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Upscaling surveillance of tick-borne pathogens in the French Caribbean islands

Mathilde Gondard[1,3], Sabine Delannoy[2], Valérie Pinarello[3,4], Rosalie Aprelon[3,4], Elodie Devillers[1], Clémence Galon[1], Jennifer Pradel[3,4], Muriel Vayssier-Taussat[1], Emmanuel Albina[3,4], and Sara Moutailler[1]*

[1] UMR BIPAR, Animal Health Laboratory, ANSES, INRA, Ecole Nationale Vétérinaire d'Alfort, Université Paris-Est, Maisons-Alfort, France.

[2] IdentyPath Platform, Food Safety Laboratory, ANSES, Maisons-Alfort, France

[3] CIRAD, UMR ASTRE, Petit-Bourg, Guadeloupe, France.

[4] ASTRE, University of Montpellier, CIRAD, INRA, Montpellier, France.

*Corresponding author: Dr. Moutailler Sara, ANSES, sara.moutailler@anses.fr

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Abstract

Background

Among hematophagous arthropods, ticks transmit the greater variety of pathogens of public health and veterinary importance whose (re)-emergence is recognized worldwide. However, the epidemiological situation of the Caribbean area with regard to tick-borne diseases is poorly documented, mainly focusing on livestock pathogens such as *Ehrlichia ruminantium*, *Babesia* (*bovis* and *bigemina*) and *Anaplasma marginale*. These observations underline the need to undertake new large-scale epidemiological surveys to better assess the distribution of tick-borne pathogens and anticipate the risk of (re)-emergence of tick-

borne diseases in these areas. To ease and reduce the cost of such large-scale surveys, the development of a fast and cheap high-throughput detection technics is desirable.

Methodology/Principal Findings

In this study, we first implemented a high-throughput microfluidic real-time PCR (BioMark™ dynamic arrays, Fluidigm Corporation) adapted for the large-scale screening of tick-borne pathogens in Caribbean ticks. The system developed here includes 57 designs allowing both the screening of bacteria and parasites potentially circulating in the West Indies (5 bacterial genera, 30 bacterial species, 1 parasitic phylum, 2 parasitic genera and 17 parasitic species), and the molecular identification of three tick species mainly involved in tick-borne pathogens transmission in the Caribbean (*Amblyomma variegatum*, *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus sanguineus*). Secondly, we performed an exploratory epidemiological study on 597 ticks collected in Guadeloupe and Martinique. We successfully detected expected pathogens, including *Rickettsia africae*, *Ehrlichia ruminantium*, *Anaplasma marginale*, *Babesia bigemina*, and *Babesia bovis*. In addition, the system allowed us to detect unexpected microorganisms belonging to genera of *Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., and *Leishmania* spp.

Conclusions/Significance

Our study demonstrated how high-throughput microfluidic real time PCR technology is a major improvement in large-scale epidemiological studies, giving a rapid overview of the tick-borne pathogens and microorganisms present in ticks in a given area.

Author Summary

In this study, we developed a high-throughput microfluidic real time PCR system adapted to large scale epidemiological study of tick-borne pathogens in Caribbean ticks. Regarding the lack of epidemiological data on tick-borne pathogens diversity circulating within the Neotropical area, and the risk of (re)-emergence of vector-borne diseases in such region, the need of new high throughput survey methods was paramount. The Biomark system (BioMark™ dynamic arrays, Fluidigm Corporation) implemented here allowed both the survey of 47 tick-borne pathogens and microorganisms belonging to 5 and 3

bacterial and parasitic species respectively involved in Tick-borne diseases worldwide, as well as the molecular identification of the main vector ticks in the Caribbean. We screened 137 specimens of *Amblyomma variegatum* and 460 *Rhipicephalus (boophilus) microplus* collected in Guadeloupe and Martinique. The Biomark system implemented here allowed the detection of expected tick-borne pathogens – *Ehrlichia ruminantium*, *Rickettsia africae*, *Anaplasma marginale*, *Babesia bigemina*, *Babesia bovis*, *Theileria velifera* and *Theileria mutans* – as well as the discovery of unsuspected pathogens and microorganisms belonging to the genera *Anaplasma*, *Ehrlichia*, *Borrelia*, and *Leishmania*. We demonstrated the ability of the Biomark system to give a rapid overview of the pathogens/microorganisms diversity present in ticks, thus opening new research perspectives on tick-borne pathogens epidemiology in the Caribbean.

Introduction

Among hematophagous arthropods, ticks transmit the greatest variety of pathogens of public health and veterinary importance which incidence is growing worldwide (Dantas-Torres *et al*., 2012). The French West Indies, Guadeloupe and Martinique islands, are located in the heart of the Caribbean Neotropical Zone, a cosmopolite area characterized by a tropical climate, intercontinental exchanges, and animal movements (legal or illegal trade and bird migration) that are favorable for ticks and tick-borne pathogens (TBPs) introduction and dispersal (George *et al*., 2002). The epidemiological situation of the Caribbean area with regard to the diversity of tick species and tick-borne diseases is poorly documented (Gondard *et al*., 2017). *Amblyomma variegatum* also known as Tropical Bont Tick (TBT) in the Caribbean and *Rhipicephalus (Boophilus) microplus* ("Cattle tick") are the two main tropical livestock pests since their introduction in the Caribbean through the importation of infested animals from Africa and Asia during the 18-19th century (Barré and Uilenberg, 2010). Both tick species are present in the French Antilles where they are involved in the epidemiology of TBPs of medical and veterinary importance (Morel, 1967; Uilenberg *et al*., 1983; Camus and Barré 1995; Alonso *et al*., 1992; Parola *et al*., 1999). *Rh. microplus*, a one-host tick highly specific of cattle, is responsible for the

transmission of *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina*, causing bovine anaplasmosis and babesiosis, respectively. These pathogens are considered as endemic in the Caribbean (Camus and Montenegro-James 1994; Camus and Barré 1995). *A. variegatum*, is a three-host tick species, with immature stages that can parasitize a large host range, including rodent, mongoose, bird, and adult stage that are more specific to cattle (Barré, *et al*., 1988). This tick species can transmit *Ehrlichia ruminantium* the causative agent of heartwater, a fatal ruminant ehrlichiosis. Although *A. variegatum* is present in both Martinique (mainly in the South) and Guadeloupe (widespread), *E. ruminantium* is only reported in Guadeloupe (Vachiéry *et al*., 2008). This tick species is also vector of *Rickettsia africae*, common in the Caribbean, which can induce human rickettsiosis called African Tick Bite Fever (Parola *et al*., 1999, 2003; Kelly *et al.*, 2010). Despite high level of tick infection and seroprevalence in human and cattle sera, to date only two human cases from travelers returning from Guadeloupe have been reported (Parola *et al*., 1999; Raoult *et al*., 2001). Finally, *A. variegatum* is also involved in *Theileria mutans* and *Theileria velifera* epidemiology, two cattle parasites of low and no virulence, respectively (Uilenberg 1983; Alonso *et al*., 1992). The majority of the epidemiological information available on tick-borne pathogens in the Caribbean are based on serological studies in animals or humans, or molecular biology assays (PCR, nested PCR). These studies do not survey and discriminate the whole repertoire of the infectious species involved since they are often limited to the detection of some well-known pathogens. (Estrada-Peña *et al.*, 2013; Cabezas-Cruz *et al.*, 2018). Thus, new insights into tick and TBPs epidemiology are needed to address Tick-Borne Diseases (TBD) prevalence and (re-) emergence in the Caribbean.

The recent development and use of a microfluidic real-time PCR system for rapid and concomitant detection of a large panel of TBPs has paved the way for a promising wider surveillance capacity with the implementation of a large-scale epidemiological survey of TBPs in European ticks (Michelet *et al*., 2014; Dupraz *et al*., 2017; Gioia *et al*., 2018; Gondard *et al*., 2018; Malmsten *et al*., 2018). To screen a large panel of TBPs of medical and veterinary importance that are likely to circulate within the Caribbean ticks we have implemented this technology allowing the simultaneous detection of 1 parasitic phylum, 8 bacterial and parasitic genera, 49 bacterial and parasitic species in 95 DNA tick samples, and the molecular identification of 3 well known vector tick species in a single experiment. In

this study, we screened these TBPs in 137 *A. variegatum* and 460 *Rh. microplus* collected in Guadeloupe and Martinique. We demonstrated the ability of the system to detect well-known TBPs occurring in the French Antilles, as well as unsuspected TBPs and potential new microorganisms. This new method can improve considerably the monitoring of emergent and non-emergent TBPs through large-scale survey in the Caribbean area.

Materials and methods

Tick collected in Guadeloupe and Martinique

The ticks used in this study were collected within the framework of two distinct epidemiological surveys conducted in Guadeloupe (between February 2014 and January 2015) and Martinique (between February and March 2015) respectively. In Guadeloupe, adult ticks (any species, any sex) were collected on 40 bovines originating from 22 different herds that were sampled in 9 localities situated in six different biotopes (urban area, dry coastal regions, valleys and hills, evergreen seasonal forest, sub-mountainous rain forest, swamp forest). In Martinique, engorged females of *Rh. microplus* were collected from cattle in 29 farms participating in a study on acaricide resistance in ticks. Ticks were morphologically identified at the species level (Walker *et al*., 2003). A total of 597 adult ticks were included in the study: 137 *A. variegatum* and 175 *Rh. microplus* from Guadeloupe and 285 *Rh. microplus* from Martinique (see maps, Fig 2). GPS coordinates of tick collection sites are available in Supplementary data 1. All the ticks were partially engorged, and then conserved at -80°C.

DNA extraction

For 20 mg of ticks, 1 mL of recently prepared PBS 1X was added to the sample, then ticks were washed by gentle shaking over 2-3 min at 7 Hz/s in a Tissue Lyzer (Qiagen, Germany). After discarding the supernatant, ticks were frozen at -80°C during 15-20 min. Then a steel ball was added and samples were crushed twice during 2 min at 30 Hz/s with the Tissue Lyzer (Qiagen, Germany). 450 µL of fresh PBS 1X were added to the samples. Samples were vortexed during 10 s, and then centrifuged during 2-3 min

at 8000 g. Finally, 20 µL of Proteinase K were added to 180 µL of crushed tick sample and DNA was extracted using the NucleoSpin® 96 virus Core kit (Macherey-Nagel, Germany) and the automatic platform Biomek4000 (Beckman Coulter). This protocol allows the simultaneous extraction of both DNA and RNA. Total nucleic acid per sample was eluted in 160 µl of rehydration solution and stored at −80°C until further use. *A. variegatum* ticks were individually extracted. *Rh. microplus* ticks were extracted both individually for adults and in pools of two to four small tick specimens.

Assay design

The list of pathogens to be monitored, and the sets of primers and probe required for their detection, as well as the targeted genes are shown on Table 1. Some of the oligonucleotides were specifically designed for the purpose of this study, the others came from Michelet *et al*., 2014. The newly developed oligonucleotides were validated on dilution range of positive controls including cultures, plasmids or DNA samples (Table 1) by real-time TaqMan PCRs assays on a LightCycler® 480 (LC480) (Roche Applied Science, Germany). Real-time PCR assays were performed with LightCycler® 480 Probe Master Mix 1× (Roche Applied Science, Germany), using 200 nM of primers and probes in a final volume of 12 µl and 2 µl of control DNA were added. The Thermal cycling program was as follows: 95°C for 5 min, 45 cycles at 95°C for 10 s and 60°C for 15 s and one final cooling cycle at 40°C for 10 s.

Pre-amplification of DNA samples

All the DNA samples were subject to preamplification in order to enrich the pathogen DNA content comparatively to tick DNA. The Perfecta® PreAmp SuperMix (Quanta Biosciences, Beverly, USA) was used for DNA pre-amplification, following the manufacturer's instructions. All the primers were pooled (except the ones targeting the tick species) with a final and equal concentration of 45 nM each. Pre-amplification reaction was performed in a final volume of 5 µL containing 1 µL of PerfecTa PreAmp SuperMix (5X), 1.25 µL of pooled primers mix, 1.25 µL of DNA and 1.5 µL of MilliQ water with one

cycle at 95°C for 2 min, 14 cycles at 95°C for 10 s and 60°C for 3 min. At the end of the cycling program the reactions were diluted 1:10. Pre-amplified DNAs were stored at −20°C until use.

High-throughput microfluidic real-time PCR

High-throughput microfluidic real-time PCR amplifications were performed using the BioMark™ real-time PCR system (Fluidigm, USA) and 96.96 dynamic arrays (Fluidigm, USA), that allows to perform 9,216 individual reactions in one run (Michelet *et al*., 2014). Real-time PCRs were performed using 6-carboxyfluorescein (FAM) and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression Master Mix (Applied Biosystems, USA) following the manufacturer's instructions. Cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 2-step amplification of 15 s at 95°C, and 1 min at 60°C. The BioMark™ Real-Time PCR system was used for data acquisition and the Fluidigm Real-time PCR Analysis software for C_t values determination. Three kinds of control were used per chip for experiment validation: a negative water control, to exclude contaminations; a DNA extraction control, corresponding to primers and probe targeting a portion of the 16S rRNA gene of ticks; and an internal control to check the presence of PCR inhibitors made of a DNA of *Escherichia coli* strain EDL933 added to each sample with specific primers and probe targeting the *eae* gene (Nielsen and Andersen, 2003).

Infection Rates of ticks from the French Antilles

According to the tick species and the island of origin, for each detected pathogen, infection rates (the proportion of infected ticks divided by the total number of ticks analyzed) were calculated. The majority of the samples were single specimens of ticks. However, some ticks were too small to be treated as individual ticks and were therefore pooled by 2 to 4 specimens. Thus, among the 538 samples analyzed, 49 consisted of a pool of 2 to 4 tick specimens. The final estimation of the infection rate included the pools and is therefore expressed as the minimal (assuming at least one positive ticks in the pools) and maximal (assuming all ticks positive in the pools) proportions of infected ticks out of the total number of ticks analyzed.

PCRs and sequencing for results confirmation

Conventional PCR/Nested PCR using primers targeting different genes or regions than those of the BioMark™ system were used to confirm the presence of pathogenic DNA in some field samples and positive controls (Table 2). Amplicons were sequenced by Eurofins MWG Operon (BIOMNIS-EUROFINS GENOMICS, France), and then assembled using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). An online BLAST (National Center for Biotechnology information) was used to compare results with published sequences listed in GenBank sequence databases.

Phylogenetic sequence analysis

Alignment were performed using ClustalW (Thompson *et al.*, 1994). Maximum Likelihood trees were generated by 1000 bootstrap repetitions under the Tamura-Nei model (Tamura and Nei, 1993) by MEGA7 (Kumar *et al.*, 2016). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Further information is provided in the figure legends.

Results

Implementation of the high-throughput microfluidic real-time PCR system for Caribbean tick-borne pathogens survey

The high-throughput microfluidic real-time PCR system developed for the screening of Caribbean tick-borne pathogens included 61 sets of primers and probes. Among them, 49 designs were developed for the detection of bacterial (n=32) and parasitic (n=17) species, bacterial (n=5) and parasitic genera/phylum (n=3) (Table 1). Three sets of primers and probes were developed for the molecular

identification of the three Caribbean vector ticks, including *A. variegatum*, *Rh. microplus* and *R. sanguineus* (Table 1). Finally, we developed a design targeting a conserved region of the 16S rRNA genes within ticks, called Tick spp., used as a control of DNA/RNA extraction (Table 1).

The detection ability of each designs and the effect of pre-amplification on detection signals were first checked by Taqman real-time PCRs on a LightCycler 480 apparatus using dilution range of positives controls (Table 1). Three kind of positive controls were used, including bacterial or parasitic cultures when available, DNA from infected ticks or blood samples, and plasmidic constructions as a last resort (Table 1). Except the design targeting *Borrelia burgdorferi sensu stricto* which never succeeded in detecting the positive controls even after a preamplification step, the remaining 60 designs targeting TBPs and ticks species were able to detect their target with C_t values between 6 to 38 (data not showed). Pre-amplification improved the quality of the detection, as observed in Michelet *et al.*, 2014, and was therefore validated as part of the screening protocol (Supplementary data 2).

Then, the specificity of the designs was evaluated using the Biomark system and a total of 62 positive controls (Table 1, Fig 1). 42 primers/probe sets were able to detect and amplify specifically their target using a C_t cut-off value of 30 (Fig 1). The design Tick spp**.** was able to detect *A. variegatum* and *R. sanguineus* samples, as well as the DNA of *R. sanguineus* tick present in the *Rickettsia conorii* positive control as expected (Fig 1). However, high C_t values were observed and the DNA of ticks from the *Rh. microplus* control sample and others positive controls including tick DNA (such as the controls *Borrelia lonestari*, *Anaplasma phagocytophilum*, etc.) were not detected (Fig 1). The apparent lack of sensitivity of this design was explain by the exclusion of the Tick spp. primers during the preamplification step, since the objective was to enrich pathogen DNA content comparatively to tick DNA. Following these results, the Tick spp. primers were added to the pre-amplification mix and correct amplifications for all tick samples were obtained, thus confirming the interest of this design as internal control for tick DNA extraction, at least for *A. variegatum*, *Rh. microplus*, and *R. sanguineus*. The 17 remaining designs gave false-positive results in outgroup controls in addition to their target detection. Interestingly, two kinds of unsuspected signals were observed, some related to cross-reactions with closely related species and some related to potential co-infections in controls corresponding to field samples (Fig 1). Eight designs - *Rickettsia massiliae*, *Rickettsia conorii*, *Bartonella henselae*, *Bartonella*

bacilliformis, Babesia canis vogeli, Babesia microti, Theileria parva, Hepatozoon americanum - gave positive results in outgroup controls, revealing cross-reactions with one to two closely related species (Fig 1). *Babesia ovis* and *Rickettsia rickettsii* designs gave multiple cross-reactions with closely related species or distant outgroups and thus were considered as non-specific and removed from the study (Fig 1). Finally, the seven remaining designs - *Rickettsia* spp., *Rickettsia felis, Rickettsia africae,* Apicomplexa, *Babesia bigemina, Hepatozoon* spp., *Hepatozoon canis* - gave positive results in outgroup controls linked to potential co-infection in controls corresponding to DNA from ticks collected in the field or DNA from infected blood (Fig 1). As co-infections may occur in natural tick or blood samples, these unexpected detections in biological samples could be due to natural (co-)occurrence of microorganisms rather than cross-reactions (Moutailler *et al*., 2016) (details of co-infection analysis in Supplementary data 3).

To conclude, except sets of primers and probes targeting *Borrelia burgdorferi sensu stricto*, *Babesia ovis* and *Rickettsia rickettsii* that were removed from the study, the 58 remaining designs were validated for the high throughput screening of pathogens in Caribbean ticks, taking into account the notified cross-reactions.

Large scale TBPs detection survey in ticks from Guadeloupe and Martinique

A total of 597 adults ticks were collected from cattle in Guadeloupe and Martinique. Among them, 137 *A. variegatum*, were collected only in Guadeloupe, and 460 *Rh. microplus* were collected in Guadeloupe (n=175) and Martinique (n=285). A total of 538 samples (some ticks were pooled, see Material and methods) were tested using the BioMark^{TM} system developed in this study. Following the analysis, nine samples were removed from the screening of pathogens due to an inconsistency between morphological and molecular identification of tick samples**.** At the end, the number of positive ticks and the corresponding infection rates for each pathogen detected were calculated for 137 *A. variegatum*, 166 and 281 *Rh. microplus* from Guadeloupe and Martinique, respectively (Fig 2). Conventional PCR/Nested PCR followed by amplicon sequencing were performed on several tick samples to confirm some of the results of the newly designed BioMark^{TM} system (Table 2). Percentage of identity of the sequences obtained with reference sequences available in NCBI are presented in Table 3.

Detection of known TBPs in Caribbean ticks. Seven TBPs known to circulate in the area of the Caribbean were detected in ticks from Guadeloupe and Martinique, including *R. africae, E. ruminantium, An. marginale, B. bigemina, B. ovis, T. mutans* and *T. velifera* (Fig 2).

Rickettsia spp. were only detected in ticks collected in Guadeloupe (Fig 2). *R. africae* was identified in 95.6% (131/137) of the *A. variegatum* samples (Fig 2). In contrast, *Rickettsia* spp. detected in 15.7-23.5% (26-39/166) of the *Rh. microplus* samples from Guadeloupe were not directly identified with the Biomark system (Fig 2). Thus, fourteen samples of *A. variegatum* (6/14) and *Rh. microplus* (8/14) positive for *Rickettsia* spp. were tested by nested PCR with primers targeting the ompB gene, followed by sequencing (Table 2). All the sequences recovered were identical, and displayed 100% of identity with *R. africae* (Table 3). The consensus sequence was deposited under the name *Rickettsia africae* Tick208, accession number MK049851. The absence of direct identification of *R. africae* in *Rh. microplus* ticks was probably due to a lower sensitivity of the specific target design compared to the genus target design. Indeed, *Rickettsia* spp. positive *Rh. microplus* samples displayed quite high C_t values, suggesting a low infection level that might be below the detection limit for *R. africae*. *E. ruminantium* was identified in 5.1% (7/137) of the *A. variegatum* ticks from Guadeloupe (Fig 2). We confirmed the detection of *E. ruminantium* by testing one sample of *A. variegatum* by conventional PCR targeting the 16S rRNA genes followed by amplicon sequencing (Table 2). The sequence obtained displayed 100% of sequence identity with *E. ruminantium* and were deposited under the name *Ehrlichia ruminantium* Tick116 (accession number MK049848) (Table 3, Fig 3).

An. marginale was identified in *Rh. microplus* ticks from the both Islands, with infection rates reaching 3.6-4.8% (6-8/166) and 39.5-41.3% (111-116/281) of specimens from Guadeloupe and Martinique respectively (Fig 2). We confirmed the detection of *An. marginale* by testing two samples of *Rh. microplus* by conventional PCR targeting the 16S rRNA genes followed by amplicon sequencing (Table 2). We obtained two identical sequences, deposited under the name *Anaplasma sp.* Tick283 (accession number MK049844) that displayed 100% of sequence identity with *Anaplasma* spp. including *An. marginale* (Table 3, Fig 4).

B. bigemina was detected in 0.6-1.2% (1-2/166) and 12.5-12.8% (35-36/281) of the *Rh. microplus* ticks from Guadeloupe and Martinique respectively (Fig 2). *B. bovis* was only detected in tick from Martinique, with an infection rate of 0.7% (2/281) of *Rh. microplus* samples (Fig 2). As conventional and nested PCR did not succeed in detecting those parasites, we sequenced amplicons obtained with the *B. bigemina* and *B. bovis* designs developed here and corresponding sequences were identified (accession numbers MK071738 and MK071739 respectively) (Table 3).

T. velifera and *T. mutans* were detected in both tick species from the both islands. *T. velifera* was identified in 42.3% (58/137) of the *A. variegatum* samples, 24.1-31.9% (40-53/166) and 25.6-26% (72-73/281) of the *Rh. microplus* samples from Guadeloupe and Martinique respectively (Fig 2). As well, *T. mutans* was detected in 1.5% (2/137) of the *A. variegatum* samples, and in 1.8-2.4% (3-4/166) and 1.4% (4/281) of the *Rh. microplus* samples from Guadeloupe and Martinique, respectively (Fig 2). However, neither conventional PCR nor Biomark amplicon sequencing succeeded in confirming the Biomark results. Although false signals could not be ruled out, those results may reflect a very low level of infection by these two parasites.

Detection of unexpected microorganisms in Caribbean ticks. Unexpected signals were obtained during the screening of TBPs in ticks from Guadeloupe and Martinique, including the first detection of untargeted species belonging to the genera *Anaplasma*, *Ehrlichia*, *Borrelia* and *Leishmania* (Fig 2).

Ehrlichia spp. were detected in *Rh. microplus* ticks from both islands, with infections rates reaching 4.2-6.6% (7-11/166) and 47.7-49.1% (134-138/281) in Guadeloupe and Martinique respectively (Fig 2). We tested two of the *Ehrlichia* spp. positive *Rh. microplus* samples by conventional PCR analysis targeting the 16S rRNA genes (Table 2). We obtained two identical sequences, deposited under the name *Ehrlichia* sp. Tick428 (accession number MK049849) (Table 3). The *Ehrlichia* sp. Tick428 sequence displayed 100% of sequence identity with various *Ehrlichia* species detected in different ticks species from Asia and Africa (Table 3) (Parola *et al.*, 2001, 2003; Inokuma *et al.*, 2004; Loftis *et al.*, 2006; Kang *et al.*, 2014; Lu *et al.*, 2017). Phylogenetic and genetic distance analyses were performed using fragment of the 16S rRNA genes of several *Ehrlichia* species (Fig 3). The *Ehrlichia*

sp. Tick428 sequence was found within a cluster including the other undescribed *Ehrlichia* species sequences. However, regarding the highly conserved nature of the 16S rRNA genes, we could not define more accurately the phylogenetical relationships within the *Ehrlichia* species group. Further investigations are needed to characterize this potential new *Ehrlichia* species.

In addition, in around 50% (4/8) and 18% (22/112) of the *Rh. microplus* specimens positives for *Anaplasma* spp., none of the *Anaplasma* species targeted by the Biomark system gave positive results, suggesting the presence of an unexpected or a new *Anaplasma* spp. (Fig 2). We tested two of the *Anaplasma* spp. positive *Rh. microplus* samples by conventional PCR analysis targeting the 16S rRNA genes (Table 2). We obtained two identical sequences, deposited under the name *Anaplasma* sp. Tick314 (accession number MK049845) (Table 3). This sequence displayed 100% of sequence identity with *Candidatus* Anaplasma boleense. *Candidatus* Anaplasma boleense have been described in ticks and mosquitoes in China (Kang *et al.*, 2014; Guo *et al.*, 2016). Phylogenetic and genetic distance analyses were performed using fragment of the 16S rRNA genes of several *Anaplasma s*pecies (Fig 4). *Anaplasma* sp. Tick314 sequence was found in a cluster including *Candidatus* Anaplasma boleense, *Anaplasma platys* and *Anaplasma phagocytophilum*. Few information are available regarding *Candidatus* Anaplasma boleense. Further investigations are needed to confirm the presence of this bacteria in Caribbean ticks and to decipher the epidemiology of this newly described *Anaplasma* species.

Borrelia spp. were surprisingly detected in both tick species from the both islands (Fig 2). Infection rates reached 5.1% (7/137) in *A. variegatum*, and 0.6% (1/166) and 4.3% (12/281) in *Rh. microplus* from Guadeloupe and Martinique respectively (Fig 2). None of the specific *Borrelia* species targeted, neither from Lyme disease nor Relapsing fever group, gave any positive results suggesting the occurrence of a new or unexpected *Borrelia* spp. in our samples (Fig 2). We tested 30 of the *Borrelia* spp. positive ticks by nested PCR analysis targeting the flaB genes (Table 2). Interestingly we obtained two sequences according to the tick species analyzed. *Borrelia* sp. Tick7 (accession number MK049846) sequence was recovered from one *A. variegatum* samples from Guadeloupe and displayed 90% of sequence identity with *Borrelia anserina* (X75201.1) (Table 3). *Borrelia* sp. Tick457 sequence (accession number MK049847) was recovered from four *Rh. microplus* samples from Martinique, and displayed 100% of sequence identity with *Borrelia* sp. strain Mo063b (KY070335.1), Borrelia sp. BR

(EF141022.1) and 99% of sequence identity with *Borrelia theileri* (Table 3). *Borrelia* sp. strain Mo063b (KY070335.1) and *Borrelia* sp. BR (EF141022.1) correspond to *Borrelia* spp. detected in *Rh. microplus* ticks from Madagascar and Brazil respectively, both clustering with *Bo. theileri* (Yparraguirre *et al.*, 2007; Hagen *et al.*, 2017). Phylogenetic and genetic distance analyses were performed using fragment of the flaB gene of several *Borrelia* species (Fig 5). Surprisingly, the *Borrelia* sp. Tick7 sequence recovered from the *A. variegatum* sample, and found closely related to *Bo. anserina*, displayed an intermediate position, sharing homology with both the relapsing fever and Lyme disease groups (Fig 5). Finally, the *Borrelia* sp. Tick457 sequence recovered from the *Rh. microplus* samples confirmed the previous observations, forming a cluster with Relapsing Fever *Borrelia* species encountered in hard ticks, including *Borrelia lonestari* and *Bo. theileri*, as it was found for *Borrelia* sp. strain Mo063b and *Borrelia* sp. BR (Yparraguirre *et al.*, 2007; Hagen *et al.*, 2017) (Fig 5). Further investigations are needed to confirm the presence of *Borrelia* spp. in Caribbean ticks, especially regarding the medical/veterinary impact of those bacteria.

Finally, 0.7% (2/281) *Rh. microplus* ticks from Martinique were positive for *Leishmania* spp. (Fig 2). We tested two of the *Leishmania* spp. positive ticks by nested PCR analysis targeting the small sub-unit rRNA gene (Table 2). We obtained one sequence from one sample, deposited under the name *Leishmania martiniquensis* Tick389 (accession number MK049850) (Table 3). This sequence displayed 100% of identity with both *Leishmania martiniquensis* and *Leishmania siamensis* sequences (Table 3). Studies on *Leishmania* nomenclature highlighted the fact that isolates of "*L. siamensis"* have never been officially characterized, and that this name should not be used (Pothirat *et al.*, 2014; Espinosa *et al*., 2018; Leelayoova *et al*., 2017; Steverding, 2017). Thus, as all the sequence reported as "*L. siamensis*" – except one - in databases should be considered as a synonym of *L. martiniquensis*, we assumed here the occurrence of *L. martiniquensis*.

Co-infections in ticks of Guadeloupe and Martinique. In Guadeloupe, almost all *A. variegatum* (99.3%) were infected by at least one pathogen whereas only 39.8 – 56.6% of *Rh. microplus* were infected (Supplementary material 4). In contrast, 79.4 – 81.5% of *Rh. microplus* from Martinique were infected by at least one pathogen. High percentages of the two tick species were infected by one or two

pathogens (SD4). The percentages drastically felt down from co-infection by three pathogens with less than 10% of the ticks being concerned. One and nine *A. variegatum* and *Rh. Microplus*, from Guadeloupe and Martinique respectively, were infected by four pathogens and one *Rh. microplus* from Martinique was found infected by five pathogens. Only pathogens for which a sufficient number of infected ticks were detected were further considered for possible interactions in the ticks. These pathogens are *R. africae* and *T velifera* in *A. variegatum* of Guadeloupe and *T. velifera*, *An. marginale* and *Ehrlichia spp*. in *Rh. microplus* of Martinique. In *A. variegatum,* massively infected *by R. africae,* there are up to 40.1% of the ticks infected by *T. velifera*, suggesting that this pathogen is not excluded by *R. africae*. There is no difference in the infection rates of *T. velifera* in *Rh. microplus* of Guadeloupe and Martinique (24-26%). However, the infection rates of *An. marginale* and *Ehrlichia* spp. in *Rh. microplus* of Guadeloupe were much lower than the ones observed in Martinique: 4% for both pathogens in Guadeloupe versus 40 and 48% for *A. marginale* and *Ehrlichia* spp., respectively in Martinique. The detailed analyses of the presence of *T. velifera*, *An. marginale* and *Ehrlichia* spp. in the Martinique ticks did not show any significant biases in the infection rates between single versus dual-infected ticks, suggesting that none of these pathogens can interfere with the infection by the two others.

Discussion

In this study, we developed a high-throughput microfluidic real-time PCR system tool based on the use of multiple primers/probes allowing large scale survey of bacteria and parasites potentially involved in human and/or animal tick-borne diseases in the Caribbean. The association of genus and species designs targeting TBPs improved the screening capacity of the technology, allowing not only the identification of infectious agents known to circulate in the studied area, but also the detection of unsuspected TBPs or new microorganisms belonging to the main bacterial and parasitic genera/phylum involved in Tick-borne diseases worldwide. In addition to pathogens detection, genus designs may also detect potentially new and endosymbiotic microorganisms. As ticks may harbor endosymbiotic bacterial species that belong to genera including also known TBPs, such as the *Rickettsia* and *Coxiella* genus,

confirmations are still required before suggesting the presence of a pathogenic microorganism (Ahantarig *et al*., 2013; Duron *et al*., 2015; Bonnet *et al*., 2017). Cross-reactions have been observed for some of the species specific designs, and must be taken into account when interpreting results. Due to high design constraints and a lack of available sequences in public databases, the improvement of such cross-reacting oligonucleotides remain challenging. However, non-specific signals can be identified with the concomitant use of the bacterial and parasitic genera. Finally, as morphological identification of ticks collected on the field remains challenging, we developed sets of primers and probes allowing the molecular identification of the three main tick species involved in TBDs in the Caribbean, including *A. variegatum*, *Rh. microplus* and *R. sanguineus*.

The Biomark system developed here was used for a large-scale screening of TBPs in ticks collected in Guadeloupe and Martinique. The analysis gave a comprehensive overview of the epidemiological situation of 55 TBPs, and explored the diversity of microorganisms belonging to the main bacteria and parasite genera transmitted by ticks. The system was not only able to detect known TBPs of sanitary importance in the area, suggesting a high level of detection capacity of the assay for these pathogens, but it also detected unexpected microorganisms within Caribbean ticks.

Among the known TBPs detected, we found the four main pathogens responsible of ruminant diseases in the Caribbean – currently classified as notifiable diseases by the World Organisation of Animal Health (OIE-WOAH) - including *E. ruminantium* in *A. variegatum* specimens and *An. marginale*, *B. bigemina* and *B. bovis* in *Rh. microplus*.

Surprisingly, *E. ruminantium* infection rate in *A. variegatum* reported here was lower compared to previous studies conducted between 2003 and 2005 in Guadeloupe (5.1% versus 36.7%) (Vachiéry *et al*., 2008). Although sampling strategies and study designs were different, it could be interesting to further investigate if there is indeed a decrease in tick infection rate in Guadeloupe and the epidemiological consequences in term of incidence of heartwater cases in a situation where systematic TBP surveillance and control programs have stopped since the end of the eradication programs in 2006.

In this study, we reported the first infection rate records of *B. bigemina*, *B. bovis* and *An. marginale* in the vector tick *Rh. microplus* from the French Antilles. Indeed, records of such pathogens are mostly based on seroprevalence studies in cattle (Camus and Montenegro-James, 1994; Camus and

Barre, 1995; Alonso *et al*., 1992). However, *B. bigemina* and *B. bovis* infection rates in *Rh. microplus* ticks could have been underestimated. Indeed, *B. bigemina* is mainly transmitted by nymphs of *Rh. microplus* and secondarily by adults (Alonso *et al*., 1992). As well, *B. bovis* is mainly transmitted by larvae of *Rh. microplus* (Bock *et al*., 2004). As we only analyzed adult tick specimens, the probability of parasites detection may have decreased. TBPs screening on larvae or nymphs of *Rh. microplus* should be considered when evaluating parasites infection rate in ticks. Interestingly, we obtained a lower *An. marginale* infection rate in ticks from Guadeloupe than in ticks from Martinique. The same tendency have been reported during seroprevalence studies (Camus and Montenegro-James, 1994; Camus and Barre, 1995; Alonso *et al*., 1992). Moreover, although the sampling strategy here differed between the islands, *An. marginale* positive ticks were collected on only three of the 20 animals sampled (belonging to three different livestock) in Guadeloupe and on 24 of the 29 animals sampled (belonging to 24 different livestock) in Martinique. Together, the observations suggest that *An. marginale* is more prevalent in Martinique than in Guadeloupe. Anaplasmosis can be transmitted by other vectors than ticks and some cattle breeds are known to be more susceptible than others to *Anaplasma* infection. Therefore, the difference of prevalence could be explained by the difference in Cattle population in those two islands (mainly local Creole or mixed European-Creole breed in Guadeloupe known to be more resistant to anaplasmosis than Brahman and European breeds, mainly present in Martinique (Camus and Montenegro, 1994)). In addition, other factors, like differences in the population of other vectors such as flies could contribute to this difference and deserve further investigations.

Among the known TBPs detected, we also found less virulent pathogens – almost considered as endosymbionts - such as *R. africae*, *T. velifera* and *T. mutans* in their vector tick *A. variegatum* and surprisingly in *Rh. microplus* ticks.

With almost all the specimens collected infected, here is the higher infection rate report of *R. africae* in *A. variegatum* ticks reported in the Caribbean (Parola *et al*., 1999; 2003; Robinson *et al*., 2009; Kelly *et al*., 2010). As *A. variegatum* is both the vector and the reservoir of the pathogen, with an estimated 100% transovarial transmission efficacy rate, such a high level of *R. africae* infection rate was not surprising (Socolovshi *et al*., 2009; Kelly *et al*., 2010). Interestingly, the high *R. africae* infection rates in vector ticks, associated to a very low number of African tick bite Fever cases in the Caribbean

highlight the difficulty in some cases to clearly distinct endosymbiotic microorganisms from pathogens. Considered as an endosymbiont, the biological relationship between *R. africae* and *A. variegatum* should deserve more investigation, especially regarding the impact of bacteria on vector biology and pathogen transmission (Ahantarig *et al.*, 2013; Duron *et al.*, 2017). Considered as a pathogen, strains variety and virulence of *R. africae* in the Caribbean should be investigated in order to better assess the risks and guide prevention measures, especially for the travelers (Main *et al.*, 2014).

Here is the first update concerning the presence of *T. mutans* and *T. velifera* in Caribbean ticks. Indeed, references to these parasites in the Caribbean are quite old, and no prevalence studies, neither in ticks nor in cattle have been conducted since then (Morel *et al*., 1967; Uilenberg *et al*., 1983; Rodriguez *et al*., 1989). *T. mutans* and *T. velifera* are bovine parasites usually transmitted by *A. variegatum*. The first one can be responsible of mild theileriosis, and the last one is considered as non-pathogenic. The lack of pathogenicity of these piroplasms may explain the few information available concerning their distribution and prevalence in the Caribbean. However, these parasites species may be important in theileriosis management and protection. Indeed recent study suggests that cattle chronically infected with *T. velifera* and/or *T. mutans* develop immunity and heterologous protection against other pathogenic *Theileria* species, such as *Theileria parva* (Woolhouse *et al*., 2015).

Unexpectedly, *R. africae*, *T. velifera* and *T. mutans* were also found in *Rh. microplus* ticks. Detection signal were associated to higher Cp values than those observed in *A. variegatum*. As *Rh. microplus* in contrast to *A. variegatum*, is not considered as vector of those TBPs, tick host may play a central role in the epidemiological meaning of this result. Indeed, most of the positive *Rh. microplus* were collected on the same animal than positive *A. variegatum* and ticks were partially engorged. Thus, microorganisms circulating in cattle blood, can also make positive ticks engorging on these animals (Matsimbe *et al*., 2017). As well, the presence of such TBPs in *Rh. microplus* may result from a cross-contamination during tick co-feeding (Yssouf *et al*., 2014). So, *R. africae*, *T. velifera* and *T. mutans* lightly detected in *Rh. microplus* samples could represent TBP's traces from the midgut of partially engorged ticks. Thus, screening pathogens in such ticks, could lead to the detection of pathogens present in the host blood, but not able to replicate into ticks, or to be transmitted by them (Estrada-Peña *et al.*, 2013; Yssouf *et al*., 2014; Matsimbe *et al*., 2017).

Finally, some unexpected results were obtained with genus designs, leading to the detection and nucleotides sequences recovery of *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp. and *Leishmania* spp. in ticks collected in Guadeloupe and Martinique.

Both the *Ehrlichia* sp. Tick428 sequence (accession number MK049849) and the *Anaplasma* sp. Tick314 (accession number MK049845) identified here clustered with sequences belonging to uncharacterized species. Few information are available on those uncharacterized microorganisms and confirmations are still required before suggesting the presence of new bacteria. Those observations highlight the need to set up characterization studies. Indeed, high-throughput detection technologies can highlight the presence of potential new microorganisms, but it is still necessary to be able to isolate and characterize them in order to determine if there is a sanitary risk related to their presence in ticks, especially when those microorganisms belong to bacterial genera involved in TBDs worldwide.

We reported her for the first time the detection of *Borrelia* spp. in ticks from the Guadeloupe and Martinique. *Borrelia* spp. reports in the Caribbean are scarce. In Cuba, one study suggested the presence of *Borrelia burgdorferi sensu stricto* antibodies in human serum associated with clinical cases of Lyme disease-like syndrome (Rodríguez *et al.*, 2004, 2012). However, the real seropositivity of the sample was contested (Dessau, 2012). In the US Virgin Islands, seropositivity to *Borrelia hermsii* and closely related species have been reported in association with a human case of relapsing fever (Flanigan *et al.*, 1991). Here, we were able to recover two different sequences. The *Borrelia* sp. Tick7 (accession number MK049846) sequence identified in *A. variegatum* ticks from Guadeloupe was found closely related to *Bo. anserina*, the agent of avian spirochetosis, forming an intermediate cluster between the relapsing fever and the Lyme disease group. The *Borrelia* sp. Tick457 sequence (accession number MK049847) identified in *Rh. microplus* from Martinique, clustered with uncharacterized *Borrelia* spp. detected in *Rh. microplus* ticks from Madagascar and Brazil and Relapsing Fever *Borrelia* species encountered in hard ticks, including *Borrelia lonestari* and *Bo. theileri* (Yparraguirre *et al.*, 2007; Hagen *et al.*, 2017). Interestingly, the same observations were made recently about *Borrelia* spp. found in *A. variegatum* and *Rh. microplus* ticks from Ethiopia and Côte d' Ivoire (Kumsa *et al*., 2015; Ehounoud *et al.*, 2016). As *A. variegatum* and *Rh. microplus* were imported in the Caribbean from Africa during the time of the Atlantic triangular trade, potentially new *Borrelia* species found here probably originate

from old introductions that since then have evolved within their vector over a long period (Barré and Uilenberg, 2012; Estrada-Peña *et al.*, 2018).

Finally, Leishmania spp. were detected in *Rh. microplus* specimens from Martinique, and a sequence identified as *Leishmania martiniquensis* Tick389 (accession number MK049850) was recovered. Parasites of the genus *Leishmania* are usually transmitted by female phlebotomine sand flies and are responsible of human disease called Leishmaniasis, widespread in tropical and sub-tropical areas (Steverding, 2017). *L. martiniquensis* belongs to the *Leishmania enriettii* complex and have been described in Martinique and in Thailand, where it was responsible of both cutaneous leishmaniosis and visceral leishmaniosis (Desbois *et al.*, 2014; Pothirat *et al.*, 2014; Liautaud *et al.*, 2015; Steverding, 2017). *L. martiniquensis* is suspected to be endemic in Martinique (Desbois *et al.*, 2014). Although phlebotomines and rodents are present in Martinique, neither a vector nor a reservoir of this parasite have been yet described (Desbois *et al.*, 2014). Here is the first report of *L. martiniquensis* in *Rh. microplus* ticks. The role of ticks in *Leishmania* transmission is still a debate (Dantas-Torres, 2011; Rakhshanpour *et al.*, 2017) even if *Leishmania infantum* have been detected in *R. sanguineus* ticks, and more recently *Leishmania guyanensis* have been detected in *Rh. microplus* ticks in Peru (Dantas-Torres, 2011; Rojas-Jaimes *et al.*, 2017). Although there is no evidence of vector capacity yet, and that ticks analyzed here were partially engorged, the participation of ticks in *Leishmania* epidemiology deserve further investigation, especially since *Rh. microplus* ticks could parasitizing human (Lamattina and Nava., 2016).

To conclude, although screening tools are useful for pathogen discovery in ticks, the epidemiological meaning of such results warrants further investigations. Detecting microorganisms DNA in ticks, especially in partially engorged ticks, does not necessarily mean that the tick is involved in the microorganism's life cycle and it cannot replace vector competence studies (Estrada-Peña *et al.*, 2013). Nevertheless, the detection of potentially new microorganisms in ticks from the French Antilles open new research perspectives on TBPs epidemiology in the Caribbean for the future that should start by a large-scale epidemiological survey on TBPs in ticks collected in different islands of the Caribbean area to strengthen our results.

Our study demonstrated the high ability of microfluidic real time PCR technology to give a rapid overview of the diversity of TBPs of veterinary and medical importance present in ticks from the Caribbean. This innovative high-throughput tool is a major improvement in TBPs surveillance study, allowing a rapid screening especially in regions where few epidemiological data are available.

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Author contributions

MG wrote the paper. MVT, EA, JP and SM reviewed the manuscript. EA, JP and SM designed the experiment and supervised the manuscript. MG performed the experiment. ED, VP, RA, CG, MVT contributed to sampling and sample preparation. SD contributed to technical support.

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Table 1: List of the 61 primers/probe sets and their targets constituting the Biomark system developed in this study, with the list of the positive controls used for their validation. (In grey: Design from Michelet *et al*., 2014; In white: Design developed in this study; **: include all the controls belonging to the genus described in the table and targeted by specific design). Plasmids used as control are recombinant pBluescript IISK + containing the target gene.

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
***Rickettsia* spp.**	*gltA*	Rick_spp_gltA_F	GTCGCAAATGTTCACGGTACTT	78	**, Culture of *R. slovaca*
		Rick_spp_gltA_R	TCTTCGTGCATTTCTTTCCATTG		
		Rick_spp_gltA_P	TGCAATAGCAAGAACCGTAGGCTGGATG		
Rickettsia massiliae	ITS	Ri_ma_ITS_F	GTTATTGCATCACTAATGTTATACTG	128	**Culture**
		Ri_ma_ITS_R	GTTAATGTTGTTGCACGACTCAA		
		Ri_ma_ITS_P	TAGCCCCGCCACGATATCTAGCAAAAA		
Rickettsia rickettsii	ITS	Ri_ri_ITS_F	TCTACTCACAAAGTTATCAGGTTAA	124	**Plasmid**
		Ri_ri_ITS_R	CCTACGATACTCAGCAAAATAATTT		
		Ri_ri_ITS_P	TCGCTGGATATCGTTGCAGGACTACAG		
Rickettsia conorii	*sca1*	Ri_co_sca1_F	GTAGATGCTTCATAGAATACTGC	88	***Rhipicephalus sanguineus* naturally infected**
		Ri_co_sca1_R	CCAAATTTAGTCTACCTTGTGATC		
		Ri_co_sca1_P	TCCTCCTGACGTATTAAAAGAAGCTGAAGCT		
Rickettsia africae	*sca1*	Ri_af_sca1_F	GATACGACAAGTACCTCGCAG	122	**Culture**
		Ri_af_sca1_R	GGATTATATACTTTAGGTTCGTTAG		
		Ri_af_sca1_P	CAGATAGGAACAGTAATTGTAACGGAACCAG		
Rickettsia felis	*orfB*	Ri_fel_orfB_F	ACCCTTTTCGTAACGCTTTGC	163	**Culture**
		Ri_fel_orfB_R	TATACTTAATGCTGGGCTAAACC		
		Ri_fel_orfB_P	AGGGAAACCTGGACTCCATATTCAAAAGAG		
Rickettsia typhi	*ompB*	Ri_typ_ompB_F	CAGGTCATGGTATTACTGCTCA	133	**Culture**
		Ri_typ_ompB_R	GCAGCAGTAAAGTCTATTGATCC		
		Ri_typ_ompB_P	ACAAGCTGCTACTACAAAAAGTGCTCAAAATG		
Rickettsia prowazekii	*gltA*	Ri_pro_gltA_F	CAAGTATCGGTAAAGATGTAATCG	151	**Plasmid**
		Ri_pro_gltA_R	TATCCTCGATACCATAATATGCC		
		Ri_pro_gltA_P	ATATAAGTAGGGTATCTGCGGAAGCCGAT		
***Borrelia* spp.**	23S rRNA	Bo_bu_sl_23S_F	GAGTCTTAAAAGGGCGATTTAGT	73	** **Culture of *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. recurrentis***
		Bo_bu_sl-23S_R	CTTCAGCCTGGCCATAAATAG		
		Bo_bu_sl_23S_P	TAGATGTGGTAGACCCGAAGCCGAGT		
Borrelia burgdorferi sensu stricto	*glpA*	Bo_bu_glpA_F	GCAATTACAAGGGGGTATAAAGC	206	**Culture**
		Bo_bu_glpA_R	GGCGTGATAAGTGCACATTCG		
		Bo_bu_glpA_P	TTAATTAAACGGGGTGCATTCTTCTCAAGAATG		
Borrelia anserina	*fla*	Bor_ans_fla_F	GGAGCACAACAAGAGGGAG	76	**Plasmid**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
		Bor_ans_fla_R	TTGGAGAATTAACCCCACCTG		
		Bor_ans_fla_P	TGCAAGCAACTCCAGCTCCAGTAGCT		
Borrelia lonestari	*glpQ*	Bor_lon_glpQ_F	GATCCAGAACTTGATACAACCAC	99	***Amblyomma americanum* naturally infected**
		Bor_lon_glpQ_R	TTCATCTAGTGAGAAGTCAGTAG		
		Bor_lon_glpQ_P	AGTAATATCGTCCGTCTTCCCTAGCTCG		
Borrelia parkeri	*gyrB*	Bor_par_gyrB_F	GCAAAACGATTCAAAGTGAGTCC	184	**Culture**
		Bor_par_gyrB_R	CTCATTGCCTTTAAGAAACCACTT		
		Bor_par_gyrB_P	TTAAAACCAGCAACATGAGTTCCTCCTTCTC		
Borrelia bissettii	*rpoB*	Bo_bi_rpoB_F	GCAACCAGTCAGCTTTCACAG	118	**Plasmid**
		Bo_bi_rpoB_R	CAAATCCTGCCCTATCCCTTG		
		Bo_bi_rpoB_P	AAAGTCCTCCCGGCCCAAGAGCATTAA		
Borrelia theileri	*glpQ*	Bo_th_glpQ_F	GTGCTAACAAAGGACAATATTCC	213	**Plasmid**
		Bo_th_glpQ_R	GGTTAGTGGAAAACGGTTAGGAT		
		Bo_th_glpQ_P	TATTATAATTCACGAGCCAGAGCTTGACAC		
***Bartonella* spp.**	*ssrA*	Bart_spp_ssrA_F	CGTTATCGGGCTAAATGAGTAG	118	****, Culture of *B. quintana***
		Bart_spp_ssrA_R	ACCCCGCTTAAACCTGCGA		
		Bart_spp_ssrA_P	TTGCAAATGACAACTATGCGGAAGCACGTC		
Bartonella barcilliformis	*rpoB*	Ba_ba_rpoB_F	GAAGAGTTTGTAGTTTGTCGTCA	105	**Culture**
		Ba_ba_rpoB_R	AGCAGCTACAGAAACCAACTG		
		Ba_ba_rpoB_P	TGCAGGTGAAGTTTTGATGGTGCCACG		
Bartonella henselae	*ribC*	Bar_he_ribC_F	GGGATGCGATTTAATAGTTCTAC	116	**Culture**
		Bar_he_ribC_R	CGCTTGTTGTTTTGATCCTCG		
		Bar_he_ribC_P	ACGTTATAGTAGCGAAAACTTAGAAATTGGTGC		
Bartonella vinsonii* subsp. *berkhoffii	ITS	Bar_vin_ITS_F	GGAATTGCTTAACCCACTGTTG	141	**Culture**
		Bar_vin_ITS_R	CCTTATTGATTTAGATCTGATGGG		
		Bar_vin_ITS_P2	AGAAACTCCCGCCTTTATGAGAGAAATCTCT		
***Coxiella burnetii* and *Coxiella*-like**	*Icd*	Co_bu_icd_F	AGGCCCGTCCGTTATTTTACG	74	**Culture**
		Co_bu_icd_R	CGGAAAATCACCATATTCACCTT		
		Co_bu_icd_P	TTCAGGCGTTTTGACCGGGCTTGGC		
	IS1111	Co_bu_IS111_F	TGGAGGAGCGAACCATTGGT	86	**Culture**
		Co_bu_IS111_R	CATACGGTTTGACGTGCTGC		
		Co_bu_IS111_P	ATCGGACGTTTATGGGGATGGGTATCC		
***Francisella tularensis* and *Francisella*-like endosymbionts**	*tul4*	Fr_tu_tul4_F	ACCCACAAGGAAGTGTAAGATTA	76	**Culture**
		Fr_tu_tul4_R	GTAATTGGGAAGCTTGTATCATG		
		Fr_tu_tul4_P	AATGGCAGGCTCCAGAAGGTTCTAAGT		
	fopA	Fr_tu_fopA_F	GGCAAATCTAGCAGGTCAAGC	91	**Culture**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
		Fr_tu_fopA_R	CAACACTTGCTTGAACATTTCTAG		
		Fr_tu_fopA_P	AACAGGTGCTTGGGATGTGGGTGGTG		
***Anaplasma* spp.**	16S rRNA	Ana_spp_16S_F	CTTAGGGTTGTAAAACTCTTTCAG	160	**
		Ana_spp_16S_R	CTTTAACTTACCAAACCGCCTAC		
		Ana_spp_16S_P	ATGCCCTTTACGCCCAATAATTCCGAACA		
Anaplasma marginale	*msp1b*	An_ma_msp1_F	CAGGCTTCAAGCGTACAGTG	85	**Blood of an experimentally infected cow**
		An_ma_msp1_R	GATATCTGTGCCTGGCCTTC		
		An_ma_msp1_P	ATGAAAGCCTGGAGATGTTAGACCGAG		
Anaplasma phagocytophilum	*msp2*	An_ph_msp2_F	GCTATGGAAGGCAGTGTTGG	77	***Ixodes* spp. tick naturally infected**
		An_ph_msp2_R	GTCTTGAAGCGCTCGTAACC		
		An_ph_msp2_P	AATCTCAAGCTCAACCCTGGCACCAC		
Anaplasma platys	*groEL*	An_pla_groEL_F	TTCTGCCGATCCTTGAAAACG	75	**Blood of infected dog**
		An_pla_groEL_R	CTTCTCCTTCTACATCCTCAG		
		An_pla_groEL_P	TTGCTAGATCCGGCAGGCCTCTGC		
Anaplasma bovis	*groEL*	An_bo_groEL_F	GGGAGATAGTACACATCCTTG	73	**Plasmid**
		An_bo_groEL_R	CTGATAGCTACAGTTAAGCCC		
		An_bo_groEL_P	AGGTGCTGTTGGATGTACTGCTGGACC		
Anaplasma ovis	*msp4*	An_ov_msp4_F	TCATTCGACATGCGTGAGTCA	92	**Plasmid**
		An_ov_msp4_r	TTTGCTGGCGCACTCACATC		
		An_ov_msp4_P	AGCAGAGAGACCTCGTATGTTAGAGGC		
***Ehrlichia* spp.**	16S rRNA	Neo_mik_16S_F	GCAACGCGAAAAACCTTACCA	98	**
		Neo_mik_16S_R	AGCCATGCAGCACCTGTGT		
		Neo_mik_16S_P	AAGGTCCAGCCAAACTGACTCTTCCG		
Ehrlichia canis	*gltA*	Eh_ca_gltA_F	GACCAAGCAGTTGATAAAGATGG	136	**Culture**
		Eh_ca_gltA_R	CACTATAAGACAATCCATGATTAGG		
		Eh_ca_gltA_P	ATTAAAACATCCTAAGATAGCAGTGGCTAAGG		
Ehrlichia chaffeensis	*dsb*	Eh_ch_dsb_F	TATTGCTAATTACCCTCAAAAAGTC	117	***Amblyomma americanum* naturally infected**
		Eh_ch_dsb_R	GAGCTATCCTCAAGTTCAGATTT		
		Eh_ch_dsb_P	ATTGACCTCCTAACTAGAGGGCAAGCA		
Ehrlichia ewingii	*dsb*	Eh_ew_dsb_F	CAATACTTGGAGAAGCATCATTG	111	***Amblyomma americanum* naturally infected**
		Eh_ew_dsb_R	TTGCTTATGGCTTAATGCTGCAT		
		Eh_ew_dsb_P	AAAGCAGTACGTGCAGCATTGGCTGTA		
Ehrlichia ruminantium	*gltA*	Eh_ru_gltA_F	CCAGAAAACTGATGGTGAGTTAG	116	**Culture**
		Eh_ru_gltA_R	AGCCTACATCAGCTTGAATGAAG		
		Eh_ru_gltA_P	AGTGTAAACTTGCTGTTGCTAAGGTAGCATG		
	gltA	Eh_PME_gltA_F	GCTAGTTATGAGTTAGAATGTAAAC	121	

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
Panola Mountain Ehrlichia		Eh_PME_gltA_R	TACTATAGGATAATCTTGAATCAGC		***Amblyomma americanum* naturally infected**
		Eh_PME_gltA_P	TTGCTATCGCTAAAATTCCAAGTATGATTGCG		
Neoehrlichia mikurensis	*groEL*	Neo_mik_groEL_F	AGAGACATCATTCGCATTTTGGA	96	**Blood of infected rodent**
		Neo_mik_groEL_R	TTCCGGTGTACCATAAGGCTT		
		Neo_mik_groEL_P	AGATGCTGTTGGATGTACTGCTGGACC		
Aegyptianella pullorum	*groEL*	Ae_pul_groEL_F	AGCCAGTATTATCGCTCAAGG	168	**Plasmid**
		Ae_pul_groEL_R	GCCTCACGTGCCTTCATAAC		
		Ae_pul_groEL_P	TGCTTCTCAGTGTAACGACAGGGTTGG		
Apicomplexa	18S rRNA	Apic_18S_F	TGAACGAGGAATGCCTAGTATG	104	**** Blood of Infected dog, with *B. canis rossi, B. canis canis*; Culture of *B. divergens, T. lestoquari, T. annulata***
		Apic_18S_R	CACCGGATCACTCGATCGG		
		Apic_18S_S	TAGGAGCGACGGGCGGTGTGTAC		
Babesia canis vogeli	*hsp70*	Ba_vo_hsp70_F	TCACTGTGCCTGCGTACTTC	87	**Blood of infected dog**
		Ba_vo_hsp70_R	TGATACGCATGACGTTGAGAC		
		Ba_vo_hsp70_P	AACGACTCCCAGCGCCAGGCCAC		
Babesia ovis	18S rRNA	Ba_ov_18S_F	TCTGTGATGCCCTTAGATGTC	92	**Plasmid**
		Ba_ov_18S_R	GCTGGTTACCCGCGCCTT		
		Ba_ov_18S_P	TCGGAGCGGGGTCAACTCGATGCAT		
Babesia bigemina	18S rRNA	Ba_big_RNA18S_F	ATTCCGTTAACGAACGAGACC	99	**Plasmid**
		Ba_big_RNA18S_R	TTCCCCCACGCTTGAAGCA		
		Ba_big_RNA18S_P	CAGGAGTCCCTCTAAGAAGCAAACGAG		
Babesia gibsoni	*Rap1*	Ba_gib_rap1_F	CTCTTGCTCATCATCTTTTCGG	130	**Plasmid**
		Ba_gib_rap1_R	TCAGCGTATCCATCCATTATATG		
		Ba_gib_rap1_S	TTTAATGCGTGCTACGTTGTACTTCCCAAAG		
Babesia caballi	*Rap1*	Ba_cab_rap1_F	GTTGTTCGGCTGGGGCATC	94	**Plasmid**
		Ba_cab_rap1_R	CAGGCGACTGACGCTGTGT		
		Ba_cab_rap1_P	TCTGTCCCGATGTCAAGGGGCAGGT		
Babesia bovis	*CCTeta*	Ba_bo_CCTeta_F	GCCAAGTAGTGGTAGACTGTA	100	**Plasmid**
		Ba_bo_CCTeta_R	GCTCCGTCATTGGTTATGGTA		
		Ba_bo_CCTeta_P	TAAAGACAACACTGGGTCCGCGTGG		
Babesia duncani	ITS2	Ba_du_ITS_F	ATTTCCGTTTGCGAGAGTTGC	87	**Plasmid**
		Ba_du_ITS_R	AGGAAGCATCAAGTCATAACAAC		
		Ba_du_ITS_P	AACAAGAGGCCCCGAGATCAAGGCAA		
Babesia microti	*CCTeta*	Bab_mi_CCTeta_F	ACAATGGATTTTCCCCAGCAAAA	145	**Culture**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
		Bab_mi_CCTeta_R	GCGACATTTCGGCAACTTATATA		
		Bab_mi_CCTeta_P	TACTCTGGTGCAATGAGCGTATGGGTA		
Theileria parva	18S rRNA	Th_pa_18S_F	GAGTATCAATTGGAGGGCAAG	173	**Culture**
		Th_pa_18S_R	CAGACAAAGCGAACTCCGTC		
		Th_pa_18S_P	AAATAAGCCACATGCAGAGACCCCGAA		
Theileria mutans	ITS	The_mu_ITS_F	CCTTATTAGGGGCTACCGTG	119	**Plasmid**
		The_mu_ITS_R	GTTTCAAATTTGAAGTAACCAAGTG		
		The_mu_ITS_P	ATCCGTGAAAAACGTGCCAAACTGGTTAC		
Theileria velifera	18S rRNA	The_ve_18S_F	TGTGGCTTATCTGGGTTCGC	151	**Plasmid**
		The_ve_18S_R	CCATTACTTTGGTACCTAAAACC		
		The_ve_18S_P	TTGCGTTCCCGGTGTTTTACTTTGAGAAAG		
Theileria equi	*ema1*	Th_eq_ema1_F4	CGGCAAGAAGCACACCTTC	167	**Plasmid**
		Th_eq_ema1_R4	TGCCATCGCCCTTGTAGAG		
		Th_eq_ema1_P4	AAGGCTCCAGGCAAGCGCGTCCT		
Cytauxzoon felis	ITS2	Cy_fel_ITS2_F	AAGATCCGAACGGAGTGAGG	119	**Plasmid**
		Cy_fel_ITS2_R	GTAGTCTCACCCAATTTCAGG		
		Cy_fel_ITS2_S	AAGTGTGGGATGTACCGACGTGTGAG		
***Hepatozoon* spp.**	18S rRNA	Hepa_spp_18S_F	ATTGGCTTACCGTGGCAGTG	175	******
		Hepa_spp_18S_R	AAAGCATTTTAACTGCCTTGTATTG		
		Hepa_spp_18S_S	ACGGTTAACGGGGGATTAGGGTTCGAT		
Hepatozoon canis	18S rRNA	He_can_18S_F	TTCTAACAGTTTGAGAGAGGTAG	221	**Blood of infected dog**
		He_can_18S_R	AGCAGACCGGTTACTTTTAGC		
		He_can_18S_S	AGAACTTCAACTACGAGCTTTTTAACTGCAAC		
Hepatozoon americanum	18S rRNA	He_ame_18S_F2	GGTATCATTTTGGTGTGTTTTTAAC	159	**Plasmid**
		He_ame_18S_R2	CTTATTATTCCATGCTCCAGTATTC		
		He_ame_18S_P2	AAAAGCGTAAAAGCCTGCTAAAAACACTCTAC		
***Leishmania* spp.**	*hsp70*	Leish_spp_hsp70_F	CGACCTGTTCCGCAGCAC	78	**** and Culture of *L. martiniquensis***
		Leish_spp_hsp70_R	TCGTGCACGGAGCGCTTG		
		Leish_spp_hsp70_S	TCCATCTTCGCGTCCTGCAGCACG		
Leishmania infantum	ITS	Le_inf_ITS_F	CGCACCGCCTATACAAAAGC	103	**Culture**
		Le_inf_ITS_R	GTTATGTGAGCCGTTATCCAC		
		Le_inf_ITS_S	ACACGCACCCACCCCGCCAAAAAC		
Rangelia vitalii	18S rRNA	Ra_vit_18S_F	TAACCGTGCTAATTGTAGGGC	92	**Plasmid**
		Ra_vit_18S_R	GAATCACCAAACCAAATGGAGG		
		Ra_vit_18S_S	TAATACACGTTCGAGGGCGCGTTTTGC		
***Tick* spp.**		Tick_spp_16S_F	AAATACTCTAGGGATAACAGCGT	99	******

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
	16S rRNA	Tick_spp_16S_R	TCTTCATCAAACAAGTATCCTAATC		
		Tick_spp_16S_P	CAACATCGAGGTCGCAAACCATTTTGTCTA		
Amblyomma variegatum	ITS2	Amb_var_ITS2_F	GCCAGCCTCTGAAGTGACG	117	**Wild tick from Guadeloupe**
		Amb_var_ITS2_R	TTCTGCGGTTTAAGCGACGC		
		Amb_var_ITS2_P	TCTTGCCACTCGACCCGTGCCTC		
Rhipicephalus microplus	ITS2	Rhi_mic_ITS2_F	GCTTAAGGCGTTCTCGTCG	144	**Wild tick from Galapagos Islands**
		Rhi_mic_ITS2_R	CAAGGGCAGCCACGCAG		
		Rhi_mic_ITS2_P	TAGTCCGCCGTCGGTCTAAGTGCTTC		
Rhipicephalus sanguineus	ITS2	Rhi_san_ITS2_F	TTGAACGCTACGGCAAAGCG	110	**Wild tick from France**
		Rhi_san_ITS2_R	CCATCACCTCGGTGCAGTC		
		Rhi_san_ITS2_P	ACAAGGGCCGCTCGAAAGGCGAGA		

Table 2: Primers used to confirm the presence of pathogenic DNA in tick samples, and positive controls.

Pathogen	Targeted gene	Primer name	Sequence (5' → 3')	Amplicon size (bp)	References
Rickettsia spp.	*gltA*	Rsfg877	GGGGGCCTGCTCACGGCGG	381	Regnery *et al.*, 1991
		Rsfg1258	ATTGCAAAAAGTACAGTGAACA		
	ompB	Rc.rompB.4362p	GTCAGCGTTACTTCTTCGATGC	475	Choi *et al.*, 2005
		Rc.rompB.4,836n	CCGTACTCCATCTTAGCATCAG		
		Rc.rompB.4,496p	CCAATGGCAGGACTTAGCTACT	267	
		Rc.rompB.4,762n	AGGCTGGCTGATACACGGAGTAA		
Anaplasma/Ehrlichia spp.	16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	345	Hornok *et al.*, 2008
		EHR16SR	TAGCACTCATCGTTTACAGC		
***Borrelia* spp.**	flaB	FlaB280F	GCAGTTCARTCAGGTAACGG	645	Loh *et al.*, 2016
		FlaRL	GCAATCATAGCCATTGCAGATTGT		
		FlaB737F	GCATCAACTGTRGTTGTAACATTAACAGG	407	
		FlaLL	ACATATTCAGATGCAGACAGAGGT		
Leishmania spp.	SSU rRNA	R221	GGTTCCTTTCCTGATTTACG	603	Albuquerque *et al.*, 2017
		R332	GGCCGGTAAAGGCCGAATAG		
		R223	TCCATCGCAACCTCGGTT	358	
		R333	AAAGCGGGCGCGGTGCTG		

Table 3: Homology between deposited sequences and reference sequences in GenBank. T: number of samples tested; S: number of samples obtained after sequencing; AN: Accession number; L: Lenght (bp); ID%: percentage of identity.

Biomark Id	Sequence name	T	S	AN	L	Closest homology	ID%	Reference
***Rickettsia* spp.**	*Rickettsia africae* Tick208	30	14	MK049851	248	*Rickettsia africae*	100	AF123706.1
***Leishmania* spp.**	*Leishmania martiniquensis* Tick389	2	1	MK049850	272	*Leishmania martiniquensis*	100	AF303938.1
						Leishmania siamensis	100	GQ226033.1
***Borrelia* spp.**	*Borrelia* sp. Tick7	30	1	MK049846	245	*Borrelia anserina*	90	X75201.1
	Borrelia sp. Tick457		4	MK049847	327	*Borrelia sp. BR*	100	EF141022.1
						Borrelia sp. strain Mo063b-flaB	100	KY070335.1
						Borrelia theileri	99	KF569936.1
***Anaplasma* spp.**	*Anaplasma* sp. Tick314	2	2	MK049845	245	*Candidatus Anaplasma boleense*	100	KX987335.1
Anaplasma marginale	*Anaplasma sp.* Tick283	2	2	MK049844	244	*Anaplasma marginale*	100	MH155593.1
						Anaplasma centrale	100	MF289482.1
						Anaplasma ovis	100	MG770440.1
						Anaplasma capra	100	MF000917.1
						Anaplasma phagocytophilum	100	DQ648489.1
***Ehrlichia* spp.**	*Ehrlichia* sp. Tick428	2	2	MK049849	246	*Ehrlichia spp.*	100	KY594915.1*
						Ehrlichia canis	99	KY594915.1
						Ehrlichia ewingii	99	U96436.1
						Ehrlichia chaffeensis	99	NR_074500.2
						Ehrlichia muris	99	KU535865.1
						Ehrlichia minasensis	99	NR_148800.1
Ehrlichia ruminantium	*Ehrlichia ruminantium* Tick116	1	1	MK049848	207	*Erlichia ruminantium*	100	NR_074155.1
Babesia bigemina	*Babesia bigemina* Tick222	2	1	MK071738	99	*Babesia bigemina*	100	KP710227.1
Babesia bovis	*Babesia bovis* Tick497	2	2	MK071739	100	*Babesia bovis*	99	AB367921.1

* Various *Ehrlichia* spp. including KY046298.1, MG346223.1, KX987325.1, KX987324.1, KX987323.1, KX987322.1, KX577724.1, KJ410257.1, KJ410255.1, JX402605.1, AY309970.1, AY309969.1, DQ379970.1, AF311968.1, AF311967.1 and AF497581.1.

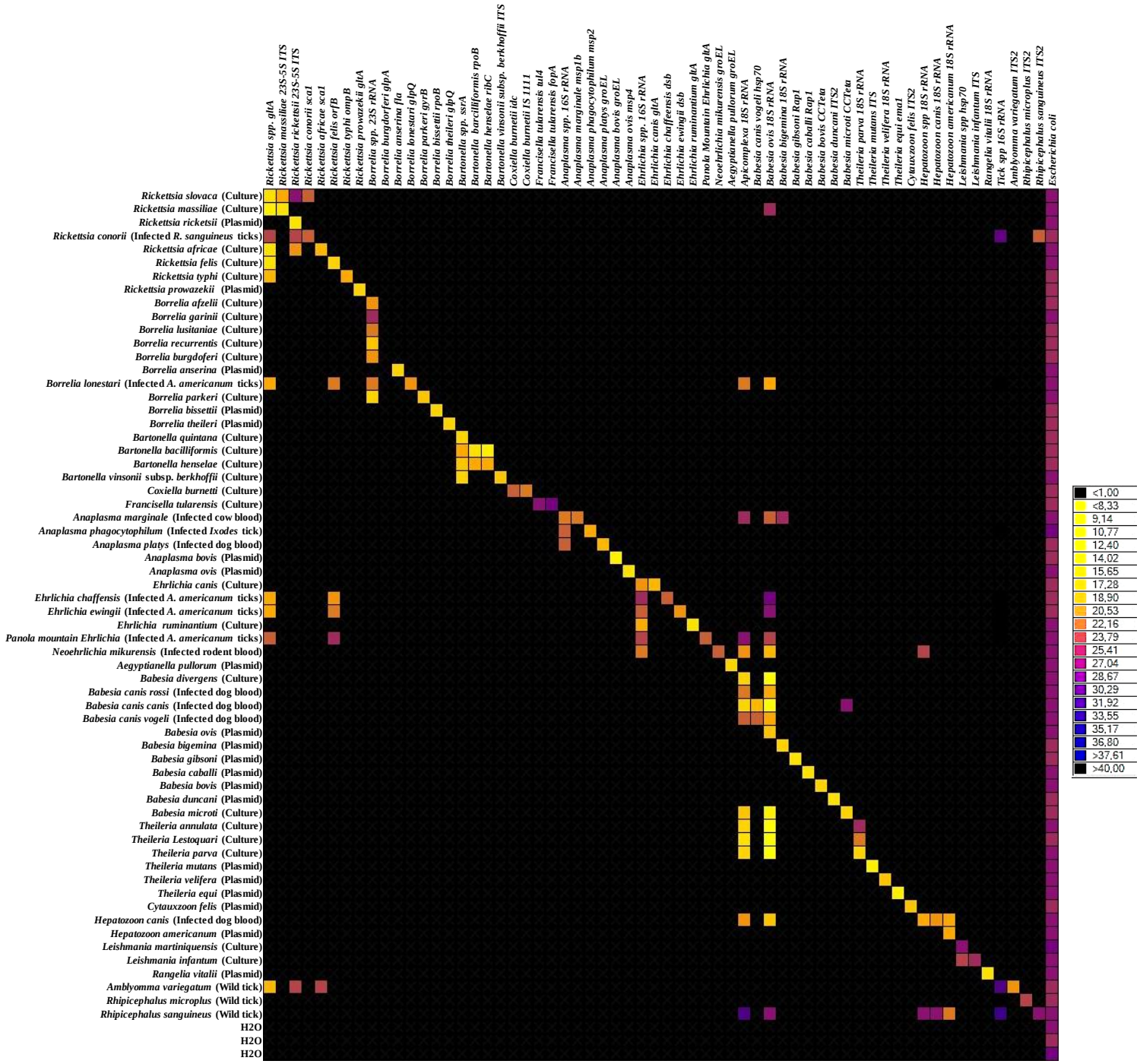
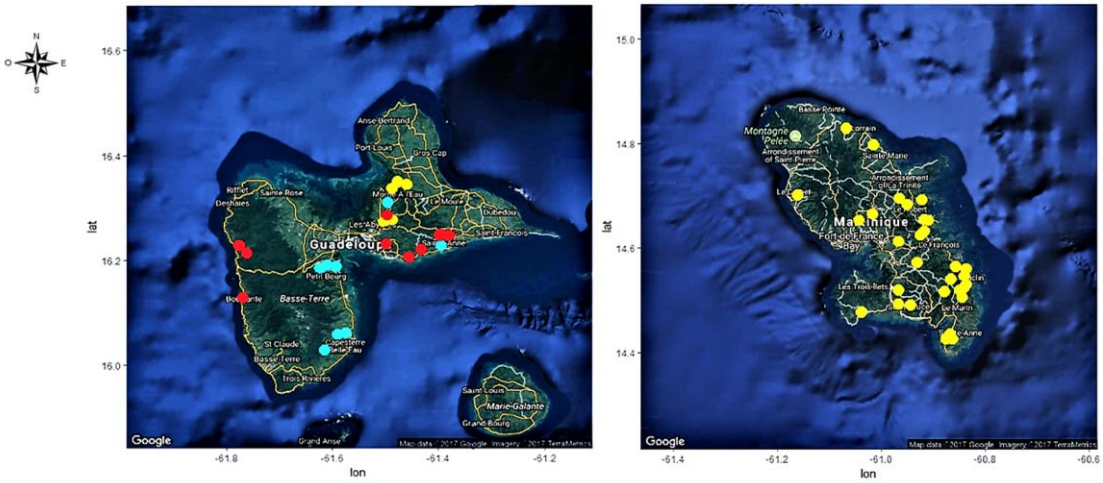


Figure 1. BioMark™ dynamic array system specificity test (96.96 chip). Each square corresponds to a single real-time PCR reaction, where rows indicate the pathogen in the sample and columns represent the target of the primers/probe set. C_t values for each reaction are represented by a color gradient; the color scale is shown on the right y-axis. The darkest shades of blue and black squares are considered as negative reactions with $C_t > 30$.



	Guadeloupe		Martinique
	Positive Amblyomma variegatum (IR)	Positive *Rhipicephalus microplus* min – max (IR)	
Anaplasma spp.	0	8 (4.8%) - 9 (5.4%)	112 (39.9%) - 116 (41.3%)
Anaplasma marginale	0	6 (3.6%) - 8 (4.8%)	111 (39.5%) - 116 (41.3%)
Ehrlichia spp.	11 (8%)	7 (4.2%) - 11 (6.6%)	134 (47.7%) - 138 (49.1%)
Ehrlichia ruminantium	7 (5.1%)	0	0
Borrelia spp.	7 (5.1%)	1 (0.6%)	12 (4.3%)
Rickettsia spp.	135 (98.5%)	26 (15.7%) - 39 (23.5%)	0
Rickettsia africae	131 (95.6%)	0	0
Leishmania spp.	0	0	2 (0.7%)
Theileria mutans	2 (1.5%)	3 (1.8%) - 4 (2.4%)	4 (1.4%)
Theileria velifera	58 (42.3%)	40 (24.1%) - 53 (31.9%)	72 (25.6%) - 73 (26%)
Babesia bigemina	0	1 (0.6%) - 2 (1.2%)	35 (12.5%) - 36 (12.8%)
Babesia bovis	0	0	2 (0.7%)

Figure 2. Number of positive ticks *Amblyomma variegatum* (out of the 137), *Rhipicephalus microplus* from Guadeloupe (out of 166) and Martinique (out of 281). Dots on the map indicate the tick collection sites in Guadeloupe and Martinique. Yellow: collection site of *Rhipicephalus (Boophilus) microplus* ticks; Red: collection site of *Amblyomma variegatum*; Blue: collection site of both tick species, sometimes on the same animal. IR: Infection rate. As some samples of *Rhipicephalus (B.) microplus* were pooled, we present minimum and maximum infection rates of infected ticks.

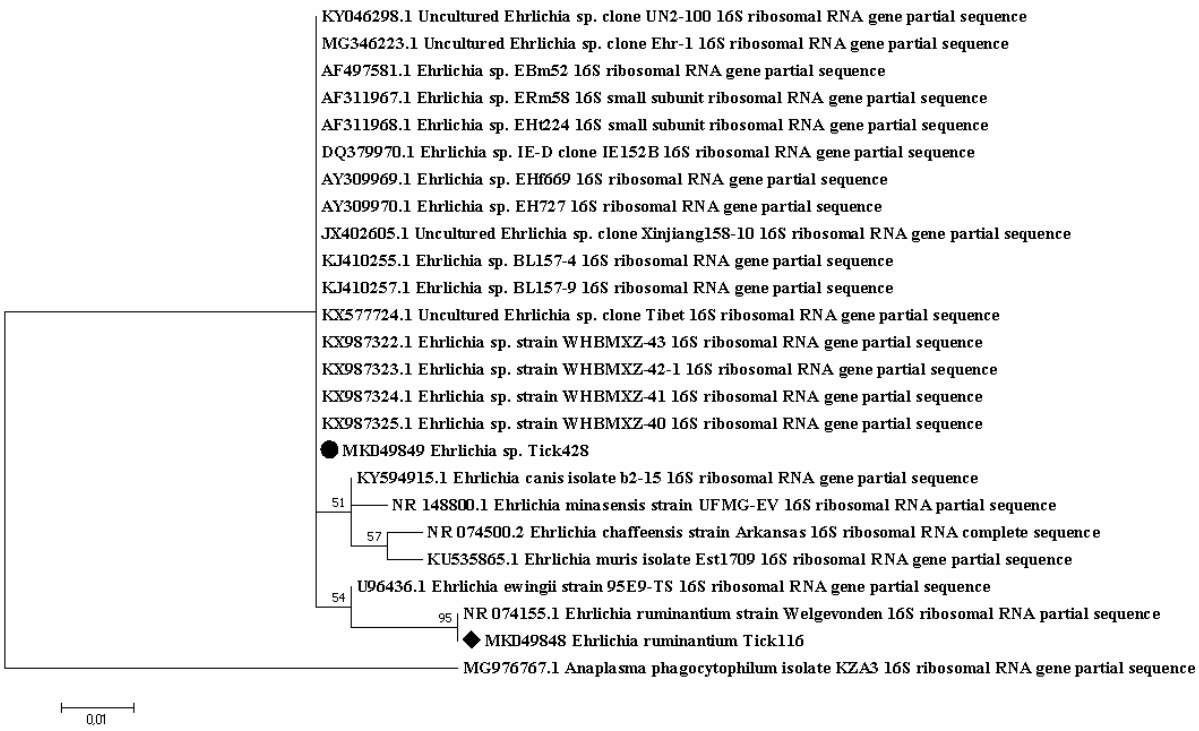


Figure 3. Phylogenetic analysis of 16S rRNA sequences of *Ehrlichia* spp. by maximum likelihood method based on the Tamura-Nei model. In the phylogenetic tree, GenBank, species designation and strain name are given. The sequences investigated in the present study are marked with a black circle (*Ehrlichia* sp. Tick428, accession number MK049849) and a black diamond (*Ehrlichia ruminantium* Tick116, accession number MK049848). The tree with the highest log likelihood (-413.76) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches (bootstrap values). The analysis involved 25 nucleotide sequences. There were a total of 206 positions in the final dataset.

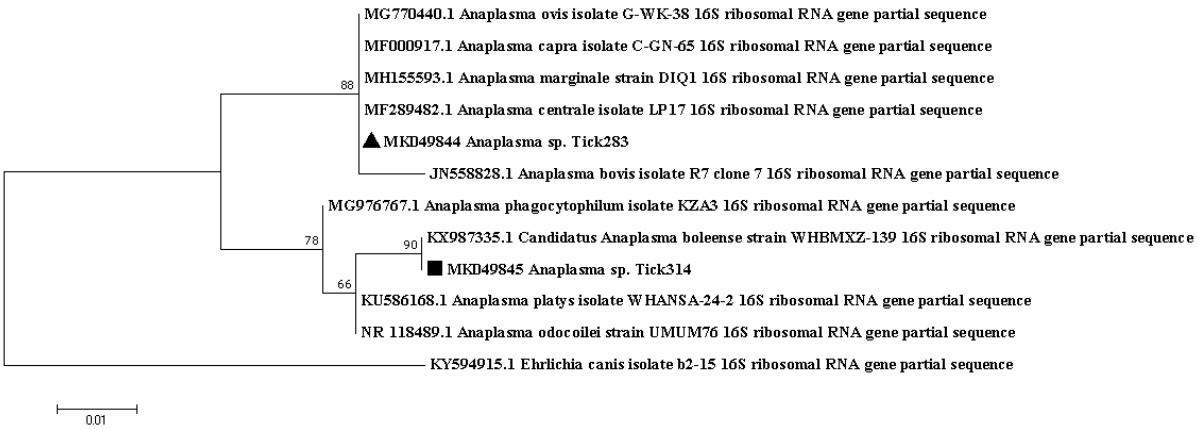


Figure 4. Phylogenetic analysis of 16S rRNA sequences of *Anaplasma* spp. by maximum likelihood method based on the Tamura-Nei model. In the phylogenetic tree, GenBank, species designation and strain name are given. The sequences investigated in the present study are marked with a black triangle (*Anaplasma* sp. Tick283, accession number MK049844) and a black square (*Anaplasma* sp. Tick314, accession number MK049845). The tree with the highest log likelihood (-473.44) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches (bootstrap values). The analysis involved 12 nucleotide sequences. There were a total of 243 positions in the final dataset.

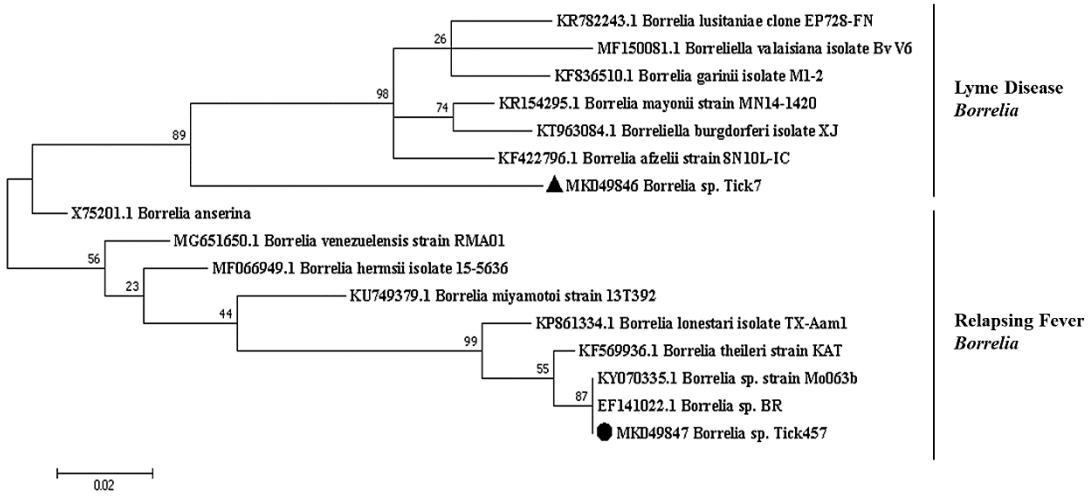


Figure 5. Phylogenetic analysis of flaB sequences of *Borrelia* sp. by maximum likelihood method based on the Tamura-Nei model. In the phylogenetic tree, GenBank, species designation and strain name are given. The sequences investigated in the present study are marked with a black circle (*Borrelia* sp. Tick457, accession number MK049847) and a black triangle (*Borrelia* sp. Tick7, accession number MK049846). The Lyme disease and relapsing fever clades of *Borrelia* are marked. The tree with the highest log likelihood (-963.24) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches (bootstrap values). The analysis involved 16 nucleotide sequences. There were a total of 245 positions in the final dataset.