A pilot study for serological evidence of hantavirus infection in human population in south India


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Background & objectives: Hantaviruses are rodent-borne viruses of the family Bunyaviridae that have been identified as aetiological agents of two human diseases, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). There are no reports of hantavirus infections in humans from India, hence this pilot study was undertaken to provide the serological evidence of hantavirus infections in humans in south India.

Methods: Serum samples were obtained from individuals with acute febrile illness and from voluntary blood donors, majority of whom were from south India. Serum samples were tested for anti-hantavirus IgM using a commercial enzyme immunoassay (EIA). Samples found positive by the EIA were tested by an indirect immunofluorescence assay (IFA) using slides coated with Seoul virus (SEOV) infected cells as substrate.

Results: Of the 152 serum samples from individuals with pyrexial illness, 23 (14.7%) were positive for anti-hantavirus IgM by EIA. In contrast, only 5.7 per cent of healthy blood donors were positive by this assay. Eighteen of the 22 (82%) EIA-positive samples from patients were positive by the IFA assay. In contrast, only 2 of the 5 (40%) blood donor EIA positive samples were positive in the IFA assay.

Interpretation & conclusion: The finding of this study indicated the possible presence of hantavirus infections in the human population of India presenting both as asymptomatic and symptomatic infections. Further studies need to be done to confirm the findings on a larger sample using molecular techniques.

Key words Enzyme immunoassay - hantavirus - IgM antibodies - pilot study

Hantaviruses are enveloped viruses with a negative-sense single stranded RNA genome and belong to the family Bunyaviridae. The spectrum of clinical symptoms caused by hantaviruses in humans varies from sub-clinical presentation to severe haemorrhagic fever with renal syndrome (HFRS) or pulmonary syndrome (HPS). Several genotypes/serotypes have been described of which at least five are pathogenic to humans. The vast majority of human hantavirus infections are asymptomatic.
The natural reservoirs of hantaviruses are small rodents and transmission to man is believed to occur via aerosolized excretions. The worldwide distribution of rodents known to harbour hantaviruses suggests great disease causing potential.

The clinical diagnosis of hantavirus infections has routinely been confirmed by immunofluorescence antibody assay (IFA) or enzyme-linked immunosorbent assays (ELISA). The presence of cross reacting antibodies makes it difficult to distinguish hantavirus species by serology. Molecular tests based on nested reverse transcriptase-polymerase chain reaction (RT-PCR) have been used for diagnosis. Primary isolation of hantaviruses is most often attempted using Vero E6 cell line in laboratories with biosafety level-3 (BSL-3) facilities.

Hantavirus nucleocapsid protein (N) antigen elicits a strong humoral response in infected patients and immunized animals. High levels of antibody to the N antigen have been detected which indicated that it could be suitable as the sole antigen for serodiagnosis.

The Thottapalayam virus, which belongs to the same family, was isolated from the spleen of a shrew captured in July 1964, in Vellore, North Arcot district, Tamil Nadu, India. Subsequently there have been no studies showing hantavirus infections in India. We undertook this study to investigate for the serological evidence of hantavirus infections in the human population by detecting anti-hantavirus IgM antibodies.

Material & Methods

Blood samples were obtained from 152 individuals who had acute febrile illness (duration <14 days). These patients were categorized into three different categories: (i) patients (n=67) with dengue-like illness but negative for dengue serology by a commercial Dengue Duo IgM and IgG Rapid Strip test; (ii) patients (n=51) with leptospirosis-like illness, but negative for leptospira serology by the microscopic agglutination test (MAT); (iii) patients (n=34) with suspected hantavirus infection. The samples were included by convenient sampling and had been submitted to the laboratory for various tests requested by the clinicians. Serum samples were stored at -20°C until testing. All the tests were conducted at the Department of Clinical Virology, Christian Medical College, Vellore. Since this was a pilot study, sample size was not calculated. All these patients presented with complaints of high grade fever with chills and rigors of <14 days duration associated with myalgia, headache and haemorrhagic manifestations like petechiae and purpuric skin rash.

In addition, 87 blood samples from voluntary blood donors were used as controls for the study. Sixteen samples were also included from patients with other viral infections as shown by corresponding IgM testing including dengue (n=10) and cytomegalovirus (n=6) (by a commercial Euroimmun kit, Germany) to serve as disease controls. All study samples were collected during a period of 15 months (July 2002 to September 2003). This study protocol was approved by the institutional research committee for scientific content and ethics.

The hantavirus IgM ELISA kit was procured from Focus Technologies (USA). This kit has been certified by ISO for human testing. This is an indirect antibody detection system; the assays were performed according to the manufacturer’s instructions. As per the manufacturer’s claim, this assay detects IgM antibodies against most of the known hantavirus subtypes including Puumala (PUUV), Hantaan (HNTV), Seoul (SEOV), Sin Nombre (SNV) and Dobrava (DOBV).

Briefly, microwell plates were coated with a mixture of baculovirus-derived recombinant nucleocapsid proteins from hantavirus strains. Serum samples (1:101 dilution) were incubated for 60 min, and after washing, anti-human IgM-horse radish peroxidase conjugate was added to the wells and incubated for 30 min. Tetramethylbenzidine was used as a substrate. After adding the stop solution, the resultant colour change was quantified by an EIA reader ELx 800 (Bio-Tek Instruments Inc. Vermont, USA). The optical density (OD) is shown to be directly proportional to the amount of antigen-specific IgM present in the sample. Sample OD readings were compared with reference cut off OD readings. Results
were reported as index values relative to the ‘cut off’ calibrator. To calculate the index values, specimen OD values were divided by the mean of the ‘cut off’ calibrator OD values.

All the samples found positive by ELISA were further tested by an indirect immunofluorescence assay (IFA) using Seoul virus (SEOV) (strain SR-11) infected Vero E6 cells as substrate. The serum samples were tested at a dilution of 1:10. Serum samples (15 µl) were added onto the 24 well slides and incubated for 30 min. The slides were then washed three times with phosphate-buffered saline (PBS, pH=7.4) and incubated with polyclonal rabbit anti-human IgM FITC-labeled conjugate (Dakocytomation) for 30 min. The slides were then washed and after mounting it with buffered alkaline glycerol, were read under the fluorescence microscope. All spots wherein 50 per cent of infected cells showing a characteristic apple green cytoplasmic fluorescence were recorded as reactive. Samples showing a positive reaction at 1:10 dilution were further diluted and tested at dilutions 1:20, 1:40, 1:80.

Data were analysed by Chi-square test using the EPI INFO (Version 6.04b), \( P<0.05 \) was considered significant.

**Results**

A total of 255 serum samples were tested for hantavirus IgM by ELISA; 28 (10.98%) of these were positive for hantavirus IgM by EIA (Table I). Twenty seven (96.4%) of the 28 positive samples were retested and produced reproducible results; one sample was insufficient for retesting. Ten of 67 (14.9%) samples negative for dengue, 9 of 51 (17.6%) negative for leptospirosis, 3 of 34 (8.8%) of those with suspected clinical hantavirus infection, and 1 of 10 positive for dengue infection, were hantavirus IgM EIA positive. Of the 87 voluntary blood donors, 5 (5.7%) were hantavirus IgM EIA positive. None of the six samples positive for CMV serology was hantavirus IgM EIA positive.

Of the 28 hantavirus IgM positive samples, only 27 could be tested by the IFA. One sample was insufficient for testing. Of these, 20 (71.4%) were positive by IFA. A representative number (n=5) of EIA negative samples were negative by IFA.

Examination of the patients’ clinical records revealed that of the 23 patients who were seropositive for hantavirus IgM, eight had other underlying diseases. Records of three patients were not available for evaluation. The remaining 12 labeled as cases of pyrexia of unknown origin (PUO) were evaluated based on the clinical history at the time of presentation, the laboratory findings and the final diagnosis (Table II).

Among the individuals with febrile illnesses (n=152, 59.6%), 14.5 per cent (95% CI: 9.5-20.75) were positive for anti-hantavirus IgM antibodies. The difference in the rate of positives between the study group and the blood donor healthy control group (n=87)
where the positivity rate was 5.7 per cent (95% CI: 2.13-12.27) was statistically significant \((P<0.05)\).

**Discussion**

This is probably the first study in India showing serological evidence of hantavirus infection in the Indian population. Clinically, the febrile illness produced by dengue virus, leptospira serotypes and hantaviruses may be indistinguishable\(^\text{11}\). The rate of IgM positive status in the febrile illness group was significantly higher than that seen in the healthy blood donors. We also found that this test did not pick up significant false positives in the samples positive for dengue or CMV IgM. It could be assumed that the positives in the control group indicated asymptomatic infections. Likewise, the hantavirus IgM seropositives in patients with other underlying diseases could be due to serological cross reactivity or asymptomatic infections. Alternatively, it could also be speculated that there are multiple hantaviruses in circulation and that at least one is of low pathogenicity.

It was noted that the mean of index values of the blood donor controls which were positive for hantavirus IgM (1.2±0.35) was lower than that of the cases (3.5±2.5). Such information could be used to establish a baseline ‘cut off’ index value to differentiate symptomatic from asymptomatic cases following screening of a larger number of individuals.

Hantavirus infections can appear clinically uncharacteristic and may mimic other syndromes. This compounds the difficulties in diagnosing hantavirus infections in areas where the disease is not endemic and clinical cases may be sporadic\(^\text{12}\). Further, less pathogenic hantaviruses may cause a greater amount of asymptomatic infections, as seen for HFRS in Europe and Asia\(^\text{13}\).

Though a good association was seen in the findings of the two serological assays, EIA and IFA, IFA was cumbersome and required specialized equipment and experienced personnel for accurate interpretation. The difference in positives between the two assays could be due to the fact that the EIA had a cocktail of baculovirus-derived recombinant nucleocapsid antigens from different hantavirus strains while in the IFA only Seoul virus antigen was used. The EIA offered the advantage of a rapid and less complicated detection system and could be done even in middle-level laboratories.

Positive serology should be interpreted very cautiously when done on single serum samples. Paired serum samples may help delineating true hantaviral infections. Further, the results of serology should be correlated with clinical features to arrive at a definitive diagnosis. Future studies need to be focused on confirmatory testing by detecting the hantaviral genome using RT-PCR in samples collected in the acute phase of illness.

Since no previous published data on hantavirus infections in India are available and not much is known about laboratory diagnosis or clinical cases, general physicians are probably unaware of diseases with hantavirus aetiology. This suggests that there could be circulation of the hantaviruses in the Indian population. Further studies need to be done to identify hantaviruses in clinical samples by molecular techniques to definitely describe the clinical picture of hantaviral infections in India. The molecular techniques will include primers that can identify Thottapalayam virus, the only hantavirus isolate from India. These techniques will also help to identify the rodent reservoirs and to elucidate the importance of potentially unknown hantaviruses in India.

**References**


