

Use of *Saccharomyces cerevisiae*-Expressed Recombinant Nucleocapsid Protein To Detect Hantaan Virus-Specific Immunoglobulin G (IgG) and IgM in Oral Fluid[∇]

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Hantaan virus is the causative agent of severe hemorrhagic fever with renal syndrome. Clinical surveillance for Hantaan virus infection is unreliable, and laboratory verification is essential. The detection of virus-specific immunoglobulin M (IgM) and IgG in serum is most commonly used for the diagnosis of hantavirus infection. Testing of oral fluid samples instead of serum offers many advantages for surveillance. However, commercial tests for hantavirus-specific antibodies are unavailable. For the detection of Hantaan virus in the oral fluid of humans, we have developed a monoclonal antibody-based capture enzyme-linked immunosorbent IgM assay (IgM capture ELISA) and indirect enzyme-linked immunosorbent IgG and IgM assays (indirect IgG and IgM ELISAs) for paired serum and oral fluid samples using the *Saccharomyces cerevisiae* yeast-expressed nucleocapsid protein of the Hantaan-Fojnica virus. The sensitivity and specificity of the oral fluid IgM capture ELISA in comparison with the results of the serum Hantaan virus IgM assay were 96.7% and of 94.9%, respectively. Thus, data on the overall performance of the oral fluid IgM capture ELISA are in close agreement with those of the serum IgM assay, and the method exhibits the potential to serve as an easily transferable tool for large-scale epidemiological studies. Data on the indirect IgM ELISA also showed close agreement with the serum IgM assay data; however, the indirect IgG ELISA displayed a lower sensitivity and a lower specificity. In conclusion, the IgM capture ELISA can be used with oral fluid instead of serum samples for the diagnosis of Hantaan virus infection.

Hantaan virus (HTNV) is the prototype species of the genus *Hantavirus* and has remained the epidemiologically most important species in the genus until now. Hantaviruses form a separate genus within the family *Bunyaviridae*, forming a group of closely related negative-stranded RNA viruses (4, 17, 25, 29). Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. HFRS occurs mainly in Europe and Asia and is associated with HTNV, Seoul virus (SEOV), Dobrava virus (DOBV), and Puumala virus (PUUV) infections (34). Usually, hantavirus pulmonary syndrome is found in the New World, where most of the cases are caused by the Sin Nombre virus (SNV) and Andes virus (ANDV) serotypes (8, 11, 15). Hantaviruses are transmitted to humans through the aerosol excreta of infected small mammals, mainly wild rodents (17). In China, HTNV together with SEOV accounts for at least 100,000 cases of hantavirus-associated HFRS each year. Approximately 150,000 HFRS cases occur annually worldwide. HTNV infection is characterized by fever, renal dysfunction, and in some cases, hemorrhagic manifestations (9, 10, 12, 13, 15).

Hantaviruses have a tripartite genome that encodes a nucleocapsid protein (N protein), two envelope glycoproteins, and a viral RNA polymerase (30). The viral N protein elicits a

strong humoral immune response in infected patients and immunized animals and has been extensively used to produce reagents for the diagnosis of hantavirus infections (1, 14, 21). Comparison of the amino acid sequences of five amino-terminal immunogenic regions of the N proteins of the HTNV, SEOV, and DOBV hantaviruses shows that they have high degrees of homology. Rabbit polyclonal sera produced against cell culture-grown virus and recombinant viral proteins were tested for their responses to five hantavirus antigens. Two types of antibody responses were generated; hence, two groups of sera could be distinguished, with HTNV, SEOV, and DOBV be attributed to the first group and SNV and PUUV to the second group (3). Schmidt and coworkers (31) investigated the cross-reactivity of rabbit sera raised against the N proteins of SEOV and other hantaviruses. The N-protein-specific antibodies induced by natural infection in human and experimental infections and immunizations have been found to be highly cross-reactive among the different hantaviruses (16, 26, 31). The N-protein-specific antibody titers in SEOV N-protein-immunized rabbits were only slightly lower than those in rabbits immunized with the N protein of the closely related HTNV and much lower than those in rabbits immunized with the N proteins of PUUV, SNV, and ANDV. Similarly, the anti-HTNV N-protein rabbit serum reacted with the SEOV and HTNV N proteins at equal titers. Anti-HTNV- and anti-DOBV-positive serum pools reacted with both SEOV and HTNV N proteins at equal endpoint titers (31).

The most reliable serological methods for the detection of HTNV-specific immunoglobulin G (IgG) and IgM antibodies

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appear to be immunoassays based on either native or recombinant N antigen (5, 6). Previously, assays for IgG and IgM detection were based on the analysis of serum samples. Oral fluid can be used instead of serum because of the similar IgG and IgM profiles in oral fluid and serum, although the concentrations are much lower in oral fluid. This approach has successfully been used for the routine diagnosis and surveillance of viral infections, including measles, mumps, rubella, and diseases caused by other viruses (7, 19, 22, 23, 24, 27, 28, 33). The sampling method is safe, noninvasive, and easier and cheaper than blood sampling, and the compliance rate is high (18). For the detection of human infections caused by HTNV, an oral fluid capture IgM enzyme-linked immunosorbent assay (ELISA) and indirect IgG and IgM ELISAs with paired serum and oral fluid samples were tested for their abilities to detect IgM and IgG against the *Saccharomyces cerevisiae* yeast-expressed N protein of Hantaan-Fojnica virus (HTNV-Foj). There are no changes in amino acid sequence between HTNV-Foj and HTNV strain 76-118 genes, except for 3 and 6 nucleotides in the S and the M segments, respectively (32). This report describes the capture ELISA and the indirect ELISA for the detection of HTNV-specific antibodies in oral fluid by using samples from suspected HFRS cases from China.

MATERIALS AND METHODS

Samples. A total of 151 paired serum and oral fluid specimens were obtained from patients suspected of being infected with HTNV in Shenyang, China, in 2004 and 2005. Twelve serum samples (from the dialysis center in Kaunas, Lithuania) tested for HTNV-specific antibodies by our in-house IgG and IgM ELISAs were negative and were used for the determination of the cutoff values of the serum ELISA. The cutoff values of the oral fluid ELISA were determined by using 12 negative oral fluid specimens obtained from healthy adult volunteers from the Institute of Biotechnology (Vilnius, Lithuania). Oral fluids were collected by using a saliva collection system (Oracol; Malvern Medical Developments, Worcester, United Kingdom). The serum and oral fluid specimens were stored at -20°C until they were required for testing. The oral fluid specimens were cleared by centrifugation at $12,000 \times g$ in a microcentrifuge for 20 to 30 s before testing.

Sample screening. Serum samples were screened for HTNV-specific IgG and IgM antibodies by using Hantavirus Hantaan IgM and IgG kits (Progen, Heidelberg, Germany). The test was performed and the results were deduced according to the manufacturer's instructions.

Recombinant antigen. Expression and purification of His-tagged recombinant HTNV-Foj, PUUV Vranica, PUUV Kazan, PUUV Sotkamo, and DOBV Slovenia N proteins from yeast cells were performed as described previously (2, 26).

MABs. Monoclonal antibody (MAB) B5D9 against the N protein of HTNV strain 76-118 (29) was purchased from Abcam, United Kingdom. MAB 7G2 against recombinant yeast-expressed hantavirus N protein was raised at the Institute of Biotechnology (A. Zvirbliene, R. Petraityte, I. Kucinskaitė, A. Gedvilaite, A. Razanskiene, J. Schmidt, M. Mertens, P. Padula, B. Hjelle, K. Sasnauskas, and R. Ulrich, unpublished data).

Indirect IgG and IgM ELISAs. Polystyrene microtiter plates (Nerbe plus; Winsin/Luhe, Germany) were coated with 100 μl per well of the yeast-expressed His-tagged HTNV-Foj N protein diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) to a concentration of 1 $\mu\text{g}/\text{ml}$, and the plates were incubated overnight at 4°C . The coated plates were blocked with 150 $\mu\text{l}/\text{well}$ of 3% bovine serum albumin (BSA) for 2 h at room temperature. The plates were twice rinsed with washing buffer, which was composed of phosphate buffered saline (PBS) containing 0.1% Tween 20.

Undiluted oral fluid specimens or serum specimens diluted 1:200 in PBS containing 1% BSA and 0.2% Tween 20 were added to the plates (100 $\mu\text{l}/\text{well}$). After 1 h of incubation at 37°C , the plates were rinsed three times with washing buffer (PBS containing 0.1% Tween 20). Peroxidase-conjugated F(ab')₂ fragments of rabbit anti-human IgG or IgM (Dako, Denmark) diluted 1:12,000 in PBS containing 1% BSA and 0.2% Tween 20 were added to the wells, and the plates were incubated for 1 h at 37°C . The plates were washed as described above. The binding of the specific antibodies was visualized by the addition of

100 $\mu\text{l}/\text{well}$ of tetramethylbenzidine (Dako TMB+ substrate-chromogen; Dako-cytomation) substrate. After 10 min of incubation at room temperature the reaction was stopped by adding 100 $\mu\text{l}/\text{well}$ of 10% sulfuric acid, and the optical density (OD) at 450 nm was measured (reference filter, 620 nm).

IgM capture ELISA. Polystyrene microtiter plates (Nerbe plus) were coated with 100 μl of rabbit antibody to human IgM (A0426; Dako) diluted 1:1,000 in coating buffer, and the plates were incubated overnight at 37°C . The plates were washed once with washing buffer (as described above) and blocked with 150 $\mu\text{l}/\text{well}$ of 3% BSA.

Undiluted oral fluid specimen or serum specimens diluted 1:200 in PBS containing 1% BSA and 0.2% Tween 20 were added to the plates (100 $\mu\text{l}/\text{well}$). After incubation for 30 min at 37°C , the plates were washed three times with washing buffer. Recombinant His-tagged HTNV-Foj N protein was added at a concentration of 2 $\mu\text{g}/\text{ml}$, and the plates were incubated at 37°C for 30 min. For the dilution of the antigen and, subsequently, of MAb and conjugate, PBS containing 1% fetal calf serum, 0.2% Tween 20, and a 5% hantavirus-negative human serum pool was used. After the plates were rinsed, 100 $\mu\text{l}/\text{well}$ of MAb to HTNV N protein was added (dilution, 1:1,000) and the plates were incubated for 30 min at 37°C . Next, 100 μl of goat anti-mouse IgG peroxidase conjugate (Bio-Rad) diluted 1:5,000 was added to each well. The plates were incubated for 30 min at 37°C . After the plates were washed, the binding of specific antibodies was visualized by adding 100 $\mu\text{l}/\text{well}$ of tetramethylbenzidine (Dako TMB+ substrate-chromogen; Dako-cytomation) substrate. The color reaction was stopped after 20 min with an equal volume of 10% sulfuric acid. The OD at 450 nm (reference filter, 620 nm) was read on an ELISA plate reader.

Western blotting. Protein samples were separated in 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) by semidry blotting. After the samples were transferred, the membrane was blocked with 5% dry milk in PBS containing 0.1% Tween 20 for 1 h. The membrane was incubated for 2 h with a human serum sample (diluted 1:100 in PBS containing 0.1% Tween 20). Thereafter, the membrane was incubated for 1 h with peroxidase-conjugated F(ab')₂ fragments of rabbit anti-human IgG or anti-human IgM (Dako) diluted 1:3,000 in PBS containing 0.1% Tween 20. The membrane was developed by adding 4-chloro-naphthol solution in Tris-buffered saline supplemented with H₂O₂ (Sigma, Germany).

RESULTS

Characterization of serum samples. Paired serum and oral fluid specimens were obtained from 151 individuals. Initially, the serum samples were tested both with commercial Progen Hantavirus Hantaan IgG and IgM kits and by our in-house indirect IgG and IgM ELISAs by using a recombinant yeast-expressed His-tagged HTNV-Foj N protein. The indirect ELISA for the detection of hantavirus-specific IgG and IgM in serum by using the recombinant His-tagged HTNV-Foj N-protein antigen has recently been developed at the Institute of Biotechnology (24a). The 151 serum samples were evaluated for the presence of antibodies to HTNV antigen by commercial immunoassays and our in-house immunoassays. Eighty-five of 151 samples tested appeared to be positive with the Progen Hantavirus Hantaan IgG kit and our indirect IgG ELISA (sensitivity of our indirect IgG ELISA, 100%). Fifty-one samples were negative by both assays (specificity of our indirect IgG ELISA, 100%), while the other 15 samples with equivocal results with the Progen Hantavirus Hantaan IgG kit were negative by the indirect IgG ELISA (Table 1). Fifteen samples with equivocal results with the Progen kit were retested by Western blotting with recombinant His-tagged N-protein antigens of different hantaviruses (three PUUV N proteins of different strains and the N proteins of HTNV-Foj and DOBV Slovenia) to clarify whether these samples contain antibodies against hantavirus antigens. All 15 samples were negative by the Western blot assay (data not shown); only the HTNV-positive control serum sample showed reactivity with the

TABLE 1. Results for the panel of 151 serum samples tested with the Progen Hantaan IgM and IgG kits and by indirect IgM and IgG ELISAs

Ig and result with Progen test kit	No. of samples with the following result by indirect ELISA with recombinant antigen:	
	Positive	Negative
IgM		
Positive	91	0
Negative	0	47
Equivocal	1	12
IgG		
Positive	85	0
Negative	0	51
Equivocal	0	15

DOBV Slovenia and HTNV-Foj antigens (Fig. 1B, lanes 4 and 5, respectively). Overall, the sensitivity and specificity of our indirect IgG ELISA were 100% for all samples except the 15 samples that showed equivocal results by the Progen IgG test. Subsequently, 151 serum samples were analyzed with the Progen IgM kit and by our indirect IgM ELISA. Ninety-one serum samples were positive and 47 serum samples were negative by both IgM assays. One sample revealed a positive reaction by the indirect IgM assay but appeared to have an equivocal result by the Progen IgM test, while the other 12 samples with equivocal results by the Progen test were negative by the indirect IgM assay (Table 1). Furthermore, samples with equivocal results with the Progen IgM kit were retested by Western blotting. Only one sample appeared to be positive by the indirect IgM ELISA and Western blotting (Fig. 1D) with the HTNV-Foj and DOBV Slovenia antigens. The other samples with equivocal results with the Progen IgM kit were negative by the Western blot assay (data not shown). Figure 1C presents the reactivities of the HTNV positive control with the DOBV Slovenia and HTNV-Foj antigens (lanes 4 and 5, respectively). Overall, the sensitivity and specificity of our indirect IgM ELISA were 100% for all except 13 samples that exhibited equivocal results by the Progen IgM test.

Comparison of HTNV-specific IgG and IgM measurements in oral fluid and the corresponding serum samples by indirect ELISA. One hundred fifty-one oral fluid specimens were analyzed by the indirect IgG and IgM ELISAs, and the results obtained were compared with those obtained for the matching serum specimens. The results were plotted to show the correlation between HTNV-specific IgG and IgM antibody titers in oral fluid specimens and the corresponding serum specimens

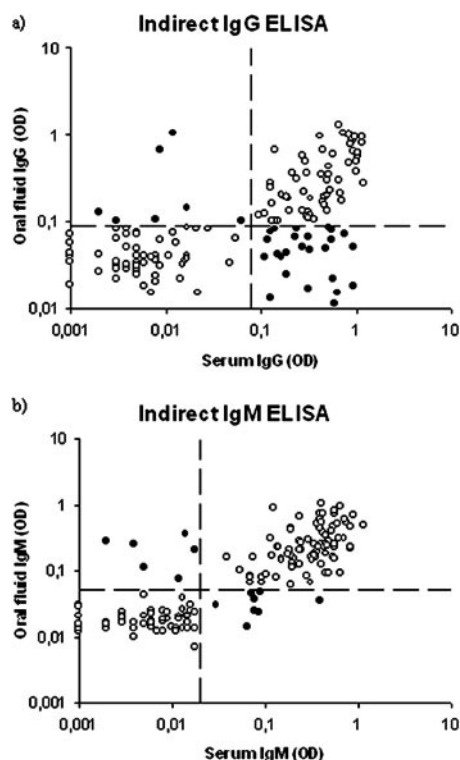


FIG. 2. Comparison of results obtained by the indirect ELISAs for the detection of HTNV-specific IgG (a) and IgM (b) antibodies in serum and oral fluid. Horizontal dotted lines, cutoff values for oral fluid assays; vertical dotted lines, cutoff values for serum assays; filled symbols, samples with discordant results.

(Fig. 2). The dotted lines represent cutoff values, estimated by using negative samples and taking the average OD value for the negative samples plus 3 standard deviations. The cutoff values for the indirect IgG and the indirect IgM ELISA serum assays were 0.08 and 0.02, respectively; those for the indirect IgG ELISA and the indirect IgM ELISA oral fluid assays (oral fluid IgG and IgM indirect ELISAs) were 0.09 and 0.05, respectively. Given that the indirect ELISA with serum is the standard assay, the data for the oral fluid IgG indirect ELISA agreed with those for the serum IgG assay for 59 of 85 serum IgG-positive samples. Seven of 66 IgG-negative serum samples were positive by the oral fluid IgG indirect ELISA. The other oral fluid specimens were negative by the indirect IgG ELISA. Overall, the sensitivity and the specificity of the oral fluid indirect IgG ELISA in comparison to the results of the serum IgG assay were 69.4% ($n = 59/85$; 95% confidence interval,

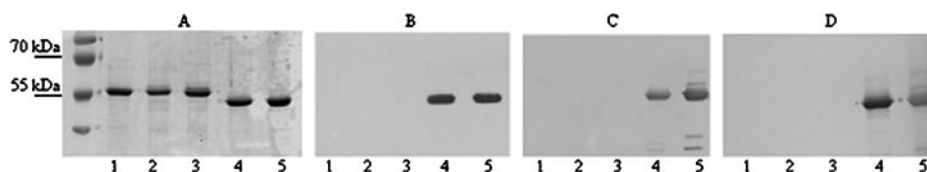


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gels (A) and Western immunoblots (B to D) of different hantavirus N proteins with HTNV-positive human sera. (B) Detection of IgG antibodies; (C and D) detection of IgM antibodies. The lanes contain recombinant yeast-expressed proteins. Lanes: 1, PUUV Vranica N protein; 2, PUUV Kazan N protein; 3, PUUV Sotkamo N protein; 4, DOBV Slovenia N protein; 5, HTNV-Foj N protein.

TABLE 2. Detection of HTNV-specific IgG antibodies by the indirect ELISA with 39 paired serum and oral fluid specimens obtained at known times after disease onset

Sample no.	Day after onset	OD at 450 nm for:	
		Serum	Oral fluid
1	2	0.196	0.18
2	2	0.239	0.08
3	4	0.13	0.056
4	4	0.593	0.011
5	4	0.129	0.156
6	5	0.317	0.124
7	5	0.781	0.07
8	5	0.19	0.044
9	5	0.548	0.079
10	5	0.64	0.015
11	5	0.429	0.049
12	5	0.139	0.102
13	6	0.325	0.046
14	6	0.316	0.064
15	6	0.126	0.013
16	6	0.097	0.116
17	6	0.956	0.018
18	6	0.565	0.022
19	6	0.126	0.274
20	7	0.511	0.084
21	7	0.539	0.23
22	7	1.288	0.274
23	7	0.11	0.039
24	8	0.28	0.22
25	8	0.152	0.042
26	9	0.966	0.642
27	9	0.187	0.024
28	9	0.455	0.649
29	10	0.654	0.289
30	11	0.306	0.359
31	11	0.953	0.838
32	11	0.565	0.592
33	12	1.15	0.804
34	12	0.86	0.478
35	12	1.054	0.578
36	12	0.475	0.191
37	16	0.94	0.051
38	17	0.897	0.766
39	17	1.173	0.932

59.6% to 79.2%) and 89.4% ($n = 59/66$; 95% confidence interval, 81.9% to 96.9%), respectively. Most of the oral fluid samples (samples 1 to 18, 20, and 23; Table 2) whose OD values at 450 nm were very low (≈ 0.05 to 0.1) by the oral fluid IgG indirect ELISA were collected on days 2 to 7 after the onset of disease (Table 2); the exceptions were samples 19, 21, and 22, whose OD values were 0.23 to 0.274. The higher OD values (0.2 to 0.9) were obtained for samples collected on days

8 to 17 after the onset of disease; for a few samples, however (samples 25, 27, and 37), the OD values at 450 nm were very low (< 0.1) (Table 2).

Among the matched oral fluid specimens of 92 serum IgM-positive serum specimens, 84 were positive by the indirect IgM test, and a 91.3% (84/92) sensitivity (95% confidence interval, 85.6% to 97%) was obtained. Six of 59 samples that were IgM negative by the indirect serum IgM ELISA were positive by the oral fluid IgM indirect assay. Therefore, the specificity of the oral fluid IgM indirect ELISA was 89.8% (53/59 samples; 95% confidence interval, 82.1% to 97.5%).

Comparison of HTNV-specific IgM measurements in oral fluid and the corresponding sera by IgM capture ELISA. Previous studies suggested that capture antibody assays may exhibit a greater sensitivity and a greater specificity than the indirect IgG and IgM assays for the detection of virus-specific antibodies (18). Our recent data on the use of recombinant hantavirus N proteins in serum ELISAs have suggested that the recombinant N proteins might be useful in antibody capture assays with oral fluid. To develop a IgM capture assay, MAbs obtained from Abcam (clone B5D9) and generated at the Institute of Biotechnology (clone 7G2) were tested. Both MAbs were first tested in the serum IgM capture assay. Data from tests with serum were compared with those from the Progen IgM and the indirect IgM ELISA tests. The results are presented in Table 3. Ninety-one serum samples were positive and 47 were negative by all tests; the differences emerged for samples with equivocal results with the Progen kit. Of 13 serum samples with equivocal results with the Progen IgM kit, 2 were positive by the IgM capture ELISA with MAb B5D9 and 6 were positive by the IgM capture ELISA with MAb 7G2. On the basis of the presumption that these samples with equivocal results were positive but the concentration of antibodies was too low for the indirect IgM assay to recognize them as positive, we can conclude that the IgM capture assay, especially that with MAb 7G2, is more sensitive.

In our recent study, the IgG capture ELISA was also used for the detection of N-protein-specific IgG antibodies, but this assay had no advantages over an indirect IgG test in terms of sensitivity and specificity (R. Petraityte and L. Jin, unpublished data).

Furthermore, all 151 oral fluid specimens were tested by IgM capture assay. The results were compared with those of the indirect IgM assay (Table 4). The sensitivity of the oral fluid IgM capture assay was higher than that of the indirect IgM assay. Among 92 indirect IgM ELISA-positive serum samples, 89 matching oral fluid samples were positive by the IgM capture assay with MAb 7G2 and 86 were positive by the IgM

TABLE 3. Detection of HTNV-specific IgM antibodies by different assays^a

Result with Progen IgM kit	No. of samples	No. of serum samples with the indicated result by the following assay:					
		Indirect IgM ELISA		Capture IgM ELISA with MAb B5D9		Capture IgM ELISA with MAb 7G2	
		Positive	Negative	Positive	Negative	Positive	Negative
Positive	91	91	0	91	0	91	0
Negative	47	0	47	0	47	0	47
Equivocal	13	1	12	2	11	6	7

^a The IgM-positive, IgM-negative, and equivocal serum samples were screened with the commercial Hantavirus Hantaan IgM kit (Progen).

TABLE 4. Detection of HTNV-specific IgM antibodies in oral fluid samples by different assays and comparison of the results obtained with matched serum samples by the indirect IgM ELISA

Result of indirect IgM ELISA with serum samples	No. of samples	No. of oral fluid samples with the indicated result by the following assay:					
		Indirect IgM ELISA		Capture IgM ELISA with MAb B5D9		Capture IgM ELISA with MAb 7G2	
		Positive	Negative	Positive	Negative	Positive	Negative
Positive	92	84	8	86	6	89	3
Negative	59	6	53	2	57	3	56

capture assay with MAb B5D9. Two oral fluid samples reacted nonspecifically with MAb B5D9 by the IgM capture assay: these positive oral fluid samples were from a group of oral fluid samples whose 59 matching serum samples were negative for hantavirus-specific IgM by the indirect IgM serum assay. Three oral fluid samples from a group of oral fluid samples whose 59 matching serum samples were negative by the indirect IgM serum assay were positive by the oral fluid IgM capture assay with MAb 7G2. In total, the oral fluid IgM capture assay with MAb 7G2 showed a 96.7% (89/92) sensitivity (95% confidence interval, 93% to 100%) and a 94.9% (56/59) specificity (95% confidence interval, 89.3% to 100%). The oral fluid IgM capture assay with MAb B5D9 showed a 93.5% (86/92) sensitivity (95% confidence interval, 88.4% to 98.6%), and a 96.6% (57/59) specificity (95% confidence interval, 92% to 100%).

DISCUSSION

This paper describes the development and evaluation of immunoassays for the detection of HTNV-specific IgG and IgM antibodies in oral fluid samples. HTNV infection accounts for a number of HFRS cases in China each year. The need for simple, robust assays for the determination of immune status is required. Ideally, the assessment of immune status should be performed painlessly with samples obtained by noninvasive methods. Four different assays were developed and tested by using clinical samples, namely, indirect IgG and IgM ELISAs based on the use of recombinant His-tagged HTNV-FoJ N protein and IgM capture ELISAs based on the use of the same recombinant protein and two different MAbs, commercial MAb B5D9 and in-house-generated MAb 7G2. The performances of the different ELISAs were assessed by testing a large panel (n = 151) of paired oral fluid and serum samples from suspected HFRS patients from China.

The results of the oral fluid IgG indirect ELISA were not sufficient: the sensitivity and the specificity of the oral fluid IgG indirect ELISA assay in comparison to the results for serum were 69.4% and 89.4%, respectively. HTNV-specific antibodies were not detected in 30.6% of the oral fluid specimens, probably due to the presence of an insufficient concentration of specific IgG antibodies in the oral fluid samples. It was previously observed that measurement of the antiviral IgG concentration in oral fluid is more complicated and that more sensitive tests are necessary. Therefore, the development of IgG antibody detection assays for use with oral fluid samples for the detection of most viral infections is still in progress (20). Hantavirus-specific IgG antibodies appear during the acute phase of HFRS, but an increase in IgG levels is observed during the early convalescent phase, 2 weeks after the onset of disease

(15). Thus, the sensitivity of the oral fluid IgG assay tended to increase with samples obtained after a longer period after onset. The higher OD values were determined with samples obtained 8 to 17 days after the onset of disease (Table 2), although the association was not significant and requires further investigation. Currently, we still have a weak understanding of the importance of hantavirus-specific IgG determination in oral fluid specimens, and further investigation in this direction would be useful.

The results of the oral fluid IgM assays were more promising than those of the IgG assay. The IgM capture assay showed better results than the simpler and faster indirect IgM assay, namely, a higher sensitivity and a higher specificity; in addition, a better correlation with the results of the tests with the matching serum samples was detected. The sensitivity and the specificity of the oral fluid IgM capture assay was quite high compared with the results of the serum IgM capture assay. The data on the capture IgM assay for paired serum and oral fluid samples correlated very well: of the 151 samples tested, 86 oral fluid samples (by the oral fluid IgM capture assay with MAb B5D9) and 89 oral fluid samples (by the oral fluid IgM capture assay with MAb 7G2) gave concordant positive results, and 57 oral fluid samples (by the oral fluid IgM capture assay with MAb B5D9) and 56 oral fluid samples (by the oral fluid IgM capture assay with MAb 7G2) gave concordant negative results (Table 4). The sensitivity of the IgM capture assay with MAb 7G2 was higher than that of the IgM capture assay with MAb B5D9: 96.7% and 93.5%, respectively. However, the specificity of the IgM capture assay with MAb 7G2 was lower than that of the IgM capture assay with MAb B5D9: 94.9% and 96.6%, respectively. The higher sensitivity of the IgM capture assay with MAb 7G2 could be due to the higher affinity of this antibody for the yeast-expressed protein (used in the present assay), whereas commercial MAb B5D9 was generated by using as an immunogen an *Escherichia coli*-expressed HTNV N protein comprising residues 76 to 118 and therefore might have a lower affinity for the yeast-expressed antigen (35).

In conclusion, the newly developed IgM capture ELISAs for the detection of virus-specific IgM antibodies in oral fluid are useful tools for the serological diagnosis of acute HTNV infection.

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