Molecular methods routinely used to detect Coxiella burnetii in ticks cross-react with Coxiella-like bacteria

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ORIGINAL RESEARCH ARTICLE

Molecular methods routinely used to detect *Coxiella burnetii* in ticks cross-react with *Coxiella*-like bacteria

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Background: Q fever is a widespread zoonotic disease caused by *Coxiella burnetii*. Ticks may act as vectors, and many epidemiological studies aim to assess *C. burnetii* prevalence in ticks. Because ticks may also be infected with *Coxiella*-like bacteria, screening tools that differentiate between *C. burnetii* and *Coxiella*-like bacteria are essential.
Methods: In this study, we screened tick specimens from 10 species (*Ornithodoros rostratus*, *O. peruvianus*, *O. capensis*, *Ixodes ricinus*, *Rhipicephalus annulatus*, *R. decoloratus*, *R. geigy*, *O. sonrai*, *O. occidentalis*, and *Amblyomma* cajennense) known to harbor specific *Coxiella*-like bacteria, by using quantitative PCR primers usually considered to be specific for *C. burnetii* and targeting, respectively, the *IS1111*, *icd*, *scvA*, *p1*, and *GroEL/htpB* genes.
Results: We found that some *Coxiella*-like bacteria, belonging to clades A and C, yield positive PCR results when screened with primers initially believed to be *C. burnetii*-specific.
Conclusions: These results suggest that PCR-based surveys that aim to detect *C. burnetii* in ticks by using currently available methods must be interpreted with caution if the amplified products cannot be sequenced. Future molecular methods that aim at detecting *C. burnetii* need to take into account the possibility that cross-reactions may exist with *Coxiella*-like bacteria.

Keywords: *Q fever*; *tick-borne diseases*; *tick endosymbiont*; *false positive*; *surveillance*; *PCR primers*

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Q fever is a worldwide zoonosis caused by *Coxiella burnetii*, a ubiquitous intracellular bacterium that infects humans and a variety of animals. Livestock, especially small ruminants, are the main sources of human infections (1–3). In domestic ruminants, Q fever's major clinical manifestations are abortions and stillbirths, whose occurrence may translate into significant economic losses (1–3). In humans, *C. burnetii* infections range from asymptomatic to severe. Acute forms of the disease may result in high fevers and severe pneumonia or hepatitis, and chronic forms are strongly debilitating and may be fatal when endocarditis develops in patients with underlying heart disease (3, 4). Both animals and humans essentially become infected through the inhalation of airborne particles contaminated with *C. burnetii* (2, 5).

Ticks are historically known to be potential vectors for Q fever. Indeed, the first strain of *C. burnetii* was isolated from a *Dermacentor andersoni* tick in the 1930s (6–8). First assigned to the genus *Rickettsia* (in which infection by arthropods is the rule), it was later recognized as the Q fever etiologic agent and is now considered as *C. burnetii* reference strain (9). Because *C. burnetii* is frequently detected in field-sampled ticks (10–13) and because laboratory experiments have revealed that at least some tick species are competent vectors (6, 14, 15), it is currently considered that ticks may act as vectors and help transmit the bacterium among wildlife and, on occasion, domestic ruminants (2, 16).

Interestingly, ticks also frequently carry *Coxiella*-like bacteria that are likely involved in mutualistic symbioses

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with their arthropod hosts (16–19). To date, there has been no indication that these tick-carried *Coxiella*-like bacteria are transmitted to vertebrates, although *Coxiella*-like bacteria have sporadically been detected in pet birds (19, 20). Recent investigations based on multilocus phylogenetic analyses and whole genome sequencing data revealed that all known *C. burnetii* strains originate within the vast group of *Coxiella*-like endosymbionts and are the descendants of a *Coxiella*-like progenitor hosted by ticks (17).

Because epidemiologic studies that aim at assessing *C. burnetii* prevalence in ticks frequently rely on DNA detection by polymerase chain reaction (PCR), it is important to make sure that these screening methods are specific for *C. burnetii*. The objective of this study was to determine whether five molecular methods frequently used to detect or characterize *C. burnetii* cross-react with *Coxiella*-like bacteria present in ticks.

Materials and methods

Selection of a panel of 20 ticks infected with specific Coxiella*-like bacteria*

Ten tick species, previously shown to harbor specific *Coxiella*-like bacteria (17), were investigated.

They were selected with the aim to represent the four clades (A–D) currently described for the *Coxiella* genus (17) (Fig. 1): clade A (*Ornithodoros rostratus*, *O. peruvianus*, *O. capensis*), clade B (*Ixodes ricinus*), clade C (*Rhipicephalus annulatus*, *R. decoloratus*, *R. geigyi*, *O. sonrai*, *O. occidentalis*), and clade D (*Amblyomma cajennense*). This panel included three tick species from which *C. burnetii* had previously been reported, namely *O. sonrai* (12), *I. ricinus* (21–23), and *R. annulatus* (24).

Two tick specimens were examined for each species. They were either obtained from breeding colonies or sampled from their host species or habitats and they were processed as previously described (17). Briefly, the ticks were first washed with sterile water to avoid external bacterial contamination. Then, DNA was individually extracted using the DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. DNA template quality was verified via PCR amplification of 18S ribosomal RNA or cytochrome oxidase 1 arthropod primers. Nested PCR assays were conducted using primers designed to amplify bacteria from the Coxiellacae family (i.e. *Coxiella* and its sister genus *Rickettsiella*) and to target the *rpoB* (DNA-directed RNA polymerase beta) gene and the *GroEL/htpB* (60 kDa chaperone heat shock protein B) gene as described elsewhere (17, 25). Sequencing of the PCR products obtained showed that each tick species was infected by a specific *Coxiella*-like bacterium that was genetically related to, but distinct from, *C. burnetii*. None of these tick DNA templates was found infected with *C. burnetii* on the basis of multilocus DNA sequencing (17).

Selection of qPCR primers thought to be specific for C. burnetii

The 20 tick specimens were tested using quantitative PCR (qPCR) methods using primers that are usually considered to be specific for *C. burnetii* (Table 1). We used TaqMan

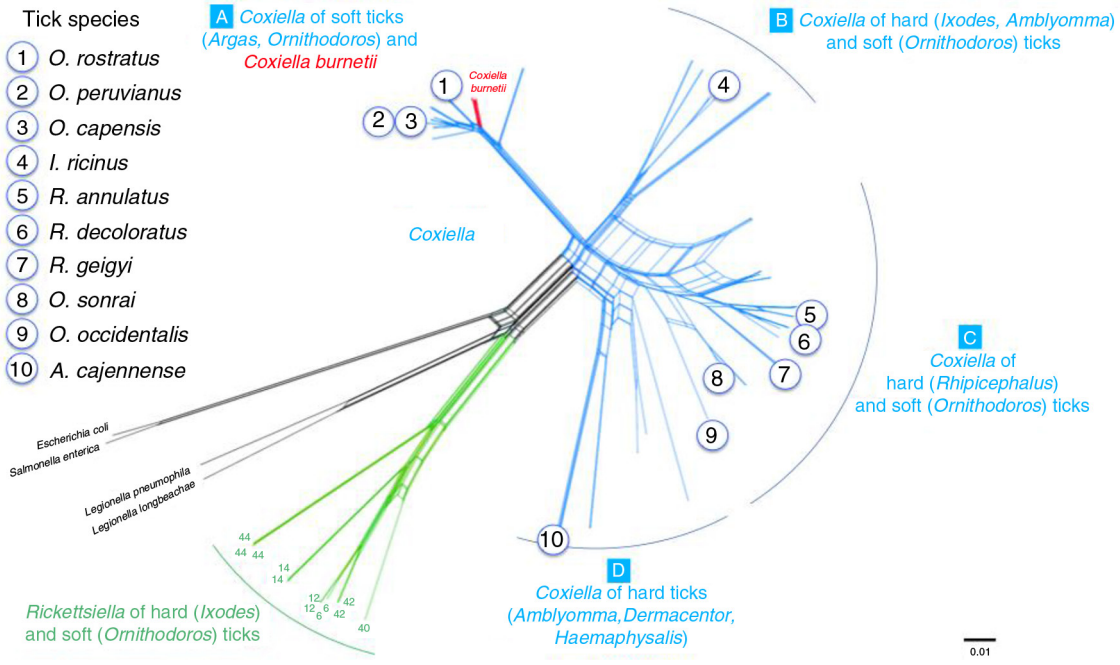

Fig. 1. Genetic relatedness of the 10 tick species used in this study using as reference the phylogenetic network published by Duron *et al.* (17) with concatenated *16S rRNA*, *23S rRNA*, *GroEL/htpB*, *rpoB*, and *dnaK* sequences for 71 tick-borne *Coxiella* strains, 15 *C. burnetii* reference strains, and several bacterial outgroups.

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Table 1. Details about the qPCR methods used in the study

Gene	Function	Primer designation	Primers and probe sequences (5′–3′)	Fragment length (bp)	Reference	% covering with the endosymbiont of *R. turanicus*[b]	% covering with the endosymbiont of *A. americanum*[c]
IS1111	Insertion sequence	Forward primer	Confidential[a]	76	(1)	58	63[d]
		Reverse primer	Confidential[a]			0	0
		Probe	Confidential[a]			0	63[d]
icd	Isocitrate dehydrogenase	Forward primer	GACCGACCCATTATTCCCT	139	(2)	84	0
		Reverse primer	CGGCGTAGATCTCCATCCA			0	0
		Probe	CGCCCGTCATGAAAAACGTGGTC			0	0
p1	Porine	Qp1-F	CGGCGATTGGCGTTTC	68	(3)	0	0
		Qp1-R	GGTTGCGGTAATGCCGTTAA			12[d]	0
		Probe	AACTGTTCAAAATCCGAAACGAGTCGCA			50[d]	0
scvA	Chromatin condensation	QscvA-F	TGGAAAGACAAAATGTCCAACAA	69	(3)	52[d]	0
		QscvA-R	GGTTAGAAGCACCCGGTCGT			0	0
		Probe	ACGTGGAAAAGACCAACG			67[d]	0
GroEL/htpB	Heat shock protein	HtpB-1	TGGCTCAAGCGATTTTGGTT	82	(4)	65[d]	0
		HtpB-2	TTATCAATACCCCGTTTCAAATCC			92[d]	0
		Probe	AAAGCCGTTATTGCTGGAATGAACCCC			70[d]	0

[a]The detailed protocol used for the amplification of *IS1111* will be soon published by Sidi-Boumedine *et al*. (in preparation) and remains meanwhile confidential; [b]GenBank accession number: CP011126; [c]GenBank accession number: CP007541; [d]sequence positions are distant from each other on the endosymbiont complete genome.

Universal PCR Master Mix (UMM 2 ×) following the amplification protocol: 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 40 PCR cycles of 95°C for 15 s and 60°C for 1 min. Two of the targeted markers – the multicopy *IS1111* insertion sequence (26, 27) and the *icd* (isocitrate dehydrogenase) housekeeping gene (26) – are frequently used in surveys that aim to estimate the prevalence of *C. burnetii* infection in ticks (16). The following genes were also targeted: *scvA* (small-cell-variant protein A), which is likely involved in chromatin condensation when the bacterium is 'sporulating', and *porine p1*, which encodes an outer membrane protein (28). Finally, we focused on a specific region of the *GroEL/htpB*, distinct from the region targeted to amplify the genome of *Coxiella*-like bacteria (17) and considered to be specific to *C. burnetii* (29). Nine Mile phase II genomic DNA (RSA 493 isolate) was used as a reference. *In silico* comparisons of the primers and probes with currently published sequences of *R. turanicus* (GenBank accession number: CP011126) and *A. americanum* (GenBank accession number: CP007541) suggested that mismatches with these symbionts were unlikely (Table 1).

Results

We found that some *Coxiella*-like bacteria, belonging to clades A and C, yield positive PCR results when screened with primers initially believed to be *C. burnetii*-specific (Table 2). Overall, DNA was amplified for at least one marker in 6 of the 10 tick species studied. The most frequently amplified marker was *IS1111*, which was detected in five different species, whereas *GroEL/htpB* and *scvA* were amplified from three species. *Porine p1* was solely amplified from a *R. geigyi* specimen, which was also positive for *htpB*, *scvA,* and *IS1111*, and displayed a particularly low C_t value ($C_t = 33$) for *IS1111*. Conversely, *icd* was not detected in any of our samples. Interestingly, we observed intraspecific variation: one of the *Coxiella*-like endosymbiont from *R. decoloratus* was positive for *IS1111*, whereas the other was positive for *scvA*. Unfortunately, because all PCR products were poorly concentrated, sequencing was unsuccessful.

Discussion

The marker we most frequently detected in ticks infected with *Coxiella*-like endosymbionts was the *IS1111* transposable element, which is routinely targeted during epidemiological surveys examining *C. burnetii* prevalence in ticks (16). We thus showed that *C. burnetii* detection assays based only on *IS1111* may lead to misidentification with *Coxiella*-like endosymbionts. The recent work of Duron (30) corroborates this finding: several genetically divergent *IS1111* copies were found widespread in many *Coxiella*-like endosymbionts, therefore showing that *IS1111* can no longer be considered specific to *C. burnetii*. These findings may explain why surveys based on *IS1111* screening occasionally report prevalence levels $>10\%$ (23, 31–33).

Our results also showed that the use of a combination of primers targeting different markers, as performed in some studies (11, 21, 34–36), is not sufficient to guarantee the specificity of *C. burnetii* detection. Indeed, up to four of our markers were detected in a same *Coxiella*-like endosymbiont. Interestingly, *icd*, which is frequently used as a PCR target in epidemiological studies (16, 34), was not amplified from our panel of *Coxiella*-like infected ticks. However, Reeves et al. (37) were able to amplify a 612-bp *icd* fragment, displaying 93% homology with *C. burnetii*, from a *Coxiella*-like bacterium that infects ticks from the *O. capensis* complex in South Carolina, USA. This result contrasts with our observation that *icd* was not amplified from the endosymbiont of *O. capensis* ticks sampled from Cape Verde and highlights the fact that the amplification of a specific genetic marker strongly depends on the PCR method (PCR, nested PCR, or qPCR) and the primer sequences used.

Table 2. C_t values obtained using qPCR for both specimens of the 10 tick species tested

Coxiella-like clade	Tick species	*IS1111*	*icd*	*GroEL/htpB*	*p1*	*scvA*
A	*O. rostratus*	–/–	–/–	–/–	–/–	–/–
	O. peruvianus	39/37	–/–	30/31	–/–	–/–
	O. capensis	–/–	–/–	35/35	–/–	–/38
B	*I. ricinus*	–/–	–/–	–/–	–/–	–/–
C	*R. annulatus*	37/38	–/–	–/–	–/–	–/–
	R. decoloratus	–/36	–/–	–/–	–/–	39/–
	R. geigyi	37/33	–/–	–/38	–/35	–/38
	O. sonrai	39/36	–/–	–/–	–/–	–/–
	O. occidentalis	–/–	–/–	–/–	–/–	–/–
D	*A. cajennense*	–/–	–/–	–/–	–/–	–/–

The sign '/' is used to separate the results obtained for the first and the second tick specimen; '–' indicates that no amplification was observed.

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More generally, negative results may be due to low detection sensitivity, which is supported by high detection thresholds for most of the genes. In particular, intraspecific variation may be due to the individual ticks having a low bacterial burden, sex- or stage-specific differences, and the presence of PCR inhibitors. In our study, it is possible that the *IS1111*-based PCR method was the most sensitive of the tests used, because several copies of this gene are likely present in the genome of *Coxiella*-like bacteria, as is the case for *C. burnetii*. This hypothesis is supported by the observation that *GroEL/htpB*, *porine p1*, and *scvA* were detected in the endosymbiont of a *R. geigyi* specimen that displayed a low C_t value for *IS1111*.

Standardizing methodology across laboratories is essential to allow comparisons among studies. Although remarkable progress has recently been made in designing new PCR-based techniques to detect *C. burnetii*, these advances have overlooked that an important genetic diversity actually exists within the *Coxiella* genus (16). In this context, it may not be surprising that the PCR primers routinely used to target *C. burnetii* actually cross-react with *Coxiella*-like bacteria. Therefore, in the future, molecular methods aiming at detecting *C. burnetii* should make sure that no cross-reaction exists not only with other abortive agents but also with *Coxiella*-like organisms. Recent full-genome sequencing data indeed not only highlighted obvious genetic similarities of *C. burnetii* with *Coxiella*-like bacteria but also revealed some mutations specific to *Coxiella*-like bacteria (18, 38). This pattern likely explains why PCR cross-reactions with *Coxiella*-like bacteria are partial and variable between markers. Interestingly, identical *IS1111* copies were found in *C. burnetii* and some *Coxiella*-like bacteria (30), suggesting that the risk of detecting *Coxiella*-like bacteria with *IS1111* primers designed to detect *C. burnetii* is very high and must not be underestimated.

PCR-based surveys that aim to detect *C. burnetii* in ticks must be interpreted with caution if the amplified DNA products are not sequenced. Unfortunately, the ratio of bacterial DNA to tick DNA is frequently low, which makes it challenging to obtain PCR products concentrated enough for direct sequencing via conventional PCR. Additionally, currently available qPCR methods, such as those used in this study, often yield very short DNA fragments that are difficult to concentrate for sequencing purposes and that correspond to rather uninformative sequences. Therefore, there is an urgent need to develop a multiplex qPCR or microchip method that would make it possible to directly differentiate *Coxiella*-like bacteria from *C. burnetii* in tick samples and to detect co-infections. Pending development of such a test, useful alternative methods include the sequencing of the *16S rRNA*, *rpoB*, and *GroEL* genes of *Coxiella* bacteria, after amplification by nested PCR, as previously described (17, 25).

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