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Short Research Communication

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Abstract

Background: Tick-borne rickettsioses have become a health concern worldwide following the increasing incidence in recent decades. However, there is limited information about these diseases in Islamic Republic of Iran.

Aim: This cross-sectional study was conducted to estimate the Rickettsia infection among ixodid ticks collected from cattle, sheep and goats in Islamic Republic of Iran.

Methods: The DNA of ixodid ticks collected from cattle, sheep and goats in 54 villages of Zanjan Province, Islamic Republic of Iran, were collected and analysed using a spectrophotometer. Rickettsial-positive samples were screened by targeting the htrA gene and fragments of gltA gene were analysed. The variables were analysed using descriptive statistics and the $\chi 2$ test was used to compare the variables.

Results: A total of 528 ticks were tested. Overall, Rickettsia infection rate was 6.44%. Nine of the 12 tick species were infected. Rickettsial positive rates in Hyalomma marginatum and Dermacentor marginatus were 21.33% and 12.77%, respectively. R. aeschlimannii, the predominant rickettsia, was detected only in Hy. marginatum. R. raoultii, R. sibirica and R.

slovaca comprised about half of the positive ticks and were recovered from more than one tick species.

Conclusion: Considering the discovery of infected ticks in the Islamic Republic of Iran, there is a need to establish a tick control programme in the country, paying attention to populations at high-risk.

Keywords: Rickettsia, spotted fever, rickettsioses, gltA gene, htrA gene, Ixodid ticks, tick-borne disease, Iran

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Background

Spotted fever group (SFG) rickettsioses, caused by a neglected group of bacteria belonging to the genus Rickettsia, are recognized as new and emerging vector-borne diseases with a worldwide distribution (1). These infections are mainly transmitted by hard ticks and maintained in nature in cycles involving tick vectors that act as reservoirs. Variations of ecological factors alter the status of human contact with infected ticks and change the transmission map of these diseases (2).

Historically, Rickettsia rickettsii, R. sibirica, R. japonica, R. conorii and R. australis were considered to be the traditional SFG rickettsiae (3). More recently, application of molecular markers, such as 17-kDa lipoprotein outer membrane antigen (htrA), citrate synthase (gltA) and rickettsial outer membrane proteins A and B (OmpA and OmpB) genes in the diagnosis of

rickettsioses, and variations of ecological factors have changed the epidemiological map of the world's SFG rickettsioses in the last decades (3,4).

Likewise, the Middle East region has undergone drastic environmental change, and several rickettsial diseases have been reported (5,6). Serological surveys have confirmed the presence of R. conorii in this region since ancient times (3), and R. aeschlimannii, R. slovaca, R. sibirica and R. raoultii have lately been reported in the Mediterranean Basin and Transcaucasia (7–9).

R. hoogstraalii, R. monacensis and R. helvetica have been reported in Turkey (10). Despite records of numerous rickettsiae in Turkey, there is limited evidence of Rickettsia infection in the neighbouring areas of the country, particularly in northwestern Islamic Republic of Iran (3). Recent cases of R. conorii israelensis and other rickettsial infection of Hyalomma asiaticum have been reported in southern Islamic Republic of Iran (11). Surveys of tick pool samples have documented the presence of R. slovaca, R. massiliae, R. sibirica, R. raoultii and R. aeschlimannii in the north and northwestern Islamic Republic of Iran (12–14).

The northwest of Islamic Republic of Iran, the gateway of Eurasia and a link between the continents, is an important area for vector and reservoir hard ticks. This region acts as a bridge between Africa, Europe and Asia for migratory birds and has proper geographical conditions for breeding migratory birds. This natural condition favours many tick infestations in both animals and humans.

SFG rickettsiosis is a mandatory notifiable disease in northwest Islamic Republic of Iran (15); however, due to under-notification and misdiagnosis of cases, the real incidence of the disease remains obscure. Identifying rickettsial infection of hard ticks in the northwest of the country may help explain the prevalence of SFG rickettsioses in this area.

Because of the clinical importance of SFG rickettsioses, the presence of large populations of ixodid ticks and their potential for infection with rickettsial pathogens underscore the need to characterize these agents in northwest Islamic Republic of Iran. Therefore, we investigated rickettsial infections in hard ticks collected from cattle, sheep and goats in the Zanjan Province of the country.

Methodology

Study area, sampling and identification of ticks

This cross-sectional study was conducted in 54 villages of Zanjan Province where residents keep domestic animals (sheep, cattle and goats), from April to November 2019 and in 2020. The villages are located on the path of the Mediterranean, West Asian, East African and Central Asian migratory bird flyways (https://www.eaaflyway.net/the-flyway/). These villages comprise lowlands, plains and highlands with altitudes of 2000 metres above sea level, respectively (Fig ure 1).

The animals' owners verbally approved the collection of ticks. Adult hard ticks were collected from the ear, neck, armpit, chest, abdomen and crissum of randomly selected animals. Feeding ticks (randomly 1–4 ticks per animal) were hand-picked using tweezers and gloves, transferred to 1.8 ml cryovial tubes and stored at -20°C until further examination. Collected ticks were identified morphologically at the species level using standard taxonomic keys (16,17).

DNA extraction and evaluation of DNA quantity and quality

The collected ticks were soaked in 70% ethanol, rinsed with sterile distilled water and dried on sterile filter paper. Individual ticks were pulverized in liquid nitrogen using a sterile mortar and pestle. Fine powder of each sample was suspended in 500 µl of lysing buffer [100 mM of Tris-HCl (pH 8.0), 0.5 mM of NaCl, 10 mM of EDTA and 1% W/V SDS], and genomic DNA (gDNA) extracted as described previously (18).

DNA samples were analysed by a spectrophotometer (NanoDrop, Thermo Fisher Scientific). Low-quality samples of

PCR amplification and identification of Rickettsia species

The 17kDF (5′-GCTCTTGCAACTTCTATGTT-3′) and 17kDR (5′-CATTGTTCGTCAGGTTGGCG-3′) primers were used for the initial detection of Rickettsia in total DNA. These primers recognize htrA gene, encoding 17-kDa outer membrane lipoprotein (4). Rickettsia-positive samples were further characterized using GLTF (5′-ATCCTATGGCTATTATGCTTGC-3′) and GLTR (5′ TACATAACCGGTGTAAAGCTGT-3′) primers as described previously (18,19).

These primers were modified based on the gltA gene of R. conorii (accession MK304547). Each 25 µl total volume of PCR reaction contained 3 µl of gDNA (25–50 ng/µl), 10 pM of each primer, 12.5 µl of 2× Mix Master Red (Amplicon, Denmark) and 7.5 µl of nuclease-free water. All amplifications were completed using the following thermal profile: initial denaturation at 95°C for

5 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 58°C for 50 seconds, and extension at 72°C for 1 minute.

A final extension cycle at 72°C for 5 minutes was performed, and reactions were cooled at 4°C. For each reaction, a negative control of ddH2O was used. PCR products were resolved by electrophoresis on a 1.5% agarose gel, run for 40 minutes at 80 V, visualized under a UV light and analysed with the 100-bp DNA ladder. PCR products were sequenced bidirectionally by Macrogen (Seoul, Republic of Korea) using the forward and reverse specific primers.

Data analysis

Variables were analysed using descriptive statistics presented as frequencies and percentages. The 95% confidence interval was used to statistically estimate proportions. The chi-squared test was used to compare variables. Calculated differences among variables were considered statistically significant for P

The obtained sequences were checked and assembled using Chromas (https://technelysium.com. au/wp/chromas/) and BioEdit 7.0 (https://bioedit.software. informer.com/7.0/). Each sequence was analysed using BLASTN (https://blast. ncbi.nlm.nih.gov/Blast.cgi) with gltA and htrA reference sequences available in the GenBank for sequence homology.

Sequence trace files were analysed with MEGA X (https://www.megasoftware.net/downloads/dload_win_gui), DNASTAR 6 (http://www.ub.edu/dnasp/downloadTv6.html), PopART Qt4.8.4 (http://popart.otago.ac.nz) and T Coffee (https://tcoffee.org/Projects/tcoffee/index.html) software for constructing phylogenetic trees and identifying haplotype groups.

Phylogenetic trees of studied groups were constructed using Neighbor-Joining and Maximum Likelihood statistical methods using the Kimura 2-parameter model under 500 bootstrap replicates. The sequences of R. prowazekii were included as an out-group.

Results

A total of 638 adult hard ticks were collected from 363 sheep, 167 cattle and 47 goats. Of the

identified ticks, 528 ticks were tested for Rickettsia infection. Most of these samples were collected from sheep and cattle (PTable 1. Overall, 4 genera and 12 species of hard ticks were identified. Hy anatolicum and Hy. marginatum ticks were the most common species, comprising 22.92% and 14.20% of the samples, respectively (P

Overall, Rickettsia infection was detected in 34 (6.44%) of 528 tested samples. The abundance of Rickettsia in studied ticks is summarized in Table 1 . Hy. marginatum, Dermacentor marginatus, Haemaphysalis sulcata and Rhipicephalus bursa had the highest infection rates (21.33%, 12.77%, 9.52% and 9.30%, respectively).

Ticks collected from goats were free of rickettsial infection, as were all ticks collected from plains areas. Rickettsial infection was highest (19.18%) in tested ticks from lowlands, and lowest (3.70%) in ticks from the highlands (P

Sequencing of htrA gene amplicons showed a ~370-bp DNA fragment size, and their multiple alignments exhibited 3 rickettsial groups that were similar to R. raoultii/sibirica, R. slovaca and R. aeschlimannii (Figure 2). Fragments of the gltA gene recovered from 34 positive ticks yielded an 842-bp size and shared more than 99.8% identity with the reference species of R. aeschlimannii, R. raoultii, R. sibirica and R. slovaca.

Table 2 shows identified species of Rickettsia infecting hard ticks in the studied area. The nucleotide sequences of the gltA gene samples were deposited in the GenBank under the accession MT293336–MT293352, MW117933–MW117936 and MW219588–MW219600.

BLASTN sequence analysis of Rickettsia fragments in 16 positive Hy. marginatum ticks showed 99% nucleotide identity with the gltA gene of the R. aeschlimannii reference strain (accession KU961540). Six ticks of De. marginatus contained the DNA of Rickettsia, in which 99% nucleotide identity was presented with the gltA gene of reference R. sibirica (accession KM288711 and HM050296) and R. raoultii (accession MK304547) in 4 and 2 samples, respectively. Of 6 positive Rh. bursa ticks, Rickettsia DNA shared 99% identity with the gltA gene of reference R. raoultii (accession MK304547) in 4 ticks and R. sibirica (accession KM288711) in 2 ticks.

Two samples each of Hy. anatolicum, Hy. detritum, Hy. dromedarii and Ha. sulcata ticks were

rickettsial positive. Rickettsia detected in the Hy. anatolicum samples shared 99% identity with R. slovaca (accession MN581989). Rickettsia obtained from the Hy. detritum and Hy. dromedarii specimens showed 98% identity with R. sibirica (accession HM050296).

Rickettsia recovered in the Ha. sulcata samples shared 99% identity with R. raoultii (accession MK304547). Based on phylogenetic analysis, rickettsial samples assembled in 2 monophyletic groups: R. aeschlimannii, the most evolved taxon, R. raoultii, its closest taxon, matched in one group; and italics and R. slovaca formed the sister group of the mentioned samples (Figure 3).

R. aeschlimannii, the predominant Rickettsia, was detected only in Hy. marginatum. Other species, R. raoultii, R. sibirica and R. slovaca, comprised about half of the rickettsial positive ticks. These Rickettsiae species infected more than one tick species (see Table 2).

Phylogenetic analysis of R. aeschlimannii gltA sequences identified 4 haplotypes: HI, HII, HIII and HIV, with the ratios of 44%, 25%, 18.5% and 12.5%, respectively (see Figure 4). Samples of the dominant haplotype (HI) and haplotype HIII were similar to the specimens from Egypt, Zambia and Senegal, as well as Mongolia, China and Kazakhstan.

HII and HIV were matched with the samples from Cyprus, Lebanon, Italy, France and Russia (Figure 5). Three types of R. raoultii matched the Chinese and Russian samples. Each of the R. slovaca and R. sibirica specimens was monotype.

The samples of R. slovaca were related to French and Slovakian specimens, and R. sibirica samples were linked to specimens from China and Mongolia, as well as Senegal (Figure 6). The fragments of R. sibirica we studied were completely conserved, and nucleotide analysis could not identify its current subspecies.

Discussion

This study revealed 4 Rickettsia species including Dermacentor, Hyalomma, Haemaphysalis and Rhipicephalus ticks from domestic animals in northwestern Islamic Republic of Iran.

Despite the fact that this region is the main global route of migratory birds, to our knowledge, there has been no comprehensive study on the prevalence of Rickettsia species in this area.

While the tick collection method applied in this study has been used previously (12,20,21), the survey of engorged ticks is a limitation that may cause interference in the results. However, the presence of infective ticks and evidence of population exposure to these ticks may have flawed the extent of subclinical and acute forms of SFG rickettsioses in the study areas.

Our findings revealed the presence of rickettsial infected ticks in both lowlands and highlands, but not in the plains. Acaricides have been a commonly used to control ticks in the study areas for several years. However, technical, operational, and financial problems have caused heterogeneity in these areas. The absence of rickettsial infection in the ticks in the plains areas may be related to the geographical isolation of livestock, and complete coverage with acaricides.

This study showed the high infection rate of R. aeschlimannii in Hy. marginatum in a restricted lowland region, the countryside of Abbar City in Tarom County. It has long been known that Hy. marginatum is the main vector of R. aeschlimannii (22), and high prevalence of this pathogen has previously been reported (23,24).

Because the high tick infection rate increases the risk of rickettsioses, the localization of infected Hy. marginatum ticks in Tarom County of Zanjan Province should be considered in future investigations. This phenomenon may have arisen from the introduction of infected vector ticks by migratory birds and/or vertebrate hosts to this region. Consequently, further studies are recommended to elucidate such a likelihood.

Our findings indicate that 3 emergent Rickettsia species – R. raoultii, R. sibirica and R. slovaca – account for about half of the rickettsial-positive ticks in northwest Islamic Republic of Iran. R. raoultii has been detected in a wide range of tick genera (3,20,21,25) and human cases of this pathogen have been reported in Eurasia (3,28).

Recently, in the taxonomic revision of Rickettsiae, R. raoultii was identified as a subspecies of R. conorii (26); however, this nomenclature is invalid (27). Recognition of R. slovaca in De. marginatus is in line with previous studies (28,29). As far as we know, this is the first report of R.

slovaca presence in Hy. anatolicum. Detection of R. raoultii versus Ha. sulcata and Rh. bursa, and R. sibirica versus Hy. spp and Rh. bursa need to be confirmed.

The presence of R. sibirica in Hy. detritum, Hy. dromedarii and Rh. bursa ticks indicates the emergence of these species in the Islamic Republic of Iran. The low infection rate of this pathogen is related to its high pathogenicity in the vector ticks (3,30).

Two subspecies, namely R. sibirica sibirica and R. sibirica mongolitimonae, have been recognized for this Rickettsia. Cases of R. sibirica sibirica have been reported from North Asia in Dermacentor ticks (31,32). R. sibirica mongolitimonae infections have occurred in a wide region of southern Europe, the Middle East, North and Southern Africa, and Central Asia. This Rickettsia has been isolated from different tick species (3,8).

The presence of such emerging species as well as other Rickettsia species in tick infestations strongly indicates the need for increased surveillance of SFG rickettsioses, development of diagnosis and treatment facilities, and vector management programmes in this area. Physicians need information on the occurrence of these diseases and related clinical symptoms to enhance their awareness and enable them to request for laboratory diagnostic confirmation if required. More efficient monitoring of these pathogens at local, national and international levels is imperative.

This study showed a relatively high level of rickettsial infection in Hy. marginatum and De. marginatus ticks. These ticks are commonly found in the Middle East and are passively transmitted over long distances by passerine migratory birds (33). The potential role of these ticks in the transmission of SFG rickettsioses should be taken into account in future investigations.

On behalf of the Iranian Ministry of Health and Medical Education and with the cooperation of the Ministry of Agriculture Jihad and Iran Veterinary Organization, public health officers should prepare health brochures that promote health culture and inform the population about potential emerging threats associated with these ticks.

Conclusion

Further investigation is needed to determine the potential role of De. marginatus and Hy.

marginatum ticks in the transmission of SFG rickettsioses. Provision of health knowledge on emerging threats of infected ticks, surveillance of SFG rickettsioses, and development of tick control programmes are recommended to improve community health.

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Competing interests: None declared.

Émergence d'infections à Rickettsia du groupe de la fièvre pourprée chez les tiques dures en République islamique d'Iran

Résumé

Contexte : Les rickettsioses transmises par les tiques sont devenues un problème de santé dans le monde entier suite à leur incidence croissante au cours des dernières décennies. Cependant, les informations concernant ces maladies sont limitées en République islamique d'Iran.

Objectif : La présente étude transversale a été menée afin d'évaluer l'infection à Rickettsia chez les tiques ixodidées prélevées sur les bovins, les ovins et les caprins en République islamique d'Iran.

Méthodes : L'ADN de ces tiques, prélevées dans 54 villages de la province de Zanjan (République islamique d'Iran) a été recueilli et analysé à l'aide d'un spectrophotomètre. Les échantillons positifs pour les rickettsies ont été sélectionnés en ciblant le gène htrA et des fragments du gène gltA ont été analysés. Les variables ont été étudiées à l'aide de statistiques descriptives et le test $\chi 2$ a été utilisé afin de procéder à leur comparaison.

Résultats : Au total, 528 tiques ont été testées. Dans l'ensemble, le taux d'infection à Rickettsia était de 6,44 %. Neuf des 12 espèces de tiques étaient infectées. Les taux positifs de rickettsies chez les tiques Hyalomma marginatum et les tiques Dermacentor marginatus étaient de 21,33 % et 12,77 % respectivement. R. aeschlimannii, la rickettsie prédominante, n'a été

détectée que chez les tiques Hy. marginatum. R. raoultii, R. sibirica et R. slovaca se retrouvaient sur près de la moitié des tiques positives et ont été isolées sur de plusieurs espèces de tiques.

Conclusion : Compte tenu de la découverte de tiques infectées en République islamique d'Iran, il est nécessaire de mettre en place un programme de lutte contre ces dernières, en accordant une attention particulière aux populations à haut risque.

WHO EMRO | Emergent spotted fever group Rickettsiae infections among hard ticks in Islamic Republic

والرِّيكِتْسِيَّة سلوفاكا، إذ وُجِدَت في أكثر من نوع من أنواع القُراد .

: في ضوء اكتشاف القُراد المصاب في جمهورية إيران الإسلامية، هناك حاجةالاستنتاجات
إلى إنشاء برنامج لمكافحة القُراد في البلاد، وإيلاء الاهتمام للفئات السكانية
الأكثر عرضة لخطر الإصابة به .

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