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Design and analytical validation of a duplex PCR for *Ehrlichia and Rickettsia* detection in ticks[¤]

Diseño y validación analítica de una PCR dúplex para la detección de <u>Ehrlichia</u> y <u>Rickettsia</u> en garrapatas.

Projeto e validação analítica de uma PCR duplex para detecção de <u>Ehrlichia</u> e <u>Rickettsia</u> em carrapatos

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Abstract

Background: *Ehrlichia* and *Rickettsia* are two major rickettsial genera transmitted by ticks that affect a number of wild and domestic animal species and human populations around the world. **Objective**: To design and validate a duplex PCR for *Ehrlichia* and *Rickettsia* in ticks. **Methods:** Assay validation included testing for sensitivity, specificity, reproducibility, and robustness of the PCR. The groEL and 23sr RNA genes were used for *Ehrlichia* and *Rickettsia*, respectively. **Results:** The limit of detection was one hundred gene copies per 50 μL of reaction for *Ehrlichia* spp, and one gene copy of *Rickettsia* per 50 μL of reaction. In general, the primers of the test only amplified in silico those bacterial agents for which they were originally designed, with the exception of the primers for *Rickettsia* that also amplified *Methylocystis* sp. The test was reproducible (intermediate precision) 96.7% of the times for both agents. The test was robust enough to tolerate concentration changes of all reagents with the exception of Taq DNA polymerase. **Conclusions:** The validation results indicated that this PCR is useful for detection in both bacterial genera and it is a good candidate for diagnostic validation.

Key words: *exclusiveness, groEL, inclusiveness, robustness, rRNA 23S.*

Resumen

Antecedentes: *Ehrlichia* spp. y *Rickettsia* spp. son dos de los principales géneros rickettsiales transmitidos por garrapatas que afectan a animales silvestres, domésticos y humanos alrededor del mundo. **Objetivo:**

Diseñar y validar una prueba PCR dúplex para *Ehrlichia* y *Rickettsia* en garrapatas. **Métodos:** La validación de la prueba incluyó ensayos de sensibilidad, especificidad, reproducibilidad y robustez. En la PCR se usó groEL y ARNr 23S como genes blanco para *Ehrlichia* y *Rickettsia,* respectivamente. **Resultados:** El límite de detección fue de 100 copias del gen por 50 µL de reacción para *Ehrlichia* spp y una copia del gen de *Rickettsia* por 50 µL de reacción. En general, los cebadores de la prueba solo amplificaron in silico los agentes bacterianos para los cuales fueron originalmente diseñados, con la excepción de los cebadores de *Rickettsia* que también amplificaron *Methylocystis* sp. La prueba fue reproducible (precisión intermedia) en un 96.7% de las veces para ambos agentes. La prueba fue suficientemente robusta como para tolerar cambios de concentración de los diferentes reactivos, con excepción de la Taq DNA polimerasa. **Conclusión:** Los resultados de validación indican que la PCR es útil para detectar ambos géneros bacterianos y podria usarse para validación diagnostica.

Palabras clave: ARNr 23S, *exclusividad, groEL, inclusividad, robustez.*

Resumen

Antecedentes*: Ehrlichia* e *Rickettsia* são dois dos principais gêneros de rickettsias transmitidos por carrapatos que infectam tanto animais selvagens quanto animais domésticos e até homens em todo o mundo. **Objetivo:** O objetivo principal foi elaborar e validar uma PCR duplex para *Ehrlichia* e *Rickettsia* em carrapatos. **Métodos:** A validação incluiu testes de sensibilidade, especificidade, reprodução e robustez. Para o PCR, utilizamos os genes groEl e 23Sr-RNA para *Ehrlichia* e *Rickettsia*, respectivamente. **Resultados**: O limite de detecção foi de 100 cópias de genes por 50 ml de reação para *Erliquia* spp e uma cópia de gene de *Rickettsia* por 50 ml de reação. Em geral, os iniciadores dos testes amplificaram em modelos computacionais os agentes bacterianos para os quais eles foram projetados, exceto os primers de *Rickettsia* que também amplificou Methylocystis sp. Os testes foram reproduzíveis (precisão intermediária) 96,7% para ambos os agentes e foram também robustos para tolerar mudanças de concentração em todos os reagentes, exceto o reagente Taq DNA polymerase. **Conclusões:** Os resultados da validação indicaram que o PCR é útil para detecção em ambos os gêneros bacterianos, portanto, um bom exame para validação diagnóstica.

Palavras-chave: *exclusividade, groEL, inclusividade, robustez, rRNA 23S.*

Introduction

Ticks are obligate ectoparasites of many vertebrate classes around the world. After mosquitoes, ticks are considered the second most important vector for human diseases (Pfäffle *et al*., 2013). In spite of the broad diversity of tick species, the majority of them belong to the genera *Amblyomma*, *Dermacentor, Haemaphysalis*, *Hyalomma*, *Ixodes,* and *Rhipicephalus*, in the Ixodidae family (Socolovschi *et al*., 2009).

Many agents transmitted by ticks are considered zoonotic. Among them it is worth mentioning the alpha-proteobacterial agents *Ehrlichia* and *Rickettsia* (Dantas-Torres *et al*., 2012; Moriello, 2007). Both are intracellular obligate gram-negative bacteria and affect a great variety of vertebrate hosts, including humans and dogs (Mansueto *et al*., 2012; Rar and Golovljova, 2011).

The *Rickettsia* genus are divided into four major groups, known as Spotted Fever (SFG), Typhus (TG), Ancestral (AG), and Transitional group (TranG)

(Quintero Vélez *et al*., 2012; Mansueto *et al.*, 2012). On the other hand, the *Ehrlichia* genus includes *E. chaffeensis*, *E. canis*, *E. ewingii*, *E. ruminantium*, *E. minasensis,* and *E. muris* (Rar and Golovljova, 2011, Cabezas-cruz *et al*., 2016). Rocky Mountain Spotted Fever (RMSF), caused by *Rickettsia rickettsii,* remains as the most deadly rickettsial human disease (Hidalgo *et al.* 2007, Quintero Vélez *et al*., 2012), while Canine Ehrlichioses (CE) caused by *Ehrlichia canis* is the most important and widespread rickettsial disease among dogs (Moriello et al, 2007).

The PCR-based methods are the best choice for detecting both rickettsial agents in ticks due to its high sensitivity and specificity (Oteo *et al.,* 2014). However, *Ehrlichia* and *Rickettsia* detection in infected ticks remains as the most challenging task in ecological studies of tick-borne agents. Some genes, such as 16S ribosomal RNA, *dsb*, *p30* and *p28,* are commonly used for molecular detection of *Ehrlichia* (Doyle *et al.*, 2005), while *gltA, ompA* and *ompB* genes are used for detection of *Rickettsia* (Oteo *et al.*, 2014; Quintero Vélez *et al.*, 2012). The identification

of rickettsial agents is a difficult process that usually requires sequencing and phylogenetic analysis of one or several bacterial genes.

Despite *Ehrlichia* and *Rickettsia* bacteriae co-infect the same ticks (Bowman, 2011; Dantas-Torres *et al.*, 2012; Dumler and Walker, 2001; Rar and Golovljova, 2011) and the numerous PCR strategies available for detection of rickettsial agents in ticks, there are no reports of PCR methodologies for simultaneously detecting both *Rickettsia* and *Ehrlichia* in ticks. Based on the aforementioned arguments, the aim of this work was to develop and validate a duplex PCR for detection of *Ehrlichia* and *Rickettsia* in DNA extracted from ticks.

Materials and methods

Rickettsial genes and oligonucleotides design

We retrieved sequences of *Ehrlichia* groESL gene and *Rickettsia* 23S ribosomal gene from genetic databases (GenBank/EMBL/DDBJ) and aligned the DNA sequences using the ClustalW algorithm (Thompson *et al.*, 1994) to identify conserved regions for primer design. The Primer-BLAST tool of NCBI (fork of Primer3 software) was used for the design and preliminary evaluation of target-specificity of primers (Ye *et al.*, 2012.). We calculated oligonucleotide properties such as dimerization potential, hairpin formation and Tm in PerlPrimer 1.1.21 software (Marshall, 2004). Primers selected for *Ehrlichia* were Ehr-GroELF 5´ CAATAGCAAGAGCCAATG 3´ and Ehr-GroELR 5´ TTAGAAGATGCTGTAGGATG 3´ and amplified to a size of 145 bp and *Rickettsia* was Rick-23SF 5´ CAGACTTACCAAACTCAATC 3´ and Rick-23SR 5´ TACGCAAGAACCCTTGGA 3´ amplified to 437 bp.

Ticks naturally infected with Ehrlichia and Rickettsia

As positive controls for *Ehrlichia-Rickettsia* duplex PCR assays, we isolated DNA from ticks positive for *Ehrlichia canis dsb* gene by PCR, and other ticks positive to *gltA* and *ompB* genes of *Rickettsia* sp. Atlantic Rainforest, these ticks were stored at the laboratory of the Centauro research group (Universidad de Antioquia). DNA of ticks

was extracted from individual complete ticks using a QIAGEN DNeasy Blood and Tissue kit according to manufacturer instructions, and it was quantified by nanodrop. DNase and RNases free water was used as a negative control and Genomic DNA of *R. rhipicephali, R. amblyommatis, R. parkeri* and *R. bellii,* kindly provided by Dr. Marcelo B. Labruna from University of São Paulo (Brazil), were also used as positive controls.

Ehrlichia and Rickettsia duplex PCR (ER-dPCR)

PCR reactions were carried out in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA, USA) in a PCR volume of 50µL with a PCR cocktail mixture as shown in Table 1. Amplification conditions were 95 °C for 1 min, followed of 40 cycles to 95 °C/30 s, 56 °C/30 s, 72 °C/30 s and a final extension step 72 °C/7 min. Electrophoresis was performed in 2% Agarose gel in TAE 1X using 0,25 µL of GelRed (Biotium) for each 10 mL of gel. Electrophoresis was run with 100 Volts during 45 minutes. GeneRuler 100 bp Plus (Thermo Scientific) was used as molecular weight marker. Photodocumentation of gels was performed with a Doc™ XR+System instrument (Bio-Rad).

Table 1. PCR cocktail for ER-dPCR.

Reagent	[Initial]	[Final]	Volume (µL)
Nuclease free water	NA	NA	30.75
PCR Buffer *	10 X	1 X	5
dNTPs**	10 mM	0.2 mM	1
Ehr-groELF ***	10 µM	0.2 µM	1
Ehr-groELR ***	10 µM	0.2 µM	1
Rick-23SF ***	10 µM	0.2 µM	1
Rick-23SR***	10 µM	0.2 µM	1
MgCl$_2$ *	25 mM	3 mM	6
Taq DNA polymerase *	5 U/µL	1.25 U	0.25
Tick DNA	100 ng/µL	300 ng	3

* Thermo Scientific; ** Invitrogen; *** Bioneer.

Detection threshold of ER-dPCR (sensitivity)

groESL amplicon of Ehrlichia canis and 23S amplicon of Rickettsia sp. Atlantic Rainforest was inserted into pDrive Cloning Vector (QIAgen). Concentration of the plasmids was adjusted to 10^8

plasmids or gene copies/µL in ten-fold serial dilutions to obtain a single copy of each gene/µL. Three replicas per dilution were tested in each PCR to determine the minimum number of copies of each rickettsial gene amplifiable by a positive PCR.

Robustness assay for ER-dPCR

The performance of the duplex PCR was assayed under three stepwise concentrations of $MgCl_2$ (2.5 mM; 3.0 mM; 3.5 mM), dNTPs (0.1 mM; 0.2 mM; 0.3 mM), Taq DNA polymerase (1.0 U; 1.25 U; 1.5 U), oligonucleotide concentration (0.2 µM; 0.4 µM; 0.6 µM), variation in temperature alignment (54, 56, 58°C), and we also tested three different brands of Taq DNA Polymerase (Thermo Scientific, Invitrogen and Bioline). Three replicas for each reagent concentration were tested in all experiments.

Repeatability and reproducibility for ER-dPCR

Repeatability was assayed by testing the same DNA sample (tick DNA with three replicas) in the ER-dPCR by the same person, along five consecutive days in the same PCR machine. The same analyst performed all PCR in the same thermocycler. Reproducibility (intermediate precision) of the ER-dPCR was tested by making the same experiments of the repeatability assay, but performed by a different person. The results of successful ER-dPCR were expressed as percentages in both assays.

Inclusiveness and exclusiveness assay of ER-dPCR

The Primer-BLAST and In Silico platforms (http://insilico.ehu.eus) were used to determine specificity of *Ehrlichia* and *Rickettsia* primers. During the evaluation of each oligonucleotide, *R. amblyommatis*, and DNA of other agents transmitted by ticks -such as *Babesia* and *Borrelia*- were also tested in order to exclude unspecific amplification.

Phylogenetic trees. phylogenetic analysis of the sequences with the amplicons downloaded from the simulation of In silico platforms was carried out for which the best model was determined, and the construction of the trees was performed by Mega 6.06 using the methods of maximum likelihood and neighbor joining with a 1000 Bootstrap replications.

Results

Detection threshold of ER-dPCR

The threshold for detection of *Ehrlichia* was 100 copies of groESL gene per 50 µL of reaction (2 gene copies per µL), and the threshold for detection of *Rickettsia* was 1 copy of 23S gene per 50 µL of reaction (0.02 gene copies per µL) (Figure 1).
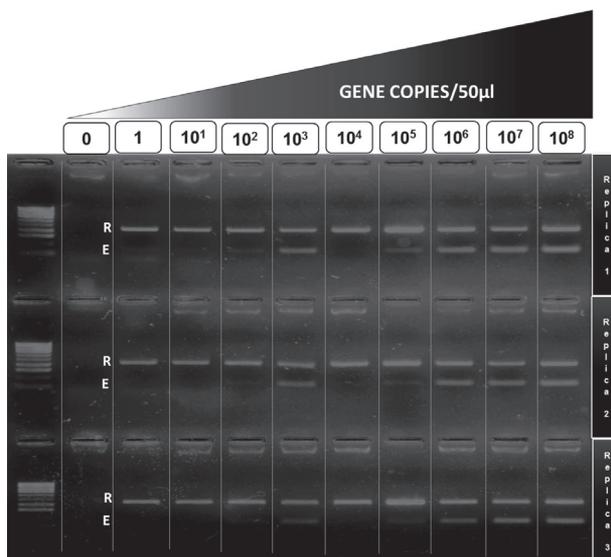

Figure 1. Detection threshold assay for ER-dPCR. R- *Rickettsia* (437 bp), E- *Ehrlichia* (145 bp).

Robustness of ER-dPCR

The duplex methodology was robust enough to allow the simultaneous amplification of both rickettsial agents in all the evaluated concentration ranges of reagents, except for the minimum value of Taq DNA Polymerase (1 U) where no amplification of any fragment was observed. In addition, the ER-dPCR successfully amplified both rickettsial agents regardless of Taq polymerase brand used (Thermo Scientific, Biolase and Invitrogen). This molecular procedure was also robust for alignment temperature changes between 54 to 58° C. Results of the robustness assay are shown in Figure 2.

Repeatability and reproducibility

Ehrlichia testing repeatability of analyst #1 was 100% (15/15), while for *Rickettsia* testing the repeatability was 93.3% (14/15). On the other hand,
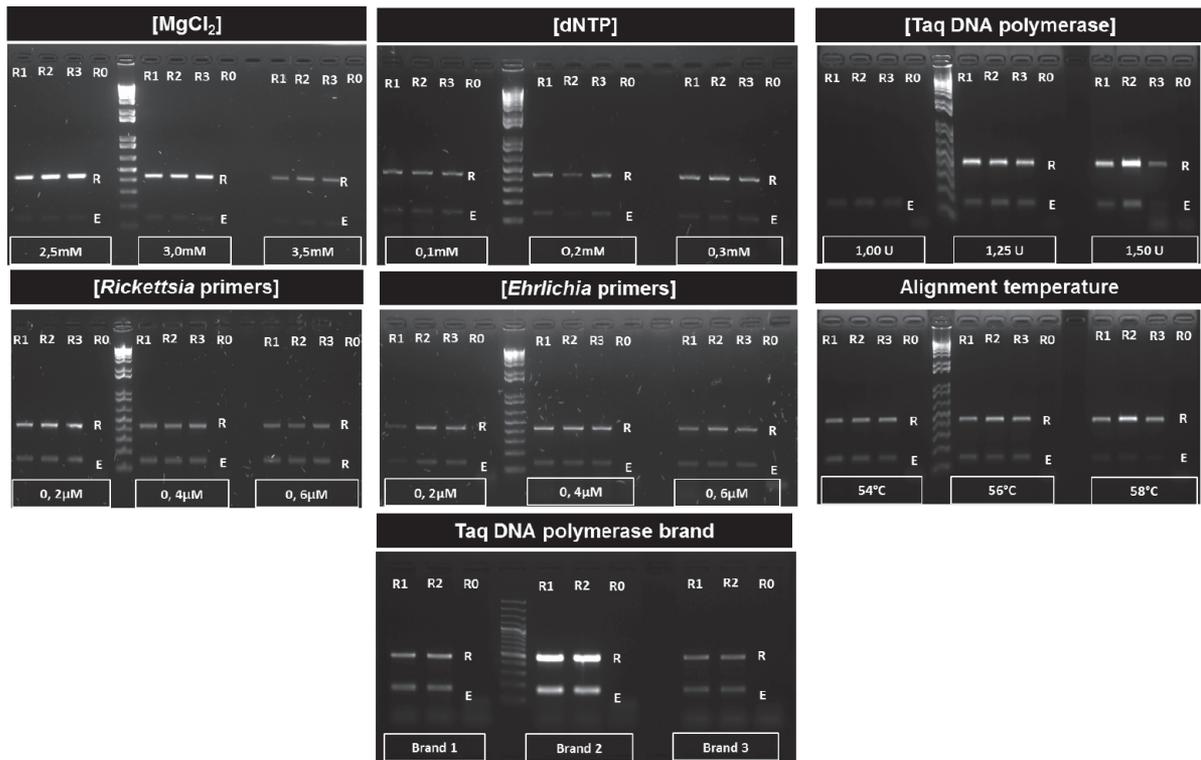
Figure 2. Robustness test of ER-dPCR. R- Band 437 pb of *Rickettsia* spp. E- band 145 pb of *Ehrlichia* spp. R1 - replica 1. R2- replica 2. R3 - replica 3. R0 - negative control.

the repeatability of analyst #2 was 93.3% for *Ehrlichia* detection (14/15) and repeatability for *Rickettsia* was 100% (15/15). The reproducibility (intermediate precision) for both agents was 96.7% (29/30), the summary of both tests is presented in Table 2.

Inclusiveness and exclusiveness assays. The Primer-BLAST tool of BCBI shows that primers Ehr-groELF/Ehr-groELR can amplify 41 sequences of different species of *Ehrlichia* genus (Supplementary file 1), and these oligonucleotides do not show cross-amplification with organisms other than *Ehrlichia*. *E. canis* infected ticks were analyzed upon availability in our laboratory (see figure 3A).

The Primer-BLAST analysis of Rick-23SF/Rick-23SR shows that they can amplify 79 sequences of different *Rickettsia* species (Supplementary file 3), potential cross-amplification was observed for one non-rickettsial target: *Methylocystis* sp. SC2 strain SC2 23S ribosomal RNA (NR_077052, 437pb). The potential cross-amplification of *Methylocystis* could be considered as irrelevant since this bacterium is a free-living microorganism so far not found in ticks. *In vitro* testing of the ER-dPCR was able to amplify *R. rickettsii, R. rhipicephali, Rickettsia* sp Atlantic Rainforest, *R. parkeri, R. amblyommatisi, R. felis*, and *R. bellii* DNA (see figure 3A). The In Silico testing shows successful amplification for all

Table 2. Repeatability and reproducibility results.

	Analyst #	Day 1	Day 2	Day 3	Day 4	Day 5	Repeatability	**Reproducibility**
Ehrlichia	1	3/3	3/3	3/3	3/3	3/3	100% (15/15)	96.6% (29/30)
	2	3/3	3/3	3/3	2/3	3/3	93.3% (14/15)	
Rickettsia	1	3/3	2/3	3/3	3/3	3/3	93.3% (14/15)	96.6% (29/30)
	2	3/3	3/3	3/3	3/3	3/3	100% (15/15)	

Rickettsia species (41 reference sequences available in the platform), no cross-amplification was observed for *Anaplasma, Ehrlichia, Wolbachia, Borrelia* and *Neorickettsia* (Supplementary file 4). Further *in vitro* evaluation shows no cross amplification of ER-dPCR in *Wolbachia, Babesia bigemina* and *Borrelia anserina* (see Figure 3B).
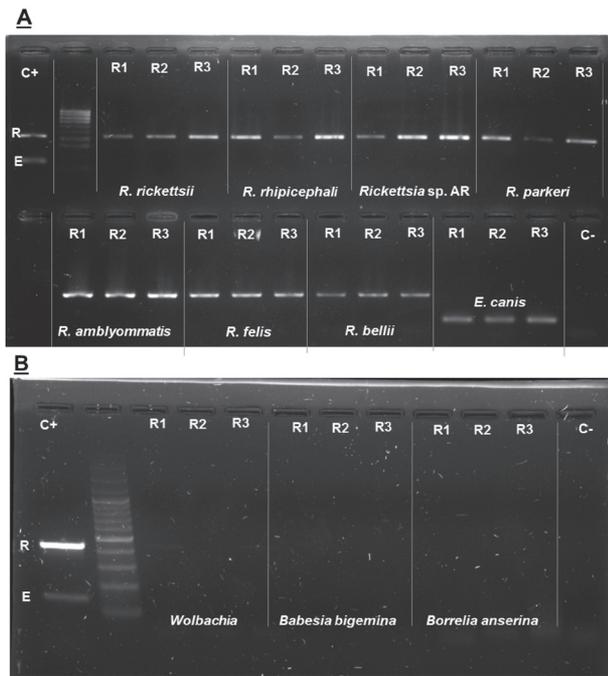

Figure 3. Inclusiveness and exclusiviness *in vitro* tests. R-band 437 pb of *Rickettsia* spp. E- band 145 pb of *Ehrlichia* spp. C+ Positive control with *Rickettsia* spp. and *Ehrlichia* spp. C- Negative control (water free of DNases and RNases). A. Inclusiveness test. B. Exclusiviness test. R1 Replica 1. R2 Replica 2. R3 Replica.

In the In Silico platform, the *Ehrlichia* primers did not allow the successful amplification of groESL gene when genomes of *Rickettsia, Wolbachia, Borrelia, Anaplasma* and *Neorickettsia* were used (Supplementary file 2). The In Silico test of Ehrlichia primers against complete genomes of *E. canis* str. Jake, *E. chaffensis* str. Arkansas, *E. muris* AS145, *E. ruminantium* str. Gardel, *E. ruminantium* str. Welgevonden CIRAD and *E. ruminantium* str. Welgevonden produces amplicons of expected size. The sequences of Ehrlichial amplicons (and *E. ewingii* AF195273) were used to build a phylogenetic tree of *groEL* gene as shown the Figure 4A.

A dendrogram of *Ehrlichia* genus using partial sequences of groEL and Neighbor-Joining algorithm retrieved from In Silico platform shows that *Ehrlichia* species can be rapidly distinguished in spite of the small fragment analyzed (Figure 4A). From the dendrogram, using the 23S *Rickettsia* partial sequences, it is obvious that species discrimination inside the SFGR is not possible (red circle), although discrimination inside the TG might be possible (Figure 4B).

Discussion

Currently, there is a large number of PCR strategies for detection of *Rickettsia* and *Ehrlichia*. These techniques are good for individual or multiplexed amplification with other tick-borne agents in conventional (Corales *et al*., 2014; Jado *et al*., 2006; Kledmanee *et al.*, 2009; Levine et al, 2010; Regnery *et al.*, 1991; Rufino *et al.*, 2013; Stevenson et al, 2003; Sumner *et al.*, 1997; Warner *et al.*, 1997), nested (Massung *et al,*, 1998), and real-time PCR (Doyle *et al*., 2005; Killmaster *et al.*, 2014; Loftis *et al.*, 2006a, 2006b, 2003; Paris *et al.*, 2008; Peleg *et al.*, 2010; Quarsten *et al.*, 2015). Among those, real-time PCR strategies represent the new standard for highly sensitive and specific detection of Rickettsial agents in ticks; however, conventional PCR still remains as the most common molecular platform for selective DNA amplification.

The most commonly used gene for detection of Anaplasmatacea agents (*Anaplasma* and *Ehrlichia*) is 16S rRNA (Killmaster *et al.*, 2014; Levine *et al.*, 2010; Loftis *et al.*, 2006b; Massung *et al.*, 1998; Peleg *et al.*, 2010; Rufino *et al.*, 2013; Warner *et al.*, 1997), whereas *dsb* gene is the standard for identification of Ehrlichial agents (Doyle *et al.*, 2005). On the other hand, *groEL* and virB9 genes are considered as alternative genes for *Ehrlichia* identification through genetic analysis (Levine *et al.*, 2010; Sumner *et al.*, 1997; Corales *et al.*, 2014; Kledmanee *et al.*, 2009). On *Rickettsia* detection, the citrate synthase gene (gltA), has been deemed as a gold standard for long time (Paris *et al.*, 2008; Quarsten *et al.*, 2015; Regnery *et al.*, 1991; Roux *et al.*, 1997; Oteo *et al.*, 2014), but other genes such as 17KDa genus common rickettsial antigen and 23S-5S internal transcribed spacer have also been used and tested for the same task
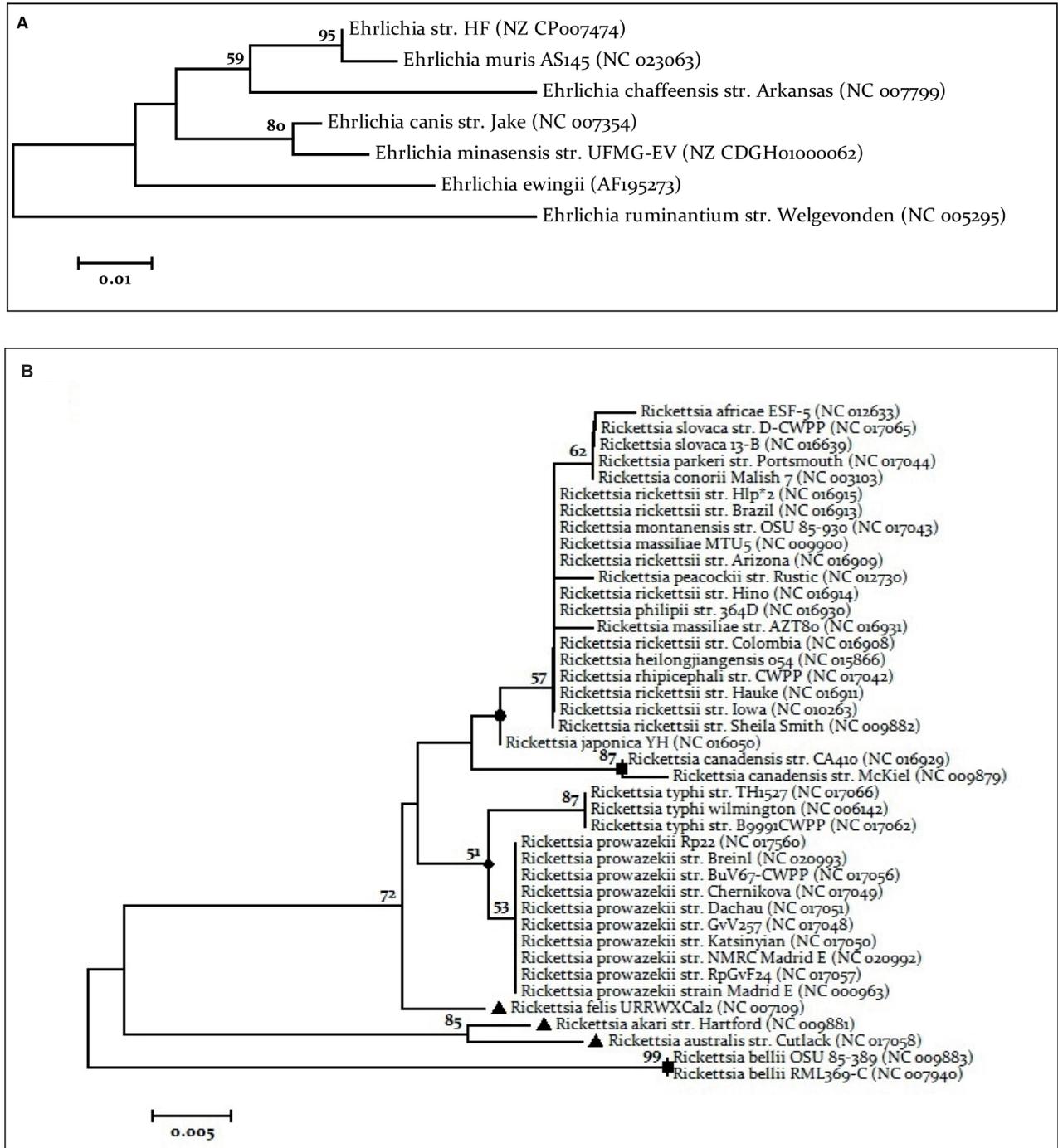
Figure 4. Phylogenetic trees. A. Phylogenetic tree *Ehrlichia* spp. Tree was performed using the downloaded sequences of the application IN SILICO PCR. Tree performed by neighbor joining using the Tamura 1992 model has invariant sites (I), with a 1000 Bootstrap replications. B. Phylogenetic tree *Rickettsia* spp. Tree was performed using the downloaded sequences of the IN SILICO PCR application. The tree performed by maximum likelihood using Kimura 2 model parameters with gamma distribution (G), with a 1000 Bootstrap replications. Circle - Spotted fever group. Square - Ancestral Group Triangle - Transitional group. Diamond- Typhus Group.

(Killmaster *et al.*, 2014; Loftis *et al.*, 2006a; Stevenson *et al.*, 2003; Jado *et al.,* 2006). In the present study, we developed a sensitive conventional PCR strategy for simultaneous and specific detection of *Ehrlichia* and *Rickettsia* in ticks.

Co-infection with multiple rickettsial agents is a common scenario in ticks. Actually, there are reports of simultaneous infection by *Rickettsia*, *Ehrlichia chaffensis* and *Ehrlichia ewingii* in *Amblyomma americanum* in Georgia (USA) (Killmaster *et al.*, 2014), and a more recent report describes the occurrence of common bacterial co-infections in *Ixodes ricinus* in France (Moutailler *et al.,* 2016). The molecular detection of co-infection by several bacterial agents would require performing a single PCR for each considered agent, or developing new multiplex and duplex PCRs assays.

The ER-dPCR has shown promising results with a detection threshold for *Rickettsia* about 0.02 copies/μL and 2 copies/μL for detection of *Ehrlichia*. Our results for *Rickettsia* detection threshold are similar to the PCR-RLB designed by Jado *et al.,* 2006 that detects up to 0.02 copies/μL, and better than the one obtained by Paris *et al.,* (2008) that reports detection up to 1 copy/uL for TGR and 2 ompB gene copies/uL for SFGR in a multiplexed SYBR-qPCR assay. On the other hand, the detection threshold of ER-dPCR for *Ehrlichia* (2 copies/uL) is similar to Taqman-qPCR reported by Doyle *et al.* (2005), that detects 1 copy/μL of any *Ehrlichia*.

Duplexed detection techniques have been developed for *E. canis* and the protozoa *B. canis vogeli* with threshold detection between 1-10 gene copies (Peleg *et al.,* 2010). Other conventional PCR assays targeting the groEL region claim to detect 2-gene copies/μL (Levine *et al.,* 2010), and in our case the *Ehrlichia* detection showed similar sensitivity to the aforementioned techniques. Moreover, other strategies focused on qPCR and conventional PCR reported a threshold detection for *Rickettsia* about 2-gene copies/μL (Killmaster *et al.,* 2014; Loftis *et al.,* 2006); according to this, our ER-dPCR is comparable to other strategies available in the scientific literature, with the advantage that this one does not lose sensitivity in detection of both rickettsial agents.

One of the major concerns regarding the use of duplexed or multiplexed PCR techniques has been the poor reproducibility and instability of the assays. However, as we showed in the repeatability and reproducibility tests (intermediate precision), the ER-dPCR is accurate enough to ensure its use in research, has promising features for its use in clinical diagnosis, and it seems not to be greatly affected by different users. We also show that ER-dPCR is highly robust and tolerates wide ranges of reagent concentration and commercial brands, with the only exception that it requires a minimal amount of Taq DNA polymerase (1,25 U per reaction) for good performance.

The inclusiveness and exclusiveness test (in silico and *in vitro*) showed that the design of the ER-dPCR is specific for *Ehrlichia* and *Rickettsia* bacterial genera and does not amplify other microorganisms commonly found in ticks around the world, such as *Anaplasma*, *Borrelia*, *Babesia* or *Wolbachia*. The phylogenetic signal of the 23S region of *Rickettsia* used in our study is good enough to differentiate among the main bacterial groups within this genus. However, it is not suitable to differentiate among SFGR species, whereas differentiation of *Ehrlichia* species using a small groEL region is possible, as demonstrated in previous studies (Sumner *et al.,* 1997). A big limitation of our work was the inability to test for other *Ehrlichia* agents, since other species of this bacterial genus are found in the neoarctic and paleartica regions and only *E. canis* is widely found across the world. The ER-dPCR described here was designed for *Ehrlichia* and *Rickettsia* detection in ticks, but it can also be applied to the study of dog populations, since none of the targets shows potential cross-amplification with carnivores. Finally, further validation of our rickettsial detection strategy must be conducted to establish the epidemiological utility of the ER-dPCR for rickettsial diseases.

In conclusion, we developed a robust duplex PCR for the reliable detection of *Ehrlichia* and *Rickettsia* agents, comparable to other high-performance single-detection strategies. To the best of our knowledge, this is the first rickettsial PCR performed with analytical validation.

Further validation of the test is planned using field collected ticks and ticks from dogs.

Acknowledgments

Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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