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Original Article

Molecular and Serological Evaluation of Hantavirus in Wild Rodents in Kohgiluyeh and Boyer-Ahmad Province, Southwest of Iran

Jamal Sarvari[1], Bahador Sarkari[2], Fatemeh Osooreh[1], Mehdi Fazlalipour[3], Mohammad Hassan Pouriayevali[3], Tahmineh Jalali[3], Mohammad Javad Ranjbar[2], *Mostafa Salehi-Vaziri[3,4]

[1]Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Department of Arboviruses and Viral Hemorrhagic Fevers (National Reference Laboratory), Pasteur Institute of Iran, Tehran, Iran
[4]Research Center for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Tehran, Iran

***Corresponding author:** Dr Mostafa Salehi-Vaziri, E-mail: mostafavaziri1985@gmail.com

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Abstract

Background: Hantaviruses are mainly transmitted to humans through the inhalation of aerosolized excreta from infected rodent reservoirs. The present study was conducted to analyze the prevalence of hantavirus infection among rodents in the Boyer-Ahmad region.

Methods: A total of 52 rodents were captured in the Boyer-Ahmad region of Kohgiluyeh and Boyer-Ahmad Province during June to November 2014, using Sherman live traps. Blood and tissue samples were obtained from the heart and lungs, respectively. Hantavirus Pool 1 "Eurasia" IgG and Pool 2 "America" ELISA IgG kits were used to detect IgG antibodies against both Old World and New World hantaviruses. Moreover, total RNA extraction was performed on the lung tissue, and a pan-hantavirus nested RT-PCR was conducted to detect hantavirus RNA.

Results: *Meriones persicus* was the most abundant species (n=25, 48%). The results of the ELISA showed that all the serum samples from the rodents were negative for antibodies against both Eurasian and American hantaviruses. Moreover, no rodent tissue samples tested positive for the hantavirus RNA by the pan-hantavirus RT-PCR.

Conclusion: Although no hantavirus infection was detected in this study, the presence of hantavirus reservoirs in Kohgiluyeh and Boyer-Ahmad Province suggests that hantavirus circulation cannot be completely ruled out. Further studies with a larger sample size are recommended.

Keywords: Hantavirus; Rodents; Kohgiluyeh and Boyer-Ahmad; Iran

Introduction

Hantaviruses are RNA viruses that have recently been classified as a separate family, *Hantaviridae*, within the order *Bunyavirales*. They have a single-stranded RNA genome divided into three segments, which encode structural and non-structural proteins (1). Hantaviruses produce long-term asymptomatic or chronic infections in various rodent species (2). A variety of small mammals, including rodents, shrews, moles, as well as bats, serve as the natural reservoirs of hantaviruses (3). Moreover, there are some reports of hantavirus seropositivity in some domestic animals, including dogs and cats, that might be infected through contact with infected small animals (4). In a single reservoir population, up to 100% of the rodents may be infected and long-lasting viral shedding occurs in the urine, feces and saliva of the infected

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animals (1). Rodent excreta can infect humans through inhalation of aerosols; however, the virus can also be transmitted through rodent bites (5, 6).

Hantaviruses are relatively resistant to environmental conditions and may remain infectious for more than 10 days at ambient temperature and over 18 days at 4 °C (7). Hantaviruses have been reported from different continents, including Europe, Asia, the Americas and more recently, from Africa (8). More than 40 strains of hantaviruses have been identified and each strain infects a specific rodent as a host reservoir (9).

Jobs involving close contact with rodents, such as agricultural, forestry, or military activities, are at increased risk of hantavirus infection (9). Hantavirus infection in humans can result in hantavirus cardiopulmonary syndrome (HCPS) in the New World (Americas) and hemorrhagic fever with renal syndrome (HFRS) in the Old World (Eurasia) (6). Both syndromes begin with influenza-like illness with nonspecific symptoms including fever, chill, headache, myalgia, nausea and gastrointestinal symptoms. HCPS is characterized by cough, dyspnea, pulmonary edema, and hypoxia, whereas HFRS is distinguished by hypotension, hemorrhage and renal involvement such as oliguria or anuria (10).

So far, more than 40 hantaviruses have been identified and some of them, such as Puumala virus (PUUV), Hantaan virus (HNTV), Seoul virus (SEOV), Dobrava virus, Sin Nombre (SNV) and Andes virus (ANDV), are pathogenic to humans (6). Nearly all epidemiological data are based on studies conducted in East Asia, Europe and the Americas, while there is a notable lack of data on hantavirus circulation in Africa, the Middle East, and the Indian subcontinent (11).

The seroprevalence of hantaviruses in small mammals varies across different regions of the world. In Malaysia (12), French Mayotte Island (13) and Italy (14), none of the tested serum samples of rodents were positive for hantavirus antibodies. In contrast, in Sweden (15), Japan (9), China (16), Korea (17) and Argentina (2), 1.4%, 1.6%, 6.89%, 7% and 11.9% of the rodents were seropositive for hantavirus, respectively.

Several studies have investigated the presence of hantavirus RNA in the tissues of infected rodents. In Japan and Sweden, Lokugamage et al. (9) and Lohmus et al. (15) reported that all examined tissues of the rodent samples were negative for hantaviruses. However, 4.5%, 3.4% and 13.8% of tissue samples of the rodents in Korea (17), Lithuania (18) and Germany (19) tested positive for hantavirus, respectively.

In the case of hantavirus infection in humans, a steady increase in the frequency of HFRS in Europe has been reported, with more than 10,000 HFRS cases annually (20). The hantavirus seropositivity in the staff of the Japan Ground Self-defense Force was only 0.48% (1 out of 207 cases) (9). A recent study in England showed that hantavirus seroprevalence was significantly higher among pet fancy rat owners (34.1%) compared to the control group (3.3%) (19). In Africa, out of the 200 febrile patients, 4 were anti-hantavirus IgM seropositive during the acute phase and IgG seropositive in the convalescent phase (21).

The first laboratory evidence of Hantavirus circulation in Iran was established by a study in 2014 in which IgM and IgG antibodies against Hantaviruses were detected in 4.5% (9 out of 200) street sweepers from central Iran (22). Later, in 2019, a nationwide study indicated Hantavirus IgM and IgG antibodies were identified in 16.8% and 3.5% of 113 Iranian patients with viral hemorrhagic fever clinical presentations, respectively (23). Additionally, a 2018 study showed a seropositivity rate of 0.5% (2 out of 385) of hantavirus IgG antibody in street sweepers in the southwest of Iran (24). There is limited published data regarding the circulation of hantavirus infections in small mammals, including rodents, in Iran. Therefore, the current study was carried

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out to investigate the molecular and serological evidence of hantavirus among rodents captured in Kohgiluyeh and Boyer-Ahmad Province, located in southwestern Iran.

Materials and Methods

Collection of rodents

Between June and November 2014, 52 rodents were captured from different areas of the Boyer-Ahmad region in Kohgiluyeh and Boyer-Ahmad Province, in the southwest of Iran (Fig.1). Sherman live traps baited with roasted almonds were used for rodent collection. The captured rodents were transported to the laboratory and their genus and species were identified based on their morphological features. Following anesthesia, blood samples were collected from the heart of each rodent and subjected to serum separation by centrifugation. Moreover, lung tissue samples were obtained from each rodent. Serum samples and lung tissues were stored at -20 °C and -70 °C, respectively, until further analysis.

Detection of anti-hantavirus antibodies by ELISA

To detect anti-hantavirus IgG antibodies in the serum samples of the rodents, Hantavirus Pool 1 "Eurasia" IgG and Pool 2 "America" ELISA IgG kits (Euroimmun, Lubeck, Germany) were used to detect IgG against both HCPS and HFRS-associated hantaviruses. According to the manufacturer's data, the sensitivity and specificity of Hantavirus Pool 1 "Eurasia" IgG ELISA kit range from 88.2 to 100% and from 94.1 to 100%, respectively. The sensitivity and specificity of Hantavirus Pool 2 "America" ELISA IgG kit are 100% and range from 88.7 to 100%, respectively.

ELISA was performed as per the manufacturer's instructions, except for the replacement of the secondary conjugated anti-human IgG by anti-mouse IgG (cat. no. PAB10764) to optimize the assay for identification of murine antibodies.

Detection of Hantavirus RNA in the tissue samples of the rodents by RT-PCR

Total RNA was extracted from rodent lung tissues, using the RNeasy Kit (QIAGEN, Hilden, Germany) based on the manufacturer's instructions. To identify viral RNA of hantaviruses in rodent tissue samples, we used a nested reverse transcription polymerase chain reaction (RT-PCR) assay as previously described (23, 25). Briefly, cDNA synthesis and the first PCR were carried out simultaneously using One-Step RT-PCR Kit (QIAgen GmbH, Hilden, Germany) in a 25µl reaction volume consisting of 5 µl 5 × Qiagen OneStep RT-PCR buffer, 1 mM of HAN-L-F1: 5'-ATGTAYGTBAGTGCWGATGC-3'' and HAN-L-R1: 5'-AACCADTCWGTYCCRTCATC-3' primers, 1 µl of Qiagen OneStep RT-PCR Enzyme Mix, 1 µl of dNTPs Mix (containing 10 mM of each dNTP), and 2.5 µl of extracted RNA. The thermal cycling program was as follows: for cDNA synthesis, 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 sec, 53 °C for 30 sec, 72 °C for 45 sec and a final extension step of 72 °C for 10 min. Then, the nested PCR was performed using the Takara ExTaq PCR kit (Takara Bio Inc., Otsu, Japan). The reaction was done in a 25µl total volume, which consisted of 2.5µl of 10X Ex Taq buffer, 2.5µl $MgCl_2$, 1µM of HAN-L-F2 5'-TGCWGATGCHACIAARTGGTC-3 and HAN-L-R2 5'-GCRTCRTCWGART GRTGDGCAA-3' primers, 0.125µl of TaKaRa Ex Taq DNA pol (250 U), 2µl dNTPs Mix (containing 10 mM of each dNTP) and 2µl of the first PCR product. The thermal cycling program was as follows: 5 min at 94 °C followed by 40 cycles of 94 °C for 30 sec, 53 °C for 45 sec and 72 °C for 30 sec with a final extension step of 72 °C for 6 min. The nested PCR products (390 bp) were subjected to 2% agarose gel electrophoresis and visualized by safe stain (Yekta Tajhiz Azma, Iran). In each run, Nova hantavirus RNA (a gift from the Institut de Systématique, Evolution, Biodiversité, Paris, France) and nuclease-free water were used as positive and negative controls, respectively.

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Results

Among the 52 rodents studied, *Meriones persicus* was the most frequently observed species (n=25, 48%). Other species identified among the trapped rodents included *Apodemus sylvaticus* (n=10, 19%), *Rattus norvegicus* (n=8, 15%), *Rattus rattus* (n=7, 13%), *Arvicola terrestris* (n=1, 1.9%) and *Calomyscus bailwardi* (n=1, 1.9%). No anti-hantavirus antibodies were identified in the sera of any of the trapped rodents, using ELISA assays. Moreover, no hantavirus RNA was detected in any of the tissue samples of the rodents examined by the pan-hantavirus nested-PCR method. Table 1 presents the molecular and serological findings for hantavirus detection in the trapped rodents, grouped by species.

Table 1. IgG ELISA and RT-PCR results by species and sex of rodents captured in the Boyer-Ahmad region of Kohgiluyeh and Boyer-Ahmad Province, between June and November 2014. The table shows the distribution of captured rodent species, including frequency, mean body weight, sex ratio, and results of hantavirus IgG ELISA and RT-PCR. All tested specimens were negative for hantavirus infection

Rodent Species	Frequency (n)	Weight mean±SD (g)	Gender		America ELISA IgG assay	Eurasia ELISA IgG assay	Pan-Hantavirus RT-PCR
			Female	Male			
Apodemus sylvaticus	10	55.5± 9.2	4	6	Negative	Negative	Negative
Arvicola terrestris	1	145	0	1	Negative	Negative	Negative
Calomyscus bailwardi	1	90	1	0	Negative	Negative	Negative
Meriones persicus	25	65.8 ± 13.9	14	11	Negative	Negative	Negative
Rattus norvegicus	8	284.3 ± 16.5	3	5	Negative	Negative	Negative
Rattus rattus	7	202.1 ± 12.8	2	5	Negative	Negative	Negative

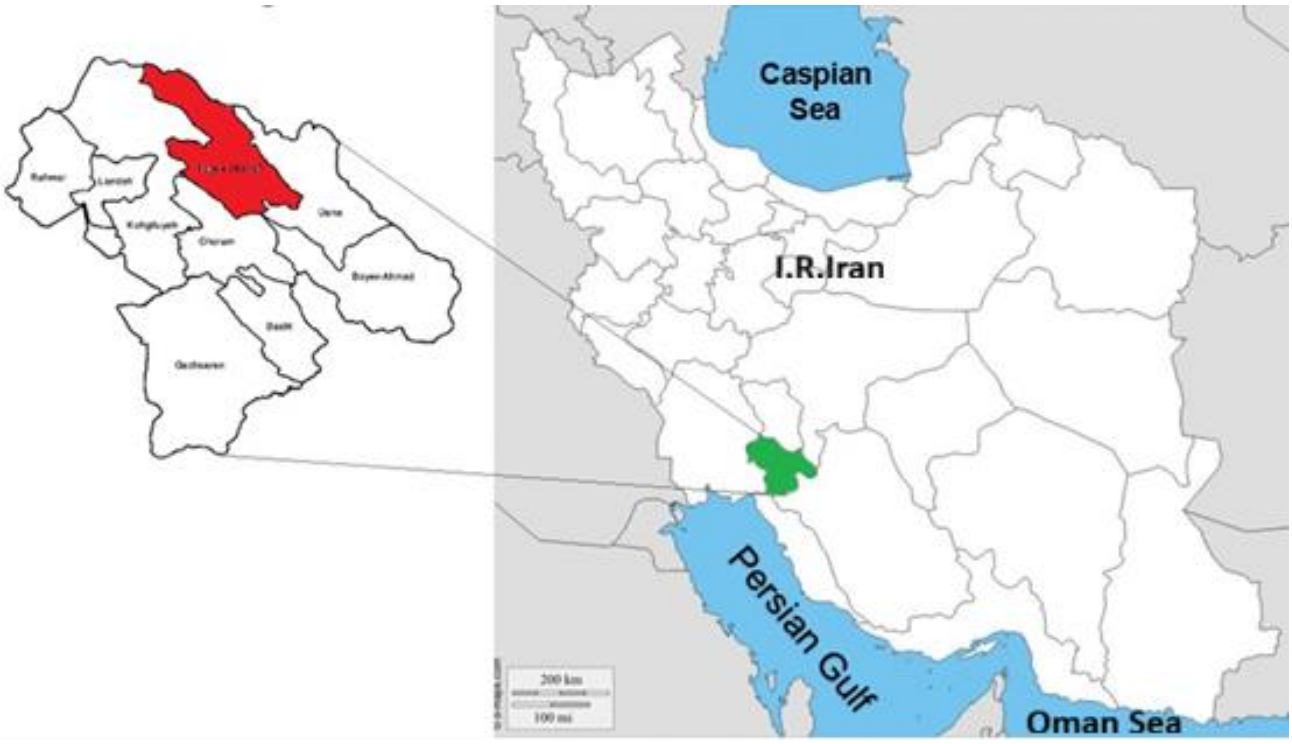

Fig. 1. Map of Iran showing the study area (Boyer-Ahmad District in Kohgiluyeh and Boyer-Ahmad Province)

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Discussion

The findings of this study showed that none of the rodents captured in the study area were exposed to hantavirus as determined by IgG ELISA for both Old World and New World hantaviruses. In line with our findings, Filippone et al. reported that none of the mammals from French Mayotte island tested positive for hantavirus antibodies (13). Moreover, Hamdan et al. reported that none of the 53 tested serum samples of rodents were seropositive in Malaysia (12). On the other hand, in Argentina and China, 11.9% and 6.89% of the rodents were seropositive for hantaviruses, respectively (2, 16). Klein et al. (17) reported that 8.2% of *Apodemus agrarius*, 4.2% of *Micromys minutus* and 1.5% of *Crocidura lasiura* were infected with hantaviruses (Hantaan virus (HTNV)) in Korea. Lohmus et al. detected hantavirus-specific antibodies in *Apodemus flavicollis* in mice in the suburbs of the cities of Uppsala and Stockholm, Sweden (15). In Japan, Lokugamage et al. (9) showed that 1.0%, 1.1%, 6.7%, and 3.6% of *Apodemus*, *Rattus norvegicus*, *Rattus rattus* and *Clethrionomys rufocanus* were seropositive for Hantavirus antibodies, respectively.

Pan-hantavirus nested-PCR assay showed that all lung tissue samples were negative for hantavirus RNA in our study. Similar to the findings of this study, Lokugamage et al. (9) reported that all rodent tissue samples from Japan were negative for hantavirus RNA. Moreover, in Sweden, Lohmus et al. (15) reported that hantavirus RNA genome was not detected in any of the tissue samples of the rodents. On the other hand, in the French Mayotte island, 18% of *Rattus rattus* were found to be positive for hantavirus RNA (13). In Klein et al.'s study, Hantaan virus (HTNV) RNA was detected in 4.5% of the captured rodents in Korea (17). Additionally, 5.6% and 13.8% of the tissue samples of the rodents in Lithuania (18) and Germany (19) were positive for hantavirus RNA, respectively.

Despite the negative results of the present study, there is strong and convincing evidence that hantavirus is present in Iran. Several independent studies have confirmed active circulation of hantavirus in the country's human and animal populations. In a nationwide study of 113 patients with suspected viral hemorrhagic fevers, exposure to Old World hantaviruses, particularly Puumala and Hantaan viruses, was reported; 16.8% of cases had IgM antibodies and 3.5% had IgG antibodies against hantaviruses (23). Serological surveys among municipal sweepers in Iran, known as a high-risk group for hantavirus infections, reported low prevalence of anti-hantavirus antibodies in the cities of Isfahan (4.5%) and Shiraz (0.5%) (22, 24). Small rodent surveillance in East Azerbaijan Province led to the detection of Tula orthohantavirus (TULV) RNA in a forest mouse (*Dryomys nitedula*) using a pan-hantavirus nested RT-PCR method (26). This finding represents the first genetic evidence of hantavirus in Iranian wildlife. Recently, in a 2023 study in Tehran, Seoul virus (SEOV) was detected in 12% of an urban rat population, *Rattus norvegicus*, which is the first report of this rodent-borne virus in the country (27).

Since hantavirus prevalence depends on the population dynamics, as well as on agricultural and climate changes (28, 29), ongoing molecular and serological studies are essential for the prevention and control of this rodent-borne virus, not only in humans, but also in rodent populations (3).

The results also showed that among the collected rodents, *Meriones persicus* was the most prevalent species, followed by *Apodemus sylvaticus*, *Rattus norvegicus*, *Rattus rattus*, *Arvicola terrestris*, and *Calomyscus bailwardi*. In previous studies, Filippone et al. showed Hantavirus genomic RNA in 18% of *Rattus rattus* from French Mayotte island, Indian Ocean (13). Xiaokang Sun et al. revealed the presence of Seoul hantavirus in *Rattus losea* and

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Rattus norvegicus in southern China between 2011 and 2013 (30). Similarly, Gerardo Rubén Cueto et al. detected a rate of 11.9% hantavirus seropositivity in *R. norvegicus* in Buenos Aires City, Argentina (2).

Around 79 rodent species have been identified in Iran (31, 32). Besides the families *Muridae* and *Cricetidae*, the mid-day gerbil (*Meriones meridianus*) has been reported in northeastern Iran. The presence of *Rattus norvegicus* has been documented in Turkmen plains, Tehran, Khuzestan, Razavi, Khorasan, and East Azerbaijan Provinces. Field mice (genus *Apodemus*) exhibit considerable diversity in the northern and western regions of Iran. The water vole (*Arvicola amphibious*) has been observed in these regions. Both the black rat (*Rattus rattus*) and *R. norvegicus* have been found throughout Iran.

Our study did not detect any hantavirus antibodies or RNA in the rodents sampled, but interpretation of negative results requires caution. Several factors could account for the lack of detection, including small sample size, sampling outside of peak transmission seasons, and limitations of modified serological assays that may not be sensitive enough for the detection of hantavirus antibodies in rodents. Also, host susceptibility to viral infection varies, and the dominant species in our study may not be efficient reservoirs for hantaviruses, unlike the *Rattus* or *Apodemus* species known to be vectors of hantavirus in other countries. Limited geographic coverage also reduces the likelihood of detecting low-prevalence infections. Available evidence from Iran, including positive seroepidemiology in patients suspected of viral hemorrhagic fevers, occupational exposure in urban sweepers, detection of Tula virus in *Dryomys nitedula* and Seoul virus in *Rattus norvegicus* in Tehran, indicates that hantaviruses are circulating in the country. Therefore, our negative results do not mean that there is no risk but rather highlight the need for larger studies with greater seasonal and geographical coverage and integrating rodent and human surveillance to better understand the ecology of hantaviruses in Iran.

Conclusion

Although no hantavirus infection was observed in this study, the presence of hantavirus reservoirs in Kohgiluyeh and Boyer-Ahmad Province suggests the circulation of the virus cannot be completely ruled out, and therefore, further studies with a larger sample size are recommended.

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Ethical Considerations

This research has been approved by the Ethics Committee of Shiraz University of Medical Sciences under the ethics code IR.sums.med.rec.1396.S37.

Conflict of Interest Statement

The authors declare there is no conflict of interest.

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