AUTHOR QUERIES

DATE  6/24/2016
JOB NAME  TROPMED
ARTICLE  160199
QUERIES FOR AUTHOR  Kai Ling Kho et al.

THIS QUERY FORM MUST BE RETURNED WITH ALL PROOFS FOR CORRECTIONS

AU1: Please provide the city name in the location of the manufacturer of “Co-amoxiclav (IV).”

AU2: Please check whether the edit made to the sentence “However, specific identification of rickettsiae…” retains the intended meaning.

AU3: Please confirm whether the Authors’ addresses are set correctly. Also, please confirm the address “Department of Medicine, Jalan Universiti, Kuala Lumpur, Malaysia.” Should it be changed to “Jalan University”?

AU4: Please provide the city name in the location of the manufacturer “GENET BIO” and “GeneAll Biotechnology.”

AU5: Please provide the location (city and state) of the manufacturer “Applied Biosystems.”

AU6: Please confirm whether the expansion inserted for “BLAST” is OK.
Case Report: Spotted Fever Group Rickettsioses and Murine Typhus in a Malaysian Teaching Hospital

Kai Ling Kho, Fui Xian Koh, Harvinder Kaur Lakhbeer Singh, Hafizatul Anis Mohamed Zan, Sasheela Ponnampalavanar, Anjanna Kukreja, and Sun Tee Tay

1Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; 2Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract. Limited information is available on the etiological agents of rickettsioses in southeast Asia. Herein, we report the molecular investigation of rickettsioses in four patients attending a teaching hospital in Malaysia. DNA of Rickettsia sp. RF2125, Rickettsia typhi, and a Rickettsia closely related to Rickettsia raoultii was detected in the blood samples of the patients. Spotted fever group rickettsioses and murine typhus should be considered in the diagnosis of patients with nonspecific febrile illness in this region.

INTRODUCTION

Spotted fever group (SFG) rickettsioses and murine typhus have been regarded as important vector-borne diseases worldwide. Various species of SFG rickettsiae, including Rickettsia sibirica, Rickettsia helongiangensis, Rickettsia japonica, Rickettsia conorii, Rickettsia honei, Rickettsia tsutsugamushi, and Rickettsia rickettsii have been implicated in human infections in the Asia-Pacific region. Rickettsia felis is an emerging flea-borne pathogen which has been reported in a wide variety of arthropods from more than 20 countries on five continents. Rickettsia typhi, the causative agent of murine typhus, is maintained in a biological cycle involving rats (Rattus spp.) as reservoirs, and the oriental rat flea (Xenopsylla cheopis) as the main arthropod vector. The clinical manifestations of patients with SFG rickettsioses and murine typhus are nonspecific and are difficult to be differentiated from other febrile diseases such as malaria, dengue, leptospirosis etc. Despite the seroepidemiologic data, study on the clinical aspects of rickettsioses in Malaysia is limited. This study reports the molecular investigation of rickettsioses in four adult patients attending a Malaysian teaching hospital. The demographic, clinical, and laboratory features of these patients are reviewed in this study. This study was approved by the University Malaya Medical Center Ethics Committee (MEC reference no. 830.6 and no. 944.20).

CASES

A 73-year-old lady (patient B), living on a farm at the east coast of Malaysia, was admitted to our hospital in June 2010 with febrile illness and underlying interstitial pulmonary fibrosis. During admission, she presented with the complaints of backache, anorexia, diarrhea, abdominal pain, reduced exercise tolerance, and productive cough. She developed atrial fibrillation and was hypotensive. Chest examination revealed bilateral lung crepitation. Her examination was otherwise normal and there was no rash or eschar. The patient was thrombocytopenic with raised plasma levels of urea, creatinine, and aspartate aminotransferase (Table 1). Hypoalbuminemia was also noted. The thorax computed tomography scan showed extensive patchy consolidation, bilateral pleural effusion, and mediastinal lymphadenopathy, suggesting severe pneumonia. Septic workup including blood and stool cultures did not reveal any bacterial growth. Examination of peripheral blood smear for malaria parasites and serological tests for dengue and hepatitis C were negative. His fever persisted despite treatment with ceftriaxone. A rickettsiosis was suspected. He was then started on doxycycline and the temperature subsided within 24 hours. On a follow-up visit after a week, he was well and his platelet count and liver enzymes were normal. Retrospective analysis of rickettsial serology for acute serum sample showed that the patient was positive to two rickettsial antigens available for testing (R. typhi [IgM = 1:256, IgG ≥ 1:2048]; R. rickettsii [IgM < 1:64, IgG = 1:256]) using an indirect immunofluorescence assay (Focus diagnostic, Cypress, CA). A 4-fold increase in the IgG titer (1:1024) against R. rickettsii was noted for the convalescent sample (collected after 12 days) of the patient. High IgM and IgG titers (≥ 2048) against R. typhi were detected in the convalescent samples.

Molecular investigation revealed the amplification of rickettsial citrate synthase gene (gltA) and the 135-kDa outer membrane protein gene (ompB) from the patient’s acute blood samples. Sequence analysis of the gltA (GenBank accession no.: KU255716, 399/402 nucleotide [nt], 99.3%) and ompB (GenBank accession no.: KU255717, 772/772 nt, 100.0%) gene fragments shows the closest match with Rickettsia sp. RF2125 (GenBank accession no.: AF516333 and JX183538), and next with those of R. felis type strain URRWXCal2 (GenBank accession no.: CP000053, 394/402 nt [98.0%] for gltA and 764/824 nt [92.7%] for ompB).

A 72-year-old lady (patient B), living on a farm at the east coast of Malaysia, was admitted to our hospital in June 2010 with febrile illness and underlying interstitial pulmonary fibrosis. During admission, she presented with the complaints of backache, anorexia, diarrhea, abdominal pain, reduced exercise tolerance, and productive cough. She developed atrial fibrillation and was hypotensive. Chest examination revealed bilateral lung crepitation. Her examination was otherwise normal and there was no rash or eschar. The patient was thrombocytopenic with raised plasma levels of urea, creatinine, and aspartate aminotransferase (Table 1). Hypoalbuminemia was also noted. The thorax computed tomography scan showed extensive patchy consolidation, bilateral pleural effusion, and mediastinal lymphadenopathy, suggesting severe pneumonia. Septic workup including blood and stool cultures did not reveal any bacterial growth. Examination of peripheral blood smear for malaria parasites and serological tests for Leptospira, Mycoplasma, and Legionella were all negative. She was treated for septic shock and was started on amoxicillin and clavulanic acid (Co-amoxiclav [IV], KALP, India) and azithromycin. However, due to poor response and worsening symptoms, her antibiotics were changed to piperacillin–tazobactam and doxycycline.
### Table 1

The demographic, hematology, and blood chemistry profiles of patients investigated in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Occupation</th>
<th>Hemoglobin (g/L)</th>
<th>White blood cells (10⁹/L)</th>
<th>Platelets (10⁹/L)</th>
<th>Creatinine (mg/dL)</th>
<th>Bilirubin (μmol/L)</th>
<th>Albumin level (g/L)</th>
<th>Alkaline phosphatase (U/L)</th>
<th>Alanine aminotransferase (U/L)</th>
<th>Aspartate aminotransferase (U/L)</th>
<th>Tick bite history</th>
<th>Rash/eschars</th>
<th>Molecular detection of Rickettsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>15</td>
<td>Male</td>
<td>Student</td>
<td>12.6</td>
<td>4.8</td>
<td>120</td>
<td>1.2</td>
<td>11</td>
<td>32</td>
<td>129</td>
<td>141</td>
<td>112</td>
<td>No</td>
<td>No</td>
<td>Rickettsia typhi</td>
</tr>
<tr>
<td>Patient B</td>
<td>73</td>
<td>Female</td>
<td>Farmer</td>
<td>13.3</td>
<td>6.8</td>
<td>51</td>
<td>3.2</td>
<td>12</td>
<td>12.9</td>
<td>122</td>
<td>126</td>
<td>125</td>
<td>No</td>
<td>No</td>
<td>Rickettsia closely related to R. raoultii</td>
</tr>
<tr>
<td>Patient C</td>
<td>33</td>
<td>Female</td>
<td></td>
<td>12.6</td>
<td>4.8</td>
<td>120</td>
<td>1.2</td>
<td>11</td>
<td>32</td>
<td>129</td>
<td>141</td>
<td>112</td>
<td>No</td>
<td>No</td>
<td>Rickettsia closely related to R. raoultii</td>
</tr>
<tr>
<td>Patient D</td>
<td>42</td>
<td>Male</td>
<td></td>
<td>12.6</td>
<td>4.8</td>
<td>120</td>
<td>1.2</td>
<td>11</td>
<td>32</td>
<td>129</td>
<td>141</td>
<td>112</td>
<td>No</td>
<td>No</td>
<td>Rickettsia closely related to R. raoultii</td>
</tr>
</tbody>
</table>

En dashes (–) indicate that data are not available.

---

**DISCUSSION**

The impact of rickettsioses as the leading causes of treatable fever of unknown origin has been documented in south-east Asian countries. However, as most studies were based on serological diagnosis, information is lacking on the genetics and biology of pathogenic rickettsiae in this region. Although rickettsioses have been known to occur in Malaysia for many years, data is scarce on *Rickettsia* spp. associated with human infections. Since the first report of *R. felis* infection amongst rural residents of the central Thai–Myanmar border, the rickettsiae have been identified from febrile patients in several Asian countries, including Korea, Thailand, and Laos. This study reports for the first time the detection of *Rickettsia* sp. RF2125, a *R. felis*-like organism (RFLO) in the blood sample of a Malaysian febrile patient by molecular method. However, specific identification of rickettsiae is not possible merely by IFA alone due to serological cross-reactivity, especially when high-endpoint
titers were noted for more than one rickettsial antigen. The diagnosis of spotted fever was confirmed based on the observation of a 4-fold rise in the IgG antibody titers against R. rickettsii. The high IgG titers against R. rickettsii and R. typhi in the convalescent sample suggest cross-reactivity between both rickettsial antigens. In addition, mixed infections due to spotted fever and typhus group rickettsiae can also complicate the interpretation of serological results, as reported in some studies in southeast Asia. Recent zoonotic surveillance studies showed the detection of Rickettsia sp. RF2125 in cat fleas and cynomolgus monkeys in Malaysia. It will be interesting to investigate whether R. rickettsii and R. slovaca in cats and cynomolgus monkeys are associated with murine typhus is variable (nonpruritic, maculopapular) and has been reported in 20% of the patients in China who had painful rashes around the site of tick bites, but no lymphadenopathy. T he pre sentation of a 4-fold rise in the IgG antibody titers were noted for more than one rickettsial antigen. The diagnosis of spotted fever was confirmed based on the observation of a 4-fold rise in the IgG antibody titers against R. rickettsii. The high IgG titers against R. rickettsii and R. typhi in the convalescent sample suggest cross-reactivity between both rickettsial antigens. In addition, mixed infections due to spotted fever and typhus group rickettsiae can also complicate the interpretation of serological results, as reported in some studies in southeast Asia. Recent zoonotic surveillance studies showed the detection of Rickettsia sp. RF2125 in cat fleas and cynomolgus monkeys in Malaysia. It will be interesting to investigate whether R. rickettsii and R. slovaca in cats and cynomolgus monkeys are associated with murine typhus is variable (nonpruritic, maculopapular) and has been reported in 20% of the patients in China who had painful rashes around the site of tick bites, but no lymphadenopathy. The presence of R. raoultii in Dermacentor, Haemaphysalis, and Amblyomma ticks has been reported in China, Japan, Thailand, and Malaysia. In this study, the infections caused by R. raoultii were considered mild as both patients recovered without any specific medication for rickettsioses. The typical features such as eschar and neck lymphadenopathy were not noted. Although the rash is a typical feature of rickettsioses, only one patient (A) presented with petechial rash. Rash can be difficult to see especially in patients with darker complexion. Rash (mostly maculopapular) has been reported in R. felis infection; however, a lack of cutaneous rash amongst Senegalese patients has been reported. The rash associated with murine typhus is variable (nonpruritic, macular, or maculopapular) and has been reported in 20–80% of infected patients. For R. rickettsii infection, localized rashes around sites of tick bites has been described in two (100%) patients in China, but only one (20%) of the five patients in France diagnosed with R. rickettsii infection developed rash. The most severe presentation noted in this study was pneumonia and septic shock in the patient diagnosed with murine typhus. However, as the patient also had underlying interstitial pulmonary fibrosis and precipitated by the existing lung pathology, it is difficult to conclude that her respiratory problems were solely related to murine typhus. Severe pulmonary manifestations of murine typhus are rare, but has been reported from travelers returning from Thailand and Indonesia. It has been reported that elderly patients have more severe clinical manifestations, as evidenced by a higher complication rate and longer duration of fever. In conclusion, the molecular investigations in this study suggest Rickettsia sp. RF2125, R. typhi, and a Rickettsia closely related to R. raoultii as the etiological agents for rickettsioses in four Malaysian patients. The finding of human cases and surveillance of possible vectors and animal reservoirs will improve our knowledge on the transmission of the newly identified rickettsiae.


SUPPLEMENTAL INFORMATION

Materials and methods. Molecular detection of rickettsial DNA. Two hundred microliters of the whole blood samples from each patient were used for DNA extraction using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the instructions of the manufacturer. The samples were first screened for rickettsial DNA using polymerase chain reaction (PCR) primers CS78/CS323 (gltA) or CS-239/CS-1069 (gltA-1) targeting 410 base pairs (bp) and 830 bp of the rickettsial citrate synthase gene, respectively. Positive samples were then subjected to amplification using primers Rr190.70p/Rr190.602n, targeting a 532-bp fragment of the 190-kDa outer membrane protein gene (ompA), and primers 120-M59/120-807, targeting a 866-bp fragment of 135-kDa outer membrane protein gene (ompB). All PCR assays were performed in a final volume of 20 μL containing 2 μL of DNA template, 1× ExPrime Taq DNA polymerase (GENET BIO, South Korea), and 0.2 μM of each primer, in a Veriti thermal cycler (Applied Biosystems). DNA extracted from Rickettsia conorii antigen slides (Fuller Laboratories, Fullerton, CA) was used as positive control for the PCR assay. Sterile distilled water was used as the negative control in each PCR reaction. PCR products were purified using a GeneAll Expin™ Combo GP kit (GeneAll Biotechnology, South Korea). The purified DNA was then subjected to sequencing on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems), using both forward and reverse primers of each PCR assay. The sequences obtained were subjected to Basic Local Alignment Search Tool analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous sequences in the GenBank database.

SUPPLEMENTAL REFERENCES