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Evidence of the presence of two hantaviruses in Slovenia, Yugoslavia

Tatjana Avšič-Županc[1], M. Likar[1], Suzana Novakovič[1], Borut Čižman[2], Alenka Kraigher[3], G. van der Groen[4], R. Stojanovič[5], Mirčeta Obradovič[5], Ana Gligič[6], and J. W. LeDuc[7]

[1] Institute of Microbiology, Medical Faculty of Ljubljana, Ljubljana,
[2] Department of Nephrology, University Clinical Center, Ljubljana, and
[3] Institute of Public Health and Social Welfare, Ljubljana, Yugoslavia
[4] Institute of Tropical Medicine, Antwerp, Belgium
[5] Military Medical Academy, Belgrade, and
[6] Institute of Immunology and Virology, Belgrade, Yugoslavia
[7] U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, U.S.A.

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Summary. Thirty-three cases of hemorrhagic fever with renal syndrome (HFRS) have been serologically confirmed in Slovenia during the last five years. The clinical picture varied from mild to severe, with a mortality rate of 3%. Serum samples from 240 patients suspected of having HFRS were tested by immunofluorescence using four different hantaviral antigens. Three reactivity patterns were observed. Using capture enzyme immunoassay, IgM was detected in 100% acute-phase serum samples of patients. The distribution of hantaviral infections in small mammals was examined in two natural foci of HFRS. Two hantaviruses were found in these mammals. In one area, *Clethrionomys glareolus* was the predominant species, and most of their sera reacted to highest titer with Puumala virus; mild illness was diagnosed in this area. In the second location, *Apodemus flavicollis* predominated, and most of their sera reacted to highest titer with Hantaan virus; severe illness was diagnosed in this area.

Introduction

Hemorrhagic fever with renal syndrome (HFRS) is an acute infectious disease with a variety of clinical manifestations [4, 21]. It is caused by viruses belonging to the family *Bunyaviridae*, genus *Hantavirus*, [15]. The prototype of this group is Hantaan virus, isolated in Korea in 1976 [9]. The severe form of HFRS is more often seen in the Far East [10]. In Europe, the mild form, known as nephropathia epidemica (NE) is found in Scandinavia [7] and western Europe [20]. Evidence for infection of wild, small mammals and of humans by hantaviruses has been reported nearly worldwide. Recognition of the number of small mammal reservoirs for hantaviruses has dramatically increased [19].

In Yugoslavia the first HFRS case was reported in 1952 [16], and several epidemics occurred between 1962 and 1986 [6]. The presence of HFRS in Slovenia, in northwestern Yugoslavia, was first reported in 1989 [1].

In this paper we report hantaviral antigen in lungs and antibodies to hantaviruses in sera of small mammals in three natural foci of HFRS in Slovenia. In addition, we present data on the prevalence of antibodies to these viruses in human sera, data indicating that at least two hantaviruses are present in Slovenia.

Materials and methods

Trapping procedure and locations

Small mammals were live-trapped in fields and forests surrounding villages in which HFRS cases had been diagnosed between 1985 and 1989. Study sites included Murska Sobota in northeast Slovenia, Novo mesto and Črnomelj in southern Slovenia, and a third location, Vipava, as an area where, to now, there is no evidence of hantaviral infections in humans (Fig. 1). Animals were identified by species and exaguinated in the field. Sera were diluted

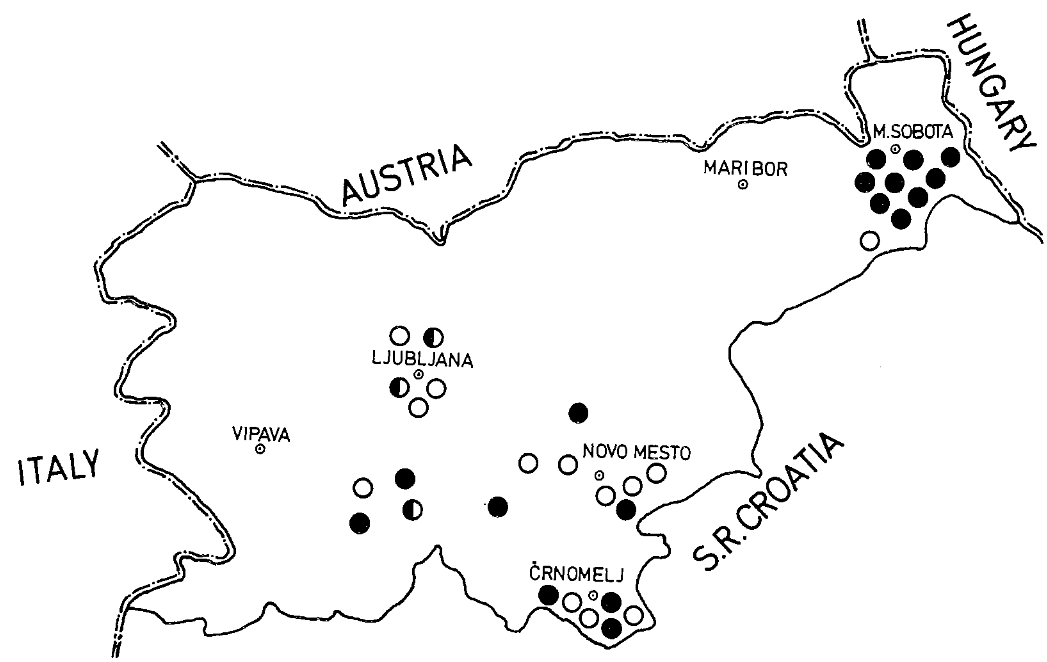

Fig. 1. Geographic distribution of 33 HFRS patients in Slovenia. ● Hantaan serotype, ○ Puumala serotype, ◐ Unidentified serotype

1:16 with phosphate buffer saline (PBS) pH 7.2 and stored at −20 °C until they were tested for antibody. Lung tissues were removed aseptically and stored in liquid nitrogen.

Trap sites in Murska Sobota consisted of corn and grape fields. Grasslands, sedges and bogs are characteristic for this part of Slovenia. The area of Novo mesto and Črnomelj is characterized by hilly-woodland area with rivers between.

Detection of hantavirus antigen

Cryostat-cut sections (4 μm thick) of lung tissue from small mammals were examined for the presence of hantaviral antigen by an indirect immunofluorescent antibody technique (IFA) [2]. Enzyme-linked immunosorbent assay (ELISA) for detection of hantavirus antigen in lung suspensions was also used [12].

Serological techniques

Human and animal sera were tested at a single 1:16 dilution by IFA using spot slides of Vero E-6 cells infected with hantaviruses as described previously [18]. Hantaviruses used in the study were: prototype Hantaan (strain 76-118), Puumala (strain Hällnäs B1), Prospect Hill (strain Prospect Hill 1) and Seoul (strain SR-11). Sera producing characteristic stippled fluorescence in the cytoplasma of infected cells were considered positive. All positive sera were diluted 2-fold and titrated for end-points; titers were expressed as the reciprocal of the highest dilution of serum giving typical focal cytoplasmic staining. Human IgM antibodies to Hantaan virus (HTN) and Puumala virus (PUU) were measured using an enzyme immunosorbent assay according to methods published previously [12–14]. An immunoblot method as described [1], was used for confirmation.

Results

Human serology

Between August 1985 and October 1989, 320 acute and/or convalescent sera were tested from 240 patients with clinical disease compatible with HFRS in Slovenia. Sera were stored at −20 °C until they were tested for antibody. Since then, 33 clinically diagnosed cases of HFRS have been serologically confirmed. Clinical pictures varied from mild to severe; the overall mortality rate has been 3% [3].

Sera from 240 patients suspected of having HFRS were examined by IFA using four hantaviruses and 33 cases of HFRS were serologically confirmed. Three reactivity patterns were observed. Table 1 presents nine examples of these patterns. Highest titers to HTN antigen and lower titers to PUU antigen were found in patients 1–3. Higher titers to PUU than to HTN virus were seen in patients 4–6. Sera of patients 7–9 reacted with almost equal (within two-fold) titers to all hantaviral antigens used. IFA positive human sera were confirmed by immunoblotting.

Patients from Novo mesto generally had more severe HFRS than patients residing elsewhere in Slovenia. Sera from two patients (Table 2, patients A and B) with severe disease collected at different time intervals and

Table 1. IFA antibody to four hantaviruses in serum samples from nine representative patients with HFRS in Slovenia

Patient	Reciprocal fluorescent antibody titer to				IB[b]	
	HTN[a]	PUU	SEO	PH	HTN	PUU
1	256	16	256	64	+[c]	NT[d]
2	1024	128	2048	512	+	+
3	2048	64	1024	16	+	−
4	16	2048	512	1024	−	+
5	64	2048	128	256	NT	+
6	256	2048	32	512	−	+
7	64	64	64	32	+	−
8	256	256	256	256	+	NT
9	2048	2048	1024	512	+	+

[a] HTN Hantaan
PUU Puumala
SEO Seoul
PH Prospect Hill
[b] *IB* Immunoblot
[c] +Positive reaction of a convalescent-phase patients serum with the electrophoretically blotted 50 kDa nucleoprotein of HTN or PUU virus on vitro cellulose strips. Serum dilution was 1/50
[d] *NT* Not tested

Table 2. IgM and IgG antibody to Hantaan and Puumala viruses in serum samples from two patients with HFRS in Murska Sobota and from six patients from Novo Mesto

Patient (days after onset)	Antibody titer			
	EIA (IgM)		IFA (IgG)	
	HTN	PUU	HTN	PUU
A (10)	>12800	200	512	128
(30)	6400	NT[a]	1024	128
(60)	800	NT	1024	32
B (6)	6400	<100	256	<16
(14)	>25000	400	1024	64
(90)	1600	<100	512	32
C (7)	200	6400	<16	1024
D (13)	800	3200	256	1024
E (21)	1600	3200	64	2048
F (14)	400	1600	32	2048
G (20)	100	400	128	512
H (14)	800	3200	64	2048

[a] *NT* Not tested

tested for hantaviral antibody, had significantly higher IgM and IgG titers to HTN virus than to PUU virus. Patients from Murska Sobota generally suffered a milder clinical picture and when their sera were tested by IFA their reactivity was higher to PUU antigen than to HTN. The same reactivity pattern was seen with IgM antibody (Table 2, patients C–H).

Small mammal survey

Of 93 animals collected in Murska Sobota, 12 (13%) had antibody by the IFA test. The highest positive rate was found in bank voles, *C. glareolus* (10/34). Hantaviral antigen was found in 18 (19%) animals (Table 3).

In Novo mesto and Črnomelj, animals were collected three times during 1987 and 1989. Seventeen (12%) of 146 animals from this region had IFA antibodies to hantaviruses (Table 3). Antigen prevalence in small mammals (24/146 = 15%) was slightly lower than in mammals from Murska Sobota (18/93 = 19%). *Apodemus flavicollis* was the predominant species infected with hantaviruses in Novo mesto and Crnomelj.

Although HFRS has yet to be diagnosed in residents of Vipava, seroprevalence (25%) and antigen prevalence (28.1%) of small mammals was

Table 3. Prevalence of hantaviral antibodies and antigens in small mammals captured at three locations in Slovenia

Species	Location								
	Murska Sobota			Novo Mesto/ Črnomelj			Vipava		
	T[a]	Ab[b]	Ag[c]	T	Ab	Ag	T	Ab	Ag
Rodents									
Apodemus flavicollis	21	2	4	75	12	16	6	1	4
A. sylvaticus	23	0	1	32	2	4	6	1	0
A. microps	2	0	0	1	0	0	0		
A. agrarius	2	0	0	0			7	4	2
Clethrionomys glareolus	34	10	11	6	0	1	0		
Mus musculus	3	0	2	14	3	3	13	2	3
M. agrastris panonicus	4	0	0	0			0		
Pitymys subterraneus	0			1	0	0	0		
Insectivores									
Crocidura suaveolens	1	0	0	10	0	0	0		
Sorex araneus	2	0	0	1	0	0	0		
Sorex spp.	1	0	0	0			0		
Total	93	12	18	146	17	24	32	8	9
% positive		13%	19%		12%	15%		25%	28%

[a] *T* Number of animals tested
[b] *Ab* Number with antibody
[c] *Ag* Number with antigen

high (Table 3). *Mus musculus* was the most abundant species in this region, but *A. agrarius* and *A. flavicollis* were the species most frequently found infected.

Discussion

Hemorrhagic fever with renal syndrome is a classical clinical syndrome, the etiology of which can be confirmed by virus isolations and antibody studies. Differences between clinical manifestations of this disease in different areas of the world are associated with the strain of hantavirus present. In Asia and the far eastern U.S.S.R. the disease is generally more severe than that observed in western Europe and Scandinavia. Korean hemorrhagic fever and epidemic hemorrhagic fever are severe forms of the disease and are associated with Hantaan virus; nephropathia epidemica, a milder form of the disease is associated with Puumala virus; a less severe form has been attributed to Seoul virus. In Yugoslavia, sporadic cases, small outbreaks, or extensive epidemics occur essentially every year in natural foci of HFRS [6]. Results of a previous study provide substantial evidence for the occurrence of HFRS in Slovenia [1].

Studies of antigen classification of hantaviruses has been done using a variety of methods [11, 17]. Cross-plaque reduction neutralization and IFA tests have permitted serotype classification of hantavirus infections in humans and rodents. Although antibody response to the four serotype (HTN, SEO, PUU, and PH) were sufficiently distinct to permit classification of hantavirus infection in most of our HFRS patients, results with some sera (Table 1) were not clear. Lee et al. [11] described similar observations with sera from HFRS patients from southern Yugoslavia. Based on the geographical distribution of the HFRS patients in Slovenia, it is clear there are at least two natural foci of this disease (Fig. 1).

Almost all patients from Murska Sobota had milder clinical course of the disease, similar to that described for NE in Scandinavia, for which the etiologic agent is PUU virus [7, 13]. More recently, HFRS due to Puumala or a closely related virus has been recognized in France, Belgium, Federal Republic of Germany, and southern Yugoslavia [5, 19].

In Novo mesto and Črnomelj rodents were captured around patients' homes and in the surrounding woods, and *A. flavicollis* and *A. sylvaticus* were the most abundant species captured. *A. flavicollis* was the dominant species infected with hantaviruses. Nine (64%) HFRS cases in this region of Slovenia showed a severe clinical picture with pronounced hemorrhagic manifestations. The one patient who died, did so on the fifth day after onset. When sera from patients with severe HFRS were tested for antibody to hantaviruses, a significant rise of titers was observed by ELISA (IgM) and IFA (IgG) test to Hantaan antigen.

A. flavicollis is widely distributed throughout the region where Balkan HFRS cases have been recorded. Our results support the presumption that this species is the principal maintenance host for a HTN-like virus, which causes severe forms of HFRS [5, 8].

In Vipava we found high antibody and antigen prevalence in *A. agrarius* and *A. flavicollis*. These data suggest that some inapparent infections with hantaviruses probably exists here also.

Our results provide evidence that at least two hantaviral serotypes circulate simultaneously in Slovenia. In geographic areas where more than one hantavirus circulates, it is important to distinguish between the two, because the prognosis for patients is quite different. Therefore, it is necessary to use diagnostic methods that provide adequately discriminatory results.

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Authors' address: Dr. Tatjana Avšič-Županc, Institute of Microbiology, Medical Faculty of Ljubljana, Zaloska 4, YU-61105 Ljubljana, Yugoslavia.

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