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3 1 **Development of a duplex real-time PCR for the detection of *Rickettsia* spp. and**
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5 2 **typhus group rickettsia in clinical samples**

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2
3 23 **ABSTRACT**
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6 24 Molecular diagnosis using real-time polymerase chain reaction (PCR) may allow earlier
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8 25 diagnosis of rickettsiosis. We developed a real-time duplex PCR that amplifies (i) DNA of any
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10 26 rickettsial species and (ii) DNA of both typhus group rickettsia, i.e. *R. prowazekii* and *R.*
11
12 27 *typhi*. Primers and probes were selected to amplify a segment of the 16S rRNA gene of
13
14 28 *Rickettsia* spp. for the pan-rickettsial PCR and the citrate synthase gene (*gltA*) for the typhus
15
16 29 group rickettsia PCR. Analytical sensitivity was 10 copies of control plasmid DNA per
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18 30 reaction. No cross-amplification was observed when testing human DNA and 22 pathogens
19
20 31 or skin commensals. Real-time PCR was applied to 16 clinical samples. Rickettsial DNA was
21
22 32 detected in the skin biopsies of 3 patients. In one patient with severe murine typhus, the
23
24 33 typhus group PCR was positive in a skin biopsy from a petechial lesion and seroconversion
25
26 34 was later documented. The two other patients with negative typhus group PCR suffered from
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28 35 Mediterranean and African spotted fever, respectively; in both cases, skin biopsy was
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30 36 performed on the eschar. Our real-time duplex PCR showed a good analytical sensitivity and
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32 37 specificity, allowing early diagnosis of rickettsiosis among 3 patients, and recognition of
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34 38 typhus in one of them.
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39 INTRODUCTION

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

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

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

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3 62 **METHODS**
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6 63 Development of the real-time PCR
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9 64 Primers and probes were designed using Primer3 software (Rozen & Skaletsky, 2000)
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11 65 starting from alignments of the 16S rRNA and of the citrate synthase (*gltA*) genes obtained
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13 66 for the different rickettsial species available in the GenBank database
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15 67 (<http://www.ncbi.nlm.nih.gov/genbank/>).
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18 68 For the *Rickettsia* spp. PCR, a forward primer Rsp-F1 (5'-CGCAACCCTCATTCTTATTTGC-
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20 69 3'), a second forward primer Rsp-F2 (5'-CGCAACCCTTATTCTTATTTGC-3'), a reverse
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22 70 primer Rsp-R (5'-TGCTACAATGGTGTTCACAGAGG-3') and a MGB probe (minor-groove
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24 71 binder) labeled with 5'FAM (6-carboxyfluorescein) Rsp-Probe (5'-FAM-
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26 72 TAAGAAAAGCTGCCGGTGATAAGCCGGAG-BHQ-3') were designed to amplify a 149-bp
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28 73 fragment of the 16S rRNA gene of all *Rickettsia* spp. This fragment was chosen because
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30 74 several rickettsial species including *R. conorii*, *R. africae*, *R. rickettsii*, *R. slovaca* and *R.*
31
32 75 *akari* show an identical sequence in the selected 16S rRNA fragment (Fournier, *et al.*, 2003).
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34 76 Since typhus group rickettsia differs from other rickettsial species at position 1097, the
35
36 77 second forward primer was added.

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39 78 For the typhus group rickettsia PCR, the citrate synthase gene *gltA* was targeted, since it is
40
41 79 less conserved among rickettsial species. To amplify *R. prowazekii* DNA, the forward primer
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43 80 Rtp-F (5'-TTCGGATTGCTGGCTCATCA-3') and the reverse primer Rtp-R (5'-
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45 81 GCTAAAGCTAAAGATAAGAATGATCCATTT-3') were designed. To amplify *R. typhi* DNA
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47 82 the primers Rtt-F (5'-TACGAATTGCTGGCTCATCA-3') and Rtt-R (5'-
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49 83 GCTAAAGCTAAAGACAAAATGATCCATTT-3') were added. Only one MGB probe was
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51 84 designed labelled with 5'TET (tetrachlorofluorescein phosphoramidite), Rt-Probe (5'-TET -
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53 85 ATCCTTTTGCATGTATTAGCACTGGTATTGCATCA--BHQ-3'), to detect both species. All
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55 86 primers and probes were prepared by Eurogentec (Belgium)
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3 87 PCR amplification and products detection were performed with ABI Prism 7900 Sequence
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5 88 Detection system (Applied Biosystems, Rotkreuz, Switzerland) during 45 cycles. The
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7 89 reactions were performed with 0.2 μ M of each primer, 0.1 μ M of probe and 10 μ l 2x TaqMan
8
9 90 universal master Mix (Applied Biosystem) and 5 μ l DNA sample (final volume 20 μ l). Cycling
10
11 91 conditions were 2 min at 50°C, 10 min at 95°C, followed by 45 cycles during 15s at 95°C and
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13 92 2 min at 60°C.

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16 93 To obtain positive controls for both PCR and to allow quantification, 4 plasmids were
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18 94 constructed using rickettsial DNA from 3 species: DNA from a skin biopsy positive for *R.*
19
20 95 *conorii* subsp. *israelensis* (Boillat, *et al.*, 2008) and *R. prowazekii* DNA and *R. typhi* DNA
21
22 96 extracted from two strains grown in cell culture (kindly provided by Prof. Didier Raoult,
23
24 97 Université de la Méditerranée, Marseille, France). Thus, for each PCR, we obtained two
25
26 98 positive control plasmids: (i) *R. conorii* and *R. typhi* for the pan-rickettsial real-time PCR; (ii)
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28 99 *R. typhi* and *R. prowazekii* controls for the typhus group real-time PCR. The genomic DNA
29
30 100 was amplified using the polymerase AmpliTaq Gold (Applied Biosystems, Zug, Switzerland).
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32 101 PCR products were cloned using the TOPO TA Cloning® kit (Invitrogen, Basel, Switzerland).
33
34 102 After isolation of plasmidic DNA using the QIAprep Spin Miniprep Kit (Qiagen,
35
36 103 Kombrechtikon, Switzerland), quantification was performed on a Nanodrop ND-1000 (Witech,
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38 104 Littau, Switzerland).

105 Analytical sensitivity, reproducibility and specificity

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

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2
3 113 Prof. Didier Raoult, Université de la Méditerranée, Marseille, France): *R. africae*, *R. conorii*,
4
5 114 *R. felis*, *R. rickettsii*, *R. slovaca*, *R. prowazekii* and *R. typhi*.

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8 115 Conversely, the specificity of the typhus group PCR was confirmed using the DNA from the
9
10 116 spotted-group rickettsiae.

117 Clinical samples

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

124

125 **RESULTS**

126 Analytical sensitivity, reproducibility and specificity

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

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3 138 at a positive control concentration of 10 copies/reaction for both plasmids (figure 2A).
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5 139 Specificity was high, as there was no amplification when testing DNA of *R. africae*, *R. conorii*,
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7 140 *R. felis*, *R. rickettsii*, and *R. slovaca*.
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10 141 Clinical application

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12 142 The duplex real-time PCR was applied to 16 specimens taken from 13 patients: 8 skin
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14 143 biopsies, 4 EDTA blood samples, 3 cerebrospinal fluids (CSF), and one pericardial fluid.
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16 144 Rickettsial DNA was detected in three samples from three different patients, whose clinical
17
18 145 characteristics are summarized in Table 2. In one patient, typhus group rickettsia real-time
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20 146 PCR was also positive. This 43-year old man presented with fever, petechiae, pulmonary
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22 147 infiltrates, acute renal failure, encephalopathy and hyperbilirubinemia after a stay in Tunisia,
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24 148 where he was in contact with animals in his family farm. At admission both leptospirosis and
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26 149 rickettsiosis were suspected and he was promptly treated with ceftriaxone and doxycycline.
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28 150 Our duplex real-time PCR performed on a biopsy of a petechial cutaneous lesion (taken 2
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30 151 days after treatment start) allowed diagnosis of typhus. By contrast, EDTA blood tested
31
32 152 negative for rickettsial DNA. During the course of illness, renal-replacement therapy was
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34 153 necessary and he developed heart failure with an ejection fraction of 25%. After 14 days of
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36 154 treatment, he recovered without sequelae. Seroconversion against *Rickettsia* spp. was
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38 155 documented 17 and 37 days after onset of symptoms for IgM (titer 1/1024) and IgG (titer
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40 156 1/128), respectively, but due to cross-reaction, identification at species level was not
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42 157 possible. A presumptive diagnosis of murine typhus was considered based on the clinical
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44 158 presentation, the zoonotic exposure and the PCR results.
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48 159 The two other patients with a positive pan-rickettsial PCR and negative typhus group PCR
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50 160 were clinically and epidemiologically diagnosed with Mediterranean spotted fever and African
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52 161 tick bite fever, after a travel to Sardinia (Southern Italy) and South Africa, respectively. Both
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54 162 presented with fever, headache and an inoculation eschar, while only the patient with
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56 163 Mediterranean spotted fever exhibited a rash and a severe disease requiring hospitalisation.
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3 164 Diagnosis was established in both patients by a PCR done on the biopsy of the inoculation
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5 165 eschar.

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8 166 Ten patients with negative duplex real-time PCR results presented with various clinical
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10 167 syndromes: encephalitis (2), fever and rash (1), fever in a returning traveller (1), fever of
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12 168 unknown origin in a HIV-positive patient (1), hemophagocytosis syndrome (1), acute liver
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14 169 failure and pericardial tamponade (1), skin nodules (1), bilateral lung infiltrates (1). In one
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16 170 case, no clinical information was available. Five patients had a history of travel to an endemic
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18 171 region. Serology for *Rickettsia* spp. was performed in 6 cases and was negative or showed a
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20 172 pattern of past infection.

21 22 23 173 **DISCUSSION**

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26 174 A duplex real-time PCR targeting all rickettsial species and the typhus group rickettsiae was
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28 175 developed to detect rickettsial DNA in clinical samples and to identify agents of typhus. The
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30 176 test was sensitive for at least 10 DNA copies per reaction and exhibited a good
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32 177 reproducibility. Its application to clinical samples (skin biopsies) from patients with clinical
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34 178 suspicion of rickettsiosis allowed diagnosis of spotted fever in two cases, and recognition of
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36 179 murine typhus in another case.

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39 180 Timely diagnosis of rickettsiosis can be challenging, since seroconversion occurs usually in
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41 181 the convalescent phase (Brouqui, *et al.*, 2004). For example, by using indirect fluorescent
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43 182 antibody assay, diagnostic titers of *R. typhi* antibodies are found 15 days after onset of
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45 183 symptoms (Dumler, *et al.*, 1991). For *R. conori* and *R. africae* median time to IgM
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47 184 seroconversion is even longer (16 and 25 days, respectively) (Fournier, *et al.*, 2002).

48
49 185 Therefore, since the first report of use of molecular methods for the detection of rickettsiosis
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51 186 (Tzianabos, *et al.*, 1989), several assays have been developed. Primers have usually
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53 187 targeted outer membrane protein genes *ompA* (Fournier & Raoult, 2004) and *ompB* (Paris, *et*
54
55 188 *al.*, 2008), the citrate synthase gene *gltA* (Roux, *et al.*, 1997) and the 17-kD protein gene
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57 189 (Leitner, *et al.*, 2002). In this work, we selected the 16S rRNA gene for the *Rickettsia* spp

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3 190 PCR and were able to target a gene region that was conserved among all rickettsial species.
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5 191 The *gltA* gene was chosen for the typhus group rickettsia, due to its higher variability.
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8 192 Due to its broad spectrum and the low discriminative power of the 16S rRNA gene, our pan-
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10 193 rickettsial PCR is not able to precisely identify the rickettsia at species level. Species
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12 194 identification is warranted, since several species can be responsible of the same clinical
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14 195 picture. Practically, we recommend DNA amplification and sequencing of *gltA* and *ompA*
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16 196 genes (Fournier & Raoult, 2004), which allow identification at species and subspecies level
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18 197 (Boillat, *et al.*, 2008).
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21 198 Despite the limited number of samples tested and their heterogeneity, the clinical experience
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23 199 with the new duplex real-time PCR is encouraging. Since its development in 2007, we could
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25 200 confirm the clinical suspicion of rickettsial infection in 3 cases. Moreover, the test allowed
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27 201 rapid identification of typhus in a patient with a severe febrile illness after a stay in Tunisia.
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29 202 Clinical presentation was non-specific, and in particular leptospirosis was suspected initially
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31 203 because of the triad of rash, hyperbilirubinemia and acute renal failure. This case illustrates
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33 204 that clinical recognition of rickettsiosis may be difficult and that empirical treatment with
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35 205 doxycycline is indicated in case of severe illnesses in returning travelers.
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38 206 It should be noted that only skin biopsies were positive in this series. PCR has been
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40 207 successfully applied to blood and serum samples (Leitner, *et al.*, 2002, Choi, *et al.*, 2005) ,
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42 208 arthropod vectors (Karpathy, *et al.*, 2009), but most studies have used skin biopsies
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44 209 (Fournier & Raoult, 2004). Biopsies of the inoculation eschar, when present, have the best
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46 210 diagnostic efficiency (Fournier & Raoult, 2004). In case of rickettsioses that are not
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48 211 associated with an eschar (e.g. murine typhus), skin biopsy should be performed on skin
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50 212 lesions (maculopapular or petechial lesions), since endothelial cells are the site of
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52 213 multiplication of rickettsiae (Walker, *et al.*, 2003) and since these skin lesions generally result
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54 214 from local rickettsial multiplication. As highlighted in this case, PCR of a skin lesion may
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56 215 remain positive even after a few days of doxycycline treatment.
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3 216 In conclusion, we have developed a duplex real-time PCR for the direct detection of
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5 217 rickettsial DNA and for the identification of typhus group rickettsia. Optimal use of the assay
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7 218 includes its application to skin biopsy of patients presenting a clinical picture and
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9 219 epidemiological features compatible with a rickettsial infection. Furthermore, the broad range
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11 220 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
<i>Aspergillus fumigatus</i>	Clinical specimen
<i>Aspergillus terreus</i>	Clinical specimen
<i>Candida albicans</i>	ATCC 90028
<i>Candida glabrata</i>	Clinical specimen
<i>Chlamydia pneumoniae</i>	Clinical specimen
<i>Chlamydia trachomatis</i>	Clinical specimen
<i>Corynebacterium pyogenes</i>	Clinical specimen
<i>Escherichia coli</i>	ATCC 25922
Herpes simplex virus 1	Clinical specimen
Herpes simplex virus 2	Clinical specimen
<i>Kingella kingae</i>	Clinical specimen
<i>Lactobacillus</i> spp.	Clinical specimen
<i>Mycoplasma pneumoniae</i>	Clinical specimen
<i>Neisseria lactamica</i>	Clinical specimen
<i>Neisseria subflava</i>	Clinical specimen
<i>Neisseria weaveri</i>	Clinical specimen
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Staphylococcus aureus</i>	ATCC 43300
<i>Staphylococcus epidermidis</i>	Clinical specimen

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

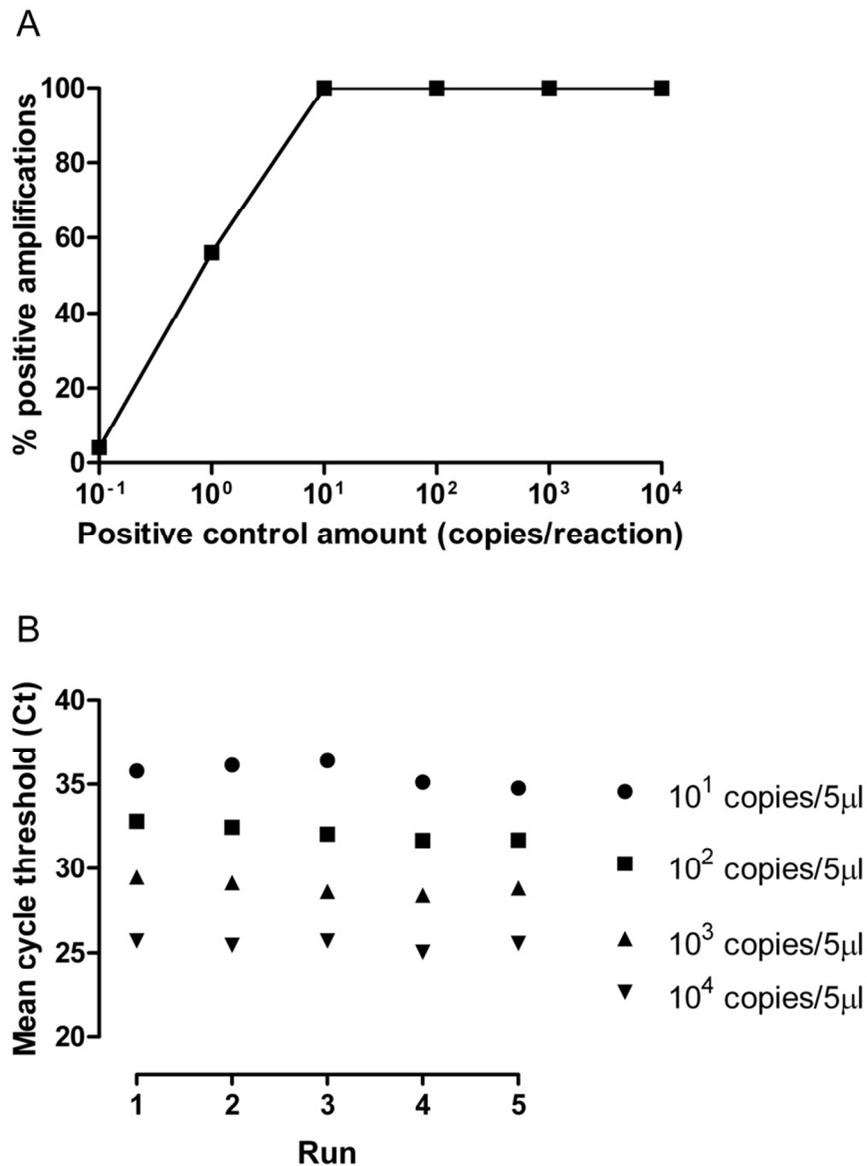
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Table 2.

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	<i>Rickettsia</i> 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

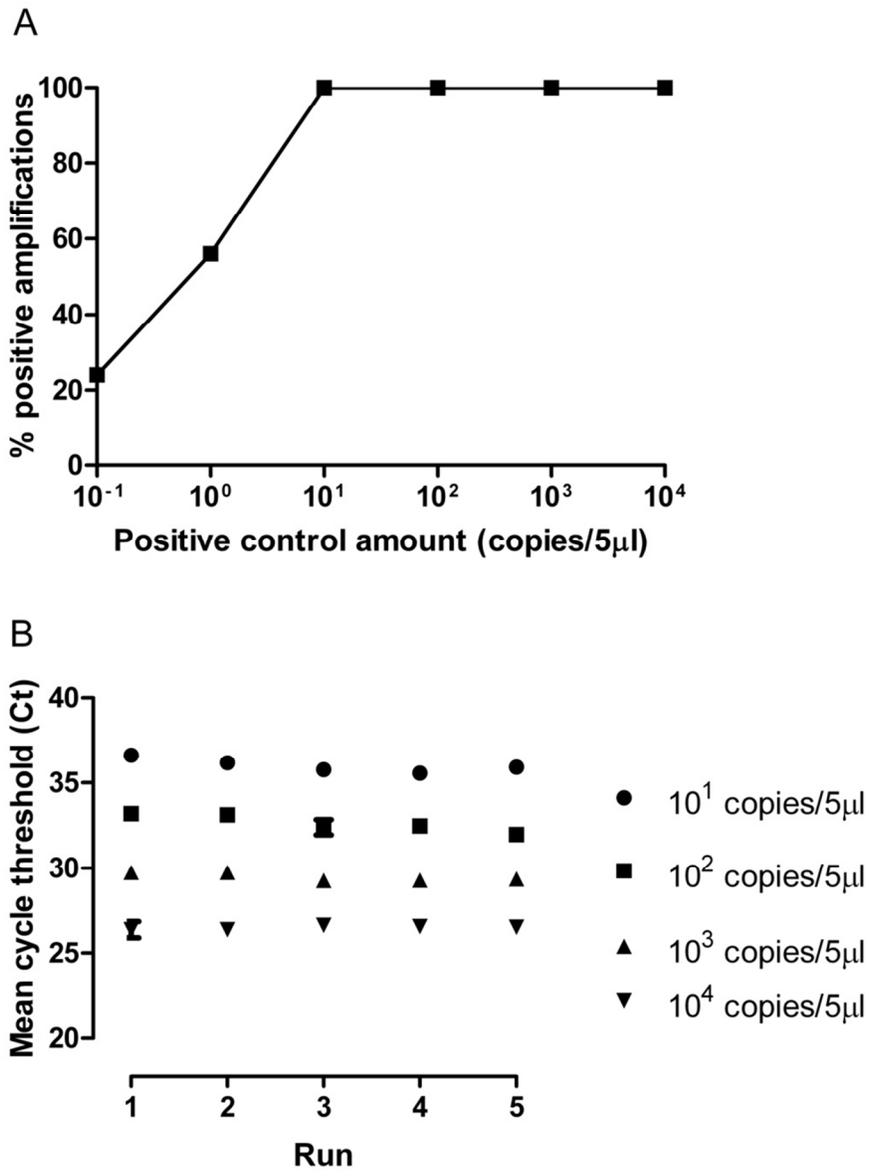
ELT: elevated liver enzymes. NA: not available. MSF: Mediterranean Spotted Fever. ATBF: African Tick-Bite Fever



Sensitivity and reproducibility of the pan-rickettsial real-time PCR (*R. conorii* positive control plasmid). A. Analytical sensitivity. B. Inter-run and intra-run reproducibility 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)

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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 reproducibility 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.
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