Determination of scrub typhus in negative patients for dengue and rickettsia

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RESUME

The scrub typhus is an endemic disease from Asia caused by a bacteria called *Orientia tsutsugamushi* which is characterized by the presence of general discomfort, headache, body aches and muscle pain, fever, rash, and enlarged lymph nodes, similar to symptoms of many other diseases as dengue or rickettsia. In Latin America the presence of *O. tsutsugamushi* has already been reported; nevertheless, as in México the presence of this bacteria has not been detected, is not considered as a part of molecular diagnosis consequently we decided to identify the presence of clinical cases of scrub typhus in negative patients for dengue and rickettsia. The sample was established by 21 patients from public and private hospitals of Yucatán from January until June of 2017, under suspicion of dengue and rickettsia but serologically negative in these. The DNA was extracted and amplified for the gen STA-56, it showed a band in level between 300-400 pb where there was the positive control. With these results through the PCR exists the possibility of having positive cases of *O. tsutsugamushi*; however, for its low quality in the visualization, those should be confirmed by genetic sequencing.

Keywords: *Orientia tsutsugamushi*, dengue, rickettsia, polymerase chain reaction, negative diagnosis, febrile illness, rash.

1 INTRODUCTION

Scrub typhus is an acute febrile illness transmitted by vector¹. His agent, *Orientia tsutsugamushi*, is an intracellular bacteria transmitted by the sting of mites of the genus *leptotrombidium* associated principally with rodents of the genus *Rattus* (common rat)².

The *Rickettsiaceae* family includes two genus, the genus *Rickettsia* and the genus *Orientia*. This

phylogenetic relation is the cause of the infections produced by *Rickettsia* and *Orientia*³ share a very similar clinical symptoms between them, giving like consequent the typical manifestations of high fever, general discomfort, headache, body aches, muscle pain, rash, and enlarged lymph nodes⁴.

Scrub typhus is an endemic disease from Asia⁵. Until 2006, infections caused by *Orientia* had not been confirmed beyond Asia and the Pacific; however, in that year were two cases of infection reported with *Orientia* species in the Middle East and Chile¹. Land, sea, and air trade, together with global mobility, may be responsible for the spread of these endemic diseases across the globe.

In Mexico, no cases of *Orientia* infection have been reported until the date⁶; however, in the state of Yucatan, a number of suspicious cases have been detected in the hospital admission with the clinical manifestations of rash fever, where the first diagnosis impressions, dengue and rickettsia, have been previously ruled out by the main diagnostic tests. The clinical records of these patients and the environment in which they lives indicate the coexistence with vectors and ectoparasites that could suggest, starting from the antecedent in Chile, a possible infection by *O. tsutsugamushi*, explaining in this way the similarity of the clinical manifestations with rickettsiosis⁷ and the negativity of the laboratory tests; reason why we took on the task of identifying, through DNA tests, the presence of *O. tsutsugamushi* in the blood samples of these patients.

2 MATERIALS AND METHODS

This cross-sectional study was carried between January 2017 and June 2017 in the Inter-Institutional Unit of Clinical and Epidemiological Research (IIUCE) belonging to the Faculty of Medicine of the Autonomous University of Yucatan, in the city of Merida, Yucatan, Mexico.

2.1 SAMPLE COLLECTION

A total of 21 blood samples were recollected from patients with symptoms suggestive of rash fever who gave negative results to the dengue and rickettsia tests and which were collected during the period from January to June 2017 in the city of Merida, Yucatan. These patients were selected from the General Hospital "Dr. Agustín O'Horán", the "ISSSTE Regional Mérida" and private hospitals referred to the Inter-Institutional Unit of Clinical and Epidemiological Research (IIUCE) for molecular diagnosis.

2.2 DNA EXTRACTION

DNA was extracted from each of the samples using the Zymoclean™ Gel DNA Recovery Kit and following the established protocol⁸, obtaining a total of 50 µl of DNA for each blood sample.

2.3 MOLECULAR DIAGNOSIS BY END-POINT PCR

Once the DNA was extracted, polymerase chain reaction (PCR) was performed with the aim of amplifying a common region of gene for all *O. tsutsugamushi* species, being chosen as the STA-56 gene⁹. For each diagnosis sample was used 12.5 µl of DreamTaq Green Polymerase, 0.25 µl of the first Pr1 (5' - TAC ATT AGC TGC AGG TAT GAC-3') and 0.25 µl of the first Pr2 (5' - AAT TCT TCA ACC AAG CGA TCC-3')¹⁰. Subsequently 7 µl of nuclease-free water was added, finally, 5 µl of the DNA extracted from the respective sample was added, making a total of 25 µl for each diagnosis. 21 diagnostic samples and 2 controls were prepared, one negative using 5 µl nuclease-free water instead of 5 µl diagnostic DNA and one positive using 4 µl nuclease-free water and 1 µl pure *O. tsutsugamushi* DNA.

The 23 samples were processed by the MULTIGENE thermocycler under standardized conditions for 160 minutes. Once the PCR was completed, the products obtained underwent electrophoresis in 1.5% agarose gel under the conditions of 80 V for 90 minutes. At the end of this process, the agarose gel was immersed in ethidium bromide (EtBr) for 10 minutes; subsequently was washed with a water distiller for another 10 minutes. Once the bath with the distilled water was finished, the gel was developed in the Gel Doc XR (Bio-Rad) system using UV light and capturing the image using computer software.

3 RESULTS

After analyzed them by UV light, one band was detected at the position of 317 bp in 4 of the 21 samples, just where the positive control of the *Orientia tsutsugamushi* STA-56 gene was located (Figure 1); the negative control as the remaining samples no showed visual data of any band presence.

Figure 1. PCR results beyond UV light

The analysis of the reference notes and clinical records of the four patients with molecular evidence of *Orientia* show us that all patients presented fever $>39^{\circ}\text{C}$ with at least 7 days of evolution. The four patients presented asthenia, adynamia, general discomfort, nausea, myalgia, arthralgias, and maculopapular rash. In two patients hyperbilirubinemia was found with direct pattern exteriorizing jaundice in skin and conjunctiva.

In the physical examination, hepatomegaly was found only in one patient. Complete blood count found leukocytosis in all four patients. Abnormalities in coagulation like PT prolongation and thrombocytopenia were found in two patients. In only one patient was death recorded days after the blood extraction.

4 DISCUSSION

In the present study, the PCR test was directed at the 59 kDa gene, which is a specific protein exclusive to *Orientia tsutsugamushi*. This gene has four domains that are hypervariable responsible for antigenic variation.⁹

In our study the visualization of the band in the mentioned samples was only for the contrast adjust through the software since at first glance was difficult visualize, in such a way we can not conclude that bands belongs to STA-56 gen but neither we can discard it, since electrophoresis clearly produces a product of similar size between 300-400 bp.

There are many factors that have influenced this phenomenon, the DNA quality analyzed, the researcher technique for the PCR or it could have been variations in the numbers of base pairs between the diagnostics and the STA-56 gen proportioned by the GenBank.

Guo HB *et al* (1997) mentioned that PCR amplification of *O. tsutsugamushi* from the blood often lacks sensitivity because hemoglobin, porphyrin and other factors can inhibit PCR, although it is possible to obtain and process blood to avoid inhibitors¹¹; on the other hand, it has been noted that a large number of positive serological cases were negative for PCR, which may indicate a low sensitivity of this test, either due to the low copy numbers or the excess of the DNA of the host as various studies established ^{12,13}, this could be the reason why visualize the bands was difficult.

5 CONCLUSION

Despite the low quality of the visualization, in patients with negative results for dengue and rickettsia, we concluded that there exists the possibility of having positive cases of scrub fever by the results of the PCR since we can observe the band between 300-400 pb, matching with the clinical symptoms and laboratory patterns of infection by *O. tsutsugamushi*. We suggest another test of detection with the blood samples including a sequence by Sanger method to verify the percent of similitude of the obtained products compares with *O. tsutsugamushi* data from GenBank.

If confirmed by a sequencing method, the first molecular evidence of the disease would be established in the State of Yucatan, in order to integrate and consider it within vector-borne diseases, thus establishing a timely diagnosis and treatment, as well as directing measures towards primary prevention.

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