Journal of Medical Virology 33:26–32 (1991)

Identification of Hantavirus Serotypes by Testing of Post-Infection Sera in Immunofluorescence and Enzyme-Linked Immunosorbent Assays

J. Groen, H.G.M. Jordans, J.P.G. Clement, E.J.M. Rooijakkers, F.G.C.M. UytdeHaag, J. Dalrymple, G. Van der Groen, and A.D.M.E. Osterhaus

Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, Bilthoven (J.G., E.J.M.R., F.G.C.M.U., A.D.M.E.O.) and Department of Internal Medicine, Medical Spectrum Twente, Enschede (H.G.M.J.), The Netherlands; Military Hospital Brussels, Belgium (J.P.G.C.); Department of Viral Biology, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland (J.D.); and Institute of Tropical Medicine, Antwerp, Belgium (G.V.d.G.)

Serum samples were collected from 27 individuals who had been infected with a member of the genus *Hantavirus* in the Netherlands or Belgium during the last 15 years. These samples were tested in an immunofluorescence assay (IFA) and two enzyme-linked immunosorbent assay (ELISA) systems, using different virus strains that represented each of the four recently proposed serotypes of this genus. The serum samples from 11 individuals who had been infected through contacts with laboratory rats showed the highest reactivities with Hantaan virus (serotype I) and SR-11 (serotype II) in the IFA and ELISA systems. The samples of 16 individuals who had probably been infected through contacts with wild rodents showed the highest reactivities with Hällnäs virus (serotype III) in the IFA. All except two of these also showed the highest reactivity with Hällnäs virus in the two different ELISA systems.

KEY WORDS: Hantavirus, Hällnäs virus, serological systems, serotype analysis

INTRODUCTION

Hantavirus (HV) is a newly defined genus of the family Bunyaviridae, for which at least four different serotypes have been proposed [Lee et al., 1985b; Goldgaber et al., 1985; Dantes et al., 1987; Lee and Van der Groen, 1989; Sugiyama et al., 1987; Van der Groen et al., 1987]. Members of this genus are transmitted to man by direct or indirect contacts with subclinically infected rodents which serve as a reservoir. Most of these have been associated with serious infections in man, causing haemorrhagic fever with renal syndrome (HFRS). The proposed serotypes are more closely associated with the genus of the reservoir host than with geographical areas: *Apodemus* (serotype I), *Rattus* (serotype II), *Clethrionomys* (serotype III), and *Microtus* (serotype IV). Serotypes I and II are generally associated with more severe clinical manifestations in man, causing mortality rates up to 10%, whereas serotype III generally causes milder symptoms and a mortality of less than 1% [Lee and Van der Groen, 1989; Koolen et al., 1989; Tsai, 1987]. Infections with serotype IV have so far not been associated with clinical symptoms in humans [Lee and Van der Groen, 1989; Childs et al., 1987]. Human infections due to contact with wild rodents in Europe are generally caused by serotype III [Tsai, 1987; Osterhaus et al., 1989; Van der Groen et al., 1983]. For the differentiation of HV serotypes, several assay systems using monoclonal and polyclonal antibodies have been used. These include immunofluorescence assay (IFA), plaque reduction neutralization assay, Western blot analysis, enzyme-linked immunosorbent assays (ELISA), precipitation, and haemagglutination inhibition tests [Lee et al., 1985b; Goldgaber et al., 1985; Dantes et al., 1987; Takahashi et al., 1986; Zoller et al., 1989]. For the determination of the serotypes that have infected certain individuals, IFA on convalescent sera has been the most widely used assay. Recently we described different ELISA systems for the serology of HV infections in humans [Groen et al., 1989]. In the present paper we compared two of these assays—an indirect ELISA and an inhibition ELISA—with an IFA for the determination of serotypes that had been incriminated in recent human cases of HV disease in the Netherlands and Belgium. In both ELISA and IFA systems it was shown that human cases acquired through contacts with laboratory rats were associated with infections with serotypes I or II,

Accepted for publication September 13, 1990.

Address reprint requests to A.D.M.E. Osterhaus, Head of the Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

whereas in most of the cases in which a contact with wild rodents could be assumed, a closer association with serotype III was found.

MATERIALS AND METHODS

Virus and Antigen-Preparations

HV, serotype I (strain HV76-118) [Lee et al., 1978], serotype II (strain SR-11) [Kitamura et al., 1983], serotype III (strain Hällnäs) [Yanagihara et al., 1984], and serotype IV (strain Prospect Hill) [Lee et al., 1985a] were propagated in Vero E6 cells. When 80% of the cells were infected, as determined by IFA, the infected cell cultures were solubilized by treatment with 1% Triton X-100 and sonicated twice for 15 seconds as described previously [Groen et al., 1989]. The supernatants were used as antigen after storage at −20°C.

Serum Samples

Serum samples were collected from 11 individuals who had been infected with HV after contacts with laboratory rats in the Netherlands or Belgium in 1981. Their clinical histories have been described elsewhere [Desmyter et al., 1983; Osterhaus et al., 1984]. Sixteen serum samples were collected from individuals who had developed HV disease after infection probably due to contact with wild rodents in the Netherlands or Belgium during the last 15 years as previously documented [Tsai, 1987; Osterhaus et al., 1989; Van der Groen et al., 1983; Clement and Van der Groen, 1987; Lahaije et al., 1989]. Rodent antisera specific for serotypes I and II were generated by experimental intranasal infection of two groups of six specific pathogen-free rats, housed in separate pressurized glove boxes. Six weeks later all the rats were bled by cardiac puncture, their sera were pooled per group and used as reference antisera. For practical reasons, antisera against serotypes III and IV were raised in rabbits. Six New Zealand white laboratory rabbits were infected by a single intramuscular injection of Vero E6 cell propogated Puumala virus (serotype III) or Prospect Hill virus. Animals were bled from the marginal ear vein at three-day intervals from six to eight weeks post-infection and all sera were pooled before dispensing as references reagents.

Indirect Immunofluorescence (IFA)

An IFA was performed, essentially as described previously [Groen et al., 1989]. In brief, infected Vero E6 cells were collected and suspended in growth medium containing 10% (v/v) dimethylsulfoxide at a density of 3.5×10^6 cells/ml. After gamma irradiation (2.5×10^6 rad), the suspension was stored in 2 ml volumes at −70°C until use. After thawing, the cell suspension was centrifuged for 5 min at 580 × g, the cells were washed three times in PBS, and then resuspended in PBS containing 5% FCS. This suspension was seeded on Teflon-coated drop slides (Wellcome, Beckenham, UK), fixed with −70°C ethanol, and stained with either human, rabbit, or rat sera as the first antibody. The second antibody used was a rabbit anti-human, swine anti-rabbit (Dakopatts, Glostrup, Denmark), or goat anti-rat (Nordic, Tilburg, The Netherlands) Ig preparation conjugated with fluorescein isothiocyanate. Results were considered positive when a characteristic dot-like immunofluorescence pattern was observed in the cytoplasm of infected cells. Titers were expressed as the reciprocal of the dilution of primary antibody still giving clear immunofluorescence.

Indirect ELISA

The indirect ELISA was performed in a microtiter system essentially as the cell-ELISA that we described previously [Groen et al., 1989], using the respective virus antigens described above instead of fixed infected cells. In brief, antigen was coated to microtiter plates in phosphate buffered saline (PBS pH 7.2) after determination of the optimal dilution by checkerboard titration using sera from rats and rabbits raised against the different serotypes. After adding a 1:100 diluted human serum sample, plates were incubated for 1 hr at 37°C and binding of the human Ig was detected by adding a sheep anti-human Ig peroxidase conjugate (Amersham International, Amersham, UK). Plates were developed by adding tetramethylbenzidine as a substrate and optical density was measured at 450 nm (OD_{450}). Rat sera were diluted 1:30 and rabbit sera 1:100, goat anti-rat Ig peroxidase (Cappel Cooper Biomedical, West Chester, PA) and goat anti-rabbit Ig peroxidase (Sigma, St. Louis, MO) were used as conjugate. The results obtained with each serum were expressed as the difference between the OD_{450} obtained with HV antigen and the OD_{450} obtained with control Vero E6 antigen. The OD_{450} of negative human sera (n = 485) never exceeded 0.05.

Complex Trapping Blocking (CTB) ELISA

A CTB ELISA was performed in a microtiter system as previously described [Groen et al., 1989]. In brief, plates were coated with an optimal dilution of a Protein-A purified IgG fraction of a serum from an individual who had been infected with serotype II [Desmyter et al., 1983; Osterhaus et al., 1984]. Serial twofold dilutions of the sera to be tested were incubated in the plates together with an optimal dilution of serotype II HV antigen. Subsequently the reduction of antigen binding, as detected by a horseradish peroxidase conjugate of the same serum used as a capture antibody, was measured. Antibody titers were expressed as the reciprocal of the highest serum dilution giving at least 40% reduction of the OD_{450} obtained with negative human sera. The same test was used for the titration of antibodies against serotype III, using the IgG fraction of an individual who had been infected with serotype III as capture antibody and conjugate and serotype III HV antigen. The sensitivity, specificity, and reproducibility of the indirect ELISA as well as the CTB ELISA has been described [Groen et al., 1989].

RESULTS

Reactivities of Post-Infection Sera With Different HV Serotypes in IFA

A comparison of the reactivities in IFA of the sera from individuals who had been infected with HV through contacts with laboratory rats (n = 11) or through contacts with wild rodents (n = 16) and of the used antisera is shown in Figure 1. The antibody titers of the sera from laboratory-associated human cases proved to be about the same against serotypes I and II and generally higher than against serotypes III and IV (Fig. 1a–e). The titers of the sera from the individuals infected through contacts with wild rodents proved to be generally higher against serotype III than against the other serotypes (Fig. 1b,d,f). The serum pool of the rats experimentally infected with serotype I only showed reactivity with type I (titer 20) and not with the other serotypes (Fig. 1d,e,f). The serum pool of the rats experimentally infected with serotype II showed the same titer against serotypes I and II (titer 180), and no reactivities with the other two serotypes (Fig. 1b,d,c,f). The rabbit serum raised against serotype III showed the highest titer (titer 1620) against serotype III (Fig. 1b,d,f), a titer of 540 against serotype IV (Fig. 1c,e,f), and was negative (titer <20) against the other two serotypes. The rabbit serum raised against serotype IV showed the same reactivities with serotypes II, III, and IV (titer 540) (Fig. 1b,c,d,e,f) and had a titer of 180 against serotype I.

Reactivities of Post-Infection Sera With Different HV Serotypes in Indirect ELISA

Generally the same results were obtained when the same collection of sera tested in the IFA was assayed in the indirect ELISA (Fig. 2). Sera from the laboratory-associated human cases reacted about the same with serotypes I and II (Fig. 2a,b,c) and generally better than with serotypes III and IV (Fig. 2b,c,d,e). Also the reactivities of sera from individuals infected through contact with wild rodents in the Netherlands and Belgium proved to be generally higher against serotype III than against the other serotypes (Fig. 2b,d,f) in this

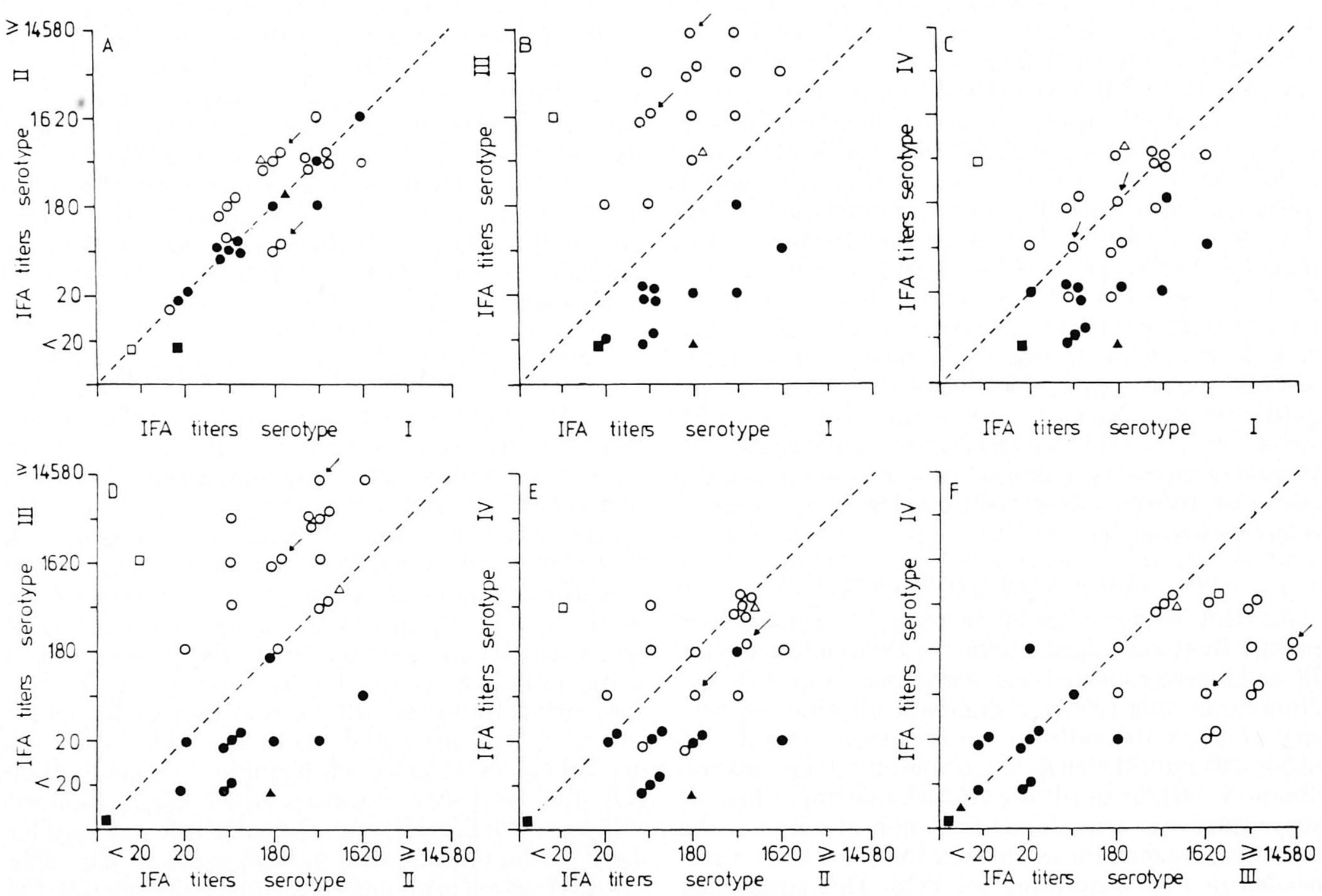

Fig. 1. Comparison of IFA antibody titers in sera from laboratory- and non-laboratory-associated HV infections against four different serotypes of HV. ●, sera from laboratory-associated human HV infections; ○, sera from non-laboratory-associated human HV infections; ■, serum pool of laboratory rats infected with serotype I (strain HV76-118); ▲, serum pool of laboratory rats infected with serotype II (strain SR-11); □, serum from laboratory rabbit infected with serotype III (strain Puumala); △, serum from laboratory rabbit infected with serotype IV (Strain Prospect Hill). Arrows: samples showing different patterns of reactivities in IFA and ELISA.

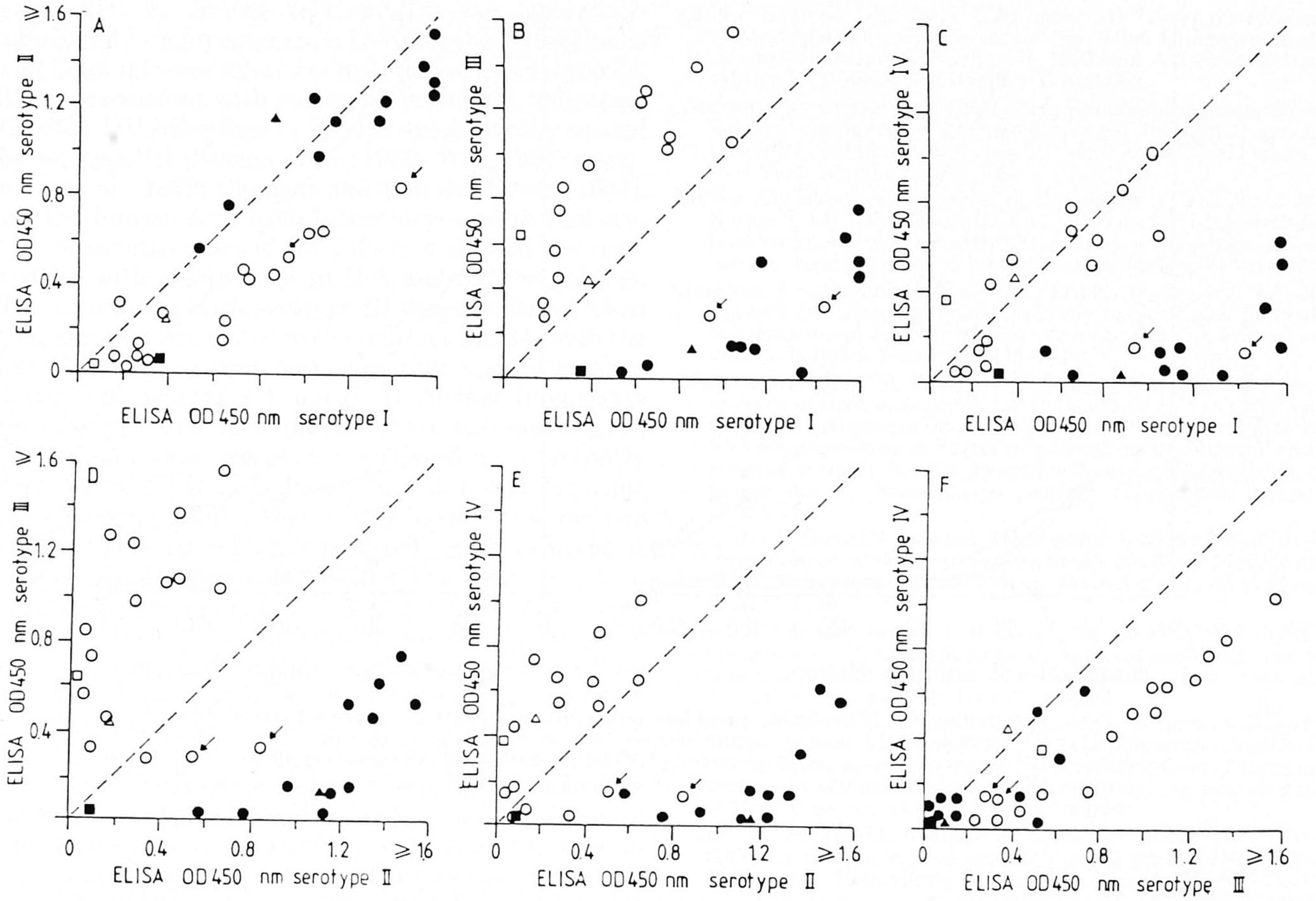

Fig. 2. Comparison of indirect ELISA OD_{450} in sera from laboratory- and non-laboratory-associated HV infections against four different serotypes of HV. ●, sera from laboratory-associated human HV infections; ○, sera from non-laboratory-associated human HV infections; ■, serum pool of laboratory rats infected with serotype I (strain HV76-118); ▲, serum pool of laboratory rats infected with serotype II (strain SR-11); □, serum from laboratory rabbit infected with serotype III (strain Puumala); △, serum from laboratory rabbit infected with serotype IV (strain Prospect Hill). Arrows: samples showing different patterns of reactivities in IFA and ELISA.

assay. However, two of these sera indicated with arrows in Figure 2 reacted better against serotypes I and II than against serotype III (Fig. 2b,c,d,e). The reactivities of the rat antisera raised against serotypes I and II, respectively, were also higher against both serotypes I and II in this assay than against serotypes III and IV (Fig. 2a,d,e,f). The rabbit anti-serotype III serum reacted specifically with serotype III antigen. (Fig. 2b,d,f). The reactivity of serum raised in rabbits against serotype IV was only slightly higher against serotype IV than against the other serotypes (Fig. 2d,e,f).

Reactivities of Post-Infection Sera in CTB ELISA

To further confirm the specificities of the reactivities measured with the human sera in IFA and indirect ELISA with the different virus strains, a third assay system, the CTB ELISA, which is based on a different principle, was examined. Since discrepant values between IFA and the indirect ELISA were found with two human sera, serotypes I and II versus serotype III (see arrows in Figs. 1, 2), CTB ELISAs were carried out with the human sera using serotypes II and III as antigens. The reactivities of the antigens of the different serotypes with the human anti-serotypes II and III IgG preparations were first evaluated in the absence of serum, in order to determine the specificities of these IgG preparations (Fig. 3). Antigens of serotypes I and II reacted better with the anti-serotype II IgG preparation than the other two antigens (Fig. 3a), whereas antigen of serotype III reacted better with the anti-type III IgG preparation than the other antigens (Fig. 3b). Testing of the human sera in the CTB-ELISA against serotypes II and III showed similar results (Fig. 4) as obtained in the indirect ELISA (Fig. 2b): sera from laboratory associated human cases reacted better with serotype II than with serotype III, whereas the reactivities of the sera from non-laboratory associated human cases reacted generally better with serotype III than with serotype II. However, the same two samples of the non-laboratory-associated human cases, which exhibited higher values against serotype II than against serotype III in the indirect ELISA, also showed higher titers against serotype II in the CTB ELISA.

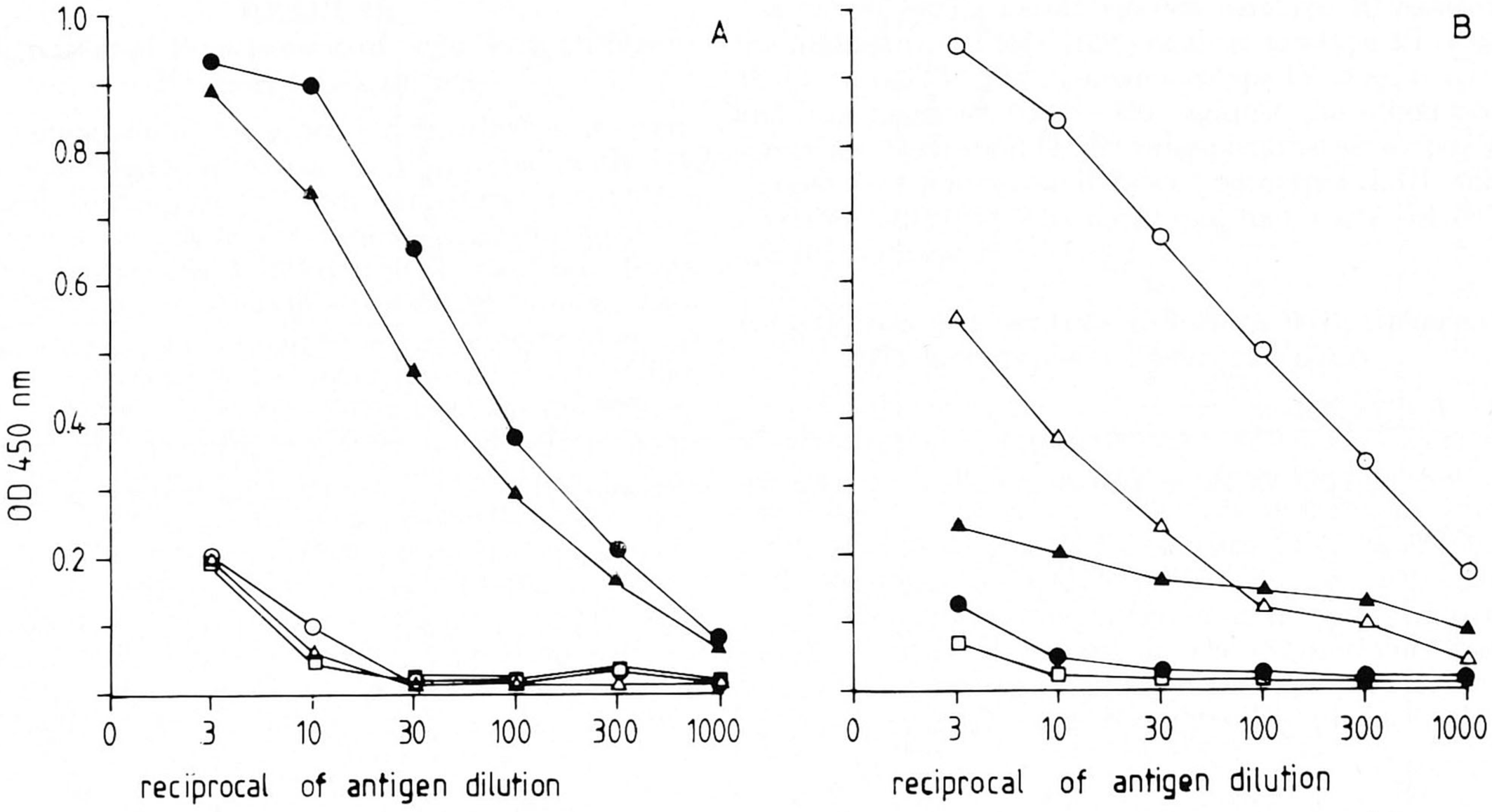

Fig. 3. Reactivities in ELISA of IgG prepared from sera from two individuals infected with HV serotype II (**A**) or HV serotype III (**B**), used as capture antibody and peroxidase conjugate with antigens of four different HV serotypes. ▲——▲ , serotype I (strain HV76-118); ●——●, serotype II (strain SR-II); ○——○. serotype III (strain Hällnäs); △——△ , serotype IV (strain Prospect Hill) and □——□, control Vero E6 antigen.

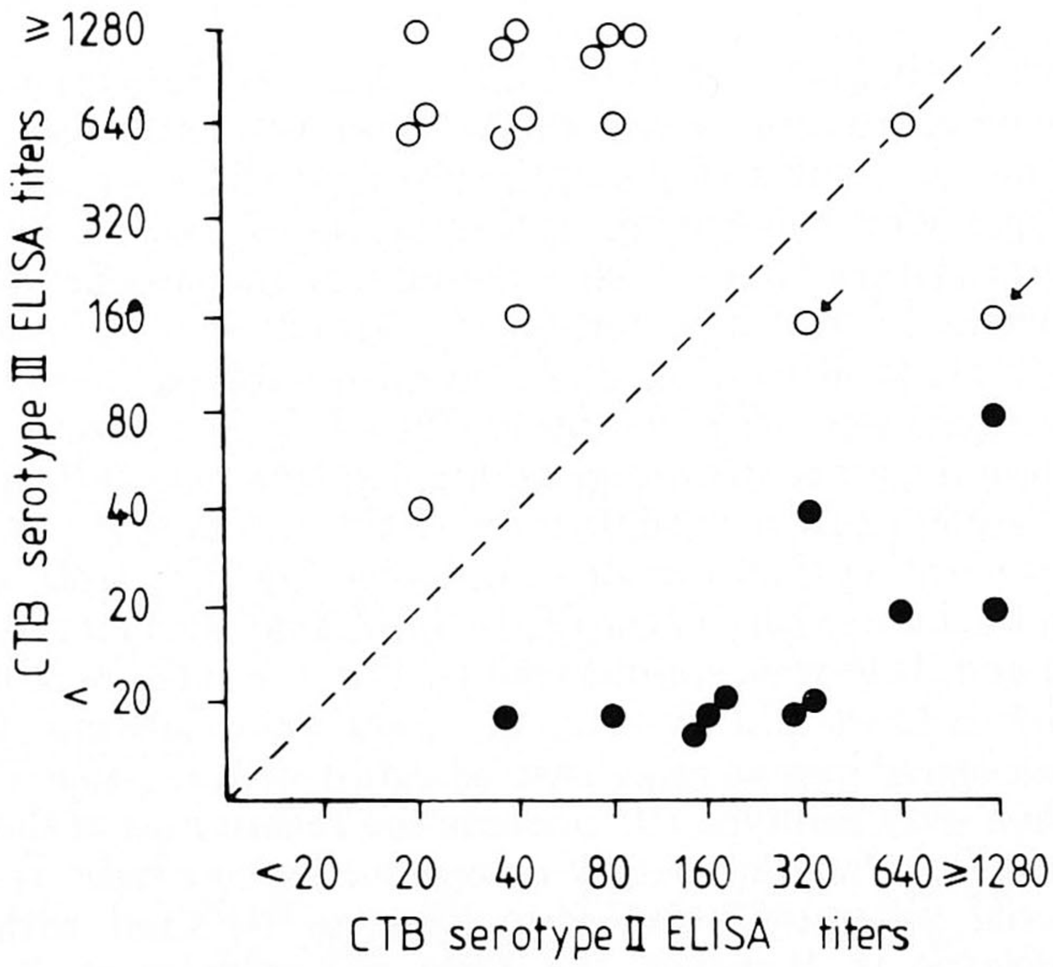

Fig. 4. Comparison of CTB ELISA antibody titers of sera from laboratory- and non-laboratory-associated human HV infections against serotype II and serotype III, ●, laboratory-associated human HV infections; ○, non-laboratory-associated HV infections. Arrows: samples showing different patterns of reactivities in IFA and ELISA.

DISCUSSION

In the present study, we have evaluated different serological methods for the retrospective identification of HV serotypes by testing post-infection sera. For this purpose the IFA has been the most generally used assay. Disadvantages of this test include problems with standardization, subjectivity of interpretation, and the laboratory manipulations involved. Comparison of the results of this assay with those of a recently described indirect ELISA [Groen et al., 1989] performed with a collection of human and animal post-infection sera showed that the indirect ELISA gave essentially the same results, with few exceptions. All of the laboratory-associated human infections from the Netherlands and Belgium could be related to infections with serotypes I or II in both assay systems. This reconfirms the nature of these infections [Desmyter et al., 1983; Osterhaus et al., 1984] and also confirms the close antigenic relationship between serotypes I and II. This was also shown by the results obtained with the rat anti-serotypes I and II sera (Figs. 1 and 2) and by showing that antigens of these serotypes reacted better with the anti-serotype II IgG preparation than the other anti-

gens (Fig. 3). In the IFA, all the non-laboratory-associated human cases (n = 16) from the Netherlands and Belgium were related to infection with serotype III. This is consistent with earlier observations, indicating that the HV infections in Europe are generally caused by serotype III [Koolen et al., 1989; Tsai, 1987; Osterhaus et al., 1989; Clement and Van der Groen, 1987]. All the human sera from laboratory- and non-laboratory-associated cases of HV infection showed low reactivities with serotype IV in IFA and indirect ELISA. The association with serotype III was also found when the samples were tested in the indirect ELISA, with the exception of sera from two cases that seemed to react better with serotypes I and II. To further investigate the serotype that had infected these two individuals, the human sera were also analysed in a recently described CTB ELISA, based on a different principle [Groen et al., 1989]. Also in this assay the same two samples showed a higher reactivity with serotype II than with serotype III. Whether this discrepancy in reactivities in IFA and ELISA of these two sera indeed reflects differences in the serotypes of the infecting HV must be evaluated with caution. Anamnestic evaluation of their possible infection histories did not indicate that these individuals might have acquired their HV infection outside the Netherlands or Belgium. Also no data about the reservoir rodent species from which they could have acquired the HV infection are available. In this light it is interesting to note that recently in Yugoslavia a high incidence of unusually severe human HV infections has been reported [Lee and Van der Groen, 1989; Avsic-Zupanc et al., 1989; Gligic et al., 1988], which was apparently caused by a serotype closely related to serotype I. Further studies are required to define the HV serotypes circulating in Europe and to determine whether e.g. antigen fixation methods used in different serological assay systems significantly influence the results obtained in each of the respective tests.

In conclusion it can be stated that non-laboratory-associated HV infections in the Netherlands and Belgium are generally caused by infection with serotype III and that the indirect ELISA described is a useful tool for the serological identification of HV serotypes using post-infection human sera. Clear advantages of this assay over the IFA are that it can easily be incorporated in automated screening systems, can be evaluated objectively, and is easy to standardize.

ACKNOWLEDGMENTS

We kindly acknowledge Mr. A. Lefèvre and Mr. G. Hoofd for technical assistance, and Ms. C. Kruyssen and Ms. M.C. Eskens for help in preparing the manuscript.

REFERENCES

Avsic-Zupanc T, Cizman B, Glicgic A, Hoofd G, Van der Groen G (1989): Evidence for hantavirus disease in Slovenia, Yugoslavia. Acta Virologica 33:327–337.

Childs JE, Glass GE, Korch GW, Leduc JW (1987): Prospective seroepidemioloy of hantaviruses and population dynamics of small mammal communities of Baltimore, Maryland. American Journal of Tropical Medicine and Hygiene 37:648–662.

Clement J, Van der Groen G (1987): Actu Hantavirus nephropathy in Belgium: Preliminary of a sero-epidemiolocal study. In: Amerio A, Coratelli P, Campese VH, Massry SG (eds): "Acute Renal Failure." New York: Plenum, pp 251–263.

Dantes JR, Okuno Y, Tanishita O, Takahashi Y, Takahashi M, Kurata T, Lee HW, Yamanishi K (1987): Viruses of hemorrhagic fever with renal syndrome (HFRS) grouped by immunoprecipitation and hemagglutination inhibition. Intervirology 27:161–165.

Desmyter J, Johnson KM, Deckers C, Leduc JW, Brasseur F, Van Ypersele de Strihou C (1983): Laboratory rat associated outbreak of haemorrhagic fever with renal syndrome due to hantaan-like virus in Belgium. Lancet 11:1445–1448.

Gligic A, Obradovic M, Stojanovic R, Hlaca D, Antonijevic B, Arnautovic A, Gaon J, Frusic M, Lee P, Goldgaber D, Yanagihara R, Gibbs CJ Jr, Gajdusek DC, Svedmyr A (1988): Hemorrhagic fever with renal syndrome in Yugoslavia: Detection of hantaviral antigen and antibody in wild rodents and serological diagnosis of human disease. Scandinavian Journal of Infectious Diseases 20:261–266.

Goldgaber D, Gibbs CJ, Gajdusek DC, Svedmyr A (1985): Definition of three serotypes of Hantaviruses by a Double Sandwich ELISA with Biotin-Avidin Amplification System. Journal of General Virology 66:1733–1740.

Groen J, Van der Groen G, Hoofd G, Osterhaus ADME (1989): Comparison of immunofluorescence and enzymelinked immunosorbent assays for the serology of hantaan virus infectons. Journal of Virological Methods 23:195–203.

Kitamura T, Morita C, Komatsu T, Sugiyama K, Arikawa J, Shiga S, Takeda H, Akao Y, Imaizumi K, Oya A, Hashimoto N, Urasawa S (1983): Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. Japanese Journal of Medical Science and Biology 36:17–25.

Koolen MI, Jansen JLL, Assmann KLM, Clement J, Liebergen FJHM (1989): A sporadic case of acute hantavirus nephropathy in the Netherlands. Netherlands Journal of Medicine 35:25–32.

Lahaije JJM, Lustermans FATHWBH, Gravekamp C, Groen J, Osterhaus ADME (1989): Another case of hantavirus infection in the Netherlands. Nederlands Tijdschrift voor Geneeskunde 40:133.

Lee HW, Lee PW, Johnson KM (1978): Isolation of the etiologic agent of Korean hemorrhagic fever from wild urban rats. Journal of Infectious Diseases 146:638–644.

Lee HW, Van der Groen G (1989): Hemorrhagic fever with renal syndrome. Progress in Medical Virology 36:62–102.

Lee PW, Amyx KL, Yanagihara R, Gajdusek DC, Goldgaber D, Gibbs CJ (1985a): Partial characterization of prospect-hill virus isolated from meadow voles in the United States. Journal of Infectious Diseases 158:826–829.

Lee PW, Gibbs CJ, Gajdusek DC, Yanagihara R (1985b): Serotypic classification of hantavirus by indirect immunofluorescent antibody and plaque reduction neutralization test. Journal of Clinical Microbiology 22:940–944.

Osterhaus ADME, Spijkers I, Van Steenis G, Van der Groen G (1984): Hantavirusinfecties in Nederland. Nederlands Tijdschrift voor Geneeskunde 52:128.

Osterhaus ADME, Groen J, UytdeHaag FCGM, Van Steenis G, Van der Groen G, Clement J, Jordans JGM (1989): Hantavirus nephropathy in the Netherlands. Lancet August 5:338–339.

Sugiyama K,Morikawa S, Matsuura Y, T Kachenko EA, Morita C, Komatsu T, Akao Y, Kitamura T (1987): Four serotypes of haemorrhagic fever with renal syndrome viruses identified by polyclonal and monoclonal antibodies. Journal of General Virology 68:979–987.

Takahashi Y, Okuno Y, Yamanouchi T, Takada N, Yamanishi K (1986): Comparison of immunofluorescence and hemagglutination inhibition tests and enzyme linked immunosorbent assay for detection of serum antibody in rats infected with hemorrhagic fever with renal syndrome virus. Journal of Clinical Microbiology 24:712–715.

Tsai TF (1987): Hemorrhagic fever with renal syndrome clinical aspects. Laboratory Animal Science 37:419–427.

Van der Groen G, Piot P, Desmyter J, Colaert J, Muylle L, Tkachenko

EA, Ivanov A, Verhagen R, Ypersele de Strihou C (1983): Seroepidemiology of hantaan related virus infections in Belgian populations. Lancet 2:1493–1494.

Van der Groen G, Yamanishi K,Mccormick J, Lloyd G, Tkachenko EA (1987): Characterization of hantaviruses using monoclonal antibodies. Acta Virologica 34:499–503.

Yanagihara R, Svedmyr A, Amyx HL, Lee PW, Goldgaber D, Gajdusek DC, Gibbs CJ, Nystrom JR, Nystrom K (1984): Isolation and propagation of nephropathia epidemica virus in bank voles. Scandinavian Journal of Infectious Diseases 16:226–228.

Zoller L, Scholz J, Stohwasser R, Giebel LB, Sethi KK, Bautz EKF, Darai G (1989): Immunoblot analysis of the serological response in hantavirus infections. Journal of Medical Virology 27:231–237.