical changes in the course of the disease, in order to ensure a consistent and timely treatment.


- This article is important because it shows the highlights of the medical practice in relation to emerging infectious diseases and discusses the need for better diagnostic tools desirable in tropical countries of low incomes.


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