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SCIENCE DIRECT®
Microbes and Infection 5 (2003) 769–773
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Evidence of the presence of Seoul virus in Cambodia

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Received 18 October 2002; accepted 15 April 2003

Abstract

A study was conducted in agricultural and urban areas in Cambodia to assess the presence of hantaviruses in rodent populations. In 1998, rodents were trapped in two villages and in Phnom Penh city around market places and a rubbish dump. IgG antibodies to Hantaan virus were detected in 54 (8.2%) rodents among 660 tested: 6.4% (13/203) among roof rats (*Rattus rattus*), 20.9% (39/187) among Norway rats (*R. norvegicus*), 16.7% (2/12) among unidentified *Rattus* species and none in 183 Polynesian rats (*R. exulans*) or in 75 bandicoot rats (*Bandicota* sp.). The presence of the viral genome was detected by a reverse transcription-PCR amplifying part of the sequence coding for the nucleoprotein in the S segment, in 87% of the seropositive rodents. Thirty-one representative cDNAs were sequenced. Phylogenetic studies of the sequences indicated a close relationship with Seoul virus. However, the Cambodian Seoul virus sequences clustered within two different phylogenetic lineages, one associated with *R. rattus* and the other with *R. norvegicus*.

Keywords: Seoul virus; *Hantavirus*; Cambodia; Rodent

1. Introduction

The genus *Hantavirus* (family Bunyaviridae) includes more than 20 species of viruses, many of which are etiological agents of hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia or cause hantavirus pulmonary syndrome in North and South America. Transmission of hantaviruses occurs through inhalation of virus-contaminated aerosols of rodent excreta [1–3]. Each virus is closely associated with a principal natural rodent host from the family Muridae, with the exception of Thottapalayam virus, which was isolated from an insectivorous, *Suncus murinus* (shrew), in India. Hantaviruses responsible for HFRS are closely associated with Murinae and Arvicolinae rodents, and those responsible for hantavirus pulmonary syndrome in the New World are transmitted by Sigmodontinae rodents, which are not present in Eurasia or Africa [1–3].

Except for Thailand, where the presence of Seoul virus (SEOV) and Thailand virus (THAIV) were reported, very little information on hantavirus circulation has been described in Southeast Asia, and in Cambodia in particular [4–8]. In Thailand, SEOV is associated with the roof rat (*Rattus rattus*) and the Norway rat (*Rattus norvegicus*), and THAIV with the Norway rat and the great bandicoot rat (*Bandicota indica*) [4]. All the Muridae rodent species reported in Cambodia belong to the subfamily Murinae. However, the *Apodemus* genus, host of Hantaan virus (HTNV) in Asia and Dobrava in Europe has not been described in the country [3,9]. More than 20 species are probably present: the genera *Mus*, *Rattus* and *Bandicota* principally are found in urban and agricultural areas and the genera *Berylmys*, *Maxomys*, *Niviventer* and *Leopoldamys*, in tropical forests [9].

In the absence of information on hantaviruses in Cambodia, a field study was carried out to assess the presence of these viruses among Cambodian rodents.

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DOI: 10.1016/S1286-4579(03)00149-7

2. Materials and methods

2.1. Rodent study sites

The study was conducted in agricultural and urban areas including two rice-growing rural villages in Kandal province near the Mekong River and markets and a rubbish dump in Phnom Penh city. The first village, Chamcar Kouy (N 11°28'55" latitude, E 104°43'38" longitude 20 km southwest of Phnom Penh), is located in a non-flooded area. The second village, Thnal Boat (TB) (N 11°49'52" latitude, E 104°49'8" longitude, 40 km north of Phnom Penh), is located in a seasonally flooded area. The advantages of those sites were that paddy farming is the most representative agricultural activity in Cambodia, as well as that the villages are near Phnom Penh.

In Phnom Penh city, rodents were trapped in several market places and in human settlements next to the municipal rubbish dump in the south of the city.

2.2. Rodent trapping and sampling procedures

Small mammal trapping was undertaken six nights per week from March to May 1998 and mid-September to mid-December 1998. Every 2 weeks trapping sites were moved, and within these sites traps were moved every two or three nights. Animals were collected early in the morning after trapping and transferred to Institut Pasteur du Cambodge, where they were euthanized with chloroform.

Species identification was done using a regional taxonomic identification key [9]. Blood, kidneys, liver and lung were collected. Serum samples and organs were stored in cryovials at –20 and –70 °C, respectively.

2.3. Antibody assay

ELISA was used to detect antibodies (IgG) to hantavirus as previously described [10]. Positive antigen was produced on Vero E6 cells infected with HTN76 118 strain. Sera from rodents were tested at a dilution of 1:100 using peroxidase-labeled purified goat antibodies to rat IgG (γ) (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, USA).

2.4. RNA extraction from organs, reverse transcription (RT) and nested PCR amplification

Approximately 50 mg of liver were ground in a microbiological safety cabinet, then homogenized in 1 ml of Trizol reagent (Life Technologies, Gaithersburg, MD, USA), and RNA was extracted according to the manufacturer's instructions. Dry RNA was dissolved in 200 µl of RNase-free water, incubated for 10 min at 55 °C, and used for RT. Amplification was performed according to the method of Papa et al. [11] using sets of primers previously designed to detect part of the N coding region in the S RNA of hantaviruses associated with rodents of the subfamily Murinae. When the products of the second round contained non-specific bands together with the specific product, the second round of the nested PCR was repeated with a dilution of the RT-PCR product from the first round.

When viral sequences were not detected in the liver of a seropositive rat, its kidneys and lungs were assayed successively.

2.5. Sequencing of PCR amplicons and sequence analysis

Specific DNA fragments were purified after migration in agarose gels using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Sequencing was performed using the dye termination cycle sequencing technique (Applied Biosystems, Foster City, CA, USA) and an ABI 377 sequencer.

Sequences were analyzed with the MacMolly program and aligned with the Clustal W program. Phylogenetic trees of nucleotide sequences were constructed using the neighbor joining method implemented in Clustal W (1.81) (Genetics Computer Group, Madison, WI). When different samples contained the same sequence, only one representative was used. To determine the robustness of the tree 1000 bootstrap replicates were used. Sequences from 16 cDNA products were deposited at the EMBL library under serial accession numbers AJ 427498–AJ 427513.

3. Results

3.1. Evidence of antibody to HTNV in urban and agricultural rodents

Six hundred and sixty rodents were caught alive in the course of 5902 trap-nights and their serum samples were collected. Two hundred and three were identified as roof rats (*R. rattus*), 187 were Norway rat (*R. norvegicus*), 183 were Polynesian rats (*R. exulans*), 12 belonged to *Rattus* species, 70 were great bandicoot rats (*B. indica*) and 5 were bandicoot rats (*Bandicota* sp.).

IgG antibodies reacting with HTNV antigens by ELISA were detected in 54 (8.2%) rodents: 13 (6.4%) roof rats, 39 (20.9%) Norway rats, 2 (16.6%) of the *Rattus* sp. (which were likely juveniles of one of the two seropositive species).

3.2. Identification of hantavirus species infecting the seropositive rats

Of the 54 seropositive rats, 47 were positive by nested RT-PCR when their organs (liver, kidney or lung) were analyzed for the presence of the viral genome: 9 of the 13 roof rats, 36 of the 39 Norway rats and 2 of the 12 *Rattus* sp. As expected, no product was amplified from the organs of eight of the seronegative rodents. Among the 47 amplified products, 31 cDNAs representative of both the rat species and the site of the capture were sequenced.

Nucleotide sequences of the 599 base-pair cDNAs from the S segment were used for phylogenetic analyses. Se-

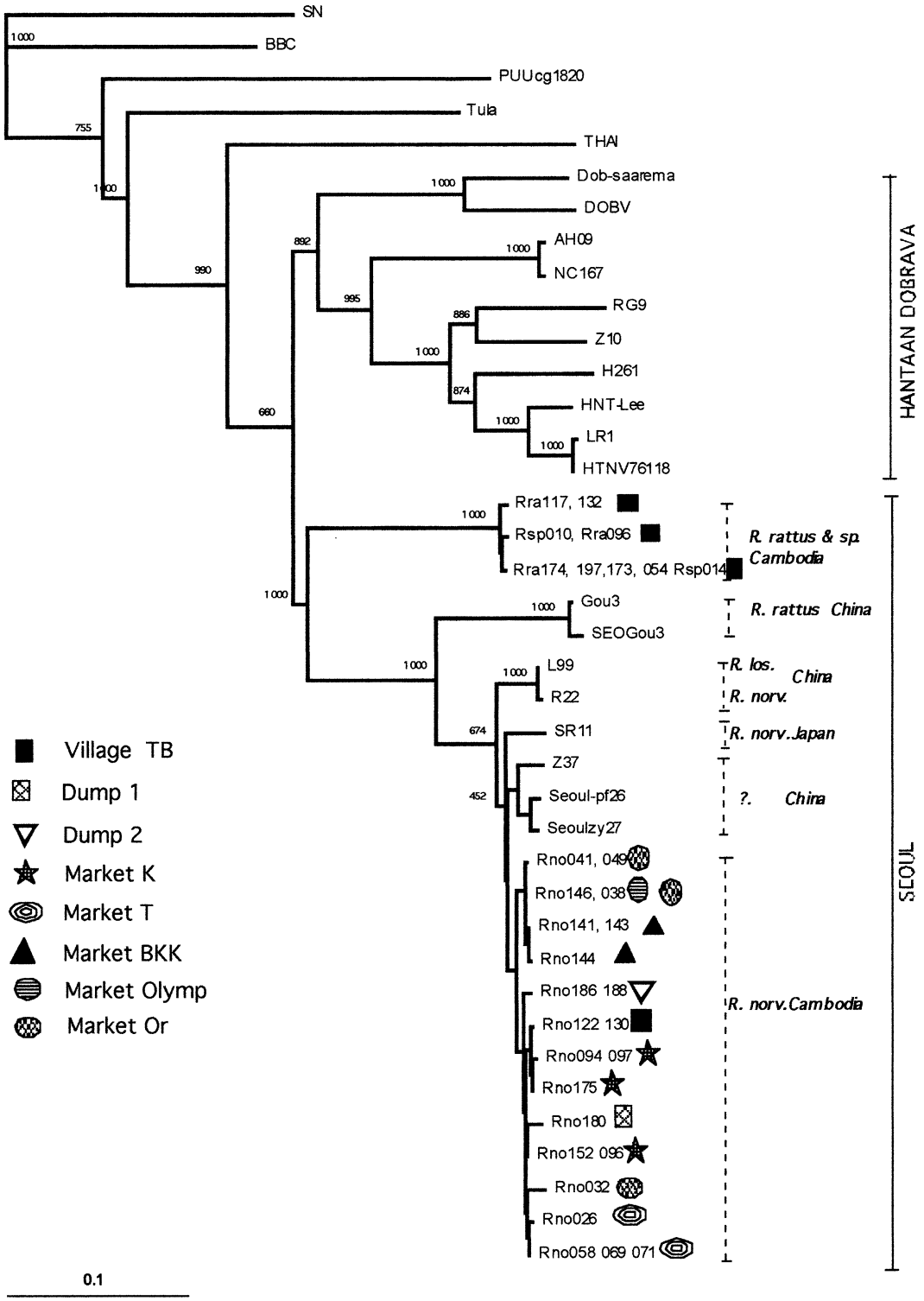

Fig. 1. Phylogenetic relationships of Cambodian hantaviruses using the neighbor joining method on the 599 nucleotide-long sequence of the S segment. Bootstrap values >600, determined from 1000 replicates, are indicated at the branch point. The S sequences with their accession numbers retrieved from the EMBL database are from Wang et al. [12]. The following sequences were compared: Gou3 strain AF 184 988 and AB 027 522; Z10, AF 184 987; L99, AF 288 299; RG9, AF 288 296; R22, AF 288 295; Seoul zy27, AF 406 965; and Seoul pf26, AB 027 522. The rodent from which an RT-PCR fragment was sequenced is indicated as follows: Rra (*R. rattus*) Rsp (*Rattus* species) or Rno (*R. norvegicus*), the number representing the reference recorded during trapping. Abbreviations for the hantaviruses: DOBV: Dobrava, SNV, Sin nombre, BBCV: Black Creek Canal, PUUV: Puumala, THAIV: Thailand, HNTV: Hantaan, SEOV: Seoul.

quences of HTNV- and SEOV-representative members of the Old World hantaviruses circulating in Asia were included. All the viral sequences detected in Norway rats were closely related to each other, differing only by at most, 2.2%. This held true also for the sequences from roof rats and *Rattus* sp. which vary only by as much as 0.8%, whereas the divergence between the two groups of sequences extends to 27.8–29.4%. The phylogenetic tree (Fig. 1) indicates that all the sequences clustered within two lineages. One is composed of SEOV from the roof rat and *Rattus* sp. and the other of SEOV from the Norway rat. As expected, the sequences found in the Norway rats clustered with recognized SEOV strains SR11, R22 and L99, which were also detected in the Norway rat or from the lesser rice-field rat (*R. losea*) [12]. It should be noted that the genetic divergence between the viruses carried by roof rats and Norway rats cannot be due to geographical distance. All the roof rats (and *Rattus* species) forming the newly identified lineage were sympatric (captured in the same village) with the two Norway rats Rno122 and Rno130 present in the other cluster.

4. Discussion

This study is the first report demonstrating the presence of a hantavirus in Cambodia. The presence of SEOV in Cambodia is not surprising. Its reservoirs, the Norway rat and the roof rat, are present in the country, and distribution of this virus is reported worldwide. THAIV, isolated from the Norway rat and the great bandicoot rat, was not identified among the sequences we obtained from the Norway rats. All bandicoot rats (*Bandicota* sp.) trapped during this study were seronegative. The HTNV lysate (test) antigen could provide a sensitive assay for hantaviruses principally associated with murid rodents [13]. Therefore, if a great bandicoot rat specimen were infected with THAIV, it would have been detected by our serological assay. So far, there is no data on hantavirus associated with the Polynesian rat (*R. exulans*). All the samples we tested from these rats were seronegative. Interestingly, a recent serological study performed in Thailand reported the circulation of hantavirus in 10 of 302 Polynesian rats, but the virus was not identified [6].

Compared with other hantaviruses, less is known about the sequence diversity of SEOV strains. This virus is transmitted by roof rats and Norway rats, but the genetic diversity of the *Rattus*-borne viruses remains unclear. In this study, we detected hantavirus sequences from both *Rattus* species and found that they belong to distinct phylogenetic groups, highly supported by the bootstrap values and correlating with their host carriers. All our sequences from Norway rats cluster together with SR11, the original isolate of SEOV from a Norway rat [14], and with strains isolated from Norway and lesser rice-field rats in China. This cluster also comprises human isolates from China. All sequences detected in roof rats or in *Rattus* sp. captured near Phnom Penh form a different cluster. Interestingly, strain Gou3, also isolated from a roof rat in China, was not located within this cluster but formed a third lineage. Two similar sequences of this latter virus were retrieved from sequence data bases. Both strains were included in this analysis. The sequence difference may have been caused by passage history. Recently, Wang and colleagues [12] reported a genetic analysis of SEOV strains isolated in China and found five subtypes closely related to each other, strain Gou3 representing one of these subtypes. Their analysis was performed mainly with M segment sequences. However, a tree based on partial S sequences composed of only a few representative strains seems to confirm the existence of at least four SEOV subtypes. It should be noted that all the *Rattus*-borne isolates analyzed by Wang et al. were derived from Norway rats, Gou3 strain being the only isolate from roof rats. Here we show that the hantavirus RNA sequences found in Cambodian *R. rattus* were clearly distinct from Gou3, indicating that at least two subtypes of SEOV carried by *R. rattus* circulate in Asia. The existence of SEOV subtypes may represent a geographic clustering or a complex evolution of *R. rattus*-borne and *R. norvegicus*-borne hantaviruses, due to the worldwide dissemination of rats infected with SEOV.

Rodent species which colonize the tropical forest were not investigated during our study. Since all Cambodian provinces are now accessible, it will be interesting to carry out field studies and search for the presence of hantaviruses in the tropical forest.

Acknowledgements

The authors thank Ms. Chantary Keo and Mr. Hoy Sam Serei Chan for their help in trapping and dissecting the animals, Dr. Hervé Bourhy for helpful discussion on the phylogenetic analyses, and Mr. Daniel Coudrier for excellent technical assistance. The authors are grateful for help and encouragement from the Cambodian Health Authorities. This work received financial support from the “Actions Concertées des Instituts Pasteur”.

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