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Assessment of a quantitative multiplex 5′ nuclease real-time PCR for spotted fever and typhus group rickettsioses and *Orientia tsutsugamushi*

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INTRODUCTION

Scrub typhus, murine typhus and spotted fever group rickettsioses are re-emerging causes of acute febrile illness in south and southeast Asia [1,2], whereas spotted fever group and typhus group rickettsioses are globally distributed. Data from the United States suggest both increasing incidence and serious under-reporting (fewer than a quarter of cases) [3]. Lack of accurate, rapid diagnostic tools has limited assessment of the global burden, distribution and clinical features of rickettsioses, including the pathogenicity of different species [4]. Paired sera, the current gold standard, at best informs epidemiology, because diagnoses can be made in retrospect only if clinical suspicion, follow-up and laboratory infrastructure enable a convalescent blood sample to be obtained and tested [5]. Serology also does not reliably distinguish among species. Because rickettsial illnesses clinically mimic other causes of undifferentiated fever that require different treatment, better diagnostic tools are needed to guide management and avert needless morbidity and mortality [1,2]. To address this need, we sought to develop a quantitative, multiplex 5′-nuclease real-time PCR assay to diagnose spotted fever (SFGR) and typhus group (TGR) rickettsioses, as well as *Orientia tsutsugamushi* (OT), the cause of scrub typhus.

METHODS

We used AlleleID 6 (PREMIER Biosoft, Palo Alto, CA, USA) software to design minor groove binding primers and probes. Our gene targets included a consensus *ompA* sequence (prepared by ClustalX alignment of 23 SFGR *ompA* sequences), the *Rickettsia typhi* genus wide 17-kDa lipoprotein precursor gene, and the *O. tsutsugamushi* 56-kDa major outer membrane protein gene.

After preparing and optimising primers, DNA from cultured bacteria was amplified, cloned, and the plasmid quantity determined. Optimisation and determination of analytical sensitivity was accomplished with DNA from plasmid clones of multiple species (*R. akari*, *R. conorii*, *R. felis*, *R. parkeri*, *R. prowazekii*, *R. rickettsii*, *R. typhi* and *O. tsutsugamushi* Kato, Karp and Gilliam) that was detected with SYBR Green.

The assay was further optimised after integration of probes. The final result was a 3-colour Taqman (5′-nuclease) assay for the BioRad iQ5 PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). FAM was chosen to detect the *ompA* gene common to all SFGR, Texas Red to detect the 17-kDa lipoprotein precursor gene of *Rickettsia* spp. with preferential detection of TGR spp. (*R. typhi* and *R. prowazekii*), and HEX to broadly detect the 56-kD antigen gene of *Orientia* spp. (OT).

The specificity of the assay was assessed using DNA from a variety of genomic bacterial and protozoan species (*Anaplasma phagocytophilum*, *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Neorickettsia helminthoeca*; *Borrelia burgdorferi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus*; and *Plasmodium falciparum*) that can cause acute febrile syndromes and bloodstream infections. Additionally, normal human DNA and human HL-60 promyelocytic leukaemia DNA were used.

Finally, we assessed the sensitivity of the assay to detect DNA in a blood matrix. We spiked genomic DNA from spotted fever group *Rickettsia* (including *R. akari*, *R. conorii*, *R. felis*, *R. montanensis*, *R. parkeri*, *R. peacockii* and *R. rickettsii*), TGR (*R. typhi* and *R. prowazekii*), and *Orientia* spp. (Kato, Karp and Gilliam strains of OT) into blood. Multiplex validation was performed using all three primer and probe sets and total DNA from *Rickettsia* and *Orientia* spp. spiked into human blood.

RESULTS

Multiplex analytical sensitivity for all cloned targets was linear over a range of 2–2 × 10^5 copies, or as few as 2000 target copies/mL of each were detected. Genomic DNA from all SFGR and TGR species as well as OT strains was detected. Multiplexing the assays did not result in a loss of sensitivity of the assay for SFGR, TGR or OT. The assays all had very similar efficiencies and correlations (FAM efficiency 119% and R = 0.99, Texas Red 115% and 0.998, and HEX 106% and 0.998). Multiplex analytical specificity showed no cross-

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reactions among genomic DNA from SFGR, TGR and OT, nor detected genomic DNA of other bacterial pathogens in human blood.

CONCLUSIONS

To be useful clinically, a tool for the diagnosis of rickettsial infections must be able to detect the pathogen accurately and quickly to enable targeted therapy. An ideal epidemiologic tool would also be able to distinguish between species, so that the distribution of existing *Rickettsia* species and their vectors may be better understood, and new species and ecologic niches uncovered [5]. This multiplex quantitative PCR assay holds the promise of doing both. It detects as few as two SFGR, TGR or OT DNA gene copies in blood, distinguishes between them and other closely-related rickettsiae (*Ehrlichia* and *Anaplasma*), and can be conducted in <3 h. The clinical test characteristics of this assay need to be determined to assess its diagnostic effectiveness. Validation of the assay will require prospective collection of clinical samples that can be tested by the current diagnostic standard (paired serology) and by PCR with blinded interpretation of testing. Concomitant prospective collection of clinical data will also be important, so that clinical and laboratory results can be correlated.

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