Vet. Res. 37 (2006) 245–253

DOI: 10.1051/vetres:2005055

Original article

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

Lénaïg HALOS[a], Maria MAVRIS[a], Gwenaël VOURC'H[b], Renaud MAILLARD[c], Jacques BARNOUIN[b], Henri-Jean BOULOUIS[a], Muriel VAYSSIER-TAUSSAT[a]*

[a] UMR 956 INRA/AFSSA/ENVA/UPVM, Microbiologie, École Nationale Vétérinaire d'Alfort, 7 avenue du Général de Gaulle, 94700 Maisons-Alfort, France
[b] Unité d'Épidémiologie Animale, INRA, 63122 Saint-Genès-Champanelle, France
[c] Unité de Pathologie du Bétail, École Nationale Vétérinaire d'Alfort, Maisons-Alfort, France

(Received 8 July 2005; accepted 10 November 2005)

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.

* Corresponding author: mvayssier@vet-alfort.fr

(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

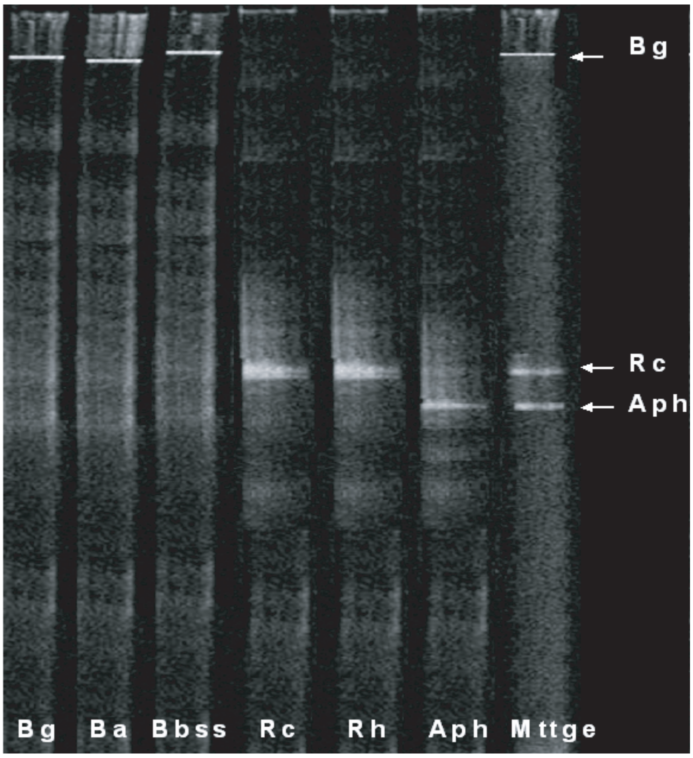

Figure 1. Design of the reference pathogen marker. SYBR-green stained TTGE fingerprints of 16S rDNA fragments of tick-borne bacterial reference strains. Bg: *Borrelia garinii*; Ba: *Borrelia afzelii*; Bbss: *Borrelia burgdorferi* ss; Rc: *Rickettsia conorii*; Rh: *Rickettsia helvetica*; Aph: *Anaplasma 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	Proteobacteria , Alphaproteobacteria, Rickettsiales	SFG *Rickettsia* sp. (*Ixodidae* tick)	100
DQ065806	Uncultured *Anaplasma phagocytophilum* sequence IRB04Z1		Anaplasma phagocytophilum (clinical sample)	98
DQ065804	Uncultured *Rickettsiale* bacterium sequence IRRB9F1		*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	Proteobacteria, Gammaproteo bacteria — Enterobacteriales	*Escherichia* sp. (gut cells)	100
DQ065819	Uncultured *Ixodes ricinus* tick associated bacterium sequence IRN4D5	Proteobacteria, Gammaproteo bacteria — Legionellales	*Legionellales* bacterium (environment)	95
DQ065805	Uncultured *Coxiellaceae* bacterium sequence IRB04Y3D2		*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

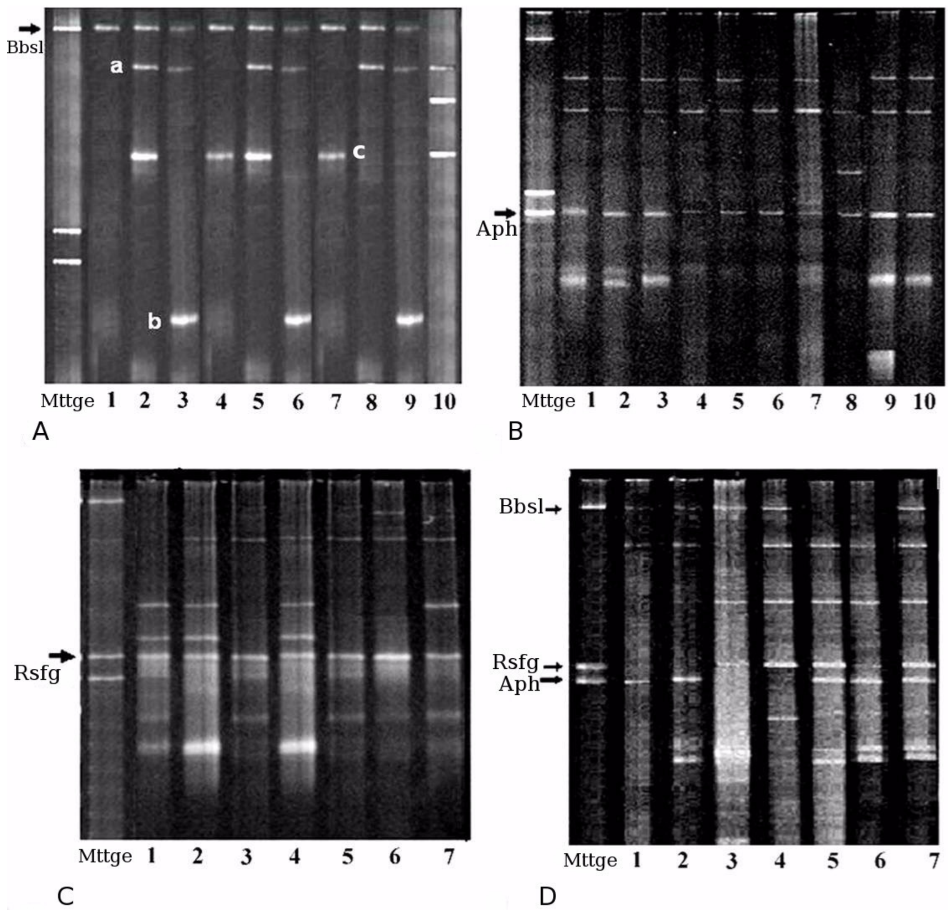

Figure 2. Use of broad-range PCR-TTGE for detection of DNA from bacterial tick-borne pathogens in extracts from pooled *I. ricinus* nymphs. (A) 16S rDNA TTGE fingerprints of 10 pools with a positive PCR for *B. burgdorferi* sl (Bbsl). (B) 16S rDNA TTGE fingerprints of 10 pools with a positive PCR for *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 parameters (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 bacteria [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 potentially belong to pathogenic agents. In our samples, we found sequences related to known tick-associated bacteria, such as a Rickettsiales bacterium, IRicES1, considered 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 *Rhodococcus* 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. ricinus* 18S rDNA [18]. Nevertheless, this aspecific amplification did not limit amplification 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-infection 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.

ACKNOWLEDGEMENTS

We thank students, Mathieu Soulage, Delphine Filiputti and Franck Bonnamy for their excellent technical assistance, Dr Benoit Jauhlac (IBS, Strasbourg, France) and Dr Jean Marc Rolain (Unité des rickettsies, Marseille, France) for having kindly provided bacterial reference strain DNA. This work was funded as part of the Transzoonose project of the National Institute for Agricultural Research (INRA).

REFERENCES

[1] Belongia E.A., Epidemiology and impact of coinfections acquired from *Ixodes ticks*, Vector Borne Zoonotic Dis. 2 (2002) 265–273.

[2] Beninati T., Lo N., Sacchi L., Genchi C., Noda H., Bandi C., A novel alpha-Proteobacterium resides in the mitochondria of ovarian cells of the tick *Ixodes ricinus*, Appl. Environ. Microbiol. 70 (2004) 2596–2602.

[3] Bosshard P.P., Santini Y., Gruter D., Stettler R., Bachofen R., Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis, FEMS Microbiol. Ecol. 31 (2000) 173–182.

[4] Comer J.A., Paddock C.D., Childs J.E., Urban zoonoses caused by *Bartonella, Coxiella, Ehrlichia*, and *Rickettsia* species, Vector Borne Zoonotic Dis. 1 (2001) 91–118.

[5] Courtney J.W., Kostelnik L.M., Zeidner N.S., Massung R.F., Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi,* J. Clin. Microbiol. 42 (2004) 3164–3168.

[6] Elnifro E.M., Ashshi A.M., Cooper R.J., Klapper P.E., Multiplex PCR: optimization and application in diagnostic virology, Clin. Microbiol. Rev. 13 (2000) 559–570.

[7] Fukatsu T., Nikoh N., Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera), Appl. Environ. Microbiol. 66 (2000) 643–650.

[8] Halos L., Jamal T., Vial L., Maillard R., Suau A., Le Menach A., Boulouis H.J., Vayssier-Taussat M., Determination of an efficient and reliable method for DNA extraction from ticks, Vet. Res. 35 (2004) 709–713.

[9] Jenkins A., Kristiansen B.E., Allum A.G., Aakre R.K., Strand L., Kleveland E.J., Van de Pol I., Schouls L., *Borrelia burgdorferi* sensu lato and *Ehrlichia* spp. in *Ixodes* ticks from southern Norway, J. Clin. Microbiol. 39 (2001) 3666–3671.

[10] Ji N., Peng B., Wang G., Wang S., Peng X., Universal primer PCR with DGGE for rapid detection of bacterial pathogens, J. Microbiol. Methods 57 (2004) 409–413.

[11] Klappenbach J.A., Saxman P.R., Cole J.R., Schmidt T.M., rrndb: the ribosomal RNA operon copy number database, Nucleic Acids Res. 29 (2001) 181–184.

[12] Liveris D., Gazumyan A., Schwartz I., Molecular typing of *Borrelia burgdorferi* sensu lato by PCR-restriction fragment length polymorphism analysis, J. Clin. Microbiol. 33 (1995) 589–595.

[13] Marconi R.T., Garon C.F., Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis, J. Clin. Microbiol. 30 (1992) 2830–2834.

[14] Massung R.F., Slater K., Owens J.H., Nicholson W.L., Mather T.N., Solberg V.B., Olson J.G., Nested PCR assay for detection of granulocytic ehrlichiae, J. Clin. Microbiol. 36 (1998) 1090–1095.

[15] Muyzer G., DDGE/TGGE a method for identifying genes from natural ecosystems, Curr. Opin. Microbiol. 3 (1999) 317–322.

[16] Parola P., Raoult D., Ticks and tickborne bacterial diseases in humans: an emerging infectious threat, Clin. Infect. Dis. 32 (2001) 897–928.

[17] Regnery R.L., Spruill C.L., Plikaytis B.D., Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes, J. Bacteriol. 173 (1991) 1576–1589.

[18] Schabereiter-Gurtner C., Lubitz W., Rolleke S., Application of broad-range 16S rRNA PCR amplification and DGGE fingerprinting for detection of tick-infecting bacteria, J. Microbiol. Methods 52 (2003) 251–260.

[19] Schwan T.G., Piesman J., Vector interactions and molecular adaptation of Lyme disease and relapsing fever spirochetes associated with transmission by ticks, Emerg. Infect. Dis. 8 (2002) 115–121.

[20] Sparagano O.A., Allsopp M.T., Mank R.A., Rijpkema S.G., Figueroa J.V., Jongejan F., Molecular detection of pathogen DNA in ticks (Acari: Ixodidae): a review, Exp. Appl. Acarol. 23 (1999) 929–960.

[21] Stanek G., Steere F., Lyme Borreliosis, Lancet 362 (2003) 1639–1647.

[22] Wilson K.H., Blitchington R.B., Greene R.C., Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction, J. Clin. Microbiol. 28 (1990) 1942–1946.

[23] Yoshino K., Nishigaki K., Husimi Y., Temperature sweep gel electrophoresis: a simple method to detect point mutations, Nucleic Acids Res. 19 (1991) 3153.