zcd00207/zcd2881d07z | xppws | S=1 | 12/27/06 | 4/C Fig: 5 | Art: 0347-06

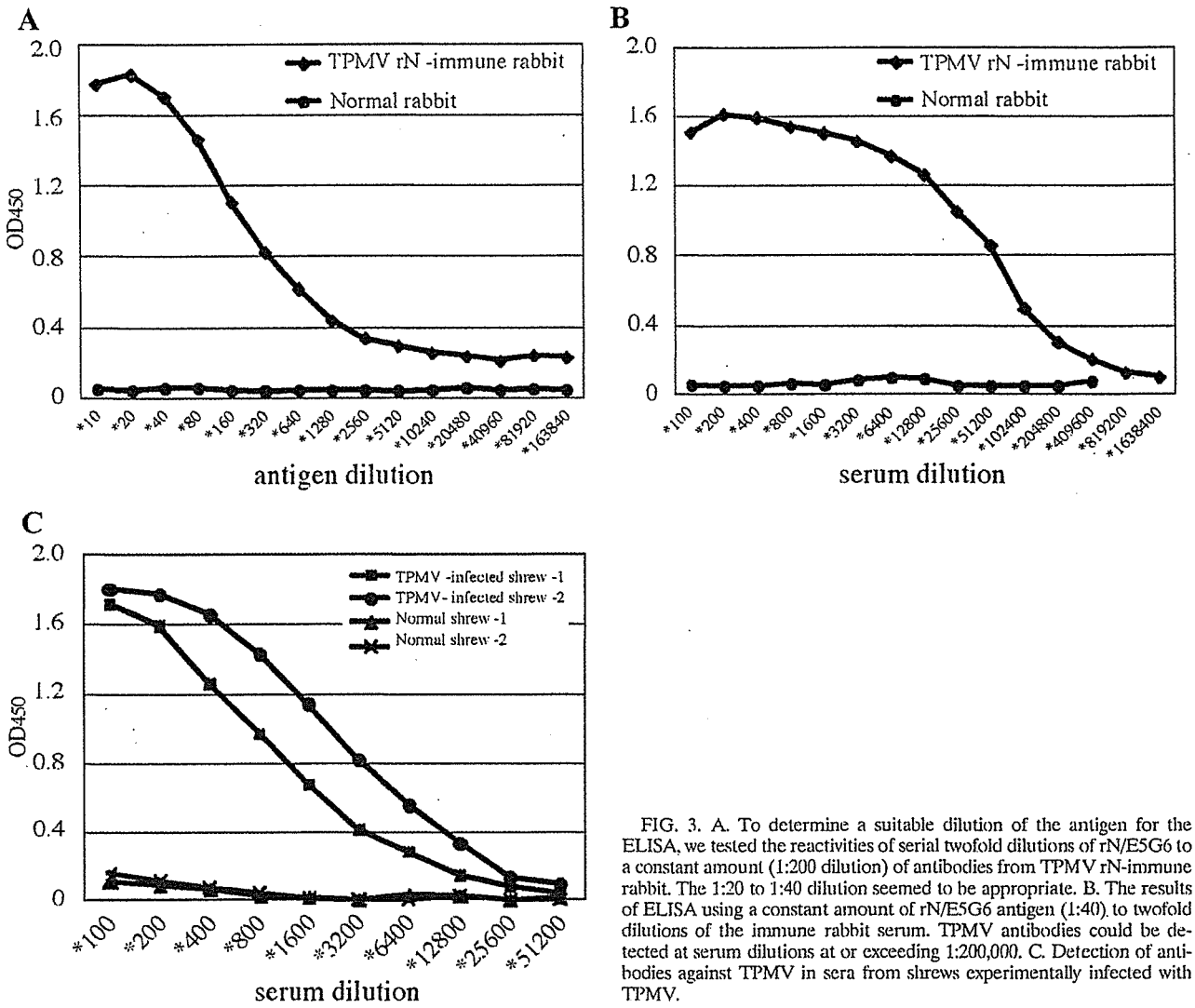


FIG. 3. A. To determine a suitable dilution of the antigen for the ELISA, we tested the reactivities of serial twofold dilutions of rN/E5G6 to a constant amount (1:200 dilution) of antibodies from TPMV rN-immune rabbit. The 1:20 to 1:40 dilution seemed to be appropriate. B. The results of ELISA using a constant amount of rN/E5G6 antigen (1:40) to twofold dilutions of the immune rabbit serum. TPMV antibodies could be detected at serum dilutions at or exceeding 1:200,000. C. Detection of antibodies against TPMV in sera from shrews experimentally infected with TPMV.

E6 cell antigen. So, we succeeded in producing TPMV rN with the E5/G6 epitope, which has the antigenicity of TPMV as well as reactivity with MAb E5/G6.

Developing the E5/G6 capture ELISA system for TPMV. Using the TPMV rN/E5G6 antigen, we developed an IgG antibody-detecting capture ELISA system, according to methods described previously (1, 7, 8, 18). Figure 3A shows the results of an ELISA with twofold dilutions of TPMV rN/E5G6 to a constant dilution of rN-immune rabbit sera (1:200 dilution), and Fig. 3B shows the results of an ELISA with a constant amount of TPMV rN/E5G6 antigen (1:40 dilution) to serial twofold dilutions of rN-immune rabbit sera. The ELISA assay system using the TPMV rN/E5G6 antigen detected anti-TPMV antibodies with high sensitivity. Results with sera from shrews experimentally infected with TPMV also supported the sensitivity of this assay system (Fig. 3C).

In addition, we compared the antigenic cross-reactivities of TPMV and other hantaviruses using this ELISA system (Table 2). In the reactions with each homologous combination, the optical density value was remarkably high. Although the heterologous combinations showed a variety of reactivities, according to the antigenic similarity between viruses, TPMV antigen did not cross-react with other antihantavirus antibodies. This result indicated that rN/E5G6 is a useful tool for the specific detection of anti-TPMV antibodies.

Serological survey of TPMV infection among febrile patients in Thailand. Employing the newly developed capture ELISA

TABLE 2. Cross-reactivities in capture ELISA among TPMV and representative disease-causing hantaviruses

Source of antigen	Cross-reactivity of immune rabbit serum to:			
	HTNV	PUUV	SNV	TPMV
HTNV	0.781	0.453	0.037	0.015
PUUV	0.671	1.487	0.669	0.000
SNV	0.614	1.362	1.672	0.036
TPMV	0.011	0.007	0.002	1.578

zcd00207/zcd2881d07z | xppws | S=1 | 12/27/06 | 4/C Fig: 5 | Art: 0347-06

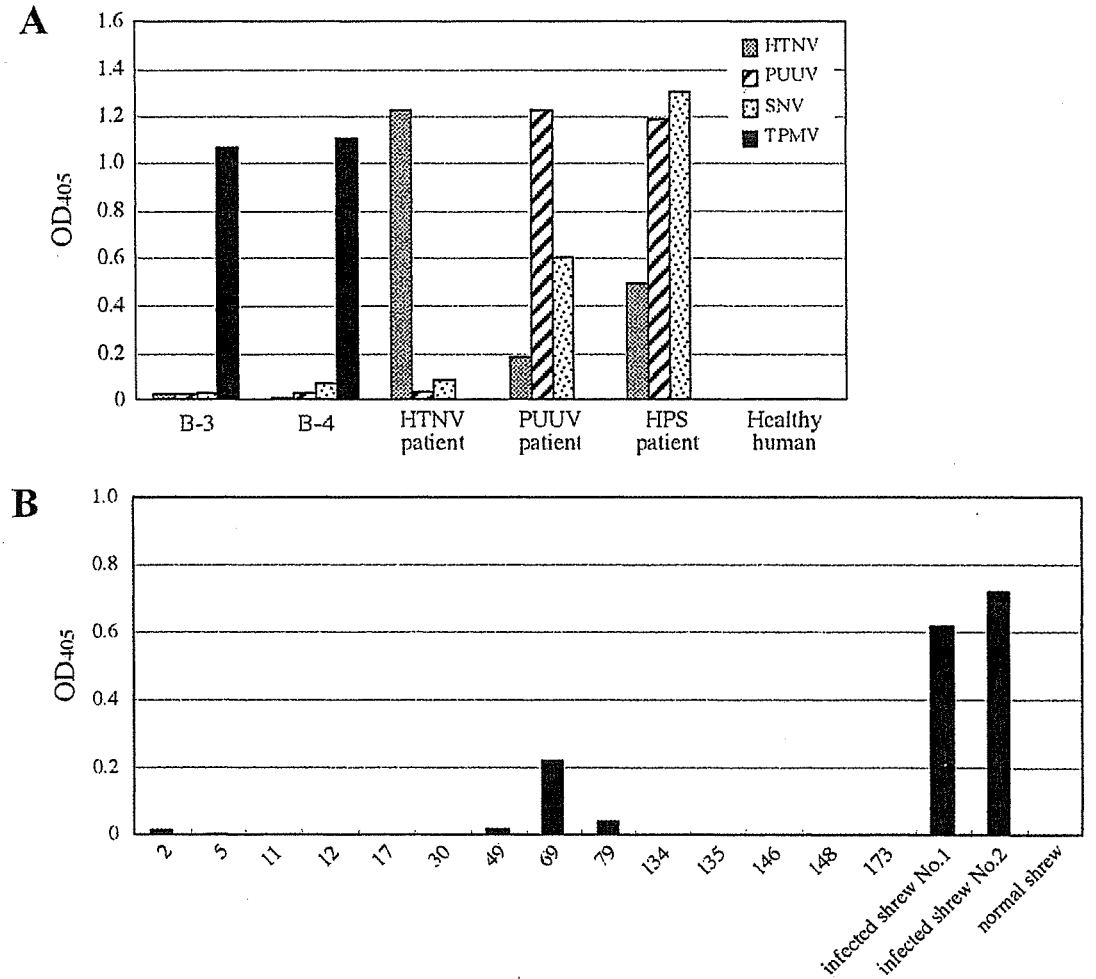


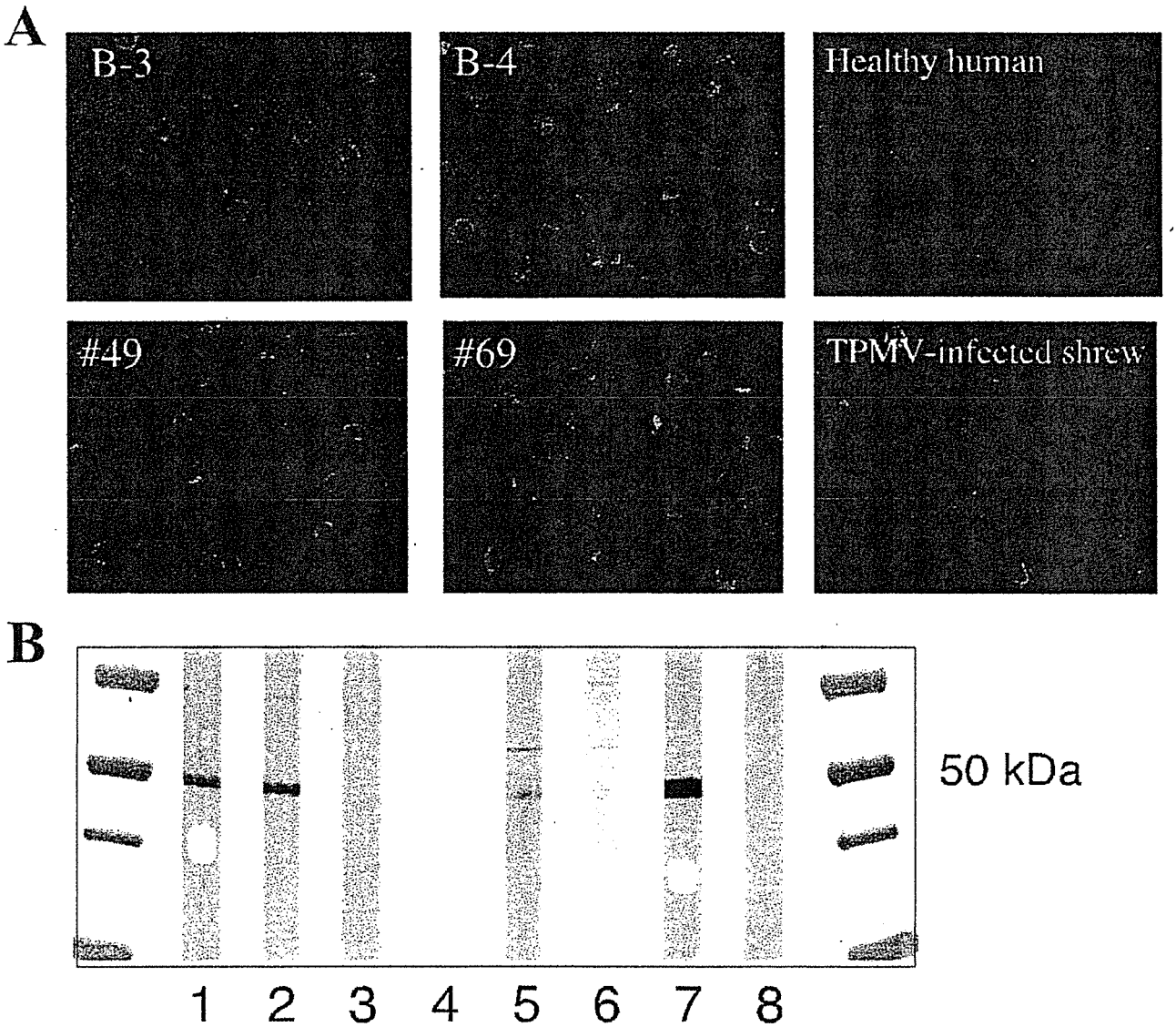
FIG. 4. Serological surveys for TPMV infection in Southeast Asia. We examined 478 sera from patients with fever in Thailand and found two sera (B-3 and B-4 from the same patient at different phases of illness) with anti-TPMV IgG antibodies. Results of the ELISA are shown in panel A. Sera B-3 and B-4 reacted with TPMV antigen. HTNV, PUUV, and HPS patient immune sera served as positive controls. In addition, we tested sera from 14 wild shrews (*Suncus murinus*) captured in Indonesia in 2005 (B). Serum no. 69 was positive for anti-TPMV IgG antibodies, while sera no. 2, 49, and 79 were weakly positive.

system, we tested 478 serum samples from patients with fever in Thailand who were serologically negative for leptospirosis and dengue fever. Each serum was tested with whole HTNV, F4 PUUV, SNV, and TPMV rN for serotyping (Fig. 4A). Serum samples no. B-3 and no. B-4, which were from the same patient during different phases of illness, were positive for anti-TPMV IgG antibodies. Sera from seven other cases were weakly positive to HTNV (data not shown). Sera no. B-3 and no. B-4 also reacted with TPMV-infected Vero E6 cells by the IFA test and F5 Western blot analysis (Fig. 5A and B) and by FRNT using T3/AQ:D native TPMV (Table 3). However, virus-specific IgM was not detected (data not shown). Therefore, this patient may have been infected with TPMV previously, although it is not clear if he had shown symptoms.

This anti-TPMV-antibody-positive patient was a 58-year-old Laotian male who fell ill in Laos and came to a hospital in Nongkhai Province, along the border of Thailand and Laos, in April 2005. He presented with high fever, chills, headache, cough, sore throat, vomiting, diarrhea, abdominal pain, and exhaustion. The patient recovered fully after being hospitalized for several weeks. However, these symptoms were not necessarily related to TPMV infection directly, because he lacked IgM against TPMV. Unfortunately, no information is available about his occupation or his exposure to shrews or wildlife.

Serological survey of TPMV infection in wild shrews captured in Indonesia. Of sera collected from 14 shrews captured in Indonesia in 2005, one (no. 69) was positive for anti-TPMV IgG antibodies by ELISA. Sera from three other shrews (no. 2, 49, and 79) were weakly positive by ELISA (Fig. 4B). By contrast, in the IFA test using TPMV-infected Vero E6 cells as antigen, sera no. 49 and 69 were positive (Fig. 5A), whereas sera no. 2 and 79 were negative. Sera no. 49 and 69 were also positive by Western blotting analysis using TPMV-infected Vero E6 cell antigens (Fig. 5B). Only no. 49 was positive by FRNT (Table 3).

zcd00207/zcd2881d07z | xppws | S=1 | 12/27/06 | 4/C Fig: 5 | Art: 0347-06



4 COLOR

AQ: E FIG. 5. A. Results of IFA test using TPMV-infected Vero E6 cell antigens. As a positive shrew serum control, serum from a shrew experimentally infected with TPMV was used. Sera no. 49 and 69 were positive against TPMV antigen. But sera no. 2 and 79 were negative by the IFA test (data not shown). B. Western blot analysis of TPMV-positive sera with TPMV antigen. The human positive sera B-3 and B-4 in ELISAs also reacted with TPMV-infected Vero E6 antigen by Western blot analysis. On the other hand, for shrews, only no. 49 and 69 showed a band at 50 kDa with TPMV antigen, and sera no. 2 and 79 did not. Lanes 1 (B-3) and 2 (B-4) are human positive samples in ELISA; lane 3 (no. 2), lane 4 (no. 49), lane 5 (no. 69), and lane 6 (no. 79) are shrew positive samples in ELISA; lane 7 (serum from a shrew experimentally infected with TPMV) is a positive control. Lane 6 (normal shrew serum) is a negative control.

TABLE 3. FRNT with native TPMV in human and shrew sera[a]

Serum no.	FRNT titer
Human	
B-3	40
B-4	80
(−)	<40
Shrew	
49	80
69	<40
(+)	320
(−)	<40

[a] Human (−) is a serum from a healthy individual as a negative control. Shrew (+) is a serum from a shrew experimentally infected with TPMV as a positive control, and (−) is a serum from a normal uninfected shrew.

DISCUSSION

Long unclassified, TPMV is now known to be a member of the genus *Hantavirus*. Surprisingly little is known about TPMV, however, despite the fact that its isolation predates that of Hantaan virus. For example, until very recently sequences of the full-length S-, M- and L-genomic segments of TPMV were not known. Also, although TPMV was isolated from tissues of a musk shrew, the identity of its natural reservoir host has remained shrouded in some uncertainty, with some believing that TPMV must represent spillover from a rodent host. The dearth of information about TPMV can largely be attributed to

the lack of systematic, well-designed studies focusing on its epizootiology and pathogenicity. One of the barriers to conducting such studies has been the unavailability of highly sensitive and specific serological assays.

To address this limitation, we first compared the antigenic profile of TPMV with those of representative hantaviruses, which segregate into three groups according to the subfamilies of their rodent reservoir hosts: that is, Murinae-, Arvicolinae- and Sigmodontinae-associated hantaviruses (13, 16). Viruses in each group have antigenic properties similar to each other's (5, 7). As determined by the IFA test using MAb and polyclonal immune sera, TPMV had the most divergent antigenic profile among hantaviruses, which conforms to data from an earlier report using the plaque reduction neutralization test (5). Moreover, immune serum, prepared by inoculating BALB/c mice with TPMV, had a high IFA titer against TPMV of 1:12,800. However, in Western blot analysis, the mouse immune serum did not detect TPMV antigen in TPMV-infected Vero E6 cell lysates or in TPMV rN antigen prepared with *E. coli*, whereas other hantavirus N proteins were detected by mouse serum immunized with the respective hantavirus (data not shown). These data suggest that TPMV induces either no or very low levels of linear epitope-recognizing antibodies in mice. The antigenic difference of TPMV N from that of other hantaviruses indicated a requirement for a new ELISA system for the serological diagnosis of TPMV infection.

We have developed an E5/G6 capture ELISA system which has excellent specificity and sensitivity profiles for the diagnosis of hantavirus infection (1, 7, 8, 18). In this ELISA system, each rN antigen is captured in wells coated with MAb E5/G6. Since TPMV seemed to have no affinity to MAb E5/G6, we inserted several amino acid mutations into the region corresponding to the E5/G6 epitope of TPMV N. Because antibodies against the E5/G6 epitope are not induced in hantavirus-infected patient sera and E5/G6 does not compete with other antibodies induced by hantavirus infections (18), we expected that inserting amino acid-altering point mutations within this region would not change its antigenicity. Finally, we succeeded in developing an E5/G6 capture ELISA which can identify TPMV rN-immune rabbit sera and sera of shrews experimentally infected with TPMV with high specificity.

We previously proposed that three kinds of whole-length rN antigens of HTNV, PUUV, and SNV were required for the serological diagnosis of rodent-borne hantavirus infections (7). Now, by adding TPMV rN/E5G6, it is possible to diagnose both rodent- and insectivore-borne hantavirus infections. Using these four rN antigens, we examined 478 serum samples from patients with high fever in Thailand who were serologically negative for leptospirosis and dengue virus and found two anti-TPMV IgG antibody-positive sera from a single individual. Anti-TPMV IgG antibodies in these sera were confirmed by IFA, Western blotting, and FRNT. Because the patient came to the hospital after his condition had worsened, the relationship between his illness and TPMV infection could not be accurately determined. Thus, while this case suggests the infectivity of TPMV for humans, its pathogenicity for humans remains uncertain.

In testing sera from 14 wild shrews captured in Indonesia in 2005, one sample (no. 69) reacted strongly against TPMV, and three other samples (no. 2, 49, and 79) reacted weakly by ELISA. In the IFA test, using TPMV-infected Vero E6 cells as the antigen, two of these sera (no. 49 and 69) were positive, and this was confirmed by Western blot analysis. However, only serum no. 49 neutralized TPMV by FRNT, suggesting the possible existence of TPMV variants or other antigenically distinct insectivore-borne hantaviruses in nature. To fully demonstrate that shrews are the natural reservoir of TPMV, it is necessary to survey additional species and detect the viral genome using RT-PCR assays in the future.

This is the first report of TPMV infection serologically confirmed with both humans and shrews. Our data indicate that TPMV can infect humans and is maintained in musk shrews as its natural host. The availability of newly developed serological assays for TPMV will facilitate future studies aimed at further elucidating the epizootiology and molecular phylogeny of insectivore-borne hantaviruses. Moreover, such studies will provide important insights about the role of TPMV and TPMV-like hantaviruses in the pathogenesis of febrile illnesses.

ACKNOWLEDGMENTS

M.O. is a research fellow of the Japan Society for the Promotion of Science (JSPS) and was supported by JSPS Research Fellowships for Young Scientists. This work was also supported in part by a grant from the 21st Century COE Program, "Program of Excellence for Zoonosis Control," and Grants-in-Aid for Scientific Research and the Development of Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

REFERENCES

1. **Araki, K., K. Yoshimatsu, M. Ogino, H. Ebihara, A. Lundkvist, H. Kariwa, I. Takashima, and J. Arikawa.** 2001. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. J. Clin. Microbiol. 39:2397–2404.
2. **Arikawa, J., A. L. Schmaljohn, J. M. Dalrymple, and C. S. Schmaljohn.** 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J. Gen. Virol. 70:615–624.
3. **Carey, D. E., R. Reuben, K. N. Panicker, R. E. Shope, and R. M. Myers.** 1971. Thottapalayam virus: a presumptive arbovirus isolated from a shrew in India. Indian J. Med. Res. 59:1758–1760.
4. **Chu, Y. K., G. Jennings, A. Schmaljohn, F. Elgh, B. Hjelle, H. W. Lee, S. Jenison, T. Ksiazek, C. J. Peters, P. Rollin, and C. Schmaljohn.** 1995. Cross-neutralization of hantaviruses with immune sera from experimentally infected animals and from hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome patients. J Infect. Dis. 172:1581–1584.
5. **Chu, Y. K., C. Rossi, J. W. Leduc, H. W. Lee, C. S. Schmaljohn, and J. M. Dalrymple.** 1994. Serological relationships among viruses in the Hantavirus genus, family Bunyaviridae. Virology 198:196–204.
6. **Meyer, B. J., and C. S. Schmaljohn.** 2000. Persistent hantavirus infections: characteristics and mechanisms. Trends Microbiol. 8:61–67.
7. **Miyamoto, T.** 2004. Antigenic characterization of Sin Nombre virus and establishment of serodiagnosis of hantavirus pulmonary syndrome. Jpn. J. Vet. Res. 52:54–55.
8. **Morii, M., K. Yoshimatsu, J. Arikawa, G. Zhou, H. Kariwa, and I. Takashima.** 1998. Antigenic characterization of Hantaan and Seoul virus nucleocapsid proteins expressed by recombinant baculovirus: application of a truncated protein, lacking an antigenic region common to the two viruses, as a serotyping antigen. J. Clin. Microbiol. 36:2514–2521.
9. **Nichol, S. T., B. J. Beaty, R. M. Elliott, R. Goldbach, A. Plyusnin, C. S. Schmaljohn, and R. B. Tesh.** 2005. Bunyaviridae, p. 695–716. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy: classification and nomenclature of viruses. Eighth report of the International Committee on the Taxonomy of Viruses. Elsevier Academic Press, San Diego, Calif.
10. **Ogino, M., K. Yoshimatsu, H. Ebihara, K. Araki, B. H. Lee, M. Okumura, and J. Arikawa.** 2004. Cell fusion activities of Hantaan virus envelope glycoproteins. J. Virol. 78:10776–10782.
11. **Okumura, M., K. Yoshimatsu, K. Araki, B. H. Lee, A. Asano, T. Agui, and J. Arikawa.** 2004. Epitope analysis of monoclonal antibody E5/G6, which binds to a linear epitope in the nucleocapsid protein of hantaviruses. Arch. Virol. 149:2427–2434.
12. **Pattamadilok, S., B. H. Lee, S. Kumperasart, K. Yoshimatsu, M. Okumura,**

I. Nakamura, K. Araki, Y. Khoprasert, P. Dangsupa, P. Panlar, B. Jandrig, D. H. Kruger, B. Klempa, T. Jakel, J. Schmidt, R. Ulrich, H. Kariwa, and J. Arikawa. 2006. Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am. J. Trop. Med. Hyg. 75:994–1002.

13. **Plyusnin, A.** 2002. Genetics of hantaviruses: implications to taxonomy. Arch. Virol. 147:665–682.
14. **Schmaljohn, C., and B. Hjelle.** 1997. Hantaviruses: a global disease problem. Emerg. Infect. Dis. 3:95–104.
15. **Schmaljohn, C. S.** 1996. Molecular biology of hantaviruses. p. 63–90. *In* R. M. Elliott (ed.), The Bunyaviridae. Plenum Press, New York, N.Y.
16. **Wang, H., K. Yoshimatsu, H. Ebihara, M. Ogino, K. Araki, H. Kariwa, Z. Wang, Z. Luo, D. Li, C. Hang, and J. Arikawa.** 2000. Genetic diversity of hantaviruses isolated in china and characterization of novel hantaviruses isolated from Niviventer confucianus and Rattus rattus. Virology 278:332–345.
17. **Xiao, S. Y., J. W. Leduc, Y. K. Chu, and C. S. Schmaljohn.** 1994. Phylogenetic analyses of virus isolates in the genus Hantavirus, family Bunyaviridae. Virology 198:205–217.
18. **Yoshimatsu, K., J. Arikawa, M. Tamura, R. Yoshida, A. Lundkvist, B. Niklasson, H. Kariwa, and I. Azuma.** 1996. Characterization of the nucleocapsid protein of Hantaan virus strain 76-118 using monoclonal antibodies. J. Gen. Virol. 77:695–704.
19. **Yoshimatsu, K., J. Arikawa, R. Yoshida, H. Li, Y. C. Yoo, H. Kariwa, N. Hashimoto, M. Kukinuma, T. Nobunaga, and I. Azuma.** 1995. Production of recombinant hantavirus nucleocapsid protein expressed in silkworm larvae and its use as a diagnostic antigen in detecting antibodies in serum from infected rats. Lab. Anim. Sci. 45:641–646.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Vaccine 24 (2006) 2928–2934
Vaccine
www.elsevier.com/locate/vaccine

A pseudotype vesicular stomatitis virus containing Hantaan virus envelope glycoproteins G1 and G2 as an alternative to hantavirus vaccine in mice

Byoung-Hee Lee, Kumiko Yoshimatsu, Koichi Araki, Megumi Okumura, Ichiro Nakamura, Jiro Arikawa*

Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan

Received 28 June 2005; received in revised form 18 November 2005; accepted 14 December 2005
Available online 6 January 2006

Abstract

We examined whether a vesicular stomatitis virus (VSV) pseudotype bearing the hantavirus envelope glycoproteins (GPs) G1 and G2 (VSVΔG*HTN) could be used as a safe and effective alternative to native hantavirus. Mice were immunized with purified particles of VSVΔG*HTN. After the second immunization, all mice produced anti-GP antibody as detected in ELISA and a neutralization test. After the third immunization, the mice were challenged with Hantaan virus. Neither anti-NP antibody production nor Hantaan virus-specific CD8 T-cell reactions were detected in these mice. The present study demonstrated the potential of using a pseudotype VSV system as a tool for developing a hantavirus vaccine.

Keywords: Envelope; Hantavirus; Pseudotype

1. Introduction

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent-borne viral zoonoses caused by viruses in the *Hantavirus* genus of the *Bunyaviridae* family. Among the 22 currently registered virus species within the genus, the Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus, and Puumala virus (PUUV) are causative agents of HFRS in Eurasia, while the Sin Nombre virus, Andes virus, New York virus, and other related viruses are known to cause HPS in North and South America [1].

Each virus species is carried by a specific rodent species in nature, thus restricting the virus' prevalence to the regions populated by these rodents [1,2]. HFRS has been reported throughout Eurasia and particularly in China, where tens of thousands of cases are reported annually [3]. In addition, thousands of cases have been reported in Europe and Far East Asia [4], and several hundred have been reported in both North and South America [1]. The mortality rates of HFRS and HPS patients are 0.1–10% and around 40%, respectively [1]. Thus, both HFRS and HPS are important zoonoses from a public health perspective.

Hantaviruses contain a single-stranded, negative-sense RNA genome that is divided into three segments, designated large (L), medium (M), and small (S) [5]. The L segment encodes RNA-dependent RNA polymerase. The S segment encodes nucleocapsid protein (NP), and the M segment encodes a glycoprotein (GP) precursor that is cotranslationally cleaved into the envelope proteins G1 and G2 [5]. The G1 and G2 proteins form projections on the virion surface, induce low-pH dependent cell fusion [6], and are the targets of neutralizing antibodies [7,8].

Inactivated HTNV, SEOV, or PUUV vaccines prepared from the viruses grown in suckling mouse brain or tissue culture cells have been developed in Korea and China [9–11]. Although limited efficacy has been observed, attempts have been made to develop recombinant protein-based vaccines

* Corresponding author. Tel.: +81 11 706 6905; fax: +81 11 706 7879.
E-mail address: j_arika@med.hokudai.ac.jp (J. Arikawa).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.12.040

that reduce or eliminate biohazard risks during preparation as well as achieve higher efficacy. Immunizations with recombinant G1 and G2 proteins expressed by baculovirus and vaccinea virus induce very low or negligible amounts of neutralizing antibody [12–14]. Although the antibody titers are high, this low efficacy for inducing neutralizing antibody may be attributable to the incorrect conformation of the recombinant G1 and G2 compared to the native proteins.

We recently expressed the G1 and G2 of HTNV using a mammalian cell expression vector. In addition, by supplying the G1 and G2 proteins *in trans* to the infected cells with recombinant vesicular stomatitis virus (VSV), whose envelope G protein was altered to enhanced green fluorescent protein (eGFP), we generated the pseudotype VSV (pVSV), which possesses the HTNV envelope proteins G1 and G2 (VSVΔG*HTN) [15]. Infection by VSVΔG*HTN was inhibited by neutralizing monoclonal antibodies (MAbs) and patient sera. The neutralizing antibody titers were virtually identical to those measured with native virus. These results indicated that the structure and function of the expressed G1 and G2 effectively mimics the native viral proteins. Based on these results, we examined the applicability of pVSV as a possible tool for producing inactivated vaccine that may induce neutralizing antibody more efficiently than the previously examined recombinant proteins.

2. Materials and methods

2.1. Viruses and cells

HTNV strain 76–118 was propagated in the Vero cell E6 clone (ATCC C1008). The 293T cell is derived from the human embryonic kidney cell line 293 and contains the simian virus 40 large T antigen. The 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 0.45% glucose and 10% heat-inactivated fetal calf serum (FCS). The recombinant baculovirus for expressing HTNV viruses G1 and G2 was kindly supplied by Dr. Connie S. Schmaljohn [12] and was propagated in high five™ cells.

2.2. Mice

Specific-pathogen-free, 6-week-old female BALB/c/slc mice were obtained from SLC (Hamamatsu, Japan). All mice were treated in accordance with the laboratory animal control guidelines of our institute, which conform to those of the US. National Institutes of Health. All experiments were conducted in a BSL class P3 facility.

2.3. Production of pseudotype VSV

Pseudotype VSV containing HTNV G1 and G2 (VSVΔG*HTN) or the G protein of VSV (VSVΔG*G) was prepared in 293T cells as previously described [15]. Briefly, 36 h after the transfection of 293T cells with expression vectors based on pCAGGS/MCS containing the coding information of the glycoproteins of HTNV, SEOV, or G protein of VSV, the cells were infected with VSVΔG*G at a multiplicity of infection (MOI) of one for 1 h at 37 °C. VSVΔG*G, whose genome had the enhanced GFP gene instead of the G protein, was kindly provided by Dr. Michael A. Whitt. The 293T cell monolayer was then washed with 1% heat-inactivated FCS–PBS three times, and culture medium was added. After a 24-h incubation at 37 °C in a CO_2 incubator, the culture supernatant was clarified by low-speed centrifugation and stored at −80 °C.

2.4. Purification of pseudotype VSV

The culture supernatant containing pseudotype VSV was purified by ultracentrifugation at 100,000 rpm for 1 h through a 20–60% sucrose cushion in TNE buffer (10 mM Tris, 135 mM NaCl, 2 mM EDTA) with a type-50 titanium rotor in a Beckman L-80 ultracentrifuge (Beckman Instruments, Palo Alto, CA). Virions were recovered from the sucrose cushion and measured as the hemagglutination (HA) activity of goose erythrocytes [16,17].

2.5. Titration of pseudotype VSV

Vero E6 cells grown on eight-well slides were infected with 50 μl of serially diluted virus stock. After a 1-h adsorption period, the inoculum was removed; fresh culture medium was added, and the cells were incubated at 37 °C in a CO_2 incubator. At 16 h postinfection, the cells were fixed with 10% formalin in PBS for 10 min at room temperature, washed with distilled water, and air-dried. Fluorescent GFP-expressing cells were counted under a fluorescence microscope. Because pseudotype VSV is unable to produce infectious progeny, the numbers of GFP-positive cells were regarded as infectious units (IU).

2.6. Immunization and sample collection

Eight mice were immunized subcutaneously three times at intervals of 3 and 2 weeks after the first immunization (Fig. 1) with purified particles of VSVΔG*HTN

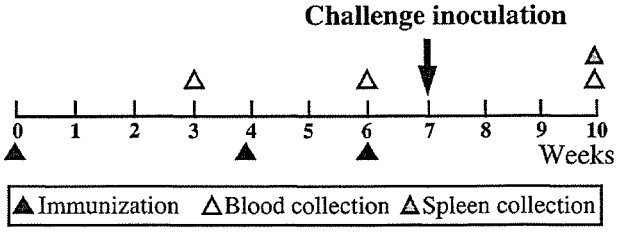


Fig. 1. Schedule of experiment. Immunizations with VSVΔG*HTN, VSVΔG*G, or PBS are shown as closed triangles. The collection of serum samples is shown as open triangles.

(1×10^7 IU/mouse/50 μl) mixed with 50 μl of CpG DNAadjuvant (ImmunEasy Mouse Adjuvant; Qiagen GmbH, Hilden, Germany). The adjuvant contained immunostimulatory CpG™ DNA short oligonucleotides that had unmethylated cytosine–guanine dinucleotides within a certain base context. Four mice were immunized with VSVΔG*G as controls. Fifty microliters of blood was collected from the mice under ether anesthesia, at the intervals described in Fig. 1 by retro-orbital plexus puncture. The blood was immediately mixed with 200 μl of PBS and centrifuged to remove blood cells. The supernatant represented an approximately 1:10 dilution of serum. The sera were inactivated by exposure at 56 °C for 30 min. All serum specimens were stored at −30 °C prior to antibody titration. After 3 weeks of HTNV inoculation, the spleen was collected under ether anesthesia. Spleen single-cell suspensions were obtained using previously published methods [18] and stored at −80 °C in freezing medium (Cell Banker; Jujin, Tokyo, Japan) prior to $CD8^+$ T cell analysis.

2.7. HTNV infection

Seven groups of six, 6-week-old female BALB/c mice were inoculated intraperitoneally with various doses (10^{-3} to 10^{-8} dilution) of HTNV diluted in PBS, and the antibody responses were examined 3 weeks after inoculation. Based on the ratio of seropositive mice, the ID_{50} of HTNV was determined as 0.127 focus-forming units (FFU). Mice were inoculated with 4.0 FFU of HTNV in the challenge experiment.

2.8. Purification and papain digestion of MAb clone 11E10-2-2

MAb clone 11E10-2-2, which recognizes G2 of HTNV [7], was purified by protein A column chromatography (Affi–Gel, MOPS II kit; Bio-Rad, Hercules, CA). To prepare the Fab fragment of the MAb, purified IgG was digested with papain (P-3125; Sigma–Aldrich, St. Louis, MO) as previously described [19]. Briefly, papain digestion was performed with an enzyme-to-antibody ratio of 1:400 in standard PBS buffer containing 3 mM EDTA and 10 mM-cysteine at 37 °C. The reaction was stopped with the addition of fresh iodoacetamide solution to a final concentration of 10 mM followed by incubation in the dark at 4 °C for 30 min. The digested MAb was then passed through protein A columns under the same experimental conditions used for the purification of IgG. The unbound fraction (Fab fragment) and bound fractions (Fc fragment and undigested IgG) were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

2.9. ELISA

To detect antibody specific to HTNV GP, ELISA was performed using the Fab fraction of MAb 11E10-2-2, which was directed to HTNV GP as the capture antibody. The 96-well microtiter plates (FALCON 3915; Falcon, Franklin Lakes, NJ) were coated with Fab of MAb 11E10-2-2 (50 μg/ml) in PBS and incubated at 4 °C overnight. After three washes with PBS containing 0.05% Tween-20 (PBS-T; Wako, Osaka, Japan), High Five™ cell lysate containing recombinant GP as previously described [20] was added to the plates for 1 h at 37 °C. After washing three times with PBS-T, the wells were filled with Block Ace (Yukijirushi, Tokyo, Japan), incubated at room temperature for 30 min, and washed again. The mouse sera were diluted at 1:200 with ELISA buffer containing PBS-T with 0.5% BSA (A-4503; Sigma–Aldrich) and were then added to the wells. The plates were incubated at 37 °C for 1 h and washed three times with PBS-T. Bound antibodies were detected with 0.5 μg/ml protein A–horseradish peroxidase conjugate (Biogenesis, San Leandro, CA) in ELISA buffer. Wells were developed with 100 μl of substrate solution, which contained 10 mg *o*-phenylenediamine (Sigma–Aldrich) plus 6 ml of 0.02% H_2O_2 (Wako). After a 15-min incubation in the dark at room temperature, the absorbance at 450 nm was read using a microplate spectrophotometer (Spectra Max 340; Molecular Devices, Sunnyvale, CA). For ELISA to detect antibody specific to NP, recombinant HTNV NP was used as the antigen. The ELISA procedure was as previously described [21].

2.10. Focus reduction neutralization test

The focus reduction neutralization test (FRNT) using HTNV and Vero E6 cells was performed as previously described [22]. The FRNT titer was expressed as the reciprocal of the highest dilution that gave a reduction of greater than 80% in the number of infected cell foci.

2.11. Detection of HTNV-specific $CD8^+$ T cells

To detect HTNV-specific $CD8^+$ T cells, we used flow cytometry to assay for intracellular gamma interferon (IFN-γ) of $CD8^+$ T cells stimulated by incubation with HTNV-infected antigen-presenting cells, as previously described [18].

3. Results

3.1. ELISA and FRNT antibody responses in mice immunized with pseudotype VSV

The antibody responses of mice immunized with pseudotype VSVs were measured by ELISAs for specific antibodies to HTNV GP (Fig. 2) or to HTNV NP (Fig. 3). Although FRNT antibody was not detected in any mice except one at 3 weeks after the first immunization with VSVΔG*HTN, a low level of anti-GP antibody was detected in a few mice compared to control mice immunized with VSVΔG*G or PBS (Table 1, Fig. 2). After the second immunization, all mice

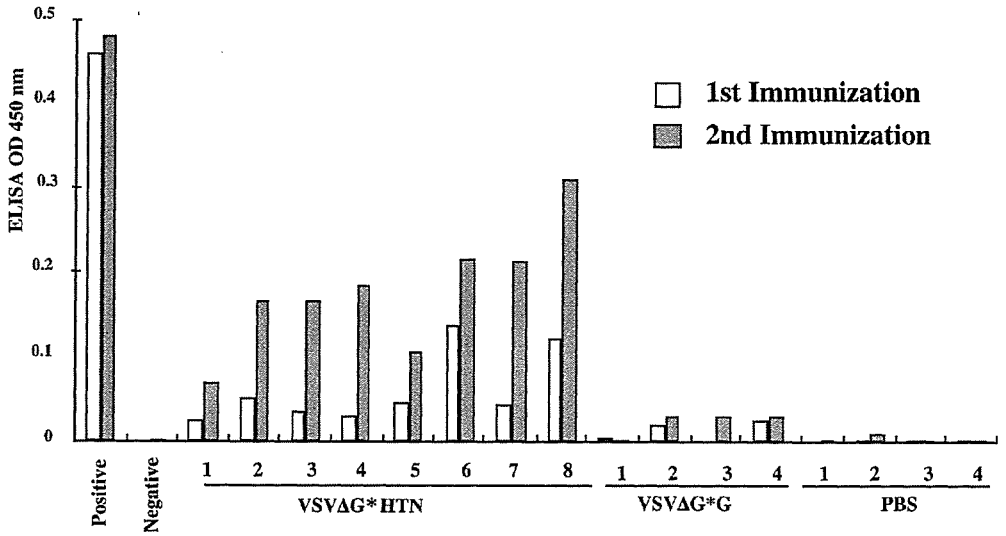


Fig. 2. Antibody responses against hantavirus GP before challenge inoculation. Anti-GP antibodies were measured using ELISA; the recombinant GP was expressed in insect cells by the baculovirus vector system as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally inoculated with HTNV. The negative control was serum from an uninfected mouse.

immunized with VSVΔG*HTN possessed both FRNT antibody and anti-GP antibody as detected by ELISA (Table 1, Fig. 2). However, NP-specific antibody induction was not detected in any of the mice inoculated with VSVΔG*HTN, VSVΔG*G, or PBS alone (Fig. 3). These results indicated that VSVΔG*HTN was able to induce GP-specific antibody in mice.

The neutralizing activity of the GP-specific antibody was measured using the 80% FRNT. As summarized in Table 1, all eight mice immunized with VSVΔG*HTN developed FRNT antibody at titers ranging from 1:40 to 1:160 within 2 weeks after the second immunization. All control mice inoculated with either VSVΔG*G or PBS alone were negative for FRNT antibody.

3.2. Evaluation of protective immunity induced by pseudotype VSV for preventing HTNV challenge in mice

To assess the protective immunity, mice were challenged with HTNV at 1 week after the third immunization (Fig. 1). Although exceptional fatal infection models in adult mice have been reported [23,24], hantavirses are generally non-pathogenic to mature rodents. In this study, we used a general HTNV strain. Therefore, it is difficult to evaluate protective immunity. To differentiate the antibody response induced by immunization from that induced by HTNV infection, seroconversion against NP accompanied with the establishment of infection was used as an index for protection (Fig. 2). In addition, the HTNV-specific $CD8^+$ T cell response was also

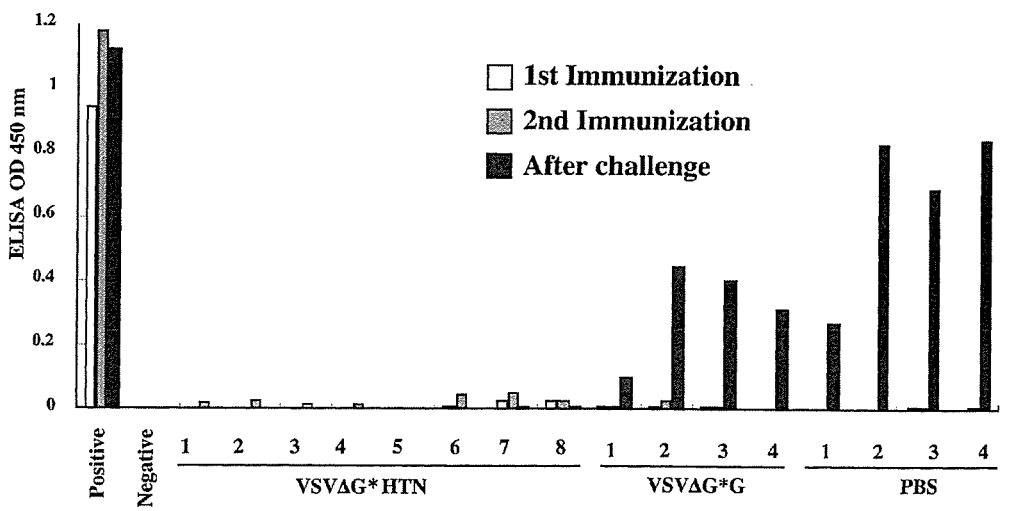


Fig. 3. Antibody responses against HTNV N protein after challenge inoculation. Anti-N protein antibodies were measured using ELISA; recombinant NP was expressed in *Escherichia coli* as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally inoculated with HTNV. The negative control was serum from an uninfected mouse.

Table 1
Neutralizing antibody responses of mice immunized with VSVΔG*HTN

Antigen	Mouse ID	Titer for 80% FRNT			
		1st imm.	2nd imm.	Post-challenge	Protection
VSVΔG*HTN	1	<40	40	40	Yes
	2	<40	40	80	Yes
	3	<40	40	80	Yes
	4	<40	40	80	Yes
	5	40	80	80	Yes
	6	<40	160	160	Yes
	7	<40	160	80	Yes
	8	<40	80	40	Yes
VSVΔG*G	1	<40	<40	40	No
	2	<40	<40	160	No
	3	<40	<40	160	No
	4	<40	<40	80	No
PBS	1	<40	<40	80	No
	2	<40	<40	80	No
	3	<40	<40	80	No
	4	<40	<40	80	No

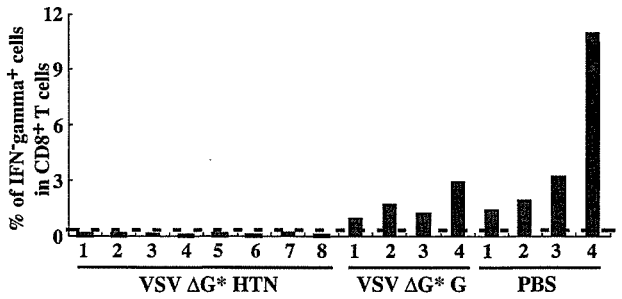


Fig. 4. HTNV-specific $CD8^+$ T cell responses after challenge inoculation. HTNV-specific $CD8^+$ T cells were counted as IFN-γ-producing cells; values are expressed as percent of total splenic $CD8^+$ T cells. A horizontal broken line indicates the limitation of the background level in this assay.

measured as an index of protection. All mice immunized with VSVΔG*HTN remained negative for anti-NP antibody in ELISA, and no detectable level of HTNV-specific $CD8^+$ T cells was observed in spleen cells at 3 weeks after the challenge (Fig. 4), indicating that the mice were protected from HTNV infection. In contrast, seroconversion was detected in VSVΔG*G-immunized mice and in PBS control mice. Furthermore, a significant number of HTNV-specific $CD8^+$ T cells was observed only in the control mice. These results indicate that protective immunity was not induced in these mice, confirming that immunization with VSVΔG*HTN conferred protective immunity.

4. Discussion

In general, virus proteins that are targeted by vaccines are observed on the outer surfaces of the virions or the envelope proteins. In addition, it is well-known that the structure of virus like particles (VLPs) in combination with envelope proteins strengthens their immunogenicity. Therefore, the combination of VLPs and envelope proteins was expected to be an effective material for vaccine development. VLPs with recombinant hepatitis B surface proteins expressed in yeast provided a remarkably successful application for vaccine development [25]. Chimeric HBc particles with the hantavirus NP sequence have been reported to induce high antibody titer to NP, as well as to confer protective immunity in mice models [26]. However, similar applications have not been reported with hantavirus GPs.

In our previous study, we succeeded in producing VSV pseudotypes bearing hantavirus GPs, and these GPs were considered to have structures similar to those of native hantavirus GPs based on neutralization pattern profiling of Mabs and polyclonal antibodies [15]. Furthermore, because the pseudotype VSV lacks a gene for GP, it also lacks replication activity. Therefore, we anticipated that the pseudotype virion could be applicable as a safe alternative to authentic virion for use in vaccines. In this study, we used the particle of pseudotype VSVΔG*HTN as a type of VLP and examined its antigenicity in a mouse model.

Epitope analyses of GPs using MAbs against G1 and G2 showed that both G1 and G2 were associated with the FRNT-related epitope. The FRNT-related epitope was composed of partially overlapping epitopes on G1 and G2 [7,27,28] and might be related to the inhibition of the membrane fusion step [6]. In this study, we measured only anti-GP2 antibody in the ELISA because of the difficulty with the methodology. Because the FRNT epitopes on G1 and G2 overlap each other, it was expected that anti-G2 antibody was related to the rise in anti-G1 antibody. Actually, anti-G2 antibodies were correlated to FRNT titers as shown in Table 1.

As observed in this study, the pseudotype VSVΔG*HTN was able to induce neutralizing antibody with titers comparable to those reported for inactivated vaccines in humans as well as for recombinant proteins in the mouse model [10,11,29]. Furthermore, the pseudotype VSVΔG*HTN conferred protective immunity for hantavirus challenge in the mouse model. The fact that an HTNV-specific $CD8^+$ T-cell response was not observed in immunized mice after challenge also confirmed that the induced neutralizing antibody alone was effective for protection from the challenge inoculation. This is the first successful application of pseudotype VSV as a type of VLP for the induction of protective immunity. Few studies on the application of pseudotype virus as a vaccination antigen have been reported. In hepatitis type C virus (HCV), pseudotype virus was developed and applied to analyze virus–cell interactions and the assembly of virus particles [30,31]. Beyene et al. [32] tried to apply pseudotype VSV incorporating the envelope glycoprotein of HCV to vaccine development. In their experiment, neutralization antibody against pseudotype virus was successfully induced; however, there is no system for detecting neutralizing and protective activity against authentic HCV in vitro or in vivo [32].

Although an inactivated hantavirus vaccine has been developed and a protective efficacy comparable to those

of other virus vaccines has been reported, low efficacy for the induction of neutralizing antibody remains an important aspect to be overcome [9–11,29]. Further studies are needed to clarify the potential of the pseudotype VSV with HTNV GPs as a useful tool for the development of hantavirus vaccine. It has been reported that the M protein of VSV strongly induces budding. The characteristics of VSV M protein suggest that the pseudotype VSV system could be applied to other viruses for which VLP development has been unsuccessful.

Owing to the structural complexity of HTNV GP, a component vaccine made from recombinant GP proteins has not been developed. However, DNA vaccine trials have been reported [8] in which the DNA vaccine was able to induce a significantly high level of FRNT antibody in animals; this indicates that the recombinant GP expressed in mammalian cells possesses sufficient antigenicity to induce FRNT antibody. These results also imply that the fixation step during the preparation of inactivated hantavirus vaccine may be responsible for the preservation of antigenicity of the inactivated vaccine. The pseudotype virion used here could be developed as a useful tool with which to evaluate fixation methods for virus particles. By comparing the immunogenicity of unfixed and fixed pseudotype virions, we were able to estimate the damage done to GP molecules by fixation during the preparation of the hantavirus vaccine. Careful examination of the appropriate fixation conditions for inactivated vaccines may lead to higher efficacy.

The pseudotype VSV used in this study can replicate to express reporter genes, such as GFP, and exhibit a cytopathic effect. Therefore, for the practical application of pseudotype virion to vaccine, the development of VLPs lacking a replication system and containing HTNV GPs and VSV M protein should be pursued.

Acknowledgments

We thank Dr. C.S. Schmaljohn for providing the recombinant baculovirus for expressing the HTNV GPs. This study was partly supported by a grant from the 21st Century COE Program of Excellence for Zoonosis Control from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Schmaljohn C, Hjelle B. Hantaviruses—a global disease problem. Emerg Infect Dis 1997;3(2):95–104.
[2] Plyusnin A, Vapalahti O, Lundkvist A. Hantaviruses: genome structure, expression and evolution. J Gen Virol 1996;77(11):2677–87.
[3] Song G. Epidemiological progresses of hemorrhagic fever with renal syndrome in China. Chin Med J (Engl) 1999;112(5):472–7.
[4] Lee HW. Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome. In: Elliott RM, editor. The bunyaviridae. New York: Plenum Press; 1996. p. 253–67.
[5] Schmaljohn CS, Hooper JW. Bunyaviridae: the viruses and their replication. In: Knipe DM, editor. Fields virology. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 1581–602.
[6] Ogino M, Yoshimatsu K, Ebihara H, Araki K, Lee BH, Okumura M, et al. Cell fusion activities of Hantaan virus envelope glycoproteins. J Virol 2004;78(19):10776–82.
[7] Arikawa J, Schmaljohn AL, Dalrymple JM, Schmaljohn CS. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J Gen Virol 1989;70(3):615–24.
[8] Hooper JW, Kamrud KI, Elgh F, Custer D, Schmaljohn CS. DNA vaccination with Hantavirus M segment elicits neutralizing antibodies and protects against Seoul virus infection. Virology 1999;255(2):269–78.
[9] Hantavirus Vaccine Development. Bull World Health Organ 1996;74(3):336–37.
[10] Lee HW, Ahn CN, Song JW, Baek LJ, Seo TJ, Park SC. Field trial of an inactivated vaccine against hemorrhagic fever with renal syndrome in humans. Arch Virol Suppl 1990;1:35–47.
[11] Song G, Huang YC, Hang CS, Hao FY, Li DX, Zheng XL, et al. Preliminary human trial of inactivated golden hamster kidney cell (GHKC) vaccine against haemorrhagic fever with renal syndrome (HFRS). Vaccine 1992;10(4):214–6.
[12] Schmaljohn CS, Arikawa J, Dalrymple JM, Schmaljohn AL. Expression of the envelope glycoprotein of Hantaan virus with vaccinia and baculovirus recombinants. In: Kolakofsky D, Mahy BWJ, editors. Genetics and pathgenicity of negative strand viruses. Amsterdam, NewYork: Elsevier; 1989. p. 58–66.
[13] Schmaljohn CS, Chu YK, Schmaljohn AL, Dalrymple JM. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. J Virol 1990;64(7):3162–70.
[14] Yoshimatsu K, Yoo YC, Yoshida R, Ishihara C, Azuma I, Arikawa J. Protective immunity of Hantaan virus nucleocapsid and envelope protein studied using baculovirus-expressed proteins. Arch Virol 1993;130(3–4):365–76.
[15] Ogino M, Ebihara H, Lee BH, Araki K, Lundkvist A, Kawaoka Y, et al. Use of vesicular stomatitis virus pseudotypes bearing hantaan or seoul virus envelope proteins in a rapid and safe neutralization test. Clin Diagn Lab Immunol 2003;10(1):154–60.
[16] Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 1958;7(5):561–73.
[17] Okuno Y, Yamanishi K, Takahashi Y, Tanishita O, Nagai T, Dantas Jr JR, et al. Haemagglutination-inhibition test for haemorrhagic fever with renal syndrome using virus antigen prepared from infected tissue culture fluid. J Gen Virol 1986;67(Part 1):149–56.
[18] Araki K, Yoshimatsu K, Lee B-H, Kariwa H, Takashima I, Arikawa J. Hantavirus-specific $CD8^+$ T cell responses in newborn mice persistently infected with Hantaan virus. J Virol 2003;77(15):8408–17.
[19] Casale E, Wenisch E, He XM, Righetti PG, Snyder RS, Jungbauer A, et al. Crystallization of the Fab from a human monoclonal antibody against gp 41 of human immunodeficiency virus type I. J Mol Biol 1990;216(3):511–2.
[20] Yoshimatsu K, Lee BH, Araki K, Morimatsu M, Ogino M, Ebihara H, et al. The multimerization of hantavirus nucleocapsid protein depends on type-specific epitopes. J Virol 2003;77(2):943–52.
[21] Lee BH, Yoshimatsu K, Araki K, Ogino M, Okumura M, Tsuchiya K, et al. Detection of antibody for the serodiagnosis of hantavirus infection in different rodent species. Arch Virol 2003;148(10):1885–97.
[22] Araki K, Yoshimatsu K, Ogino M, Ebihara H, Lundkvist A, Kariwa H, et al. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. J Clin Microbiol 2001;39(7):2397–404.
[23] Asada H, Balachandra K, Tamura M, Kondo K, Yamanishi K. Cross-reactive immunity among different serotypes of virus causing

haemorrhagic fever with renal syndrome. J Gen Virol 1989;70(Pt 4):819–25.
[24] Wichmann D, Grone HJ, Frese M, Pavlovic J, Anheier B, Haller O, et al. Hantaan virus infection causes an acute neurological disease that is fatal in adult laboratory mice. J Virol 2002;76(17):8890–9.
[25] Hollinger FB, Troisi CL, Pepe PE. Anti-HBs responses to vaccination with a human hepatitis B vaccine made by recombinant DNA technology in yeast. J Infect Dis 1986;153(1):156–9.
[26] Ulrich R, Lundkvist A, Meisel H, Koletzki D, Sjolander KB, Gelderblom HR, et al. Chimaeric HBV core particles carrying a defined segment of Puumala hantavirus nucleocapsid protein evoke protective immunity in an animal model. Vaccine 1998;16(2–3):272–80.
[27] Wang M, Pennock DG, Spik KW, Schmaljohn CS. Epitope mapping studies with neutralizing and non-neutralizing monoclonal antibodies to the G1 and G2 envelope glycoproteins of Hantaan virus. Virology 1993;197(2):757–66.
[28] Kikuchi M, Yoshimatsu K, Arikawa J, Yoshida R, Yoo YC, Isegawa Y, et al. Characterization of neutralizing monoclonal antibody escape mutants of Hantaan virus 76118. Arch Virol 1998;143(1):73–83.
[29] Yamanishi K, Tanishita O, Tamura M, Asada H, Kondo K, Takagi M, et al. Development of inactivated vaccine against virus causing haemorrhagic fever with renal syndrome. Vaccine 1988;6(3):278–82.
[30] Matsuura Y, Tani H, Suzuki K, Kimura-Someya T, Suzuki R, Aizaki H, et al. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology 2001;286(2):263–75.
[31] Meyer K, Basu A, Ray R. Functional features of hepatitis C virus glycoproteins for pseudotype virus entry into mammalian cells. Virology 2000;276(1):214–26.
[32] Beyene A, Basu A, Meyer K, Ray R. Hepatitis C virus envelope glycoproteins and potential for vaccine development. Vox Sang 2002;83(Suppl. 1):27–32.

Microbiol. Immunol., 48(2), 103–109, 2004

First Isolation of *Bartonella henselae* Type I from a Cat-Scratch Disease Patient in Japan and Its Molecular Analysis

Soichi Maruyama*[,1], Koichi Izumikawa[2], Mayumi Miyashita[1], Hidenori Kabeya[1], Takeshi Mikami[1], Hirotsugu Yamanouchi[2], Eisuke Sasaki[2], Hiroshi Yoshida[3], and Kinichi Izumikawa[2]

[1]*Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252–8510, Japan,* [2]*Izumikawa Hospital, Nagasaki, Nagasaki 859–1504, Japan, and* [3]*Department of Medicine, Yame General Hospital, Fukuoka, Fukuoka 834–0034, Japan*

Received September 12, 2003; in revised form, October 27, 2003. Accepted November 20, 2003

***Abstract*: We isolated *Bartonella henselae* from an inguinal lymph node of a 36-year-old male patient with cat-scratch disease. The patient had many areas of erythema on his body, swelling of the left inguinal lymph nodes with pain and slight fever. The diagnosis was made on the basis of polymerase chain reaction for *B. henselae* DNA from the lymph node biopsies and blood sample, and isolation of the organism, histology of the lymph node and serology with an indirect immunofluorescent antibody test. We also analyzed the genome profiles for five strains of 90 isolates from the lymph node by pulsed-field gel electrophoresis after *Not*I endonuclease digestion. We found two different genomic profiles. These results suggest that the patient had been either co-infected or re-infected with two genetically different strains of *B. henselae*.**

***Key words*: *Bartonella henselae*, Cat-scratch disease, Isolation, Pulsed-field gel electrophoresis**

Cat-scratch disease (CSD) is a worldwide zoonosis primarily caused by *Bartonella henselae* (5, 11, 20, 24, 33, 34), but *Bartonella clarridgeiae* has also been linked to a few human cases of CSD (17, 19). The organism also causes bacillary angiomatosis (15, 31) and peliosis (32) in immunocompromised individuals and bacteremia in immunocompetent individuals. The clinical manifestation of CSD is mainly characterized by pyrexia, papules on the site of the cat scratch or bite and unilateral lymphadenopathy, while cats themselves, the major reservoir of CSD, are asymptotic carriers, showing long-term bacteremia with antibody formation (1, 16).

The number of human clinical cases of CSD has been increasing worldwide. Most cases are diagnosed based on clinical manifestations, the history of contacts with cats, serology (14, 20, 24, 26, 28, 33) or PCR-based methods for examining the biopsy specimens (2, 10, 28). However, the isolation of the organism from patients is relatively difficult and only a few cases of the organism isolation have been reported worldwide (7, 9, 18, 25), because suitable material is not available, the culture conditions are not appropriate or the organism shows fastidious, cytozoic and slow-growth properties. The isolation and establishment of *Bartonella* strains are extremely important for both etiological diagnosis and epidemiological and genetical analysis of the isolates. Though many clinical cases of CSD have been reported in Japan (20, 24, 33), there are to date no available reports of the isolation of *B. henselae* from human cases in Japan.

B. henselae type II was detected in 18% of the isolates from CSD patients in the Netherlands (3), and 94% of cat isolates in Germany (27). In contrast, Maruyama et al. showed that type I is the predominant gene type in domestic cats in Japan (21). However, no data was available for the prevalence of gene type on the isolates from CSD patients in Japan.

In this study, the authors describe a typical CSD case and the isolation of *B. henselae* from the patient in

**Address correspondence to* Dr. Soichi Maruyama, Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan. Fax: 0466–84–3636. E-mail: maruyama@brs.nihon-u.ac.jp

Abbreviations: bp, base pair; CSD, cat-scratch disease; CFU, colony forming unit; CRP, C-reactive protein; CT, computed-tomography; γ-GTP, gamma glutamyl transpeptidase; IFA, indirect immunofluorescence antibody; LAP, leucine amino peptidase; Mbp, mega base pair; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis.

Japan for the first time. Furthermore, the authors investigate the genomic properties of the isolates by pulsed-field gel electrophoresis (PFGE) and suggest that the patient has been either co-infected or possibly re-infected with two genetically different strains of *B. henselae*.

Case Report

A 36-year-old previously healthy male had persistent multiple areas of erosive erythema with severe itching on his legs and body for about 30 days and swelling of the left inguinal lymph node with pain. He was admitted to Izumikawa Hospital on September 15, 2001 for further examination and treatment. At the time of admission, his body temperature was 37.1 C and medical examination was negative except for multiple systemic erosive eruptions, cat scratches on his legs (Fig. 1) and the swollen appearance and tenderness of the left inguinal lymph node. He had been keeping a cat infested with fleas for 3 months before admission. Laboratory findings showed an elevated gamma-GTP (154 IU) and LAP (589 IU), and he was positive for CRP. The computed-tomography X-ray films of the patient's left inguinal region showed a 30×40 mm area of lymphadenopathy containing a 10×10 mm low-density area (Fig. 2). A left inguinal lymphoidectomy was performed on the second day of admission, and a 50 mm×50 mm lymph node and a 12 mm×12 mm lymph node were removed (Fig. 3). The patient started receiving 100 mg/day of minomycin intravenously for 5 days after the lymphoidectomy, followed by 50 mg/day by oral administration for 4 days. The low-grade fever and pain in the inguinal regions were diminished during the treatment, and the CRP value turned out to be negative. The patient was discharged on September 24, 2001 without no complications.

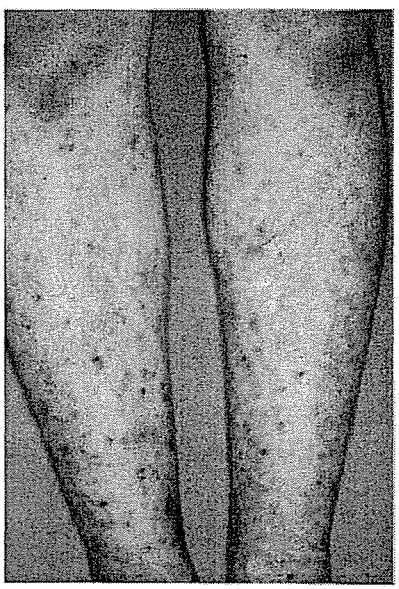


Fig. 1. Many cat scratches, fleabites and areas of erosive erythema are observed on both legs of the patient.

Materials and Methods

Clinical samples. The lymph node specimen was submitted to the culture of *Bartonella* species and to histopathological examinations by using the Warthin-Starry stain. The blood and serum samples collected just before the surgical operation were also sent for the laboratory diagnosis of CSD. The blood samples were collected in EDTA tubes and submitted to the culture and genetical examination of *Bartonella* species. Flea samples were collected from the cat and submitted for genetical and bacteriological examination of *Bartonella* species. The blood, serum and lymph node from the patient and the flea samples were sent to the Laboratory of Veterinary Public Health, Department of Veterinary

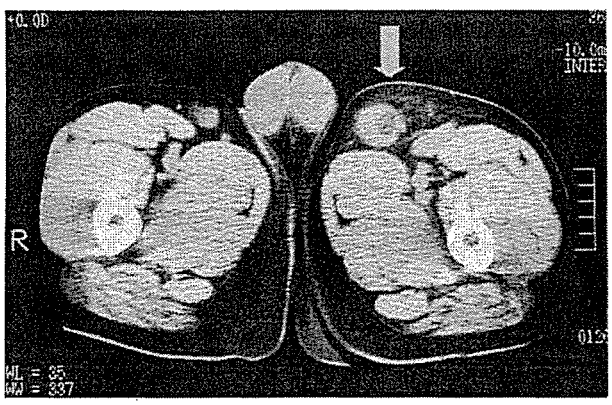


Fig. 2. The computed-tomography X-ray film shows 30×40 mm of lymphadenopathy containing a 10×10 mm low-density area in the left inguinal region (arrow).

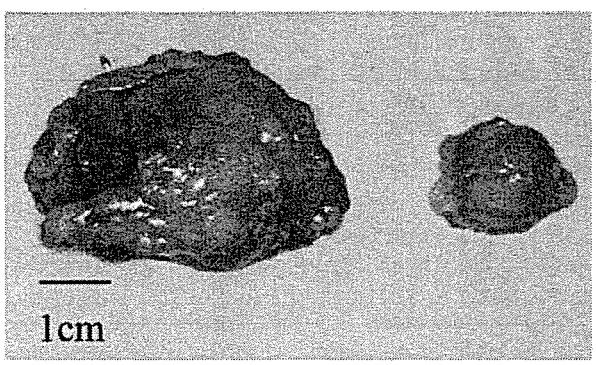


Fig. 3. Two inguinal lymph nodes, one 50 mm×30 mm and one 12 mm×12 mm, were removed from the patient on the second day of hospitalization by surgical operation.

Medicine, College of Bioresource Sciences, Nihon University. All the samples were stored at −70 C until they were examined.

Indirect immunofluorescence antibody (IFA) test. The serum was inactivated at 56 C for 30 min. The antibody titer to *B. henselae* was examined by IFA test with strain *B. henselae* Houston-1. The IFA test procedure was as reported previously (20).

Isolation of Bartonella *species.* The frozen blood sample in an EDTA tube was thawed at room temperature and centrifuged at 3,800 rpm for 70 min. After centrifugation, the supernatant was removed and 120 µl of supplemented Medium 199 (Gibco, U.S.A.) was added to the sediment, which was mixed well. The mixture was inoculated to two 7% rabbit blood-agar plates, which were incubated at 35 C in a 5% CO_2 atmosphere for 4 weeks.

One gram of the lymph node specimen was aseptically minced, and 1 ml of sterile phosphate-buffered saline (PBS) was added to the specimen. The mixture was then homogenized well with a sterile glass homogenizer. A volume of 100 µl of homogenate was inoculated to two 7% rabbit blood-agar plates and incubated at 35 C in a 5% CO_2 atmosphere for 4 weeks.

For flea samples, 5 fleas collected from the cat were dipped in 70% (vol/vol) ethanol in a 1.5-ml sterile microcentrifuge tube and left for 15 min at room temperature. The tube was centrifuged at 12,000 rpm for 3 min and the ethanol was removed. A volume of 1 ml of PBS was added to the tube and washed twice as same procedure stated above. A volume of 200 µl of supplemented Medium 199 was added and homogenized with a small sterile plastic homogenizer for a conical 1.5-ml tube. A 100-µl volume of the homogenate was used for the isolation of *B. henselae* and the rest for the DNA extraction.

Extraction of DNA from the homogenate of lymph node, blood and fleas was performed with a QIAamp Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. For *Bartonella* suspected isolates, the DNA was extracted by InstaGene DNA purification matrix (Bio-Rad, U.S.A.).

Identification of Bartonella *species.* Five colonies suspected to be *Bartonella* on the agar plates were subcultured and subjected to identification of *Bartonella* species by species-specific PCR of 16S–23S rRNA intergenic region sequences (13). In the PCR, amplification of template DNA with 5'- CCTTCGTTTCTCTTTCTTCA-3' and 5'-AACCAACTGAGCTACAAGCC-3' as forward and reverse primers, respectively, resulted in amplified products corresponding to those of the predicted size, namely, 154 bp (*B. clarridgeiae*), 260 bp (*B. vinsonii* subsp. *berkhoffii*) and 172 bp (*B. henselae*). Amplifications were performed in an iCycler (Bio-Rad) by a timed-release PCR protocol, as follows: 10 min of incubation at 20 C, followed by 2 min denaturation at 95 C and then 45 cycles of 1 min of denaturation at 95 C, 1 min of annealing at 60 C and 30 sec of extension at 72 C. PCR products were differentiated by gel electrophoresis.

The 16S rRNA gene typing of *B. henselae*, type I or type II, was performed by PCR as previously reported (21). Briefly, 1 µl of the extracted DNA sample was added to 17 µl of reaction mixture (10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$) containing 0.5 µM of each set of 16SF and BH1 or 16SF and BH2 primers, 0.8 mM dNTP and 2.5 U of *Taq* polymerase. The DNA amplification was performed with initial denaturation (95 C, 3 min), followed by 30 cycles of denaturation (95 C, 20 sec), annealing (56 C, 30 sec) and extension (73 C, 1 min), with a single final extension step (73 C, 5 min). The amplified PCR product was subjected to electrophoresis in a 3% agarose (Agarose 21, Nippon Gene, Japan). When a specific band of 185 bp was detected with each specific primer set, the strain was identified as type I or type II.

Pulsed-field gel electrophoresis. Five *Bartonella* strains isolated from the patient were cultivated on 5% rabbit blood agar plates at 35 C in 5% CO_2 for 4–7 days. The bacteria grown on the agar plates were harvested and suspended in sterile distilled water and washed twice by centrifugation at 12,000 rpm for 5 min. The turbidity of the suspension was adjusted to McFarland # 6. Agarose plugs were prepared by adding 1 ml of cell suspension to the same amount of 2% low-melting-point agarose (Bio-Rad). The solidified agarose plugs were treated using procedures reported previously (22). The restriction enzyme *Not*I (Roche, Germany) was used for the analysis of genomic DNA patterns of the isolates. The molecular size of each isolate was calculated with the Windows version of Lane Multi Screener, version 2.0 (Atto Co., Ltd., Japan).

Results

The *B. henselae*-specific IgG antibody was detected in the patient serum samples at titers of 1:64 to strain Houston-1.

Histopathological examination showed severe granulomatous lymphadenitis. The lymph node was infiltrated with neutrophils, and abundant necrotic lesions and small bacilli suspected to be *Bartonella* species were observed in the tissue (Fig. 4).

B. henselae DNA was found in the blood and lymph node specimens from the patient and in fleas from the cat by species-specific PCR (Fig. 5). Colonies suspected of being *Bartonella* species were isolated from the homogenate of the lymph node specimen at the number

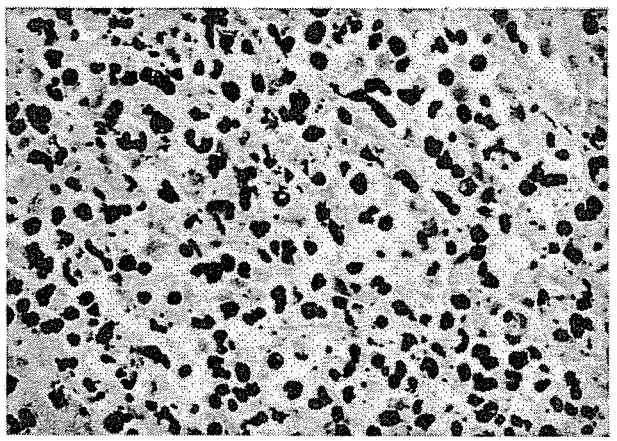


Fig. 4. Warthin-Starry stain of a lymph node from the patient showing many necrotic lesions with neutrophil infiltrations and small bacilli suspected of being *Bartonella* (×60).

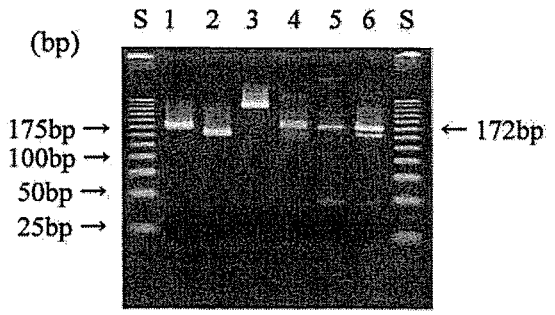


Fig. 5. Detection of *Bartonella* DNA by species-specific PCR. S, DNA size standard; lane 1, *B. henselae* ATCC49882; lane 2, *B. clarridgeiae* ATCC51734; lane 3, *B. vinsonii* subsp. *berkhoffii* ATCC51672; lane 4, blood of the patient; lane 5, inguinal lymph node of the patient; lane 6, fleas collected from the patient's cat.

of 90 colony forming units (CFU)/g. Five colonies (IZM1–IZM5) were identified as *B. henselae* by species-specific PCR (Fig. 6). No bacteria were isolated from the blood of the patient or the flea samples.

The extracted DNA from the isolates and flea samples yielded a 16S rRNA gene type I fragment by PCR with primers 16SF and BH1, while no specific band of 185 bp was observed with primers 16SF and BH2.

Five *B. henselae* isolates from the lymph node were submitted to PFGE analysis and digested by the restriction enzyme *Not*I. Isolates IZM1 (lane 1), IZM4 (lane 4) and IZM5 (lane 5) showed a similar genomic DNA pattern, while the patterns of IZM2 (lane 2) and IZM3 (lane 3) were different from those of the other three isolates (Fig. 7). The genome size of *B. henselae* isolates was calculated to be 1.7 Mbp (strains IZM1, IZM4 and IZM5) and 1.9 Mbp (strains IZM2 and IZM3) by the software used.

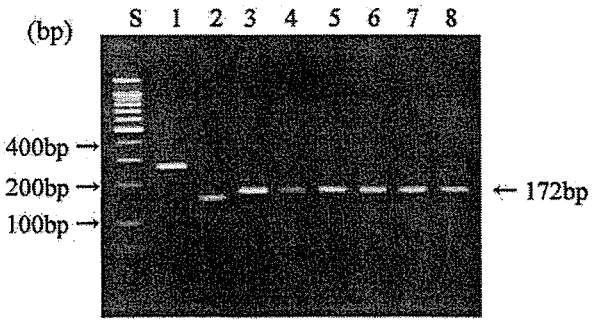


Fig. 6. Identification of *Bartonella* isolates from the inguinal lymph node of the patient by species-specific PCR. S, DNA size standard; lane 1, *B. vinsonii* subsp. *berkhoffii* ATCC51672; lane 2, *B. clarridgeiae* ATCC51734; lane 3, *B. henselae* ATCC49882; lane 4, IZM1; lane 5, IZM2; lane 6, IZM3; lane 7, IZM4; lane 8, IZM5.

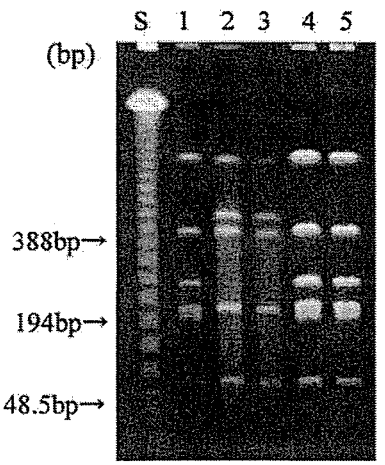


Fig. 7. DNA analysis of *Bartonella* isolates from the patients after *Not*I digestion by pulsed-field gel electrophoresis. S, DNA size standard (Lambda ladder); lane 1, IZM1; lane 2, IZM2; lane 3, IZM3; lane 4, IZM4; lane 5, IZM5.

Discussion

This is the first report of the isolation of *Bartonella henselae* from a CSD patient in Japan. The patient showed a significant antibody elevation in reaction to strain Houston-1 after typical clinical manifestation of CSD, and the DNA of *B. henselae* was also detected from the blood and lymph node specimen. Thus, the patient was clinically, serologically, histopathologically, genetically and bacteriologically diagnosed as having CSD.

La Scola (18) reported that only 5 of 290 patients with suspected CSD were positive for isolation, while 73 patients were diagnosed by serology or genomic detection among definitively diagnosed CSD patients. In

Australia, seventeen *B. henselae* strains were isolated from the 83 PCR-positive human specimens (9). In our previous study, though genetical and/or serological diagnosis was successful in three suspected CSD patients, no *Bartonella* species was isolated from the patients (20). Thus, an isolation of *B. henselae* from CSD patients is seldom accomplished, because suitable materials are not available, the culture conditions are not appropriate or the organism shows fastidious, cytozoic and slow-growth characteristics. In the present case, the patient had received no treatment with any antibiotics before sample collection. Antibiotic treatment seems to make isolation and establishment of strains difficult in CSD patients.

As reported previously, CSD patients were more likely to own a kitten under 12 months old (5). Several investigations have also suggested that young cats are strongly associated with bacteremia and seropositivity to *B. henselae* (6, 11, 21, 23). In the present case, the patient had also kept a kitten 2 months before the onset of the disease and had many cat scratches. These data indicate that cats are the significant natural reservoir of the organism, and cat ownership and close contact are the most important epidemiological risk factors for CSD (5, 14, 20).

A number of CSD patients have owned kittens infested with cat fleas (*Ctenocephalides felis*), and *B. henselae* strains and the DNA from fleas have been found on carrier cats (4, 15). *B. henselae* was isolated from the blood and the fleas on a cat of a patient with CSD in Australia (8), although this patient had no history of a bite or scratch from the cat and no primary lesion on the skin. Higgins et al. (12) have shown that *B. henselae* can survive in the gut of a flea up to 9 days after infection. The present patient had many fleabites on his body, and *B. henselae* DNA was also detected in the flea samples obtained from the patient's cat. These data suggest that fleas may play some role in the transmission of *B. henselae* from infected cat to human.

The prevalence of the 16S rRNA gene type of *B. henselae* was different in patients depending upon the country where patients lived. In the Netherlands, *B. henselae* type I was more prevalent than type II in the isolates from CSD patients (3). Zeaiter et al. (35) reported that among the 107 patients of France, 64 (59.8%) were infected with the Marseille genogroup (type II) and 43 (48.2%) were infected with the Houston-1 genogroup (type I) when examined by PCR, while there were no significant differences between the genogroups epidemiologically or clinically. In Germany, *B. henselae* type II was isolated more frequently from cats than type I (27), but 23 of the 39 PCR-positive lymph nodes from the patients were found to belong to genotype I and 9 (23%) to genotype II by type-specific PCR (30). In Australia, out of 17 *B. henselae* strains isolated from CSD patients, 13 (76.5%) isolates belonged to the Houston-1 genotype and 4 (23.5%) belonged to the Marseille genotype (9). Although we had, unfortunately, no opportunity to isolate the organism from the cat implicated in our patient, *B. henselae* type I was isolated from the inguinal lymph node and identical DNA was detected from the blood of the patient and fleas from the cat. In our previous study, we found that most infected cats in Japan harbored *B. henselae* type I in their blood (21). These data suggest that our patient's cat harbored *B. henselae* type I, which may be the predominant genotype in CSD patients in Japan.

To date, there is no available data indicating the infective dose of *B. henselae* in CSD patients. In this study, 90 CFU/g of the organism was detected from the lymph node specimen. This suggests that the infective dose of *B. henselae* is relatively low, which may be why it is difficult to isolate the organism from clinical specimens of CDS patients.

In PFGE analysis, the genomic DNA size of *B. henselae* isolated from the patient was found to be 1.7 Mbp and 1.9 Mbp by digestion with *Not*I enzyme. Previous study also showed that the genome size of *B. henselae* isolates from cats in Germany, the United States, France and Japan varied from approximately 1.5 Mbp to 2.9 Mbp (22, 29). The genome size of the isolates obtained from our patient is in agreement with previous data of isolates from cats. Sander et al. (30) reported three *B. henselae* variants in human lymph node specimens by PCR and explained the wide variation of genomic size of the organism by the eventual presence of double bands by *Sma*I endonuclease digestion, especially in the high-molecular-weight range above 194 kbp. Zeaiter et al. (35) also identified two *B. henselae* genogroups using the *groE* sequences and four genotypes using *Pap31* sequences. Thus, several genetic variants were found in the strain of *B. henselae*. In our previous study with cat strains from France, the United States and Japan, some cats were co-infected with genetically different *B. henselae* strains (22). In the present PFGE analysis, two different genome patterns were obtained from five *B. henselae* isolates, which suggests that our patient has either been co-infected or possibly re-infected with two genetically different strains of *B. henselae* type I.

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 14360193) from the Japan Society for the Promotion of Science.

References

1) Abbott, R.C., Chomel, B.B., Kasten, R.W., Floyd-Hawkins, K.A., Kikuchi, Y., Koehler, J.E., and Pedersen, N.C. 1997. Experimental and natural infection with *Bartonella henselae* in domestic cats. Comp. Immunol. Microbiol. Infect. Dis. **20**: 41–51.
2) Anderson, B., Sims, K., Regnery, R., Robinson, L., Schmidt, M.J., Goral, S., Hager, C., and Edwards, K. 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J. Clin. Microbiol. **32**: 942–948.
3) Bergmans, A.M., Schellekens, J.F., van Embden, J.D., and Schouls, L.M. 1996. Predominance of two *Bartonella henselae* variants among cat-scratch disease patients in the Netherlands. J. Clin. Microbiol. **34**: 254–260.
4) Bergmans, A.M., de Jong, C.M., van Amerongen, G., Schot, C.S., and Schouls, L.M. 1997. Prevalence of *Bartonella* species in domestic cats in the Netherlands. J. Clin. Microbiol. **35**: 2256–2261.
5) Carithers, H.A. 1985. Cat-scratch disease. An overview based on a study of 1,200 patients. Am. J. Dis. Child. **139**: 1124–1133.
6) Chomel, B.B., Abbott, R.C., Kasten, R.W., Floyd-Hawkins, K.A., Kass, P.H., Glaser, C.A., Pedersen, N.C., and Koehler, J.E. 1995. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. J. Clin. Microbiol. **33**: 2445–2450.
7) Dolan, M.J., Wong, M.T., Regnery, R.L., Jorgensen, J.H., Garcia, M., Peters, J., and Drehner, D. 1993. Syndrome of *Rochalimaea henselae* adenitis suggesting cat scratch disease. Ann. Intern. Med. **118**: 331–336.
8) Flexman, J.P., Lavis, N.J., Kay, I.D., Watson, M., Metcalf, C., and Pearman, J.W. 1995. *Bartonella henselae* is a causative agent of cat scratch disease in Australia. J. Infect. **31**: 241–245.
9) Fournier, P.E., Robson, J., Zeaiter, Z., McDougall, R., Byrne, S., and Raoult, D. 2002. Improved culture from lymph nodes of patients with cat scratch disease and genotypic characterization of *Bartonella henselae* isolates in Australia. J. Clin. Microbiol. **40**: 3620–3624.
10) Goral, S., Anderson, B., Hager, C., and Edwards, K.M. 1994. Detection of *Rochalimaea henselae* DNA by polymerase chain reaction from suppurative nodes of children with cat-scratch disease. Pediatr. Infect. Dis. J. **13**: 994–997.
11) Hamilton, D.H., Zangwill, K.M., Hadler, J.L., and Cartter, M.L. 1995. Cat-scratch disease—Connecticut, 1992–1993. J. Infect. Dis. **172**: 570–573.
12) Higgins, J.A., Radulovic, S., Jaworski, D.C., and Azad, A.F. 1996. Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera: Pulicidae). J. Med. Entomol. **33**: 490–495.
13) Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., and Breitschwerdt, E.B. 2000. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. J. Clin. Microbiol. **38**: 1717–1722.
14) Kikuchi, E., Maruyama, S., Sakai, T., Tanaka, S., Yamaguchi, F., Hagiwara, T., Katsube, Y., and Mikami, T. 2002. Serological investigation of *Bartonella henselae* infections in clinically cat-scratch disease-suspected patients, patients with cardiovascular diseases, and healthy veterinary students in Japan. Microbiol. Immunol. **46**: 313–316.
15) Koehler, J.E., Glaser, C.A., and Tappero, J.W. 1994. *Rochalimaea henselae* infection. A new zoonosis with the domestic cat as reservoir. J. Am. Med. Assoc. **271**: 531–535.
16) Kordick, D.L., Wilson, K.H., Sexton, D.J., Hadfield, T.L., Berkhoff, H.A., and Breitschwerdt, E.B. 1995. Prolonged *Bartonella* bacteremia in cats associated with cat-scratch disease patients. J. Clin. Microbiol. **33**: 3245–3251.
17) Kordick, D.L., Hilyard, E.J., Hadfield, T.L., Wilson, K.H., Steigerwalt, A.G., Brenner, D.J., and Breitschwerdt, E.B. 1997. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J. Clin. Microbiol. **35**: 1813–1818.
18) La Scola, B., and Raoult, D. 1999. Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: a 5-year experience (1993 to 1998). J. Clin. Microbiol. **37**: 1899–1905.
19) Margileth, A.M., and Baehren, D.F. 1998. Chest-wall abscess due to cat-scratch disease (CSD) in an adult with antibodies to *Bartonella clarridgeiae*: case report and review of the thoracopulmonary manifestations of CSD. Clin. Infect. Dis. **27**: 353–357.
20) Maruyama, S., Kabeya, H., Nogami, S., Sakai, H., Suzuki, J., Suzuki, H., Sugita, H., and Katsube, Y. 2000. Three cases of cat scratch disease diagnosed by indirect immunofluorescence antibody assay and/or polymerase chain reaction of 16S rRNA gene of *Bartonella henselae*. J. Vet. Med. Sci. **62**: 1321–1324.
21) Maruyama, S., Nakamura, Y., Kabeya, H., Tanaka, S., Sakai, T., and Katsube, Y. 2000. Prevalence of *Bartonella henselae, Bartonella clarridgeiae* and the 16S rRNA gene types of *Bartonella henselae* among pet cats in Japan. J. Vet. Med. Sci. **62**: 273–279.
22) Maruyama, S., Kasten, R.W., Boulouis, H.J., Gurfield, N.A., Katsube, Y., and Chomel, B.B. 2001. Genomic diversity of *Bartonella henselae* isolates from domestic cats from Japan, the USA and France by pulsed-field gel electrophoresis. Vet. Microbiol. **79**: 337–349.
23) Maruyama, S., Kabeya, H., Nakao, R., Tanaka, S., Sakai, T., Katsube, Y., and Mikami, T. 2003. Seroprevalence of *Bartonella henselae, Toxoplasma gondii*, FIV and FeLV infections in domestic cats in Japan. Microbiol. Immunol. **47**: 147–153.
24) Murakami, K., Tsukahara, M., Tsuneoka, H., Iino, H., Ishida, C., Tsujino, K., Umeda, A., Furuya, T., Kawauchi, S., and Sasaki, K. 2002. Cat scratch disease: analysis of 130 seropositive cases. J. Infect. Chemother. **8**: 349–352.
25) Reed, J.B., Scales, D.K., Wong, M.T., Lattuada, C.P., Jr., Dolan, M.J., and Schwab, I.R. 1998. *Bartonella henselae* neuroretinitis in cat scratch disease. Diagnosis, management, and sequelae. Ophthalmology **105**: 459–466.
26) Regnery, R.L., Olson, J.G., Perkins, B.A., and Bibb, W. 1992. Serological response to "*Rochalimaea henselae*" antigen in suspected cat-scratch disease. Lancet **339**: 1443–1445.
27) Sander, A., Buhler, C., Pelz, K., von Cramm, E., and Bredt, W. 1997. Detection and identification of two *Bartonella*

henselae variants in domestic cats in Germany. J. Clin. Microbiol. **35**: 584–587.
28) Sander, A., Posselt, M., Oberle, K., and Bredt, W. 1998. Seroprevalence of antibodies to *Bartonella henselae* in patients with cat scratch disease and in healthy controls: evaluation and comparison of two commercial serological tests. Clin. Diagn. Lab. Immunol. **5**: 486–490.
29) Sander, A., Ruess, M., Bereswill, S., Schuppler, M., and Steinbrueckner, B. 1998. Comparison of different DNA fingerprinting techniques for molecular typing of *Bartonella henselae* isolates. J. Clin. Microbiol. **36**: 2973–2981.
30) Sander, A., Posselt, M., Bohm, N., Ruess, M., and Altwegg, M. 1999. Detection of *Bartonella henselae* DNA by two different PCR assays and determination of the genotypes of strains involved in histologically defined cat scratch disease. J. Clin. Microbiol. **37**: 993–997.
31) Welch, D.F., Hensel, D.M., Pickett, D.A., San Joaquin, V.H., Robinson, A., and Slater, L.N. 1993. Bacteremia due to *Rochalimaea henselae* in a child: practical identification of isolates in the clinical laboratory. J. Clin. Microbiol. **31**: 2381–2386.
32) Welch, D.F., Pickett, D.A., Slater, L.N., Steigerwalt, A.G., and Brenner, D.J. 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. J. Clin. Microbiol. **30**: 275–280.
33) Yoshida, H., Kusaba, N., Omachi, K., Miyazaki, N., Yamawaki, M., Tsuji, Y., Nakahara, K., Sumino, M., Noudomi, M., Shimokawa, Y., and Tanikawa, K. 1996. Serological study of *Bartonella henselae* in cat scratch disease in Japan. Microbiol. Immunol. **40**: 671–673.
34) Zangwill, K.M., Hamilton, D.H., Perkins, B.A., Regnery, R.L., Plikaytis, B.D., Hadler, J.L., Cartter, M.L., and Wenger, J.D. 1993. Cat scratch disease in Connecticut. Epidemiology, risk factors, and evaluation of a new diagnostic test . N. Engl. J. Med. **329**: 8–13.
35) Zeaiter, Z., Liang, Z., and Raoult, D. 2002. Genetic classification and differentiation of *Bartonella* species based on comparison of partial ftsZ gene sequences. J. Clin. Microbiol. **40**: 3641–3647.

JOURNAL OF CLINICAL MICROBIOLOGY, July 2006, p. 2499–2506 Vol. 44, No. 7
0095-1137/06/$08.00+0 doi:10.1128/JCM.00498-06

Multispacer Typing To Study the Genotypic Distribution of *Bartonella henselae* Populations

Wenjun Li,[1] Bruno B. Chomel,[2] Soichi Maruyama,[3] Lynn Guptil,[4] Anna Sander,[5] Didier Raoult,[1] and Pierre-Edouard Fournier[1]*

Unité des Rickettsies, IFR 48, CNRS UMR 6020, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France[1]; Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616[2]; Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan[3]; Veterinary Clinical Sciences, Purdue University, West Lafayette, Indiana 47907[4]; and Institute for Medical Microbiology and Hygiene, University of Freiburg, 79104 Freiburg, Germany[5]

Received 7 March 2006/Returned for modification 24 April 2006/Accepted 14 May 2006

Bartonella henselae*, a worldwide fastidious bacterium, has a feline reservoir and is pathogenic for humans. However, the relationship between human and cat isolates of *B. henselae*, as well as its population dynamics and geographic heterogeneity, is not fully understood, in part because of the absence of appropriate typing methods. Multilocus sequence typing (MLST), the most discriminatory genotyping method for *B. henselae*, identified seven genotypes and suggested that human isolates arose from a limited number of cat isolates. Herein, we estimated the discriminatory power of multispacer typing (MST) by studying 126 *B. henselae* cat isolates from various areas of Europe, Asia, and the United States. We identified the nine most variable intergenic spacers conserved by both *B. henselae* and *Bartonella quintana* genomes. By comparing the sequences obtained from these nine spacers for each studied isolate, we identified 39 MST genotypes. The distribution of isolates into MST genotypes matched their phylogenetic organization into four clusters. MST showed that European and Asian isolates were different, in contrast with American isolates, but failed to identify pandemic strains. Our study demonstrated that MST is a powerful method for genotyping *B. henselae* at the strain level and may serve in studying the population dynamics of this bacterium and understanding the relationships between cat and human isolates. Finally, we provide a free-access MST-Rick online software program (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst) that investigators may use to compare their own MST sequences to our database.

Bartonella henselae is a gram-negative, fastidious bacterium associated with cats. Its transmission among cats is mediated by the cat flea, *Ctenocephalides felis* (7). Infected cats may remain bacteremic for long periods, thus playing a major role as a reservoir for the bacterium (6, 24). Human infection occurs through cat scratches or bites (22) and presents as cat scratch disease (2), bacillary angiomatosis (23), peliosis hepatis (32), endocarditis (18), or a variety of other, less frequent manifestations (14).

Although criteria exist for classifying *Bartonella* isolates as new species (27), there is a need for a method able to reliably identify *B. henselae* at the strain level. Such a method would allow investigation of the relationships between cat and human isolates, the question of whether epidemic strains occur in cats, and the geographic heterogeneity of *B. henselae* isolates. Various methods have been proposed for typing *Bartonella* isolates (10, 12, 19, 20, 26, 29, 34). Of these, sequence-based methods have the advantages of being applicable to clinical or environmental specimens and producing reproducible and comparable results. On the basis of comparison of 16S rRNA gene sequences, *B. henselae* isolates were classified into two main genotypes, i.e., types I and II. This gene was considered a useful delineation among isolates because the two genotypes also exhibited different serotypes and possessed consistently distinguishable protein profiles (26). Sequences from the *ftsZ* (12), *gltA* (10), 35-kDa protein-encoding (26), *groEL* and *pap31* (34) genes, and from the 16S-23S intergenic spacer (20), later permitted the identification of three, two, two, four, and six genotypes, respectively, that did not exactly match 16S rRNA gene types. To date, the most discriminatory typing method for *B. henselae* isolates is multilocus sequence typing (MLST) incorporating nine genes (21). This method distinguished seven genotypes among 37 human and cat isolates and suggested that lateral gene transfer occurs among *B. henselae* isolates (21). Although these investigators and others suggested that human infection is caused by a limited number of specific *B. henselae* genotypes (4, 10, 21), the discriminatory power of the genotyping methods that they used and the small number of *B. henselae* isolates that they studied were insufficient to allow any statistically significant conclusions to be drawn. Therefore, a genotyping tool with greater discriminatory power for genotyping *B. henselae* at the strain level is needed to investigate the diversity and population structure of this bacterium.

Recently, we applied a new genotyping method to *Bartonella quintana*, i.e., multispacer typing (MST) (13). This method allows genotyping of bacteria at the strain level. MST, initially

* Corresponding author. Mailing address: Unité des Rickettsies, IFR 48, CNRS UMR 6020, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France. Phone: (33) 04.91.38.55.17. Fax: (33) 04.91.83.03.90. E-mail: Pierre-Edouard.Fournier@medecine.univ-mrs.fr.