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Development of a duplex real-time PCR for the detection of *Rickettsia* spp. and typhus group rickettsia in clinical samples

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ABSTRACT

Molecular diagnosis using real-time polymerase chain reaction (PCR) may allow earlier diagnosis of rickettsiosis. We developed a real-time duplex PCR that amplifies (i) DNA of any rickettsial species and (ii) DNA of both typhus group rickettsia, i.e. *R. prowazekii* and *R. typhi*. Primers and probes were selected to amplify a segment of the 16S rRNA gene of *Rickettsia* spp. for the pan-rickettsial PCR and the citrate synthase gene (*gltA*) for the typhus group rickettsia PCR. Analytical sensitivity was 10 copies of control plasmid DNA per reaction. No cross-amplification was observed when testing human DNA and 22 pathogens or skin commensals. Real-time PCR was applied to 16 clinical samples. Rickettsial DNA was detected in the skin biopsies of 3 patients. In one patient with severe murine typhus, the typhus group PCR was positive in a skin biopsy from a petechial lesion and seroconversion was later documented. The two other patients with negative typhus group PCR suffered from Mediterranean and African spotted fever, respectively; in both cases, skin biopsy was performed on the eschar. Our real-time duplex PCR showed a good analytical sensitivity and specificity, allowing early diagnosis of rickettsiosis among 3 patients, and recognition of typhus in one of them.

INTRODUCTION

Rickettsial diseases are worldwide emerging arthropod-borne zoonoses that are caused by small obligate intracellular gram-negative rods. They are traditionally divided into the spotted fever group, the typhus group and the scrub typhus group (Parola*, et al.*, 2005).

Microbiological diagnosis of rickettsiosis is usually established by serology, as isolation in cell culture or animals is difficult and dangerous for the laboratory personnel, and immunohistochemistry is not widely available. However, since IgM increase takes 15 to 26 days, serological diagnosis is usually retrospective, thus limiting the clinical impact of diagnosis (Fournier*, et al.*, 2002). Moreover species identification is limited by cross-reactions.

Molecular diagnosis using polymerase chain reaction (PCR) allows earlier diagnosis of rickettsiosis and species identification. Thus, several PCR assays targeting various rickettsial genes have been developed in order to accelerate the diagnosis of rickettsiosis. While some targeted several species (Leitner*, et al.*, 2002, Fournier & Raoult, 2004), other were designed to detect only a single rickettsial species (Choi*, et al.*, 2005, Karpathy*, et al.*, 2009). Since several rickettsiae can be responsible of the same clinical syndrome, a broader spectrum is warranted. In addition, the biodiversity of rickettsial species is likely underestimated and some yet unknown species might also be pathogenic (Parola*, et al.*, 2005). Moreover, the recognition of typhus group rickettsiosis is clinically and epidemiologically relevant, since these infections may be associated with a worse prognosis than spotted fevers (Dumler*, et al.*, 1991, Bechah*, et al.*, 2008). We therefore developed a real-time duplex PCR that amplifies DNA of any rickettsial species and both typhus group rickettsia, i.e. *R. prowazekii* and *R. typhi*.

METHODS

Development of the real-time PCR

Primers and probes were designed using Primer3 software (Rozen & Skaletsky, 2000) starting from alignments of the 16S rRNA and of the citrate synthase (*gltA)* genes obtained for the different rickettsial species available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

For the *Rickettsia* spp. PCR, a forward primer Rsp-F1 (5'-CGCAACCCTCATTCTTATTTGC-3'), a second forward primer Rsp-F2 (5'-CGCAACCCTTATTCTTATTTGC-3'), a reverse primer Rsp-R (5'-TGCTACAATGGTGTTTACAGAGG-3') and a MGB probe (minor-groove binder) labeled with 5'FAM (6-carboxyfluorescein) Rsp-Probe (5'-FAM-TAAGAAAACTGCCGGTGATAAGCCGGAG-BHQ-3') were designed to amplify a 149–bp fragment of the 16S rRNA gene of all *Rickettsia* spp. This fragment was chosen because several rickettsial species including *R. conorii*, *R. africae*, *R. rickettsii*, *R. slovaca* and *R. akari* show an identical sequence in the selected 16S rRNA fragment (Fournier*, et al.*, 2003). Since typhus group rickettsia differs from other rickettsial species at position 1097, the second forward primer was added.

For the typhus group rickettsia PCR, the citrate synthase gene *gltA* was targeted, since it is less conserved among rickettsial species. To amplify *R. prowazekii* DNA, the forward primer Rtp-F (5'-TTCGGATTGCTGGCTCATCA-3') and the reverse primer Rtp-R (5'-GCTAAAGCTAAAGATAAGAATGATCCATTT-3') were designed. To amplify *R. typhi* DNA the primers Rtt-F (5'-TACGAATTGCTGGCTCATCA-3') and Rtt-R (5'-GCTAAAGCTAAAGACAAAAATGATCCATTT-3') were added. Only one MGB probe was designed labelled with 5'TET (tetrachlorofluorescein phosphoramidite), Rt-Probe (5'-TET -ATCCTTTTGCATGTATTAGCACTGGTATTGCATCA--BHQ-3'), to detect both species. All primers and probes were prepared by Eurogentec (Belgium)

PCR amplification and products detection were performed with ABI Prism 7900 Sequence Detection system (Applied Biosystems, Rotkreuz, Switzerland) during 45 cycles. The reactions were performed with 0.2 μM of each primer, 0.1 μM of probe and 10μl 2x TaqMan universal master Mix (Applied Biosystem) and 5 μl DNA sample (final volume 20 μl). Cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 45 cycles during 15s at 95°C and 2 min at 60°C.

To obtain positive controls for both PCR and to allow quantification, 4 plasmids were constructed using rickettsial DNA from 3 species: DNA from a skin biopsy positive for *R. conorii* subsp. *israelensis* (Boillat*, et al.*, 2008) and *R. prowazekii* DNA and *R. typhi* DNA extracted from two strains grown in cell culture (kindly provided by Prof. Didier Raoult, Université de la Méditerranée, Marseille, France). Thus, for each PCR, we obtained two positive control plasmids: (i) *R. conorii* and *R. typhi* for the pan-rickettsial real-time PCR; (ii) *R. typhi* and *R. prowazekii* controls for the typhus group real-time PCR. The genomic DNA was amplified using the polymerase AmpliTaq Gold (Applied Biosystems, Zug, Switzerland). PCR products were cloned using the TOPO TA Cloning® kit (Invitrogen, Basel, Switzerland). After isolation of plasmidic DNA using the QIAprep Spin Miniprep Kit (Qiagen, Kombrechtikon, Switzerland), quantification was performed on a Nanodrop ND-1000 (Witech, Littau, Switzerland).

Analytical sensitivity, reproducibility and specificity

To assess analytical sensitivity of both real-time PCR, 10-fold dilutions of the 4 positive control plasmids were tested in 5 independent runs and in 5 replicates. Intra- and inter-run reproducibility was assessed by comparing mean threshold cycle (Ct) and standard error of the mean of replicates obtained in the 5 runs. Analytical specificity of the pan-rickettsial PCR was tested using DNA extracted from 22 pathogens and skin commensals, including bacteria, fungi and virus (Table 1). In addition, the broad-range of the pan-rickettsial PCR was investigated using the following rickettsial DNA (grown in cell culture and provided by

Prof. Didier Raoult, Université de la Méditerranée, Marseille, France): *R. africae, R. conorii*, *R. felis*, *R. rickettsii*, *R. slovaca, R. prowazekii* and *R. typhi*.

Conversely, the specificity of the typhus group PCR was confirmed using the DNA from the spotted-group rickettsiae.

Clinical samples

The new rickettsia duplex real-time PCR, i.e. the pan-rickettsial PCR and the typhus group rickettsia PCR was applied to various samples taken from patients with clinical suspicion of rickettsiosis. With one exception, tissue samples were not fixed. DNA was extracted from fresh clinical samples using MagNA Pure LC automated system (Roche) with the MagNA Pure LC DNA isolation kit I (Roche). DNA was extracted from 200μl of sample and eluted in a final volume of 100 μl.

RESULTS

Analytical sensitivity, reproducibility and specificity

Sensitivity and reproducibility of the pan-rickettsial real-time PCR are shown in figure 1. Fourteen of 25 replicates (56%) were positive with a *R. conorii* plasmid positive control concentration of 1 copy/reaction, and all replicates were positive at a concentration of 10 copies. Similar results were obtained with the *R. typhi* positive control (data not shown). Intra- and inter-run reproducibility was high for both *R. conorii* and *R. typhi* positive controls (figure 1B and 2B). The average difference between ten-fold dilutions was 3.24 and 3.17 cycles when testing the *R. conorii* and *R. typhi* plasmid, respectively. No cross-amplification was observed with the different microorganisms tested (Table 1). Moreover, with this PCR, we obtained an excellent positive amplification with all the different rickettsial species investigated: *R. africae, R. conorii*, *R. felis*, *R. rickettsii*, R. *slovaca* , *R. prowazekii* and *R. typhi* (with Ct values ranging from 18.8 to 24.8). Sensitivity of typhus group PCR was 100%

at a positive control concentration of 10 copies/reaction for both plasmids (figure 2A). Specificity was high, as there was no amplification when testing DNA of *R. africae, R. conorii*, *R. felis*, *R. rickettsii*, and *R. slovaca*.

Clinical application

The duplex real-time PCR was applied to 16 specimens taken from 13 patients: 8 skin biopsies, 4 EDTA blood samples, 3 cerebrospinal fluids (CSF), and one pericardial fluid. Rickettsial DNA was detected in three samples from three different patients, whose clinical characteristics are summarized in Table 2. In one patient, typhus group rickettsia real-time PCR was also positive. This 43-year old man presented with fever, petechiae, pulmonary infiltrates, acute renal failure, encephalopathy and hyperbilirubinemia after a stay in Tunisia, where he was in contact with animals in his family farm. At admission both leptospirosis and rickettsiosis were suspected and he was promptly treated with ceftriaxone and doxycycline. Our duplex real-time PCR performed on a biopsy of a petechial cutaneous lesion (taken 2 days after treatment start) allowed diagnosis of typhus. By contrast, EDTA blood tested negative for rickettsial DNA. During the course of illness, renal-replacement therapy was necessary and he developed heart failure with an ejection fraction of 25%. After 14 days of treatment, he recovered without sequelae. Seroconversion against *Rickettsia* spp. was documented 17 and 37 days after onset of symptoms for IgM (titer 1/1024) and IgG (titer 1/128), respectively, but due to cross-reaction, identification at species level was not possible. A presumptive diagnosis of murine typhus was considered based on the clinical presentation, the zoonotic exposure and the PCR results.

The two other patients with a positive pan-rickettsial PCR and negative typhus group PCR were clinically and epidemiologically diagnosed with Mediterranean spotted fever and African tick bite fever, after a travel to Sardinia (Southern Italy) and South Africa, respectively. Both presented with fever, headache and an inoculation eschar, while only the patient with Mediterranean spotted fever exhibited a rash and a severe disease requiring hospitalisation.

Diagnosis was established in both patients by a PCR done on the biopsy of the inoculation eschar.

Ten patients with negative duplex real-time PCR results presented with various clinical syndromes: encephalitis (2), fever and rash (1), fever in a returning traveller (1), fever of unknown origin in a HIV-positive patient (1), hemophagocytosis syndrome (1), acute liver failure and pericardial tamponade (1), skin nodules (1), bilateral lung infiltrates (1). In one case, no clinical information was available. Five patients had a history of travel to an endemic region. Serology for *Rickettsia* spp. was performed in 6 cases and was negative or showed a pattern of past infection.

DISCUSSION

A duplex real-time PCR targeting all rickettsial species and the typhus group rickettsiae was developed to detect rickettsial DNA in clinical samples and to identify agents of typhus. The test was sensitive for at least 10 DNA copies per reaction and exhibited a good reproducibility. Its application to clinical samples (skin biopsies) from patients with clinical suspicion of rickettsiosis allowed diagnosis of spotted fever in two cases, and recognition of murine typhus in another case.

Timely diagnosis of rickettsiosis can be challenging, since seroconversion occurs usually in the convalescent phase (Brouqui*, et al.*, 2004). For example, by using indirect fluorescent antibody assay, diagnostic titers of *R. typhi* antibodies are found 15 days after onset of symptoms (Dumler*, et al.*, 1991). For *R. conori* and *R. africae* median time to IgM seroconversion is even longer (16 and 25 days, respectively) (Fournier*, et al.*, 2002). Therefore, since the first report of use of molecular methods for the detection of rickettsiosis (Tzianabos*, et al.*, 1989), several assays have been developed. Primers have usually targeted outer membrane protein genes *ompA* (Fournier & Raoult, 2004) and *ompB* (Paris*, et al.*, 2008), the citrate synthase gene *gltA* (Roux*, et al.*, 1997) and the 17-kD protein gene (Leitner*, et al.*, 2002). In this work, we selected the 16S rRNA gene for the *Rickettsia* spp

PCR and were able to target a gene region that was conserved among all rickettsial species. The *gltA* gene was chosen for the typhus group rickettsia, due to its higher variability.

Due to its broad spectrum and the low discriminative power of the 16S rRNA gene, our pan-rickettsial PCR is not able to precisely identify the rickettsia at species level. Species identification is warranted, since several species can be responsible of the same clinical picture. Practically, we recommend DNA amplification and sequencing of *gltA* and *ompA* genes (Fournier & Raoult, 2004), which allow identification at species and subspecies level (Boillat*, et al.*, 2008).

Despite the limited number of samples tested and their heterogeneity, the clinical experience with the new duplex real-time PCR is encouraging. Since its development in 2007, we could confirm the clinical suspicion of rickettsial infection in 3 cases. Moreover, the test allowed rapid identification of typhus in a patient with a severe febrile illness after a stay in Tunisia. Clinical presentation was non-specific, and in particular leptospirosis was suspected initially because of the triad of rash, hyperbilirubinemia and acute renal failure. This case illustrates that clinical recognition of rickettsiosis may be difficult and that empirical treatment with doxycycline is indicated in case of severe illnesses in returning travelers.

It should be noted that only skin biopsies were positive in this series. PCR has been successfully applied to blood and serum samples (Leitner*, et al.*, 2002, Choi*, et al.*, 2005) , arthropod vectors (Karpathy*, et al.*, 2009), but most studies have used skin biopsies (Fournier & Raoult, 2004). Biopsies of the inoculation eschar, when present, have the best diagnostic efficiency (Fournier & Raoult, 2004). In case of rickettsioses that are not associated with an eschar (e.g. murine typhus), skin biopsy should be performed on skin lesions (maculopapular or petechial lesions), since endothelial cells are the site of multiplication of rickettsiae (Walker*, et al.*, 2003) and since these skin lesions generally result from local rickettsial multiplication. As highlighted in this case, PCR of a skin lesion may remain positive even after a few days of doxycycline treatment.

In conclusion, we have developed a duplex real-time PCR for the direct detection of rickettsial DNA and for the identification of typhus group rickettsia. Optimal use of the assay includes its application to skin biopsy of patients presenting a clinical picture and epidemiological features compatible with a rickettsial infection. Furthermore, the broad range format of the pan-rickettsial PCR may allow the identification of new rickettsial species.

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Table 1.

List of DNA of strains tested to investigate the specificity of the rickettsial PCR.

Species	**Source/strain**
Aspergillus fumigatus	Clinical specimen
Aspergillus terreus	Clinical specimen
Candida albicans	ATCC 90028
Candida glabrata	Clinical specimen
Chlamydia pneumoniae	Clinical specimen
Chlamydia trachomatis	Clinical specimen
Corynebacterium pyogenes	Clinical specimen
Escherichia coli	ATCC 25922
Herpes simplex virus 1	Clinical specimen
Herpes simplex virus 2	Clinical specimen
Kingella kingae	Clinical specimen
Lactobacillus spp.	Clinical specimen
Mycoplasma pneumoniae	Clinical specimen
Neisseria lactamica	Clinical specimen
Neisseria subflava	Clinical specimen
Neisseria weaveri	Clinical specimen
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 43300
Staphylococcus epidermidis	Clinical specimen

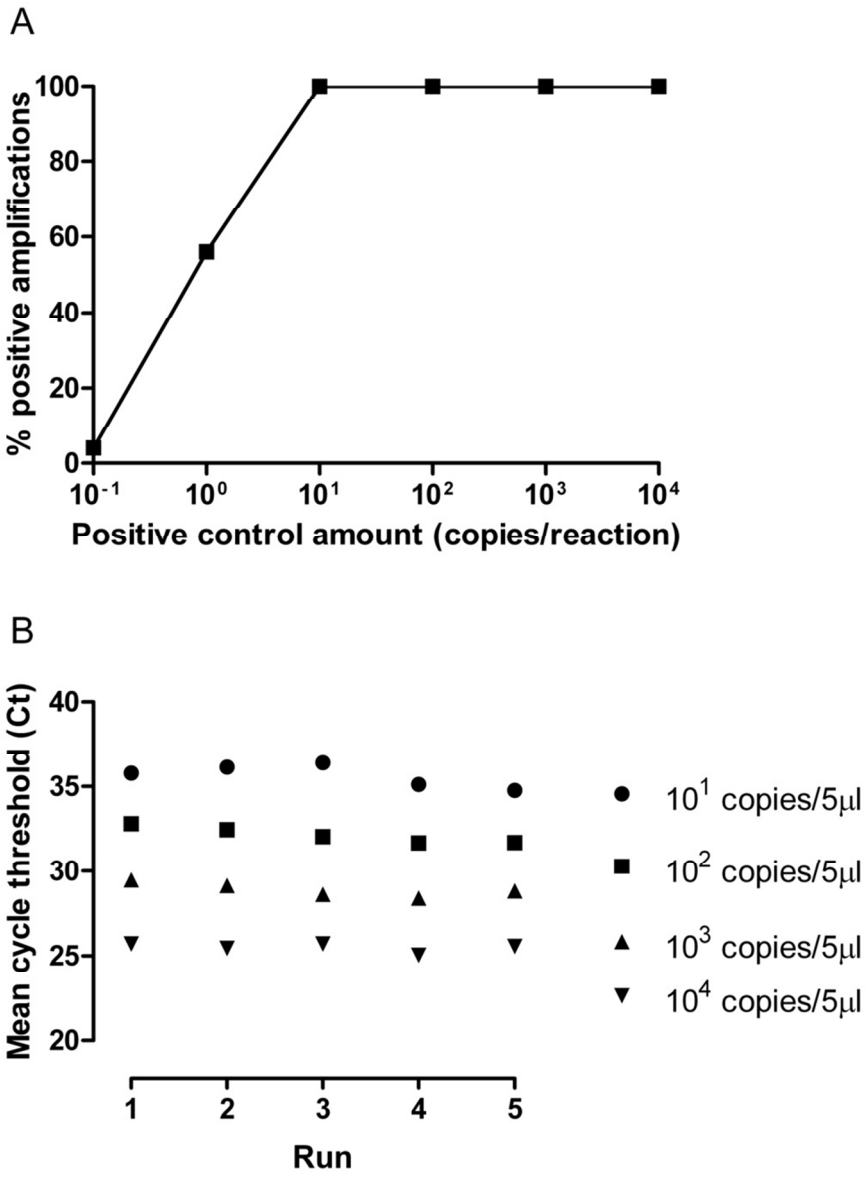
Streptococcus mitis	Clinical specimen
Streptococcus pyogenes	Clinical specimen
Varicella zoster virus	Clinical specimen
Human DNA	Human Genomic DNA (Roche Diagnostics, Basel, Switzerland)

Table 2.

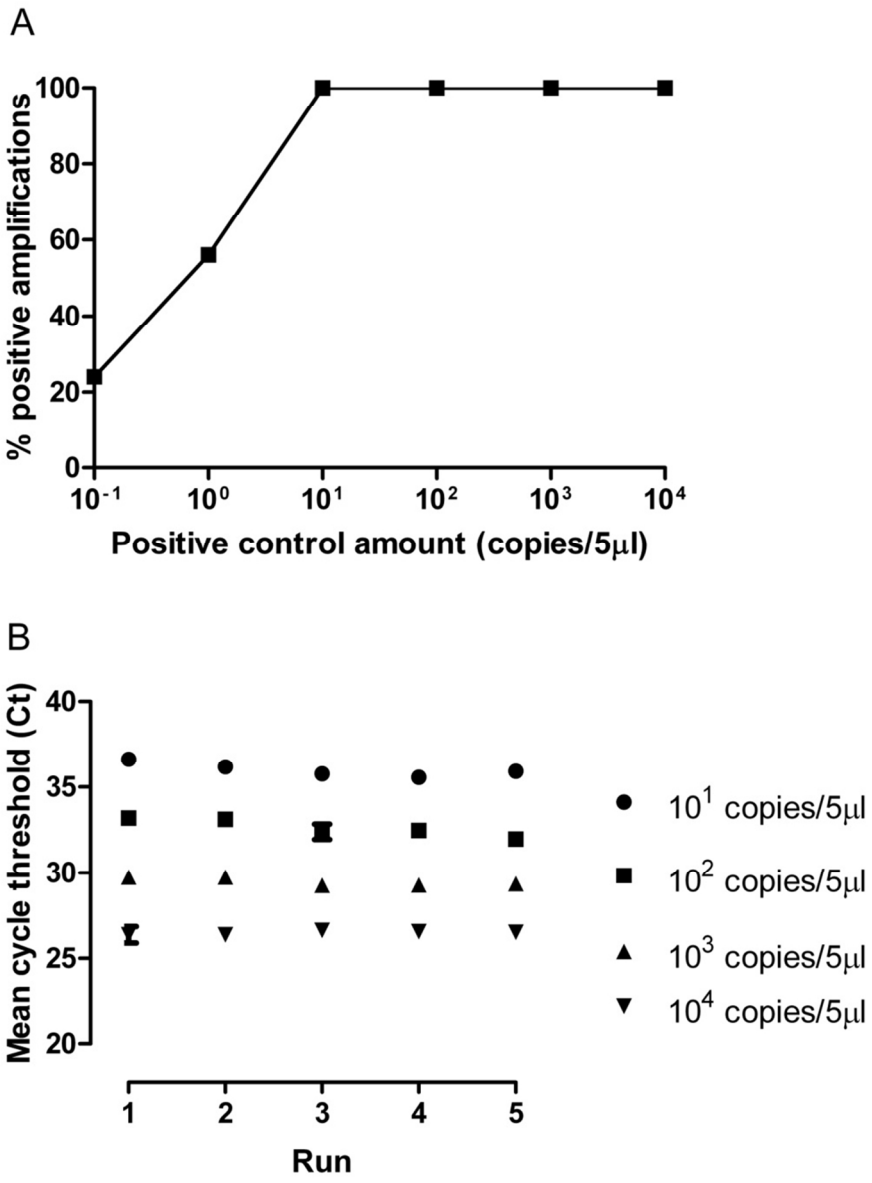
Clinical characteristics and results of laboratory investigations of the three patients with positive PCR.

Patient no	Age	Sex	Clinical presentation	Laboratory findings	Complications	Travel history	Sample	*Rickettsia* spp PCR, DNA copies/ml (mean Ct value)	Typhus group PCR DNA copies/ml (mean Ct value)	Serology	Final diagnosis
1	35	F	Fever Headache Rash Eschar	Leucopenia Thrombocytopenia ELT	None	Italy	Skin biopsy	7886 (31.4)	Negative	NA	MSF
2	41	F	Fever Headache Eschar	NA	None	South Africa	Skin biopsy	791 (35.9)	Negative	Negative	ATBF
3	43	M	Fever Headache Myalgia Dysphagia Cough Rash	Thrombocytopenia Elevated creatinine ELT Hyperbilirubinemia	Acute renal failure Encephalopathy Myocarditis	Tunisia	Skin biopsy	137 (39.5)	171 (40.1)	Seroconversion after 17 days	Murine typhus

ELT: elevated liver enzymes. NA: not available. MSF: Mediterranean Spotted Fever. ATBF: African Tick-Bite Fever



Sensitivity and reproducibility of the pan-rickettsial real-time PCR (R. conorii positive control plasmid). A. Analytical sensitivity. B. Inter-run and intra-run reproductibility assessed using 101 to 104 positive control plasmid copies/reaction (copies/5microl) in 5 independent runs. Error bars represent the standard error of the mean of replicates.
124x166mm (300 x 300 DPI)



Sensitivity and reproducibility of the typhus group rickettsia real-time PCR (R. typhi positive control plasmid). A. Analytical sensitivity. B. Inter-run and intra-run reproductibility assessed using 101 to 104 positive control plasmid copies/5microl in 5 independent runs. Error bars represent the standard error of the mean of replicates.
122x164mm (300 x 300 DPI)