Original article

Broad-range PCR-TTGE for the first-line detection of bacterial pathogen DNA in ticks

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Abstract – Ticks are known or suspected vectors for a wide range of bacterial pathogens. One of the first steps for tick-borne risk assessment is the detection of these pathogens in their vectors. In the present study, a broad-range PCR amplification of the eubacterial gene encoding the 16S rRNA gene combined with Temporal Temperature Gradient gel Electrophoresis (TTGE) was evaluated as a method allowing the one-step detection of bacterial pathogen DNA in ticks. Firstly, DNA extracts from bacteria known to be tick-borne pathogens, i.e., *Borrelia burgdorferi* lato sensu, *Anaplasma phagocytophilum,* Spotted Fever Group (SFG) *Rickettsia* spp., were used to establish a TTGE pathogen DNA reference marker. Secondly, we used broad-range PCR-TTGE to detect the presence of DNA from these three pathogens in 55 DNA extracts from pools of 10 nymphal *Ixodes ricinus* ticks, which have been previously shown to carry DNA from at least one of those bacteria by specific PCR. Among the 20 *B. burgdorferi* specific-PCR samples, 15 (75%) were also found to be positive using PCR-TTGE. Sixteen of the seventeen (94%) *Rickettsia* spp. PCR-specific samples were positive using PCR-TTGE detection and all PCR-specific positive extracts (11/11, 100%) for *A. phagocytophilum* were also positive using PCR-TTGE. Moreover, we identified unexpected bacterial sequences that were not related to any of the three pathogens such as a sequence related to *Spiroplasma* sp. Thus, broad-range PCR-TTGE allowed the single step detection of DNA from up to 3 pathogens in the same co-infected samples as well as detection of DNA from unexpected bacteria.

Ixodes ricinus / *Borrelia burgdorferi* **sensu lato** / *Anaplasma phagocytophilum* / *Rickettsia* **spp**. / **bacterial diversity**

1. INTRODUCTION

Many tick-borne bacteria are considered as emerging pathogens [16] such as *Borrelia burgdorferi* lato sensu (sl), the agent of Lyme borreliosis, the most significant human tick-borne disease in the Northern Hemisphere, and *Anaplasma phagocytophilum,* the agent of animal and human anaplasmosis and spotted fever group *Rickettsia* spp.

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(SFG) [4, 16, 20, 21]. A single tick may transmit multiple pathogens [1, 9, 16] but little is known about the co-infection frequency between bacteria infecting ticks [1, 16].

One of the first steps for assessing the risk for tick-borne diseases is the detection of pathogens in their vectors. PCR amplification of pathogen DNA using species-specific primers is now the standard for pathogen detection in ticks [16, 20]. However, PCR assays can be time-consuming, labor-intensive and expensive, particularly when testing for multiple pathogens in a large number of samples. Multiplex PCR assays are an alternative but their optimization is often difficult [6] and they have only been used for the detection of DNA for a maximum of two tick-borne pathogens [5].

Broad-range PCR, using primers that target highly-conserved regions of genes common to all bacteria, e.g. 16S ribosomal RNA (16S rDNA), allows the simultaneous amplification of DNA from all bacteria present in one sample in a single-step [22]. The different amplicons are then cloned and subsequently sequenced, or separated on the basis of their sequence. Temporal Temperature Gradient gel Electrophoresis (TTGE) is one technique that allows the sequence-specific separation on the basis of the GC content of amplicons by using the denaturing conditions of an increasing temperature [3, 23]. This technique, as well as the very similar DGGE (Denaturing Gradient Gel Electrophoresis), are commonly used to determine the bacterial profile of different biotopes such as soil and lakes [3, 15] and have also been successfully applied to study the microflora of *Ixodes ricinus* ticks [18]. Although they have been proposed to detect fish-borne pathogenic bacteria [10], these techniques have not yet been used for the detection of bacterial pathogens in ticks. Samples such as ticks generally carry several bacteria that can be pathogenic for humans and animals. Therefore, universal detection techniques result in multiple PCR products. Thus, the prerequisite for distinguishing pathogen PCR products within complex profiles is to build a pathogen reference marker.

Using a broad-range PCR-based technique combined with TTGE separation, we first set up a pathogen reference marker for the detection of three tick-borne bacteria DNA: *B. burgdorferi* sl, *A. phagocytophilum,* and *Rickettsia* spp. Secondly, we evaluated the feasibility of this technique to simultaneously detect DNA from the three bacteria in ticks. Finally, we identified the other broad-range PCR fragments not related to any of the three target bacterial pathogens.

2. MATERIALS AND METHODS

2.1. Bacterial genomic DNA

Genomic DNA from (i) *A. phagocytophilum* and *R. helvetica* extracted using the Genomic DNA Isolation Kit (Qiagen, Hilden, Germany); (ii) *B. garinii, B. burgdorferi* stricto sensu (ss) and *B. afzelii* provided by the Institut de Bactériologie of Strasbourg, France, and (iii) *Rickettsia conorii* obtained from Unité des Rickettsies, Marseille, France, were used to design a "pathogen DNA" reference marker.

2.2. Tick DNA extracts

Tick DNA extracts were obtained, as previously described [8], from 55 pools of 10 *Ixodes ricinus* nymphs collected by flagging vegetation in the Auvergne region (France). For all DNA extracts, previous specific-PCR data for *B. burgdorferi* sl, *A. phagocytophilum* and S.F.G. *Rickettsia* spp., were available. With specific-PCR, 20 pools were positive for *B. burgdorferi* sl only (amplification of a 357 bp fragment of 16S rDNA from *B. burgdorferi* sl [13]), 11 for *A. phagocytophilum* only (amplification of a 546 bp fragment of 16S rDNA from *A. phagocytophilum* [14]), and 17 for *Rickettsia* spp. only (amplification of a 381 bp fragment of citrate synthase gene from

Table I. Correlation between the positive specific-PCR and TTGE results for the detection of *Borrelia burgdorferi* sl, *Anaplasma phagocytophilum,* and SFG *Rickettsia* spp. in pools of ten *Ixodes ricinus* nymphs.

Pathogens detected by specific PCR in pools	Number of PCR-specific positive pools	Number of PCR-TTGE positive pools	Closest BLAST match obtained from TTGE band; accession number; (% similarity of the sequence)	Reference to figure
B. burgdorferi sl	20	15	*Borrelia burgdorferi* isolate St4; AY083501; (98%)	2A
A. phagocytophilum	11	11	*A. phagocytophilum*; AY281809; (99%)	2B
Rickettsia SFG spp.	17	16	*Rickettsia* SGF sp.; AY158006; (99%)	2C
B. burgdorferi sl and *A. phagocytophilum*	2	2	NS	2D Lanes 1 and 2
B. burgdorferi sl and *Rickettsia* SFG spp.	2	2	NS	2D Lanes 3 and 4
A. phagocytophilum and *Rickettsia* SFG spp.	2	2	NS	2D Lanes 5 and 6
B. burgdorferi sl, *A. phagocytophilum* and *Rickettsia* SFG spp.	1	1	NS	2D Lanes 7

NS: Not sequenced.

Rickettsia spp. [17]). Two pools were positive for both *A. phagocytophilum* and *B. burgdorferi* sl, two were positive for both *A. phagocytophilum* and *Rickettsia* spp., and two were positive for both *Rickettsia* spp. and *B. burgdorferi* sl. One pool was positive for the three pathogens, *B. burgdorferi* sl, *A. phagocytophilum* and *Rickettsia* spp. (Tab. I).

2.3. Polymerase chain reactions

A fragment of approximately 180-bp of eubacterial 16S rDNA was amplified with a broad-range eubacterial primer set 350f (5'-CTCCTACGGGAGGCAGCAGT-3') and PC535 (5'-GTATTACCGCGGCT-GCTGGCA-3') from all DNA extracts. For TTGE analyses, the 350f primer possessed an additional GC-clamp at the 5' extremity (5'-CGCCCGCCGCGCGCGGCGGGCG-GGGCGGGGGCCGGGGGG-350F-3'), which prevented strand dissociation at high temperature during electrophoresis [15]. Each 50-μL reaction contained 0.5 μmol/μL of each primer, 2.5 mM of each dNTP, 5 μL of 10X PCR buffer, 1 U of *Taq* DNA polymerase (Takara, Shiga, Japan) and 5-μL of the DNA extract. Cycling conditions were one denaturing cycle (8 min, 95 °C), followed by 30 cycles of denaturing (1 min, 94 °C), annealing (1 min, 52 °C) and extension (1 min, 72 °C) and a final extension step (10 min, 72 °C).

2.4. Temporal Temperature Gradient Gel Electrophoresis

For sequence-specific separation of PCR products, the TTGE DCode System (Bio-Rad, Marnes-la-Coquette, France) was used. Gel electrophoresis was performed for 16 h

in 0.5 × TE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH8), 7 M urea, with 10% acrylamide-bisacrylamide (37.5:1) gels at a constant voltage of 55 V and with a temperature gradient from 63 °C to 70 °C at a constant temperature increment of 0.4 °C/h. After electrophoresis, the gel was incubated using the sensitive SYBR green nucleic acid gel staining method (Amresco, Solon, USA) and DNA fragments were visualized under ultraviolet light.

2.5. Sequencing of TTGE fragments and sequence analyses

TTGE bands were excised and the DNA was eluted with 50 μL of Elution buffer EB (Qiagen) for 3 h at 55 °C before PCR amplification with the same eubacterial primer set except that the primer 350f was used without the GC clamp. The reaction conditions were similar to those described above. PCR products were sequenced (Qiagen). Sequences were compared with known sequences listed in the GenBank nucleotide sequence databases. The BLAST search option of the National Center for Biotechnology Information (NCBI) (internet site http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database.

3. RESULTS

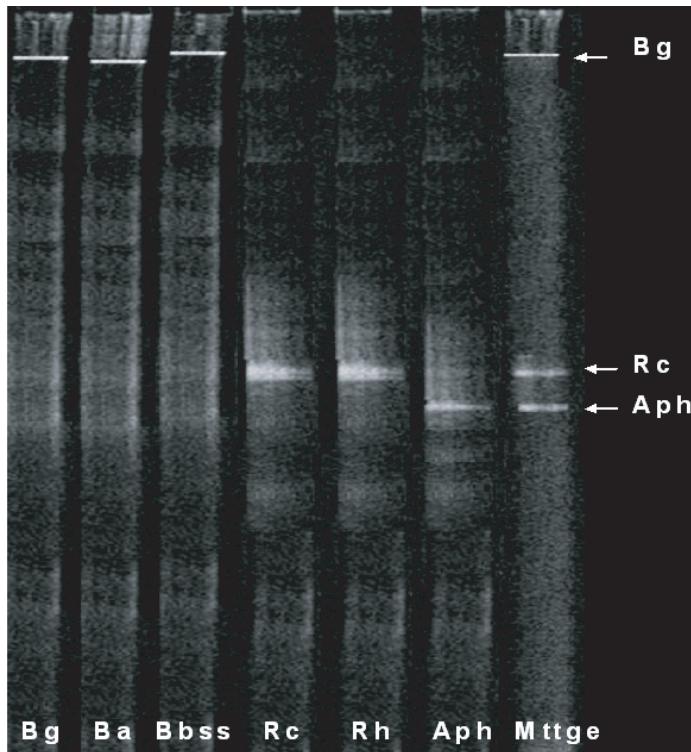### 3.1. Design of a reference pathogen DNA marker

Amplified fragments obtained from *B. burgdorferi* ss (198 bp; 45% GC), *B. afzelii* (197 bp; 45% GC), *B. garinii* (194 bp; 45% GC), *R. conorii* (170 bp; 52% GC), *R. helvetica* (170 bp; 52% GC), and *A. phagocytophilum* (173 bp; 54% GC), were electrophoresed using TTGE (Fig. 1). We defined a specific "front rate" (FR) for each fragment, as the distance of that fragment to the well, divided by the distance of the *A. phagocytophilum* fragment to the well.

The three *B. burgdorferi* sl species showed similar profiles characterized by unique fragments with a FR of 0.1 (Fig. 1, lanes Bg, Ba, Bbss). *R. conorii* and *R. helvetica* had unique fragments with identical migration profiles and a FR of 0.9 (Fig. 1, lanes Rc, Rh). *A. phagocytophilum* had a single fragment with a FR of 1 (Fig. 1, lane Aph). Migration profiles of three genera were always reproducible from one migration to another and distinct from each other. A reference pathogen DNA marker, named "Mttge", was then designed using *B. garinii*, *R. conorii,* and *A. phagocytophilum* 16S rDNA fragments (Fig. 1, lane Mttge). Mttge was used for the detection of DNA from pathogens in tick samples by profile comparison.

3.2. Validation of the use of broad range PCR-TTGE for the detection and co-detection of *B. burgdorferi* sl, *A. phagocytophilum* and *Rickettsia* spp. DNA in ticks

Broad-range PCR amplification of the 16S rDNA V3 region was carried out on the 55 tick DNA extracts and the resultant PCR fragments were separated by TTGE. The profiles were compared to Mttge in order to detect the presence of the three tick-borne pathogens in tick DNA samples (Fig. 2).

The results of broad-range PCR-TTGE were compared to those obtained by specific PCR detection. TTGE detection correlated with positive specific-PCR results in 15/20 (75%) DNA extracts for *B. burgdorferi* sl, 16/17 (94%) DNA extracts for *Rickettsia* spp. and all extracts (11/11; 100%) for *A. phagocytophilum* (Tab. I and Fig. 2A, 2B, 2C). All (7/7) samples positive with specific-PCR for 2 or 3 of the targeted pathogens showed the expected bands in their TTGE profile (Tab. I and Fig. 2D). Among the 55 samples, all found to be negative using specific-PCR for *B. burgdorferi*, *A. phagocytophilum* or *Rickettsia* spp. were also negative with broad-range PCR-TTGE. At least two bands were excised for

Figure 1. Design of the reference pathogen marker. SYBR-green stained TTGE fingerprints of 16S rDNA fragments of tick-borne bacterial reference strains. Bg: *Borrelia garinii*; Ba: *Borrelia afzelii*; Bbss: *Borrelia burgdorferi* ss; Rc: *Rickettsia conorii*; Rh: *Rickettsia helvetica*; Aph: *Anaplasma phagocytophilum*. Mttge represented the reference marker made by addition of (from top to bottom of gel) *Borrelia garinii, Rickettsia conorii*, and *Anaplasma phagocytophilum* 16S rDNA TTGE fingerprints.

each detected pathogen, and amplified as described above. PCR products were sequenced (Qiagen) and the sequences obtained were related to the expected pathogens (Tab. I).

3.3. Use of broad range PCR-TTGE for the detection of other bacterial DNA in ticks

All TTGE profiles obtained showed numerous fragments not related to any of the three target bacterial pathogens. We excised and sequenced these fragments for some of the samples (Tab. II). The sequences were related to arthropod symbionts such as *Spiroplasma* sp. (Fig. 2A; band a) and a Rickettsiales bacterium, IRicES1, considered as a symbiont of *I. ricinus* (Fig. 2A; band b). They were also related to environmental bacteria such as *Rhodococcus* sp. and *Mycobacterium* sp. and one sequence was related to uncultured and unknown eubacteria. One sequence was identified as 18S rDNA of *I. ricinus* (Fig. 2A; band c).

Sequences related to *Spiroplasma* sp., IRicES1 or *I. ricinus* 18S rDNA were each present in more than 40% of the samples. All other sequences were found in less than 10% of the samples.

Table II. GenBank accession number, classification and closest identified phylogenic relatives for the PCR fragments obtained after broad-range PCR-TTGE.

GenBank accession number	Denomination	Bacterial phylum, class, and order	Closest identified phylogenic relatives (origin)	Percent similarity
DQ065811	Uncultured *Borrelia* sp. sequence IRN34D1	Spirochaetes, Spirochaetales	*Borrelia burgdorferi* isolate St4 (*Ixodes ricinus* ticks)	97
DQ065810	Uncultured *Rickettsia* sp. sequence IRN11D2		SFG *Rickettsia* sp. (*Ixodidae* tick)	100
DQ065806	Uncultured *Anaplasma phagocytophilum* sequence IRB04Z1		*Anaplasma phagocytophilum* (clinical sample)	98
DQ065804	Uncultured *Rickettsiale* bacterium sequence IRRB9F1	Proteobacteria, Alphaproteobacteria, Rickettsiales	*Rickettsiales* bacterium *IricES* (*Ixodes ricinus* symbiont)	100
DQ065816	Uncultured *Rickettsiale* bacterium sequence P46Y1D2		*Rickettsiales* bacterium (endosymbiont of *Acanthamoeba* UWC8)	97
DQ065808	Uncultured *Wolbachieae* bacterium sequence IRB15Y1D1		*Wolbachia* sp. (*Xenopsylla cheopis* symbiont)	100
DQ074440	Uncultured *Anaplasmataceae* bacterium sequence P87Y4D1		*Ehrlichia*-like sp. "Schotti variant" (*Ixodes ricinus* ticks)	98
DQ065817	Uncultured *Enterobacteriale* bacterium sequence P02Y2D3	Enterobacteriales	*Escherichia* sp. (gut cells)	100
DQ065819	Uncultured *Ixodes ricinus* tick associated bacterium sequence IRN4D5	Proteobacteria, Gammaproteo bacteria	*Legionellales* bacterium (environment)	95
DQ065805	Uncultured *Coxiellaceae* bacterium sequence IRB04Y3D2	Legionellales	*Coxiellaceae* bacterium (*Folsomia candida* symbiont)	94
DQ065809	Uncultured *Spiroplasma* sp. sequence IRN29D1	(Low GC gram-positive bacteria) Firmicutes, Mollicutes, Entomoplasmatales	*Spiroplasma* sp. (*Antonina crawii* symbiont)	100
DQ065807	Uncultured *Mycobacterium* sp. sequence IRB04Z1D3	(High GC gram-positive bacteria) Actinobacteria, Actinomycetales	*Mycobacterium* sp. (environment)	100
DQ065813	Uncultured *Rhodococcus* sp. sequence IRP28Y5D2		*Rhodococcus* sp. (environement, soil)	97
DQ065815	Uncultured eubacterial sequence IRP28Y5	Eubacteria	No taxon with similar sequence	–
		Ixodes ricinus 18S rRNA gene		100
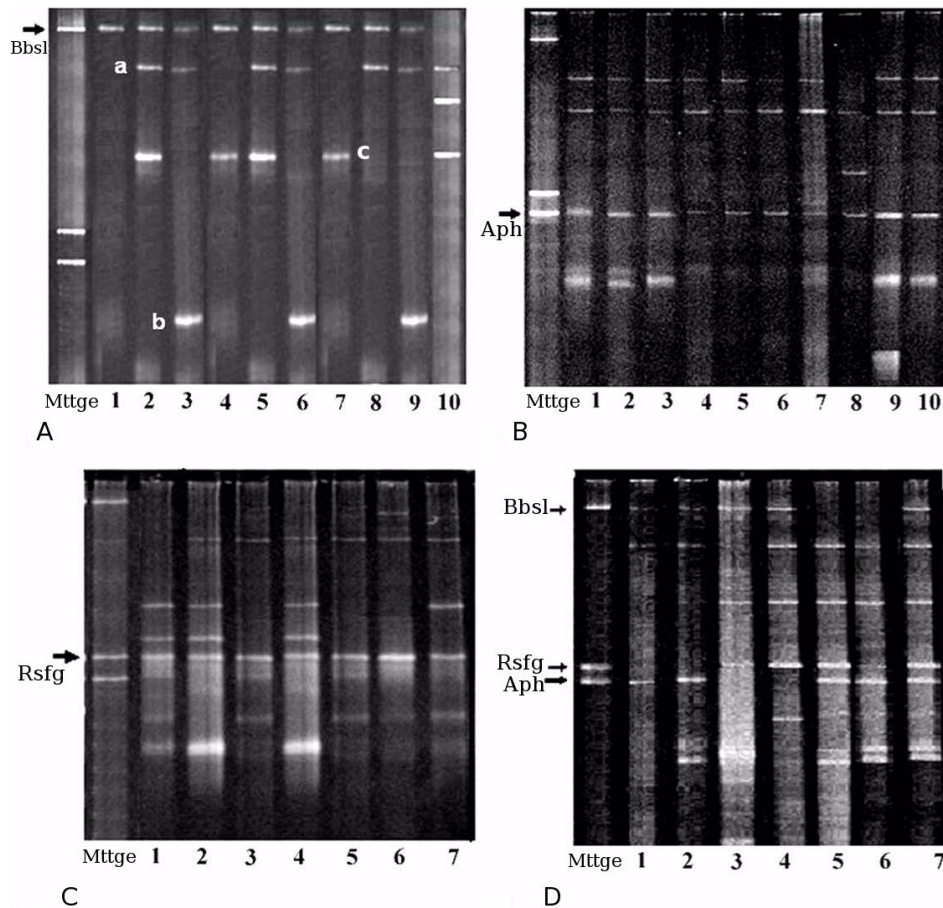
Figure 2. Use of broad-range PCR-TTGE for detection of DNA from bacterial tick-borne pathogens in extracts from pooled *I. ricinus* nymphs. (A) 16S rDNA TTGE fingerprints of 10 pools with a positive PCR for *B. burgdorferi* sl (Bbsl). (B) 16S rDNA TTGE fingerprints of 10 pools with a positive PCR for *A. phagocytophilum* (Aph). (C) 16S rDNA TTGE fingerprints of 7 pools with a positive PCR for SFG *Rickettsia* spp. (Rsfg). (D) Lanes 1 and 2: 16S rDNA TTGE fingerprints two pools PCR positive for both *B. burgdorferi* sl and *A. phagocytophilum*; Lanes 3 and 4: 16S rDNA TTGE fingerprints of two pools PCR positive for both *B. burgdorferi* sl and *Rickettsia* spp.; Lanes 5 and 6: 16S rDNA TTGE fingerprints of two pools PCR positive for both *A. phagocytophilum* and Rickettsia spp. Lane 7: 16S rDNA TTGE fingerprints of one pool PCR positive for *B. burgdorferi* sl *A. phagocytophilum* and *Rickettsia* spp. "Mttge" represents the reference marker. a: Band excised and sequenced corresponding to *Spiroplasma* sp. b: Band excised and sequenced corresponding to the Rickettsiales bacterium IRicES1. c: Band excised and sequenced corresponding to *I. ricinus* 18S rDNA.

4. DISCUSSION

Broad-range PCR-TTGE was proven to be adapted for the detection of tick-borne bacterial DNA whether belonging to pathogens or not. It is therefore of great interest for global tick-borne risk assessment as a first-line detection tool for the screening of tick populations.

We observed consistent results between the specific-PCR and broad-range PCR-TTGE

for *A. phagocytophilum* and *Rickettsia* spp.
(Tab. I). For *Borrelia burgdorferi* sl, all
negative samples using specific-PCR were
also found to be negative using PCR-
TTGE, while 5/20 PCR specific positive
samples were not detected as positive using
PCR-TTGE. These results were not improved
by modifying the TTGE migration parame-
ters (voltage, polyacrylamide concentration,
temperature variation). This low sensitivity
could be due to the qualitative and limiting
measure of visually detecting PCR bands of
low intensity within complex profiles. An
automated standardized reading process
would improve sensitivity and accuracy.
Moreover, preferential amplification of 16S
rDNA of some bacterial taxons [18] could
also explain these biases. Indeed, the
number of *B. burgdorferi* sl is known to be
low in questing nymphs [19]. In addition,
the genome of *B. burgdorferi* sl contains
one single copy of the 16S rDNA gene [12]
while reaching up to 15 copies in some bac-
teria [11]. An alternative for increasing the
sensitivity for *B. burgdorferi* sl detection
would be to target another pertinent gene.
This entails an important setting up process
with the risk of losing the sensitivity for
other target bacterial pathogens.

Interestingly, broad range PCR-TTGE
detection can result in the identification of
untargeted bacterial DNA that could poten-
tially belong to pathogenic agents. In our
samples, we found sequences related to
known tick-associated bacteria, such as a
Rickettsiales bacterium, IRicES1, consid-
ered as a symbiont of *I. ricinus* [2], but also
sequences related to bacterial symbiont of
arthropods, such as *Spiroplasma* sp. [7], not
associated with ticks to date. We also found
sequences that have already been described
in ticks such as the sequences related to
environmental bacteria such as *Rhodococ-
cus* sp. or arthropod symbiotic bacteria
(*Coxiellaceae*) [18]. One sequence was
identified as 18S rDNA of *I. ricinus*. This
artefactual amplification has also been
shown in a previous similar study and is
supposed to be related to the complete
annealing of the reverse primer to the *I. rici-*
nus 18S rDNA [18]. Nevertheless, this
aspecific amplification did not limit ampli-
fication of bacterial DNA.

Broad-range PCR-TTGE allowed co-
detection of 2 or 3 pathogens in one pool
and could effectively be applied to detect
several pathogens in a single tick. Thus, it
offers a powerful alternative for co-infec-
tion studies. When precise identification of
a pathogen is required, subsequent steps
could include specific PCR analyses of
samples of interest. It could also allow the
study of the relationships between tick-
infecting bacteria.

In conclusion, broad-range PCR-TTGE
offers new opportunities for the first line
detection of bacterial pathogens in ticks in
the context of their natural ecology.

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