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Cross-sectional survey on hantavirus seroprevalence in Canton St. Gallen, Switzerland

Detlev Schultze[a], Walter Fierz[b], Hans C. Matter[c], Sergej Bankoul[d], Matthias Niedrig[e], Andreas Schmiedl[f]

[a] Institute for Clinical Microbiology and Immunology, St. Gallen, Switzerland
[b] LogoLab, Zürich, Switzerland
[c] Federal Office of Public Health, Bern, Switzerland
[d] Swiss Armed Forces, Medical Services Directorate, Bern-Ittigen, Switzerland
[e] Robert Koch Institute, Berlin, Germany
[f] Progen Biotechnik GmbH, Research and Development, Heidelberg, Germany

Summary

Background and objectives: In 2002 the first endemic hantavirus infection in Switzerland was detected only by chance following a broad spectrum of diagnostics. This raised the question, whether Hantavirus infection should be included in the differential diagnosis of febrile illness of patients in Switzerland. In order to estimate the frequency of hantavirus infections in Switzerland, this survey on hantaviral seroprevalence was conducted in the Canton St. Gallen.

Methods: A total of 1693 sera from farmers, forestry workers, and young soldiers as well as blood donors, as a cross-section of the average adult population of the Canton St. Gallen, were screened for hantavirus-specific antibodies by a microsphere-based assay. All volunteers with positive screening results obtained a questionnaire for assessment of details of previous rodent encounter and illnesses compatible with hantavirus infection.

Results: This first survey on hantavirus-specific IgG in populations of eastern Switzerland revealed low seroprevalence-rates not significantly different among populations with higher risk for hantavirus infection (0.0%–1.9%) and the average adult population (0.5%).

Conclusions: As hantavirus infections among different populations are rare, and no evidence for hantaviral nephropathy could be found, serological investigation of suspected endemic hantavirus infection in eastern Switzerland should be confined to patients with acute nephropathy and/or a history of recent rodent encounter.

Key words: hantavirus; seroprevalence; microsphere-based duplex immunoassay; Switzerland

Introduction

Hantaviruses are carried by rodents worldwide with transmission to humans, causing an estimated 60 000 to 100 000 hospitalised cases of hantavirus disease annually. Extended knowledge on the existence of a wide range of unique hantaviruses, circulating in different geographical areas, has marked these agents as "emerging viruses" and asks for an increased awareness. Hantaviruses are transmitted to humans via inhalation of aerosolized excreta, urine and saliva, of silently lifelong infected carrier rodents [1–5]. Case reports suggest, that bites may also play a role in transmission [6, 7].

Occupations that favour human-rodent contact are associated with a higher risk of infection by hantaviruses such as forestry workers, farmers and military personnel [8, 9]. Evidence of several serotypes of hantaviruses in Europe was put forward in 1982 by Lee et al. [10]. In Central Europe, the prevailing Puumala hantavirus causes nephropathia epidemica, a usually mild form of hemorrhagic fever with renal syndrome without major hemorrhages, with the southernmost Puumala hantavirus infection been found in Greece [9, 11]. Whereas Puumala virus has been found to be the cause of HFRS all throughout Europe with high incidence in European Russia, Northern Scandinavia and Finland, Dobrava hantavirus has been shown to cause a large number of cases of severe disease on the Balkan Peninsula with sporadic

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cases reported from Central Europe [12–15]. Infections by Saaremaa virus are reported mainly in eastern and central Europe and symptoms are similar to nephropathia epidemica caused by Puumala virus. Dobrava and Saaremaa virus are genetically and antigenically very closely related and were previously thought to be variants of the same virus [9].

In Germany, hantaviral infection is now reported as the most common endemic rodent-borne human illness, when compared with tularemia, lymphocytic choriomeningitis, and leptospirosis. Furthermore, hantavirus is suspected to be the prevailing cause of renal failure associated with infectious diseases in central Europe [16]. By contrast, no data are available about the incidence of these rodent-borne infections in Switzerland (Swiss Federal Office of Public Health, http://www.bag.admin.ch).

Until recently, endemic hantavirus infection has never been proven in Switzerland. Only in 2002 a hantavirus infection of a child was mereley detected by chance following a broad spectrum of diagnostics [7]. This first case and the suspicion of more undetected endemic cases in Switzerland [17] raised the question, whether Hantavirus infection should be included in the differential diagnosis of febrile illness of patients in Switzerland. In order to estimate the frequency of hantavirus infections in Switzerland, this cross-sectional survey on hantaviral seroprevalence has been conducted in the Canton St.Gallen, on the assumption that hantavirus antibodies are detectable even decades after infection [18, 19].

Different populations with presumed risks of occupational exposure to hantaviruses were included in the survey as well as blood donors from the Regional Blood Donation Centre, Swiss Red Cross, St.Gallen, as a cross-section of the average adult population of the Canton St. Gallen, with its 457'289 inhabitants (year 2004).

In this study, screening for hantavirus-specific antibodies was done by a microsphere-based assay, that enabled the simultaneous measurement of Hantaan- and Puumala virus-nucleocapsid IgG in sera.

Volunteers, material and methods

Participants sera were collected prospectively between October 2002 and May 2003, with the exception of sera from recreational hunters from eastern Switzerland that were collected during January 1993 and December 1996 [20]. Our hypothesis is that in populations with a higher likelihood of rodent encounter, hantavirus infections are more likely to occur than in the average adult population. Farmers, forestry workers, and young soldiers at the end of their military service of 3 months were asked to take part in the study. Farmers were students, grown up on farms, of two cantonal vocational schools. Sera of forestry workers were collected during their meetings and additionally from students of the vocational school.

Especially members of the armed forces are traditionally forming an important contingent with increased risk of hantaviral disease [8, 21]. Hantaviruses can be serologically differentiated into two major subgroups: the Hantaan virus-like and the Puumala virus-like hantaviruses. Depending upon their crossreactivity, Hantaan-antibody assays may also detect infection with viruses carried by Murinae rodents [e.g. Dobrava, Saaremaa and Seoul virus], whereas Puumala hantavirus – antibody test systems show cross-reactivity with a group of viruses carried by Arvicolinae rodents (e.g. Tula virus). Thus, antigens of both viruses are regarded as sufficient to cover all strain types, including the New World Sin Nombre hantavirus [5].

Blood was drawn into tubes without additives and aliquots of serum samples were held at 4 °C for a maximum of 1 week, than frozen at –30 °C. Sera were shipped on dry ice.

For the validation of the microsphere-based assay for detection of Hantaan virus-nucleocapsid IgG, 49 acute-phase or convalescent-phase sera from patients with HFRS from the People's Republic of China and from Greece, positive for Hantaan virus-specific antibodies in IgG ELISA (Progen, Heidelberg, Germany), were used. Additionally, 29 sera of blood donors, and 24 sera of patients with rheumatoid arthritis from Germany, all negative by Hantaan virus-IgG ELISA were tested.

Likewise, validation of the microsphere-based assay for detection Puumala virus-nucleocapsid-IgG was done by use of 22 sera of European patients with acute or past Puumala virus infection, positive for Puumala virus-antibodies in Puumala virus IgG ELISA (Progen, Heidelberg, Germany), 31 negative sera of blood donors and 24 sera of patients with rheumatoid arthritis with no detectable Puumala virus-antibodies in IgG ELISA, respectively.

The sample size calculation was based on a comparison of exposure to hantaviruses measured by seroprevalence in different populations. Based on known seroprevalences in other Central European countries [22–27] an exposure of 5% in the populations with higher risk of exposure to hantaviruses and of 0.5% in blood donors was assumed. With a power of 0.8, an alpha value of 0.05, and a ratio of seropositive samples for the higher risk group to the blood donors of 10:1, 94 volunteers from each population and 940 blood donors would have been needed [28].

Finally, a total of 1710 sera from 1029 blood donors, 382 farmers, 104 forestry workers, 104 soldiers, and 91 hunters were collected.

Due to the small number of seropositive cases, the exact probability of finding the observed numbers was calculated using the Fisher exact probability test for 2×2 contingency tables. A probability smaller than $p = 0.05$ indicates strong evidence against the null hypothesis that occupation is not likely to influence the proportion of seropositives. 95% binomial confidence intervals (95%CI) for the prevalence in each of the 5 populations were calculated using SAS 9.1 software (SAS Institute, Cary, NC, USA)

All volunteers had agreed upon obtaining a questionnaire for assessment of details of previous rodent encounter and illnesses compatible with hantavirus infection, in case of having been tested positive.

The study protocol was approved by the Ethics Committee of the Canton St.Gallen (EKSG 02/64), and written informed consent was obtained from all participants in the study.

Hantavirus-specific duplex microsphere-based immunoassay (MIA)

Recombinant Puumala- and Hantaan virus-nucleocapsid proteins were produced and purified as described previously [29, 30]. Bovine serum albumin was obtained from Roche (Mannheim, Germany). The Fcγ-fragment was derived from Dianova (Hamburg, Germany). Antigens were coupled to microspheres (Luminex Corp., Austin, Texas) by using an N-hydroxysulfosuccinimide enhanced carbodiimide-mediated coupling reaction [31]. The Hantaan virus antigen was coupled to nominal microsphere number 21, Puumala virus antigen to microsphere number 19, bovine serum albumin to microsphere number 45 and the Fcγ-fragment to microsphere number 61. A total volume of 500 µl of each antigen was added to the respective microspheres. Microsphere concentrations were determined using a hemacytometer (Fisher Scientific GmbH, Nidderau,Germany) and diluted in PBS, containing 0.1% bovine serum albumin and 0.09% sodium azide to generate a Puumala virus-, Hantaan virus-, bovine serum albumin-, and Fcγ-coated microsphere mix (8000 microspheres total, 2000 microspheres of each type).

In the seroprevalence study the MIA was performed as a two-step indirect procedure, using the same lot of prepared beads as for validation. Participant's sera and the Puumala- and Hantaan virus-IgG positive controls were diluted 1:3000 in assay buffer, as an optimal dilution obtained from separate experiments (data not shown). 25 µl of each dilution were transferred into the wells of a 96-well microtiter plate. 25 µl of the microsphere mix (8000 microspheres total, 2000 microspheres of each type) were added to each well. The microtiter plate was covered with foil and incubated for 1 h at 37 °C in darkness. R-Phycoerythrin-conjugated anti-human IgG antibodies, diluted in assay buffer to an optimized concentration (data not shown) were added to each well. After a second 1 h incubation step, 25 µl PBS containing 0.8% formaldehyde was added to each well. The samples were subsequently analyzed on a Luminex100 instrument using software, calibration microspheres, and sheath fluid supplied by the manufacturer. The median fluorescence intensity (MFI) of fluorochrome-conjugated secondary antibodies bound to individual microspheres is derived from flow analysis of 100 microspheres per type and well. Collected data, in median fluorescence intensity (MFI) units, are processed with StatView for Windows.

The conjugation of recombinant hantavirus nucleocapsid proteins of the Puumala and Hantaan virus serotype to distinguishable fluorescent microspheres provided the basis for a duplex immunoassay to detect human antibodies induced by hantavirus infection. Fcγ and bovine serum albumin coupled microsphere populations served as internal assay controls. The duplex immunoassay was performed at 37 °C without wash steps and measured anti-Puumala and anti-Hantaan virus nucleocapsid protein IgG in diluted human serum samples simultaneously in a standardized 2.5 h procedure.

The MFI obtained with 49 Hantaan virus-IgG positive sera ranged from 635 to 21093 MFI (mean 8763, SD 6299), and for 53 Hantaan virus-IgG negative sera from 24 to 734 MFI (mean 142, SD 100) (data not shown). By choosing a cutoff of 442 MFI (mean + 3×SD) one false positive result with a Hantaan virus-IgG negative serum (MFI = 734) was obtained. Accordingly, a sensitivity of 100% (95%CI, 92.8–100%) and a specificity of 98% (95%CI, 89.0–100%) was calculated for the Hantaan virus-IgG duplex immunoassay.

Likewise, the MFI values for Puumala virus positive and negative control sera were established. Twenty-two sera from patients with Puumala virus infection yielded MFI from 690 to 13728 (mean 4738, SD 3832), and MFI of 31 negative sera of blood donors and 24 sera of patients with rheumatoid arthritis ranged from 1 to 531 (mean 129, SD 82). By selecting 378 MFI as cut off (mean + 3×SD) one false positive result was found. According to these results, a sensitivity of 100% (95%CI, 84.6–100%) and a specificity of 98.2% (95%CI, 90.3–100%) was calculated. For both assay validations, results from repeated experiments were compared to demonstrate good inter-operator reproducibility (data not shown). The signal intensity for bovine serum albumin- and Fcγ-conjugated control microspheres demonstrated no false positive or false negative results, respectively.

Bunyavirus immunoblot

All serum samples with MFI above cutoff, i.e. 442 MFI by Hantaan virus- and 378 MFI by Puumala virus-MIA were additionally analyzed by Bunyavirus immunoblot IgG Mikrogen, Munich). The Bunyavirus immunoblot contains recombinant antigens, i.e. combined Puumala + Hantaan virus antigens, separate antigens of Puumala, Hantaan, Dobrava, and Seoul virus antigen and has previously been found to be sensitive (90%) and specific (100%) in hantavirus diagnostics external quality assurance [32] and in the diagnosis of nephropathia epidemica [33].

Results

Seventeen out of 1710 sera from this cross-sectional survey showed signal intensities above cutoffs for Hantaan and Puumala virus-MIA and additionally on bovine serum albumin-coated (n = 9) and uncoated microspheres (n = 8). They thus demonstrated bovine serum albumin- and plastic-binding capacities that inhibit detection of specific binding to Hantavirus antigens and were excluded from data analysis.

Of the remaining 1693 sera, 25 (1.5%) reacted positive by Puumala virus-MIA, 8 (0.5%) by Hantaan virus-MIA, and 31 (1.8%) coincidentally by both MIA.

Results for Puumala – and for Hantaan virus-MIA positive sera ranged from 380 to 2974 MFI (mean 734, SD 572) and from 460 to 1904 MFI (mean 758, SD 364), respectively. Sera negative by Puumala- and Hantaan virus-MIA showed MFI from 0 to 374 (mean 132, SD 60) and from 0 to 436 (mean 129, SD 57), respectively.

Eleven (17.2%) of 64 MIA-positive sera were reactive by Bunyavirus immunoblot. Although the prevalence of positive sera confirmed by Bunyavirus immunoblot in blood donors (0.5%) was different from farmers (0.8%), soldiers (1.9%), hunters (1.1%), and forestry workers (0%), differences were not significant (p = 0.45, p = 0.13, p = 0.40, and p = 1.0, respectively) (table 1 and 2).

Table 1

Number of sera reactive by microsphere-based immunoassay (MIA) and Bunyavirus immunoblot IgG.

Participants	Total	Blood donors	Farmers	Forestry workers	Soldiers	Hunters
number	1693	1020	379	100	103	91
Sera reactive by						
Puumala virus MIA	25	16	6	0	2	1
Hantaan virus MIA	8	3	1	2	0	2
Puumala and Hantaan virus MIA	31	22	6		1	1
total (%)	64 (3.8)	41 (4.0)	13 (3.4)	3 (3.0)	3 (2.9)	4 (4.4)
Sera reactive by						
Bunyavirus immunoblot IgG	11	5	3	0	2	1
% (95% CI)	0.6	0.5 (0.1–1.1)	0.8 (0.1–1.7)	0 (0.0–4.0)	1.9 (0.02–6.8)	1.1 (0.03–6.0)

Table 2

Participants and reactivity of sera.

Participants			Microsphere-based immunoassay [MFI]1		Bunyavirus immunoblot IgG reactivity			
Sex	Age	Contact to rodents during professional/ leisure acitivities	PUU cutoff = 378	HTN cutoff = 442	PUU + HTN	PUU	HTN	SEO
Blood donors								
m	51	–/+	454	–	+	+	–	–
m	42	–/+	691	623	+	–	–	–
m	42	–/+	–	505	+	+	–	–
f	52	–/–	1624	1361	+	–	–	–
f	38	–/+	472	509	+	–	–	–
Farmers								
m	20	+/+	787	–	–	+	–	–
m	20	+/+	427	–	–	+	–	–
m	32	+/+	824	562	–	+	–	–
Soldiers								
m	20	–/+	2437	471	+	–	–	–
m	20	+/–	395	–	–	+	–	–
Hunters, recreational								
m	un	un /+	–	466	+	+	–	–

1 MFI = median fluorescence intensity, mean results from serum analysis in duplicate; + = positive, – = below cutoff (MIA) or not reactive by Bunyavirus immunoblot, respectively; PUU = Puumala, HTN = Hantaan, SEO = Seoul virus antigen; un = unknown.

The mean age of participants with confirmed-positive sera was 33.7 years, ranging from 20 to 52 years, with a male to female ratio of 4.5 to 1. All but one seropositive participant reported as having had contact to rodents during professional or leisure activities, i.e. staying in cabins infested by mice. None of them recalled a feverish illness with apparent involvement of the kidneys. All positive participants had travelled outside Switzerland, but none of them recalled a feverish illness while travelling.

Discussion

In this study we used a two-tiered system with screening by MIA and confirmation of reactives by Bunyavirus immunoblot.

Among 64 MIA-positive of 1693 sera (3.8%) 11 could be confirmed by Bunyavirus immunoblot. Among 1682 sera negative by MIA or Bunyavirus immunoblot, a ratio of 30 false positive sera by Puumala virus- and 34 by Hantaan virus-MIA could have been expected, given its unspecificity of 1.8% and 2.0%, respectively. This is in the order of magnitude of 53 MIA-positive, but Bunyavirus immunoblot IgG non-reactive sera found in this study.

Results of positive sera were lower in study pa-

tients (mean 734 MFI for Puumala virus-MIA, mean 758 MFI for Hantaan virus-MIA) than in sera for validation of Puumala- and Hantaan virus-MIA (mean 4738 and 8763 MFI, respectively). For the latter purpose, sera were selected from patients with acute hantaviral illness or during convalescence. Although we do not know how long hantavirus-specific antibodies are detectable by MIA after acute infection, it can be assumed that titers will drop over years, thus explaining the lower titers in study patients without symptoms of hantaviral illness.

The Bunyavirus immunoblot has not been used as a screening test due to its high manual operation time. The ratios of Bunyavirus immunoblot-confirmed positive sera found among the normal adult population (0.5%) and among populations with a higher likelihood of rodent encounter (0.0%–1.9%) differ from the originally assumed values (0.5% and 5%), which results in less power when testing the null hypothesis. Thus, this study provides an estimate of seroprevalence but can't say whether occupation has an influence in it or not.

In neighbouring Austria, a serosurvey on patients from the internal medicine revealed an overall prevalence of 1.2% (n = 1215), ranging from 0.2% to 1.8% in different areas of the country [23]. A serosurvey in Slovakia demonstrated Puumala- and/or Hantaan virus-IgG in 0.84% sera of the average population (n = 2133), ranging form 0.54% (western/central) to 1.91% (eastern), with significantly more positive sera from forestry workers (5.88%; n = 153) than those from the general population of eastern Slovakia [26]. Among residents of Germany the overall Hantavirus-specific seroprevalence was about 1.63%, ranging from 0.8% in north-east to 3.12% in southwest Germany, thereby significantly different either in certain areas (e.g. Reutlingen in Baden-Würtemberg) or between professionally exposed forest workers and the average population [27]. By contrast, two independently conducted studies in Germany revealed only minor differences between forestry workers and the normal population [4, 34].

In this study, seroprevalence of Bunyavirus immunoblot-confirmed Hantavirus-specific IgG among blood donors (0.5%), as a surrogate for the adult normal population is at or below the lower limit of magnitude found in studies from other central European countries [22–27]. This low seroprevalence is in agreement with the absence of published cases of nephropathia epidemica and clinically unspecific hantavirus illness in eastern Switzerland so far.

All except one confirmed seropositive participant of our study reported known risks for acquisition of hantavirus infection, i.e. staying in cabins infested by mice [35]. But none of them recalled a feverish illness with apparent involvement of the kidneys. This is in agreement with the fact, that asymptomatic or non-specific mild infections outnumber the symptomatic, characteristic infections, e.g. HFRS [2, 24, 36], with a presumed ratio of symptomatic disease in about 10% of those infected [37].

The male to female ratio among confirmed seropositive participants in this study was 4.5/1 and thus in the order of male to female ratio of 2/1 to 5/1 in clinical cases of nephropathia epidemica in Fennoscandia, France and Korea, respectively [9, 36].

In this report, we describe a MIA for the detection of hantavirus-IgG, that uses no washing steps und can be automatically performed in a 2.5h procedure. The duplex MIA for detection of Hantaan- and Puumala virus-IgG in human serum can be multiplexed, allowing the simultaneous detection of different hantavirus serotype-IgG in one assay. Furthermore, inclusion of bovine serum albumin-coated and uncoated microspheres enables the multiplexed assay to detect bovine serum albumin- and plastic-binding capacities of sera that might cause false-positive results in ELISA.

In conclusion, prevalence of hantavirus-specific IgG in different populations of the Canton St. Gallen is low in comparison to other central European countries. As hantavirus infections among the population of the Canton St. Gallen are seemingly rare events, routine serological investigation should be confined to patients with acute nephropathy and appropriate laboratory findings, e.g. hematuria, leucocyturia, tubular cell casts, elevated serumcreatinine, leucocytosis, and thrombocytopenia [3, 38]. Additionally, other causes of nephropathy, like infection by *Leptospira interrogans* or intake of non-steroidal anti-inflammatory drugs should be considered [36].

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Correspondence:
Detlev Schultze
Institute for Clinical Microbiology and Immunology
Frohbergstr. 3
CH-9001 St.Gallen
Switzerland
E-Mail: detlev.schultze@ikmi.ch,

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