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Dinara – New Natural Focus of Hemorrhagic Fever with Renal Syndrome in Croatia

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Aim. To investigate the characteristics and determine risk factors for hantanvirus infection in natural focus of hemorrhagic fever with renal syndrome (HFRS) on the Dinara Mountain, where outbreak of disease emerged among Croatian soldiers in 1995, and to describe the features of HFRS acquired on the Dinara mountain and determine the scale of the largest HFRS epidemic so far in Croatia.

Methods. During 1996, small mammals were captured in the region of Dinara Mountain where infected Croatian soldiers had sojourned. By taxonomic classification of 42 captured small mammals, three species were determined: 23 yellow-necked mouse, 9 wood mouse, and 5 bank vole. Hantavirus antigen was determined in the lungs of the captured animals by means of direct immunofluorescence assay. The most important features of HFRS were retrospectively determined in 37 soldiers with HFRS treated in the Department for Infectious Diseases of the Split University Hospital. The degree of inapparent exposure to infection was determined by indirect immunofluorescence in 103 soldiers sojourning in this region of natural focus with no apparent signs of HFRS. Epidemiological questionnaire included 50 soldiers with negative serum antibodies, as well as 33 available out of total 37 soldiers with HFRS. Chi-square test was used to determine risk factors.

Results. Hantavirus was found in the lungs of 5/42 (12%) captured animals. Mild form of the disease, with few hemorrhagic symptoms and pronounced renal insufficiency, was present in 19/37 patients. The epidemiological questionnarie determined the following risk factors for hantanvirus infection in this focus: service in artillery corps ($p=0.040$), sleep in wooden barracks ($p=0.004$), station in forest biotope ($p=0.037$), usage of natural camouflage ($p=0.024$), smoking ($p=0.010$), and the presence of rodents in the place of housing ($p<0.001$).

Conclusion. A new natural focus of HFRS in Croatia, and the first one in Dalmatia, was defined by seroepidemiologic, mamologic, and virologic analysis. The risk factors for infection in the new focus have been identified. Our patients suffered from a mild form of HFRS, which predominates in south-eastern Europe, without lethal outcome.

Key words: *disease reservoirs; hantavirus; hantavirus infections; hemorrhagic fever with renal syndrome; military personnel; rodentia; risk factors; war*

Hemorrhagic fever with renal syndrome is an acute infectious disease caused by a virus from the *Bunyaviridae* family, *Hantavirus* genus. It is a natural focus zoonosis. The infection is spread by inhalation of aerosol formed by the excretions of mouse-like rodents of the *Apodemus*, *Chletrionomys*, *Microtus,* and *Ratus* genus (1). The principal clinical characteristics of hemorrhagic fever with renal syndrome are fever, hemorrhages, and renal insufficiency. Mortality is low, up to 10%, in contrast to Hantavirus pulmonary syndromes where it reaches 50% (2). The disease is common among soldiers during training and in war (3). The limited knowledge of natural foci of hemorrhagic fever with renal syndrome in Croatia, together with persistent military activities, contributed to an increased number of cases during the war in Croatia (4). In Croatia hemorrhagic fever with renal syndrome was described for the first time in 1954 in four patients (5). The first cases of hemorrhagic fever with renal syndrome in military personnel in Croatia were described among soldiers of former Yugoslav Federal Army in 1989 near Velika Gorica, with 14 diseased (6). Among soldiers of Croatian Army, seven cases of hemorrhagic fever with renal syndrome appeared during 1991 and 1992 (7), and 125 cases in 1995. The disease broke out in the previously known foci in west Slavonia and on the mount of Mala Kapela, and, for the first time, on the mountain of Dinara in 1995 (8). The appearance of hemorrhagic fever with renal syndrome among soldiers on the mountain Dinara in 1995 prompted us to investigate whether it was a case of a new focus. Thirtyseven out of 66 soldiers who got infected with hantavirus on the Dinara Mountain were treated at the Department for Infectious Diseases of the Split University Hospital.

Patients and Methods

Small Mammals

In September 1996, small mammals were captured in the region of the Dinara Mountain in an area between North geographic latitudes N43^{0}57′45′′ and N44^{0}04′50′′, and East geographic longitudes E16^{0}31′28′′ and E16^{0}35′27′′, where the disease had been registered among the Croatian soldiers in 1995. During three consecutive nights, 700 rodent traps were placed on three localities (Gnjat with 350, Crni Bunari with 300, and Kolibine with 50 traps) at the height of 1,300 m above sea level. The traps were placed on different routes at the distance of five steps, in the late afternoon, and emptied at twilight on the same day and the following day at dawn. A total of 42 mouse-like rodents were caught on that occasion (Gnjat 19, Crni Bunari 19, and Kolibine 4 animals). The animals were taxonomically classified immediately upon capture. Out of 37 animals that belonged to rodents, 32 were from the mice family and 5 from the vole family, whereas the remaining 5 were insectivores from the mole family. That same day the samples of lung tissues of the animals were taken in the Livno Medical Center and frozen to –70 ^{0}C. Hantavirus antigen was determined on kryosections of the lungs by the standard direct immunofluorescence technique at the Virology Department of the Croatian Institute of Public Health in Zagreb.

Patients

Thirty-seven patients with hemorrhagic fever with renal syndrome treated during 1995 at the Department for Infectious Diseases of the Split University Hospital were included in the study. All patients had previously sojourned on the territory of the Dinara Mountain where mammals were caught. They were all members of the Croatian Army. Their median age was 28 years (range 18-45). Their epidemiological, clinical, and laboratory data were collected, systematized, and retrospectively analyzed. The severity of their disease was estimated according to the incidence of hemorrhagic manifestations (conjunctival injection, petechial rash, hematoma, and melena) and serum creatinine value (normal range 35-115 μmol/L). According to the severity of their clinical features, they were divided into three groups: a group with mild, a group with moderate, and a group with serious clinical features. The group with mild clinical features included patients with no hemorrhagic manifestations and serum creatinine value <700 μmol/L. The group with moderate clinical features included patients with hemorrhagic manifestations and serum creatinine ranging between 700-1,000 μmol/L. The patients with severe clinical feature had hemorrhagic manifestations and serum creatinine value >1000 μmol/L. The sera of 35/37 patients were tested by indirect immunofluorescence method. The serum was obtained by centrifugation of 5 ml of blood, frozen to –20 °C, and then sent to the Virology Department of the Croatian Institute of Public Health in Zagreb, where the indirect immunofluorescence tests were performed. Only one titer dilution was used and a characteristic fluorescent pattern at 1:16 titer was considered positive.

Control Group

The control group consisted of 103 soldiers who sojourned in natural foci and who did not develop clinically apparent hemorrhagic fever with renal syndrome. The mean age of the group was 30 years (range 19-53).

Their sera were collected and serologically tested by indirect immunofluorescence to determine unapparent infection. Determination of unapparent infection degree is crucial for the assessment of the valence of new focus.

Epidemiological Questionnaire

The questionnaire included the following variables: age, sex, location of sojourn, smoking habits, military occupation, distance from the front line, bivouac, sleeping place in the barracks, trench digging, camouflage use, dust in and out of the place of housing, rodent presence, and direct contact with rodents and biotope in the region of station. According to their military occupation, subjects were divided into three groups: infantry (48/83), artillery (14/83), and logistics/commanding staff (21/83).

Epidemiological questionnaire included two groups of examinees: 50 healthy soldiers and 33 soldiers suffering from hemorrhagic fever with renal syndrome. A group of healthy soldiers was formed by selection of every second seronegative person from the control group. The mean age of the 50 healthy examined persons was 30 years (range 20-53). The group of soldiers with hemorrhagic fever with renal syndrome included 33 of 37 soldiers who were treated at the Department of Infectious Diseases of the Split University Hospital in 1995. The mean age of 33 patients was 27 years (range 19-45). Data related to examinees were collected by the questionnaire and direct examination of both the patient and the control group.

Detection of the Virus Antigen

Hantaan virus antigen was proved by the direct immunofluorescence technique on the lung tissues of captured small mammals.

Frozen (-70 °C) lung tissue samples were cut into ≈3mm blocks, than cut to 4μm by cryostat (American optical corp, Buffalo, NY, USA). Lung sections were fixed to cleaned glass slides by exposure to cold anhydrous acetone (Alkaloid, Skopje, Macedonia) for 8 min. Sections examined for hantavirus antigen were incubated with 30 μl of 1:40 dilution of convalescent human sera and control sections with normal human sera. After 30 min of incubation at 37 °C, slides were washed, stained, and incubated with fluorescein isothiocyanate (FITC)-conjugated immunoglobulin to human IgG (Institute of Immunology, Zagreb, Croatia). Slides were examined for hantavirus antigen by fluorescent microscopy (400X Labor-Lux D, Ernst Leitz Wetzkar GmbH, Hildeshein, Germany) (9). Tissue was considered positive if a characteristic fluorescent pattern was clearly present in any section stained with hantavirus antiserum and if no fluorescence was seen in the negative control tissue.

Detection of the Virus Antibodies

Hantavirus antibodies were determined by indirect immunofluorescence testing.

Hantaan antigen-containing Vero E6 cells were prepared on slides with 12 circular areas ("spots"), fixed in anhydrous acetone, air-dried, and stored at –70°C until use. Thirty μl of each serum were tested at dilution 1:16. Samples were deposited on a spot and incubated at 37 °C for 30 min. The slides were washed and FITC-conjugated; after anti-immunoglobulin (Institute of Immunology, Zagreb, Croatia) was added, the incubation followed. Slides were examined by fluorescent microscopy (400X Labor-Lux D, Leitz Wetzkar GmbH, Hildeshein, Germany). Sera were considered positive if a characteristic fluorescent pattern was clearly present and if no fluorescence was seen in the negative control sera (9).

Statistical Analysis

We used EPY INFO 6 program (WHO, Geneva, Switzerland) for statistical analysis and performed the Mantel-Haenszel chi-square test.

Results

Small Mammals

Among 42 small mammals captured in 700 traps, the most common species was yellow-necked mouse (n = 23), followed by the wood mouse (n = 9), and bank vole (n = 5). Presence of hantavirus was confirmed in lung tissues of 5 animals, belonging to all three species: yellow–necked mouse (3/23), wooden mouse (1/9), and bank vole (1/5) (Table 1). Among captured small mammals hantavirus carriage was proved in five.

Patients

Thirty-seven (37/66) soldiers infected on the Dinara Mountain were hospitalized at the Department for Infectious Diseases of the Split University

Table 1. Captured animals according to trapping site and virus carriage

Species	No. of positive animals/ No. of captured animals			
	trapping site			
	woods	mountain planes	woods + rocky ground	total
Yellow-necked mouse (*Apodemus flavicollis*)	2/9	1/13	0/1	3/23
Wood mouse (*Apodemus sylvaticus*)	1/5	0/3	0/1	1/9
Bank vole (*Clethryonomys glareolus*)	0/1	0/2	1/2	1/5
Bicolored white-toothed shrew (*Crocidura leucodon*)	0/2	0	0	0/2
Scilly shrew (*Crocidura suaveolens*)	0/1	0	0	0/1
Alpine shrew (*Sorex alpinus*)	0	0/1	0	0/1
Pygmy shrew (*Sorex minutus*)	0/1	0	0	0/1
Total	3/19	1/19	1/4	5/42

Hospital. Additionally, 29 patients were treated ambulatory or in other medical care units in Croatia. The peak of the epidemic occurred in March and April 1995, when 23 out of 37 patients were treated.

Mild clinical form of hemorrhagic fever with renal syndrome was found in 19/37 patients, moderate in 11/37, and severe in 7/37. The impaired renal function (creatinine >124 μmol/L) was recorded in 34/37 cases.

Indirect immunofluorescence test was positive in 33/35 patients.

Control Group

In the control group, the indirect immunofluorescence test was positive in 6 out of 103 (5.8%) examinees.

Epidemiological Questionnaire

The incidence of hemorrhagic fever with renal syndrome among the artillery (9/14), infantry (18/48), and logistics/commanding staff (6/21) did not differ significantly ($p=0.094$). Comparison showed that the incidence of hemorrhagic fever with renal syndrome among artillery soldiers (9/14) was significantly higher than the incidence among other military personnel all together (24/69) ($p=0.040$). The incidence of hemorrhagic fever with renal syndrome was significantly higher among soldiers who used to sleep in wooden barracks (25/47) than in those who slept in dug-outs (2/18) or containers (6/18) ($p=0.004$). The bunks in wooden barracks were placed either on the floor or one meter above it. However, there was no significant difference between soldiers who slept on the floor (21/55) and those who slept on the bunks (12/28) ($p=0.682$). Soldiers who were stationed in the forest biotope had hemorrhagic fever with renal syndrome more frequently (27/57) than those who were stationed in other biotopes (rocky ground and high planes) (6/26) ($p=0.037$). There was a higher statistical probability for soldiers to get hemorrhagic fever with renal syndrome if they used natural camouflage (27/56) instead of artificial one (6/27) ($p=0.024$). Incidence of hemorrhagic fever with renal syndrome among soldiers with smoking habits was significantly higher (28/33) than among non-smokers (29/50) ($p=0.010$). Soldiers who noticed the presence of rodents (19/29) in their huts were infected with hantaviruses considerably more often than those who did not notice rodents (14/54) ($p<0.001$) (Table 2).

Table 2. Risk factors for hantavirus infection on the Dinara

Risk factors	Cases (n = 33) yes	Cases (n = 33) no	Controls (n = 50) yes	Controls (n = 50) no	p
Service in artillery corps	9	24	5	45	0.040
Housing in wooden barracks	25	8	22	28	0.004
Station in forest biotope	27	6	30	20	0.037
Using natural camouflage	27	6	29	21	0.024
Smoking	28	5	29	21	0.010
Presence of rodents in the place of housing	19	14	10	40	<0.001
Indoor dust	26	7	30	20	0.075
Sleeping on the ground in barracks	21	12	34	16	0.682
Using uncontrolled water and snow	18	15	36	14	0.104

Discussion

The occurrence of 125 reported clinical cases of hemorrhagic fever with renal syndrome in Croatia in 1995, out of which 66 among Croatian soldiers who sojourned on the Dinara Mountain, aroused suspicion that there could exist a "new", still not registered natural focus of that disease. In our investigation hantavirus antigen was established in the lung tissues of 5/42 (12%) mouse-like rodents caught on the Dinara Mountain, which indicates a high focus valence (10). In the research in the Plitvice region in 1989, hantavirus antigen was confirmed in 9/45 (5.4%) rodents, in the surroundings of Velika Gorica in 1990 in 7/141 (4.9%) (11), and in the Novska area in 1994 in 12/110 (10.9%) rodents (7). Findings of six unapparent hantavirus infections among 103 control subjects who spent certain period of time on the mountain Dinara in 1995 when the epidemic broke out speaks in behalf of a high natural focus activity and allows us to conclude that this was indeed a new focus of the hemorrhagic fever with renal syndrome.

A catch of 42 animals in 700 traps indicated low rodent population density in the area. Whereas bank vole was the most frequently captured species of small mammals in the Plitvice region in 1989, wood mouse in Velika Gorica in 1990 (11), and stripped field mouse in the surroundings of Novska in 1994 (7), the most common species in our investigation was the yellow-necked mouse. Bearing in mind different biotopes, these differences could have been expected. In our research, the exemplars of high alpine fauna (alpine shrew), residing at altitudes of over 1,000 m above sea level, were caught (12). This finding is in accordance with the location of the Dinara focus at 1,260-1,807 m above sea level, making it the focus of hemorrhagic fever with renal syndrome at the highest altitude in Croatia. The highest focus described so far in southeast Europe is in Greece

(Tsepalovo, 2,200 m) (13). We must emphasize that the exemplars of scilly shrew (*Crocidura suaveolens*) and bicolored white-toothed shrew (*Crocidura leucodon*), previously seen at altitudes up to a maximum of 800 meters (12), were trapped on the mountain Dinara.

Based on the polymerase chain reaction (PCR) analysis of the patients' sera during the epidemic in 1995, Markotić et al (14) proved that two Hantaviruses could be found in Croatia: Dobrava and Puumala. The infections caused by Dobrava were more common on the Dinara Mountain, whereas those caused by Puumala hantavirus could be found on Mala Kapela. That same year both viruses were also found in Bosnia and Herzegovina (15). There was no mortality recorded among our patients, but that is not surprising because milder hemorrhagic fever with renal syndrome infections prevail in the south-east Europe (1).

It is interesting that the epidemic on the Dinara broke out in winter with an average monthly temperature of -3.7°C, under almost same conditions as described in 1942 in Finland (16). It is obvious that the abundance of food and waste, as well as warm barracks and the surrounding area, were an attractive "bait" for the rodents. Some risk factors for hemorrhagic fever with renal syndrome infection in such conditions were defined by the epidemiological questionnaires. The main risk factor was the visible presence of rodents in the soldiers' dwelling-places, which is in accordance with findings of Dixon et al (17). Significantly higher percentage of infection was recorded in soldiers who were stationed in woods. This finding can be explained by better habitat conditions for rodents in the forest biotope, and thus higher rodent population density (10). The rate of hemorrhagic fever with renal syndrome infection among the artillery soldiers proved to be the highest, what can be related to the static quality of their service and therefore longer exposure to infected aerosol. Similar results were obtained by Pons et al (18). It is not surprising that the incidence of hemorrhagic fever with renal syndrome infection was significantly higher among soldiers who lived in inadequate, and for rodents very permeable, wooden barracks than among those staying in metal containers. Dixon et al (17) showed that the quality of accommodation was significantly related to the incidence of infection. Besides, hemorrhagic fever with renal syndrome was more frequent among smokers than non-smokers. This result can be explained by facts that inhalation is the main route of infection, and that smokers have damaged respiratory epithelium. Similar result was obtained by Korpela et al (19), but without statistical significance.

In conclusion, the results of this research, together with the previous ones conducted in Croatia, point out that Croatia, except its Adriatic coast and islands, could be an endemic area of hemorrhagic fever with renal syndrome. A more complete insight into the situation related to natural hemorrhagic fever with renal syndrome foci in Croatia (Fig. 1) could be achieved by seroepidemiologic survey of hantavirus antibodies in the human population and evidence of hantavirus carriage among rodents in the entire country.

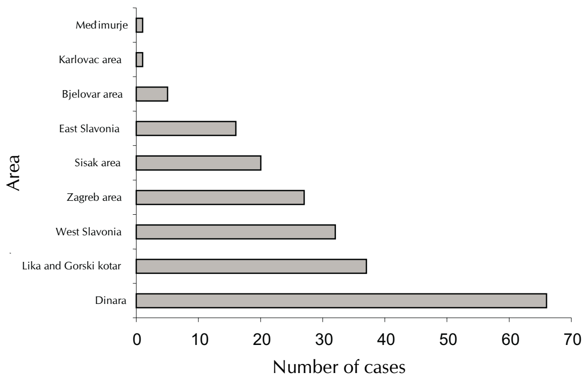

Figure 1. Areas and numbers of hemorrhagic fever with renal syndrome cases in Croatia from 1986 to 2000.

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