INVESTIGATION OF HANTAVIRUS INFECTIONS AMONG CCHFV NEGATIVE CASES IN THE WESTERN BLACK SEA REGION OF TURKEY

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ABSTRACT

Objective: The Hantaviruses and Crimean Congo hemorrhagic fever virus (CCHFV) are members of the Bunyaviridae family. Hantavirus infections cause two main febrile diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Hantaviruses are transmitted by rodents. In 2009, a hantavirus outbreak occurred in the Western Black Sea region of Turkey. For the last 10 years, the prevalence of CCHFV has also been high in this region.

Methods: We screened patients’ samples for the presence of hantavirus IgM and IgG using ELISA on 42 clinically suspect CCHF cases which were CCHFV real-time-RT-PCR and anti-CCHFV IgM negative without a tick-bite history. The current study was carried out on samples sent between March and September of 2008. SELISA-seropositivity was further evaluated using immunofluorescence assay, immunoblotting, and FRNT (focus reduction neutralization test). Real-time-PCR was performed to genotype the seropositive cases.

Results: Anti-Hantavirus IgM and IgG positivity were detected in 3 samples (7.1%) by ELISA and immunoblotting tests. Two had a 1:640 titer against Puumala virus (PUUV) strain by FRNT, while all three samples were found negative for Dobrova virus (DOBV). Thus, hantavirus-specific antibodies could be confirmed detected in 2 out of 42 sera (4.8%) by FRNT.

Conclusions: The authors postulate that Hantavirus infection should be taken into consideration in patients with clinically suspected CCHFV infection. This study also shows that Hantavirus was circulating in the area before the 2009 hantavirus outbreak in the Western Black Sea region of Turkey.

Key words: Hantavirus, CCHFV, ELISA, real time-RT-PCR, FRNT, Turkey.

Received February 18, 2014; Accepted May 19, 2014

Introduction

Hantaviruses belong to the Bunyaviridae family, which includes five genera: Bunyavirus, Phlebovirus, Nairovirus, Tospovirus, and Hantavirus. The Bunyavirus family comprises a number of arthropod-borne viruses (arboviruses, while the Tospoviruses are plant viruses, and the Hantaviruses rodent-borne1). The viruses within the genus Hantavirus causes various febrile diseases: hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, and hantavirus cardiopulmonary syndrome (HCPS) in the Americas2. Hantaviruses are transmitted by rodents and insectivores. The infection in humans is transmitted via aerosols of infected rodents’ saliva, feces and urine1,2. Recently published data have revealed that also bats are possible hantavirus vectors3.

Hantaviruses are negative-sense, single-stranded enveloped RNA viruses with a genome of three single-stranded RNA segments designated S (small), M (medium) and L (large)1. Clinically, hantavirus infections range from subclinical to fatal and can be separated into five main phases: febrile,
hypotensive, oliguric, diuretic and convalescent\(^{(4)}\). The febrile phase of hantavirus infection lasts for 3-7 days, accompanied by headache and abdominal pain\(^{(5)}\).

Hantavirus infection is a global public health problem with approximately 50,000 to 150,000 annual cases reported worldwide. Most cases (about 50,000) are caused by Hantaan virus (HTNV) and Seoul virus (SEOV) in Asia. In addition, approximately 200 cases are diagnosed annually as HCPS in the United States\(^{(6)}\). Puumala virus (PUUV) and Dobrava virus (DOBV) have caused the majority of the HFRS cases in Europe\(^{(4)}\).

Each Hantavirus is transmitted predominantly by its own natural host, whether rodent or insectivore\(^{(7,8)}\). Of the approximately 2,000 existing rodent species, only about 5% so far been tested for the presence of Hantaviruses\(^{(9)}\). The most common route of hantavirus infection is via inhalation of infectious aerosolized saliva or excreta originating from infected rodents\(^{(10)}\). Humans can become infected by exposure to contaminated dust from rodent nests or droppings\(^{(4,6,10)}\). Human to human transmission is not generally occurring\(^{(11)}\) except for the Andes virus (ANDV) in Argentina\(^{(12)}\).

There are three distinct genetic groups of rodent hosts: Murinae, Arvicolinae, and Sigmodontinae rodents. HTNV, SEOV and DOBV viruses causing more severe forms of HFRS are carried by Murinae rodents mainly found in Asia and the Balkans. PUUV, hosted by Arvicolinae voles in most European countries, causes a milder form of HFRS. The Hantaviruses in the Americas are mainly transmitted by rodents of the subfamilies Sigmodontinae and Neotominae\(^{(13)}\). In Europe, three Hantaviruses (PUUV, DOBV, and Saaremaa SAAV) are known to cause HFRS. PUUV and SAAV generally cause milder HFRS, but DOBV infections often also induce hemorrhagic complications\(^{(14)}\).

PUUV, DOBV and SAAV viruses are carried by the bank vole (Myodes glareolus), yellow-necked mouse (Apodemus flavicollis) and striped field mouse (Apodemus agrarius), respectively. SEOV is carried by Rattus norvegicus and R. rattus in Europe, but only a few cases have been reported so far\(^{(14,15)}\). Co-evolution has been suggested for the Hantaviruses and their vectors\(^{(7,8)}\). Several hantavirus-carrying rodent species have been identified in Turkey, which also hosts 45 rodent species and 17 endemic insectivore species\(^{(16,17)}\).

In 2009, a hantavirus outbreak occurred in the Western Black Sea region of Turkey from January to May\(^{(18)}\). In this study, we investigated anti-hantavirus ELISA IgM and IgG positivity in samples from hospitalized clinically suspected Crimean Congo hemorrhagic fever (CCHF) cases without a tick-bite history from the same geographical area as the earlier Hantavirus outbreak. These patients’ sera samples had been sent before the outbreak, in 2008, to the Public Health Institute of Turkey (PHIT), Department of Microbiology Reference Laboratories (formerly, the Refik Saydam National Public Health Agency).

**Materials and methods**

**Samples**

After the 2009 hantavirus outbreak on the Western Black Sea coast (specifically Bartin and Zonguldak provinces), it was decided to investigate the presence of hantavirus infections among the CCHF negative (ELISA IgM, IgG and PCR), but clinically suspected CCHF samples. According to the case report forms, there was no evidence of tick-bite history in the selected cases’ anamnesis. For all samples, an ‘in-house’ one-step real-time PCR for CCHFV was performed as in Yapar et al.\(^{(19)}\). Anti-CCHFV IgM and IgG assays were performed according to the CDC (Centers for Diseases Control and Prevention, US) protocol\(^{(20)}\). 42 serum samples stored at -80\(^{0}\)C were selected matching the conditions listed above from the Virology Reference and Research Laboratory of PHIT. Of the 42 samples, 26 were from Kastamonu, 10 from Karabük, two from Bartin, two from Bursa, one from Bolu and one from the Zonguldak provinces (Figure 1). Only seven samples, sent from Kastamonu province, had follow-up serum samples taken 10-16 days after the initial sampling. This study includes the results for these follow-up samples. Only seven samples, sent from Kastamonu province, had follow-
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**Anti-hantavirus IgM and IgG ELISA**

Commercial hantavirus ELISA tests (Focus Diagnostics, DxSelect, Cypress, CA, USA), which detect antibodies of the most clinically relevant pathogenic strains of Hantaviruses: SEOV, HTNV, PUUV, DOBV, and SNV were used according to the manufacturer. The results were reported as index values (I.V.) relative to the cut-off calibrator. To calculate I.V.s, optical density (O.D.) values of the specimens are divided by the mean of the cut-off calibrator absorbance values. An I.V. of > 1.10 is indicative of the presence of IgM and IgG hantavirus specific antibodies.

**Immunoblotting assay for hantavirus antibodies**

An immunoblotting assay was used for confirmation of the ELISA IgG and IgM positive samples. The Hanta Profile 1 EUROLINE (Euroimmun, Luebeck, Germany) tests were performed according to the manufacturer’s protocol. The strip provides a semi-quantitative assay for detection of human antibodies of the IgG class for three different hantavirus serotypes: PUUV, DOBV and HTNV. The processed strips were visually evaluated from (0) to (+++). Medium (+) to strongly (++/++++) colored bands were considered to indicate positivity.

**RNA extraction for molecular analysis**

RNA was extracted from serum samples using a commercial extraction kit (QIAamp viral RNA mini extraction kit, QIAGEN), following the manufacturer’s instructions.

**Real-time RT-PCR assay for hantaviruses**

The Hantavirus Renal Syndrome General-type I&II Real Time RT-PCR kit (Shanghai ZJ Bio-Tech Co., Ltd., China) was used for the detection of the Hantavirus genome in serum using the ABI Prism 7500 real time PCR system (Applied Biosystems, CA, USA). The following protocol was used for real time-PCR: 45 °C for 10 min., 1 cycle; 95 °C for 15 min., 95 °C for 15 sec., 60 °C for 60 sec., and 40 cycles.

**Focus reduction neutralization test (FRNT) for seropositive samples**

As PHIT lacked the technical capability at the time of the study, the three seropositive samples tested as PUUV serotype by hantavirus immunoblotting were sent to Sweden for confirmation by FRNT tests. For analysis by FRNT, the PUUV strain Sotkamo(21) and the DOBV strain Slovenia(22) were used. The test was performed as described earlier(23). Briefly, sera were serially diluted and mixed with an equal volume of diluted virus, containing 30-70 focus-forming units/100 μl. The mixture was incubated at 37°C for 1 h. and subsequently inoculated into six-well tissue culture plates containing confluent Vero E6 cell monolayers. The wells were overlaid with a mixture of agarose and tissue culture medium and incubated for 9 (DOBV) or 13 days (PUUV). The agarose was removed from the wells and the cells fixed in methanol. Rabbit anti-hantavirus sera, followed by peroxidase labeled goat antibodies to rabbit IgG (BioRad Laboratories, Hercules, CA), were added to indicate virus-infected cells. 3, 3', 5, 5'-tetramethylbenzidine substrate (Sigma) was used as substrate and foci were enumerated. An 80% reduction in the number of foci compared to the virus control was used as the criterion for virus neutralization titers.

**Ethical approval**

This investigation was designed and conducted after gaining the official permission of the RSNPHA Scientific Board, Ankara and approval from the Erciyes University Ethical Board, Kayseri, Turkey.

**Results**

The serum samples were drawn from 22 male (mean age 51) and 20 female patients (mean age: 54 years). According to the patients’ declaration of their occupations, there was one housewife with no link to cattle-breeding, 7 wives of cattle-breeders, 6 retired people, 5 cattle-breeders, 5 housewives with unspecified additional occupation, 4 farmers of which 3 were also involved in cattle-breeding, 3 students, 2 merchants, 1 forestry worker, 1 unemployed person, 1 nurse, 1 carpenter and 2 patients with unspecified occupations.

Thrombocytopenia, fever and hemorrhage were reported in 88.1%, 66.7%, and 7.4% of the patients respectively. All the samples from patients without tick-bite history were selected in order to exclude a diagnosis of CCHF. The patients’ sera had been sent to our laboratory between March and September of 2008 for diagnosis of CCHF.
Seven follow-up samples were also sent from the Kastamonu province’s suspected CCHF patients.

Anti-Hantavirus IgM and IgG positivity were detected in three (7.1%) of the samples by ELISA. Two had a 1:640 titer against PUUV strain while all three were negative for DOBV.

After screening for antibodies by ELISA and immunoblotting tests, hantavirus-specific antibodies were confirmed by FRNT in 2 (4.8%) of the 42 samples.
The seropositive patients’ occupations were all housewife with no link to cattle breeding or farming. These patients were living in the Karabük province, which borders both Bartin and Zonguldak, where the Hantavirus outbreak occurred in 2009 (Figure 1).

According to the case report forms of these confirmed patients, one of them had hemorrhage, one had only thrombocytopenia, while one had both fever and thrombocytopenia. Table 1 summarizes the characteristics and laboratory findings of the 2 hantavirus cases.

<table>
<thead>
<tr>
<th>Characteristics of patient</th>
<th>1st confirmed case</th>
<th>2nd confirmed case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Occupation</td>
<td>Housewife – no cattle breeding</td>
<td>Farmer</td>
</tr>
<tr>
<td>Urban/Rural Area</td>
<td>Urban</td>
<td>Urban</td>
</tr>
<tr>
<td></td>
<td>(but he visited rural areas 2 weeks prior to DOS)</td>
<td></td>
</tr>
<tr>
<td>Time interval between the day of onset of symptoms (DOS) and sampling (days)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Onset of infection</td>
<td>April 2008</td>
<td>June 2008</td>
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</tbody>
</table>

Laboratory findings

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<thead>
<tr>
<th>ELISA</th>
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<tbody>
<tr>
<td>Anti-Hantavirus IgM</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Anti-Hantavirus IgG</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<th>Immunoblotting</th>
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<tbody>
<tr>
<td>Anti-Hantavirus IgM</td>
<td>Positive (PUUV ++)</td>
<td>Positive (PUUV ++)</td>
</tr>
<tr>
<td>Anti-Hantavirus IgG</td>
<td>Positive (PUUV +)</td>
<td>Positive (PUUV +)</td>
</tr>
</tbody>
</table>

| Real time RT-PCR for Hantavirus | Negative | Negative |
| FRNT for PUUV                   | Positive (1:640) | Positive (1:640) |
| FRNT for DOBV                   | Negative (< 40)  | Negative (< 40)  |

Table 1: Laboratory findings in the confirmed hantavirus cases

Discussion

Hantaviruses are rodent- or insectivore-borne emerging viruses. The rodent-borne viruses cause two important human diseases, HFRS in Asia and Europe, and HCPS in the Americas(25). HFRS cases usually occur in rural areas with the condition being an occupational hazard for forest workers, farmers, shepherds, military personnel and woodcutters(1,4).

The majority of PUUV-associated cases occur in the fall, while most DOBV-caused HFRS cases are registered in late spring and summer(19).

HFRS outbreaks due to PUUV are associated with an increased population of the bank vole (Myodes glareolus), the main natural rodent host for PUUV(1,4). Thus, changes in rodent population density influence the scale of hantavirus infection outbreaks in humans(1,2,24).

At the same time, elevated average temperatures in West-Central Europe have also been associated with more frequent PUUV outbreaks, through high food production (mast years) and subsequently higher bank vole densities(2,24).

The bank vole, the carrier of PUUV, has a distribution range that includes most of Europe and is present in Turkey as well(26). Human disease incidence and epidemiological patterns vary greatly across the continent(27).

In Turkey, PUUV infections in rodents was demonstrated for the first time using immunofluorescence assays in Microtus voles in two regions. Of 330 rodents captured in Izmir, Trabzon and Rize by Laakkonen et al in 2004(25), PUUV antibodies were found in four out of 65 Microtus voles. One of them (Microtus guentheri lydiius) originated from Izmir, while three (one Microtus robori and two Microtus rossiaemeridionalis) were captured around Trabzon. Laakkonen et al were not able to amplify RNA from these samples by RT-PCR.

Kavukcu et al. reported that hantavirus seropositivity was 7.3% among cases with nephropathy but 2.6% in cases without nephropathy or any infectious disease. From this, they concluded that hantavirus disease was only present in the Aegean Region and Anatolia(28).

Since the first reported outbreak of hantaviruses in Turkey in 2009(18), sporadic cases have been detected in several Eastern Black Sea region provinces, such as Giresun and Ordu, and in rural areas of Istanbul in the Marmara region(27,28).

In order to investigate further, we planned a hantavirus study of CCHF suspected but CCHFV negative sera samples (n: 42) collected in 2008. In the current study, two samples (4.8%) were found to be PUUV positive by FRNT. These serological results show that there were hantavirus cases occurring before the 2009 outbreak. One of the two hantavirus seropositive patients had gross hemorrhages, and one had hematuria in their anamnesis forms. In cases of PUUV serotype, hemorrhages are seen in only 2% of samples(18). One of our patients had only fever and another had both fever and thrombocytopenia. According to the literature, HFRS cases usually occur in rural areas and are
generally seen in farmers, forest workers and shepherds\(^8\).

In the current study, one of the two seropositive patients was a farmer as expected from the literature. The two patients became ill in late spring or summer and had not been abroad in their anamnesis during the preceding 2 months. HFRS cases are also known to coincide with increased agricultural activities\(^1\).

After the hantavirus outbreak in 2009, a National Hantavirus Study Group was formed in Turkey by the Ministry of Health. In June 2009, a total of 173 rodents and 2 shrews were collected from 7 sites in rural areas of Bartın Province, where suspected human hantavirus cases had been reported. Using RT-qPCR, Oktem et al found 23 DOBV RNA-positive rodents from the outbreak area, including 10 A. flavicollis mice, 12 A. uralensis mice, and 1 Rattus rattus rat\(^1\).

According to the results of the current study, hantavirus infection should be considered in serological and real time-PCR-negative CCHF suspected patients in the Black Sea region in Turkey. The current findings also show that there were positive hantavirus human cases before the 2009 outbreak emerged. This is the first report in the literature to use neutralization tests to confirm historical hantavirus human infections in Turkey.

References

24) Heyman P, Thoma BR, Marié J-M, Cochez C, Essbauer SS. In search for factors that drive hantavirus epi-
Acknowledgements

This study was funded by the RSNPHA Scientific Board (BAP Project). We thank the laboratory personnel of the Virology Laboratory of RSNPHA for excellent technical assistance, and the Primary Health Care Directorate of the Turkish Ministry of Health, Provincial Health Directorates, for their collaboration and for organizing the sample collection.

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