Am. J. Trop. Med. Hyg., 51(1), 1994, pp. 102–108
Copyright © 1994 by The American Society of Tropical Medicine and Hygiene

ISOLATION OF THE CAUSATIVE AGENT OF HANTAVIRUS PULMONARY SYNDROME

L. H. ELLIOTT, T. G. KSIAZEK, P. E. ROLLIN, C. F. SPIROPOULOU, S. MORZUNOV, M. MONROE, C. S. GOLDSMITH, C. D. HUMPHREY, S. R. ZAKI, J. W. KREBS, G. MAUPIN, K. GAGE, J. E. CHILDS, S. T. NICHOL, AND C. J. PETERS

Special Pathogens Branch, Molecular Pathology and Ultrastructure Activities, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, and Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, Georgia and Fort Collins, Colorado

Abstract. Investigation of a recent outbreak of acute respiratory illness in the southwestern United States resulted in the recognition of a new disease, hantavirus pulmonary syndrome (HPS) with high mortality. Different animals and cell lines were used in attempts to isolate the causative agent. A previously unknown hantavirus was passaged in laboratory-bred deer mice, recovered from lung tissues of a deer mouse, *Peromyscus maniculatus,* and propagated in the E6 clone of Vero cells. Virus antigen was readily detected in the infected cells by an indirect immunofluorescence assay, using convalescent-phase sera from HPS patients. By electron microscopy, the virus was shown to have the typical morphologic features of members of the genus *Hantavirus,* family *Bunyaviridae.* Virus sequences corresponded to those previously detected by a nested reverse transcriptase-polymerase chain reaction assay of hantavirus-infected specimens from rodents and humans. This newly recognized virus, the etiologic agent of HPS, has been tentatively named Muerto Canyon virus.

An outbreak of unexplained acute respiratory illness in the southwestern United States in May–June 1993 led to the recognition and description of a new disease, hantavirus pulmonary syndrome (HPS) and a new and unique agent serologically and genetically related to, but distinct from, Prospect Hill virus.[1] Through December 31, 1993, 53 cases of HPS (60% case-fatality ratio) had been reported to the Centers for Disease Control and Prevention (CDC) from 14 states,[2] and these cases had been confirmed using serologic tests, a nested reverse transcriptase–polymerase chain reaction (RT-PCR) with specifically designed sets of primers, and/or immunohistochemical analysis of fixed tissues. Rodent trapping in the area of the initial cases demonstrated that the deer mouse, *Peromyscus maniculatus,* was the most common species trapped, with an antibody prevalence in excess of 30%, implicating it as the principal rodent host of the virus in the disease-endemic area.[3]

The need for a virus isolate for pathogenesis studies and the development of homologous serologic tests prompted two approaches: expression of viral proteins through molecular biological techniques in *Escherichia coli,* baculovirus, or vaccinia,[4] and propagation of the causative virus in cell cultures. We report here the isolation of the virus associated with HPS from human and rodent specimens and propagation of this agent in the E6 clone of Vero cells after passage of a rodent tissue specimen in laboratory-colonized *P. maniculatus.*

MATERIALS AND METHODS

Safety and handling of samples. Due to the hazardous nature of the agent, all steps of the homogenization of autopsy tissue samples, cell inoculation, and the RNA extraction and purification were performed in a certified laminar flow biocontainment hood within a Biosafety Level (BSL) 3 containment laboratory. In addition, all animal manipulations were done in the CDC BSL 4 laboratory. All animals were maintained under American Association for the Accreditation of Laboratory Animal Care guidelines. All protocols were reviewed and approved by the CDC Animal Resources Branch. Rodent sera and organs were collected in the field using special precautions for handling potentially infected rodents (i.e., full-face respirators equipped with high-efficiency particulate air (HEPA) filters, disposable gowns, gloves, and shoe covers). An

individual set of instruments was used to collect samples from each rodent to avoid sample cross-contamination. Specimens were stored in liquid nitrogen and shipped to CDC on dry ice. Human specimens were collected in different hospitals and sent directly or through state laboratories to CDC on dry ice (tissues) or wet ice (blood and serum). All specimens were stored at −80°C before processing.

Enzyme-linked immunosorbent assay (ELISA). The IgG ELISA was performed by coating the plate directly with a basic buffer detergent extract of infected (Hantaan [prototype: 76-118], Seoul [prototype: 80-39], Puumala [Sotkamo for IgG tests, French strain (90-13) for IgM tests], and Prospect Hill [PH-1]) and uninfected Vero E6 cells. Sera were initially diluted 1:100 followed by four-fold dilutions through 1:6,400 in 5% skim milk in phosphate-buffered saline (PBS)-Tween and allowed to react with the antigen-coated wells. Bound IgG was detected with mouse anti-human IgG (γ-chain specific; Accurate Chemical, Westbury, NY) or a mixture of goat anti-rat and goat anti-*Peromyscus* IgG conjugated to horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD). Optical densities at 410 nm (OD_{410}) were recorded on a microplate spectrophotometer and the OD_{410} of the uninfected, antigen-coated well was subtracted from its corresponding virus antigen-coated well to yield the adjusted OD_{410}. Later in the study, an *E. coli* recombinant nucleocapsid antigen was used in the same ELISA format, accompanied by an appropriate negative control antigen.[4]

Antibody detection. Slides for the indirect fluorescent antibody (IFA) test were prepared from suspensions of infected and uninfected Vero E6 cells using the four prototype strains of hantavirus (Hantaan, Seoul, Puumala, and Prospect Hill) as previously described.[5] Sera were tested using two-fold dilutions and bound IgG was detected with anti-human IgG (sheep anti-human total IgG, ref. MF01; Wellcome Diagnostics, Dartford, UK), or anti-mouse conjugate (rabbit anti-mouse IgG; Cappel Laboratories, West Chester, PA). When gerbils or mice were tested, a 1:1 mixture of anti-mouse and anti-hamster sera was used. The cross-reactive anti-nucleocapsid mouse monoclonal antibody GB04-BF07 and normal mouse ascitic fluid were used as controls.

Antigen detection (polyclonal and monoclonal antibody testing). Slides with cells scraped from inoculated flasks and lung slide impressions from inoculated animals were air-dried and gamma-irradiated (1.10^6 rads) and fixed in acetone. Virus antigen was detected by IFA using HPS convalescent-phase serum (#9302207), rabbit polyclonal anti-Prospect Hill virus, rabbit anti-Puumala sera, and mouse monoclonal antibody (GB04-BF07) with a broad reactivity against all known hantaviruses (including the one involved in this outbreak), and specific anti-species fluorescein isothiocyanate conjugates.

Reverse transcriptase–polymerase chain reaction. Rodent and human samples were tested using a nested RT-PCR assay.[1] Special precautions were taken to avoid RNA template or PCR product cross-contamination (tissue homogenization and RNA extraction and purification, RT-PCR assay, and PCR product electrophoresis and sequence analysis were done in separate laboratories; laminar flow containment hoods were used in the first two steps). Specific nested PCR DNA products were extracted from agarose gels, and dye deoxynucleotide cycle sequence analysis was carried out (Applied BioSystems, Foster City, CA, and [1]). Estimation of relative virus RNA quantities in samples was performed by a single-step RT-PCR[6] after 10-fold dilution of template RNAs.

Cell culture. The E6 cell line, a cloned line of Vero cells (ATCC Vero clone CRL 1586), was used for all studies. Cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. After inoculation and adsorption of the inoculum, maintenance medium (2% FBS) was added.

Isolation was also attempted in other cell lines (A549 [human lung carcinoma], J77 [mouse monocyte/macrophage], HLF [human embryonic lung fibroblasts], L929 [mouse connective tissue], MRC5 [human fetal lung fibroblasts], MEC [human endothelial], N3T3 [mouse embryo], and 3C5 [transformed *Clethrionomys* cell line]) and in *P. maniculatus* primary adult kidney and primary embryo cells.

Cell cultures in T-25 flasks were inoculated with 0.1 ml of a 10% tissue suspension, maintained at 37°C with the medium changed every seven days, and blind-passed three times at intervals of two weeks. At the time of passage, the supernatant was poured into a conical centrifuge tube, and the remaining cells were trypsinized

and then mixed with the supernatant. One-third of this mixture was passed into fresh cultures and one-third was frozen; the cells in the remaining third were gently pelleted and mixed with 0.2 ml of saline, and slides were prepared for the IFA.

Animal passage. Originally, isolation in animals was attempted in suckling ICR mice (SM), young guinea pigs (Hartley, 300–500 g), and weanling outbred mongolian gerbils. The animals were inoculated intracranially (IC) and intraperitoneally (IP) with 0.02 ml of a 10% tissue suspension. Guinea pigs and gerbils were inoculated IP and/or IP and intranasally (IN) with 0.1 ml of a 10% tissue suspension. Laboratory-bred *P. maniculatus* were obtained from the *Peromyscus* Stock Center (Clemson, SC) and Dr. Suellen A Van Ooteghem at the University of West Virginia (Morgantown, WV). Both colonies were derived from wild-captured animals. All animals were confirmed to be serologically negative for hantavirus antibodies before use in the isolation procedures. *Peromyscus maniculatus* were inoculated IP and IN with 0.1 ml of tissue suspension. Animal manipulations, cocultivations, and explants were performed in a BSL 4 laboratory.

Tissue suspensions (10%) were prepared by grinding tissue samples with sterile sand in PBS with 0.4% bovine serum albumin and antibiotics. The sand was allowed to settle (not centrifuged) and tissue fragments were included in the suspensions inoculated or stored at −70°C.

The first samples received from the outbreak were inoculated IC and IP into two litters of SM. One litter was killed on day 7 postinoculation and the second litter on day 14. Organ homogenates (10%) of these mice were inoculated into a second group of SM. When these tissues were RT-PCR negative, passage in SM was discontinued. Guinea pigs were inoculated IP with samples received at the beginning of the outbreak only and tested for antibody for two months. At the end of this two-month period, they were killed and their tissues were tested for viral RNA presence by RT-PCR. Young gerbils were inoculated IP and IN with 0.1 ml of tissue suspension, three animals per sample. These animals were killed in 7–9 days and blind-passed twice. The presence of virus was monitored by RT-PCR and the presence of antibody by IFA.

Five *P. maniculatus* per sample were inoculated with tissue suspensions. One animal per group was killed on days 7, 9, 12, and 15; the fifth animal was held for antibody determination at 30 days. Lung, liver, kidney, and spleen tissues were harvested and frozen, but only the lung tissues were passaged into additional *P. maniculatus*. For each of these harvests, 10% crude lung tissue suspensions were cocultivated with Vero E6 cells and one or more other cell lines. These cocultures were incubated 14 days between each of three blind passages. In some instances, explants of lung tissue were used instead of coculture. This was done by gently breaking up lung tissue and suspending it in EMEM, incubating two weeks, and then passing into Vero E6 cells, plus splitting the growing explanted tissue. Spleen and kidney tissues were also sometimes cocultivated or explanted. Isolations of virus from throat swabs, urine, fecal material, and blood from *P. maniculatus* were also attempted.

Electron microscopy (EM). For thin-section EM, E6 cells were washed with PBS, glutaraldehyde-fixed in situ, then scraped and pelleted. Cell pellets were postfixed in buffered osmium tetroxide, en bloc stained with uranyl acetate, dehydrated, and embedded in Epon-substitute and araldite resins as previously described.[7] Sections were stained with uranyl acetate and lead citrate.

Tissue culture supernatants were prepared for negative-stain EM by fixation in glutaraldehyde, clarification by centrifugation, and pelleting the supernatant through a 30% sucrose gradient cushion. Pellets were resuspended in PBS, applied to glow-discharge pretreated formvar-carbon grids, blotted, and stained with 0.05% uranyl acetate or with 2% phosphotungstic acid adjusted to pH 6.5 with KOH.[8]

RESULTS

Isolation. Isolation attempts were made either on ELISA antibody-positive human and rodent tissues after confirmation of the presence of viral nucleic acids by RT-PCR or on some RT-PCR–positive ELISA-antibody negative (RT−PCR+/Ab−) specimens to avoid possible interference by antibody. Fifty-five samples from 13 confirmed human cases, 30 samples from seven clinically suspected but laboratory-unconfirmed patients, and samples from 50 different rodents were inoculated on Vero E6 cells. Some of these samples were also inoculated on several other

TABLE 1
*Sequence of the virus isolation**

Passage level	Days	% IFA+	RT-PCR direct	RT-PCR nested
Original sample #9302702, Pm lung			Pos	Pos
Inoculation IP in Pm-1	9	–†	Pos	ND
Lungs passed in Pm-2	12	–†	ND	Pos
Lungs cocultivated on Vero E6-1	15	5–10	ND	ND
Vero E6-2	14	2–5	Neg	ND
Vero E6-3	13	5–10	ND	ND
Vero E6-4	11	60–80	Pos‡	ND
Vero E6-5	10	60–80	ND	ND

* IFA = indirect fluorescent antibody test; RT-PCR = reverse transcriptase–polymerase chain reaction; Pm = *Peromyscus maniculatus*; Pos = positive; IP = intraperitoneally; ND = not done; Neg = negative.
† Impression smears, no quantification.
‡ The sequence of the RT-PCR fragment was identical to the original sample sequence.

cell lines. All attempts using direct cell culture inoculations were unsuccessful; neither cytopathogenic effect nor antigen detected by immunofluorescence was evident after serial passages. Inoculation of human RT-PCR–positive specimens in gerbils (12 specimens), SM (six specimens), and guinea pigs (six specimens) were unsuccessful; that is, there was no death or seroconversion or RT-PCR–positive lung tissues. Specimens from six suspected human cases and four rodents (2× RT-PCR+/Ab+, 2× RT-PCR+/Ab−) were inoculated into *P. maniculatus.* From two of the six human cases, the passage of the suspected virus was confirmed by RT-PCR–positive results for the collected lung tissues of the first and second passages, but no virus was isolated on subsequent cell culture passage. From two of the four rodent lungs, passage of the suspected virus was confirmed by RT-PCR–positive results for the collected lung tissues of the first and second passages. In one case only was the virus successfully passed in Vero E6 cells after two passages in *P. maniculatus* (Table 1). After four cell passages, 80% of the Vero E6 cells had antigen by IFA after 10–15 days postinfection. Intracytoplasmic viral inclusions were detected using either anti-nucleocapsid mouse monoclonal antibody or convalescent-phase serum from an HPS patient. Staining was not observed with negative control sera. In a subsequent passage, using the supernatant of the fifth Vero E6 cell passage, only 2–3% of the cells were infected (IFA-positive) by day 8, a two-fold increase in the number of infected cells was observed each subsequent day, and 100% of the cells were infected by day 14.

Semiquantitative PCR. The sequences of 241 nucleotides of the RT-PCR product derived from the original rodent lung suspension and the second and fifth Vero E6 passages were identical. Furthermore, a 100-fold increase in nucleic acid concentration between the second and fourth passage in E6 cells was demonstrated.

Electron microscopy. On thin sections of infected Vero E6 cells, numerous extracellular virus particles composed of strands of nucleocapsid surrounded by an envelope with surface projections[9] were seen (Figure 1). The extracellular particles had an average diameter of 107 nm (range 80–133 nm). Inclusion bodies and a possible virus antigen layer were also seen in some cells. Hantavirus-like particles with a typical grid-like pattern[8] were seen in concentrated supernatants of infected Vero E6 cells by negative staining (Figure 2).

DISCUSSION

Hantaviruses have historically been difficult to isolate from human or rodent specimens. Successful passage of Hantaan virus from wild-caught *Apodemus agrarius* to laboratory-reared *Apodemus* was only reported in 1978, despite numerous previous attempts.[10] In 1981, Hantaan virus was first propagated in a cultured human cell line.[11] Puumala virus, the causative agent of nephropathia epidemica, was isolated in 1984 from *Clethrionomys glareolus* rodents in Vero E6 cells directly[12] or after several passages in laboratory-bred bank voles and mongolian gerbils.[13]

We have clearly demonstrated the successful isolation and propagation of a virus from the lungs of a wild-caught *P. maniculatus* after am-

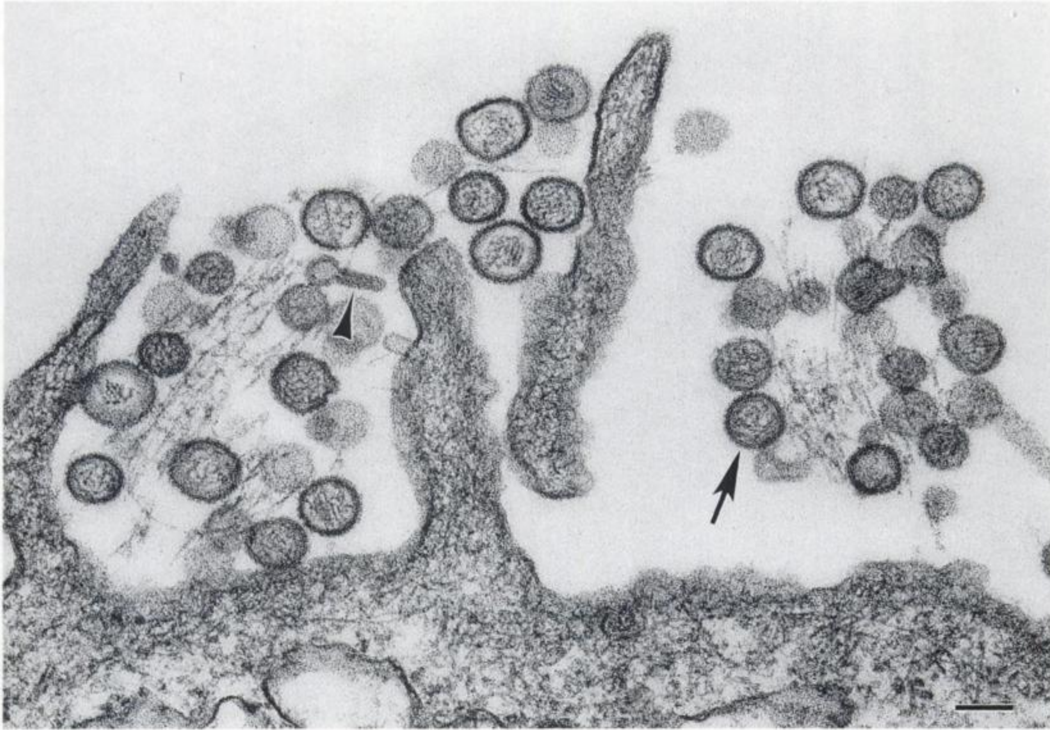

FIGURE 1. Electron micrograph of Vero E6 cells showing extracellular virus particles (**arrow**) composed of strands of nucleocapsid surrounded by an envelope with fuzzy surface projections. Rod-shaped tubules are also present (**arrowhead**). Bar = 100 nm.

plification by rodent-to-rodent transmission, followed by passage in Vero E6 cells. Classic inclusion bodies were seen by IFA in infected cell cultures and virus particles showed typical hantavirus morphologic features. The virus, before tissue culture adaptation, did not replicate in rodent species or cell lines other than *P. maniculatus* or Vero E6, despite numerous attempts using RT-PCR-positive human or rodent samples. Usually, human isolates are very difficult to obtain with Hantaan, Puumala, and Seoul viruses, and despite several attempts, we were also unable to isolate a virus, directly in cell culture or after rodent amplification, from any human specimen tested.

The nucleotide sequences of RT-PCR products obtained from the original sample and after several rodent and cell culture passages showed complete identity. Furthermore, the wild-caught rodent from which the isolate was made was trapped in a house in which a patient with HPS resided. Nucleotide sequences of the RT-PCR products obtained from autopsy tissues of this human case and this rodent were found to be identical to those of the adapted virus reported here.

The virus was tentatively named Muerto Canyon virus after a geographic landmark near the *P. maniculatus* capture site and submitted for registration to the Information Exchange Subcommittee of the American Committee on Arthropod-borne Viruses. The Vero E6 cell–propagated virus can be used to make standard diagnostic reagents for IgM and IgG ELISAs. As demonstrated with the *E. coli*–expressed recombinant nucleoprotein of this virus, the use of homologous antigens will probably increase the specific signal when compared with heterologous viruses, including Prospect Hill virus, which had previously been identified to be the most reactive with the sera from infected patients and rodents. Preliminary diagnostic findings with prototypic virus suggest that it may be possible to diagnose infection earlier in the clinical course of patients presenting to hospitals with suspected HPS.

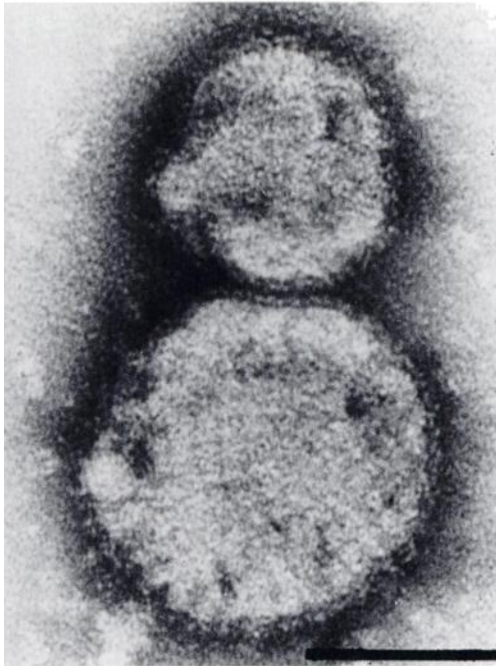

FIGURE 2. Negative-stained electron micrograph of the virus showing typical grid-like pattern (2% phosphotungstic acid, bar = 100 nm).

Acknowledgments: We thank Dr. Bo Niklasson for supplying the 3C5 (*Clethrionomys*-transformed) cell lines used for the isolation attempts. This work was presented in part at the Conference on Hantaviral Disease, Prevention and Management, Albuquerque, NM, November 1993.

Financial support: C. F. Spiropoulou and S. Morzunov are supported by a U.S. Department of Agriculture Animal Molecular Biology National Research Initiative (grant 90-37266-5473) through the University of Nevada, Reno.

Authors' addresses: L. H. Elliott, T. G. Ksiazek, P. E. Rollin, C. F. Spiropoulou, S. Morzunov, M. Monroe, S. T. Nichol, and C. J. Peters, Special Pathogens Branch, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, GA 30333. C. S. Goldsmith, C. D. Humphrey, and S. R. Zaki, Molecular Pathology and Ultrastructure Activities, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, GA 30333. J. W. Krebs and J. E. Childs, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, GA 30333. G. Maupin and K. Gage, Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Fort Collins, CO 80522.

Reprint requests: P. E. Rollin, Special Pathogens Branch, NCID/DVRD/MS G14, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333.

REFERENCES

1. Nichol ST, Spiropoulou C, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, Sanchez A, Childs J, Zaki S, Peters CJ, 1993. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. *Science 262*: 914–917.
2. Centers for Disease Control, 1994. Update: hantavirus pulmonary syndrome - United States. *MMWR Morb Mortal Wkly Rep 43*: 45–48.
3. Childs JE, Ksiazek TG, Spiropoulou C, Krebs JW, Morzunov S, Maupin GO, Gage KL, Rollin PE, Sarisky J, Enscore R, Peters CJ, Nichol ST, 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. *J Infect Dis:* (in press).
4. Feldmann H, Sanchez A, Morzunov S, Spiropoulou C, Rollin PE, Ksiazek TG, Peters CJ, Nichol ST, 1993. Utilization of autopsy tissue RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. *Virus Res 30*: 351–367.
5. Johnson KM, Elliott LH, Heyman DL, 1981. Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human serosurveys. *J Clin Microbiol 14*: 527–529.
6. Rodriguez LL, Letchworth GJ, Spiropoulou CF, Nichol ST, 1993. Rapid detection of vesicular stomatitis virus New Jersey serotype in clinical samples by using polymerase chain reaction. *J Clin Microbiol 31*: 2016–2020.
7. Mollenhauer HH, 1964. Plastic embedding mixtures for use in electron microscopy. *Stain Technol 39*: 111–114.
8. Martin ML, Lindsey-Regnery H, Sasso DR, McCormick JB, Palmer EL, 1985. Distinction between *Bunyaviridae* genera by surface structure and comparison with Hantaan virus using negative stain electron microscopy. *Arch Virol 86*: 17–28.
9. Tao H, Zin Yi C, Tung Xin Z, Se Mao X, Chang Shou H, 1985. Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome (HFRS). I. Some peculiar aspects of the morphogenesis of various strains of HFRS virus. *Intervirology 23*: 97–108.
10. Lee HW, Lee PW, Johnson KM, 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. *J Infect Dis 137*: 298–308.
11. French GR, Foulker RS, Brand OM, Eddy GA, Lee HW, Lee PW, 1981. Korean hemorrhagic fever: propagation of the etiologic agent in a

cell line of human origin. *Science 211*: 1046–1048.
12. Niklasson B, LeDuc JW, 1984. Isolation of the nephropathia epidemica agent in Sweden. *Lancet i:* 1012–1013.
13. Yanagihara R, Goldgaber D, Lee PW, Amyx HL, Gajdusek DC, Gibbs CJ Jr, 1984. Propagation of nephropathia epidemica in cell culture. *Lancet i:* 1013.