First Molecular Detection of Human Pathogen Rickettsia Raoultii in Ticks from Republic of Korea

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Research

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Abstract

**Background:** Rickettsial diseases, associated with the spotted fever group (SFG), constitute a growing number of newly identified rickettsia pathogens and their tick vectors, in various parts of the world. At least 15 distinct tick species owing to six genera have shown the presence of *Rickettsia raoultii*. Here, we report the detection of *R. raoultii* in ticks from the Republic of Korea (ROK).

**Methods:** A total of 35 ticks, collected from patients of tick bites in Gwangju Metropolitan City, Jeollanam Province, ROK. The ticks were identified through their molecular, morphological, and taxonomic characteristics. All samples were screened by nested polymerase chain reactions of their outer membrane protein (*ompA*) and citrate synthase (*gltA*) genes. The amplified products were sequenced and their phylogenetic analyses were carried out.

**Results:** Sequencing data showed that the DNA sequences of *R. raoultii* found in the three *H. longicornis* ticks. All 3 tick samples were 99.4-100% analogous to the previously reported partial sequences of *ompA* of *R. raoultii* strains CP019435 and MF002523, forming a single clade with the reference strains.

**Conclusions:** Present study provides the first description and molecular identification of *R. raoultii* detected in *H. longicornis* ticks in ROK. This observation extends the geographical distribution of *R. raoultii*. Screening of human samples for this pathogen will provide information about the prevalence of rickettsial infection in this region.

1. **Background**

Tick-borne diseases are a growing medical concern worldwide. Ticks are considered as the main reservoir and vectors of rickettsia, a set of obligately intracellular bacteria, and responsible for transmission of rickettsial diseases to humans. This zoonosis represents both the oldest known and recently identified vector-borne infectious disease [1]. The causative agents belong to the genus *Rickettsia* and are presently classified into four groups: the spotted fever group (SFG), the typhus group, the *Rickettsia bellii*, and the *Rickettsia canadensis* group [2]. SFG rickettsiae constitutes of newly identified *Rickettsia* species around the world. In the last few decades, numerous species of tick-borne rickettsiae, previously thought to be non-pathogenic, are recognized as human pathogens [2].

In 1999, three novel rickettsial genotypes, RpA4, DnS14, and DnS28 were recognized in ticks from Russia [3]. Consequently, with genotypic and phenotypic analysis of their characteristics, these bacteria were recognized as novel species of SFG rickettsiae, and in 2008, these were named *Rickettsia raoultii* [4]. The major clinical manifestations of *R. raoultii* include scalp eschar and neck lymphadenopathy (SENLAT). Initially, these were called as dermacentor borne necrosis erythema and lymphadenopathy (DEBONEL) or tick-borne lymphadenopathy (TIBOLA) [2]. To date, *R. raoultii* has been identified in many Asian and European countries [5–8]. In 1999, *Dermacentor nuttalli* and *Rhipicephalus pumilio* ticks collected from the southern part of the former Soviet Union were shown to carry these bacteria [3], and thereafter, other species of *Dermacentor* ticks (i.e., *D. reticularis, D. marginatus, D. silvarum, and D. niveus*) from various
parts of the former Soviet Union, France, Spain, and Germany, were also reported to harbor them [5, 9]. Subsequently *R. raoultii* were detected in other hard ticks too, such as *Haemaphysalis, Rhipicephalus, Hyalomma*, and *Amblyomma*, predominantly in Europe and Asia [10].

The aim of this study was to determine the existence of *R. raoultii* in ticks, and to examine the presence and circulation of this pathogen in tick populations in ROK. We found *R. raoultii* in *Haemaphysalis longicornis* ticks. To the best of our knowledge, this is the first molecular evidence of *R. raoultii* in ticks from the ROK.

2. Methods

2.1. Tick sampling and classification

In 2018, 35 ticks collected from patients with a history of tick bites in Gwangju Metropolitan City, Jeollanam Province, ROK. These were identified on the basis of their molecular, morphological, and standard taxonomic characteristics. Briefly, the ticks were first decontaminated in 70% ethanol, rinsed twice with sterile phosphate-buffered saline (PBS), and dried on sterile filter paper. Each sample was then placed in a hard-tissue-grinding MK28 tube (Bertin Technology, Rockville, MD, USA) containing 800 µL of PBS and 1X PC/SM (penicillin and streptomycin). Subsequently, ground using FastPrep®-24 Classic instrument (MP Biomedicals, Solon, OH, USA), and stored at −80 °C until DNA extraction. For molecular identification, the genomic DNA was subjected to mitochondrial 16S rRNA gene, was amplified through PCR and analyzed [11].

2.2. DNA extraction

Total genomic DNA was extracted from 150 µL of the tick homogenate, using a QIAamp Tissue & Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and eluted in 50 µL volume. The samples were stored at -20 °C until their polymerase chain reaction (PCR) amplification.

2.3. PCR amplification

To analyze the presence of *Rickettsia* species in ticks, the bacterial DNA samples were subjected to nested PCR (N-PCR), designed to target the outer membrane protein A (*ompA*) and citrate synthase (*gltA*) genes. The primers used for the reactions and the product sizes are shown in Table 1. The reactions were carried out in 20 µL volume, comprised of 16 µL of distilled water, 1 µL of each primer (10 pmol/µL), and 2 µL of genomic DNA template using AccuPower® PCR PreMix (Bioneer Corp., Korea). PCR was carried out using AB thermal cycler (Applied Biosystems, Foster City, CA, USA). A positive control with *R. conorii* DNA, and negative control with distilled water instead of template DNA were also included in each set of PCR. The amplified products were visualized by electrophoresis on a 1.2% agarose gel containing ethidium bromide.
Table 1
Oligonucleotide primers used to perform PCR in this study to detect molecular targets of *Rickettsia* species.

| Target | Primers | Nucleotide sequence (5’-3’) | Fragment length | Reference |
|--------|---------|-----------------------------|----------------|----------|
| ompA   | RR190.70F | ATGGCGAATATTTCTCCAAAAA      | 634 bp (first) | [12]     |
| RR190.701R | GTTCCGTTAATGGGCAGCATCT       |               |                |          |
| RR190.70F | ATGGCGAATATTTCTCCAAAAA      | 535 bp (nested) | [13]          |
| RR190.602R | AGTGCAGCGATTCCGTCCCCCT      |               |                |          |
| gltA   | GLTA1F  | GACGGTGATAAAGGAATCTTG       | 1022 bp (first)|          |
| GLTA1R | CATTTCTTTCCATTGTGCCATC      |               |                |          |
| GLTA2F | CTACGAACTTACCCTATTAG        | 446 bp (nested) | [13]          |
| GLTA2R | GACCCAAAACCTTAACCTAAC      |               |                |          |

2.4. Phylogenetic analysis

The PCR products were purified with QIAquick PCR purification kits (QIAGEN, Hilden, Germany) and sequenced by Solgent Inc. (Daejeon, Korea). To analyze the percentage of similarity, the resulting sequences were correlated for identity with sequences from GenBank, using the Basic Local Alignment Search Tool (BLAST) program. Neighborhood-joining method was employed to build a phylogenetic tree with ClustalW of the MegAlign Program (DNASTAR, USA). Stability of this phylogenetic tree was tested with bootstrap analysis (1,000 reiterations) by the Kimura 2-parameter method.

3. Results

The molecular, morphological, and taxonomic characteristics revealed that 4 of the 35 ticks were *Ixodes nipponensis*, 14 were *Amblyomma testudinarium*, and 17 were *Haemaphysalis longicornis* (Table 2). PCR products were amplified from 35 ticks for the identification of SFG rickettsial disease, targeting the *ompA* and *gltA* genes. Sequencing data of positive samples, targeting the *ompA* gene, revealed one distinct rickettsial species in three of the *H. longicornis* ticks, which were identified as *R. raoultii*. Morphological and taxonomic characteristic showed the these ticks were adult female. Conversely, the results of PCR targeting the *gltA* gene did not detect any distinct rickettsial species.
Table 2
Tick species analyzed in this study by stages of development and sex, collected from patients of tick bite

| Tick species | *Haemaphysalis longicornis* | *Amblyomma testudinarium* | *Ixodes nipponensis* |
|--------------|-----------------------------|---------------------------|---------------------|
| Developmental stage | | | |
| Adult female | 13 | - | 4 |
| Adult male | - | 5 | - |
| Nymph | 1 | 9 | - |
| Larva | 3 | - | - |
| Total No. | 17 | 14 | 4 |

Even though these three ticks were harvested from patients, PCR results of blood samples obtained from respective patients did not show the *R. raoultii* infection nor did they show any symptoms specific to such an infection. *R. raoultii* sequence of the *ompA* gene from all 3 tick samples were 99.4–100% homologous to the previously reported partial sequence of *ompA* from *R. roultii* IM-16 strain isolated from CP019435 and MF002523. In the phylogenetic analyses, neighborhood-joining tree of rickettsial species indicated that the present isolates formed a single clade with *R. raoultii* reference strains (Fig. 1). The bootstrap analyses statistically supported the main clustered sequence.

4. Discussion

Tick-borne rickettsiosis results from a member of genus *Rickettsia*, of the family *Rickettsiaceae* and the order *Rickettsiales* [2]. Clinically, SFG rickettsioses are associated with headache, pyrexia, myalgia, localized lymphadenopathy, characteristic eschar, and rash. Following the tick bite, *R. raoultii* has known to be presents with a clinical syndrome characterized by scalp eschars and neck lymphadenopathy. In 2010, to collectively explain this clinical manifestation, the syndrome was termed as “SENLAT” [14]. To date, 14 distinct tick species from six genera, including *Dermacentor* (*D. nuttallii, D. reticulatus, D. silvarum*, and *D. marginatus*), *Ambylomma* (*A. helvolum*), *Haemaphysalis* (*H. concinna, H. japonica, H. erinacei*, and *H. longicornis*), *Hyalomma* (*Hy. asiaticum* and *Hy. lusitanicum*), *Ixodes* (*I. persulcatus* and *I. ricinus*), and *Rhipicephalus* (*Rh. pumilio* and *Rh. turanicus*) have shown the presence of *R. raoultii* DNA [15, 16]. A report has also shown its presence in *Melophagus ovinus*, a louse fly or sheep ked [17]. *Dermacentor* ticks are considered as the main hosts and natural reservoirs of *R. raoultii* all over Europe and in a few countries of Asia, including China and Mongolia [18].

In the ROK, the first evidence of the existence of SFG rickettsia in ticks was reported in 2003, followed by the first case of SFG rickettsioses (Japanese spotted fever) in a patient in 2005 [19, 20]. Over the period of 16 years, various species of SFG rickettsial agents from ticks (*R. japonica, R. monacensis*, and *R*
R. rickettsii) and humans (R. japonica and R. monacensis) have been identified in the ROK [19–25] Till date, R. raoultii was not identified in this region, and the present study reports the first detection of R. raoultii belonging to the genus H. longicornis. Previously, only one study from China indicated the presence of R. raoultii in H. longicornis ticks [16]. The strains of R. raoultii, including Marne, 8/9 Karaganda, KhabarovskT, Shayman, and IM 16, have been documented in Europe, Russia, and China [4, 9]. The present phylogenetic tree showed that the positive samples formed a distinct clade with high (100) bootstrap value with R. raoultii IM 16 strain found in China. The nucleic acids of R. japonica, R. monacensis, and R. rickettsii have been identified in H. longicornis from ROK [19, 23]. According to a recent study, H. longicornis ticks are the most prevalent species in ROK (88.9%) with its geographical distribution nationwide [25]. Despite its identification in multiple tick species, reports on human infections are still lacking. The infection of R. raoultii in patients has been reported in Europe, the Far East of Russia, and a few cases in China [26]. Another study from China [18] identified R. raoultii DNA in clinical samples, besides the positive serological reports in patients from other countries. Based on these findings, R. raoultii has been considered as a human pathogen [27].

Present observations indicate the emergence of R. raoultii in ticks from ROK and warrant additional survey for this pathogen in a broader range of ticks. Furthermore, public health providers and physicians should use diagnostic tests of R. raoultii in patients of suspected rickettsioses. A special drive should be undertaken to investigate the presence of this emerging pathogen in human cases

5. Conclusions

This study confirms the first identification of R. raoultii in ticks from the ROK. Detection of Rickettsial specie in H. longicornis ticks, suggests that these ticks may act as a potent vector for this pathogen in ROK. This observation broadens our knowledge of the geographical spread of R. raoultii. Although no human clinical infection has been reported, its relatively strong pathogenicity is a substantial threat and a major concern for public health in this region. More extensive research and surveillance in this regard are essential.

6. List Of Abbreviations

SFG: Spotted Fever Group

ROK: Republic of Korea

*ompA*: outer membrane protein A

*gltA*: citrate synthase

SENLAT: scalp eschar and neck lymphadenopathy

DEBONEL: dermacentor borne necrosis erythema and lymphadenopathy
TIBOLA: tick-borne lymphadenopathy
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
N-PCR: Nested - polymerase chain reaction
BLAST: Basic Local Alignment Search Tool

7. Declarations

Ethics approval and consent to participate:
Not applicable.

Consent for publication:
Not applicable.

Availability of data and materials:
The datasets analyzed during the current study are available in the GenBank-database with the accession numbers CP019435 and MF002523.

Competing interests:
The authors declare that they have no competing interests.

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Authors' contributions:
Dong-Min Kim designed and coordinated the study and contributed to drafting and reviewing the manuscript during the course of submission. Misbah Tariq and Jun-Won Seo collected the data, wrote the manuscript, and revised the draft during the course of submission. You Mi Lee constructed the phylogenetic tree and performed the molecular analysis. Choon-Mee Kim carried out the molecular
analysis, wrote the manuscript, and also revised the draft during the course of submission. Na Ra Yun and Da-Young Kim helped to draft the manuscript. All the authors read and approved the final version of the manuscript.

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