Ticks and Tick-borne Diseases 7 (2016) 1029–1034

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Comparative value of blood and skin samples for diagnosis of spotted fever group rickettsial infection in model animals

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 23 May 2016
Accepted 25 May 2016
Available online 26 May 2016

Keywords:
Rocky Mountain spotted fever
Spotted fever rickettsiosis
Diagnosis
Skin biopsies
Guinea pig

ABSTRACT

The definitive diagnosis of spotted fever group (SFG) rickettsioses in humans is challenging due to the retrospective nature and cross reactivity of the serological methods and the absence of reliable and consistent samples for molecular diagnostics. Existing data indicate the transient character of bacteremia in experimentally infected animals. The ability of arthropod vectors to acquire rickettsial infection from the laboratory animals in the absence of systemic infection and known tropism of rickettsial agents to endothelial cells of peripheral blood vessels underline the importance of local infection and consequently the diagnostic potential of skin samples. In order to evaluate the diagnostic sensitivity of rickettsial DNA detection in blood and skin samples, we compared results of PCR testing in parallel samples collected from model laboratory animals infected with *Rickettsia rickettsii*, *Rickettsia parkeri* and *Rickettsia slovaca*-like agent at different time points after infection. Skin samples were collected from ears – away from the site of tick placement and without eschars. Overall, testing of skin samples resulted in a higher proportion of positive results than testing of blood samples. Presented data from model animals demonstrates that testing of skin samples from sites of rickettsial proliferation can provide definitive molecular diagnosis of up to 60–70% of tick-borne SFG rickettsial infections during the acute stage of illness. Detection of pathogen DNA in cutaneous samples is a valuable alternative to blood-PCR at least in model animals.

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1. Introduction

Spotted fever group (SFG) of the genus *Rickettsia* consists of a large number of diverse arthropod-borne intracellular bacterial species described worldwide and maintained by fleas, ticks, and mites. The list includes endosymbiotic bacteria of the arthropod vectors as well as known pathogens, causing spotted fever group rickettsioses (Walker 1989). At least 9 named SFG *Rickettsia* species are endemic to the United States (Denison et al., 2014). Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii* is the most severe rickettsial illness of humans distributed throughout North, Central, and South America. It is transmitted by tick vectors, including *Dermacentor variabilis*, *Dermacentor andersoni* (in North America), several *Amblyomma* spp. (in Mexico, Central and South America), and *Rhipicephalus sanguineus* s.l. *Rickettsia parkeri* transmitted by *Amblyomma maculatum* is distributed throughout the southeastern and mid-Atlantic United States. It causes moderately severe illness, which shares features with both RMSF and rickettsial pox, including a maculopapular rash and the occurrence of inoculation eschars (Paddock et al., 2008). *Rickettsia slovaca*-like agent has recently been found in and isolated from *D. variabilis* ticks (Killmaster et al., 2016). Pathogenicity of this agent in humans has not been established, but it causes mostly subclinical infection in guinea pigs (Zemtsova et al., 2016). Domestic and synanthropic animals are exposed to and infected with rickettsial pathogens as well and may serve as sentinels for assessment of the human risk (Walker 1989; Case et al., 2006; Milagres et al., 2010; McQuiston et al., 2011; Pacheco et al., 2011).

Both diagnosis and surveys of rickettsial infections in domestic animals currently rely primarily on serology (Breitschwerdt et al., 1985; Maggi et al., 2014), just like the clinical diagnosis in humans, where at least 4-fold increase in titers is expected between acute and convalescent serum samples (Chapman et al., 2006). Because it normally takes several weeks from the onset of the disease for the antibody titers to reach diagnostic levels, serological diagnosis is retrospective and provides a confirmation of rickettsial infection only after the patient's recovery or postmortem. Moreover, pathogen species identification is complicated by serological cross reactivity between SFG *Rickettsia* spp. Molecular methods are capable of providing better specificity, as well as real-time diagnosis of clinical cases. When molecular diagnosis of RMSF is attempted,

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http://dx.doi.org/10.1016/j.ttbdis.2016.05.011
1877-959X/Published by Elsevier GmbH.

it is usually done using acute-stage blood samples. However, SFG rickettsiae primarily target endothelial cells and normally do not infect the circulating blood cells. For example in dogs infected with SFG agents either experimentally or naturally, rickettsial DNA may be detected in the blood only intermittently (Labruna et al., 2009; Levin et al., 2012; Levin et al., 2014). Periods when rickettsial DNA is detectable in the blood of infected in animals are be short in duration, not consistent with the dynamic or severity of clinical symptoms, and is affected by external factors such as initiation of antibiotic treatment (Labruna et al., 2009; Levin et al., 2014). On the other hand, the fact that naïve ticks acquire *Rickettsia* from infected laboratory animals while the presence of rickettsial DNA cannot be detected by blood-PCR and feeding distantly from the infected ticks, emphasizes the importance of infection in the skin and local mechanisms in rickettsial horizontal transmission (Zemtsova et al., 2010). This corresponds with the previous observation that immunostaining of skin biopsy specimens of rash lesions can identify rickettsiae in 70% of human RMSF patients while blood-PCR was "unacceptably insensitive" (Walker 1995).

Detection of rickettsial DNA has been increasingly reported using eschar tissues or swabs of eschars, although the diagnostic sensitivity of these types of specimens in comparison with traditional blood samples has not been ascertained (Lakos 2002; Wang et al., 2009; Bechah et al., 2011; Mouffok et al., 2011; Myers et al., 2013). Yet, *R. rickettsii* infections are not usually associated with inoculation eschars, which are rarely observed even in human RMSF patients (Walker et al., 1981; Chen and Sexton 2008), where they would be easier to find than in fur covered animals.

Here we assess whether rickettsial DNA may be identified in skin samples not associated with either eschar, or the site of tick attachment and compare the diagnostic sensitivity of rickettsial DNA detection between blood and skin samples, using guinea pigs as model animals.

2. Materials and methods

Animal studies were conducted at a facility fully-accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All procedures and husbandry were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (8th edition). Animal protocols were pre-approved by the Centers for Disease Control Institutional Animal Care and Use Committee (IACUC) and monitored by a veterinarian stationed on-site.

Analyses presented here combine PCR results from animals used for multiple studies in vector and reservoir competence of SFG *Rickettsia* spp. The choice of pathogen isolates and the inoculation dosages had been adjusted so not to cause any mortality in the model animals. Therefore, presented results are from cases of relatively mild, nonlethal, or subclinical rickettsial infections.

One to two month-old specific pathogen free male Hartley guinea pigs weighing 400–500 g were acquired from Charles River Laboratories (http://www.criver.com). Animals were infected with *R. rickettsii* (isolates BSF-Di6 and AZ3), *R. parkeri* (isolate Longleaf), or *R. slovaca*-like agent (*D. variabilis* isolate (Killmaster et al., 2016). Pathogens were introduced either via a tick-bite or through the needle-inoculation route – intraperitoneally. For tick-borne infections, guinea pigs were exposed to 10–20 nymphal ticks (30–50% prevalence of infection) placed into feeding bags glued onto animals' backs. Needle-inoculated animals were each injected with spleen/liver tissue homogenate containing a standard infectious dose of 10^5 DNA copies of a specific *Rickettsia* sp. Tissue homogenates were prepared by grinding liver and spleen of previously infected guinea pigs in Snider-1 buffer (20% tissue suspension) and cryopreserved in liquid nitrogen as inoculation-ready aliquots until used.

Guinea pigs were observed for the duration of one to three weeks depending on the goal of an individual study. During the observation period, the core temperature and signs of infection of each animal was recorded daily. In guinea pigs infected with *R. rickettsii,* the core temperature usually rose above 39.5 °C at 5–9 days after infection depending on the strain of the pathogen and the mode of infection; and the fever period lasted for 2–7 days. Majority of guinea pigs infected with either *R. parkeri* or *R. slovaca*-like agent exhibited only subfebrile temperatures.

Paired blood and skin samples were collected for PCR 2–3 times per week. Skin samples consisted of 2 mm punch biopsies weighing 2 mg taken from an ear, and EDTA-anticoagulated blood samples (100 μl) were collected from a lacerated ear vein. At the end of each individual study, all guinea pigs were euthanized and 5 mg samples of internal organs (liver, spleen, and lung) were also collected for PCR. Blood, skin, and internal tissue samples were immediately stored at −20 °C until DNA extraction. DNA from skin and internal tissues was extracted using the DNeasy Blood & Tissue kit, and DNA from blood samples was extracted with FlexiGene DNA Kit (Qiagen, Gaithersburg, Maryland, USA) according to the manufacturer's protocols with the final elution volume of 100 μl for all samples. The presence of rickettsial DNA was detected by SYBR green-based PCR assay targeting a 154-bp fragment of the r*Omp*A gene as described (Eremeeva et al., 2003). All samples were tested in duplicates using 5 μl of the eluted DNA for each reaction. Accordingly, DNA equivalents of 5 μl of whole blood, 0.1 mg of skin tissue, 0.5 mg of internal tissues were represented in each PCR reaction.

In addition, a serum sample was collected from each animal at the end of the study and tested by IFA for the presence of anti-rickettsial IgG antibodies to confirm an infection. IFA was performed on guinea pig sera using FITC labeled goat anti-guinea pig IgG (γ) conjugate diluted per manufacturer's recommendations (KPL, Inc. Gaithersburg, Maryland, USA) and homologous rickettsial antigens.

Proportions of positive skin and blood samples per day post-infection (DPI) were compared using the paired *t*-test with the hypothesized mean difference = 0 and $\alpha = 0.05$.

3. Results

For the purpose of the current comparative analysis, we removed from the data sets any animals that did not become infected as well as all asynchronous samples when both the blood and skin biopsies were not collected at the same time. In total, we compared PCR results of 437 pairs of samples from 161 guinea pigs infected with *R. rickettsii,* 74 pairs of samples from 31 guinea pigs infected with *R. parkeri*, and 80 pairs of samples from 24 guinea pigs infected with *R. slovaca*-like agent. Numbers of paired skin and blood samples tested at different time points post-infection varied from 1 to 42 depending on the pathogen and mode of infection (Fig. 1). Overall, rickettsial DNA was detected in 38.6% of skin samples and in 13.4% of blood samples. Rickettsial DNA was also detected in internal tissues, collected at the time of euthanasia 6–22 DPI, from 109 (67.7%), 24 (77.4%), and 9 (37.5%) of the guinea pigs infected with *R. rickettsii, R. parkeri,* and *R. slovaca*-like agent respectively.

3.1. *Detection of* Rickettsia rickettsii *in skin and blood samples*

Out of 437 paired samples collected from *R. rickettsii*-infected guinea pigs for up to three weeks after infection, 173 (39.6%) of skin samples and 74 (16.9%) of blood samples contained rickettsial DNA detectable by PCR ($p = 0.00017$). The frequency of *R. rickettsii*