Western Blotting Using Recombinant Hantaan Virus Nucleocapsid Protein Expressed in Silkworm as a Serological Confirmation of Hantavirus Infection in Human Sera

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(Received 3 July 1995/Accepted 26 August 1995)

ABSTRACT. Recombinant Hantaan virus nucleocapsid protein expressed in silkworm larvae was applied as a serological diagnostic antigen in Western blots (WB) of human sera. The sensitivity of this method was similar to that of the IFA test. Hemorrhagic fever with renal syndrome (HFRS) and nephropathia epidemica diagnosed by their cross-reactivity in WB. The specificity of this method was higher than that of IFA test because the background was low. Sera that exhibited high background staining in the IFA test were readily diagnosed with this method. We recommended WB using recombinant Hantaan virus nucleocapsid antigen as a confirmatory procedure for the serodiagnosis of hantavirus. — **KEY WORDS:** baculovirus, HFRS, serodiagnosis.

J. Vet. Med. Sci. 58(1): 71–74, 1996

Viruses of the hantavirus genus of the bunyaviridae family include the etiologic agent of hemorrhagic fever with renal syndrome (HFRS) [2] and hantavirus pulmonary syndrome (HPS) [6]. HFRS is a rodent-borne viral zoonosis characterized by fever, hemorrhagic manifestations and renal disorder [3]. A variety of wild rodents as well as laboratory rats are sources of human infection. HPS is an acute form of adult respiratory distress with high mortality [13]. The deer mouse, *Peromyscus maniculatus*, is the principal rodent reservoir for the virus [9]. Therefore, field and laboratory workers are considered to be the group at highest risk for hantavirus infection from the perspective of an occupational hazard.

Since HFRS varies from the subclinical to the fatal, serological detection of a specific antibody is essential for diagnosis. The indirect immunofluorescent antibody (IFA) test is the most widely used serodiagnostic test for hantavirus infection [4]. However, the test occasionally has sensitivity and specificity problems due to strong background staining which may overwhelm the specific reaction or producing dots closely resembling the specific profiles. In addition, a P3 containment laboratory is required to prepare the antigen from culture cells infected with live virus.

To eliminate these problems, recombinant DNA technologies have been used to prepare diagnostic antigens [7, 10, 11]. We applied recombinant baculovirus expressed Hantaan virus nucleocapsid protein (NP) as the antigen in Western blotting (WB) for the serological confirmation of hantavirus infection in laboratory rats [12]. Here, we applied the recombinant NP-based WB (rNP-WB) to the serological diagnosis of human sera and compared its sensitivity and specificity to that of the IFA test.

A total of 21 patient sera were obtained from Dr. Y-X. Yu, of the National Institute for the Control of Pharmaceutical and Biological Products, China. They were considered to

be infected with Hantaan virus. A total of 3 positive sera were provided by Dr. Y. Nishimune of the Research Institute for Microbial Diseases, Osaka University. These sera were infected with Seoul type hantavirus during a laboratory-type hantavirus infection. A total of 23 serum samples from patients with nephropathia epidemica, were provided by Dr. B. Niklasson of the Swedish Institute for Infectious Disease Control. All sera were confirmed seropositive by IFA and FRNT (focus reduction neutralization test) before being given to us. A total of 244 sera from a control group with no symptoms were used as negative controls. A total of 45 sera from patients in the United Arab Emirates (UAE) with high fever of unknown etiology, were provided by Drs. M.K. Ijaz and T. Alkarmi of the Department of Medical Microbiology, Faculty of Medicine and Health Science, UAE, through Dr. H-K. Ooi of the Department of Parasitology, Faculty of Veterinary Medicine, Hokkaido University, Japan. The IFA test proceeded as described. Hantaan 76-118, SR-11 and Sotkamo strains were used as representative strains of Hantaan, Seoul and Puumala serotypes, respectively. IFA titers of more than 1:32 were regarded as IFA positive. Western blotting test proceeded essentially as described [12]. Recombinant Hantaan 76-118 NP expressed in baculovirus was used as the antigen. Serum specimens that exhibited distinct WB bands at 48, 30 and 25 kDa at a 1:10 dilution were regarded as positive.

To compare the relative sensitivity of the IFA test and rNP-WB, three groups of patient sera were diagnosed by both procedures (Table 1). The sensitivity of the IFA test with Hantaan virus-infected Vero cells was exactly the same with patient sera infected with Hantaan, Seoul and Puumala viruses as that defined with homologous antigen. The sensitivity of WB was the same as that obtained by Hantaan-IFA, except for one false negative reaction in a serum sample from Sweden, infected with Puumala virus. As shown in Fig. 1, sera from China, Japan, and Sweden represented distinct WB bands, though the bands generated by nephropathia epidemica samples are apparently less intense. Since the IFA titers of Chinese patient sera

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distributed from 1:32 to more than 1:2048 to Hantaan antigen (data not shown), the IFA test with Hantaan antigen and rNP-WB considered to exhibit the same sensitivity in regard to detecting the antibody in human sera. Similar results were obtained by using serial sera from experimentally infected rats [12]. The antigenic cross-reactivity of Hantaan virus antigen to heterologous antibody has been determined by means of ELISA and WB using

Table 1. Comparison of the sensitivity of the IFA test and Western blotting with recombinant baculovirus-expressed Hantaan virus NP in patient sera infected with Hantaan, Seoul or Puumala virus

Patient sera from	No. of sera	Nos. of pos sera by			
		IFA to		WB to	
		HTN ^{a)}	SR ^{b)}	PUU ^{c)}	HTN NP ^{d)}
China (Hantaan)	21	21	21	NT	21
Japan (Seoul)	3	3	3	NT	3
Sweden (Puumala)	23	23	NT	23	22

IFA test with Vero E6 cells infected with Hantaan 76-118^{a)}, SR-11^{b)} and Sotkamo^{c)} strains. IFA titers of 1:32 or more were regarded as positive. WB with recombinant NP of HTN expressed by baculovirus systems^{d)}. Number of sera that exhibited distinct WB bands at 48, 30 and 25 kDa at a serum dilution of 1:10.

antigens were prepared from live virus [8, 14]. The present results using various human sera with recombinant Hantaan virus NP are consistent with our previous results in that the rNP-WB detected heterologous antibody in immune sera. Although patient sera from hantavirus pulmonary syndrome (HPS) were not included in this study, NP antigen is conserved in Hantaan and Four corner virus (FCV) [9]. Thus, WB was useful for serological confirmation of hantavirus infection over the serotypes.

The relative specificity was compared between the two tests using 244 control sera. No positive results were obtained by WB. The IFA test was less specific with 9 (3.7%) false-positive reactions with intense background staining or dot-like patterns. With the UAE sera, there was an apparently higher rate of false positive reactions (11 sera, 24.4%) in the IFA test, whereas WB showed no reaction (Table 2). In addition, all IFA titers were less than 1:128 and FRNT titer to SR-11 were less than 1:32. The mechanisms, by which non specific IFA staining is caused, remain unclear. However, sera with high nonspecific IFA backgrounds also tended to bind cellular components in WB with infected Vero cell lysate [12]. Thus, the nonspecific reaction in IFA may not be completely eliminated.

Figure 2 shows typical examples of WB profiles at dilutions from 1:10 to 1:10,240 and IFA profiles at a dilution of 1:32 using patient sera from Japan (Patients A and B).

Fig. 1. Western blotting profiles of patient sera. NHS, healthy human sera; HFRS-China, 5 HFRS patient sera from China; HFRS-Japan, 3 HFRS patient sera from Japan; NE-Sweden, 4 NE patient sera from Sweden, as described in the text.

Patient A was a laboratory worker who contracted HFRS from rats during laboratory type HFRS outbreak. Patient B developed symptoms of high fever and hepatic insufficiency which were similar to that of HFRS patient. Both sera gave intense IFA reactions which were difficult to distinguish from the real one. However, sera from Patient A exhibited WB bands, while the sera of Patient B gave no reaction. Serum from Patient B did not neutralize the infectivities of Hantaan and Puumala as well as SR-11 viruses. Thus, the Patient B was diagnosed as negative for Hantavirus

infection. Hantavirus infection is a zoonosis from infected rodents and therefore is regarded as occupational risk for outside plant workers [5] and animal caretakers in laboratories [1]. Therefore, establishment of diagnostic procedure for serological confirmation is of importance for minimizing social concern.

We thank Dr. Y. Nishimune of the Research Institute for Microbial Diseases, Osaka University, Dr. B. Niklasson of Swedish Institute for Infectious Disease Control, Drs. M.K. Ijaz and T. Alkarmi of Department of Medical

Fig. 2. IFA and Western blots profiles from two individual (Patients A and B). IFA profiles from two individuals at a dilution of 1:32 are shown in the upper panels A and B. Western blots are shown in the lower portion. Lanes 1, 2, 3, 4, 5 and 6 correspond to serum dilutions of 1:10, 1:40, 1:160, 1:640, 1:2560 and 1:10240, respectively.

Table 2. Comparison of the specificity of the IFA test and Western blotting with recombinant baculovirus-expressed Hantaan virus NP in sera from Japanese who had no previous illness related to HFRS and from the UAE who had a medical history of a febrile disease

Sera from	No. of sera	Nos. of pos sera by		
		IFA ^{a)}	WB	FRNT ^{b)}
Japan	244	9 (3.7%)	0	0
UAE	45	11 (24.4%)	0	0

a) Number of sera that exhibited high backgrounds or granular IFA staining.

b) Number of sera that exhibited 80% focus reduction against SR-11 at serum dilution 1:32.

Microbiology, Faculty of Medicine and Health Science, UAE, through Dr. H-K. Ooi of Department of Parasitology, Faculty of Veterinary Medicine, Hokkaido University, Japan, for providing patient sera. This study was partially supported by a Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

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