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.

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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).

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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

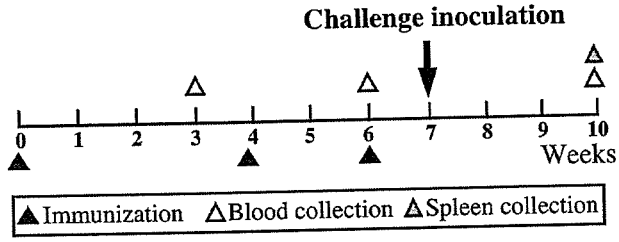

Fig. 1. Schedule of experiment. Immunizations with VSVΔG*HTN, VSVΔG*G, or PBS are shown as closed triangles. The collection of serum samples is shown as open triangles.

(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

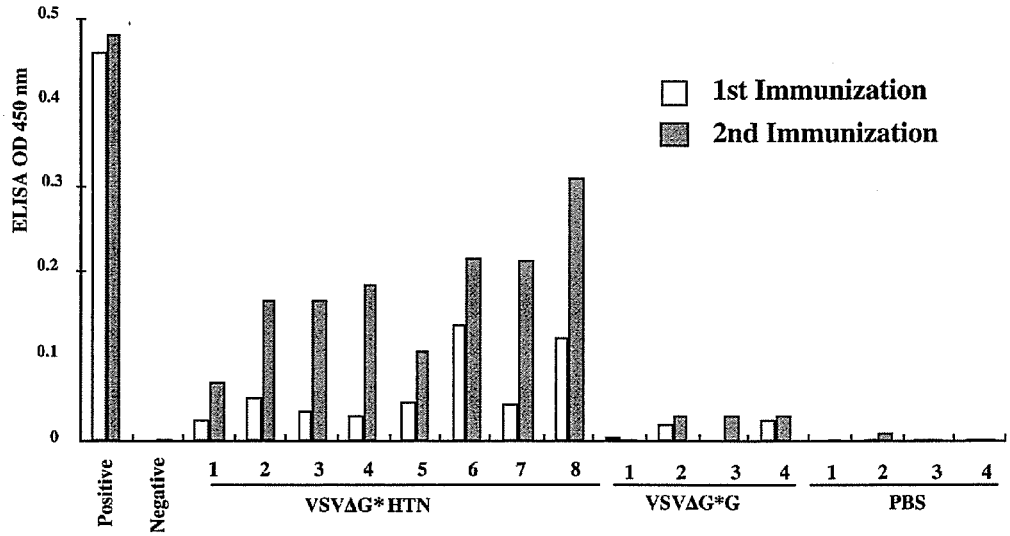

Fig. 2. Antibody responses against hantavirus GP before challenge inoculation. Anti-GP antibodies were measured using ELISA; the recombinant GP was expressed in insect cells by the baculovirus vector system as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally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

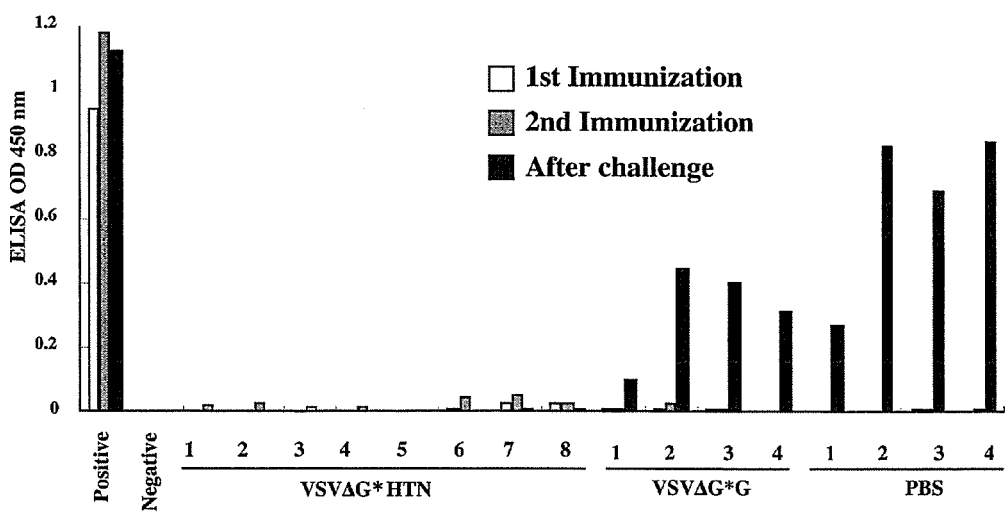

Fig. 3. Antibody responses against HTNV N protein after challenge inoculation. Anti-N protein antibodies were measured using ELISA; recombinant NP was expressed in *Escherichia coli* as described in Section 2. The positive control was serum from a hyperimmune mouse experimentally inoculated with HTNV.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

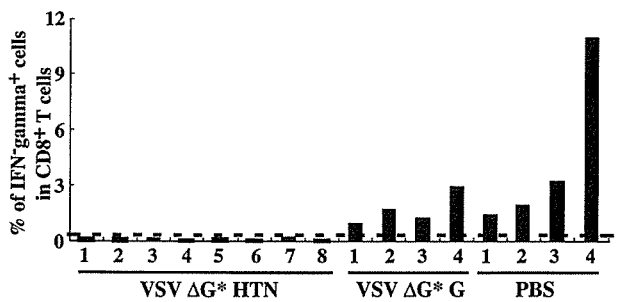

Fig. 4. HTNV-specific $CD8^+$ T cell responses after challenge inoculation. HTNV-specific $CD8^+$ T cells were counted as IFN-γ-producing cells; values are expressed as percent of total splenic $CD8^+$ T cells. A horizontal broken line indicates the limitation of the background level in this assay.

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.

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Short communication

Characterization of Th1 activation by *Bartonella henselae* stimulation in BALB/c mice: Inhibitory activities of interleukin-10 for the production of interferon-γ in spleen cells

Hidenori Kabeya [a,*], Akiko Yamasaki [a], Mikako Ikariya [a], Rie Negishi [a], Bruno B. Chomel [b], Soichi Maruyama [a]

[a] *Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Science, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan*
[b] *Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA*

Received 30 May 2006; received in revised form 28 July 2006; accepted 7 August 2006

Abstract

This study was conducted to analyze cytokine production mechanisms in mice after *Bartonella henselae* stimulation. BALB/c mice were inoculated intraperitoneally with 3×10^6 colony forming units of *B. henselae* (Houston-1 strain) twice at 10-day interval. Spleen cells were harvested from the mice and stimulated with the organisms. Following the stimulation, interferon-gamma (IFN-γ) and interleukin-4 (IL-4), IL-10, IL-12 and tumor necrosis factor-alpha (TNF-α) were measured in the culture supernatants of the spleen cells by ELISA. The spleen cells specifically secreted IFN-γ, but not IL-4, indicating that T helper 1 (Th1) cells were activated following *B. henselae* stimulation. In addition, IL-10 and TNF-α productions were also detected in the culture supernatants of spleen cells. Neutralization of IL-10 in the culture supernatants significantly enhanced the production of IFN-γ from the spleen cells stimulated with *B. henselae*. These results indicate that *B. henselae* predominantly stimulated Th1 cells and resulted in secreting IFN-γ, however the production was partially inhibited by IL-10, which was produced simultaneously.

Keywords: *Bartonella henselae*; IL-10; IFN-γ; Mice; TNF-α

1. Introduction

Although high levels of bacteremia with *Bartonella henselae* were found in cats for several months to a few years, most infected cats remain asymptomatic (Kordick et al., 1999). Immune responses against *B. henselae* infection in cats have mainly been studied from the standpoints of humoral immunity (Freeland et al., 1999). Analysis of the immune responses, especially cell-mediated immunity (CMI) is indispensable to understand the mechanisms by which the organisms are eliminated from infected animals, since

* Corresponding author. Tel.: +81 466 84 3636; fax: +81 466 84 3636.
E-mail address: kabeya@brs.nihon-u.ac.jp (H. Kabeya).

0378-1135/$ – see front matter
doi:10.1016/j.vetmic.2006.08.010

B. henselae is an intracellular pathogen (Kordick and Breitschwerdt, 1995). However, little is known about cytokine responses against *B. henselae* in cats as well as in humans (Kabeya et al., 2006; Papadopoulos et al., 2001). The cat naturally *B. henselae*-infected were shown to promote IL4, but not IFN-γ mRNA expression when the emergence of relapsing bacteremia, suggesting that the selective induction of Th2 immune responses may contribute to establishing the persistent infection of *B. henselae* in naturally infected cats (Kabeya et al., 2006).

It has been reported that despite the fact that mice are not the natural reservoir of *B. henselae*, we used a mouse model to analyze the cellular immune response against *B. henselae*, because it has been well known the immune system of such laboratory animals (Arvand et al., 2001; Kabeya et al., 2003; Karem et al., 1999). Previous investigations have been performed to examine the immune responses against *B. henselae* stimulation in BALB/c or C57BL/6 mice (Arvand et al., 2001; Karem et al., 1999). *B. henselae* elicited cell-mediated immune responses mainly $CD4^+$ helper T cells 1 (Th1) in immunocompetent mice. Karem et al. (1999) also reported that *B. henselae*-primed BALB/c mice induced delayed-type hypersensitivity and the secretion of interferon-gamma (IFN-γ) mediated by $CD4^+$ Th1. However, the mechanism by which $CD4^+$ Th1 response in *B. henselae*-primed mice is induced remains unclear. The aim of this study was to better understand how a Th1 response is specifically induced in experimentally *B. henselae*-inoculated mice through analysis of cytokine expression profiles.

2. Materials and methods

2.1. Bacterial strains

B. henselae strain Houston-1 (ATCC49882) was used in this study. The strain was cultured on heart infusion agar (HIA) plates (DIFCO, USA) containing 5% defibrinated rabbit blood at 35 °C in an atmosphere of 5% CO_2 for 1 week. The bacterial cells were re-suspended in PBS and the concentration was adjusted to OD of 1.0 at 600 nm. Ten-fold serial dilutions were made with PBS and 100 μl of each diluted suspension were plated on two HIA plates containing 5% defibrinated rabbit blood to determine the colony-forming unit (CFU) of the inoculums.

2.2. Experimental animals

Female BALB/c mice were purchased from Nihon CLEA Corp. and used for the experiment at the age of 5 weeks. Three animals were caged together and kept under specific-pathogen-free conditions throughout the study. Animal care was carried out in accordance with the guidelines for the care and use of laboratory animals by College of Bioresource Sciences, Nihon University.

2.3. Inoculation of mice with B. henselae

Three of BALB/c mice were used for each experimental group. The mice were inoculated intraperitoneally twice at 10-day interval with 1 ml of the bacterial suspension containing 3.0×10^6 CFU of *B. henselae* Houston-1 suspended in PBS.

2.4. Induction of cytokine production from mice spleen cells

Spleen was harvested from three mice per group and suspended in complete RPMI medium consist of RPMI1640 (Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 200 U/ml penicillin, and 200 μg/ml streptomycin at a concentration of 2×10^6 cells/well of 24-well microplate. The cells were stimulated with *B. henselae* Houston-1 (2×10^6 to 2×10^8 CFU/well) with or without anti-mouse interleukin-10 (IL-10) rat monoclonal antibody (ENDOGEN, MA, USA) or IgG_1 rat isotype control (R and D systems Inc., MN, USA) at a concentration of 40 μg/ml for 24–120 h at 37 °C. Following cultivation, the concentrations of secreted cytokines (IFN-γ, IL-4, IL-10, IL-12 and tumor necrosis factor α; TNF-α) in culture-supernatants were measured by using commercial ELISA kits (Quantikine, R and D systems Inc.).

2.5. Statistical analysis

Differences between the amounts of cytokine in culture conditions (stimulation with versus without stimulant, or stimulation with live versus inactivated organisms) were determined by Student's *t*-test. *P*-values of <0.05 were regarded as significant.

3. Results

3.1. Analysis of cytokine production from spleen cells of B. henselae primed mice following in vitro stimulation

Cytokine production in the culture-supernatant of spleen cells of *B. henselae* Houston-1 primed BALB/c mice was assessed following stimulations with or without antigens at a ratio of cells to bacteria was 1:10 for 24–120 h (Fig. 1). A marked production of IFN-γ (1002.2 pg/ml) was observed following the antigen stimulation for 48 h and reached a plateau over 3000 pg/ml 72 h after the stimulation, while the production was kept at low levels (9.6–10.0 pg/ml) without *B. henselae* stimulation. By contrast, IL-4 productions showed low levels from 12.3 to 31.8 pg/ml in the culture with stimulation and from 11.1 to 13.7 pg/ml in the culture without stimulation throughout the experimental period.

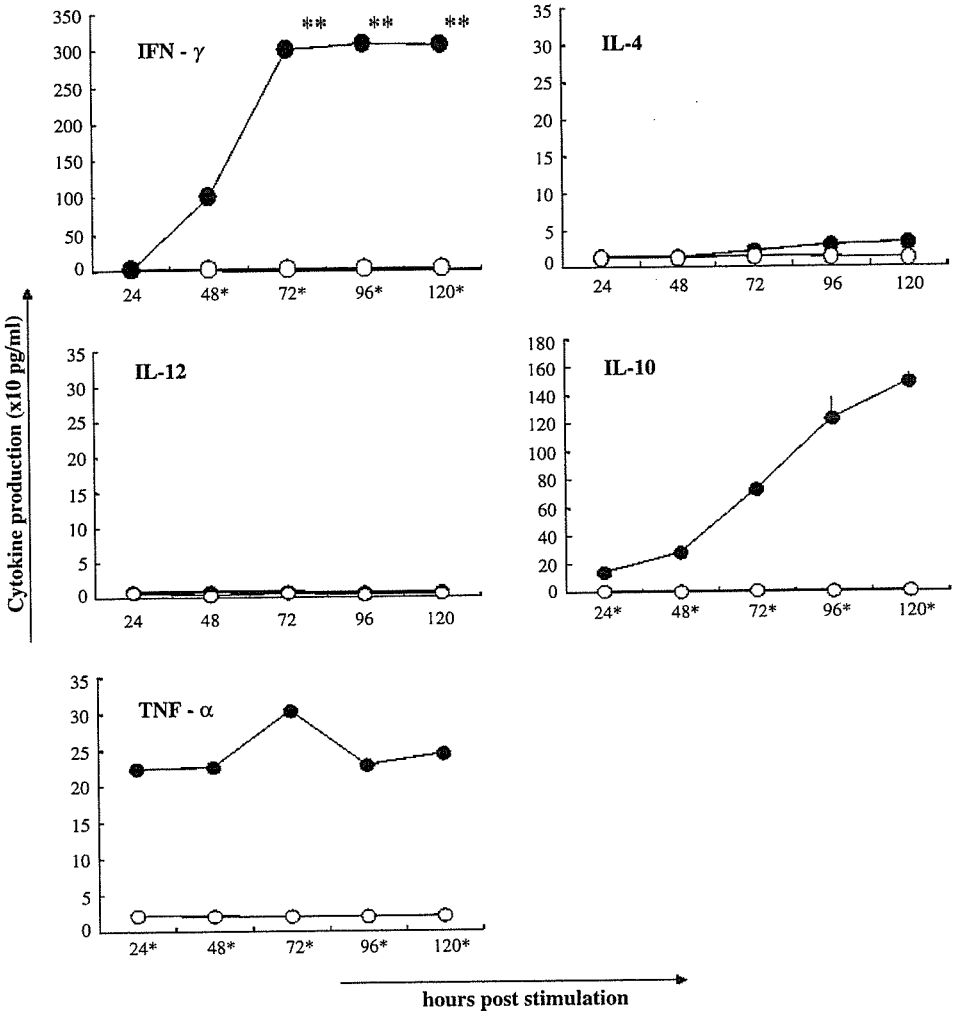

Fig. 1. Cytokine secretion in culture-supernatants of spleen cells from *B. henselae* primed mice. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with (closed circle) or without (open circle) the organism. Ratio of cells to bacteria was 1:10 from 24 to 120 h. Following cultivation, culture-supernatants were collected and the concentrations of cytokines, such as IFN-γ, IL-4, IL-12, IL-10 and TNF-α were quantitated, using ELISA. (*) $P < 0.01$; (**) production was over 3000 pg/ml.

In order to analyze the mechanisms of specific production of IFN-γ from the spleen cells of *B. henselae* primed mice, we assessed the productions of IL-12 and IL-10 which were representative cytokines of inducing and suppressing Th1 responses, respectively (Fig. 1). The spleen cells did not produce IL-12 both with and without *in vitro* antigen stimulations (5.5–8.3 pg/ml). However, production of IL-10 and TNF-α was specifically increased in the culture supernatants of antigen stimulated spleen cells. The amounts of TNF-α production were small (223.4–302.9 pg/ml), however, the cells secreted TNF-α as early as 24 h after stimulation. Production of IL-10 gradually increased from 141.6 to 1487.0 pg/ml following stimulation, although they remained at low levels from 2.7 to 7.3 pg/ml without stimulation.

3.2. Cytokine production from the primed mice spleen cells by stimulating with live or heat-inactivated B. henselae Houston-1

To examine factors involved in the induction of IFN-γ, IL-10 and TNF-α from *B. henselae* primed mice spleen cells, heat-inactivated organisms were used for *in vitro* stimulation and assessed the cytokine production (Fig. 2). The spleen cells were stimulated

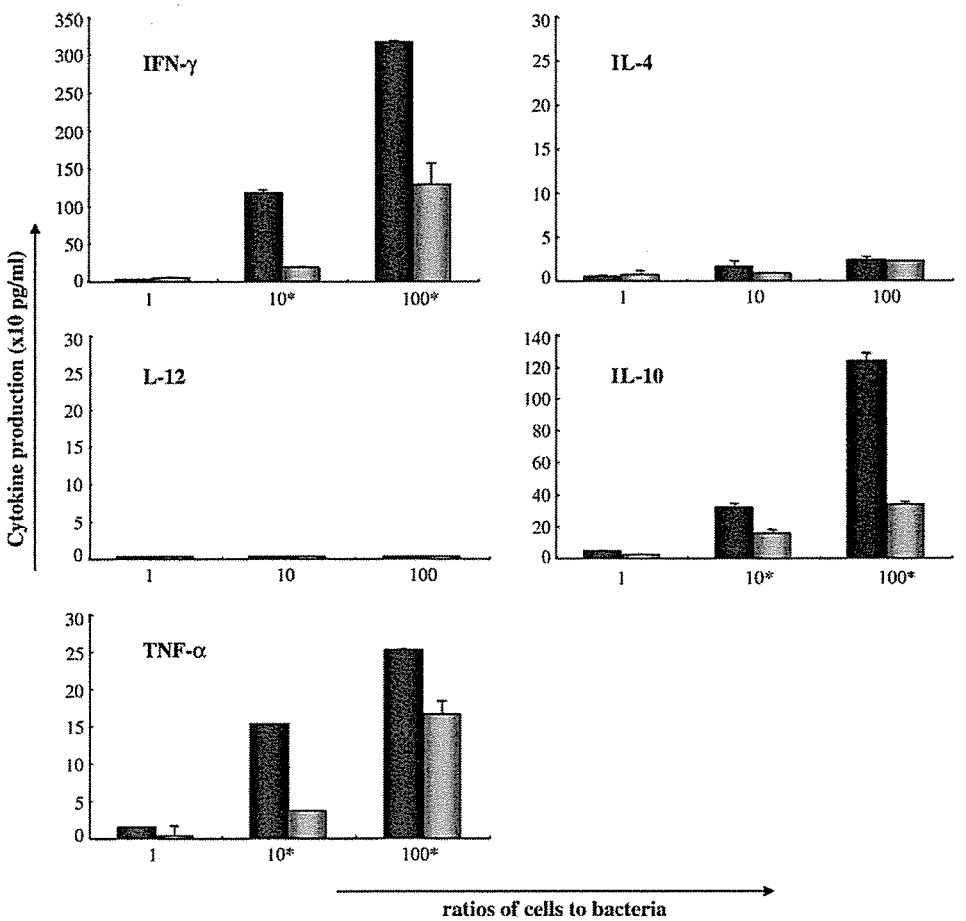

Fig. 2. Cytokine secretion by *in vitro* stimulation with live or heat-inactivated *B. henselae* in culture-supernatants of spleen cells from *B. henselae* primed mice. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with live (closed bar) or heat-inactivated (gray bar) *B. henselae* Houston-1. Ratios of cells to bacteria were from 1:1 to 1:100 for 120 h. Following cultivation, culture-supernatants were collected and cytokines, including IFN-γ, IL-4, IL-12, IL-10 and TNF-α were quantitated, using ELISA. (*) $P < 0.05$.

with live or heat-inactivated *B. henselae* Houston-1 at different concentrations (ratios of cells to bacteria from 1:1 to 1:100) for 120 h and the amount of the secreted cytokine was calculated by ELISA. The cells produced IFN-γ, IL-10 and TNF-α by stimulating with both live and heat-inactivated organisms in an antigen dose-dependent manner. However, significantly higher levels of production were observed in the cells stimulated with live organisms than the cells stimulated with heat-inactivated organisms ($P < 0.05$). At a ratio of spleen cells to bacteria equal to 1:100, the cells produced IFN-γ, IL-10 and TNF-α at concentrations of 3165.6, 1234.8 and 252.5 pg/ml, respectively when stimulated with live organisms. In comparison, stimulations with heat-inactivated organisms induced production of lower amounts of cytokines (1267.7, 334.3 and 165.2 pg/ml, respectively). Little IL-4 and IL-12 were detected in any cases of stimulation (IL-4: 5.4–21.8 pg/ml, IL-12: 2.4–2.9 pg/ml).

3.3. The effects of IL-10 on the IFN-γ production from B. henselae Houston-1—primed mice spleen cells

To clarify the effects of IL-10 on the IFN-γ production from *B. henselae*-stimulated mice spleen cells, neutralizing antibodies to mouse IL-10 were added to the cultures and the amount of IFN-γ was assessed (Fig. 3). Addition of the neutralizing antibody dramatically enhanced the IFN-γ production (66345.8 pg/ml) compared with cells without the neutralizing antibodies (34227.4 pg/ml), and with cells in which the IgG1 rat isotype control antibody was added (28588.4 pg/ml).

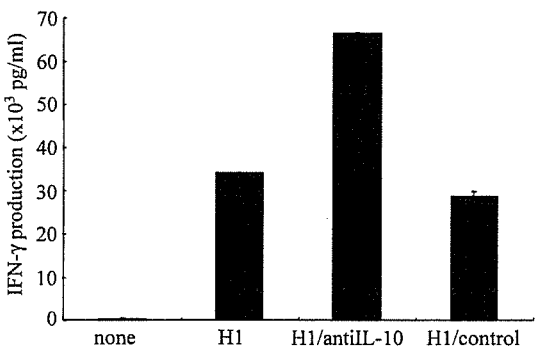

Fig. 3. Effects of neutralization of IL-10 and antigen-specificities on the production of IFN-γ. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with or without *B. henselae* Houston-1 (H1) at ratio of cells to bacteria was 1:10. When starting the cultivation with *strain* Houston-1, the other wells were cultured in the presence of anti mice IL-10 neutralization (H1/anti IL-10) or isotype control antibodies (H1/control). Following cultivation, culture-supernatants were collected and IFN-γ was quantitated, using ELISA.

4. Discussion

In this study, we demonstrated a specific IFN-γ production from *B. henselae* (Houston-1) -primed mice spleen cells following *in vitro* stimulation with the bacteria. Karem et al. (1999) showed that spleen cells from *B. henselae* primed BALB/c mice secreted large amounts of IFN-γ after stimulation with the same organisms. Spleen cells from unprimed BALB/c mice also produced IFN-γ after *in vitro* stimulation with *B. henselae* although the amount was smaller than those of the primed mice. These results indicate that the production was induced by antigen-specific lymphocytes as well as nonspecific cell-mediated immune responses, including activated macrophages.

Resto-Ruiz et al. (2003) also reported the *B. henselae*-specific IFN-γ secretion from spleen cells of primed A/J mice. They found that *in vitro* stimulation with *B. henselae* also resulted in the significant production of IL-12 from spleen cells of the mice. IL-12 is known to induce Th1-related immune responses, including inductions of IFN-γ by NK and T cells, and resulted in stimulating cytotoxic T lymphocytes and NK cells (Trinchieri, 1998). In contrast, we did not detect any significant production of IL-12 from the spleen cells. This might be caused by the differences of the mice strains used in these experiments. The other possible reason to explain the difference is the ratio of cell to bacteria for the stimulation of spleen cells. We used the ratio of cell to bacteria at 1:1 to 1:100 for *in vitro* stimulation, while Resto-Ruiz et al. applied the ratio of 1:500 (Resto-Ruiz et al., 2003).

Patients with cat-scratch disease also showed no induction of IL-12 in their serum (Papadopoulos et al., 2001). However, the levels of circulating IL-2, IL-6 and IL-10 were shown to be significantly higher in patients with CSD than in healthy individuals

(Papadopoulos et al., 2001). In addition, the production of some inflammatory cytokines such as TNF and IL-1β, and IL-6 was markedly higher in the patients infected with *B. quintana* than those of uninfected individuals. It has also been reported that patients with *B. quintana*-bacteremia showed specific increase of IL-10 production by mononuclear cells (Capo et al., 2003). Because IL-10 is known to suppress several inflammatory mediators, overproduction of IL-10 in bacteremic patients with *B. quintana* may result in establishing a persistent infection as well as other infectious diseases (McGuirk and Mills, 2002). In the present study, we also observed IL-10 secretion from spleen cells of *B. henselae*-primed BALB/c mice and the secretion increased gradually following stimulation with the organisms. Furthermore, secretion of TNF-α was observed just 24 h after *in vitro* stimulation of spleen cells with the organisms and reached a plateau at the same time. Thus, *Bartonella* species, including *B. henselae* stimulate mononuclear cells and/or lymphocytes and the cells secrete IL-10 as well as some inflammatory cytokines, such as TNF-α and IFN-γ. In a mouse model, it has been shown that nitric oxide derived from macrophages activated by IFN-γ play a significant role in eliminating *B. henselae* from infected cells (Musso et al., 2001). We showed that IL-10 induced by stimulation with *B. henselae* partially suppressed the secretion of IFN-γ following the stimulation with the organisms. These results suggest that the organisms may escape the host immune responses, such as the activation of macrophages by inhibiting the induction of IFN-γ by inducing IL-10, simultaneously. However, it has been shown that persistent infection of *B. henselae* does not last for long periods in mice, suggesting that these modulations, such as inhibition of the production of IFN-γ may not affect the infectivity of *B. henselae* in mice. Supporting this hypothesis, *Bartonella* species shows strict host specificities in mice (Kosoy et al., 2000).

Arvand et al. (2001) reported that heat-killed *B. henselae* stimulated spleen cells of the primed C57BL/6 mice and induced proliferation of $CD4^+$ T cells and IFN-γ production. In the present experiment, large amounts of cytokines, including IFN-γ, IL-10 and TNF-α were detected when the spleen cells of BALB/c mice were stimulated with live *B. henselae*, whereas heat-killed organisms induced lower amount of cytokines. These results clearly suggest that some heat-stable components of *B. henselae* as well as secreted factors from live bacteria and/or invasion into cells may be involved in the induction of the cytokines in mice. Recent studies have revealed that *B. henselae* uses type IV secretion systems for intercellular delivery of effector molecules that modify host cellular functions in favor of the pathogen (Schmid et al., 2004). It has also been proven that the type IV secretion systems of *B. henselae* mediates invasion, a NF-κB-dependent pro-inflammatory activation (Schmid et al., 2004). Thus, type IV secretion systems may be involved in the induction of cytokines in mice.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research (No. 14360193) and Academic frontier project from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and Academic Frontier Project "Surveillance and Control for Zoonoses" from The Ministry of Education, Culture, Sports, Science and Technology.

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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.

developed for *Yersinia pestis* (11), was also applied with success to strains of other human pathogens, including *Rickettsia conorii* (15), *Rickettsia prowazekii* (35) and *Coxiella burnetii* (16). MST was developed with the assumption that intergenic spacers are more variable than genes for genotyping bacteria at the strain level. In this study, to estimate the usefulness of MST for studying the population genetics of *B. henselae*, we applied it to a large collection of cat isolates.

MATERIALS AND METHODS

Study design. One hundred twenty-six *B. henselae* cat isolates of various geographic origins were incorporated in this study (Table 1). All 38 European isolates were grown in our laboratory. For the other 88 isolates, from the United States and Asia, we studied DNA extracted by two of the authors (B.B.C. and L.G.) from their isolates.

***Bartonella henselae* culture and DNA extraction.** *B. henselae* isolates were cultivated on Columbia agar with 5% sheep blood (BioMerieux, Marcy l'Etoile, France) at 37°C in 5% CO_2 (Genbag CO_2 system; BioMerieux). Genomic DNA of *B. henselae* strains was extracted by using the Chelex procedure as previously described (9) or the QIAmp Tissue kit following the manufacturer's recommendations (QIAGEN, Hilden, Germany).

Selection of target sequences. We aligned the genomic sequences of *B. henselae* (GenBank accession number BX897699) and *B. quintana* (BX897700) by using the BLASTn (1) and GenomeComp (33) software programs to identify conserved pairs of consecutive genes. Then, intergenic sequences were aligned using the CLUSTAL W program (31). We classified intergenic spacers conserved by both genomes, with sizes ranging from 150 to 600 bp, by degree of similarity and then selected the 20 most variable spacers (detailed in Table 2).

PCR amplification and sequencing. Primers were designed to amplify the 20 most variable spacers fulfilling the above criteria using the Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for amplifying the 20 most variable spacers were selected within genes flanking the selected spacers and are listed in Table 2. All primers were obtained from Eurogentec (Seraing, Belgium). Their specificity was predicted by comparison with GenBank using the BLASTn software (1). PCRs were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, Mass.). One nanomolar concentration of each DNA preparation was amplified in a 25-μl reaction mixture containing 50 pM of each primer; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg, Md.); 1 U of eLONGase polymerase (Invitrogen); 1 μl of eLONGase buffer A; and 4 μl of eLONGase buffer B. The following conditions were used for amplification: an initial 3 min of denaturation at 94°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 1 min at 68°C. Amplification was completed by holding the reaction mixture for 10 min at 68°C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore, Saint-Quentin en Yvelines, France) as recommended by the manufacturer. PCR products were sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit with Amplitaq polymerase FS (Perkin-Elmer, Coignieres, France) as described by the manufacturer. Sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sterile water was used as a negative control in each PCR assay. Sequences from each genotype were checked twice in both directions to ensure the reliability of the MST method.

Sequence analysis and phylogenetic analysis. Nucleotide sequences were edited using the Autoassembler package (Perkin-Elmer). For each intergenic spacer, a genotype was defined as a sequence exhibiting unique mutations. MST genotypes were defined as unique combinations of spacer genotypes. Multiple alignment of sequences was carried out using the CLUSTAL W software (31). Phylogenetic analysis of the studied isolates was obtained using the neighbor-joining and maximum parsimony methods within the MEGA 3 software (25). For this purpose, sequences of the selected spacers were concatenated. To facilitate sequence comparison with our MST sequences, we developed an online site named MST-Rick. This site contains a local BLAST to help scientists compare their sequences to our database.

Statistical tests. The genotypic variability of *B. henselae* isolates according to their geographic origin was estimated using Fisher's exact test. A difference was considered significant when P was <0.05.

Nucleotide sequence accession numbers. The different genotypes for the discriminatory spacers have been deposited in the GenBank database under accession numbers DQ383226 to DQ383270.

RESULTS

MST genotyping. One thousand four hundred thirteen intergenic spacers were found conserved by *B. henselae* and *B. quintana* genomes. Among them, 293 had a size ranging from 150 to 600 bp. We tested the 20 most variable (S1 to S20) of these 293 spacers among the 126 *B. henselae* cat isolates available. Nine of the spacers (S1 to S9) were found highly variable among these isolates (Table 2). The tRNA-Ala/GCA-tRNA-Ile/AUC spacer (S1), flanked by two tRNA genes, was found to be the most variable spacer among the nine tested, with five variable nucleotide positions and a 15-bp sequence fragment presenting either as a single copy or repeated up to five times, depending on the isolate (Fig. 1; Table 3). Sequences from the S1 spacer classified the 126 isolates into nine genotypes. The BH2865724-*dut* spacer (S2), with 14 variable nucleotide positions, was the second most variable spacer and allowed the 126 tested isolates to be classified into seven genotypes (Table 3). The *dnaJ*-related protein–*cobS* spacer (S3) held eight variable nucleotide positions and classified the 126 isolates into six genotypes (Table 3). The *pssA*-oxidoreductase (S4) and *carB*-cold shock protein (S5) spacers had nine and five variable nucleotide positions, respectively, and classified the 126 isolates into five genotypes each (Table 3). The *alr-gcvP* (S6) and *ftsK*-oxidoreductase spacers (S7) contained eight variable nucleotide positions each and classified the 126 isolates into four genotypes each (Table 3). The BH2864883-BH2864884 (S8) and *acpP2*-malate oxidoreductase (S9) spacers harbored eight and four variable nucleotide positions, respectively, and classified the 126 isolates into three genotypes each (Table 3). In total, 69 variable nucleotide positions were found within the nine intergenic spacers (Table 3). Each variable nucleotide was checked three times to ensure the reliability of MST. Only two alleles at each variable position were found, with the exception of position 256 within the *alr-gcvP* spacer. At this position, 117 isolates had a thymine (types 2 and 3), compared to a cytosine in five European isolates (type 1) and a guanine in four American isolates (type 4) (Table 3). By combining the genotypes obtained from each variable spacer, the 126 tested isolates could be classified into 39 MST genotypes (Table 1). Each of the 39 genotypes was identified based on sequence specificities from either a single spacer or a combination of a maximum of seven spacers (Fig. 2). Sequences from each genotype from the nine spacers were added to the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

Among the 39 MST types, 24 MST types (types 1, 3, 4, 6, 8 to 12, 14, 16, 19 to 21, 23, 26, 28 to 31, 34, 36, 37, and 39) contained only one isolate each and five MST genotypes (types 17, 24, 27, 32, and 33) contained only two isolates each (Table 1). The 19 Asian isolates were distributed into 12 MST genotypes, compared to 6 ($P < 0.01$) and 24 ($P = 0.03$) MST types for the 38 European and 69 American isolates, respectively (Fig. 3). Among the 39 MST genotypes, 10 (types 6, 10, 20, 21, 23, 32, and 36 to 39), 21 (types 1, 3, 4, 7 to 9, 11, 14 to 19, 24 to 30, and 34), and 5 (types 4, 9, 12, 22, and 31) genotypes were specific to Asian, American, and European isolates, respectively.

A significant difference in genotypic diversity was found between *B. quintana* (4 MST types out of 71 isolates) (13), and

TABLE 1. List of *B. henselae* isolates incorporated in this study and corresponding genotypes

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
Amber	USA[b]	5	1	1	1	2	2	2	1	1	5
Aron	USA	7	2	5	4	1	2	1	1	3	7
BisQuick	USA	5	2	6	5	2	2	2	1	1	35
Budda	USA	5	1	1	1	2	2	2	1	1	5
Buster Brown	USA	5	1	1	2	2	2	2	1	1	25
Cleo	USA	5	1	1	1	2	2	2	1	1	5
Kody	USA	4	5	5	5	1	2	1	1	3	27
Earl Grey	USA	5	1	1	2	2	2	2	1	1	25
Erick	USA	5	1	1	1	2	2	2	1	1	5
Faleen	USA	5	1	1	1	2	2	2	1	1	5
Gigi	USA	4	5	5	4	1	2	1	1	3	15
Jackie	USA	5	2	1	1	2	2	2	1	1	16
Junior	USA	5	1	1	1	2	2	2	1	1	5
Kelly	USA	8	2	5	4	1	2	1	1	3	8
Kodie	USA	5	1	1	1	2	2	2	1	1	5
Lathious	USA	5	2	6	5	2	2	2	1	1	35
Levi	USA	5	1	1	1	2	2	2	1	1	5
Mew Mew	USA	7	2	5	4	1	2	1	1	3	7
Mitzi	USA	5	1	1	1	2	2	2	1	1	5
Mokka	USA	5	1	1	1	2	2	2	1	1	5
Molly	USA	5	1	1	2	2	2	2	1	1	25
Norman	USA	5	1	1	1	2	2	2	1	1	5
Patches	USA	7	2	5	4	1	2	1	1	3	7
Pyewacket	USA	5	1	1	1	2	2	2	1	1	5
Rafiki	USA	5	1	1	1	2	2	2	1	1	5
Rocket	USA	7	2	5	4	1	2	1	1	3	7
Rum Tum	USA	5	1	1	1	2	2	2	1	1	5
Sabrina	USA	5	2	6	5	2	2	2	1	1	35
Sadie	USA	4	5	5	4	1	2	1	1	3	15
Saki	USA	5	1	1	1	2	2	2	1	1	5
Sam	USA	9	2	5	4	1	2	1	1	3	9
Samantha	USA	5	1	1	1	2	2	2	1	1	5
Sassy	USA	1	1	1	1	2	2	2	1	1	1
Shannon	USA	5	1	1	5	2	2	2	1	1	26
Simba	USA	3	1	1	1	2	2	2	1	1	3
Sinbad	USA	5	1	1	1	4	2	2	1	1	29
Spaz	USA	5	1	1	1	2	2	2	1	1	5
Sunday	USA	5	2	1	5	2	2	2	1	1	28
Sweetie	USA	5	1	1	2	2	2	2	1	1	25
Tabatha	USA	5	1	1	1	2	2	2	1	1	5
Tasha	USA	3	1	6	3	5	4	4	3	2	18
Timothy	USA	5	1	1	1	2	2	2	1	1	5
Toby	USA	5	1	1	1	2	2	2	1	1	5
Tori	USA	4	5	5	5	1	2	1	1	3	27
Zipper	USA	5	2	6	5	2	2	2	1	1	35
Zoe	USA	5	1	1	1	2	3	2	1	1	30
Newmans	USA	4	2	5	4	1	2	2	1	3	33
White	USA	5	2	6	5	2	2	2	1	1	35
Lavery	USA	5	7	6	1	2	2	1	2	1	17
Rae	USA	5	7	6	1	2	2	2	2	1	34
Fairminer	USA	5	2	6	5	2	2	2	1	1	35
Shaw-Lamon	USA	5	2	6	5	2	2	1	1	1	24
Moyle	USA	5	7	6	1	2	2	1	2	1	17
Linnan	USA	5	2	6	5	2	2	1	1	1	24
Silcock	USA	5	2	6	5	2	2	2	1	1	35
Hunt	USA	5	2	6	5	2	2	2	1	1	35
Eichtais	USA	5	2	6	5	2	2	2	1	1	35
Taylor	USA	5	2	6	5	2	2	2	1	1	35
Ramm	USA	5	2	6	5	2	2	2	1	1	35
USA1	USA	5	1	1	1	2	2	2	1	1	5
USA4	USA	4	1	1	1	2	2	2	1	1	4
USA6	USA	4	5	5	4	1	2	1	1	3	15
USA7	USA	3	1	6	3	5	4	4	3	2	18
USA8	USA	5	1	1	1	2	2	2	1	1	5
USA11	USA	4	1	5	4	1	2	1	1	3	19

Continued on following page

TABLE 1—*Continued*

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
USA12	USA	3	3	6	3	5	4	4	1	2	11
USA15	USA	4	2	5	4	1	2	1	1	3	14
USA16	USA	3	1	6	3	5	4	4	3	2	18
USA17	USA	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.32	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.33	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.34-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.35	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.50-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.52-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.54-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.55-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 56-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.57-M	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC.58-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 59-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.67	France	5	1	1	1	2	2	4	2	1	31
UR.BH.M.NHC.72-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 77-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.78-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.79-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 80-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 82-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.84-M	France	2	4	1	1	3	1	3	3	2	2
UR.BHM.M.NHC.128	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.129	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.130	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC 154	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 155	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 156	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 159	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 161	France	2	4	1	1	3	1	3	3	2	2
FR96/BK7	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK26II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK36	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK36II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK75	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK77	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK78	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK79	Germany	2	4	1	1	3	1	3	3	2	2
ZF-1	France	3	5	3	5	2	2	2	1	3	12
FIZZ	Switzerland	5	1	1	1	2	2	2	1	1	5
J1	Japan	5	2	6	5	2	2	2	1	1	35
J4	Japan	5	2	5	4	1	2	1	1	3	23
J5	Japan	8	2	5	4	1	2	2	1	3	32
J6	Japan	5	2	6	5	2	2	2	1	1	35
J7	Japan	5	2	6	5	2	2	2	1	1	35
J8	Japan	5	2	6	5	2	2	2	1	1	35
P1	Philippines	5	2	6	5	2	2	2	2	1	38
P2	Philippines	5	2	6	5	2	2	2	2	1	38
P4	Philippines	5	2	6	5	2	2	2	2	1	38
P5	Philippines	5	2	6	5	2	2	1	2	1	37
P6	Philippines	4	2	5	4	1	2	1	2	3	36
P7	Philippines	3	2	5	5	2	2	2	1	1	10
P8	Philippines	3	2	6	5	2	2	2	1	1	21
T1	Thailand	5	2	6	5	2	2	2	1	3	39
T3	Thailand	8	2	5	4	1	2	2	1	3	32
T5	Thailand	6	2	5	4	1	2	2	1	3	6
T6	Thailand	4	2	5	4	1	2	2	1	3	33
T7	Thailand	5	2	6	5	2	2	2	2	1	38
T8	Thailand	5	1	6	5	2	2	2	2	1	20

[a] The description of intergenic spacers S1 to S9 and the primers used for their amplification and sequencing are given in Table 2.
[b] USA, United States.