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Development of a pan-rickettsial molecular diagnostic test based on recombinase polymerase amplification assay

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ABSTRACT

Rickettsioses are zoonotic vector-transmitted bacterial infections leading to flu-like symptoms and can progress to severe illness in humans. The gold standard for diagnosis of rickettsial infections is the indirect immunofluorescence assay, a serological method which is not suitable for pathogen identification during the acute phase of the disease. Therefore, several real-time PCR assays were developed. These assays are very sensitive, but require high-equipped laboratories and well-trained personnel. Hence, in this study, a rapid point-of-need detection method was developed to detect all *Rickettsia* species. The 23S and 16S rRNA genes were targeted to develop a recombinase polymerase amplification (RPA) assay. Both 23S and 16S_RPA assays required between seven to ten minutes to amplify and detect one or ten DNA molecules/reaction, respectively. The 16S_RPA assay detected all tested species, whereas the 23S_RPA assay identified only species of the spotted fever and transitional rickettsial groups. All results were compared with real-time PCR assays directed against the same rickettsial genes. The RPA assays are easy to handle and produced quicker results in comparison to real-time PCRs. Both RPA assays were implemented in a mobile suitcase laboratory to ease the use in rural areas. This method can help to provide rapid management of rickettsial infections.

Introduction

Rickettsia spp. are nonmotile, pleomorphic and obligate intracellular Gram-negative bacteria that are transmitted by various vectors, such as ticks, fleas, body lice and mites [1]. Rodents, opossums, cats, dogs, deer and, as in the case of *R. prowazekii*, also humans act as reservoirs [2]. Serologically, the genus *Rickettsia* is divided into three groups, while by genome sequencing into four: spotted fever, typhus, ancestral and transitional groups [3]. Initially, *Rickettsia tsutsugamushi* (now *Orientia tsutsugamushi*) was placed in a fifth group ("scrub typhus group"), but was later on removed and now forms its own genus [4].

Rickettsial infections in humans are characterized by non-specific flu-like symptoms with high fever, headache and sometimes rash. In infections with spotted fever group rickettsiae, an eschar is located at the site of the arthropod bite. Despite the good treatment responses with doxycycline [1,5], typhus group rickettsiae and *R. rickettsii* can cause severe illness and fatal complications when misdiagnosed [6]. Moreover, the Center for Disease Control and Prevention (CDC) lists *Rickettsia prowazekii*, a member of the typhus group, as a bioterrorism agent.

The indirect immunofluorescence assay (IFA) is applied to detect anti-rickettsia antibodies [7], but it is not suitable to recognize early acute cases as seroconversion is delayed. Alternatively, many real-time PCR assays were established to specifically identify the genome of different rickettsial species [8]. However, real-time PCR can only be used in highly equipped laboratories and performed by trained personnel. Thus, a simple molecular diagnostic test is needed to identify infected cases at point of need, especially in low resource settings, where most human cases emerge.

Many isothermal amplification methods have been developed to allow DNA amplification and detection using unsophisticated devices. One of these methods is the recombinase polymerase amplification

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Table 1
List of microorganism DNA tested in the RPA assays and real-time PCR.

ID	Name of Microorganism	Number of samples	Provider	Real-time PCR	RPA		
				16S	23S	16S	23S
1	*Nocardia asteroids (43757)*	1	German Collection of Microorganisms and Cell Cultures (DSMZ)	–	–	–	–
2	*Streptococcus agalactiae (2134)*	1		–	–	–	–
3	*Enterococcus faecalis (20478)*	1		–	–	–	–
4	*Listeria monocytogenes (15675)*	1		–	–	–	–
5	*Clostridium perfringens (756)*	1		–	–	–	–
6	*Escherichia coli (30083)*	1		–	–	–	–
7	*Staphylococcus aureus (1104)*	1		–	–	–	–
8	*Pseudomonas aeruginosa (939)*	1		–	–	–	–
9	*Leishmania infantum*	1	American Type Culture Collection, Manassas, USA	–	–	–	–
10	*Leishmania tropica*	1		–	–	–	–
11	*Dengue virus*	1	Institut Pasteur of Dakar, Senegal	–	–	–	–
12	*Yellow fever virus*	1		–	–	–	–
13	*Zika virus*	1		–	–	–	–
14	*Chikungunya virus*	1		–	–	–	–
15	*Plasmodium falciparum*	13	University of Ibadan, Nigeria	–	–	–	–
16	*Leptospira ballum*	1	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Germany	–	–	–	–
16	*R. rickettsii*	1	Bernhard Nocht Institute Hamburg, Germany	+	+	+	+
17	*R. conorii*	1		+	+	+	+
18	*R. africae*	5		+	+	+	+
19	*R. felis*	1		+	+	+	+
20	*R. prowazekii*	1		+	+	+	–
21	*R. typhi*	2		+	+	+	–
22	*Orientia tsutsugamushi*	1		–	+	+	–
23	*R. slovaca*	1		+	+	+	+
24	*R. helvetica*	1	Bundeswehr Institute of Microbiology, Munich, Germany	+	+	+	+
25	*R. monacensis*	1		–	+	+	+

For the cross reactivity study, non-rickettsial species DNA samples contain more than 10^7 DNA molecules/reaction. While rickettsial standard DNA samples have 10^5-10^4 DNA molecules/reaction. The concentration of DNA in the Rickettsial clinical samples were around 10^2 DNA molecules/reaction.

(RPA) assay, which provides results within 15 min. The recombinase, the single strand binding protein and the strand displacing DNA polymerase are the key factors in the amplification phase of the RPA. Specific detection of the amplicons is guaranteed *via* the exo-probe, which slice at the mimic basic site upon binding to the complementary sequence. The emitted fluorescence signal can be measured in real-time using a portable reader [9].

This study, therefore, describes the development of a point of need detection system for *Rickettsia* spp. based on RPA assay technology.

Material and methods

Molecular rickettsial DNA standard and pathogen nucleic acid

The molecular rickettsial DNA standard containing both 16S and 23S genes (nt 893603–893406 and 939869–940110 on GenBank accession number CP001612.1) was synthesized by GENEART GmbH (Regensburg, Germany) and a serial tenfold dilution (10^6-10^0 DNA molecules/µl) was prepared as previously described [10]. DNA from various rickettsial species, other pathogens and clinical samples that were used in the assays are listed in Table 1. Clinical rickettsia positive sample were obtained from whole blood (1x *R. typhi*) and skin lesions (1x *R. slovaca* and 4x of *R. africae*).

RPA oligonucleotides

For the development of the rickettsial RPA assay, 16S and 23S rRNA gene regions were selected for primer and probe design. In total, five forward and five reverse primers and two exo-probes were designed and tested to select the best combination, which produces the highest RPA assay sensitivity and specificity (Fig. S1). All oligonucleotides were purchased from TIB MOLBIOL (Berlin, Germany).

RPA assay conditions

The TwistAmp exo or exo-RT kit (TwistDx Ltd, Cambridge, UK) were used. For one reaction mix, 29.5 µl rehydration buffer, 10.7 µl H_2O, 2.1 µl of each primer (10 µM) and 0.6 µl of 10 µM exo-probe were added into the reaction tube containing the freeze-dried pellet. The 2.5 µl of 280 nM magnesium acetate and 1 µl of extracted DNA or RNA were pipetted into the lid of the tubes of exo or exo-RT kits, respectively. After brief mixing and centrifugation, the strips were placed immediately into the tube scanner ESEQuant (QIAGEN Lake Constance GmbH, Stockach, Germany). The reaction was then incubated at 42 °C for 15 min. A mixing and centrifugation step was conducted after 230 s. The FAM channel signal was measured using tube scanner ESEQuant and analysed with the Tubescanner studio software (version 2.07.06, QIAGEN Lake Constance GmbH, Stockach, Germany), which determines the threshold and first derivative.

Analytical sensitivity and specificity

The best primer combination for each RPA assay was determined by using a DNA molecular standard at a concentration of 10^5 DNA molecules/µl. A dilution range from 10^6 to 10^0 molecules/µl of the rickettsial standard DNA was applied to test the analytical sensitivity or limit of detection of each RPA assay. The cross-reactivity was tested using pathogen nucleic acids listed in Table 1, and human genome provide by the University of Ibadan, Nigeria.

Statistical analysis

A semi-logarithmic regression of the data set of the eight RPA runs on 10^6-10^0 DNA molecular standard was calculated with GraphPad PRISM 7 software (GraphPad Software Inc., San Diego, California) and probit regression analyses was performed using STATISTICA software (StatSoft, Hamburg, Germany) to calculate the limit of detection in 95% of the cases.