Original Article

Serologic Investigation of Hantavirus Infection in Patients with Previous Thrombocytopenia, and Elevated Urea and Creatinine Levels in an Epidemic Region of Turkey

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SUMMARY: The first cases of Hantavirus infection in Turkey were reported in early 2009 in the Zonguldak and Bartin provinces. The aim of this study was to investigate the presence of Hantavirus antibodies in patients who had clinical and laboratory findings that were potentially associated with Hantavirus infection prior to the epidemic in Bartin in 2009. After screening 314,577 medical records from between 2007 and 2009, the clinical and laboratory data for 442 patients meeting the criteria of coexistent thrombocytopenia, and elevated urea and creatinine levels were transferred to a statistical program. Home visits were made to 170 patients, 84 of whom consented to participate in the study. The participants completed a questionnaire and provided a blood sample. Commercial anti-Hantavirus IgG and IgM ELISA and immunoblotting assays were used, with seropositive samples being confirmed by focus reduction neutralization tests (FRNT). ELISA and/or immunoblotting assays detected 10 positive samples; however, only 7 of these were recorded as positive by FRNT. FRNT positivity was significantly associated with female sex, the presence of a barn near to the house, and working in a forest ($P < 0.05$).

In a Hantavirus endemic region, physicians must keep in mind that thrombocytopenia, and elevated urea and creatinine levels may indicate Hantavirus infection.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent-borne zoonotic diseases caused by the Hantavirus genus from the Bunyaviridae family. Humans are infected through inhalation of contaminated aerosols originating from the pulmonary secretions, saliva, and urine of infected rodents (1-4).

HFRS manifests as mild, moderate, or severe disease, depending on the causative virus (5). In general, after infection with Hantaan virus (HTNV) or Dobrava-Belgrade virus (DOBV), there is a 2- to 3-week incubation period followed by a typically 5-phase clinical course that includes febrile, hypotensive, oliguric, diuretic, and convalescent periods (6). Puumala virus (PUUV) infection usually induces a mild form of HFRS called nephropathia epidemica, although it can occasionally result in a more serious form of the disease characterized by renal failure and circulatory shock. PUUV is endemic in northern Europe (Norway, Sweden, Finland, and Russia) and parts of central Europe (France, Belgium, and Germany) (7).

The number of patients infected with hantaviruses has reached levels which pose a threat to public health worldwide. Each year, approximately 150,000 to 200,000 patients are hospitalized for HFRS worldwide, with more than 10,000 patients diagnosed with HFRS in Europe (1). Approximately 200 cases of HPS are reported annually in the United States. Since most Hantavirus infections are asymptomatic (5:1–10:1), the number of reported cases does not represent actual infection rates (1). Therefore, seroepidemiologic studies are required to detect Hantavirus infection levels more accurately.

The first cases of Hantavirus infection in Turkey were reported in early 2009 in the Western Black Sea Region (Zonguldak and Bartin provinces). Patients presented clinically with high fever, restlessness, abdominal pain, diarrhea, and altered consciousness, with subsequently thrombocytopenia and acute renal failure developing during the following week. Hantavirus antibodies were found in the serum samples of these patients using indirect fluorescent antibody tests and immunoblotting techniques (8). Seroprevalence of PUUV was found in 5.2% of at-risk groups for Hantavirus infection in Bartin (8).
The aim of this study was to investigate the presence of Hantavirus antibodies in patients admitted to Bartin State Hospital prior to the 2009 epidemic with coexistent thrombocytopenia, and elevated urea and creatinine levels, clinical and laboratory findings that are associated with Hantavirus infection.

MATERIALS AND METHODS

Subjects and samples: Clinical and laboratory data were evaluated in light of current literature, and the case definition criteria for the European Network for Diagnostics of “Imported” Viral Diseases (ENIVD) were reviewed (9,10). The clinical and laboratory parameters indicative of Hantavirus infection according to the current literature and ENIVD criteria are thrombocytopenia, and elevated creatinine, urea, lactate dehydrogenase, and C-reactive protein levels. In this study, thrombocytopenia was defined as a platelet count ≤150,000/mm³ and elevated creatinine and urea levels were defined as ≥1.6 mg/dl and ≥40 mg/dl, respectively. The study was performed from May to July 2011, and the blood samples were collected during the same period. All sera samples collected from patients were sent to the Public Health Institute of Turkey (PHIT), Ankara, under cold-chain conditions.

A total of 314,577 medical records from the Bartin State Hospital electronic database from January 1, 2007 and December 31, 2009 were screened. Clinical and laboratory data from 442 patients ≥18 years old who met the criteria for coexistent thrombocytopenia, and elevated urea and creatinine levels were transferred to a statistical program (SPSS version 17). Of these patients, 272 who were alive and who had not been subsequently diagnosed with chronic renal failure and/or nephropathy were included in the study. Home visits were made to 170 patients whose home addresses were recorded in the database; of whom, 84 consented to participate in the study, and provided informed consent. A questionnaire was completed using a face-to-face interview method. Blood samples (5–10 ml) were taken from each participant and sent to the laboratory in compliance with cold-chain condition regulations (Fig. 1).

Serology: The serum samples were transported at +4°C. All sera were then stored at −25°C at PHIT, Microbiology Reference Laboratory, Ankara, Turkey, until further analysis. ELISA and immunoblotting tests were performed at PHIT. The focus reduction neutralization tests (FRNT) were performed on seropositive samples at the Swedish Institute for Communicable Disease Control and Karolinska Institutet, Stockholm, Sweden. (i) Anti-Hantavirus IgG and IgM ELISA: Commercial ELISA IgG and IgM kits (Focus Diagnostics, DxSelectTM, Cypress, CA, USA) were used to screen samples. These kits detect antibodies for Hantavirus strains including: Seoul virus, HTNV, PUUV, DOBV, Saarema virus (SAAV), and Sin Nombre virus. According to the manufacturer’s guidance, samples with an index value >1.1 are considered IgG or IgM positive to one or more Hantavirus species. (ii) Immunoblotting assay: For confirmation of the ELISA IgG and/or IgM positive samples, commercial immunoblotting assays for IgG and IgM (Hanta Profile 1 EUROLINE, Euroimmun, Germany) were used. The strips provide a quantitative assay to detect the IgG and IgM class of 3 different Hantaviruses: PUUV, DOBV, and HTNV. The strips were visually evaluated and graded from 0 to +3. (iii) FRNT: The PUUV Sotkamo strain (11), SAAV Saaremaa strain (12), and DOBV Slovenia strain (13) were used for FRNT analyses. The tests were performed as previously described by Lundkvist et al. (14). In this protocol, the ELISA and/or immunoblotting seropositive samples were serially diluted and mixed with an equal volume of diluted virus, containing 30–70 focus forming units/100 ml. The mixture was incubated at 37°C for 1 h, and subsequently inoculated into 6-well tissue culture plates containing confluent Vero E6 cell monolayers. The cell culture wells were overlaid with a mixture of agarose and tissue culture medium, and incu-
bated for 9 days for DOBV and SAAV, and 13 days for PUUV. The agarose was then removed from the wells and the cells were fixed in methanol. Rabbit anti-Hantavirus sera, followed by peroxidase-labeled goat antibodies to rabbit IgG (BioRad Laboratories, Hercules, CA, USA) were added to indicate virus-infected cells. We used 3,3′,5,5′-tetramethylbenzidine (Sigma, Saint Louis, MO, USA) as the substrate and foci were enumerated. An 80% reduction in the number of foci was used as the criterion for virus neutralization titers as compared to the virus control.

**Statistical analysis:** The statistical analysis of the results was carried out using SPSS for Windows (version 17.0). Correlates between seropositivity and variables were calculated with Fisher’s exact test. All P values were 2-tailed and the statistical significance set at $P < 0.05$.

**RESULTS**

Of the 84 participants, 64.3% were men and 48.8% were $\geq 71$ years of age. Chronic diseases were reported by 61.9% of participants (coronary artery disease/congestive heart failure, hypertension, diabetes mellitus, kidney stones, allergic bronchitis, hepatitis C, hypothyroidism, benign prostatic hypertrophy, chronic obstructive pulmonary disease, hypercholesterolemia, renal dysfunction, or renal colic). The most frequently reported chronic diseases were coronary artery disease/congestive heart failure (25%) and hypertension (23.1%). The social and demographic data and health status of the participants are presented in Table 1.

The housing conditions and daily activities of the participants are presented in Table 2. Of the participants, 72.6% lived in detached houses, 89.2% had a house with garden, and 9.4% had pets in their houses. Of the participants, 83.3% used tap water, 77.4% had a wood store attached to their house, and 46.4% had a barn. Of the daily activities investigated, 69.0% of the participants grew their own vegetables/fruit, 58.3% collected wild food, 51.2% had seen a mouse, rabbit, or other rodent around their house, 44.0% worked in the forest, and 42.9% had undertaken excursions, sport, or picnics in the forest.

ELISA and immunoblotting IgG and/or IgM detected 10 samples positive for Hantavirus. Seven of which were also found to be seropositive according to FRNT. Six were positive for PUUV and 1 was positive for DOBV. Thus, the FRNT results were similar to the immunoblotting results (Table 3). Anti-Hantavirus IgG positivity was found by ELISA and immunoblotting assays on 7 of the FRNT positive samples. The IgM antibody was detected in only 2 cases by immunoblotting and in 1 case by ELISA. Table 3 presents the complete ELISA, immunoblotting assay, and FRNT results.

Investigation into the associations between the FRNT results and age, sex, town, type of house, presence of a garden, and activities of daily living indicated that Hantavirus antibody positivity with FRNT was significantly associated with sex ($n = 7$, all men; $P = 0.047$), the presence of barn near to the house ($n = 6$; $P = 0.046$), and in working a forest ($n = 6$; $P = 0.04$).

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**Table 1.** Distribution of social and demographic data and health status of the participants who enrolled in the study with thrombocytopenia, and elevated levels of urea and creatinin between 2007 and 2009 who consented to participate in the study

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\leq 30+$</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>31–50</td>
<td>7</td>
<td>8.3</td>
</tr>
<tr>
<td>51–70</td>
<td>33</td>
<td>39.3</td>
</tr>
<tr>
<td>$\geq 71$</td>
<td>41</td>
<td>48.8</td>
</tr>
</tbody>
</table>

Mean ± SD = 65.6 ± 15.3 Median = 70.0 Min-max = 18–86

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>54</td>
<td>64.3</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>35.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reported chronic diseases</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>32</td>
<td>38.1</td>
</tr>
<tr>
<td>Yes</td>
<td>52</td>
<td>61.9</td>
</tr>
<tr>
<td>Coronary artery disease/congestive heart failure</td>
<td>13</td>
<td>25.0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>12</td>
<td>23.1</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>11</td>
<td>21.2</td>
</tr>
<tr>
<td>Other diseases(1)</td>
<td>16</td>
<td>30.8</td>
</tr>
</tbody>
</table>

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Table 3. FRNT results and conclusion of ELISA and immunoblotting assay IgG and/or IgM positive samples

<table>
<thead>
<tr>
<th>Season</th>
<th>ELISA1) IgM immunoblotting</th>
<th>IgM immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Results</td>
<td>Conclusion</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>2007-II Winter</td>
<td>PUUV/DOBV</td>
<td>Previous infection</td>
</tr>
<tr>
<td></td>
<td>PUUV 0.476</td>
<td>PUUV</td>
</tr>
<tr>
<td></td>
<td>DOBV-HTNV</td>
<td>DOBV</td>
</tr>
<tr>
<td></td>
<td>1:160 (PUUV)</td>
<td>1:160 (DOBV)</td>
</tr>
<tr>
<td>2008-II Autumn</td>
<td>PUUV/DOBV</td>
<td>Previous infection</td>
</tr>
<tr>
<td></td>
<td>PUUV 0.048</td>
<td>PUUV</td>
</tr>
<tr>
<td></td>
<td>DOBV-HTNV</td>
<td>DOBV</td>
</tr>
<tr>
<td></td>
<td>1:160 (PUUV)</td>
<td>1:160 (DOBV)</td>
</tr>
<tr>
<td>2009-II Winter</td>
<td>PUUV/DOBV</td>
<td>Previous infection</td>
</tr>
<tr>
<td></td>
<td>PUUV 0.094</td>
<td>PUUV</td>
</tr>
<tr>
<td></td>
<td>DOBV-HTNV</td>
<td>DOBV</td>
</tr>
<tr>
<td></td>
<td>1:160 (PUUV)</td>
<td>1:160 (DOBV)</td>
</tr>
</tbody>
</table>

The seasonal distribution of clinical presentation for the 7 cases that were seropositive for Hantavirus antibodies according to FRNT were: 57.1% (n = 4) in autumn, 28.6% (n = 2) in winter, and 14.3% (n = 1) in spring.

**DISCUSSION**

Diseases caused by hantaviruses have attracted a lot of attention recently in Turkey, as they have in most European countries. The incidence rate of Hantavirus-associated disease is estimated to be approximately 40/100,000 per year. However, the actual rate is probably 7–8 times higher because of the asymptomatic nature of Hantavirus infection and its flu-like symptoms in most individuals. Only an estimated 20–30% of infected patients are diagnosed serologically (7,15,16).

A literature review has reported that the seroprevalence of Hantavirus infections varies between 0.5 and 33.3% in some Asian countries, and from 0 to 24.0% in some European countries (1).

HFRS is endemic to the Balkan Peninsula and Russia. The phylogeny of DOBV and epidemiology of infection in rodents and humans in Greece has been reported (17). Akritidis et al. presented a case report indicating a reappearance of viral HFRS in northwestern Greece in 2008 (18). Severe HFRS cases caused by DOBV have also been reported in Bulgaria (19), Georgia (20) and Russia (21). PUUV is very common in Eastern parts of Europe, the Balkan Peninsula, and Russia (2,4,7). As it lies between Asia and Europe, Turkey could be a bridge with respect to Hantavirus infection (22).

According to research from 2009 in Bartin, the region of our study, Ig-G seroprevalence was found to be 5.2% in at-risk groups, such as forest workers, farmers, and hunters (8). The aim of our study was to investigate the frequency of seropositivity in patients whose past clinical and laboratory findings were possibly compatible with Hantavirus infection using patient records from between 2007 and 2009 from Bartin State Hospital. Our findings indicate higher Hantavirus seroprevalence in Bartin (11.9%) compared to the previous report (5.2%) in the at-risk population (8). Our results also suggest that the Hantavirus was in circulation and infection was present in this region before the 2009 outbreak. A similar study conducted in 2007 in western Turkey found seroprevalence rates of 7.3% in patients with nephropathy compared to 2.6% in the control group (23).

Of the 10 samples found positive by ELISA, 6 were found to be PUUV serotype and 1 was found to be DOBV in the FRNT test. Three of the serum samples (2007-II, 2008-IV, and 2009-I) that were found to be positive by ELISA and the immunoblotting test were negative according to FRNT. ELISA assays are normally used to screen for serologic diagnosis of Hantavirus infection. However, false positivity can be high with ELISA methods. The discrepancy shown here suggests either false positivity arising from a greater sensitivity of the screening test or the presence of a Hantavirus serotype not covered by the FRNT test, which only includes PUUV, SAAV, and DOBV serotypes. However, in the acute phase, the sensitivity of FRNT is not considered to be high. For example, the 2007-II sample showed high levels of IgM antibodies and an immunoblotting posi-
tive pattern to the DOBV-HTNV antigen, but it was FRNT negative.

Although the screened records were from 2007–2009, IgM seropositivity showed a weaker reaction than IgG seropositivity, which can be attributed to the collection of serum samples in 2011. That is, all positive results were evaluated as indicating previous infection. Therefore, it can be claimed that the disease has been present in this region since 2007, and that seropositivity developed when patients attended the hospital between 2007 and 2009. A study on rodents in the Bartin region in May 2009, following the outbreak, showed that they were carrying DOBV (24).

The roles of humans and animals in the epidemiology of diseases caused by Hantavirus are poorly understood (25). Although some risk factors are known, for example, age, sex, and living in a rural region (4,26,27), evidence is conflicting (28,29). Some studies have shown that seropositivity is significantly higher in men than in women, and some have reported sex as a risk factor for Hantavirus infection (7,29,31). In one retrospective study, which investigated the clinical signs of 75 patients with Hantavirus infection aged 16–82 years, the infection rate was 2.5 times higher in men than in women (10). Yet, other studies have shown no association between sex and frequency of Hantavirus infection (27,30). There is also a lack of consensus regarding the etiology of this sex imbalance, although some hypotheses suggest hormonal, behavioral, immunologic, or genetic factors (31).

A study in Egypt found no relationship between Hantavirus infection and age, sex, or place of living. Rather, the main risk factor was contact with rodent urine or feces (32). Similar to some previous reports, our results indicate that seropositivity is significantly associated with having a barn near to the house. A study performed in the Bartin region in 2009 demonstrated that contact with rodents generally occurred around houses and barns, while greenhouses and lofts were also risk factors (33). Patients were thought to have been exposed to Hantavirus through contact with rodent urine and feces. Zeitze et al. (34) reported an association between dirtiness of the home surroundings and Hantavirus infection. We did not find a relationship between seropositivity and encountering dead rodents in the residence, barn/loft, basement, attic, or wood store. However, a previous study of 1,386 patients in Brazil reported that “to see mouse or dead body in or around the house” was a risk factor for IgG positivity (35). Ventilating enclosed areas and using protective equipment during domestic cleaning is known to reduce contact with rodent urine and feces. Preventing rodents from entering living spaces also reduces the risk of infection (7).

Hantavirus infection is known to affect forest workers, campers, soldiers, hunters and people living in cottages (7). Farmers are also at risk because of increased contact with rodents and their surroundings (4,30,37,38). Crowcroft et al. (39) reported that Hantavirus infection was 6.1 times more frequent in people spending more than 16 h a day in forests (OR = 1.9–19.5; P = 0.03). The seropositivity of forest workers was significantly higher than the other patients in our study.

Hantavirus outbreaks are thought to be related to increases in rodent populations (40), which can be attributed to plentiful food and climate factors (41,42). However, other reports claim that Hantavirus infection rates are more closely related to changes in virus infectivity, loss of forests, increased cultivated areas, and individual risk factors than increased numbers of rodents (36,43). Yet, it is also known that rodent populations fluctuate significantly with the ecological cycle, and that climate influences this cycle (7). For example, Olsson et al. (44) demonstrated that human cases in October-November-December are related to rodent intensity in September. Two peaks in HFRS cases are seen annually in northern countries, with a minor peak in August and a major peak in October–February (45,46). In line with previous work, we found that most cases were seen in autumn and winter (Table 3). It is also known that the number of reported HFRS cases increase after a hot summer has led to a rich seed season for beech and oak trees (47,48). In the Bartin region, 2008 and 2009 were significantly wetter than previous years, leading to particularly rich seed seasons.

After the Hantavirus outbreak in Turkey in 2009, a National Hantavirus Study Group was founded. In June 2009, a total of 173 rodents (106 Apodemus sp., 49 Myodes sp., 11 Rattus rattus, and 7 others) and 2 shrews were collected from 7 sites in rural Bartin where suspected human Hantavirus cases had been reported. The study group found the DOBV-Belgrade virus genome by RT-qPCR in the collected rodent population (23 of 173 rodents). The ELISA assay found that 11 (6.4%) and 36 (20.8%) animals contained Hantavirus-reactive IgM and IgG, respectively (24).

In conclusion, this study adds to the evidence that, in an endemic region, physicians must keep in mind that thrombocytopenia, and elevated urea and creatinine levels may indicate Hantavirus infection. People who live close to forests should take precautions against infection. Moreover, integration of hantaviruses into current surveillance systems would be an important step towards improving public health.

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Conflict of interest None to declare.

REFERENCES
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