Hemorrhagic Fever with Renal Syndrome in the Dolenjska Region of Slovenia—A 10-Year Survey

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This report describes the first investigation of clinical findings for a larger series of patients with hemorrhagic fever with renal syndrome (HFRS) who were infected with Dobrava virus. From 1985 to 1995, 38 patients with serologically confirmed HFRS were hospitalized at the regional hospital in Novo mesto in the Dolenjska region of Slovenia. On the basis of results of serological examination, 24 patients had Dobrava virus infection, and 14 patients had Puumala virus infection. Complete clinical data were available for 31 patients. Eleven patients underwent hemodialysis for treatment of acute oliguric or anuric renal failure. Four patients, all infected by Dobrava virus, had signs of shock and severe bleeding. Three severely ill Dobrava virus–infected patients died of hemorrhagic complications. We have demonstrated that Dobrava and Puumala viruses coexist in a single region of endemicity and are capable of causing HFRS with significant differences in severity.

The genus Hantavirus of the family Bunyaviridae comprises at least 15 viruses, including those that cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. Each hantavirus is carried primarily by a specific rodent or insectivore host and is transmitted by inhalation of virus-contaminated aerosols of rodent excreta [1, 2]. Because of the virus-rodent association, each hantavirus has a characteristic geographic distribution. HFRS, which is caused by Han-taen (HTN), Seoul (SEO), Dobrava (DOB), and Puumala (PUU) viruses, occurs endemically on the Eurasian continent, whereas hantavirus pulmonary syndrome caused by Sin Nombre and related viruses occurs in the Americas [3–5]. Both syndromes involve a sudden onset of high fever, headache, and myalgia. HFRS may appear as a mild, moderate, or severe disease with renal impairment as the predominant organ manifestation. The mortality rate varies from <0.5% for HFRS caused by PUU virus to ~5% to 10% for HFRS caused by HTN virus [6]. Hantavirus pulmonary syndrome is characterized by acute noncardiac pulmonary edema and is associated with a mortality rate of ~50%; capillary leakage in hantavirus pulmonary syndrome is localized exclusively to the lungs, rather than to the retroperitoneal space, and the kidneys are usually not affected [7].

Slovenia is situated in the northern part of the Balkan Peninsula. Evidence of the circulation of PUU and DOB viruses throughout the Balkans has been collected recently [5, 8]. The presence of HFRS in Slovenia was first reported in 1954 [9]. Since then, 110 cases occurring sporadically or in small epidemics have been documented [authors’ unpublished data]. Both severe and mild clinical courses of the disease are seen, with an overall mortality rate of 4.5% [10]. The presence of two different hantaviruses responsible for human infections in Slovenia has already been reported [11]. Broad epidemiological studies have shown that 10% to 35% of the rodents captured in areas in Slovenia where HFRS is endemic have antibodies to hantavirus [12].

In 1988, DOB virus was isolated from the lungs of a yellow-necked field mouse (Apodemus flavicollis) captured in the village of Dobrava (Dolenjska region, Slovenia) where a number of cases of severe HFRS had occurred. Complete genetic and antigenic characterization identified DOB virus as a unique hantavirus [13]. Recent reports have shown that DOB virus is the etiologic agent of the severe form of HFRS that occurs in the Balkans [5, 8, 14].

In this report, we describe the clinical presentation of patients with HFRS who were hospitalized from 1985 to 1995 at the General Hospital in Novo mesto in the Dolenjska region of Slovenia. HFRS is known to be highly endemic in this southern region, where nearly one-half of all Slovenian patients with HFRS have been registered. Until now, it has been unclear which viruses are associated with HFRS of different severity in Slovenia. Serological diagnosis of hantavirus disease in 38 patients was established by native viral and/or recombinant hantavirus nucleocapsid protein–based ELISA [11, 15]. Serum
samples from these patients were retrospectively analyzed by the focus reduction neutralization test (FRNT) for antibodies to HTN, PUU, DOB, and SEO viruses. The data revealed that DOB and PUU viruses were the causative agents of the infections. Patients were categorized into the DOB or PUU virus infection group, and their clinical data were analyzed to ascertain possible differences in the clinical courses with regard to the infecting virus.

**Patients and Methods**

*Patients.* Thirty-one patients with HFRS who were hospitalized at the General Hospital in Novo mesto between 1985 and 1995 were included in a retrospective study. Case records of the 31 patients were reviewed. The clinical diagnosis was confirmed serologically by testing acute-phase serum samples (obtained 3–18 days after the onset of disease) with native HTN, DOB, SEO, and PUU virus antigens for the presence of specific IgM and IgG antibodies. Data including demographic characteristics, clinical manifestations, laboratory parameters, and possible complications during the course of illness were collected from the patients’ records. With the exception of the fatal cases, where autopsy results were collected and analyzed, patients were examined at least once after recovery. Follow-up examinations involved assessment of general health condition, urine analysis, hematological examinations, and blood pressure control. Convalescent-phase serum samples were collected (1–60 months after recovery) from all recovered patients for the analysis of neutralizing antibodies specific to hantavirus.

**Detection of specific antibodies.** Serum samples were initially tested by indirect fluorescent antibody analysis with use of spot slides of hantavirus-infected Vero E6 cells and by an EIA as described previously [11]. IgM and IgG antibodies specific to hantavirus were also detected in serum samples by an ELISA with antigens consisting of truncated (amino acids 1–117) recombinant nucleocapsid protein from HTN, DOB, SEO, and PUU hantaviruses produced by Escherichia coli [15]. Briefly, 96-well microtiter plate wells were coated with 0.2 μg of the antigens. The plates were washed and blocked, and the patients’ serum samples (diluted 1:400 in dilution buffer with *E. coli* extract; the latter was used to adsorb unspecifically reactive antibodies) were added. Horseradish peroxidase–conjugated goat antibody to human IgG (A-6029; Sigma, St. Louis) or horseradish peroxidase–conjugated goat F(ab’)2 to human IgM (A-4290; Sigma), followed by substrate, was used to detect binding of specific antibody.

Antibody activity was expressed as the net absorbance at 450 nm (i.e., absorbance for an antigen-coated well − absorbance for a control well). A net absorbance value of >0.15 was considered positive. The cutoff level for both IgG and IgM ELISAs was based on repeated testing of a battery of 100 human control serum samples negative for the presence of antibodies to hantaviruses by means of indirect fluorescent antibody analysis of hantavirus-infected cells. The mean absorbance values for these 100 serum samples ± 3 SDs never exceeded 0.15 in repeated experiments. For the detection of IgM antibody specific to hantavirus, serum samples were pre-treated with RF-Absorbent (Behring-Werke, Marburg, Germany).

To identify the serotype of the infecting virus, end point titers of neutralizing antibodies in convalescent-phase serum samples were determined by FRNT as described earlier [16]. Serum samples were serially diluted and mixed with an equal volume containing 30–70 focus-forming units (FFU) of virus/100 μL. The mixtures were incubated for 1 hour and inoculated into six-well plates containing confluent Vero E6 cell monolayers. After adsorption for 1 hour, the wells were overlaid with agarose. Plates were incubated for 9 days (HTN and SEO viruses) to 12 days (DOB and PUU viruses). Polyclonal rabbit antiserum specific to hantavirus, followed by horseradish peroxidase–labeled goat antibodies and substrate, were used for detection of virus-infected cells. An 80% reduction in the number of foci was used as the criterion for virus neutralization titers.

**Statistical analysis.** Data between groups were compared with the Student’s *t* test.

**Results**

**Epidemiological data.** Between 1985 and 1995, 38 patients with serologically confirmed HFRS were hospitalized at the General Hospital in Novo mesto, a hospital serving a population of ~100,000 people. Complete clinical documentation was available for 31 patients, and their case records were reviewed and analyzed. The mean age of the patients was 29.5 years, and three of the 31 patients were female. The patients primarily had outdoor occupations (e.g., farmers, woodworkers, drivers, and hunters). Most cases (22 of 31) occurred between May and August, although single cases were diagnosed throughout the year.

**Serological findings.** In 35 of 38 cases of HFRS, hantavirus infection was confirmed by ELISA for IgM antibody specific to hantavirus. Three clinically suspected cases were serologically confirmed retrospectively (by high levels of specific IgG antibodies in convalescent-phase serum samples), when serological diagnosis was introduced in 1986. The results of testing of a representative number of the patients’ serum samples are displayed in table 1. When acute-phase serum samples were tested for the presence of IgM antibody specific to hantavirus, two different patterns were observed: serum samples with responses to HTN, DOB, and SEO virus antigens that were >10 times the net absorbance value and serum samples with high IgM reactivities only to PUU virus antigen. Similar results were obtained when specific IgG antibody reactivity was analyzed in acute-phase and early convalescent–phase serum samples (data not shown).

To determine which hantaviruses were the actual causative agents of HFRS in the Dolenjska region, convalescent-phase
serum samples were end point titrated by FRNT (table 1). In two of three fatal cases, the causative virus was identified by genetic analysis by means of nested reverse transcriptase PCR analysis of RNA extracted from postmortem tissue specimens. Sequence data of the amplified partial M segment revealed 5% differences compared with the prototype DOB virus (data not shown). The results revealed 19 DOB virus and 12 PUU virus infections. Therefore, patients were divided into DOB and PUU virus infection groups for further clinical and laboratory evaluations.

Clinical manifestations. The clinical findings for the affected persons varied considerably. The most common findings were fever, chills, malaise, myalgia, pain in the lumbar and abdominal regions, headache, dizziness, vomiting, diarrhea, oliguria, and blurred vision (transient myopia) (table 2). Physical signs included conjunctival injection, face and neck flushing, retroorbital edema, petechiae of the soft palate, and hematomas of the skin. Other symptoms were coma, impaired consciousness, and delirium. In one of the patients with DOB virus infection, peripheral facial nerve palsy occurred 7 days after admission.

Four patients with the most severe clinical manifestations (all from the DOB virus infection group) developed signs of shock. All four patients required resuscitation, which was successful in one case, while the remaining three died 12 to 48 hours after admission to the hospital. Two patients from the PUU virus infection group had brief hypotension that was corrected with administration of vasopressors. None of the patients in the PUU virus infection group had signs of severe bleeding.

Abnormalities revealed by laboratory examinations were present in all of the patients. The most prominent abnormalities were thrombocytopenia, leukocytosis, accelerated erythrocyte sedimentation rate, and hemococoncentration (table 3). Serum transaminase levels as signs of liver involvement were elevated in 13 of 19 patients in the DOB virus infection group and in seven of 12 patients in the PUU virus infection group. Significantly higher levels of alanine aminotransferase were observed in patients with DOB virus infection (table 3). Elevated levels of serum lipase and amylase confirmed acute pancreatitis in two of the DOB virus–infected patients and in two of the PUU virus–infected patients.

Renal failure. Impairment of renal function was demonstrated in 30 (97%) of 31 patients already at the time of admission to the hospital. Proteinuria and erythrocytes and leukocytes in urinary sediment were found for all patients with renal impairment. The DOB virus–infected patients had significantly higher mean levels of serum creatinine (617.3 mmol/L) than did the PUU virus–infected patients (394.7 mmol/L) (table 3). Serum urea concentrations were elevated in 18 of 19 patients in the DOB virus infection group and in 11 of 12 patients in the PUU virus infection group.

Oliguric renal failure (urinary output, <400 mL) was seen in nine (47%) of the DOB virus–infected patients and in only two (17%) of the PUU virus–infected patients. Nine of 19 patients in the DOB virus infection group and two of 12 patients in the PUU virus infection group underwent hemodialysis. One patient in the DOB virus infection group had no signs of impaired renal function. After an initial febrile phase, this patient developed acute pancreatitis.

Hemorrhagic manifestations. Macroscopic hemorrhagic manifestations were present only in the DOB virus–infected patients; these manifestations were indicated by bleeding from
Table 2. Frequency of signs and symptoms in patients with HFRS who were from the Dolenjska region in Slovenia: DOB virus infection group vs. PUU virus infection group.

<table>
<thead>
<tr>
<th>Symptom or sign</th>
<th>DOB virus–infected patients (n = 19)</th>
<th>PUU virus–infected patients (n = 12)</th>
<th>Total (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body temperature, &gt;38.5°C</td>
<td>16 (84)</td>
<td>11 (92)</td>
<td>27 (87)</td>
</tr>
<tr>
<td>Fever</td>
<td>10 (53)</td>
<td>8 (67)</td>
<td>18 (58)</td>
</tr>
<tr>
<td>Headache</td>
<td>12 (63)</td>
<td>5 (42)</td>
<td>17 (55)</td>
</tr>
<tr>
<td>Back pain</td>
<td>16 (84)</td>
<td>10 (83)</td>
<td>26 (84)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>14 (74)</td>
<td>7 (58)</td>
<td>21 (68)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>9 (47)</td>
<td>4 (33)</td>
<td>13 (42)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>15 (79)</td>
<td>8 (67)</td>
<td>23 (74)</td>
</tr>
<tr>
<td>Diaphoresis</td>
<td>9 (47)</td>
<td>4 (33)</td>
<td>13 (42)</td>
</tr>
<tr>
<td>Blurred vision</td>
<td>8 (42)</td>
<td>10 (83)</td>
<td>18 (58)</td>
</tr>
<tr>
<td>Subconjunctival injection</td>
<td>12 (63)</td>
<td>6 (50)</td>
<td>18 (58)</td>
</tr>
<tr>
<td>Hemorrhagic complications</td>
<td>5 (26)</td>
<td>0</td>
<td>5 (16)</td>
</tr>
<tr>
<td>Pleural or abdominal effusion</td>
<td>8 (42)</td>
<td>0</td>
<td>8 (26)</td>
</tr>
<tr>
<td>Shock</td>
<td>4 (21)</td>
<td>2 (17)</td>
<td>11 (35)</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>9 (47)</td>
<td>2 (17)</td>
<td>11 (35)</td>
</tr>
<tr>
<td>Renal failure (oliguric)</td>
<td>9 (47)</td>
<td>2 (17)</td>
<td>11 (35)</td>
</tr>
<tr>
<td>Death</td>
<td>3 (16)</td>
<td>0</td>
<td>3* (8)</td>
</tr>
</tbody>
</table>

NOTE: DOB = Dobrava; HFRS = hemorrhagic fever with renal syndrome; PUU = Puumala.
* Denominator, 38 (total no. of patients with serologically confirmed HFRS).

Discussion

The epidemiology of HFRS in the Balkan Peninsula is complex because of the coexistence of different rodent species, animals constituting the reservoirs of various hantaviruses that cause different clinical courses of HFRS in humans. We have already reported evidence of the presence of two hantaviruses in Slovenia that lead to milder and more severe forms of disease.

Table 3. Selected abnormalities revealed by laboratory examination in patients with HFRS who were from the Dolenjska region in Slovenia: DOB virus infection group vs. PUU virus infection group.

<table>
<thead>
<tr>
<th>Laboratory finding</th>
<th>DOB virus–infected patients (n = 19)</th>
<th>PUU virus–infected patients (n = 12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean thrombocyte count (normal, 150–350) in ( \times 10^9/L )</td>
<td>32.8* (68)</td>
<td>67.3 (75)</td>
<td>&lt;.05³</td>
</tr>
<tr>
<td>Mean WBC cell count (normal, 4–10) in ( \times 10^9/L )</td>
<td>18.3 (53)</td>
<td>14.5 (50)</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Mean AST level (normal, &lt;36) in U/L</td>
<td>100.2 (68)</td>
<td>67.8 (66)</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Mean ALT level (normal, &lt;42) in U/L</td>
<td>122.4 (68)</td>
<td>59.4 (67)</td>
<td>&lt;.05¹</td>
</tr>
<tr>
<td>Mean serum creatinine level (normal, 44–106) in mmol/L</td>
<td>617.3 (95)</td>
<td>394.7 (100)</td>
<td>&lt;.001¹</td>
</tr>
<tr>
<td>Mean serum urea level (normal, 2.8–6.7) in mmol/L</td>
<td>28.2 (95)</td>
<td>22.3 (92)</td>
<td>&gt;.05</td>
</tr>
</tbody>
</table>

NOTE. ALT = alanine aminotransferase; AST = aspartate aminotransferase; DOB = Dobrava; HFRS = hemorrhagic fever with renal syndrome; PUU = Puumala.
* Mean values of the most markedly abnormal value for each patient.
¹ Percentage of patients with abnormalities.
³ Statistically significant result.
of illness [11, 17–19]. Because of the significant serological crossreactivity between several hantaviruses (e.g., HTN, DOB, and SEO viruses), it has been unclear which hantaviruses are the cause of HFRS in this area. Previous studies have shown that neutralization assays are the only serological assays presently available that allow specific typing of the infecting hantavirus [5, 20].

On the basis of our results of FRNT of convalescent-phase serum samples from hantavirus-infected patients, it was obvious that DOB and PUU viruses are the causative agents of HFRS in the region of endemicity in Slovenia. This report is the first in which the clinical features of the disease are grouped with respect to the infecting hantavirus.

Disease caused by hantaviruses in Slovenia is present in two major forms: mild illness closely resembling nephropathia epidemica caused by PUU virus (which is mainly seen in Fenoscandia and western Russia [21]) and severe illness caused by DOB virus, the clinical course of which is similar to that described in the Far East [22]. Although the presence of HFRS in Slovenia has often been reported, physicians still rarely recognize the disease. Patients are frequently directed to the hospital with clinical diagnoses such as acute pyelonephritis, glomerulonephritis, febrile illness, and acute abdomen. In this study, blurred vision was observed relatively often at admission and was regarded as acute transient myopia.

Patients in the DOB virus infection group had a more pronounced predisposition for hemorrhage. The thrombocyte count was significantly lower in patients with DOB virus infection than in those with PUU virus infection. It is evident that thrombocytopenia is not the sole cause of hemorrhage in HFRS (although thrombocytopenia was found frequently in patients in the PUU virus infection group), as none of the PUU virus–infected patients had severe hemorrhages. Elevated serum transaminase levels were found in 68% of our patients. In general, the elevated level was moderate and was not related to the course of the disease as reported by some investigators [23, 24].

Four patients with DOB virus infection developed signs of shock due to general vasodilatation, increased vascular permeability with loss of fluid, and erythrocytes in the third compartment and intercellular space. In these patients, sudden cardiac arrest occurred despite a relatively stable hemodynamic stage. Immediate resuscitation was successful only in one patient, although this patient had severe hemorrhage in the pericardium and pleural cavity. Autopsy results revealed the characteristic changes in the kidneys, pituitary gland, and right atrium that are usually seen in fatal HFRS cases reported in the Far East [25, 26].

Renal function impairment was evident in all patients except one. The serum creatinine level, but not the serum urea level, was significantly higher in patients in the DOB virus infection group than in those in the PUU virus infection group. Oliguric renal failure was much more frequent in patients with DOB virus infection than those with PUU virus infection, and patients in the DOB virus infection group underwent hemodialysis more frequently than did those in the PUU virus infection group. Among the DOB virus–infected patients, the mortality rate was 16%, which is similar to rates reported from Asia (where the mortality rates associated with HTN virus infection were 5% to 15% [6]). No patients with PUU virus infection died. Our data are in accordance with the results from Fenoscandia, where a mortality rate of <0.1% has been reported for patients infected with PUU virus [27, 28].

In conclusion, we have demonstrated that two different hantaviruses coexist in a single region of endemicity in Slovenia (only DOB and PUU viruses have been found to circulate in this region) and are capable of causing disease of clearly different severity. Although our study included a small number of patients, our data suggest that infection with DOB virus causes a severe form of HFRS, which may cause acute oliguric renal failure, hemorrhagic complications, and death. On the other hand, infections with PUU virus are generally less severe, causing nonoliguric renal failure and no hemorrhagic complications. We believe that early serological determination of the infecting virus is important for the prognosis of the course of the disease.

References


