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A retrospective study of hantavirus infection in Kinmen, Taiwan

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In this retrospective study, 140 serum samples collected from 85 scrub typhus-negative patients in Kinmen Island in 2000 were tested for antibodies to hantavirus using enzyme-linked immunosorbent assay. Seven patients (8.23%) were confirmed as having hantavirus infection as demonstrated by increased hantavirus-specific immunoglobulin M and/or immunoglobulin G antibodies in their convalescent serum samples. Analysis of indirect immunofluorescence assay showed that Seoul type was the etiologic agent. Serosurvey of rodents caught in the resident township of these hantavirus-infected human cases showed that the seroprevalence of antibodies to hantavirus among *Rattus norvegicus*, *Mus musculus,* and *R. flavipectus* was 50% (4/8), 20% (1/5), and 2% (7/348), respectively. Molecular analysis showed that these reservoir hosts carried a Seoul type hantavirus. To our knowledge, this is the first report demonstrating indigenous hantavirus cases in Kinmen.

Key words: Enzyme-linked immunosorbent assay, hantavirus, polymerase chain reaction, seroepidemiologic studies, Taiwan

Hantaviruses were known as the etiologic agents of hantavirus pulmonary syndrome (HPS) caused primarily by Sin Nombre virus, an historic pathogen newly identified in 1993 in the USA [1-4], and of hemorrhagic fever with renal syndrome (HFRS) or epidemic hemorrhagic fever (EHF), an infectious disease that occurs in regions that cover almost the entire Eastern Hemisphere including Korea, Japan, Soviet Russia and Eurasia [5-9]. In China, 40,000-100,000 cases of HFRS were reported annually and hantavirus infection posed a serious health problem [10]. Rodents are the main reservoir hosts and substantially responsible for the transmission of hantaviruses, with a unique and specific geographic distribution [11-14]. Hantaviruses may be transmitted through inhalation aerosol of contaminated feces, urine or saliva [15] or exposure to or bite by infected rodents [16-18].

Kinmen, an island of 150 km^2 and population of about 59,000, is geographically closer to China than to Taiwan to which it is ascribed. Although HFRS cases were not previously reported from Kinmen, the composition of rodents on Kinmen is quite similar to those of Fu-chien province of China, a neighboring region, where HFRS has been a prevalent disease since the 1970s [19]. Previous seroepidemiologic investigation indicated a prevalent distribution of hantavirus in rodent populations in Taiwan including Kinmen [20-22].

Scrub typhus disease, which is transmitted by bite of chigger mite infected with *Orientia tsutsugamushi*, has been reported from various Asia-Pacific regions ranging from southeastern Siberia and northern Japan to northern Australia and is common in some parts of Taiwan [23]. It had been listed as a reportable disease in Taiwan since 1955. The disease features include an acute febrile illness characterized by a typical primary lesion (eschar), rash and nonspecific symptoms such as fever, chills, cough and malaise. In addition to the transmission of scrub typhus, chigger mites from rodents may play an important role in the proliferation and transmission of hantavirus based on the studies that chigger mite had been demonstrated as a vector of HFRS [24,25].

Kinmen Island is an endemic area of scrub typhus disease with hundreds of suspected cases reported. Among these, relatively few were confirmed and the etiology of the rest remained unknown. Kinmen Island has similar HFRS-causing rodent populations to those of Fu-chien province of China. This retrospective study investigated the occurrence of hantavirus infection in scrub typhus-negative patients.

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Materials and Methods

Human serum samples

266 serum samples were collected from 153 patients with suspected scrub typhus as reported by the County and Military Hospitals of Kinmen Island to the Center for Disease Control (CDC) of the Department of Health in Taiwan between 1 January and 12 December 2000. Among these, 56 (36.6%) cases were confirmed to have scrub typhus disease. The remaining 140 sera including 55 paired serum samples from the acute and convalescent phases and 30 acute phase serum samples from 85 scrub typhus-negative patients (immunofluorescence antibody [IFA] testing against Karp, Kato and Gilliam strains of *O. tsutsugamushi*) were used for this study.

Indirect enzyme-linked immunosorbent assay

Serum samples were tested for antibodies reactive with hantavirus recombinant nucleocapsid protein antigens according to the protocol provided by the manufacturer (Focus Technologie, Cypress, CA, USA) with slight modification [26]. Briefly, 100 μL of 1:100 diluted human sera-positive, sera-negative and cut-off controls in phosphate-buffered saline (PBS) 0.1% Tween 20 (PBST) containing 1% bovine serum albumin (BSA) and 5% normal rabbit serum (diluent buffer) were added to the 8 wells strip coated with mixed recombinant nucleocapsid proteins of Hantaan (HTN), Seoul (SEO), Puumala (PUU), Dobrava (DOB), and Sin Nombre strains of hantavirus and incubated at 37°C for 1 h. After 4 cycles of washing with PBST, 100 μL of 1:5000 diluted alkaline phosphatase (ALP)-labeled goat anti-human immunoglobulin M (IgM) or immunoglobulin G (IgG) conjugates (Zymed Laboratories Inc., San Francisco, CA, USA) was added and reacted at 37°C for 1 h. After washing, 100 μL of substrate reagent BluePhos (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) was added for color development. The optical density (OD) was measured at the wavelength of 630 nm with a MRX II, 96-well microplate reader (Dynex Technologies, Chantilly, VA, USA). For each run of the enzyme-linked immunosorbent assay (ELISA) tests, the cut-off control was included and used to determine an index value of each sample. The index value was expressed as the OD of the sample divided by the OD of the cut-off control. Index values greater than 1.1 were considered positive, below 0.9 were considered negative and values between 0.9 and 1.1 were regarded as equivocal, according to the manufacturer's instructions.

Capture IgM ELISA

Ninty six-well polystyrene MaxiSorp surface microtiter plates (A/S Nunc, Denmark) were coated overnight at 4°C with 5 μg /mL, 100 μL/well of goat anti-human IgM antibodies (μ-chain specific) [Jackson Immunochemicals, West Grove, PA, USA] in 0.1 M carbonate buffer Na_2CO_3/$NaHCO_3$, pH 9.5. After washing with PBST, the following reagents were subsequently added and incubated: 200 μL of PBS 1% BSA, 100 μL of 1:100 diluted serum sample, sera-positive, sera-negative and cut-off controls in diluent buffer, and 50 μL of horseradish peroxidase (HRPO)-labeled specific hantavirus antigen (Chang-tien Hsin-Ko Services, China). The incubation time between each step was 1 h at 37°C, followed by 4 cycles of washing with PBST. Color development was done by adding tetramethylbenzidin substrate solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA). OD reading and interpretation of the results were performed as described for the indirect IgM and IgG ELISA.

IFA

Slides coated with Vero E6 cells infected with hantavirus (HTN, SEO or PUU) mixed with non-infected Vero E6 cells (Progen Biotechnik, Heidelberg, Germany) were used for serotyping of human IgM and IgG antibodies against hantavirus. Briefly, serum specimens were first screened at 1:40 dilution for total IgM and IgG antibodies against hantavirus. The reactive specimens were then titrated to the endpoint by 2-fold serial dilution and applied separately on the HTN, SEO or PUU slides in a humid chamber for 30 min at 37°C. After washing with PBS buffer, the appropriately diluted fluorescein isothiocyanate-labeled goat anti-human IgM or IgG (Zymed Laboratories Inc.) was added on the slides and incubated for 30 min. Evans blue dye was then used as counterstain for the Vero E6 cells. After washing and adding mounting fluid and placement of a cover slip, the slides were examined under a fluorescence microscope (Leica DM LB HC, Leica Microsystems, Wetzlar GmbH, Germany). The samples were considered positive when yellow-green fluorescing hantavirus particles were seen in the cytoplasm of the red-stained Vero E6 cells as defined by the manufacturer's instructions. A positive reaction was defined if the IgG antibody titer had a 4-fold or greater rise or IgM antibody titer ≥1:80.

Rodent collection

During mid-February, April, and June 2000, rodents were trapped on one day of each month according to

the handling guidelines in the 5 selected townships which had a higher number of scrub typhus patients in 1997-1998 [27]. A total of 100 spring-loaded rodent traps baited with sweet potato and peanut were set in the afternoon with 20 in a selected residence, and 80 in the surrounding shrub areas and collected the next morning. The species and gender of captured animals were identified. After the animal was anesthetized with Zoletil 50 (Fa. Virbac, Carros, France), blood was removed by cardiac puncture, and stood at room temperature for 1 h. After centrifugation, sera were collected, aliquoted, and kept frozen at –70°C for later analysis.

Seroprevalence of hantavirus infection in rodents

Serum specimens were tested for IgG antibody reactive to hantavirus using the same methods as in the indirect IgM and IgG ELISA, except that 1:1000 diluted ALP-labeled goat anti-mouse IgG (Zymed Laboratories Inc.) was used instead of 1:5000 AP-labeled goat anti-human IgG conjugate.

Reverse transcriptase-polymerase chain reaction

Rodent serum specimens were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR. Viral RNA was extracted by the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Briefly, a total of 140 μL mouse serum was incubated with 560 μL of lysis buffer for RNA extraction buffer for 10 min. After viral particle lysis, the sample was mixed with 560 μL ethanol and applied to the spin column provided, centrifuged and washed twice with buffer AW1 and AW2. RNA was then eluted from the column using 70 μL of elution buffer for RNA elution and used in RT-PCR and nested-PCR assays, which employed M segment type-specific first-round primers [+2548 GATATGAATGATTG(T/C)TTTGT and –2859 CCATCAGGGTCT(T/C)TTCCT] and second-round primers [+2590 TGTATAATTGGGAC(T/A) GTATCTAA and –2751 GCAAAGTTACATTT(T/C) TTCCT] [4].

Analysis of PCR reaction products

A 10 μL volume of the PCR reaction product was electrophoresed on a 2.5% agarose gel. The gel was stained with ethidium bromide and examined for bands of the appropriate size. Products of nested PCR were sequenced and analyzed by the ABI PRISM 377-96 sequencer and ABI PRISM 3100 genetic analyzer (Applied Biosystems, CA, USA). Nucleotide sequences were compared using the Basic Local Alignment Search Tool (BLAST) search programs of the National Center for Biotechnology Information, US National Library of Medicine to detect the relationships among sequences which shared only isolated regions of similarity of M segments of hantavirus [28].

Results

A total of 140 serum samples from the 85 scrub typhus-negative patients were screened for hantavirus-specific antibodies using ELISA. Seven patients (8.23%) had positive results. The results from capture IgM ELISA showed that hantavirus-specific IgM antibodies were presented in both acute and convalescent phase sera of 2 of these patients (patients 1 and 3) while the other 5 patients showed seroconversion of specific IgM antibody (Table 1). Indirect IgG ELISA showed that these 7 patients had either seroconversion (patients 1, 2, 4, 5, 6 and 7) or increase (patient 3) of hantavirus-specific IgG antibodies from acute to convalescent phase sera. Hantavirus-specific IgM antibody was detected as early as day 3 after the onset of symptoms. Analysis of hantavirus-specific IgM and IgG antibodies in the serum samples of these patients by IFA at 1:40 dilution showed that all were non-reactive to HTN and PUU hantavirus antigens but positive to SEO type. Two-fold serial dilutions starting at 1:40 showed that all of these patients had a greater than 4-fold rise in hantavirus-specific IgG antibodies to SEO type (Table 1). The results suggested that all 7 patients had current hantavirus infection by a Seoul type hantavirus. All of these 7 patients were males aged from 20 to 28 years, and were enrolled in the military for a 2- to 3-year service in Kinhu township, Kinmen. Symptom onset occurred in April through July in 6 patients and in November in 1 patient (Fig. 1).

The reservoir hosts for distribution of hantavirus in Kinmen was determined by evaluation of a total of 385 rodents, including 2 orders, 2 families, 3 genera and 5 species caught in February, April and June 2000, in 5 townships of Kinmen Island. The sera were first screened by ELISA to determine the seroprevalence of hantavirus infection. The results showed that the hantavirus IgG-positive rates of *R. norvegicus*, *R. flavipectus*, and *Mus caroli* were 50.0% (4/8), 2.0% (7/348) and 20.0% (1/5), respectively (Table 2). RT-PCR analysis of sera of hantavirus-infected rodents showed that the overall hantavirus carrier rate of infected rodents was 50% (6/12), including 4 *R. norvegicus* and 2 *R. flavipectus* spp. The nucleotide sequences obtained

Table 1. Serology of hantavirus infected human cases among scrub typhus-negative patients in Kinmen, 2000

Case	Gender	Age (years)	Date of onset	Days after onset	Indirect ELISA[a]		Capture IgM ELISA[a]	IFA titer[b] (Seoul)		IFA (Hantaan)	IFA (Puumala)
					HTN-IgM	HTN-IgG		HTN-IgM	HTN-IgG	IgM+IgG	IgM+IgG
1	Male	21	4-09-2000	3	0.653	0.078	0.463	(–)	80	(–)	(–)
				26	2.145	1.988	1.042	80	1280	(–)	(–)
2	Male	21	4-21-2000	3	0.06	0.069	0.155	(–)	(–)	(–)	(–)
				42	0.903	1.389	0.908	(–)	1280	(–)	(–)
3	Male	28	5-25-2000	4	2.452	1.442	1.274	80	(–)	(–)	(–)
				27	2.591	2.623	1.396	80	2560	(–)	(–)
4	Male	21	6-08-2000	3	0.38	0.071	0.204	(–)	(–)	(–)	(–)
				93	0.464	0.932	0.857	(–)	640	(–)	(–)
5	Male	23	6-13-2000	2	0.161	0.067	0.108	(–)	(–)	(–)	(–)
				23	1.582	1.856	0.417	(–)	1280	(–)	(–)
6	Male	22	7-31-2000	1	0.159	0.057	0.245	(–)	(–)	(–)	(–)
				72	0.971	1.554	1.346	(–)	640	(–)	(–)
7	Male	20	11-26-2000	1	0.068	0.051	0.102	(–)	(–)	(–)	(–)
				23	2.043	1.516	0.945	(–)	1280	(–)	(–)
Cut-off control					0.356	0.379	0.246				

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HTN = Hantaan; IgM = immunoglobulin M; IgG = immunoglobulin G; IFA = immunofluorescence antibody

[a]Data indicate the optical density at 630 nm. Results were considered positive when the index value was greater than 1.1.

[b]Antibody titers were expressed as reciprocals of the highest serum dilutions that showed definite specific fluorescence to Seoul hantavirus antigen; (–) indicates that serum was non-reactive in IFA test at 1:40 dilution for screening test.

from RT-PCR and nested PCR reaction products indicated that the reservoir host carried Seoul hantaviruses (data not shown).

Discussion

Previous studies found no evidence of confirmed HFRS cases in Taiwan, with the exception of 4 imported cases in 1995-1997 [20-22,29]. After the identification of 4 indigenous HFRS cases through the syndrome report program of hemophagocytosis in 2001, HFRS and HPS were included as reportable infectious diseases by Taiwan CDC, from December 3, 2001 [30]. One of these 4 cases, a 38-year-old man was confirmed to be the first case in Matsu, an islet located closer to China than to Taiwan and situated north east of Kinmen Island [30]. In this retrospective study, we found hantavirus infection in 7 of 85 scrub typhus-negative patients in Kinmen. The ELISA results showed that these 7 patients had either seroconversion or increase of hantavirus-specific IgM and/or IgG antibodies from acute to convalescent phase sera. The IFA test identified SEO as the etiologic agent in these patients (Table 1). All patients were males aged between 20 and 28 years, and were members of the Taiwan military garrison stationed in Kinhu township on Kinmen Island. The temporal distribution of these cases was April (2 cases), May (1), June (2), July (1) and November (1). This distribution reflected a spring and autumn seasonality for hantavirus infection, which corresponded to reported seasonal epidemics of HFRS in Fu-chien province of China [10]. This study provided the first evidence of indigenous hantavirus human cases ever found in Kinmen.

The relatively high percentage (8.23%) of confirmed human cases of hantavirus infection reported in this study might be related to the clinical samples analyzed, since similar symptoms were found for scrub typhus and hantavirus infection. The spectrum of disease severity for hantavirus infection ranged from unapparent or mild to severe or fatal [31-37]. Predominantly found in the Eastern Hemisphere, HFRS is characterized by fever, renal failure, and sometimes by hemorrhagic manifestations. The clinical symptoms of HFRS are more severe when caused by HTN than by SEO type, which is the prevailing strain in Taiwan and probably accounted for the mild infection of cases in this study [22,38]. Kao et al reported an average 6.2% infection rate of Hantaan virus in Taiwan from a total of 6536 human serum samples collected from residents of 19 townships covering 4 different ethnic groups [39]. A higher seropositive rate was found among Aborigines on the Orchid Islets (11.5%) and Fukien Taiwanese on the Penghu Islets (11.6%), while the lowest rate was observed among Hakka Taiwanese in

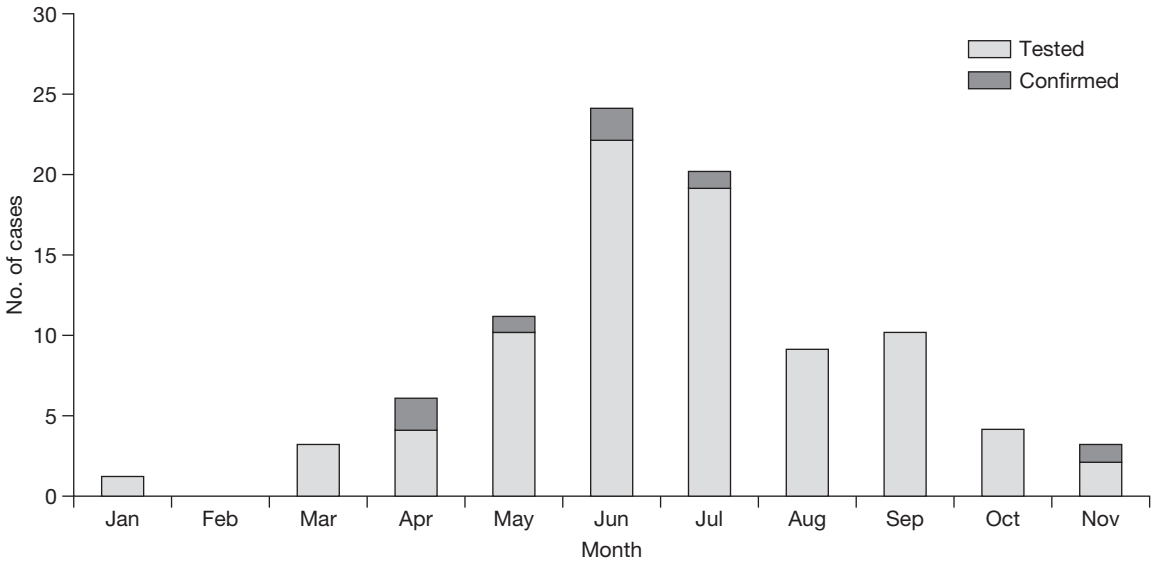

Fig. 1. Temporal distribution of hantavirus-infected human cases in Kinmen Island, Taiwan, 2000. In total, 140 serum samples from 85 scrub typhus-negative patients were tested for hantavirus-specific immunoglobulin M and immunoglobulin G antibodies using enzyme-linked immunosorbent assay.

the south of Taiwan (2.5%). Using a similar indirect immunofluorescent antibody technique, Chen et al reported a much lower infection rate among certain high-risk groups in Taiwan [21]. The positive rates were 1.55%, 3.45%, 1.42%, and 5.56% for garbage collectors, animal handlers, patients with febrile illness of unknown origin, and field rats, respectively. The discrepancies among these results might be partly due to the use of different reagents and serum samples tested.

It is reasonable to assume that the temporal distribution of human cases should be correlated with the population structures of rodents, and their distributions and seasonal fluctuations. A recent survey of murine-like animals from July 2001 to December 2002 in Kinmen showed that the trap rate started to increase from April and peaked in June (19.6%) [40]. Each serotype of hantavirus is associated with a single rodent host species or genus [11-14,31]. In this study we found that on Kinmen Island, *R. flavipectus* predominantly infested the fields, while *R. norvegicus* was detected in relatively small numbers. Nevertheless, *R. norvegicus* was the rodent species most frequently infected, with an average antibody prevalence of 50% (4/8) [Table 2], while all *R. rattus* and *S. murinus* were seronegative. Due to the relatively small number of rodents analyzed in this study, further study with a larger sample size is needed to provide more conclusive information. However, our results support previous findings of an extremely low hantavirus seroprevalence in *R. rattus* and *S. murinus* [22].

Table 2. Hantavirus infection in rodents collected in the resident township (Kinhu) and nearby townships of 7 patients infected with hantavirus in Kinmen, 2000

Species	No. specimens tested (no. seropositive)						No. PCR tested (no. positive)
	Township						
	Kinchen	Kinhu[a]	Kinnin	Kinsha	Leiyu	Total	
Rattus flavipectus	71 (2)	84 (1)	79 (2)	70 (1)	44 (1)	348 (7)	7 (2)
Rattus rattus	3 (0)	0	3 (0)	1 (0)	2 (0)	9 (0)	0
Rattus norvegicus	0	1 (1)	1 (0)	1 (0)	5 (3)	8 (4)	4 (4)
Mus caroli	0	3 (1)	0	2 (0)	0	5 (1)	1 (0)
Suncus murinus	1 (0)	5 (0)	3 (0)	2 (0)	4 (0)	15 (0)	0
Total	75 (2)	93 (3)	86 (2)	76 (1)	55 (4)	385 (12)	12 (6)

Abbreviation: PCR = polymerase chain reaction

[a]Township where the 7 men with hantavirus infection resided. They were all members of the Taiwan military garrison stationed in Kinmen.

Previous findings that hantavirus-specific RNA was detected in lung tissue of 92% of seropositive rodents, but not in seronegative rats indicate that hantaviruses establish persistent infections in their rodent hosts [12]. Due to cost considerations and lack of tissue specimens, only serum specimens of seropositive rodents were tested for RT-PCR in this study. We found that 100% (4/4) of seropositive *R. norvegicus* and 28.5% (2/7) of *R. flavipectus* possessed hantavirus-specific RNA indicating active hantavirus infection (Table 2). The positive rate of hantavirus-carrier rodent hosts might have been underestimated in this study since lung tissue samples usually contain a higher hantavirus titer than serum samples. Molecular diagnosis demonstrated that the reservoir hosts carrying the SEO hantaviruses had 98-99% similarity to L99, R22 and HB55 viral strains among the 160-bp nucleotide sequences obtained from the nested PCR reaction (GenBank accession no. AY934503). The L99, R22 and HB55 strains were categorized as subgroup #1 SEO viruses in the phylogenetic trees for hantaviruses reported to have caused mild hantavirus infection in China [37]. Our results suggest that hantavirus infection has been under-reported in Kinmen Island and other parts of Taiwan. This study has also demonstrated that SEO type hantavirus is widely distributed in humans and rats in Kinmen. Physicians and public health personnel should be aware of the possibility that hantavirus and scrub typhus may co-circulate in both mite and rodent infested areas.

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