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Diagnostics of tick-borne rickettsioses in Germany: A modern concept for a neglected disease

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Abstract

Tick-borne rickettsioses in humans occur worldwide and are caused by obligate intracellular bacteria belonging to the spotted-fever group (SFG) within the genus *Rickettsia* (*R.*). These tick-borne rickettsioses are among the most underdiagnosed vector-borne diseases in Germany: Due to the variety of unspecific clinical signs, they are not easily recognised. The clinical picture ranges from subclinical to fatal courses, but may be difficult to differentiate from other febrile conditions without specific tests. Even to date, diagnosis is either based on clinical findings, a record of tick exposure, or indirect serological detection of the pathogens. Herein, we briefly discuss modern diagnostic tools for important tick-borne rickettsial infections with emphasis on new molecular diagnostic assays. As one example, we present a novel real-time polymerase chain reaction (PCR) protocol that facilitates genus-specific, rapid, and sensitive detection of rickettsial pathogens. A conserved region of the rickettsial citrate synthase gene (*gltA*) is amplified and detected by a 5′-nuclease probe in a LightCycler instrument. Sensitivity was consistently high at less than 23 genome copies per reaction. This detection system has been evaluated both as a useful tool in epidemiological investigations in ticks and in human diagnostics. We describe a rational diagnostic approach for the detection of tick-borne human rickettsioses which consists of that real-time PCR, isolation of rickettsiae in cell culture, multi-locus sequence typing, and serology. Its implementation recently led to the first isolation and characterisation of *R. africae* in Germany from a patient returning from Zimbabwe. In conclusion, tick-borne rickettsioses should be considered in patients presenting with fever, headache, and rash following a tick bite. Further studies are needed to determine the epidemiology and clinical importance of these pathogens in Germany.

Keywords: Human rickettsioses; Rickettsia; Real-time PCR; Fluorogenic 5′-nuclease assay; Citrate synthase; Germany

Introduction

Rickettsioses are acute febrile diseases caused by rickettsiae. Rickettsiae are obligate intracellular bacteria and are found worldwide. Various arthropods, including fleas, mites, lice, and ticks can act as vectors and reservoirs. At least 15 tick-transmitted species belonging to the spotted-fever group (SFG) within the genus *Rickettsia* (*R.*) are known to cause human disease (Parola et al., 2005). Tick-borne rickettsioses generally start as acute non-specific illnesses with a sudden onset of headache, fever, and myalgia. An eschar is frequently present at the inoculation site and a petechial rash may appear. Depending on the anamnesis the clinical

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differential diagnosis includes several bacterial, viral, and parasitic infections. In Germany, only limited data are available on autochthonous or imported cases of tick-transmitted rickettsial diseases. In recent studies, a significant prevalence rate of *R. helvetica* in ticks has been found in Bavaria and Baden-Wuerttemberg (Hartelt et al., 2004; Wölfel et al., 2006). Further PCR-based results indicate endemicity of *R. felis* and *R. sibirica* in certain regions of southern Germany (Wölfel et al., unpublished data). Other tick-borne rickettsiae like *R. conorii* and *R. africae* have been identified as causative agents of imported diseases in North American and European travellers (Jensenius et al., 2006), however, detailed data on the incidence of rickettsioses among German travellers are not available. It is very likely that the diagnosis of rickettsiosis, endemic as well as imported cases, is frequently missed in Germany, largely owing to a lack of diagnostic consideration by physicians and inappropriate laboratory testing. In the past, the diagnosis of a rickettsial illness was based on retrospective serological tests.

The *Proteus* OX-19 agglutination test, historically also known as Weil-Felix reaction (Felix, 1916), is based on the detection of cross-reacting antibodies to rickettsial antigens. It is unreliable due to its low sensitivity and non-specific reactions and is therefore no longer recommended for serodiagnostics in humans anymore.

Indirect immunofluorescence assays (IFA) have been most sensitive and specific (Philip et al., 1976), but usually they are not positive within the first week of acute illness. The demonstration of a four-fold rise in antibody titre in convalescent serum has been a common method for (retrospective) documentation of rickettsioses. However, it is useless for the decision on initial treatment and – due to its cross-reactivity between all tick-borne rickettsiae – for the identification of the causative *Rickettsia* species. Western blotting in conjunction with cross-adsorption assays (La Scola et al., 2000) is a useful but rarely applied tool to circumvent the last-mentioned drawback. It should be mentioned that cases of confirmed rickettsioses have been described without an increase in antibody titre in any stage of the disease (Carpenter et al., 1999; Fournier et al., 2005).

In order to isolate the causative rickettsia(e) from human clinical specimen Vero (African green monkey kidney) and L-929 (subcutaneous areolar and adipose tissue of mouse), cell lines are routinely used by the authors of the present paper and other specialized centres worldwide (Tringali et al., 1986; Birg et al., 1999). However, even by using a shell-vial culture-based system (Marrero and Raoult, 1989), isolation requires at least 7–14 days in antibiotic-free cell cultures. Furthermore, appropriate biosafety conditions have to be available for isolation and growth of various rickettsial species. A broad range of sample materials (skin or eschar biopsy, buffy coat, and even ticks) can be used for cell culture isolation.

A reliable species identification of SFG rickettsiae can be achieved by PCR-based assays. Various primer sets have been developed and published for that purpose: Initially the 16S rRNA gene has frequently been used (Weisburg et al., 1989), but since this gene is highly conserved, unequivocal identification cannot always be obtained (Roux et al., 1997). Other genomic targets like the citrate synthase-encoding gene (*gltA*), the outer membrane protein-encoding genes A and B (*ompA*, *ompB*), the 'gene D' (*sca4*), and the 60-kDa heat shock protein (*groEL*) have been identified to allow more sensitive and significant identification of rickettsiae. Reliable primer sets are summarised in Table 1. It should be noted that in contrast to SFG rickettsiae all rickettsiae belonging to the typhus-group (*R. prowazekii* and *R. typhi*) lack the *ompA* gene.

The PCR assays mentioned above are not very sensitive and some require either an initial step of rickettsial multiplication in cell culture or a nested-PCR step. We herein present a real-time PCR assay based on the *gltA* gene developed to improve rapid PCR diagnosis of rickettsial disease in human samples. Its analytical sensitivity was compared to another real-time PCR assay that was recently published (Stenos et al., 2005). Since its development in 2005, our real-time PCR assay has been used in our lab for clinical diagnostics and recently led to the first isolation and characterisation of *R. africae* in Germany from a patient returning from Africa. We describe a rational diagnostic approach for the detection of tick-borne human rickettsioses consisting of the above-mentioned real-time PCR, cell culture, multi-locus sequence typing, and serology.

Materials and methods

Clinical case report

A 52-year-old male German patient fell ill with fever, headache, and myalgia one day after returning from a trip to South Africa in January 2006. Clinical examination showed low-grade fever (38.2 °C), a macular rash at the chest and the legs, and a necrotic black lesion surrounded by an erythematous halo identified at the right calf. A skin biopsy from the presumed eschar, serum, and whole blood were sent to our lab to test for possible rickettsial infection. The patient was treated with doxycycline (200 mg once a day for 7 days), whereupon his clinical condition improved rapidly.

Bacteria

The following bacterial species were used for validation of the real-time PCR assay: *Rickettsia helvetica*, *R. honei*, *R. rickettsii*, *R. typhi*, *R. africae*, *R. conorii*,