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Detection of Nephropathia Epidemica (Puumala Virus)-Specific Immunoglobulin M by Enzyme-Linked Immunosorbent Assay

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Nephropathia epidemica (NE), a less severe form of hemorrhagic fever with renal syndrome, is caused by Puumala virus (PUU). This communication reports the development of a μ-capture enzyme-linked immunosorbent assay (ELISA) for detection of specific immunoglobulin M (IgM) antibodies to PUU virus in human sera. Acute- and early-convalescent-phase sera (collected 1 to 41 days after disease onset) from 29 Swedish patients with clinical NE were tested for PUU virus-specific IgG and IgM antibodies by the indirect immunofluorescence test and ELISA, respectively. Late-convalescent-phase serum was also collected from 18 of these patients 3 to 24 months postinfection and assayed. The IgM ELISA values were strongly positive in sera collected during the first 2 months; at 3 to 9 months, they were negative or in the lower range of significance, and at 24 months all sera were negative. Paired sera from NE patients often fail to show seroconversion or a significant titer rise when tested by indirect immunofluorescence. Since all acute- and early-convalescent-phase sera were positive by IgM ELISA, this test could become an important tool for early diagnosis of acute human NE infections.

Nephropathia epidemica (NE), a member of the hemorrhagic fever with renal syndrome (HFRS) complex, was first described in Sweden by Zetterholm and Myhrman in 1934 (8, 20). Until recently, this human disease has been defined only by clinical symptoms: sudden onset of fever, abdominal or low back pain, renal involvement, and spontaneous recovery (5, 13).

The etiological agent of NE, Puumala (PUU) virus, was isolated in 1983 from a bank vole (*Clethrionomys glareolus*) (10). This virus isolate made serological confirmation of clinical cases possible and provided a tool for seroepidemiological surveys.

Detection of specific antibodies to PUU virus antigen with an indirect immunofluorescence test (IFT) has been used to confirm the NE diagnosis in several routine laboratories. The presence of specific antibodies in acute- as well as convalescent-phase sera correlates well with the clinical diagnosis. However, in previous studies as well as in the present study, paired sera from NE patients often fail to show seroconversion or a significant titer rise (9, 12).

Since antibody prevalence rates in areas of Sweden where NE is highly endemic may reach more than 30% in the older age groups, the presence of specific immunoglobulin G (IgG) may reflect a previous infection and have nothing to do with the patient's present condition (11). A test capable of differentiating between IgM and IgG antibodies could offer significant advantages in serological diagnosis. Towards this goal, we report the development of an enzyme-linked immunosorbent assay (ELISA) for detection of PUU virus-specific IgM antibodies.

MATERIALS AND METHODS

Serum specimens. Acute-, early-convalescent-, and late-convalescent-phase (3 to 24 month) sera (n = 74) were available from 29 Swedish patients with clinical NE. Sera from patients bled on day 5 to 24 after the onset of symptoms were also available from patients with HFRS disease; 13 from European USSR, 9 from Asian USSR (kindly provided by E. A. Tkachenko, Institute of Poliomyelitis and Viral Encephalitides, Moscow, USSR), and 5 from Korea (kindly provided by H. W. Lee, Institute for Viral Diseases, Korea University, Seoul).

Sera collected during an epidemiological investigation of apparently healthy populations in Sweden, from areas where HFRS is not (n = 48) and is (n = 122) endemic, were also tested by IgM ELISA.

Immune reagents used in IgM ELISA. Antigen was prepared from PUU virus (strain Vindeln 83-223L)-infected Vero E-6 cells (CRL 1586; American Type Culture Collection, Rockville, Md.) (10). Cultures were harvested after 20 days. Following low-speed centrifugation (2,000 rpm for 10 min), cells and supernatants were separated. Cell pellets were suspended in 1/10 of the original volume (with the supernatants as diluent), sonicated, and then centrifuged at 2,000 rpm for 10 min, and the supernatant was used as the antigen in the ELISA. A negative control antigen was prepared from uninfected cells by the same procedure.

Immune serum was prepared in a rabbit by use of affinity bead immunization (4; chromatography manual, Pharmacia Fine Chemicals AB, Uppsala, Sweden). A high IFT titer (1-year convalescent) human serum was bound to CnBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden). The beads were mixed with the antigen mentioned above, and 3 ml of this preparation was mixed with an equal volume of Freund adjuvant and inoculated into a rabbit by the intramuscular route twice at 6-week intervals. Serum was collected 9 weeks after the first injection. A purified immunoglobulin preparation was made by use of ammonium sulfate fractionation. Anti-human immonoglobulin-reactive antibodies were eliminated by affinity chromatography with beads identical to those administrated to the rabbit (3; Pharmacia manual).

Detection of PUU virus-specific IgM antibodies by ELISA. A μ-capture ELISA was used as follows. Goat anti-human

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IgM (μ-chain specific; Cappel Laboratories, catalog no. 0201-0201) diluted 1:500 in coating buffer (0.05 M sodium carbonate, pH 9.5 to 9.7) was absorbed to 96-well polystyrene microtiter plates (Cooke M 29 AR; Dynatech Laboratories) at 37°C for 2 h. Plates were consecutively treated with human test serum diluted 1:400 in ELISA buffer (phosphate-buffered saline without Mg and Ca and with 0.05% Tween 20 and 0.5% bovine serum albumin at 37°C for 1 h), virus antigen (undiluted with 0.2% normal human serum at 37°C for 1 h), rabbit anti-PUU virus immunoglobulins (diluted 1:200 in ELISA buffer at 37°C for 1 h), and goat anti-rabbit IgG (Kirkegaard and Perry, catalog no. 051506) conjugated with alkaline phosphatase (diluted 1:400 in ELISA buffer at 37°C for 1 h). *p*-Nitrophenol-phosphate (Sigma) diluted in diethanolamine buffer (1 M diethanolamine [pH 9.8], 0.5 mM $MgCl_2$) was used as the substrate. Washing between each step was done six times in washing buffer (saline with 0.05% Tween 20). The reaction was read after 30 min at room temperature in a spectrophotometer at 405 nm and expressed as optical density (OD). Optimal dilutions of all reagents used in the ELISA were determined by box titrations.

All specimens were tested in duplicate with antigen and negative control antigen. The OD was calculated as the average OD with antigen minus the average OD with negative control antigen. The border between positives and negatives was calculated as the mean of the test result of 48 known-negative sera plus 3 standard deviations. An OD of 0.040 or higher was considered positive.

To adjust for plate-to-plate and day-to-day variations in the assay, a positive control serum was included on all plates. This control had an OD of 0.700 (in the linear interval of this IgM ELISA). If the positive control serum had an OD between 0.500 and 0.900, the plate was accepted: however, all OD values on that plate were multiplied by a factor so as to set the positive control value at 0.700.

Detection of PUU virus IgG antibodies by IFT. Spot slides with PUU virus (10)- or Hantaan virus 76-118 (HTN) (17)-infected Vero E6 cells were prepared and stained as described elsewhere (9). Fluorescein isothiocyanate-conjugated sheep anti-human IgG (Wellcome Diagnostics, Temple Hill, Dartford, England; catalog no. MS03) was used to detect specific IgG.

RESULTS

Both acute- and convalescent-phase sera were available from 24 of the 29 patients investigated. The results of IgM ELISA and IgG IFT are shown in Table 1. For 15 of the patients, the acute-phase sera were collected less than 6 days after onset, and 11 samples (73%) showed a fourfold or greater titer rise by IFT. When acute-phase sera were drawn 7 to 14 days after onset of symptoms, two (22%) of nine samples revealed a significant titer rise. IgM ELISA results were positive in all acute- and early-convalescent-phase specimens. Late-convalescent-phase sera from eight patients bled at 3 to 9 months postinfection and 10 patients bled at 2 years postinfection were also tested by IFT and ELISA together with the corresponding acute- or early-convalescent-phase sera (Fig. 1 and 2). Most of the IgM ELISA values were strongly positive for samples collected during the first 2 months, but at 3 to 9 months they were in the lower range of significance or negative, and at 24 months all sera were negative.

All specimens from patients with clinical NE ($n = 74$) were also tested for rheumatoid factor (RF; Waaler-Rose hemagglutination technique), and all were found negative. All IgM-positive sera were confirmed by re-testing after absorption with sheep antibodies to human IgG (RF Absorbent; Behringewerke, Marburg, Federal Republic of Germany) (1).

A 24-month-convalescent serum (IFT titer, 2,560) was divided into three portions and mixed with equal volumes of three different RF-positive sera (RF titers of 1,000, 100, and 10, respectively) and tested for IgM by ELISA. No positive reactions were seen.

Five sera were tested in parallel with one portion that had been freeze-thawed once and one that had been freeze-thawed six times without any significant difference in OD.

TABLE 1. Results for serum samples from 24 Swedish NE patients[a]

Patient no.	Sample (days after onset of disease)	IgM ELISA[b] (OD_{405})	IFT titer[c]
1	1	86	8
	32	171	1,024*
2	2	677	1,024
	22	553	2,048
3	2	597	64
	31	182	1,024*
4	2	552	128
	41	291	1,024*
5	3	607	128
	30	595	1,024*
6	4	407	1,024
	20	178	2,048
7	4	600	16
	22	456	512*
8	4	309	256
	31	121	1,024*
9	4	433	32
	38	674	1,024*
10	5	496	32
	16	228	1,024*
11	5	207	128
	19	116	2,048*
12	5	379	128
	20	400	256
13	5	478	128
	36	315	1,024*
14	6	653	256
	18	483	1,024*
15	6	467	256
	33	118	512
16	8	281	512
	28	178	2,048*
17	8	140	1,024
	23	72	512
18	8	659	512
	15	470	256
19	9	542	1,024
	29	220	1,024
20	11	671	256
	38	83	1,028*
21	12	824	256
	23	808	256
22	13	543	1,024
	29	260	1,024
23	13	691	4,096
	35	246	2,048
24	14	700	256
	40	629	128

[a] Acute- and early-convalescent-phase sera were obtained at different intervals and tested by ELISA and IFT.
[b] A value of ≥40 is considered positive.
[c] *, ≥4-fold increase in titer.

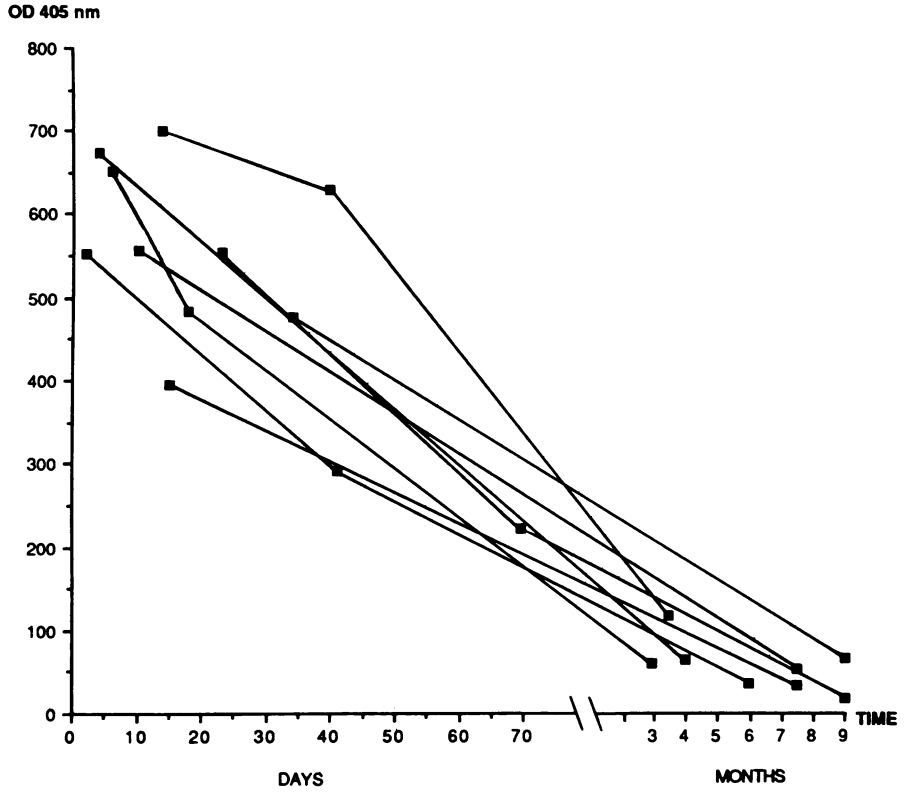

FIG. 1. Acute- and early-convalescent-phase sera and 3- to 9-month-convalescent sera from Swedish patients with clinical NE tested by IgM ELISA. Values for samples from the same patient are connected with a line.

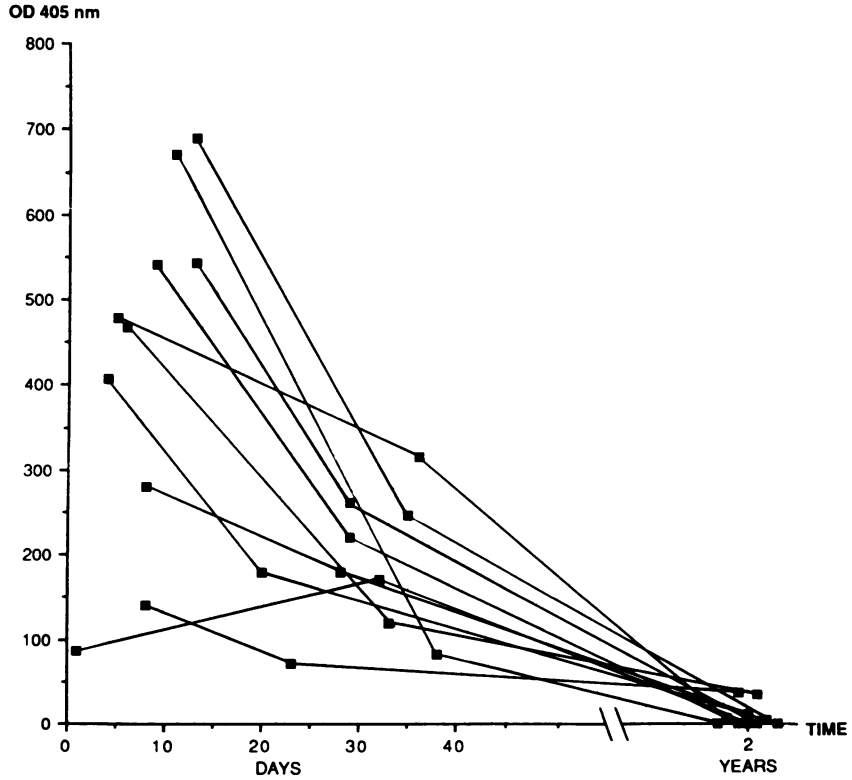

FIG. 2. Acute- and early-convalescent-phase sera and 2-year-convalescent sera from Swedish patients with clinical NE tested by IgM ELISA. Values for samples from the same patient are connected with a line.

TABLE 2. Results for samples from different geographic areas

Area and sample no.	Sample (days postonset)	IgM ELISA[a] (OD_{405})	IgG titer	
			IFT (PUU virus)	IFT (HTN virus)
Asian USSR				
1	9	0	32	8,192
2	7	34	16	8,192
3	9	28	32	8,192
4	8	22	64	8,192
5	10	2	64	2,048
6	9	58	128	8,192
7	9	54	32	8,192
8	5	1	<8	8,192
9	10	0	1,280	>32,768
Korea				
10		60	8	2,048
11		70	8	1,024
12		28	16	2,048
13		45	16	64
14		35	8	1,024
European USSR				
15	12	586	2,048	512
16	20	281	8,192	256
17	15	520	2,048	128
18	13	460	2,048	512
19	10	475	2,048	128
20	18	787	2,048	512
21	14	948	2,048	512
22	5	388	2,048	64
23	13	259	2,048	256
24	18	488	2,048	64
25	16	687	8,192	512
26	19	442	2,048	256
27	14	545	2,048	256

[a] A value of ≥40 is considered positive.

The 122 sera from a normal population in an area where NE is endemic were tested for PUU virus antibodies by IFT, and 19 were found positive. These 19 positive samples were also tested for IgM antibodies by ELISA; 18 were negative and 1 was weakly positive, with an OD of 0.100.

Acute- and early-convalescent-phase sera from patients with HFRS disease in the European part of the USSR reacted like those from NE patients, with high IgM ELISA values and high titers to PUU virus and lower titers to HTN virus by IFT. Sera from Asian USSR and Korea were weakly positive or negative by IgM ELISA and had very low titers to PUU virus but high titers to HTN virus by IFT (Table 2). The IFT data are consistent with the "one-way cross-reaction" noted in earlier studies (17, 19).

DISCUSSION

HFRS causes significant morbidity in several European and Asian countries (5, 9, 17, 19). At least two clinical forms have been recognized, with a severe form in Asia and the eastern part of Europe and a milder form in Scandinavia, Finland, and the western part of the USSR. Incidence rates vary in different parts of the world. In Sweden, an incidence of 30 cases per 100,000 inhabitants per year has been reported, while areas in the western USSR where HFRS is highly endemic report as many as 80 cases per 100,000 inhabitants (7, 9). Antibody prevalence rates also vary in different regions. Up to 30% antibody positivity has been recorded in older males living in parts of Sweden where HFRS is highly endemic (11).

The clinical picture is often diagnostic of NE. Early in the disease, however, clinicians may benefit by rapid serological confirmation. It is also possible that mild or atypical forms of the disease occur, and serological confirmation may help in defining the complete clinical range of the disease.

IFT is presently used in most clinical laboratories for diagnostic purposes. In the present study as well as in previous studies, a significant proportion of the paired sera failed to show seroconversion or a fourfold rise in titer. Since IgG antibodies occur very early (often at the time of disease onset), the absence of specific IgG helps to exclude the diagnosis of NE. The presence of PUU virus-specific IgG supports the diagnosis, but in an area with a high antibody prevalence, positive serology may be caused by earlier NE infection and is of no significance in diagnosing acute disease. A test for specific IgM antibodies offers a confirmatory diagnostic tool. The IgM ELISA is rapid (5 to 6 h with precoated plates) and often very sensitive, and the diagnosis can be made with a single serum specimen.

One problem encountered when using specific IgM detection for the diagnosis of certain diseases is that IgM antibodies may persist for a long period of time (1, 2, 6, 14). This may be due to both the sensitivity of the assay used and antibody kinetics. It is therefore important to evaluate each system to reveal its diagnostic limitations. The present IgM ELISA was positive for all sera tested during the first month postonset; at 3 to 9 months the samples were in the lower range of significance or negative, and at 2 years all sera were negative. Settergren et al. recently developed and evaluated a biotin-avidin-amplified IgM IFT method for NE patients with HTN virus as the antigen (18). When evaluating this IgM IFT, it was found that of 15 patients with clinical NE tested, 11 (73%) had detectable IgM. The duration of IgM as determined by the IFT was less than 30 days in all but one patient (18). It cannot be determined if the difference in sensitivity between the two assays is due to higher sensitivity of the ELISA method or the advantage of using a homologous system or both.

Settergren et al. suggest that freeze-thawing of specimens may have a negative effect on specific IgM and use this to explain some of the negative results obtained when testing sera collected during the first month of onset. Freeze-thawing of specimens six times or less did not affect the results of our IgM ELISA.

An earlier study by Penttinen et al. (15) found that most NE patients were RF positive during the acute phase of the disease, whereas no RF-positive samples were found in the present study. However, the previous work by Penttinen et al. was done with the latex RF test, while we used the Waaler-Rose hemagglutination technique. It has been noted in other viral infections that patients become RF positive during the acute phase of the disease (16). This phenomenon with transient RF has been documented with tests other than the Waaler-Rose hemagglutination technique. There is a theoretical possibility that an RF-positive, anti-PUU virus IgG-positive serum could give a false-positive reaction in the IgM ELISA. Mixing RF-positive serum with high-titer NE-specific IgG serum proved that RF positivity did not affect the IgM ELISA.

It should be noted that the IgM ELISA showed only a very weak reaction to or did not detect heterologous IgM antibodies when used on sera from Korean or Asian USSR patients. An ELISA for detection of specific IgM to HTN virus has been developed by J. Meegan and co-workers at the U.S. Army Medical Research Institute of Infectious Diseases. Ten acute sera from NE patients were shipped on dry ice to the United States and tested in the HTN IgM ELISA. All 10 samples were found positive by both HTN

virus IgM ELISA and PUU virus IgM ELISA (data not presented). Serological one-way cross-reaction has previously been well documented between PUU virus and HTN virus (17). This pattern appears to hold true in the IgM assays as well. In geographic areas where both viruses circulate, it is important to distinguish between the two, since the prognosis for infected patients is quite different. If treatment becomes available in the future, patients will benefit from early rapid diagnosis. The HTN virus IgM ELISA could be used as a screening test, followed by the PUU virus IgM ELISA to determine serotype in HTN virus IgM ELISA-positive specimens. A combination of the two tests would therefore offer a valuable diagnostic tool.

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