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Source / Izvornik: **World Journal of Methodology, 2021, 11, 294 - 301**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.5662/wjm.v11.i6.294

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:391304

World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 November 20; 11(6): 294-301

DOI: 10.5662/wjm.v11.i6.294

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Retrospective Study

Comparison of indirect immunofluorescence and western blot method in the diagnosis of hantavirus infections

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Author contributions: Vilibic-Cavlek T made contributions to the conception and design of the study and was involved in drafting and revising the manuscript; Stevanovic V, Savic V, Mrzljak A, and Bogdanic M were involved in collecting data and drafting and writing the manuscript; Barbic L and Tabain I revised the manuscript critically; All authors read and approved the final manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of the Croatian Institute of Public Health, No. 030-02/17-10/1.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All

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Abstract

BACKGROUND

Serologic cross-reactivity between hantaviruses often complicates the interpretation of the results.

AIM

To analyze the diagnostic value of indirect immunofluorescence assay (IFA) and western blot (WB) in the diagnosis of hantavirus infections.

METHODS

One hundred eighty-eight serum samples from Puumala (PUUV) and Dobrava (DOBV) orthohantavirus infected patients were analyzed. Serology was performed using commercial tests (Euroimmun, Lübeck, Germany).

RESULTS

Using IFA, 49.5% of acute-phase samples showed a monotypic response to PUUV, while 50.5% cross-reacted with other hantaviruses. The overall cross-reactivity was higher for immunoglobulin G (IgG) (50.0%) than for immunoglobulin M (IgM) (25.5%). PUUV IgM/IgG antibodies showed low/moderate reactivity with orthohantaviruses Hantaan (12.3%/31.5%), Seoul (7.5%/17.8%), DOBV (5.4%/

authors declare no conflict of interest.

Data sharing statement: No sharing data.

Specialty type: Medical laboratory technology

Country/Territory of origin: Croatia

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 2, 2021
Peer-review started: January 2, 2021
First decision: July 8, 2021
Revised: July 16, 2021
Accepted: August 31, 2021
Article in press: August 31, 2021
Published online: November 20, 2021

P-Reviewer: Khan S
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Guo X

28.1%), and Saaremaa (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with Hantaan (76.2%/95.2%), Saaremaa (80.9%/83.3%), and Seoul (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in most cross-reactive samples (89.5%).

CONCLUSION

The presented results indicate that WB is more specific than IFA in the diagnosis of hantavirus infections, confirming serotype in most IFA cross-reactive samples.

Key Words: Hantaviruses; Serology; Cross-reactivity; Indirect immunofluorescence; Western blot

Core Tip: Serologic cross-reactivity among hantaviruses often complicates the interpretation of the results. The overall cross-reactivity is generally higher for immunoglobulin G antibodies than for immunoglobulin M antibodies. Western blot seems to be a more specific serology method than indirect immunofluorescence assay in the diagnosis of hantavirus infections, confirming serotype in the majority of cross-reactive samples detected by indirect immunofluorescence assay.

Citation: Vilibic-Cavlek T, Barbic L, Stevanovic V, Savic V, Mrzljak A, Bogdanic M, Tabain I. Comparison of indirect immunofluorescence and western blot method in the diagnosis of hantavirus infections. *World J Methodol* 2021; 11(6): 294-301
URL: https://www.wjgnet.com/2222-0682/full/v11/i6/294.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i6.294

INTRODUCTION

Hantaviruses represent a group of serologically related rodent-borne RNA viruses that belong to the genus *Orthohantavirus* of the family *Hantaviridae*. Two different diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), are caused by hantaviruses in humans[1]. Orthohantaviruses Hantaan (HTNV), Dobrava (DOBV), Puumala (PUUV), Seoul (SEOV), and Saaremaa (SAAV) cause HFRS with varying degrees of severity. While HTNV and DOBV cause a severe form of HFRS in Asia and Europe, SEOV causes less severe disease worldwide[2,3]. SAAV is also found to be responsible for a relatively mild human disease in Europe[4]. PUUV is a causative agent of nephropathia epidemica, the mildest form of the disease, endemic in Western Europe and Scandinavia[2].

Diagnostic methods for hantavirus infections include serology, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and virus isolation [5].

Vero E6 cell culture has been used to isolate hantaviruses causing HFRS and HPS. Hantaviruses usually are not cytopathic in cultured cells; therefore, the detection of infection is confirmed using an immunofluorescence antibody test for viral antigen. Virus isolation is not performed as part of routine hantavirus diagnostics, since it is laborious and time-consuming and requires biosafety level 3 and 4 laboratories[6].

Serology is the main method for the diagnosis due to the hazardous nature of hantaviruses and a short-term viremia in infected humans[7,8]. Enzyme-linked immunosorbent assay and indirect immunofluorescence assay (IFA) are broadly used serologic tests used for detection of hantavirus immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies[9]. Immunoblot tests [western blot (WB) and line immunoassay] are also used in some laboratories[10].

Hantavirus nucleocapsid (N) protein is the major antigen in early humoral response in patients with hantavirus infection[11,12]. N protein is highly cross-reactive between different hantaviruses due to its conserved nature[11,13]. Overall, serologic cross-reactivity within the genus *Orthohantavirus* is the highest among viruses associated with (phylo)genetically closely related rodent species. DOBV is genetically and antigenetically related to other orthohantaviruses transmitted by *Murinae* rodents (Old

World mice and rats) such as HTNV, SEOV, and SAAV. PUUV is more distantly related to this group since its reservoirs belong to the *Arvicollinae* rodents (voles and lemmings)[14-16]. The interpretation of serology results is often complicated by the cross-reactivity, especially in areas where different hantaviruses co-circulate. Virus neutralization test is still the gold standard serologic test. Since this test has to be performed in biosafety level 3 laboratory, it is confined mainly to the reference laboratories[17].

Molecular diagnostic methods, including classic and real-time RT-PCR, are also widely used for the diagnosis of hantaviruses. Hantavirus RNA is detectable in blood early after the onset of symptoms; therefore, RT-PCR is a sensitive method for detecting hantavirus infections before the appearance of IgM antibodies. Primers specific for the hantavirus S and M segments have been used in different studies. The advantage of the molecular methods is that the RT-PCR product may be sequenced to identify the virus and perform phylogenetic analysis[5,18].

In Croatia, PUUV and DOBV have been demonstrated in humans[19-23], while SAAV and Tula orthohantavirus were also documented in rodents[24,25]. This study aimed to analyze the diagnostic value of IFA and WB methods in the diagnosis of hantavirus infections.

MATERIALS AND METHODS

A total of 188 serum samples from patients with serologically confirmed acute hantavirus infection (2015-2019) tested at the National Reference Laboratory for Arboviruses and Hantaviruses, Croatian Institute of Public Health were included in the study. Serologic tests were performed using a commercial IFA (Hantavirus mosaic; Euroimmun, Lübeck, Germany) to detect IgM/IgG antibodies of the most common hantaviruses: PUUV, DOBV, HTNV, SEOV, and SAAV. A fluorescence occurring as fine droplets in the cytoplasm of infected cells in a dilution 1:100 was considered a positive result.

Cross-reactive samples were further tested for hantavirus IgG antibodies using a WB (Euroline Hantavirus profile, Euroimmun). WB test strips were coated with nucleocapsid PUUV; DOBV and HTNV antigens. Band signal intensity at least as of IgG control was considered a positive result. According to the band intensity, results were interpreted as follows: strong positive-very strong band (+++); positive-medium to strong band (+/++); borderline-very weak band (+/-).

The study was approved by the Ethics Committee of the Croatian Institute of Public Health (Decision number: 030-02/17-10/1). Informed consent was obtained from all subjects included in the study.

RESULTS

PUUV was confirmed in 146 (77.6%) and DOBV in 42 (32.4%). Using IFA, 93 (49.5%) of 188 acute-phase serum samples reacted only with the homologous PUUV antigen, while in 95 (50.5%) samples, cross-reactive IgM and/or IgG antibodies were found. The overall cross-reactivity was higher for IgG antibodies (94/188; 50.0%) than for IgM antibodies (48/188; 25.5%). Among 95 cross-reactive samples, 55 (57.9%) were confirmed as PUUV and 30 (31.6%) samples as DOBV using a WB.

Cross-reactive patterns to different hantavirus antigens in PUUV- and DOBV-infected patients detected using IFA are presented in Figures 1 and 2. Among PUUV positive samples, a low/very low IgM reactivity was observed with HTNV (18/146; 12.3%), SEOV (11/146; 7.5%), DOBV (8/146; 5.4%), and SAAV (7/146; 4.8%). PUUV IgG antibodies showed a moderate reactivity with HTNV (46/146; 31.5%) and DOBV (41/146; 28.1%), while reactivity with SEOV and SAAV was low (26/146; 17.8% and 23/146; 15.7%, respectively).

In DOBV positive samples, both IgM and IgG antibodies showed a high degree of cross-reactivity. Among IgM positive samples, the highest cross-reactivity was observed with SAAV (34/42; 80.9%), 33/42 (78.6%) with SEOV, and 32/42 (76.2%) with HTNV. In 12 samples (28.5%), cross-reactive antibodies with PUUV were found. DOBV IgG antibodies showed the highest reactivity with HTNV (40/42; 95.2%). Almost equally high reactivity was found with SEOV and SAAV (36/42; 85.7% and 35/42, 83.3%, respectively), and moderate reactivity was found with PUUV (16/42; 38.1%). The majority of DOBV-positive samples (IgM 24/42, 57.1%; IgG 35/42; 83.3%) showed reactivity with all three hantavirus antigens (HTNV + SEOV + SAAV).

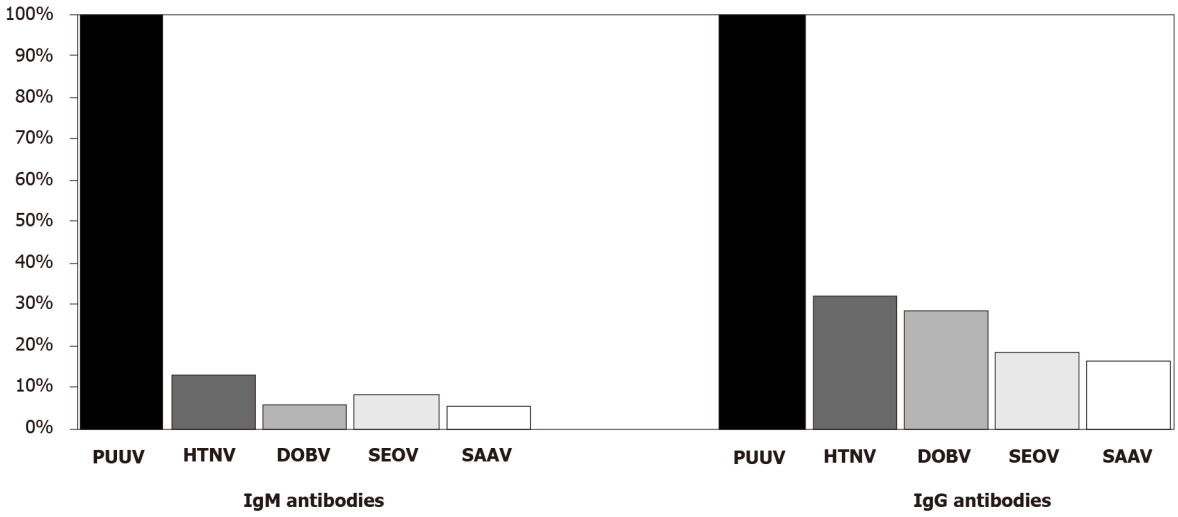

Figure 1 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Puumala-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

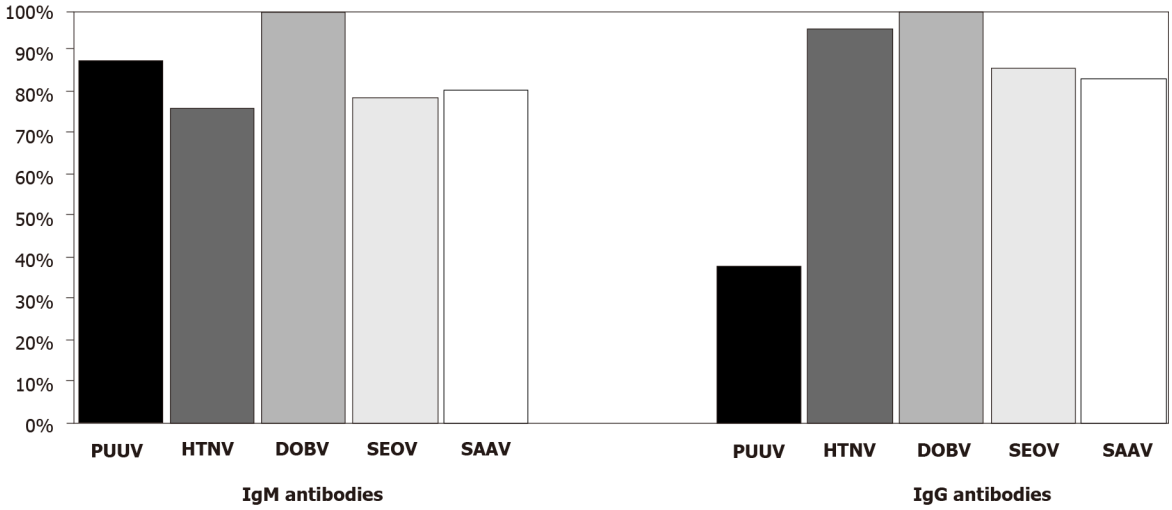

Figure 2 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Dobrava-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

Forty-six of 172 (24.5%) IgG-positive samples cross-reacted with other hantaviruses by WB. However, based on signal intensity, a very strong band to the homologous viral antigen was observed in most cross-reactive samples compared to a weak/medium band of the related hantavirus antigens (Figure 3). Among PUUV positive samples, 8 (5.5%) tested borderline to HTNV and 10 (6.8%) to DOBV. Among DOBV positive samples, 19 (45.2%) tested positive/borderline to HTNV and 5 (9.5%) to PUUV. Only 8 PUUV positive samples (5.5%) showed a very strong band to PUUV and DOBV antigens. Additionally, two DOBV positive samples (4.7%) showed a very strong band to both DOBV and HTNV antigens (Table 1). The detection of PUUV and DOBV IgM antibodies by IFA in these samples indicated acute PUUV and DOBV infection, respectively.

DISCUSSION

Results of this study indicated broadly cross-reactive patterns of hantaviruses detected by IFA, which were found to be much higher for DOBV compared to PUUV. One published multicenter study on the simultaneous detection of hantaviruses showed a high cross-reactivity of serum samples from DOBV-infected patients with SAAV, HTNV, and SEOV (60%-100%), while cross-reactivity with PUUV was moderate (up to

Table 1 Cross-reactive patterns of hantavirus immunoglobulin G antibodies by western blot

Band intensity	PUUV	HTNV	DOBV
PUUV-infected patients (n = 146)			
Strong positive (+++)[1]	-	0 (0%)	8 (5.5%)
Positive (+, ++)[2]	-	0 (0%)	0 (0%)
Borderline (+/-)[3]	-	8 (5.5%)	10 (6.8%)
DOBV-infected patients (n = 42)			
Strong positive (+++)[1]	0 (0%)	2 (4.7%)	-
Positive (+, ++)[2]	1 (2.4%)	8 (19.0%)	-
Borderline (+/-)[3]	4 (9.5%)	11 (26.2%)	-

[1]Very strong band.
[2]Medium to strong band.
[3]Very weak band.
PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan.

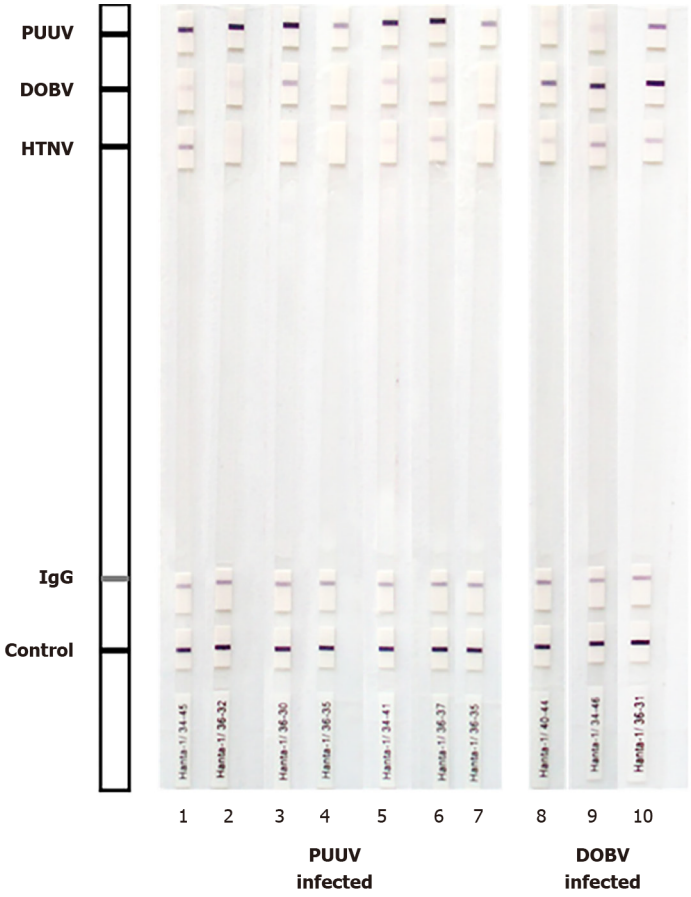

Figure 3 Western blot analysis of Puumala and Dobrava- infected patients. The test strips were coated with the affinity purified nucleocapsid Puumala (PUUV); Dobrava (DOBV) and Hantaan (HTNV) antigen. A correctly performed test for immunoglobulin (Ig)G antibodies against hantavirus antigens is indicated by a positive reaction of the control band and the IgG band. Some samples (strips 1, 3, 5, 6, 8-10) cross-reacted with other hantaviruses, however, based on signal intensity, a very strong band to the homologous virus antigen was detected compared to a very weak/weak band of the related hantavirus antigens.

43%) using IFA[26]. This study observed a remarkably high cross-reactivity for both DOBV IgM/IgG antibodies with SAAV, HTNV, and SEOV antigens (IgM 76.2%-80.9%, IgG 83.3%-95.2%). In addition, 57.1% IgM and 83.3% IgG positive samples cross-reacted with all three hantavirus antigens. These results are in accordance with the phylogenetic relatedness of hantaviruses. However, a substantial cross-reactivity was also found with PUUV (IgM 28.5%, IgG 38.1%), although PUUV is phylogenetically

distantly from DOBV.

IgM/IgG antibodies of PUUV-infected Croatian patients reacted moderately with HTNV (12.3%/31.5%). In a study by Lederer *et al*[26], even higher cross-reactivity between PUUV and HTNV IgM/IgG was found (49%/79%), while the reactivity to other tested hantaviruses was low, similar to our results.

In this study, a lower degree of cross-reactivity was also found by WB (24.5%). However, in all but 8 samples, differentiation of hantavirus serotype was possible based on powerful signal intensity to homologous antigen compared to weak/medium signal intensity to heterologous antigens. Some other studies which used WB for result confirmation showed similar results[27,28].

Since the clinical course and prognosis differ in PUUV and DOBV infection, the determination of hantavirus serotype is important for diagnosing acute HFRS cases. In addition, due to specific rodent hosts, identification of currently circulating hantavirus serotype is also useful for planning rodent control programs. Using IFA, serotype identification in seroepidemiological studies is often difficult because of extensive cross-reactivity among IgG antibodies. In DOBV infected individuals, considerable cross-reactivity was also observed between IgM antibodies. Using WB, differentiation of hantavirus serotype was possible in most cases by comparing the signal intensity in most IFA cross-reactive samples.

CONCLUSION

Although cross-reactivity among hantaviruses was detected in both IFA and WB, the results of this study showed that WB seems to be more specific than IFA, confirming hantavirus serotype in 89.5% of cross-reactive samples detected by IFA.

ARTICLE HIGHLIGHTS

Research background

The cross-reactivity among hantaviruses often complicates the interpretation of serology results, especially in areas where different hantaviruses co-circulate.

Research motivation

Data on the comparison of different serologic methods in the diagnosis of hantaviruses are scarce.

Research objectives

This study aimed to analyze the diagnostic value of indirect immunofluorescence (IFA) and western blot (WB) methods in diagnosing hantavirus infections.

Research methods

A commercial IFA was used to detect immunoglobulin M (IgM)/immunoglobulin G (IgG) antibodies to the most common orthohantaviruses: Puumala (PUUV), Dobrava (DOBV), Hantaan (HTNV), Seoul (SEOV), and Saaremaa (SAAV). Cross-reactive samples were additionally tested by a commercial WB using PUUV, DOBV, and HTNV antigens.

Research results

Using IFA, 49.5% of acute-phase serum samples reacted only with the homologous PUUV antigen, while in 50.5% samples, cross-reactive IgM and/or IgG antibodies were found. PUUV IgM/IgG antibodies cross-reacted with HTNV (12.3%/31.5%), SEOV (7.5%/17.8%), DOBV (5.4%/28.1%), and SAAV (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with HTNV (76.2%/95.2%), SAAV (80.9%/83.3%), and SEOV (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in 89.5% cross-reactive samples.

Research conclusions

WB seems to be more specific than IFA, confirming hantavirus serotype in the majority of cross-reactive samples detected by IFA.

Research perspectives

Further studies on a large sample caused by different hantavirus serotypes are needed.

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