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Pathogenicity and virulence of the present hantaviruses in Bosnia and Herzegovina: the impact on renal function.

M. Hukic¹, A. Valjevac², D. Tulumovic³, F. Numanovic⁴, P. Heyman⁵.

¹Institute of Clinical Microbiology, Clinical Center University of Sarajevo,

²Department of Physiology, Institute of Physiology and Biochemistry, Medical Faculty,
University of Sarajevo

³Clinic for Nephrology, Urology,. Dialysis and Renal Transplantation. University Clinical Centre
Tuzla

⁴Clinic for Laboratory Diagnosis, Department of Microbiology, University Clinic Centre Tuzla

⁵Research Laboratory for Vector-Borne Diseases, National Reference Laboratory for Hantavirus
infections, Queen Astrid Military Hospital. Div. Well-Being / Health /Epi&Biostat

Corresponding author:

Paul Heyman
Research Laboratory for Vector-Borne Diseases
National Reference Laboratory for Vector-Borne Diseases
Queen Astrid Military Hospital, Bruynstraat 1, B-1120 Brussels, Belgium
Tel : +32-2-2644044
Fax: +32-2-2644078
e-mail: paul.heyman@mil.be

Running title: Hantavirus infections in Bosnia and Herzegovina

Summary

Dobrava (DOBV) and Puumala (PUUV) viruses are endemic throughout the Balkans and cause high fever with renal syndrome (HFRS). The aim was to assess impact of two different HTV on renal function in HFRS patients during acute stage of illness. We also aimed to assess DOBV and PUUV distribution between symptomatic, HFRS patients and asymptomatic hantavirus antibody positive subjects. The study included 264 symptomatic, HFRS patients and 63 asymptomatic hantavirus antibody positive healthy subjects. In our study, 131 (49,6%) HFRS patients were regarded as PUUV and 69 (26,1%) as DOBV-infected patients, while in 64 (24,2%) of HFRS patients that showed all clinical and biochemical signs of HFRS, the causal hantavirus could not be determined with commercially available tests. DOBV-infected patients were associated with more requirements for haemodialysis treatment, lower diuresis and higher serum creatinine and urea values compared to PUUV-infected patients. PUUV was significantly predominant in asymptomatic hantavirus antibody positive subjects (69,8%) compared to HFRS patients. DOBV was present in 17,5% of asymptomatic subjects and interestingly, the preferential hantavirus serotype could not be determined in 12,7% of the asymptomatic antibody-positive subjects.

Key words: Puumala virus, Dobrava virus, HFRS, haemodialysis, renal failure

Introduction

Hantaviruses (family Bunyaviridae, genus Hantavirus) are enveloped RNA viruses, carried primarily by rodents or insectivores of specific host species. Three hantaviruses, Puumala (PUUV), Dobrava (DOBV) and Saaremaa (SAAV), are known to cause hemorrhagic fever with renal syndrome (HFRS) in Europe (1). HFRS is a febrile illness which generally involves haemostatic and renal disturbances. The clinical picture of HFRS is highly variable, from asymptomatic to fatal. DOBV induced infections carry a considerable mortality, whereas diseases caused by PUUV, SAAV are less severe (2). The most severe form of HFRS is caused by Hantaan virus (HTNV) in Asia and DOBV in the Balkans with a mortality rate from 3% to 12%. PUUV, the most prevalent hantavirus, causes the mild form of HFRS through central and northern Europe, the Russian Federation and the Balkans with the mortality of 0,1-0,4% (3, 4). The causes of differences in clinical severity are unknown; it may involve the virulence of individual viral strains, the infective dose, or host factors.

Bosnia and Herzegovina (B&H) has been recognized as a highly endemic region for hantavirus infections for over 50 years (5). Several HFRS outbreaks in B&H have been reported up to the present time (5, 6, 7, 8). During the war in B&H (1995), more than 300 patients, mostly soldiers from northeast Bosnia, were hospitalized with acute hantavirus disease due either to PUUV or DOBV as first documented by IgG and IgM ELISA (9) and later confirmed by focus reduction neutralization tests (10).

The target organ for PUUV and DOBV is the kidney, but the impact on renal function seems to be far more important in DOBV than in PUUV infections. Hukic et al. (11) investigated renal failure and capillary leakage in 50 patients with serological confirmed DOBV and PUUV infection admitted to the hospital during the HFRS outbreak in B&H and found that DOBV was

associated with the more severe renal function disorders compared to PUUV infection. In a recently published study from B&H by Tulumovic et al. (12) renal function was analysed during the acute phase and long term consequences in 53 DOBV and 82 PUUV infected patients who have been admitted to the hospital during the HFRS outbreak of 1995. A more severe renal impairment was observed in DOBV compared to PUUV infected patients during the acute phase. Follow up of the patients after 10 years indicated that glomerular filtration rate, although within normal range, was significantly lower in DOBV compared to PUUV patients. Reports from Croatia and Slovenia where PUUV and DOBV also coexist suggested that DOBV-infected patients exhibited significant differences in illness severity (13, 14, 15, 16). DOBV infected patients suffered more frequently from acute renal failure requiring dialysis treatment, shock, visual disturbances, and severe thrombocytopenia, hemorrhagic complications and disseminated intravascular coagulation than in patients with PUUV infection (17).

The aim was also to compare the differences in renal impairment in DOBV and PUUV infected patients with HFRS during acute stage of illness admitted to the hospital during the period from 1998 to 2002. We also aimed to compare DOBV and PUUV distribution between symptomatic HFRS patients and asymptomatic hantavirus antibody positive subjects.

Methods

Subjects

The study was designed as retrospective review of existing clinical records for 264 patients with serologically confirmed HFRS. The patients were admitted within the average of $15,7 \pm 9,2$ days post-onset-of-symptoms (POS) to the university-based tertiary centers in Bosnia and Herzegovina during the period 1998-2005. The patient's kidney function was assessed at the internal medicine departments where they were followed up and received the treatment. Exclusion criteria were the patients with the mild form of the disease who were not admitted to the hospitals and history of potentially nephrotoxic drugs abuse. Mean age of the HFRS patients was $33,5 \pm 10,65$ years; 241 (91,3%) males and 23 (8,7%) females (male/female (M/F) ratio: 10.48).

The study also included 1381 asymptomatic healthy volunteers without previous history of symptoms related to HFRS who attended health care centres for various reasons from 2000 to 2006. The subjects were selected randomly both from rural and urban areas and different regions of B&H. Mean age of asymptomatic subjects was $43,5 \pm 14,8$ years; 746 females and 635 males (M/F ratio: 1.17). Sera from asymptomatic subjects were taken according to the ethical regulation in B&H and written informed consent was obtained from all participants included in the study. Serological tests revealed that 63 (4.6%) asymptomatic subjects were hantavirus antibody positive and those were later included in the analysis.

Measurements

In HFRS patients we assessed the requirement for haemodialysis treatment, number of haemodialysis treatment, 24-hour diuresis, presence of haematuria and urine opalescence. We

also measured serum urea and creatinine levels during the acute stage of illness. 24-hour diuresis was assessed every day throughout the acute phase and the lowest 24-hour diuresis values were taken for the analysis. Serum urea and creatinine concentration was measured on the Dimension Clinical System, Dade Behring.

Serum samples from HFRS patients were taken in duplicate up to 20 to 40 days POS. Serum samples were examined for the presence of hantaviral antibodies using IgM and IgG ELISA tests reactive with DOBV, PUUV, Hantaan (HTNV) and Seoul (SEOV) viruses followed by Western Blot (WB) test. Serum samples from asymptomatic subjects were examined for the presence of hantavirus antibodies by applying IgG ELISA tests and WB test.

IgG and IgM enzyme-linked immunoassay

Hantavirus IgM and IgG antibodies were detected using a commercially available ELISA kit (Focus Technologies, California, USA). The Focus kit uses a pool of baculovirus-recombinant N-truncated protein from several hantaviruses as the antigen. The manufacturer's protocol was followed. Briefly, serum samples from patients were diluted 1:100 in duplicate and incubated for 1 h at room temperature in antigen-coated, 96-well plates. Peroxidase-coupled anti-human IgG or IgM was used as the secondary antibody, and was incubated for 30 minutes at room temperature. The substrate was added and colour development was assessed by measuring the absorbance at 450 nm. The average of both absorbance values had to be $\geq 1,100$ for a sample to be considered positive.

Bunyavirus immunoblot

All serum samples were additionally analysed applying the Bunyavirus immunoblot IgG test (Microgen, Munich, Germany). The bunyavirus immunoblot test contains recombinant antigens, i.e. combined PUUV + HTNV antigens, separate antigens of PUUV, HTNV, DOBV and SEOV antigen.

Patients were considered to be PUUV infected when there was a preferential reaction with PUUV antigen in ELISA and if –in WB- a reaction to the combined PUUV/HTNV and to the separate PUUV antigen was noted. Similar criteria were applied for DOBV infection be it that there we noted reactions against HTNV/DOBV antigens.

Patients were considered to be non-id hantavirus patients when -in ELISA and WB- there was an equal reaction to several (PUUV, HTNV, DOBV) antigens.

Statistical analysis

Data are presented as percentages or as mean \pm standard deviation. Differences in proportions were tested using Chi square test or Fishers exact test where appropriate. The difference in continuous variables between the groups was tested with t-test. All analysis were performed using the SPSS for Windows (version 16.0, SPSS Inc, Chicago, Illinois, USA).

Results

The distribution of hantavirus serotypes in 264 symptomatic, HFRS patients and in 63 asymptomatic hantavirus antibody positive subjects is shown in Table 1. In HFRS patients, PUUV accounted for 49,6% and DOBV for 26,1% of seropositive patients, while in asymptomatic subjects PUUV was detected in 44 (69,8%) of the subjects and was significantly more prominent compared to the symptomatic subjects ($\chi^2=8,5$; $p=0,014$). DOBV was detected in 11 (17,5%) hantavirus antibody positive asymptomatic subjects.

In 24,2% of the symptomatic, HFRS patients hantavirus serotype could not be defined. Interestingly, also in hantavirus antibody positive asymptomatic subjects there were 8 (12,7%) subjects in whom we could not define a specific hantavirus serotype (Table 1).

Haemodialysis treatment was required in 18 (6,9%) of the HFRS patients (Table 2).

Haemodialysis treatment was more frequently required by DOBV-infected compared to PUUV- and non-id hantavirus-infected patients ($\chi^2=10,71$; $p=0,005$) (Table 2).

The majority of the haemodialysed patients (58,8%) underwent two times or less haemodialysis treatment, 29,4% underwent 3,4 or 5 treatments while 11,8 % of the patients required 6 and/or more treatments. The number of haemodialysis treatment was not associated with the infecting hantavirus serotype ($\chi^2=3,6$; NS).

The mean diuresis value was significantly lower ($335,9\pm 100,5$ mL/day) in DOBV compared to PUUV ($615,2\pm 142,7$ mL/day; $p=0,007$) and non-id hantavirus antibody ($400,0\pm 42,0$ mL/day; $p=0,016$) positive patients, while no significant differences in mean diuresis values between PUUV and non-id HTV seropositive subjects were found (Table 2). In DOBV-infected patients

the mean serum creatinine ($610,0 \pm 433,1 \mu\text{mol/L}$) and urea ($25,4 \pm 13,6 \text{ mmol/L}$) values were significantly higher compared to PUUV-infected patients ($p < 0,05$), while no significant differences in mean serum creatinine and urea concentration between DOBV and non-id HTV infected patients were observed (Table 2). Haematuria was observed in 90% of DOBV patients, 75,8% of PUUV and in 69,6% of non-id HTV patients. Opalescent urine was observed in all DOBV infected patients (100%), in 84,3% of PUUV and 87,0% of non-id hantavirus infected patients.

Discussion

Our study findings show the circulation of PUUV and DOBV in HFRS patients in B&H. PUUV and DOBV antibodies were detected in 49,6% and 26,1% of the HFRS patients in our study. In asymptomatic hantavirus antibody positive subjects PUUV seroreactivity was significantly more prevalent in asymptomatic subjects compared to HFRS patients which suggests that PUUV is less virulent and that PUUV causes inapparent or mild infection not necessitating hospitalization.

Our study demonstrated that acute renal failure was more frequent in DOBV seropositive patients during the acute stage of hantavirus infection. In HFRS patients who underwent haemodialysis treatment due to hantavirus-induced acute renal failure a significant predominance of DOBV seropositive subjects (61,1%) was observed. Also, DOBV seropositive patients in our study had more severe renal impairment compared with the PUUV and non-id hantavirus positive patients as witnessed by the diuresis, serum urea and creatinine values during the acute stage of the illness. Our results are in line with previous reports from B&H, Croatia and Slovenia that associated DOBV with severe form of HFRS (13, 14, 15, 16, 17).

In a recently published study, Tulumovic et al. (12) analysed the clinical course and the outcome of 135 HFRS patients admitted to the hospital during the outbreak in 1995 in B&H. The authors found that DOBV seropositive patients had higher creatinine level, were frequently anuric, dialysis dependant and hypotensive compared to PUUV patients. In a study by Hukic et al. (11) diuresis, serum urea and creatinine as well as kidney size were measured in 50 patients with HFRS during the HFRS outbreak in Bosnia and Herzegovina in 2002. The enlargement of both kidneys was found in all patients. Serum creatinine and urea levels were significantly higher in DOBV compared to PUUV patients. Acute haemodialysis treatment was necessary in 28 % of the DOBV and in none of the PUUV patients. The authors did not observe significant differences in

mean diuresis values in DOBV- compared to PUUV patients. In contrast, our results show a significant difference in diuresis values between different hantavirus serotypes. DOBV patients in our study had significantly lower diuresis values compared to PUUV which can be explained with the larger study sample of the patients included in this study. Reports from Croatia also associated DOBV infection with a more severe disease than PUUV infection, but also confirmed previous findings that even PUUV infection could lead to a severe disease (13,14,17). Markotic et al. (17) observed that a significantly higher proportion of DOBV-infected patients had acute renal failure, visual disturbance, severe thrombocytopenia, and elevated levels of nonsegmented leukocytes, creatine, and total bilirubin.

HFRS is a zoonosis with sudden onset, characterized by high fever, renal insufficiency and hemorrhages. The basic pathologic and pathophysiologic disorder in HFRS is capillary damage (vasculitis). HFRS manifests depending in part on the causative virus as a mild, moderate, or severe disease, (2). The course of HFRS is usually divided into five distinct stages (febrile, hypotensive, oliguric, polyuric and convalescent). The febrile stage with sudden onset usually lasts from 3 to 7 days. The hypotensive stage lasts from one to 2 days on an average and is characterized by lower blood pressure and signs of renal failure. During the oliguric stage extensive hemorrhage may occur and urea and creatinine reach their highest values followed by the polyuric stage (18).

Tulumanovic et al. (12) in their study followed up 45 HFRS patients for 10 years and found that DOBV seropositive patients had, although within normal reference values, significantly lower glomerular filtration rates compared to PUUV patients.

Interestingly, 24,2% of the patients with typical clinical symptoms of HFRS expressed antibodies for a hantavirus which showed no preferential activity with one of the antigens present in the currently available commercial tests. Inability to define the hantaviral serotype in

symptomatic subjects could be due to the antigenic similarities among hantaviruses and cross-reactivity during the acute stage of HFRS. In serological assays, cross-reactivity is frequently seen. These cross-reactions may disturb the interpretation of serological results in diagnostic work. Neutralization tests (NT) were not applied because they are not available in B&H. The option of obtaining NT data abroad was ruled out because of stringent export procedures for biological materials.

However, in our study, 12,7% of 63 asymptomatic healthy subjects expressed hantaviral IgG antibodies that could not be identified with commercially available PUUV, HTNV, DOBV and SEOV antigens. Occurrence of antibodies for a hantavirus that cannot be identified with the currently used test both in symptomatic HFRS patients and in asymptomatic subjects implicate that further studies are needed to possibly identify another hantavirus which might be endemic throughout the Balkans.

Conclusion. Our study findings show the circulation of PUUV and DOBV in HFRS patients and in asymptomatic hantavirus antibody positive subjects in B&H. PUUV seroreactivity is more prevalent in asymptomatic subjects compared to HFRS patients. In HFRS patients DOBV is associated with severe renal impairment during the acute stage of the illness. These findings suggest that PUUV is less virulent and that PUUV causes inapparent or mild infection not necessitating hospitalization.

Table 1. Hantavirus serotype distribution in symptomatic, HFRS patients and in hantavirus antibody positive asymptomatic subjects.

	Symptomatic, HFRS	Asymptomatic
	Patients N (%)	subjects N(%)
PUUV	131 (49,6%*)	44 (69,8%*)
DOBV	69 (26,2%*)	11 (17,5%*)
Non-identified HTV	64 (24,2%*)	8 (12,7%*)
Total	264	63

*: % of the total number of patients (N= 264) and subjects (N=63)

Table 2. Clinical findings of renal function in symptomatic, HFRS patients

	PUUV	DOBV	Non-id HTV
Haemodialysis patients N(%)	4 (3,1%)	11 (15,9%)* **	3 (4,8%)
Diuresis (mL/day)	615,16±73,2	335,9±95,5 * **	400,0± 45,6
Urea (mmol/L)	18,9±11,0	25,4±13,6*	24,2±12,8
Creatinine (µmol/L)	413,2±271,5	610,0±433,1*	421,2±316,1

Data are presented as mean ± SD

* significant differences between DOBV and PUUV group; p<0,05

** significant differences between DOBV and Non-id HTV group; p<0,05

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