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Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) **102**, 70–74

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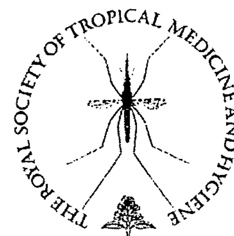

Seroepidemiological study on hantavirus infections in India

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Received 27 June 2007; received in revised form 20 September 2007; accepted 20 September 2007

KEYWORDS
Hantavirus;
Emerging pathogens;
Seroepidemiology;
Serologic tests;
Zoonoses;
India

Summary Hantaviruses are etiological agents of hemorrhagic fever with renal syndrome in many parts of Asia and Europe. There has been no documented case of hantavirus disease from India, although serological evidence exists. We investigated the prevalence of hantavirus in the Indian population and tried to identify potential risk groups for hantavirus infections. The presence of hantavirus-specific IgG antibodies was prospectively evaluated in 661 subjects belonging to different groups, i.e. patients with chronic renal disease, warehouse workers and tribal members engaged in rodent trapping. Healthy volunteer blood donors were included as a control group. Thirty-eight seropositive samples were found using a combination of a commercial ELISA followed by an indirect immunofluorescence assay. Western blot using recombinant Hantaan virus nucleocapsid antigen confirmed the presence of anti-hantavirus IgG in 28 (74%) of the 38 sera tested. This study confirms the presence of hantaviruses in India and warrants increasing awareness of the problems of emerging pathogens and the threats they may pose to the public health system.
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doi:10.1016/j.trstmh.2007.09.016

1. Introduction

Hantaviruses represent a group of emerging viruses. The genus *Hantavirus*, belonging to the family Bunyaviridae, comprises more than 20 species that can cause two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Lednicky, 2003). Almost all hantaviruses are maintained in rodents of the family Muridae, and are transmitted to humans via aerosolized urine, saliva and feces of infected rodents.

Although hantavirus infections were recognized in Asia for centuries, it was only during investigations initiated after the Korean conflict in the 1950s, during which thousands of UN soldiers were affected by HFRS, that the Hantaan virus (HTNV) serotype was isolated. The circulation of hantavirus serotypes, namely Seoul virus (SEOV) and Thailand virus (THAIV), has been demonstrated in several Southeast Asian countries, i.e. Thailand, Cambodia, Viet Nam and Indonesia (Plyusnina et al., 2004; Reynes et al., 2003; Truong et al., 2004). Recently a report from Thailand documented the first serological evidence of THAIV causing HFRS in humans (Pattamadilok et al., 2006). The Thottapalyam virus (TPMV), which was isolated from an insectivore, *Suncus murinus*, in 1964, is the only known hantavirus species indigenous to India (Cary et al., 1971).

The association of chronic renal disease and hantavirus seropositivity has frequently been speculated upon as studies conducted in the United States (Baltimore) suggested that hantavirus seropositives had higher rates of chronic renal disease and hypertensive renal disease than age-matched seronegative controls (Glass et al., 1990). Similar studies have been reported from Israel (George et al., 1998) and Egypt (Botros et al., 2004). An epidemiological study from Taiwan has reported detection of anti-hantavirus antibody in various risk groups such as garbage collectors and animal handlers (Chen et al., 1998).

Although the isolation of TPMV pre-dates that of HTNV, interest in hantaviruses was revived in India in 2005, with two reports on the serological evidence of hantavirus infections in patients with febrile illnesses (Chandy et al., 2005; Clement et al., 2006). However, seroepidemiological surveys have not been reported from India, and this study is the first attempt to investigate the epidemiology of hantavirus infections in India.

2. Materials and methods

2.1. Study population

The study subjects belonged to different groups and were recruited at the Christian Medical College, Vellore, south India. Healthy volunteer blood donors ($n = 360$) comprised the control group. The potential risk groups included 99 sera from asymptomatic Irulas, a tribal community living in Tamil Nadu, a state in south India (the tribe members are professional rat catchers and eat rats) and 51 sera from people working in warehouses (warehouse workers) in and around Vellore. An additional group was represented by 151 serum samples from patients with chronic renal disease with serum creatinine and urea levels of >1.4 mg/dl and >40 mg/dl, respectively.

The mean (±SD) age of the subjects in the Irula group was 32 (±11.2) years, and this group included 41 males and 58 females; in the warehouse workers it was 38 (±10.17) years and all were males; and in the renal disease patients it was 43 (±12.11) years and there were 47 females and 104 males. The subjects in the control group were aged 18–60 years.

The sample size was calculated based on a previous study of hantavirus infections in India (Chandy et al., 2005). The study period was from August 2004 to May 2007. Samples from healthy blood donors and patients with chronic renal disease were collected at the Department of Clinical Virology, Christian Medical College, Vellore and were included in the study by convenient sampling. All samples collected from asymptomatic Irulas and warehouse workers were included in the study. Written informed consent was obtained before collecting blood.

2.2. ELISA and immunofluorescence assay

Serological screening was done using a commercial (ISO certified) hantavirus IgG ELISA (Focus Technologies, Cypress, CA, USA) according to the manufacturer's instructions. This ELISA uses a cocktail of hantavirus antigens of HTNV, SEOV, Puumala virus (PUUV), Sin Nombre virus (SNV) and Dobrava-Belgrade virus (DOBV) to coat the polystyrene microwells and can detect IgG antibodies against these serotypes. The screening ELISA is an indirect test in which the optical density (OD) is directly proportional to the antigen-specific IgG antibodies present in the sample. The results were obtained by comparison of the sample OD readings with reference cut-off OD readings.

Results were reported as index values relative to the cut-off calibrator. To calculate index values, each sample OD value was divided by the mean of the cut-off calibrator OD values.

Sera positive by ELISA were re-tested by an indirect immunofluorescence assay (IFA) using HTNV-infected Vero E6 cells as antigens (Yoshimatsu et al., 1993). The secondary antibody used was fluorescein-isothiocyanate-conjugated rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark). For the IFA, all spots with at least half of the infected cells showing a characteristic apple green granular cytoplasmic fluorescence with $\geq$2+ intensity were scored positive.

Finally, a sample was considered positive if reactive by both ELISA and IFA. The screening ELISA and IFA were evaluated using a panel of positive and negative control sera kindly supplied by the European Network for Diagnostics of Imported Viral Diseases [ENIVD (Biel et al., 2003)].

2.3. Western blot analysis

Western blot analysis was performed on 38 positive sera using recombinant nucleocapsid protein (NP) of HTNV (Fojnica strain) as antigen (Razanskiene et al., 2004). Sera at dilutions of 1:1000 were applied to the membrane, and goat anti-human IgG conjugated with alkaline phosphatase (Genelabs Diagnostics Pte Ltd, Singapore Science Park, Singapore 118259, Republic of Singapore) at 1:1000

Table 1 Results of evaluation of the screening ELISA and Hantaan virus-immunofluorescence assay (IFA) using European Network for Diagnostics of Imported Viral Diseases (ENIVD) sera

ENIVD no.	Sample type	IgG level	Origin	Hantavirus strain	Serum status	ELISA result	IFA result
2	Positive serum	++	Sweden	Puumala	Convalescent	+	+
8	Positive serum	+	Kosovo	Dobrava	Convalescent	+	+
9	Positive serum	++	Sweden	Puumala	Convalescent	+	+
16	Positive serum	++	Sweden	Puumala	Convalescent	+	+
17	Positive serum	+	Finland	Puumala	Convalescent	–	–
4	Negative serum	–	Germany	–	Control	–	–
12	Negative plasma	–	Germany	–	Control	–	–
18	Negative serum	–	Germany	–	Control	–	–
19	Negative serum	–	Germany	–	Control	–	–

+, positive; ++, strong positive; –, negative.

dilutions was used as the secondary antibody. Thereafter the membrane was developed with substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). In the Western blot the molecular size of the expected product was about 49 kDa.

2.4. Statistical analysis

The statistical analysis was done using EpiInfo version 6.04b (CDC, Atlanta, GA, USA) to compare two categorical variables. Percentages were calculated for categorical outcomes (positives/negatives). A P-value <0.05 was considered significant.

3. Results

ENIVD sera were used to evaluate the commercial IgG ELISA and the IFA using HTNV antigen; these assays could detect anti-Puumala virus and anti-Dobrava virus IgG-positive sera. However, one ENIVD anti-Puumala virus serum (origin Finland) could not be detected by either of these assays (Table 1).

In the initial screening, 661 serum samples were tested by a commercial IgG ELISA (Table 2). Forty-seven of the 661 sera were found to be reactive in the ELISA. The majority (38/47) of the ELISA-reactive sera were also detected by IFA using HTNV antigen. Seropositivity in the Irula tribal group (11%) was significantly higher than in the control group (4%, $P < 0.05$). There was no statistically significant difference between seropositivity in the chronic renal disease patient group (7%) compared to that of the control group. The level of seropositivity in the warehouse workers (2%) was very similar to that of the control group. Sex as a demographic factor was not significantly associated with hantavirus infections in the different subject groups, suggesting that males and females are equally likely to contract hantavirus infections. The mean age of the seropositives in the renal disease patient group, the Irulas and the warehouse workers was 50 (SD ± 11.29), 29 (SD ± 8.9) and 31 years, respectively. Twenty-eight of the 38 positive sera (74%) were positive by Western blot (Table 2).

4. Discussion

Studies on hantavirus infections in India are in the early stages. The data given here represent the first attempt to characterize the epidemiology of hantavirus infections in India and strengthen previous reports on serological evidence of hantavirus infections in India (Chandy et al., 2005; Clement et al., 2006).

Forty-seven of 661 serum samples were positive by ELISA. Serology is the mainstay of diagnosis of hantavirus infections. ELISAs are highly sensitive and are the preferred diagnostic tool for serological surveys. The commercial ELISA used in this study uses a cocktail of six antigens, and can be used in areas where the circulating hantavirus species are unknown. The specificity of the hantavirus assays used in the study is acceptably good, as evaluated by the ENIVD-negative control sera. There may be problems with diagnostic sensitivity, as one ENIVD anti-Puumala IgG-positive serum was not detected by both the assays.

Table 2 Results of serological studies

Group tested	No. tested	No. positive by ELISA (%)	No. positive (ELISA and HTNV-IFA) (%)	No. positive/no. tested by Western blot (%)
Blood donors	360	19 (5)	16 (4)	11/16 (69)
Renal disease patient group	151	14 (9)	10 (7)	9/10 (90)
Irulas	99	12 (12)	11 (11)	7/11 (64)
Warehouse workers	51	2 (4)	1 (2)	1/1 (100)
Total	661	47 (7)	38 (6)	28/38 (74)

HTNV-IFA: Hantaan virus-immunofluorescence assay.

The lower seroprevalence observed in the IFA and Western blot analysis might be due to the HTNV antigen, which suggests that hantavirus species other than HTNV are circulating and causing human disease in India. Alternatively, we cannot exclude the possibility that the ELISA picked up false positives.

It has been documented that TPMV is phylogenetically and antigenically quite distinct from the other well-characterized hantaviruses (Song et al., 2007), and although antibodies against hantavirus NP are cross-reactive between different hantavirus species, we cannot speculate about the efficiency of the assays used in the study to detect antibodies against TPMV. Moreover, there may be other hantavirus species circulating in India that may be as diverse as TPMV, and in the case of an antigenic mismatch the assays used here may fail to detect seropositives. It is thus important to define the hantavirus species circulating in India and develop sensitive assays using homogeneous antigens.

In this study, the renal disease patient group appears to have a higher risk of hantavirus seropositivity compared with the control group, but the difference is not statistically significant. It is still not clear whether patients with chronic renal disease are at a higher risk of acquiring hantavirus infections or that hantavirus infections by themselves contribute to the development of chronic renal disease. These results are preliminary, and follow-up studies are needed to prove any significant association of hantavirus infections with chronic renal disease.

The tribal group in this study has a relatively high level of contact with rodents, as they are traditionally rat catchers and also eat rats. They display a high seropositivity when compared with the control group. By contrast, warehouse workers showed a low seropositivity in our study (2%). This may reflect a lower risk of contracting hantavirus infections due to the fact that many warehouses in India adopt stringent rodent-control measures.

No well-documented hantavirus case, as defined by virus isolation or molecular evidence, has been reported from India to date. However, our study confirms that one or more hantaviruses are circulating in the Indian population and indicates that the threat from emerging pathogens must be continually assessed. Studies to identify the hantaviruses that might cause problems for public health systems are important, as they will aid the development of new strategies for the prevention and control of such emerging infections.

Authors' contributions: GS and PA designed the study protocol; SC carried out the immunoassays and drafted the manuscript; KY, RGU, MM, MO and JA supplied IFA slides and Western blot strips and helped analyse results obtained; RP, GTJ, VB, JM and JM helped with the identification of subjects in the various groups and collection of samples. All authors read and approved the final manuscript. GS is guarantor of the paper.

Acknowledgements: We acknowledge the contribution of the European Network for the Diagnostics of Imported Viral Diseases (ENIVD) for kindly supplying samples of the first hantavirus External Quality Assurance (EQA) program.

Funding: This study was jointly funded by intramural research funds of the Christian Medical College, Vellore, Tamil Nadu, India (R.C. Min. No. 5230 dated 16 September 2003) and by a grant from the Indian Council for Medical Research (ICMR), New Delhi, India (Reference No./DO. No. 5/8/7/23/2004-ECD-1).

Conflicts of interest: None declared.

Ethical approval: The institutional research ethics committee of the Christian Medical College, Vellore, Tamil Nadu, India (R.C. Min. No. 5838 dated 21 February 2006) and the Indian Council for Medical Research (ICMR), New Delhi, India.

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Arch Virol (2008) 153:1605–1609
DOI 10.1007/s00705-008-0156-0

BRIEF REPORT

Lack of vertical transmission of Hantaan virus from persistently infected dam to progeny in laboratory mice

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Received: 22 June 2007 / Accepted: 9 June 2008 / Published online: 9 July 2008
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Abstract It is unclear how the hantaviruses are transferred from infected to uninfected rodents. We studied the status of persistently infected laboratory mice and examined the frequency of viral transmission to their offspring. Expression of Hantaan virus nucleocapsid protein was detected in the lungs of persistently infected dams. None of the progeny displayed viral antigen, although they were strongly positive for IgG antibodies against hantavirus. There was neither hantavirus RNA nor virus-specific IgM antibodies or virus-specific $CD8^+$ T cells in the progeny. These results did not show any indication for a vertical transmission of hantaviruses, at least in the laboratory mouse model studied.

Hantaviruses comprise the genus *Hantavirus* in the family *Bunyaviridae*. Although hantaviruses cause two serious and often fatal human diseases, viz. hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), their natural rodent hosts present no obvious clinical signs of infection. Instead, they carry the virus for long periods as reservoir animals and shed the virus into excreta such as urine, feces, and saliva [16]. An age-dependent increase in the infection rate was recently identified by epizootiological surveillance in a rodent colony [4, 6], suggesting that these viruses are maintained by horizontal transmission through close contact between adult rodents and not by vertical transmission to neonates from dams.

Several studies have demonstrated the protective effect of hantavirus-specific antibodies in rat fetuses from or neonates born to immune mothers. Neonates that received a lethal dose of Seoul virus (SEOV) strains B-1 or SR-11 intraperitoneally within 24 h or at 2 days after birth to immune dams survived [10, 23]. These results imply that the neonates were protected against infection by transfer of maternal anti-hantavirus antibodies. However, these reports did not address whether the mother rats were persistently infected during pregnancy and the nursing period. In addition, given the difficulty of analyzing young animals in nature, the existence of vertical transmission from persistently infected animals to their offspring remains unclear.

There are several animal models of persistent infection involving hantaviruses and their natural reservoir species of rodents, including SEOV-infected rats [7, 19], Hantaan virus (HTNV)-infected *Apodemus* mice [15], Black Creek Canal virus-infected cotton rats [12], and Sin Nombre virus-infected deer mice [5]. These model rodents harbor virus antigen for a long time without signs of disease, as wild rodents do. However, since there is little genetic information on natural rodent species, it has often been difficult to analyze the mechanism of persistent infection genetically. Previously, we established a persistent infection model in laboratory mice. Viral antigen was detected in the lungs of the animals until 90 days after infection without signs of disease [2], and the retention time of the viral antigen was dependent on their age at inoculation [3]. A weak antigen-specific cytotoxic T lymphocyte (CTL) response was detected in these laboratory mice following infection [2, 20]. Although laboratory mice are not the natural reservoir, the persistently infected mice mimic the natural reservoir hosts in important aspects: (1) they have

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large amounts of virus antigen in their lungs [2], (2) they do not show obvious signs of disease in the persistent infection phase [20], and (3) the virus is not eliminated, although persistently infected mice strongly express neutralizing antibodies (Table 1). Furthermore, an abundance of background information is available on experimental laboratory mice. Therefore, the experimental mouse model is useful for detailed analyses, particularly immunological characterization. In this study, we followed the status of persistently infected mice and analyzed their offspring for potential vertical transmission of HTNV infection.

Pregnant BALB/c/Slc mice and Slc:ICR mice were obtained from SLC (Hamamatsu, Japan). All animal experiments in this study were carried out under the guidance of the Hokkaido University Animal Research Committee and in accordance with their guidelines, performed in a BSL3 facility. For the production of persistently infected mice, BALB/c mice were subcutaneously inoculated with 1.3 focus-forming units (FFU) of HTNV strain 76-118 within 24 h of birth [1.3 FFU = 0.1 $NMLD_{50}$ (50% newborn mouse lethal dose)]. A total of 17 mice were examined at 1–12 weeks after infection. The antibody response in these animals was evaluated, as shown in Table 1. High HTNV-specific IgG antibody titers (titers $\geq$ 5,120) were observed at 4, 8, and 12 weeks after infection. The IgG antibodies were detected even at 23 weeks after infection (data not shown). Similarly, IgM antibodies (titer: 80–320) were continuously observed at least until 12 weeks post-infection. Neutralizing antibodies (titer: 320–640) were also observed in all animals at all investigated time points. In addition, virus was isolated from the brains or lungs of the mice until 12 weeks post-infection, although the isolation rate decreased slightly. Viral antigen in the lung started to disappear gradually from 8 to 15 weeks post-infection and completely disappeared 20 weeks post-infection (data not shown). The disappearance of viral antigen seemed to be correlated with a reduced viral load that is reflected in an increasing number of negatives in the bioassay used (Table 1). In contrast, adult mice that were intraperitoneally inoculated with 10^5 FFU of HTNV for the production of transiently infected mice showed HTNV-specific IgM antibodies only 1 week after infection (titer: 160–320, $n = 6$ mice), and the titer quickly dropped within 3 weeks after infection (titer: <40, $n = 6$). The titers of HTNV-specific IgG antibodies increased until 3 weeks after infection in these transiently infected mice (Table 1) and, usually, neutralizing antibodies also increased until 3 weeks after infection [2, 21].

Recently, we reported that in the persistent infection model, mice had a reduced number of virus-specific CTLs in their spleen, so that viral elimination was delayed [20]. It has been reported that CTLs are regulated by other immune cells, for example dendritic cells, or inflammatory

Table 1 Analysis of mice immunized with a sublethal dose of HTNV by IFA and FRNT, and virus isolation

Weeks after infection	No.	IFA titers[a]		FRNT titers[b]	Bioassay[c]	
		IgG	IgM		Brain	Lung
New born 1 week	1	<40	80	NT	NT	NT
	2	<40	160	NT	NT	NT
New born 4 weeks	1	10,240	160	640	+	+
	2	5,120	160	640	+	+
	3	5,120	80	320	−	+
	4	5,120	160	640	+	+
	5	5,120	160	320	−	−
	6	5,120	160	640	−	+
New born 8 weeks	1	10,240	160	320	+	+
	2	10,240	160	320	−	+
	3	5,120	320	640	−	−
	4	10,240	320	640	−	+
	5	20,480	320	320	+	+
New born 12 weeks	1	10,240	160	640	−	−
	2	10,240	320	320	−	−
	3	10,240	160	640	+	+
	4	10,240	160	320	−	+
Adult 1 week	1	80	320			
	2	80	160			
	3	80	160			
	4	160	320			
	5	80	160			
	6	80	160			
Adult 3 weeks	1	640	<40			
	2	640	<40			
	3	5,120	<40			
	4	640	<40			
	5	640	<40			
	6	320	<40			

Seventeen newborn mice (within 24 h after birth) and twelve 5-week-old mice were inoculated with HTNV. They were sacrificed at various time points

NT not tested

[a] Indirect immunofluorescent antibody (IFA) tests were performed using acetone-fixed smears of Vero E6 cells infected with HTNV 76-118 as antigen. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG [IgA + IgG + IgM(H + L)] or anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody. The IFA titers are expressed as the reciprocal of the highest dilution of antiserum that resulted in specific fluorescence

[b] FRNT was performed as described previously [1]. The neutralizing antibody titer was defined as the highest serum dilution that resulted in greater than 80% reduction in the number of infected cell foci

[c] The brains and lungs were removed from HTNV-infected mice. Groups of two or three newborn ICR mice were inoculated subcutaneously with 50 μl of the homogenates. The presence of the virus was assessed by seroconversion of the mice at 4 weeks after inoculation

+, all newborn ICR mouse sera were positive for HTNV. −, all newborn ICR mouse sera were negative for HTNV

cytokines after infection [11]. We suspected such a reduction in CTLs in persistent hantavirus infection in mice was also involved in these immune cells. Therefore, we have examined the infectivity to such as immune cells in spleen by histological study. Mice were anesthetized and perfused intracardially with 4% paraformaldehyde (PFA) in phosphate buffer (PB, pH 7.4), followed by brief perfusions with sucrose [13]. The spleens and lungs were stained with the Alexa Fluor 488-labeled monoclonal antibody (MAb) E5/G6 [18]. Nuclear staining was achieved using TOTO3 (Molecular Probes). Images were obtained by confocal microscopy (FLUOVIEW FV1000; Olympus, Japan). We detected N antigen in lung and spleen tissue at 2 weeks after infection in this persistent infection model. We detected high levels of N antigen in the intra-alveolar septum region (Fig. 1a), alveolar macrophages (Fig. 1b), and the elastic fiber region (Fig. 1c) of the lungs at 2 weeks after infection. Even though most of the antigen had disappeared at 8 weeks after infection, antigen remained in the intra-alveolar septum region of the lungs (data not shown). We detected N-antigen-positive cells in the marginal zone of each spleen (Fig. 1d). Generally, immune cells are distributed in the spleen marginal zone after pathogen invasion. Therefore, we have to identify N-positive cells to know whether these cells were

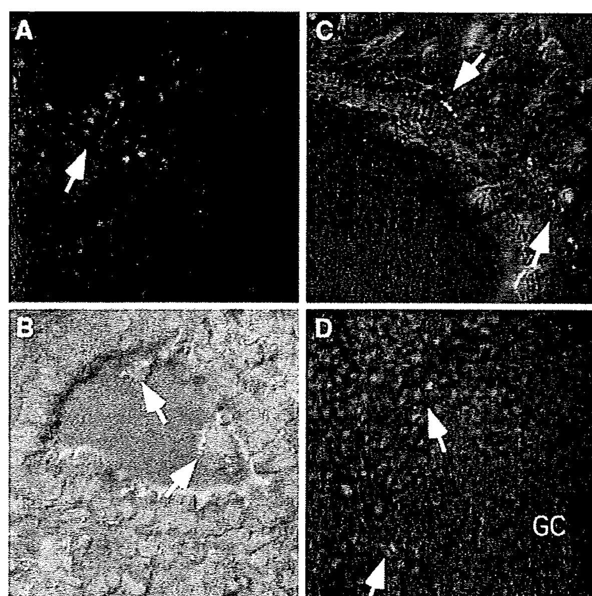

Fig. 1 Immunohistochemical analysis of persistently infected mice. Persistently infected mice were sacrificed 2 weeks after infection. The lungs and spleen were removed, fixed, sectioned, and stained with E5G6 antibody (*green*) and TOTO3 (*red*). **a** N-positive cells are observed in the intra-alveolar septum region of the lung. **b** Infected alveolar macrophages are observed in the lung. **c** Clustered N antigen is detected in the elastic fibers of the bronchiole. **d** N-positive cells are shown localized in the marginal zone of the spleen. Magnification: **a** ×20, **b** ×60, **c** ×180, **d** ×60

involved in the regulation of CTLs in persistently infected mice.

Finally, to examine the possibility of vertical transmission from persistently infected mice to their progeny, three pairs of these mice were mated, the offspring and dams were sacrificed at various time points (Fig. 2a), and tissues were examined by Western blotting and RT-PCR. We detected hantavirus N antigen and RNA in the dams' lungs (12 weeks old, $n = 2$); however, no antigen or RNA was detected in their progeny (10 days old; Fig. 2b, c, 4 weeks old; data not shown, $n = 6$). Although a high level of IgG antibody [immunofluorescent antibody (IFA) titer: 5,120] was observed until 28 days after birth and the mice maintained low IgG antibody levels until 90 days (data not shown), we could not observe IgM antibody in the infants (10 days old, 4 weeks old) even though their dams (12 weeks old, $n = 2$) had IgM antibody (Fig. 2d, data not shown). This indicates that the HTNV-specific IgG antibodies were maternally derived. To confirm a lack of infection in the infants derived from and nursed by the persistently infected dams, we used FACS analysis to prove the presence of HTNV-specific $CD8^+$ T cells. We used flow cytometry to assay the cytokines produced by $CD8^+$ T cells incubated with HTNV-infected antigen-presenting cells [20]. Antigen-negative 3-week-old (progeny 1,2) and 20-week-old (progeny 3,4) offspring from a 17-week-old HTNV-antigen-positive persistently infected dam were analyzed. No HTNV-specific IFN-γ^+ $CD8^+$ T cells were found in any of the progeny (Fig. 2e), suggesting that none of the offspring had ever been infected with HTNV. These data are in agreement with those from previous rodent studies [8, 14, 17].

The transfer of maternal antibodies from mother to progeny is well known in avian and mammalian species and is believed to protect the newborn against pathogens in the environment. Several reports have described this phenomenon for hantavirus infection [8, 14, 23]; however, there is a scarcity of studies examining the vertical transmission of hantaviruses in wild-living rodents that are persistently infected [15, 17]. To examine vertical transmission from persistently infected dams to their progeny, we studied the progeny of persistently HTNV-infected mice by Western blotting, RT-PCR, FACS analysis, and IFA. The progeny had no HTNV-antigen in their lungs, no virus-specific CTLs, and no anti-HTNV IgM antibodies, although they maintained anti-HTNV IgG antibodies until 90 days after birth. These results indicate that the progeny of persistently infected laboratory mice are immune to HTNV and that vertical transmission of HTNV from persistently infected dams to their offspring does not occur. Thus, these results suggest that the virus is transmitted horizontally in nature, from rodent to rodent, and several reports have suggested that a high dose is required for

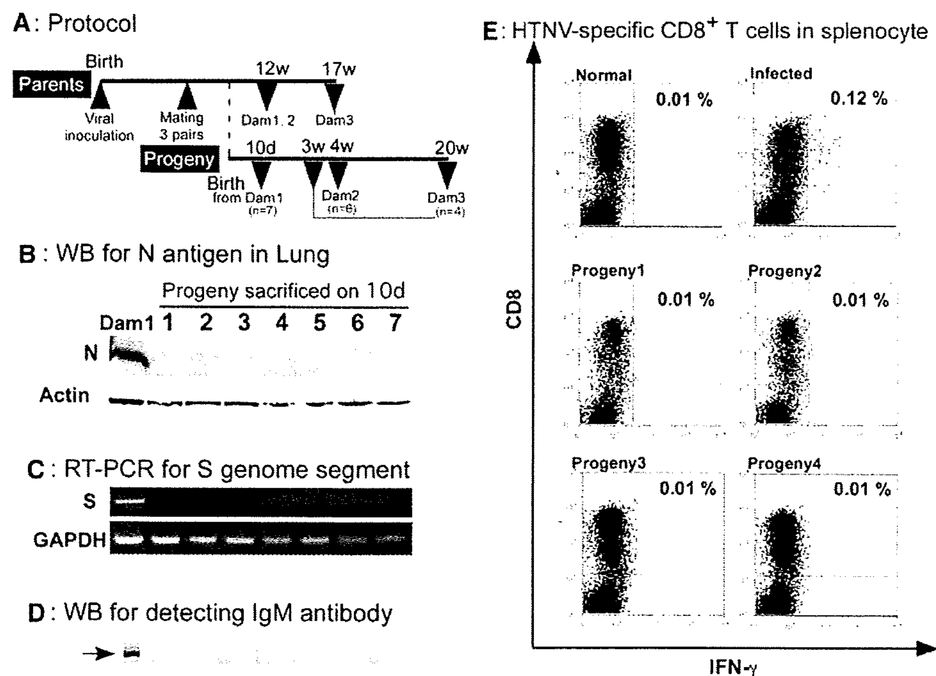

Fig. 2 Analysis of progeny from persistently infected mice. Daml possessed both N antigen and viral genomic RNA in lung, and its seven progenies lacked both viral antigen and RNA in lung. The same results were obtained from *dam2* and *dam3* and their six or four progenies (data not shown). **a** Experimental protocol. Three pairs of BALB/c parents were mated and analyzed at the time point indicated in *lane 1*; their newborns were analyzed at the time point indicated in *lane 2*. **b** Western blot (*WB*) analysis of lung lysates from persistently HNTV-infected mice and their newborn offspring [3]. The MAbs E5/G6 and ECO2 were used to detect the membrane-bound antigens [22]. Horseradish peroxidase (*HRP*)-conjugated goat anti-mouse IgG (Zymed Laboratories) was used as a secondary antibody. Actin, the control protein, was detected using mouse anti-β actin antibody (Sigma). **c** RT-PCR analysis of lungs from HNTV-infected mice and their newborn offspring to detect trace amounts of virus RNA. Lungs were homogenized and isolated by ISOGEN (NIPPON GENE). The isolated RNA was reverse transcribed using random hexamer and SuperScript II (Invitrogen). Each cDNA was amplified with hantavirus-specific primers MurS110F [5′CAGAAGGTIAIGGATGCAGA3′] and MurS1160R [5′TGGTCCAGTTGTATRCCCAT3′] or control GAPDH primers [5′TGCACCACCAACTGCTTAG3′, 5′GGATGCAGGGATGATGTTC3′]. **d** Detection of IgM antibodies by WB. Recombinant HTNV N derived from high five cells was used [1]. Sera from the progeny and their persistently infected dam were used at a 1:10 dilution. Secondary antibody is HRP-anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA). **e** Immune responses of IFN-γ-producing HTNV-specific CD8$^+$ T cells in progeny (from *dam3*). Spleen cells were cultured with HTNV-infected P388D1 cells. The cells were stained with ethidium monoazide bromide (*EMA*) (Invitrogen), anti-CD8a PE (Ly-2) antibodies (eBioscience, San Diego, CA, USA), and FITC-conjugated rat anti-mouse gamma interferon (IFN-γ) antibody (Caltag Laboratories, San Francisco, CA). IFN-γ^+ cells were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) with the gates set for EMA-negative cells. The data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA). "*Infected*" indicates that the adult BALB/c mouse inoculated with HTNV recovered (positive control). "*Normal*" denotes a normal BALB/c mouse (negative control)

horizontal transmission between rodents in experimentally persistently infected animals [9, 15]. Efficient transmission may depend on the immune status influenced by infections with other pathogens of a different nature. Here, we found that vertical transmission from persistently infected laboratory mice does not occur. To prove the absence of vertical transmission of hantaviruses, further studies should be dedicated to the influence of the load and tissue distribution of hantavirus in persistently infected dams of laboratory mice and natural rodent hosts.

Acknowledgments We thank Dr. H. Sawa and Dr. T. Kimura for their assistance with the confocal immunofluorescence microscope and cryostat. Takako Shibuya is thanked for technical help. This work was supported in part by a grant from the 21st Century COE Program of Excellence for Zoonosis Control and in part by Grants-in-Aid for Scientific Research and the Development of Science from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Veterinary Microbiology 129 (2008) 404–409

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Short communication

Virulence characteristics of *Yersinia pseudotuberculosis* isolated from breeding monkeys in Japan

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Received 6 August 2007; received in revised form 20 November 2007; accepted 28 November 2007

Abstract

Between April 2001 and 2007, 18 *Yersinia pseudotuberculosis* outbreaks occurred in breeding monkeys at 12 zoological gardens in Japan, and 28 monkeys of 8 species died. A total of 18 *Y. pseudotuberculosis* strains from the dead monkeys, comprising one strain per outbreak, were examined for serotype and the presence of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. Of the 18 *Y. pseudotuberculosis* strains, 7 (38.9%) were serotype 4b, 7 (38.9%) were serotype 1b, and there was one each of serotypes 2b, 3, 6 and 7. All the 18 strains examined harbored *virF* and *inv*. Sixteen (88.9%) strains, including the strain of serotype 7, harbored *ypmA*. However, no strain harbored *ypmB*, *ypmC* and *irp2*.

This study demonstrated that among other pathogenic factors, almost all the *Y. pseudotuberculosis* isolated from the outbreaks had the *ypm* gene encoding the superantigenic toxin, YPM. As most of the monkeys who died in those outbreaks originated from South America and other regions, where the presence of the *ypm* gene have not been reported, YPM might be the cause, or at least the most important factor for, the high mortality of the breeding monkeys infected by *Y. pseudotuberculosis* in Japan. This is also the first report of a fatal case due to *Y. pseudotuberculosis* serotype 7 infection in the world.
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Keywords: *Yersinia pseudotuberculosis*; Breeding monkey; Virulence genes; YPM

1. Introduction

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. Monkey species are especially sensitive to *Y. pseudotuberculosis*, and many fatal cases of *Y. pseudotuberculosis* infection in

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doi:10.1016/j.vetmic.2007.11.029

breeding monkeys have been reported throughout the world, including in Japan (Buhles et al., 1981; Hirai et al., 1974; Kageyama et al., 2002; MacArthur and Wood, 1983; Maruyama et al., 1983; Murata and Hama, 1992; Rosenberg et al., 1980; Sasaki et al., 1996; Taffs and Dunn, 1983; Une et al., 2003). Affected monkeys may die unexpectedly or after a very short illness, and at the present time there is no effective preventive method against *Y. pseudotuberculosis* infection. Therefore, monkey *Y. pseudotuberculosis* infection poses a serious problem for zoological gardens engaged in monkey breeding.

The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors. Pathogenic strains of *Y. pseudotuberculosis* harbor 70-kb virulence plasmid (pYV), which encodes a number of important virulence and virulence-associated proteins. Additionally, a high-pathogenicity island (HPI), encoding an iron uptake system represented by its siderophore yersiniabactin (Carniel, 1999), and *Y. pseudotuberculosis*-derived mitogen (YPM), which is a superantigenic toxin, are known to play important roles in causing severe systemic infection (Abe et al., 1997). However, it remains unclear which virulence factor is connected with the high mortality of monkeys in *Y. pseudotuberculosis* infection. In the present study, we investigated the characteristics of *Y. pseudotuberculosis* isolated from dead breeding monkeys in Japan.

2. Materials and methods

2.1. Bacterial strains

Eighteen *Y. pseudotuberculosis* strains isolated from monkeys that died in 18 outbreaks (one strain per outbreak) were analyzed. These outbreaks occurred between April 2001 and 2007 at 12 zoological gardens (A–L) in Japan, and a total of 28 monkeys of 8 species, comprising 19 squirrel monkeys (*Saimiri sciureus*), 2 hamadryas baboons (*Papio hamadryas*), 2 white-faced sakis (*Pithecia pithecia*), 1 agile gibbon (*Hylobates agilis*), 1 dusky leaf monkey (*Presbytis obscurus*), 1 orangutan (*Pongo pygmaeus*), 1 ring-tailed lemur (*Lemur catta*) and 1 ruffed lemur (*Varecia variegata*), died (Table 1). Pathological findings such as swelling of the Peyer's patch and abscesses in the spleen and liver were typical of yersiniosis. Outbreaks occurred two, three and four times in the zoological gardens C, H and G, respectively.

Table 1
Sources of *Y. pseudotuberculosis* isolated from breeding monkeys in Japan

No.	Strain	Institution	Region	Isolation month year	Source (number and species of other monkeys dead in the same outbreak)
1	NP011001	A	Kanto	April 2002	Squirrel monkey
2	NP031103	B	Kanto	November 2003	Orangutan
3	NP031101	C	Kanto	November 2003	Squirrel monkey (1 squirrel monkey)
4	NP050101	C	Kanto	January 2005	Squirrel monkey
5	NP070401	D	Kanto	April 2007	Dusky leaf monkey
6	NP031201	E	Kinki	December 2003	Squirrel monkey (2 squirrel monkeys)
7	NP040301	F	Chugoku	March 2004	Squirrel monkey
8	NP010401	G	Sikoku	April 2001	Squirrel monkey
9	NP030401	G	Sikoku	April 2003	Squirrel monkey
10	NP050102	G	Sikoku	January 2005	Squirrel monkey
11	NP051201	G	Sikoku	December 2005	Squirrel monkey
12	NP020501	H	Kyusyu	May 2002	Squirrel monkey
13	NP030601	H	Kyusyu	June 2003	Squirrel monkey
14	NP070201	H	Kyusyu	February 2007	Squirrel monkey
15	NP030701	I	Kyusyu	July 2003	Squirrel monkey (1 squirrel monkey)
16	NP050201	J	Kyusyu	February 2005	Hamadryas baboon (1 hamadryas baboon and 1 agile gibbon)
17	NP050301	K	Kyusyu	March 2005	Squirrel monkey (1 squirrel monkey)
18	NP050303	L	Kyusyu	March 2005	White-faced saki (1 white-faced saki, 1 ruffed lemur and 1 ring-tailed lemur)

2.2. Isolation and identification of Y. pseudotuberculosis

The samples (liver and spleen) collected from the dead monkeys were homogenized or suspended in phosphate-buffered saline (PBS: 7.2), and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (Fukushima et al., 1990). These PBS suspensions were incubated at 4 °C for 3 weeks and then subcultured on IN agar plate after alkali (KOH) treatment (Aulisio et al., 1980). The plates were incubated at 25 °C for 48 h. Colonies morphologically similar to those of *Yersinia* spp. were subcultured on trypticase soy agar (TSA) (BBL, Sparks, MD, USA) and submitted for biochemical examination for identification, as described elsewhere (Wauters et al., 1988).

2.3. Serotyping

Serotyping of *Y. pseudotuberculosis* isolated from the monkeys was performed by slide agglutination with a commercial rabbit anti-*Y. pseudotuberculosis* sera set (Denka-Seiken Co., Tokyo, Japan), and with the rabbit immune sera made in our laboratory. Additional serotyping was performed by PCR as described by Bogdanovich et al. (2003).

2.4. PCR detection of virulence genes

Six sets of primers, designed in Table 2, were used for detection of the virulence genes *virF*, *inv*, *ypm* (*ypmA*, *ypmB* and *ypmC*) and *irp2*. The *virF* and *irp2* genes were used as the markers for the presence of pYV and HPI, respectively. Chromosomal DNA for PCR was isolated with a Wizard Genomic DNA Purification Kit (Promega Co., Madison, WI, USA) following the manufacturer's instructions. PCRs were performed in 50 μl volumes containing 5 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer, 3 mM $MgCl_2$, 0.1 μM of each primer, and 0.5 U of Taq DNA polymerase (Promega Co., Madison, WI, USA). The PCR amplifications were carried out at 94 °C for 5 min as an initial denaturation step and then subjected to 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C for detection of *virF*, *inv*, *ypmA* and *irp2*, or at 52 °C for detection of *ypmB*, or at 49 °C for *ypmC* (Table 2), 1 min at 72 °C, followed by a final 5 min extension step at 72 °C. Amplifications were performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan). Ten microliters of the PCR amplification products were subjected to electrophoresis in a 1.5% agarose gel. A 1-kb PLUS DNA Ladder (Invitrogen Co., Carlsbad, CA, USA) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

3. Results

3.1. Serotyping of Y. pseudotuberculosis strains

By slide agglutination, 7 (38.9%) strains of the 18 were serotype 4b, 7 (38.9%) were serotype 1b, and

Table 2
Primers for PCR detection of virulence genes

Virulence factor	Taget gene	Sequence (5′–3′)	Annealing temperature (°C)	Size of product (bp)	Reference
pYV	*virF*	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	55	590	Wren and Tabaqchali (1990)
Inv	*inv*	TAAGGGTACTATCGCGGCGGA CGTGAAATTAACCGTCACACT	55	295	Nakajima et al. (1992)
YPMa	*ypmA*	CACTTTTCTCTGGAGTAGCG GATGTTTCAGAGCTATTGTT	55	350	Ito et al. (1995)
YPMb	*ypmB*	TTTCTGTCATTACTGACATTA TTTCTGTCATTACTGACATTA	52	453	Ramamurthy et al. (1997)
YPMc	*ypmA* and *ypmC*	ACACTTTTCTCTGGAGTAGCG ACAGGACATTTCGTCA	49	418	Carnoy and Simonet (1999)
HPI	*irp2*	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	55	280	Schubert et al. (1998)

there was one each of serotypes 2b, 3, 6 and 7. *Y. pseudotuberculosis* serotype 7 has not been isolated from clinical samples in humans or in animals, and thus PCR-based serotyping was used to eliminate any doubt about the serotype of strain NP030601, which was identified as serotype 7 by the slide agglutination. The PCR result of strain NP030601 matched with the above condition for serotype 7 (data not shown), eliminating any doubt about the serotype of this strain. The results of the PCR-based serotyping of the other 17 strains also matched with those of the slide agglutination (data not shown). All the *Y. pseudotuberculosis* strains isolated from the monkeys who died in the same outbreak were of the same serotype of strains chosen for analysis in this study.

3.2. Detection of virulence genes in Y. pseudotuberculosis strains

All strains were *inv* and *virF* positive, and 16 (88.9%) of the 18 strains were *ypmA* positive by PCR. Of the 2 *ypmA* negative strains, one was serotype 4b, and another was serotype 3. On the other hand, all strains were *ypmB*, *ypmC* and *irp2* negative (Table 3).

Table 3
Characteristics of *Y. pseudotuberculosis* isolated from breeding monkeys

No.	Virulence genes						Serotype
	virF	*inv*	*ypm*			*irp2*	
			ypmA	*ypmB*	*ypmC*		
1	+	+	+	−	−	−	4b
2	+	+	+	−	−	−	4b
3	+	+	+	−	−	−	4b
4	+	+	+	−	−	−	4b
5	+	+	+	−	−	−	1b
6	+	+	+	−	−	−	4b
7	+	+	+	−	−	−	4b
8	+	+	+	−	−	−	1b
9	+	+	+	−	−	−	6
10	+	+	+	−	−	−	1b
11	+	+	+	−	−	−	2b
12	+	+	−	−	−	−	4b
13	+	+	+	−	−	−	7
14	+	+	+	−	−	−	1b
15	+	+	+	−	−	−	1b
16	+	+	−	−	−	−	3
17	+	+	+	−	−	−	1b
18	+	+	+	−	−	−	1b

+: PCR positive; −: PCR negative.

4. Discussion

In the present study, the predominant serotypes of *Y. pseudotuberculosis* isolated from dead monkeys were serotypes 1b and 4b. In Japan, these serotypes have also been the predominant serotypes isolated from clinical samples, for example, of human patients, and the majority of the strains of these serotypes are highly pathogenic, with the *ypmA* (Fukushima et al., 2001). In the present study, almost all of the strains isolated from dead monkeys also had *ypmA* genes. It is known that the presence of the *ypmA* is pretty much limited to the Far East (Japan, Korea and Far-Eastern Russia), and also that it exacerbates the toxicity of *Y. pseudotuberculosis* in systemic infection in mice (Fukushima et al., 2001). Moreover, it has been reported that the clinical signs of *Y. pseudotuberculosis* infection found in the Far East include not only fever, gastroenteric symptoms, and mesenteric lymphadenitis, which are the main symptoms in Europe, but also a variety of systemic manifestations such as rash, desquamation, erythema nodosum and arthritis (Sato et al., 1983). In zoological gardens in Japan, a variety of primates are bred, including monkey species from South America, Southeast Asia or Africa, listed in Table 1, as well as the Japanese macaque (*Macaca fuscata*). It has been noted that monkeys from those regions, where the presence of *Y. pseudotuberculosis* with the *ypm* gene has not been identified, frequently die when infections with this pathogen occur, while there has been little mortality of Japanese macaques due to *Y. pseudotuberculosis* infection (Kageyama et al., 2002). Because of the persistent exposure of the Japanese macaque to *Y. pseudotuberculosis* with the *ypm* gene from ancient times they may have acquired resistance to that pathogen, unlike the imported monkeys. Thus, YPM seems to be the main cause of the high mortality of the monkeys imported from abroad.

This is the first report of isolation of *Y. pseudotuberculosis* serotype 7 from a clinical sample anywhere in the world. This serotype has been isolated from dogs, raccoon dogs, moles, wild mice and water. However, there have been no reports about *Y. pseudotuberculosis* serotype 7 isolated from samples of primate origin. Pathological analysis of the squirrel monkey, from which the serotype 7 were isolated, showed swelling of the spleen and liver and multiple

white abscesses in the spleen, and the PCR analysis demonstrated that the strain of serotype 7 also harbored pYV and *ypmA* genes. These results possibly suggest that the strain serotype 7 isolated in the present study has the same degree of pathogenicity as the other pathogenic serotypes. Therefore, we should pay attention to the possibility of humans and other animal species infected by serotype 7.

Many monkey species kept at zoological gardens are formally recognized as "threatened" by The World Conservation Union (IUCN), and their deaths pose a serious loss to the zoological gardens involved. Thus, preventive measures against *Y. pseudotuberculosis* infection in breeding monkeys should be established as soon as possible. However, most breeding monkeys kept at zoological gardens are maintained in outdoor cages or enclosures for exhibition. These conditions lead to the exposure of the monkeys to animals living in the wild, such as birds and rodents, and as *Y. pseudotuberculosis* is widely distributed in wild animals, the probability of transmission of this pathogen from those animals is very high. Moreover, it is very difficult to completely prevent wild animals from invading the cages of the monkeys, and thus the foods and water provided for the monkeys can easily become contaminated. Therefore, development of effective vaccines is important for preventing pathogenic *Y. pseudotuberculosis* infection in breeding monkeys.

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Molecular phylogeny of a newfound hantavirus in the Japanese shrew mole (*Urotrichus talpoides*)

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Communicated by Ralph M. Garruto, Binghamton University, Binghamton, NY, September 10, 2008 (received for review August 8, 2008)

Recent molecular evidence of genetically distinct hantaviruses in shrews, captured in widely separated geographical regions, corroborates decades-old reports of hantavirus antigens in shrew tissues. Apart from challenging the conventional view that rodents are the principal reservoir hosts, the recently identified soricid-borne hantaviruses raise the possibility that other soricomorphs, notably talpids, similarly harbor hantaviruses. In analyzing RNA extracts from lung tissues of the Japanese shrew mole (*Urotrichus talpoides*), captured in Japan between February and April 2008, a hantavirus genome, designated Asama virus (ASAV), was detected by RT-PCR. Pairwise alignment and comparison of the S-, M-, and L-segment nucleotide and amino acid sequences indicated that ASAV was genetically more similar to hantaviruses harbored by shrews than by rodents. However, the predicted secondary structure of the ASAV nucleocapsid protein was similar to that of rodent- and shrew-borne hantaviruses, exhibiting the same coiled-coil helix at the amino terminus. Phylogenetic analyses, using the maximum-likelihood method and other algorithms, consistently placed ASAV with recently identified soricine shrew-borne hantaviruses, suggesting a possible host-switching event in the distant past. The discovery of a mole-borne hantavirus enlarges our concepts about the complex evolutionary history of hantaviruses.

host switching | talpid | evolution | Japan

Dating from investigations conducted independently by Japanese and Russian medical scientists along opposite sides of the Amur River in the 1930s and 1940s, rodents have been suspected to harbor the etiological agent(s) of hemorrhagic fever with renal syndrome (HFRS) (1, 2). After a several decades-long impasse, the striped field mouse (*Apodemus agrarius*) was identified as the reservoir host of Hantaan virus (3), the prototype virus of HFRS (4). This seminal discovery made possible the identification of genetically distinct hantaviruses in other murinae and arvicolinae rodent species (5–12). Also, a previously unrecognized, frequently fatal respiratory disease, called hantavirus pulmonary syndrome (HPS) (13), is now known to be caused by hantaviruses harbored by neotominae and sigmodontinae rodents in the Americas, the prototype being Sin Nombre virus (SNV) in the deer mouse (*Peromyscus maniculatus*) (14). Remarkably, each of these hantaviruses appears to share a long coevolutionary history with a specific rodent host species. That is, based on phylogenetic analyses of full-length viral genomic and rodent mitochondrial DNA (mtDNA) sequences, these hantaviruses segregate into clades, which parallel the evolution of rodents in the murinae, arvicolinae, neotominae, and sigmodontinae subfamilies (15, 16).

Until recently, the single exception to the strict rodent association of hantaviruses was Thottapalayam virus (TPMV), a long-unclassified virus originally isolated from the Asian house shrew (*Suncus murinus*) (17, 18). Analysis of the recently acquired full genome of TPMV strongly supports an ancient non-rodent host origin and an early evolutionary divergence from rodent-borne hantaviruses (19, 20). Employing RT-PCR and oligonucleotide primers based on the TPMV genome, we have targeted the discovery of hantaviruses in shrew species from widely separated geographical regions, including the Chinese mole shrew (*Anourosorex squamipes*) from Vietnam (21), Eurasian common shrew (*Sorex araneus*) from Switzerland (22), northern short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex cinereus*), and dusky shrew (*Sorex monticolus*) from the United States (23, 24) and Ussuri white-toothed shrew (*Crocidura lasiura*) from Korea (J.-W. Song and R. Yanagihara, unpublished observations). Many more shrew-hantavirus associations undoubtedly exist, as evidenced by preliminary studies of *Sorex caecutiens* and *Sorex roboratus* from Russia (H. J. Kang, S. Arai and R. Yanagihara, unpublished observations) and *Sorex palustris*, *Sorex trowbridgii*, and *Sorex vagrans* from North America (H. J. Kang and R. Yanagihara, unpublished observations).

In addition to challenging the view that rodents are the sole or principal reservoirs of hantaviruses, the discovery of soricid-borne hantaviruses predicts that other soricomorphs, notably talpids, might also harbor genetically distinct hantaviruses. In this regard, hantavirus antigens have been detected by enzyme immunoassay and fluorescence techniques in tissues of the European common mole (*Talpa europea*) captured in Russia (25) and Belgium (26), but no reports are available about hantavirus infection in shrew moles. Relying on oligonucleotide primers designed from our expanding sequence database of shrew-borne hantaviruses, we have identified a hantavirus genome, designated Asama virus (ASAV), in the Japanese shrew mole (*Urotrichus talpoides*). Genetic and phylogenetic analyses indicate that ASAV is distinct but related to hantaviruses harbored by Old World soricine shrews, suggesting a very ancient evolutionary history, probably involving multiple host-switching events in the distant past.

Results

RT-PCR Detection of Hantavirus Sequences. In using RT-PCR to analyze RNA extracts, from lung tissues of three Laxmann's shrew (*Sorex caecutiens*), five slender shrew (*Sorex gracillimus*), six long-clawed shrew (*Sorex unguiculatus*), one dsinezumi shrew (*Crocidura dsinezumi*), and six Japanese shrew mole (*Urotrichus talpoides*), hantavirus sequences were not detected in shrew tissues, but were found in one of two and in two of three Japanese shrew moles (Fig. 1), captured in Ohtani (34 28′14.0° N; 136 45′46.2° E) and near

Author contributions: S.A. and R.Y. designed research; S.A., S.D.O., M.A., J.A., N.O., and R.Y. performed research; S.A. and H.J.K. contributed new reagents/analytic tools; S.A., S.D.O., H.J.K., G.M., and R.Y. analyzed data; and S.A., G.M., J.A., and R.Y. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession numbers: ASAV S segment (EU929070, EU929071, EU929072); ASAV M segment (EU929073, EU929074, EU929075); and ASAV L segment (EU929076, EU929077, EU929078].

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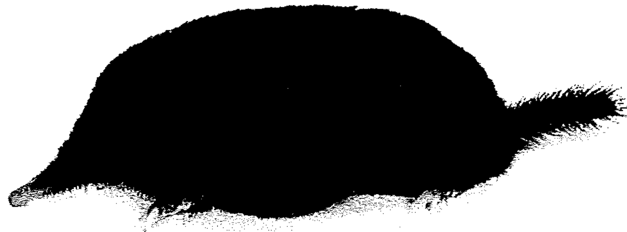

Fig. 1. Japanese shrew mole (*Urotrichus talpoides*) (family *Talpidae*, subfamily Talpinae), one of two endemic shrew mole species found only in Japan.

Asama River (34 28′12.79° N; 136 45′45.81° E), respectively, located approximately 1 km apart at an elevation of 50 m in Mie Prefecture, during February and April 2008. After the initial detection of hantavirus sequences, amplification of the S-, M-, and L-genomic segments was accomplished by using oligonucleotide primers based on conserved regions.

Nucleotide and Amino Acid Sequence Analysis. The S, M, and L segments of ASAV, as amplified from tissues of three wild-caught Japanese shrew moles, indicated an overall genomic structure similar to that of other rodent- and soricid-borne hantaviruses. The nucleotide and deduced-amino acid sequences of each ASAV genomic segment were highly divergent from that of rodent-borne hantaviruses, differing by approximately 30–40% (Table 1).

The S-genomic segment of ASAV (1,801 nucleotides for strains H4 and N9 and 1,756-nucleotides for strain N10) encoded a predicted nucleocapsid (N) protein of 434 amino acids, starting at nucleotide position 39, and a 3′-noncoding region (NCR) of approximately 465 nucleotides. The hypothetical NSs opening reading frame, typically found in the S segment of arvicolinae, neotominae, and sigmodontinae rodent-borne hantaviruses, was not found in ASAV. The interstrain variation of the S segment among the ASAV strains was negligible (1.1% at the nucleotide and 0% at the amino acid levels). In the hypervariable region of the N protein, between amino acid residues 244 and 269, ASAV differed by 18–20 and 20–22 amino acid from soricine shrew- and rodent-borne hantaviruses, respectively. Sequence similarity of the entire S-genomic segment of ASAV strains H4, N9, and N10 was higher with soricine shrew-borne hantaviruses than with hantaviruses harbored by rodents (Table 1).

Table 1. Nucleotide and amino acid sequence similarity (%) between ASAV strain N10 and representative rodent- and shrew-borne hantaviruses

	S segment		M segment		L segment	
Virus strain	1710 nt	434 aa	3604 nt	1141 aa	6126 nt	2041aa
HTNV 76–118	58.5	62.7	62.7	59.4	70.3	74.6
SEOV 80–39	63.1	62.0	62.8	59.1	70.4	74.7
SOOV SOO-1	62.8	62.9	63.3	59.7	70.2	74.3
DOBV Greece	62.2	62.2	63.0	59.3	70.2	75.7
PUUV Sotkamo	59.3	59.3	59.6	52.2	68.1	68.0
TULV 5302v	61.5	59.4	60.5	52.6	68.3	67.9
PHV PH-1	60.7	59.3	59.3	51.9	66.4	67.1
SNV NMH10	60.9	58.9	59.0	54.1	68.2	68.8
RPLV MSB89866	—	—	68.8	63.5	75.2	83.2
CBNV CBN-3	67.7	70.4	68.2	71.0	76.0	84.7
ARRV MSB73418	65.7	66.6	70.9	77.0	73.8	83.5
JMSV MSB89332	66.2	66.9	—	—	74.3	82.6
SWSV mp70	63.8	69.9	75.2	79.5	75.0	83.2
ASAV H4	98.9	100	99.3	99.6	98.2	99.6
ASAV N9	100	100	99.9	100	100	100
MJNV 05-11	57.2	46.0	56.1	44.4	65.8	61.5
TPMV VRC	58.0	45.8	57.7	43.0	64.3	62.0

Abbreviations: ARRV, Ash River virus; ASAV, Asama virus; CBNV, Cao Bang virus; DOBV, Dobrava virus; HTNV, Hantaan virus; JMSV, Jemez Spring virus; MJNV, Imjin virus; PHV, Prospect Hill virus; PUUV, Puumala virus; RPLV, Camp Ripley virus; SEOV, Seoul virus; SNV, Sin Nombre virus; SOOV, Soochong virus; SWSV, Seewis virus; TPMV, Thottapalayam virus; TULV, Tula virus. nt, nucleotides; aa, amino acids.

The 3,646-nucleotide full-length M-genomic segment of ASAV encoded a predicted glycoprotein of 1,141 amino acids, starting at nucleotide position 41, and a 183-nucleotide 3′-NCR. Four potential N-linked glycosylation sites (three in Gn at amino acid positions 138, 352, 404, and one in Gc at position 933) were found in ASAV. In addition, the highly conserved WAASA amino acid motif, which in ASAV was WAVSA (amino acid positions 649–653), was present. An interstrain variation of 0.1–0.7% and 0–0.4% at the nucleotide and amino acid levels, respectively, was found among ASAV strains H4, N9, and N10. The full-length Gn/Gc amino acid sequence of ASAV exhibited the highest similarity with Seewis virus (79.5%) from the Eurasian common shrew (Table 1).

Analysis of the nearly full-length 6,126-nucleotide (2,041-amino acid) L segment of ASAV revealed the five conserved motifs (A–E), identified among all hantavirus RNA polymerases. The overall high sequence similarity of the L segment among ASAV and rodent- and soricid-borne hantaviruses was consistent with the functional constraints on the RNA-dependent RNA polymerase (Table 1).

Secondary Structure of N Protein. Secondary structure analysis revealed striking similarities, as well as marked differences, among the N protein sequences of ASAV and 13 representative rodent- and soricid-hantaviruses. Each sequence appeared to adopt a two-domain, predominantly α-helical structure joined by a central β-pleated sheet. Whereas the length of the N-terminal domain was mostly invariant, the length of the central β-pleated sheet and of the adjoining C-terminal α-helical domain showed systematic reciprocal structural changes according to the genetic relationship and evolutionary descent of the individual sequences.

The N-terminal α-helical domain, from residues 1 to approximately 140, was composed of four helices connected by large loops (representative viruses shown in Fig. 2). The C-terminal α-helical domain, from residues 210/230 to 430, contained seven to nine helices that were connected by tighter loops (Fig. 2). And the central β-pleated region, from residues 140 to 210/230, was composed of three to five possible anti-parallel strands. Interestingly, an increasing number of strands in this section were observed when the hantaviral sequences were arranged according to their positions in the phylogenetic tree. This resulted in a widening of the central β-pleated region with a concomitant shortening of the C-terminal α-helical domain while preserving the total length of the protein. The helix adjoining the central β-sheet progressively shortened in this architectural change. These structural alterations were reversed in TPMV, which was evolutionarily more distant from the other sequences (Fig. 2).

Phylogenetic Analysis. Exhaustive phylogenetic analyses based on nucleotide and deduced amino acid sequences of the S-, M-, and L-genomic segments, generated by the maximum-likelihood (ML) method, indicated that ASAV was distinct from rodent-borne hantaviruses (with high posterior node probabilities based on 30,000 trees) (Fig. 3). Nearly identical topologies were consistently derived, by using various algorithms and different taxa and combinations of taxa, suggesting an ancient evolutionary origin. The most strikingly consistent feature was the phylogenetic position of ASAV with soricine shrew-borne hantaviruses, rather than being placed as an outgroup beyond TPMV, the prototype crocidurine shrew-borne hantavirus. That is, the prediction that a shrew mole-associated hantavirus would be phylogenetically distant from hantaviruses harbored by shrews was not validated.

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