

## A major outbreak of hantavirus infection in Belgium in 1995 and 1996

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### SUMMARY

Haemorrhagic fever with renal syndrome (HFRS) is a human disease characterized by flu-like symptoms, renal dysfunction, and in severe cases, haemorrhagic manifestations. The causative agents of HFRS are Hantaan (HTN), Seoul (SEO), Puumala (PUU) and Dobrava (DOB) hantaviruses. Hantavirus infections are of increasing importance in Europe. Outbreaks occur in Belgium with a 3- to 4-year interval with an increasing number of cases. We describe the largest outbreak so far in Belgium with 217 serologically and clinically confirmed cases in the period between October 1995 and December 1996. We demonstrated that the use of viral antigen derived from a local PUU-strain was able to detect significantly more sera positive for IgM in an immunofluorescence assay. Furthermore, although in some cases SEO, HTN and DOB antibody-reactivities were detected by ELISA, only PUU infections could be confirmed by neutralization test. The presence of an unknown hantavirus serotype circulating in Belgium should be considered.

### INTRODUCTION

Hantavirus, a genus of the *Bunyaviridae* family, comprises the aetiological agents of haemorrhagic fever with renal syndrome (HFRS). HFRS occurs in Europe and Asia, causing a disease characterized by fever, headache, gastrointestinal symptoms and renal dysfunction, the more severe forms with haemorrhagic manifestations. At least four hantaviruses cause HFRS: Hantaan (HTN), Seoul (SEO), Puumala

(PUU) and Dobrava (DOB) viruses [1–4]. The epidemiology of hantaviruses is closely linked to the ecology of their principal hosts. Rat-associated strains (SEO) have been reported to cause urban and laboratory-acquired disease, whereas strains associated with *Apodemus* sp. (HTN, DOB) and *Clethrionomys* (PUU) are linked to the rural disease type.

The type of HFRS associated with PUU infection is called nephropathia epidemica (NE) and occurs in north and west Europe [1]. The importance of NE is rapidly increasing in Europe as highlighted by several major outbreaks in the past few years [5]. We describe here an outbreak in south Belgium with 217 serologically and clinically confirmed cases in the period between October 1995 and December 1996.

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## MATERIALS AND METHODS

### Sera

A total of 1266 sera from 46 Belgian hospitals, mainly located in the south of Belgium, were sent to two laboratories, the National Reference Centre for Hantavirus Infections (Brussels) and the Virology Laboratory of the Institute for Tropical Medicine (Antwerp), and analysed by hantavirus serology.

### Serological assays

Immunofluorescence assay (IFA, IgG and IgM) for HTN (strain 76-118), PUU (strains CG 18-20 and CG 13891) by a standard IFA procedure was followed [6]. Apart from routine screening, the aim was to assess the differences in reactivity between antigens of a 'local' PUU strain and the Russian CG 18-20 strain. Since the PUU strain CG 13891 is thought to be a typical Belgian strain [7], IFA slides coated with CG 13891 infected Vero E6 cells were used for simultaneous IFA testing with strain CG 18-20 and HTN 76-118 infected Vero E6 cells. IFA slides ( $10^6$  cells/ml, 40% infected, 60% non-infected) were validated by testing against a Belgian (end-point titre for CG 13891 and CG 18-20 was 1024 and 512, respectively) and a Russian (end-point titre for CG 13891 and CG 18-20 was 512 and 2048, respectively) hantavirus IgG-positive serum. A sample was considered positive for the presence of hantavirus-specific antibodies if the titre was  $\geq 16$ .

A four-serotype ELISA IgG screening set-up for HTN (strain 76-118), PUU (strain CG 18-20), SEO (strain R22VP30) and DOB (strain 907/5) viruses, where whole cell lysate antigens were used for coating purposes whereby standard ELISA procedures were followed [8].

A three-serotype ELISA IgM screening set-up for HTN (strain 76-118), PUU (strain CG 18-20) and SEO (strain R22VP30) viruses, where standard ELISA procedures were followed [8]. Testing for DOB IgM was technically not possible due to the absence of viral antigen. Cut-off (CO) values for the ELISA tests were calculated as the mean optical density (OD) of a series of negative controls plus three standard deviations. Of each series of tests, the ratio (OD of sample/CO) was calculated to enable us to compare different series of tests. Depending on the availability of the necessary serum quantity, confirmation was performed by means of focus reduction neutralization tests (FRNT) and/or plaque reduction neutralization tests (PRNT)

for HTN, PUU, SEO and DOB [3, 9, 10]. ELISA (Progen®) based on recombinant antigens (HTN 76-118 and PUU CG 18-20 nucleocapsid protein for IgG and IgM assays), and/or Western blot (WB) assay (Chiron®) based on SNV and SEO antigens was applied to confirm the reactivity to hantaviruses of sera previously found positive by IFA or by ELISA based on whole cell lysate antigens.

### Clinical data

The clinical data, obtained from the treating physicians, including the general medical history of the patient: profession, date of onset of symptoms, date of sampling and the clinical symptoms.

The following biochemical data were collected: peak serum creatinine, nadir platelet count, peak ALT and AST, nadir serum cholesterol and HDL-cholesterol and peak serum triglycerides [11].

The criteria for hantavirus serology was a clinical picture of acute hantavirus infection; flu-like symptoms of unknown origin, unexplained acute renal failure and biological parameters indicating a severe viral infection of unknown origin.

## RESULTS

### IFA serology

Of 69 samples that reacted positive for hantavirus-specific antibodies by IFA, 52 samples (75.4%) had IgG titres that were higher for strain CG 13891 than for strain CG 18-20, while 17 samples (24.6%) had equal titres (Table 1). None of the sera were found negative for CG 13891, but positive for CG 18-20-reactive IgG antibodies. All of the 69 samples had higher IgG end-point titres to the two PUU strains when compared to the titres against HTN. The median CG 13891 IgG titre was significantly higher ( $p < 0.05$ ) than the median CG 18-20 IgG titre. Thirty-six samples (52.2%) were IgG and IgM positive, 34 sera (96.4%) had higher IgM titres to CG13891 as compared to the titre to CG-1820 while only two (5.6%) had higher IgM titres to CG 18-20. Two samples (2.9%) were CG 13891 IgM positive and CG 18-20 IgM negative while being IgG negative to both antigens, thus indicating a very recent infection. Although the median IgM titres for CG 13981 and CG 18-20 were similar, significantly more sera ( $p < 0.05$ ) were positive for CG 13891 reactive IgM. These 69 IFA-positive samples were also found positive in the PUU-ELISA assay.

Table 1. *ELISA and IFA serology findings*

IFA							
(n = 69)	IgG CG 13891	IgM CG 13891	IgG CG 18-20	IgM CG 18-20			
No. positive	68	36	59	22			
Median titre	512	32	128	32			
ELISA							
(n = 217)	HTN-IgG	HTN-IgM	PUU-IgG	PUU-IgM	SEO-IgG	SEO-IgM	DOB-IgG
No. positive	14	0	182	135	9	0	9
Median ratio*	2.2	0	2.7	3.2	3.4	0	2.4

\* Ratio, observed OD of the sample/cut-off OD.

CG 13891, PUU hantavirus isolated from a *C. glareolus* captured in Belgium.

CG 18-20, PUU hantavirus isolated from a *C. glareolus* captured in Russia.

### ELISA serology

A total of 217 samples were found positive for hantavirus-specific antibodies. A number of 182 sera (83.9%) showed the highest IgG-reactivity to PUU, while 135 (74.2%) of these PUU-IgG-positive sera were also found PUU-IgM-positive. Three sera (1.4%) were found positive for PUU-reactive IgM while being PUU-IgG-negative, indicating sampling in an early phase of the infection. Fourteen sera (6.5%) were found positive for HTN-reactive IgG while being HTN-IgM-negative, nine sera (4.1%) were found positive for SEO-reactive IgG while being SEO-IgM-negative and nine sera (4.1%) were found positive for DOB-reactive IgG, testing for DOB-reactive IgM antibodies was impossible due to absence of viral antigen (Table 1). All patients for which the first serum sample contained hantavirus-specific IgG but no detectable levels of hantavirus-specific IgM ( $n = 79$ ) were confirmed for HFRS by increasing levels of IgG assayed in a second serum sample.

For confirmation of the ELISA results obtained by the use of native viral antigens, 100 PUU-IgG- and 14 HTN-IgG-positive samples were tested by ELISA assay based on PUU recombinant nucleocapsid protein (rN) and HTN-rN antigen, respectively, and were all found positive. Ninety-one PUU-IgM-positive samples were subsequently tested in a rN  $\mu$ -capture ELISA after elimination of IgG antibodies by absorption. Again, all sera showed positive reactions.

### Western blot

For confirmation of the ELISA results of the SEO-IgG-positive sera, a WB based on SNV and SEO (strain 80/39) rN and two IgG control bands, was applied. The WB test was designed for the hantavirus

situation in the United States, thus including only SNV and SEO virus antigens.

Out of 8 SEO-IgG ELISA positives, 5 (62.5%) showed a specific reaction with the SEO rN. One of the sera was no longer available for additional tests. None of 14 HTN-IgG ELISA positive samples showed specific WB reactions with SEO, but 5 samples reacted with the SN antigen.

### FRNT/PRNT

When the serum quantity was sufficient, samples from the clinically interesting cases or sera with surprising serology results, e.g. mainly DOB reactivity, were submitted to focus reduction neutralization test (FRNT) or plaque reduction neutralization tests (PRNT). A total of 28 sera were examined by this set-up; only convalescent sera were used, since earlier findings have showed the presence of high cross-reactivity of neutralizing antibodies in acute phase sera [3]. All PUU IFA/ELISA-positive samples from patients with severe clinical symptoms were confirmed as specific for the PUU serotype. One patient that tested DOB ELISA IgG-positive on several occasions failed to demonstrate any neutralizing activity against the four hantavirus serotypes examined (PUU, DOB, HTN, SEO). Four other sera that showed DOB reactivity in ELISA could not be confirmed either. One convalescent sample, taken 34 days after onset of symptoms, reacted equally to HTN and PUU by PRNT (end-point titre of 640).

### Biochemical and clinical data

The data of major interest from 50 PUU IgG- and IgM-positive cases were compared with the mean

Table 2. Biochemical data of 50 IgM and IgG positive cases

	Platelets (1000/mm <sup>3</sup> )	Urea (mg%)	Creat, (mg%)	Total chol. (mg%)	HDL (mg%)	Trigl. (mg%)	ALT (IU/ml)	AST (IU/ml)
Norm. val.	> 150	< 50	< 1.2	< 200	< 45	< 180	< 40	< 40
Median	93	56	1.7	135	16	256	107	35
% Abn	67.6	83.3	10.0	70.8	66.7	66.7	70.0	76.2
Max	393	238	14.7	288	93	548	429	429
Min	16	20	0.8	24	3	26	3	3

Norm. val, normal value according to test procedure.

% Abn, % of patients showing abnormal values.

Max, maximum value observed in the series of patients.

Min, minimum value observed in the series of patients.

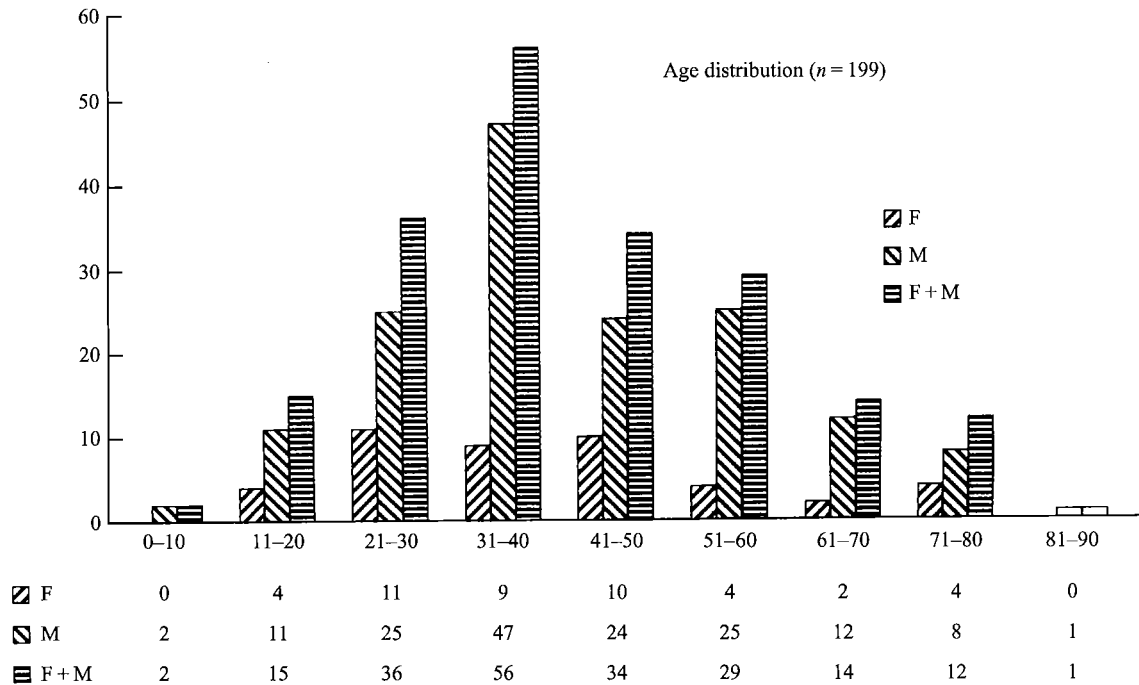


Fig. 1. Age distribution curve. Hantavirus cases sorted by age (10 year groups) and sex (F, female; M, male).

values of a series of hantavirus-negative sera and are summarized in Table 2. The most dominant abnormalities were decreased platelet count, total cholesterol and HDL-cholesterol together with increased triglycerides and ALT/AST increase. A moderate increase of urea and creatinine is indicative for renal dysfunction. These findings were in line with the previously obtained results of Colson and colleagues [11] regarding the 1992-3 epidemic.

On 50 cases detailed clinical records were obtained, the prevailing observation was acute renal failure (ARF); in 5 cases dialysis was necessary. Twelve cases were reported with myopia, 4 of which also experienced diplopia and 1 of these patients also reported photophobia. Two cases presented with non-cardiogenic pulmonary edema ( $P_{O_2}$  36, resp. 54 mmHg

on admission). One pregnant case was hospitalized with, apart from ARF, mild pulmonary problems but recovered without sequelae and delivered a normal baby. Rare complications were epistaxis in 7 cases, acalculous cholecystitis in 2 cases, Guillain-Barré syndrome in 2 cases and hematemesis in 1 case. All cases, confirmed as PUU by FRNT and/or PRNT, eventually recovered without sequelae.

### Epidemiology

A total of 217 patients were clinically and serologically confirmed for hantavirus infection. The male/female ratio was 3.62 (170 males and 47 females). The mean age was 40.8 years (2-86). The age distribution curve showed that the 31-40 years male age group was at

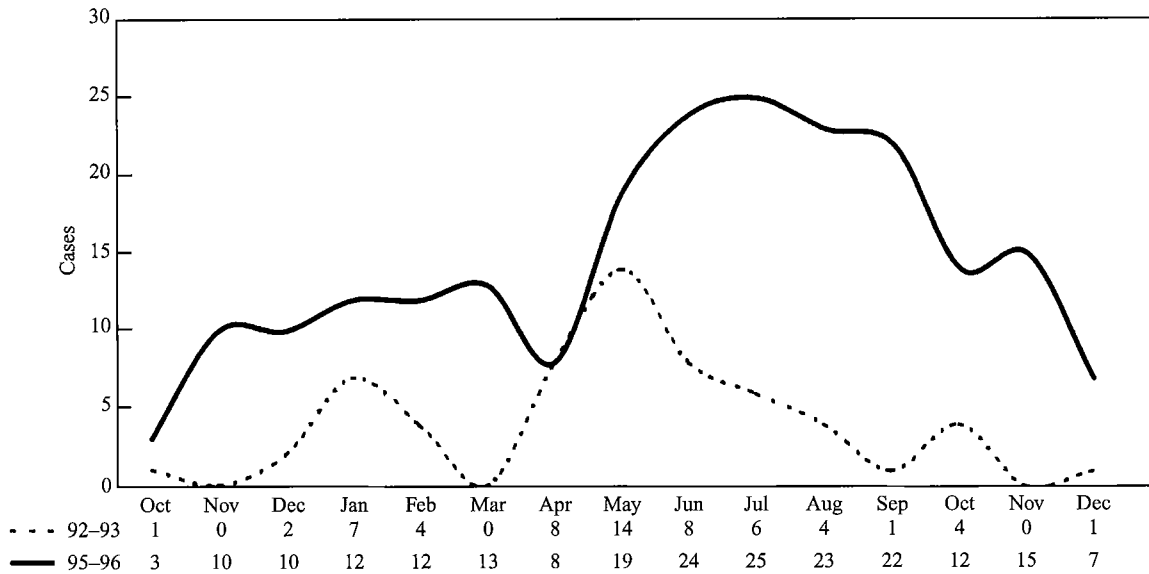


Fig. 2. Seasonal distribution curve. Cases per month, black line figures for the period from October 1995 to December 1996, dotted line figures for the period from October 1992 to December 1993.

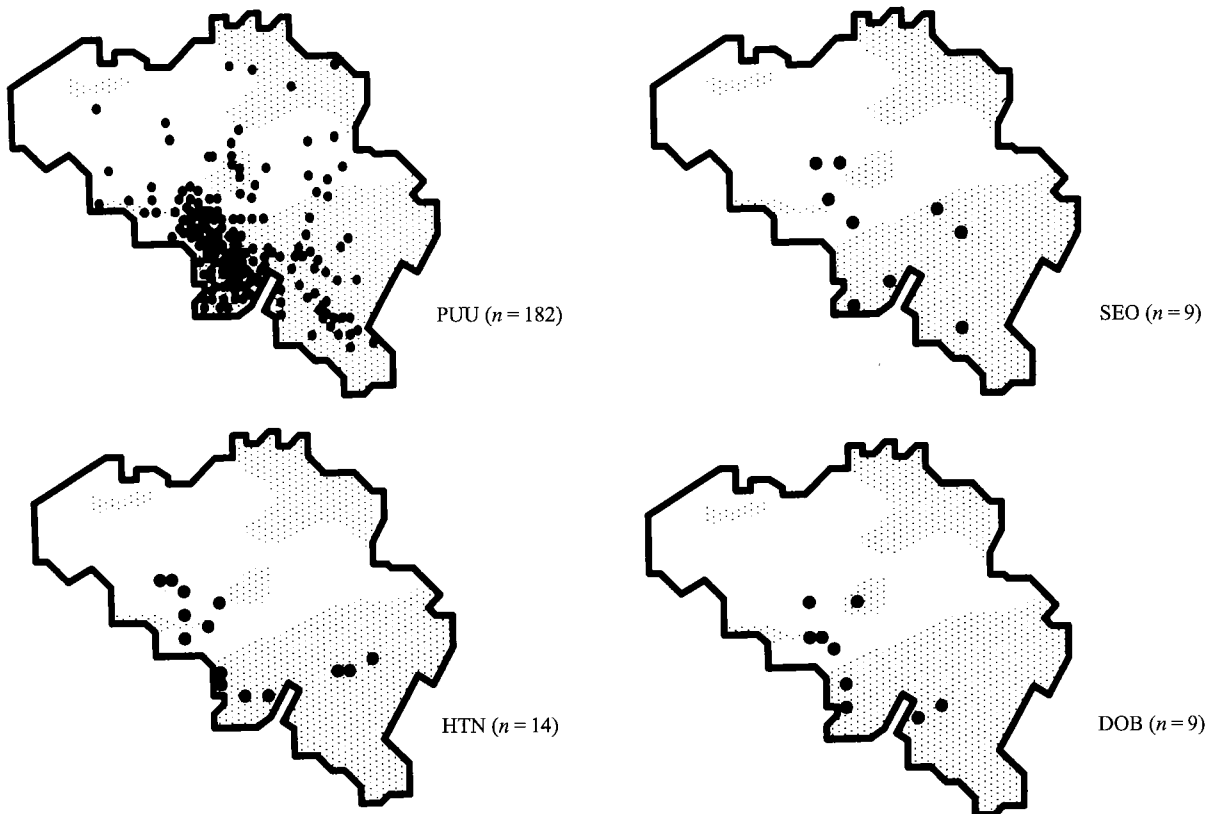


Fig. 3. Geographical distribution of hantavirus ELISA-reactive cases in Belgium. Distribution of hantavirus cases in Belgium according to antibody reactivity as demonstrated by ELISA test. The dotted areas figure for the forested areas in Belgium.

the highest risk, while in the female group the highest risk was almost evenly spread over the 21–50 years age groups (Fig. 1).

The seasonal distribution of the 217 HFRS cases from 1 October 1995 to 31 December 1996 was

slightly different from the previous outbreak (1992–3) where the distribution curve was broken down into two sharp epidemic peaks [12]; it showed a continuing epidemic period from November 1995 to March 1996, a decrease in April 1996 that was followed by the main

epidemic period, i.e. June 1996 to September 1996. October and November 1996 showed a similar, but higher peak, as October and November 1995. A sharp decrease in the number of cases in December 1996 announced the end of the epidemic (Fig. 2).

The geographical distribution of the hantavirus cases during the 1995–6 outbreak showed the highest density in the same area as the 1992–3 outbreak, i.e. the south of Belgium, near the French border. However, there was a gradual spread towards the north-west and north-east (Fig. 3).

## DISCUSSION

The 1995–6 hantavirus epidemic in Belgium took place in the same forested region, located in the southern part of the country, as the 1992–3 epidemic with 60 serologically confirmed cases during the same period of time. With 217 clinically and serologically confirmed cases, this is the most important outbreak so far described in Belgium and suggests an increasing number of hantavirus infections in the endemic area. The importance of the increased awareness of local physicians could not be assessed, but without any doubt this has contributed to the detection of the larger number of cases. The positive effect of a newsletter concerning hantavirus infection in Belgium, distributed in 1996 by the Ministry for Public Health, was demonstrated by an increased request by physicians for information about hantavirus disease.

The IFA findings, where significantly more sera tested positive for IgM against PUU strain CG 13891 than to strain CG 18-20, and where the titres for IgG against CG 13891 were also significantly higher than the titres to CG 18-20, indicate that the local PUU strain CG 13891 might be more suitable for testing suspected Belgian hantavirus cases in the future.

By ELISA, 217 patients were considered as HFRS cases. A case was considered acute when reactive by IgM serology and/or when having an IgG sero-conversion or a rising IgG reactivity (at least a twofold rise of the ratio). The relative high number of IgM-negative sera can be partially explained by the fact that sampling occurred frequently about 2–3 months after onset of symptoms. Indeed, hantavirus infection is still not the prime suspect in cases of flu-like symptoms, particularly during autumn and winter. Another possible explanation for the negative IgM serology could be that some sera were not sent until all other possibilities were explored in local

laboratories, thus not guaranteeing proper preservation of the sera ( $-20^{\circ}\text{C}$ ), which could result in a significant drop in IgM reactivity. In general, due to unidirectional cross-reactivity between HTN and PUU, HTN-reactive sera show little or no reactivity in PUU assays. However, highly cross-reactive antibody responses are seen for several individuals, also between distantly related hantaviruses such as PUU, HTN and DOB [3]. The vast majority of the PUU-positive patient sera cross-reacted only weakly or were completely negative in the HTN rN assay, which was in line with PUU as the prevailing serotype present in Belgium.

The existence of isolated HTN IgG reactivity in ELISA remains unexplained. In view of the fact that all the available samples were negative for neutralizing antibodies (we were not able to apply FRNT or PRNT on all samples, due to lack of sufficient quantities of serum), and the absence of specific IgM reactivity, we cannot rule out false positive reactions in the IgG ELISA. The presence of HTN virus has never been described in Belgium. Also, the habitat of the natural reservoir of HTN virus, *Apodemus agrarius*, does not reach as far as west-Europe. Pending confirmation, we therefore suggest cautious interpretation of such aberrant serology result.

We decided to use a WB assay based on SEO rN antigen to confirm the ELISA reactivities. Five out of eight ELISA SEO-IgG positives showed a positive reaction in WB. However, none of these eight samples were IgM positive by ELISA. Unfortunately, leptospirosis was often the first suspected agent and hantavirus serology was delayed for months thus missing the acute phase, and accordingly, potential SEO-IgM-reactive antibodies. The spread of DOB virus throughout Europe has been suggested by Plyusnin and colleagues [13] and reports by Belgian physicians regarding differences in disease severity (unpublished communications) could point in such direction. However, the disease severity not only depends on the involved hantavirus serotype; viral load, human immune responses, and the human HLA B8 allele may be associated with more or less serious symptoms [14]. Also, an increased virulence of the prevailing hantavirus strains in Belgium could be a determining factor for reported disease severity. The finding of DOB reactivity in ELISA and the subsequent non-confirmation by FRNT, where all four applied serotypes (HTN, SEO, PUU and DOB) failed to react, could be a first indication of a yet unknown hantavirus serotype existing in Belgium.

Thus, our study confirmed only PUU virus as the hantavirus serotype responsible for human hantavirus disease in Belgium. This corroborates the findings of Verhagen and colleagues, where the red bank vole (*Clethrionomys glareolus*) was shown to be the main reservoir for hantavirus in Belgium [7].

The age distribution curve showed that in the male population the 31–40 years age group was most at risk (Fig. 1), whereas in the female population the main risk is almost evenly spread over the 21–50 years age groups. This corroborates the findings of Van Loock and colleagues regarding the role of the professional activities in acquiring hantavirus infection [15]. The occurrence of only two cases (0.9%) aged under 10 years (2 and 9 years, respectively) indicated that children are not a main target age group for hantavirus infections. Although playing in forested areas and other recreational activities should bring an increased risk, there seems to exist a protective mechanism that inhibits the virus propagation in children. Only the Belgian coastal region still remains free of hantavirus cases. It must, however, be taken into account that Belgium is a relatively small country (approx. 30 000 km<sup>2</sup>) and thus patients did not necessarily come in contact with the disease in the vicinity of their homes. The geographical distribution of the hantavirus cases with aberrant serology tests, i.e. dominant HTN-, SEO-, or DOB-ELISA reactivity shows no clustering, if we define a cluster as two or more cases within a 4-week period. This observation seems to be in contradiction with the hypothesis of the existence of an unknown hantavirus in the region.

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