EXPERT REVIEWS

Diagnosis of hantavirus infection in humans

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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, Hantan virus (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

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Table 1. Main genotypes, diseases, distribution and hosts of major hantaviruses causing human disease.

Genotype	Disease	Geographic distribution	Rodent(s) involved in transmission	Ref.
Hantaan	HFRS	Asia, East Russia, South China	*Apodemus agrarius*	[1]
Seoul	HFRS	Worldwide	*Rattus norvegicus*	[44]
Puumula	Nephropathia epidemica (mild form of HFRS)	Europe, Russia, Korea	*Myodes glareolus*	[45]
Dobrava-Belgrade	HFRS	Balkans countries	*Apodemus flavicollis; A. ponticus*	[46]
Sin Nombre	HPS	North America	*Peromyscus maniculatus*	[47]
Araraquara Juquitiba	HPS HPS	Brazil	*Necromys lasiurus* *Oligoryzomys nigripes*	[48]
Andes Maciel	HPS None known	Argentina, Chile Argentina	*Oligoryzomys longicaudatus* *Necromys benefactus*	[49,50]
Rio Mamoré	HPS	Bolivia, Peru	*Oligoryzomysmicrotis*	[51,30]
Choclo virus Calabazo	HPS None known	Panama Panama	*Oligoryzomys fulvescens* Zygodontomys brevicauda	[8]
Laguna negra	HPS	Paraguay	*Calomys laucha*	[52]
Caño Delgadito	HPS	Venezuela	*Sigmodon alstoni*	[6]

HFRS: Hemorrhagic fever with renal syndrome; HPS: Hantavirus pulmonary syndrome.

infection is based on four primary categories of tests: serology, reverse transcription (RT)-PCR, immunochemistry 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 retrospective 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 (sometimes 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 286 agricultural workers in Colombia [2]. These prevalences 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.

Tests	Antigen type	Sensitivity	Specificity	Commercial name	Ref.
ELISA	Sin Nombre Seoul Hantaan Dobrava Puumala	95.1%	94.1%	Hantavirus IgG and IgMDxSelect™ ELISA Kits Focus Diagnostics	[53]
ELISA	Hantaan, Dobrava, Seoul Puumala	96.7%	98.7%	Hantavirus IgG/IgM Combo Test® (Colloidal gold immunochromatographic assay, Xiamen Biotech, China)	[31]
ELISA	Araraquara (ARAVrN)	97.2%	100%	In-house ELISA	[27]
IFA	Puumala/Hantaan	98%	91%	Progen® IFA	[20]
Immunoblot assay	Puumala, Hantaan, Dobrava-Belgrade, Seoul	96%	100%	kit Mikrogen®, Neuried, Germany	[32]

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 serum titers with the relevant hantaviruses [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 biosecurity 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.

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Table 3. PCR primers frequently used for diagnosis of hantavirus infection in Europe and America.

Virus	Primer	Nucleotide sequence primer	Local annealing
Sin Nombre	S208L S1094R	5′-CCAAACTCGGAGAACTCAAAC-3′ 5′-TCAGATGTTCCCACAGATTTTG-3	246 to 369 S
Hantaan		F-HantaS (5-ACACGAACAACAGCTCGTGAC-3_ R-HantaR (5_-AGGCTCAAGCCCTGTTGGATC-3_	262 to 283 S
Hantaan		HTNV, 5′-TGGGCTGCAAGTGC-3′, 5′- ACATGCTGTACAGCCTGTGCC-3′; HTNV, 5′-TGGGCTGCAAGTGCATCAGAG-3′ 5′-ATGGATTACAACCCCAGCTCG-3	373bp to M
Seoul		SEOV, 5′-GATATGAATGATTGYTTTGT-3′, 5′- CGATCAGGGTCTYTCCA-3′ SEOV, 5′- GATATGAATGATTGYTTTGT-3′, 5′-GCAAAGTTACATTTYTTCCT-3′	242bp to M
Andes		ANDV RTPCR Fwd primer 5′-TGGCTACAAAACCAGTTGATCCAA-3′ ANDV RTPCR Rev primer 5′-CATCCAGGACATTCCCATATCTCAAA-3′	213 - 236 to S
Araraquara and Juquitiba	N-C †	CAA AAC CAG TTG ATC AAC AGG G	213 - 236 to S
Araraquara and Juquitiba	N-S ‡	GAT GAA TCA TCC TTG AAC CTT AT	454 - 477 to S
Araraquara and Juquitiba	Gn-C	GGG CAG TAA GTG CTG AAA C	1301 - 1320 to M
Araraquara and Juquitiba	Gn-S	ACA TTT AGC AGT TTG CCA TGG G	1602 - 1625 to M

†Complementary to the RNA sequence.
‡Similar to RNA (sense) [5,36,37].

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 immunofluorescence.

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 disease. 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

Table 4. Differential diagnosis, most frequent symptoms, common laboratory findings for hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome.

Latin America			USA – Canada		Europa – Asia		
Symptoms HPS	*Differential diagnosis*	*Common laboratory findings*	*Symptoms HPS*	*Differential diagnosis*	*Common laboratory findings*	*Symptoms HFRS*	*Differential diagnosis*	*Common laboratory findings*
Fever (100%)	Dengue	Atypical lymphocytes (immunoblasts)	Fever	Dengue fever	Low platelet count	Fever (94.5%),	Scarlet fever	Increase C-reactive protein
Myalgia (85–95%)	Leptospirosis	Thrombocytopenia	Myalgia	HIV Infection	Atypical	Abdominal	Acute	Leukocytosis
Nausea (73%)	Arenavirus HF	Leukocytosis	Nausea/vomiting	Leptospirosis	lymphocytes	pain	abdomen	Thrombocytopenia
Diarrhea (40%)	Malaria	Hemoconcentration	Cough	Colorado tick	(immunoblasts)	(64.4%)	Leptospirosis	Abnormal
Dizziness	Ricketsia	Increased KPTT	Dizziness	fever	Elevated	Headache	Scrub typhus	coagulation
Headache	Pneumonia	Increased creatinine	Arthralgia	Phosphine	hematocrit	(50.7%)	Murine typhus	Increase values of
Anorexia	Mycoplasmal	Hypoprothrombinemia	Shortness of	toxicity	Elevated WBC	Conjunctival	Spotted fevers	BUN and
Vomiting	pneumonia	Increase LDH	breath	Bacterial	Left shift on	hemorrhage	Septicemia	creatinine
Abdominal	Bacterial/viral	Increase CPK		pneumonia	WBC	(74.0%),	Thrombocyto	Hypercalemia
pain	pneumonia	Increase in liver		Mycoplasmal	differential	Flank	penic	Elevation of
Pulmonary	Psittacosis	enzyme		pneumonia	Low albumin	tenderness	Purpura	hemoglobin and
edema	Trichinosis	Increased amylase		Viral	Elevated LDH	(56.2%),		hematocrit
Rash	Meningococcemia	Metabolic acidosis		pneumonia	Elevated AST	Petechiae		
Respiratory	Heart failure			Pneumonic	(SGOT)	(56.2%)		
failure	Acute abdomen			plague	Elevated ALT	Abdominal		
	Typhoid fever			Salicylate	(SGPT)	and back		
				toxicity		pain		
				with		Oliguria		
				pulmonary		Renal		
				edema		insufficiency		
				Tularemia				

AST: Aspartate aminotransferase; CPK: Creatine phosphokinase; HFRS: Hemorrhagic fever with renal syndrome; KPTT: Partial thromboplastin time; LDH: Lactacte dehydrogenase; WBC: White blood cells.
Adapted from [32,38,40].

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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 rikettsia.

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