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

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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.

Methods

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. The system developed here includes 57 designs allowing both the screening of bacteria and protozoans potentially circulating in the West Indies (5 bacterial genera, 30 bacterial species, 1 protozoan phylum, 2 protozoan genera and 17 protozoan species), and the molecular identification of three tick species mainly involved in tick-borne pathogens transmission in the Caribbean (*Amblyomma variegatum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus sensu lato*). Then, using the new high-throughput microfluidic real-time PCR system, we performed an exploratory epidemiological study on 132 specimens of *Amblyomma variegatum* and 446 *Rhipicephalus microplus* collected in Guadeloupe and Martinique.

Results

We successfully detected tick-borne pathogens expected to be present in the area – *Ehrlichia ruminantium*, *Rickettsia africae*, *Anaplasma marginale*, *Babesia bigemina*, *Babesia bovis*, *Theileria velifera* and *Theileria mutans* – as well as unsuspected pathogens and microorganisms belonging to the genera *Anaplasma*, *Ehrlichia*, *Borrelia*, and *Leishmania*.

Conclusions

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. 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.

Keywords: tick, tick-borne pathogens, molecular epidemiology, surveillance, Caribbean, microfluidic real time PCR technology.

Background

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 (TBD) is poorly documented (Gondard *et al*., 2017). *Amblyomma variegatum* also known as Tropical Bont Tick (TBT) in the Caribbean and *Rhipicephalus 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 18th-19th century (Barré and Uilenberg, 2010). Both tick species are present in the French Antilles where they are involved in the transmission 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). *R. 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 is 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 of African Tick Bite Fever related to 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

TBPs in the Caribbean is based on serological studies in animals or humans, or molecular biology testing (PCR, nested PCR). These studies did not survey nor discriminate the whole repertoire of infectious agents since they were 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 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 in European ticks has paved the way for a promising wider surveillance capacity (Michelet *et al*., 2014; Dupraz *et al*., 2017; Gioia *et al*., 2018; Gondard *et al*., 2018; Malmsten *et al*., 2018). In this study, we adapted and designed a new microfluidic real-time PCR system adapted to the simultaneous screening of the main bacteria and protozoans potentially transmitted by ticks in the Caribbean area in 95 DNA tick samples in a single experiment. Not only does the system allowed the direct detection of 49 bacterial species but it also allowed in a single experiment a broader surveillance ability of microorganisms, potentially pathogenic, by targeting the main bacterial and protozoan genera involved in human or animal vector-borne diseases (one protozoan phylum, eight bacterial and protozoan genera). In addition, the system allowed the molecular identification of the three well known tick species involved in TBDs in the Caribbean. Finally, we used the new high-throughput detection tool, to conduce a large scale screening of TBPs in 132 *A. variegatum* and 446 *R. 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 capacity of emergent and non-emergent TBPs through large-scale surveys in the Caribbean area.

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 nine 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 *R. microplus* only were collected from cattle in 29 farms participating in a study on acaricide resistance in ticks. All ticks were collected from cattle with the permission from farmers and cattle owners. Ticks were morphologically identified at the species level (Walker *et al*., 2003). A total of 578 adult ticks were included in the study: 132 *A. variegatum* and 165 *R. microplus* from Guadeloupe and 281 *R. microplus* from Martinique (see maps, Fig 2). GPS coordinates of tick collection sites are available in Additional file 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. *R. microplus* ticks were extracted both individually and in pools of two to four adult specimens when too small to be treated individually.

Assay design

The list of pathogens to be monitored, the sets of primers and probes 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.

Table 1: List of primers/probe sets constituting the Biomark system, with positive controls used for their validation.

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 up to 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 kind 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. When ticks were too small to be treated individually, they were pooled by two to four specimens. Thus, among the 523 samples analyzed, 47 consisted of a pool of two to four tick specimens. The final estimation of the infection rate also includes 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.

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		

Phylogenetic sequence analysis

Alignments were performed using ClustalW (Thompson *et al.*, 1994). Maximum Likelihood trees were generated by 1,000 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 was 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

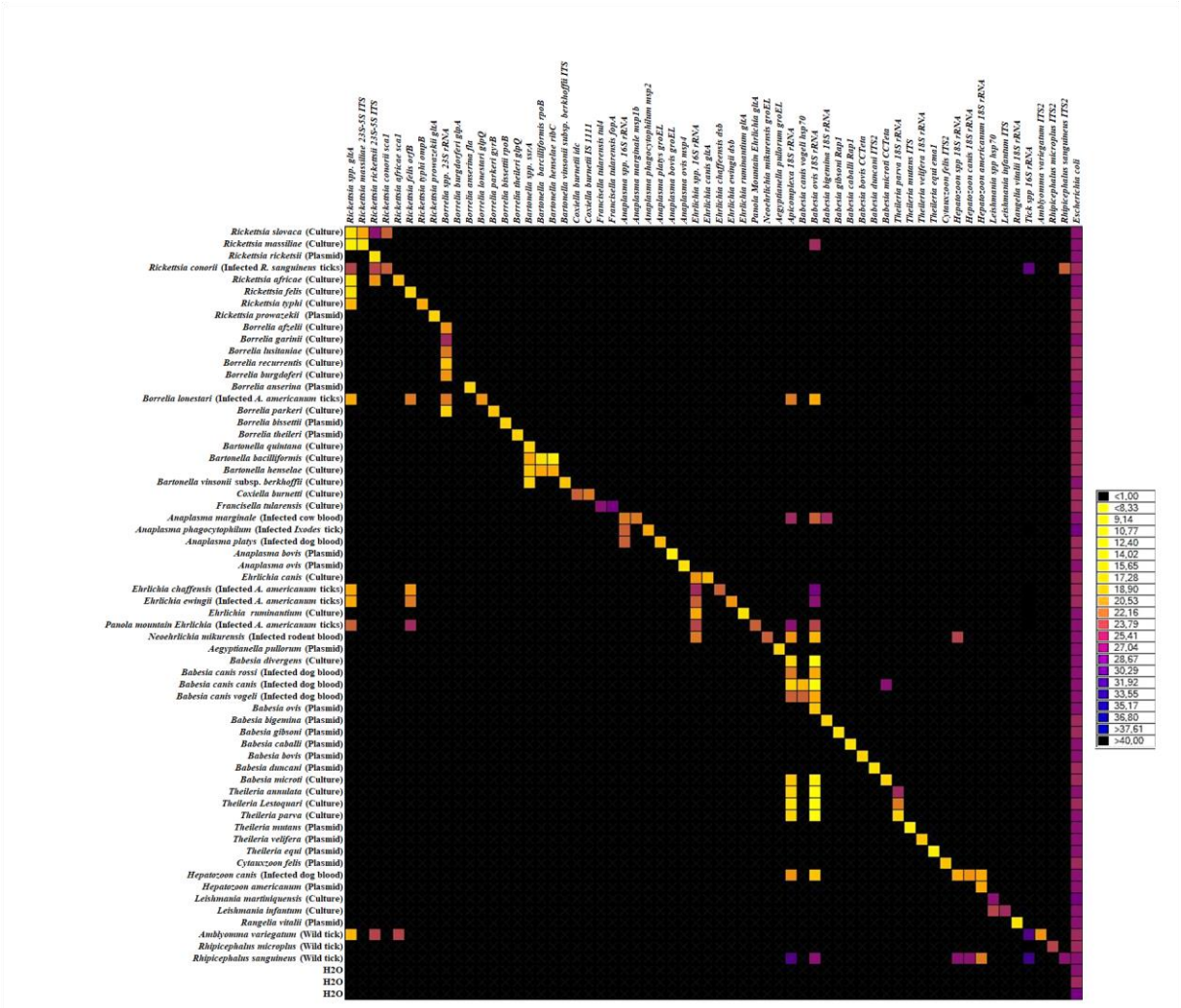
Implementation of the high-throughput microfluidic real-time PCR system for tick-borne pathogens survey

The high-throughput microfluidic real-time PCR system developed for the screening of known and potential TBPs included 61 sets of primers and probes. Among them, 49 designs were developed for the detection of bacterial (n=32) and protozoan (n=17) species, bacterial (n=5) and protozoan genera/phylum (n=3) (Table 1). Three sets of primers and probes were developed for the molecular identification of the three tick species found in the Caribbean, including *A. variegatum*, *R. microplus* and *R. sanguineus sensu lato*(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 protozoan 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 and was therefore validated as part of the screening protocol (see Additional file 2).

Then, the specificity of the designs was evaluated using the Biomark system and a total of 62 positive controls (Table 1, Fig 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 $C_t > 30$.



42 primers/probe sets were able to detect and amplify specifically their target using a C_t cut-off value of 30 and were then directly validated (Fig 1). The design Tick spp., used as tick acid nucleic extraction control, was able to detect *A. variegatum* and *R. sanguineus s.l.* samples, as well as the DNA of *R. sanguineus s.l.* tick present in the *Rickettsia conorii* positive control as expected (Fig 1). However the DNA of ticks from the *R. 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 detection ability of this designed has been corrected by the addition of the Tick spp. primers during the preamplification step, which were initially excluded since the objective was to enrich pathogen DNA content comparatively to tick DNA (data not shown).

The remaining designs gave false-positive results in outgroup controls in addition to their target detection. Interestingly, two kind 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 rest of 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 (see details of co-infection analysis in Additional file 3).

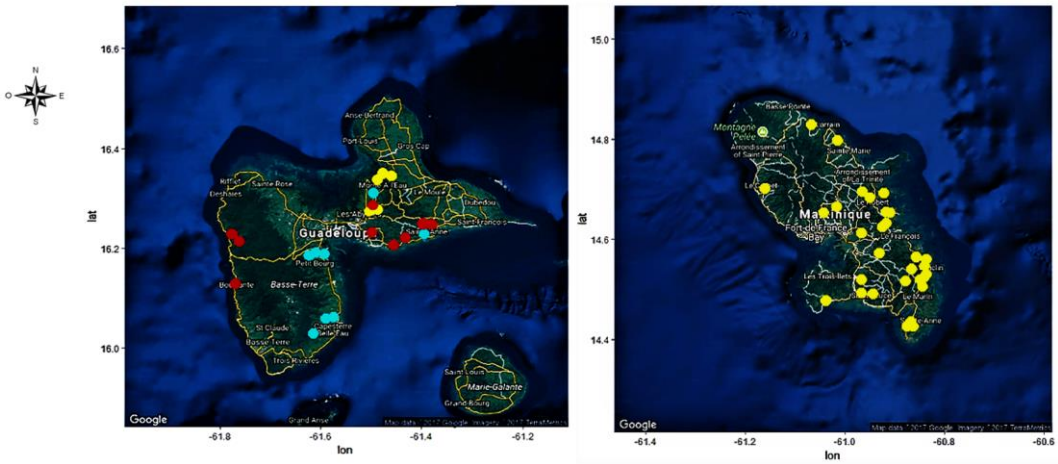
To conclude, with the exception of the sets of primers and probes targeting *Borrelia burgdorferi sensu stricto*, *Babesia ovis* and *Rickettsia rickettsii* that were finally removed from the study, the 58 remaining designs were validated for the high-throughput screening of pathogens in Caribbean ticks,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

Large scale TBPs detection survey in ticks from Guadeloupe and Martinique

A total of 578 adults ticks were collected from cattle in Guadeloupe and Martinique. A total of 523 were tested using the BioMark™ system developed in this study. The number of positive ticks and the corresponding infection rates for each pathogen detected were calculated for 132 *A. variegatum*, 165 and 281 *R. microplus* from Guadeloupe and Martinique, respectively (Fig 2). As some of the *R. microplus* samples corresponded to pool of two to four adult specimens, we reported the minimum and maximum infection rates (see Material and methods).

Figure 2. Infection rates in ticks collected in Guadeloupe and Martinique.
Number of positive ticks *Amblyomma variegatum* (out of the 132), *Rhipicephalus microplus* from Guadeloupe (out of 165) and Martinique (out of 281). Dots on the map indicate the tick collection sites in Guadeloupe and Martinique. Yellow: collection site of *Rhipicephalus 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.



	Guadeloupe		Martinique
	Positive *Amblyomma variegatum*, out of 132 (IR)	Positive *Rhipicephalus microplus*, out of 165 (IR min - max)	Positive *Rhipicephalus microplus*, out of 281 (IR min - max)
***Anaplasma* spp.**	0	**8 (4.8%) - 9 (5.5%)**	**112 (39.9%) - 116 (41.3%)**
Anaplasma marginale	0	**5 (3%) - 7 (4.2%)**	**111 (39.5%) - 116 (41.3%)**
***Ehrlichia* spp.**	**11 (8.3%)**	**7 (4.2%) - 11(6.7%)**	**134 (47.7%) - 138 (49.1%)**
Ehrlichia ruminantium	**7 (5.3%)**	0	0
***Borrelia* spp.**	**7 (5.3%)**	**1 (0.6%)**	**12 (4.3%)**
***Rickettsia* spp.**	**130 (98.5%)**	**25 (15.2%) - 38 (23%)**	0
Rickettsia africae	**126 (95.5%)**	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	**57 (43.2%)**	**39 (23.6%) - 52 (31.5%)**	**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%)**

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.

Table 3: Homology between deposited sequences and reference sequences in GenBank.

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

T: number of samples tested; S: number of samples obtained after sequencing; AN: Accession number; L: Lenght (bp); ID%: percentage of identity. * Various Ehrlichia sp. 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.

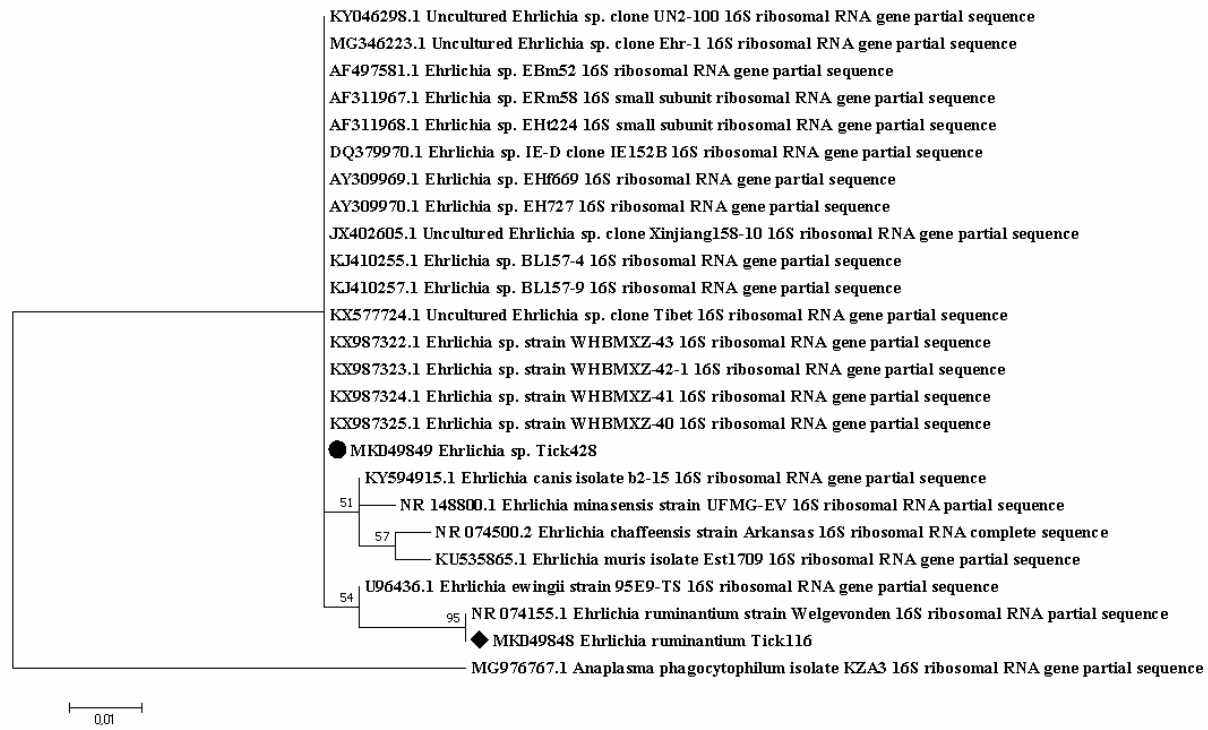
Detection of known TBPs in Caribbean ticks. Seven TBPs known to circulate in the Caribbean were detected in ticks from Guadeloupe and Martinique, including *R. africae*, *E. ruminantium*, *An. marginale*, *B. bigemina*, *B. ovis*, *T. mutans* and *T. velifera* (Fig 2).

Rickettsia spp. were only detected in ticks collected in Guadeloupe (Fig 2). *R. africae* was identified in 95.6% of the *A. variegatum* samples (Fig 2). In contrast, *Rickettsia* spp. detected in 15.7-23.5% of the *R. microplus* samples from Guadeloupe were not directly identified as *R. africae* with the Biomark^{TM} system (Fig 2). Thus, fourteen samples of *A. variegatum* (6/14) and *R. 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).

E. ruminantium was identified in 5.1% of the *A. variegatum* ticks from Guadeloupe (Fig 2). We confirmed the presence of *E. ruminantium* nucleic acids 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).

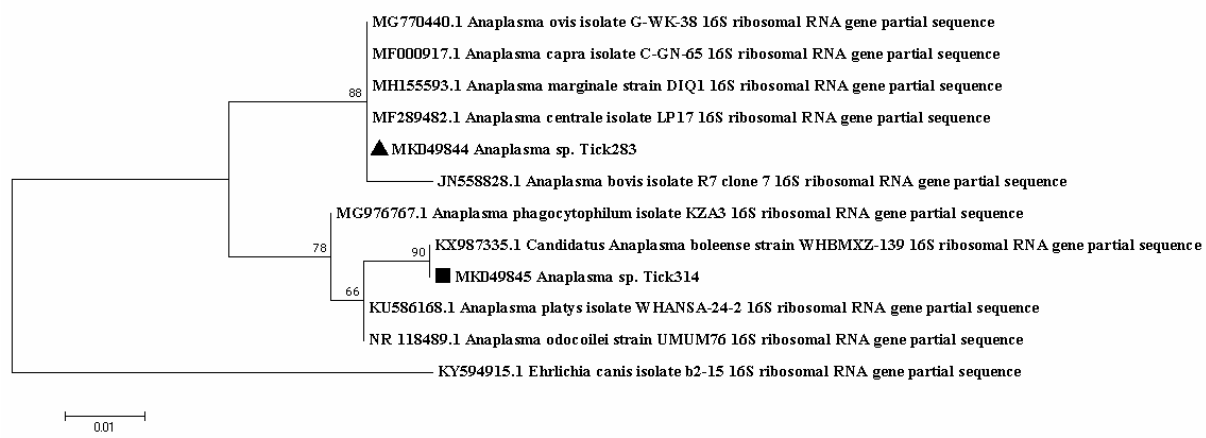
Figure 3. Phylogenetic analysis of 16S rRNA sequences of *Ehrlichia* spp.
Phylogenetic analysis of 16S rRNA sequences of *Ehrlichia* spp. by maximum likelihood method based on the Tamura-Nei model. In the phylogenetic tree, GenBank, species designation and strain name are given. The sequences investigated 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.



An. marginale was identified in *R. microplus* ticks from the both islands, with infection rates reaching 3.6-4.8% and 39.5-41.3% of specimens from Guadeloupe and Martinique, respectively (Fig 2). We confirmed the detection of *An. marginale* by testing two samples of *R. 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).

Figure 4. Phylogenetic analysis of 16S rRNA sequences of *Anaplasma* spp.
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.



B. bigemina was detected in 0.6-1.2% and 12.5-12.8% of the *R. 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% in *R. microplus* samples (Fig 2). As conventional and nested PCR did not succeed in detecting those parasites, we directly 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 and in the two islands. *T. velifera* was identified in 42.3% of the *A. variegatum* samples and in 24.1-31.9% and 25.6-26% of the *R. microplus* samples from Guadeloupe and Martinique, respectively (Fig 2). As well, *T. mutans* was detected in 1.5% of the *A. variegatum* samples, and in 1.8-2.4% and 1.4% of the *R. microplus* samples from Guadeloupe and Martinique, respectively (Fig 2). Unfortunately, neither conventional PCR nor Biomark amplicon sequencing succeeded in confirming the Biomark results.

Detection of unexpected microorganisms in Caribbean ticks. Unexpected signals were obtained during the screening of microorganisms 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 *R. microplus* ticks from both islands, with infections rates reaching 4.2-6.6% and 47.7-49.1% in Guadeloupe and Martinique, respectively (Fig 2). We tested two

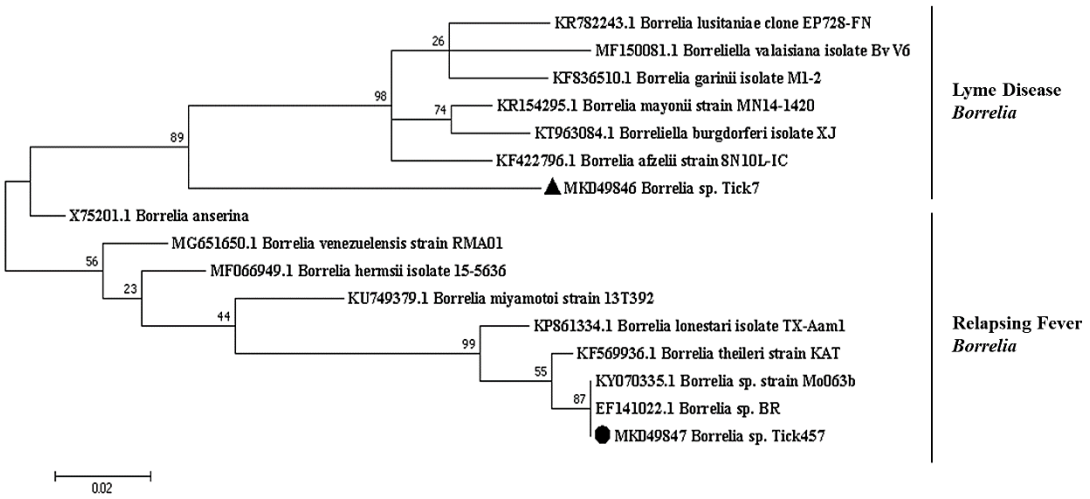
of the *Ehrlichia* spp. positive *R. microplus* samples by conventional PCR targeting the 16S rRNA genes (Table 2). We obtained two identical sequences, deposited under the name *Ehrlichia* sp. Tick428 (accession number MK049849) (Table 3). Phylogenetic and genetic distance analyses were performed using fragment of the 16S rRNA genes of several *Ehrlichia s*pecies (Fig 3). The *Ehrlichia* sp. Tick428 sequence was found within a cluster including various uncharacterized *Ehrlichia* species detected in ticks from Asia and Africa.

In addition, in around 50% and 18% of the *R. microplus* specimens positive for *Anaplasma* spp., none of the *Anaplasma* species targeted by the Biomark™ system gave signals, suggesting the presence of an unexpected or a new *Anaplasma* spp. (Fig 2). We tested two of the *Anaplasma* spp. positive *R. microplus* samples by conventional PCR 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. 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*.

Borrelia spp. was detected in both tick species from both islands (Fig 2). Infection rates reached 5.1% in *A. variegatum*, and 0.6% and 4.3% in *R. microplus* from Guadeloupe and Martinique, respectively (Fig 2). None of the specific *Borrelia* species targeted causing Lyme disease (*Borrelia burgdorferi sensu lato*) or Borrelia 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 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 *Borrelia* sp. Tick457 sequence (accession number MK049847) was recovered from four *R. microplus* samples from Martinique (Table 3). 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 *R. microplus* samples confirmed the previous observations, forming a cluster with various Relapsing Fever *Borrelia* species encountered in hard ticks, including *Bo. lonestari* and *Bo. theileri* (Fig 5).

Figure 5. Phylogenetic analysis of flaB sequences of *Borrelia* spp.
Phylogenetic analysis of flaB sequences of *Borrelia* 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 (*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.



Finally, 0.7% of *R. microplus* ticks from Martinique were positive for *Leishmania* spp. (Fig 2). We tested two of the *Leishmania* spp. positive ticks by nested PCR 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).

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 *R. microplus* were infected. In contrast, 79.4 – 81.5% of *R. microplus* from Martinique were infected by at least one pathogen. High and similar percentages of the two tick species were infected by either one or two

pathogens. The percentages drastically felt down from co-infection by three pathogens with less than 10% of the ticks infected. Respectively one and nine *A. variegatum* and *R. microplus*, from Guadeloupe and Martinique were co-infected by four pathogens and one *R. microplus* from Martinique was found infected by five pathogens. In the rest of the analysis on co-infections, only pathogens present in a sufficient number of ticks (more than 25) were further considered for possible infectious 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 *R. 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 *R. microplus* of Guadeloupe and Martinique (24-26%). However, the infection rates of *An. marginale* and *Ehrlichia* spp. in *R. 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-infection, suggesting that none of these pathogens can interfere with the infection by the two others. Details of Co-infections in ticks of Guadeloupe and Martinique are available in Additional file 4.

Discussion

In this study, a high-throughput microfluidic real-time PCR system based on the use of multiple primers/probes was developed for large scale surveys of bacteria and protozoans potentially transmitted by ticks from the Caribbean area.

The association of genus and species primer/probe 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 protozoan genera/phylum involved in TBD worldwide.

Nevertheless, as some endosymbiotic microorganisms could belong to known TBPs genera, such as *Rickettsia* and *Coxiella* genus, confirmatory testings are required before suggesting the presence of a pathogenic microorganism (Ahantarig *et al*., 2013; Duron *et al*., 2015; Bonnet *et al*., 2017).

While analyzing the specificity of the microfluidic real-time PCR system, cross-reactions have been observed for some designs targeting closely related species that 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. Here, the concomitant use of the bacterial and protozoan genera can assist non-specific signals identification.

In addition to microorganism's detection, 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*, *R. microplus* and *R. sanguineus s.l.*. As Morphological identification of ticks collected on the field remains challenging, molecular identification can assist the correct identification of the tick species analyzed.

We used the newly developed high-throughput microfluidic real-time PCR system to perform an exploratory epidemiological study of TBPs and microorganisms potentially circulating in Caribbean ticks. The analysis gave an overview of the diversity of microorganisms belonging to the main bacteria and protozoan genera potentially transmitted by ticks. It allowed both the detection of known TBPs of public and animal health importance in the area which require surveillance, and unexpected microorganisms within Caribbean ticks.

The four main pathogens responsible for ruminant diseases in the Caribbean – currently classified as notifiable diseases by the World Organisation of Animal Health (OIE) –have been detected by the microfluidic real-time PCR system, including *E. ruminantium* in *A. variegatum* specimens and *An. marginale*, *B. bigemina* and *B. bovis* in *R. microplus*.

Interestingly, *E. ruminantium* infection rate in *A. variegatum* reported in our study was much lower compared to previous studies conducted between 2003 and 2005 in Guadeloupe (5.1% versus 36.7%) (Vachiéry *et al*., 2008). Although study designs were distinct (different sampling strategy, study periods, or detection methods, etc.) and can explain this difference, it is worth investigating further whether the tick infection rate for *E. ruminantium* decreased in Guadeloupe and possibly assess the

epidemiological impact in term of incidence and prevalence of heartwater in the ruminant population. Those results are all the more surprising that systematic TBT surveillance and control programs have stopped after the end of the eradication programs POSEIDOM in 2006.

In this study, we document for the first time infection rates for *B. bigemina, B. bovis* and *An. marginale* in the vector tick *R. microplus* in 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).

R. microplus ticks are both vector and reservoir of *B. bigemina and B. bovis*, transmitting transovarially and trans-stadially the parasites (Howell, *et al.,* 2007; Chauvin *et al.,* 2009). As *R. microplus* ticks and bovine are both reservoir of the infection, the infection rates reported here seemed quite low (Howell, *et al.,* 2007; Chauvin *et al.,* 2009). The life cycle of *Babesia* spp. require complex interactions with its two host, the tick vector and the vertebrate host. The efficiency of tick acquisition, transovarial and trans-stadial transmissions of *B. bovis* and *B. bigemina* by *R. microplus*, involved in long-term persistence of *Babesia* spp. in nature, are still poorly understood and deserved further investigations (Chauvin et al 2009, Howell et al., 2009).

Interestingly, *An. marginale* was detected in *R. microplus* from the two islands but the infection rate reported in ticks from Guadeloupe seemed lower compared to Martinique. The same tendency has been reported during previous seroprevalence studies (Camus and Montenegro-James, 1994; Camus and Barre, 1995; Alonso *et al*., 1992). Anaplasmosis can be transmitted by other vectors than ticks and some cattle breeds are known to be more susceptible than others to *Anaplasma* infection (Camus and Montenegro, 1994). The difference of *Anaplasma* prevalence in ticks between the two islands could be explained by the difference in cattle populations. Indeed, there are mainly local Creole or mixed European-Creole breeds in Guadeloupe – known to be more resistant to anaplasmosis than Brahman and European breeds – which are mainly bred in Martinique (Camus and Montenegro, 1994). In addition, other factors, like differences in the population dynamics of alternate vectors such as flies could also contribute to this difference.

Among the other known TBPs detected, we also found pathogens with low impact on health in the Caribbean – almost considered as endosymbionts – such as *R. africae*, *T. velifera* and *T. mutans* in their vector *A. variegatum* and surprisingly in *R. microplus* ticks.

With almost all *A. variegatum* found infected, the *R. africae* infection rate is the higher ever 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 transovarial and trans-stadial transmission rates reaching 100%, this high level of *R. africae* infection rate is not surprising *per se* (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, highlights the difficulty in some cases to clearly distinguish between endosymbiosis and pathogenicity (Parola *et al*., 1999; Raoult *et al*., 2001). The biological relationship between *R. africae* and *A. variegatum* as well as the 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 (Ahantarig *et al.,* 2013; Maina *et al.,* 2014; Duron *et al.,* 2017). The absence of direct identification of *R. africae* in *R. microplus* ticks was probably due to a lower sensitivity of the specific target design compared to the genus target design. Indeed, *Rickettsia* spp. positive *R. microplus* samples displayed quite high C_t values, suggesting a low infection level that might be below the detection limit for *R. africae*. The unusual presence of *R. africae* in *R. microplus* tick could be explained by the co-occurrence of the two tick species, *R. microplus* and *A. variegatum*, on cattle. As the ticks here were collected partially engorged, the presence of *R. africae* in *R. microplus* may be due to bacteria circulating in cattle blood which would have been picked up by engorging ticks, or to a cross-contamination during *R. microplus* ticks co-feeding next to infected *A. variegatum* (Yssouf et al., 2014; Matsimbe *et al*., 2017).

The study provides the first update of the detection 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). The low pathogenicity of these piroplasms may explain the lack of diagnostic and the scarce information available on their distribution and prevalence in the Caribbean. However, these parasites species may play an important role in theileriosis management and protection as

chronically infected cattle can develop immunity and heterologous protection against other pathogenic *Theileria* species, such as *Theileria parva* (Woolhouse *et al*., 2015). Unfortunately, we did not succeed in confirming these results by conventional or nested PCR, which can suggest either a level of infection under the detection threshold, or simply false signals.

Finally, the high-throughput microfluidic real-time PCR system allowed the detection of unexpected and/or potentially new microorganisms, leading to the recovery of nucleotide sequences of *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp. and *Leishmania* spp. in ticks collected in Guadeloupe and Martinique.

The *Ehrlichia* sp. Tick428 sequence detected here formed a cluster with other uncharacterized *Ehrlichia* species detected in ticks from Asia and Africa (Parola *et al.*, 2001, 2003; Inokuma *et al.*, 2004; Loftis *et al.*, 2006; Kang *et al.*, 2014; Lu *et al.*, 2017). 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. The *Anaplasma* sp. Tick314 sequence was identified as *Candidatus* Anaplasma boleense, a bacteria described in ticks and mosquitoes in China (Kang *et al.*, 2014; Guo *et al.*, 2016). No further information are available regarding the epidemiology of *Candidatus* Anaplasma boleense. These observations highlight the need to set up characterization studies. Indeed, high-throughput detection technologies can highlight the presence of DNA from potentially new microorganisms, but it will be still necessary to isolate and characterize them in order first to confirm their existence and second to determine if their presence in ticks represents a risk for public or animal health.

We provided here the first report of *Borrelia* spp. in ticks from Guadeloupe and Martinique. Two different sequences have been recovered, according to the tick species analyzed. In *A. variagetum*, a sequence named *Borrelia* sp. Tick7 have been detected, closely related to *Bo. anserina*, the agent of avian spirochetosis. Both of them seemed to define an intermediate position between the relapsing fever and the Lyme disease group. In contrast, the *Borrelia* sp. Tick457 sequence found *R. microplus*, clustered with uncharacterized *Borrelia* spp., *Borrelia* sp. strain Mo063b and *Borrelia* sp. BR, detected in *R. microplus* 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

R. microplus ticks from Ethiopia and Côte d'Ivoire (Kumsa *et al*., 2015; Ehounoud *et al.,* 2016). As *A. variegatum* and *R. microplus* were imported in the Caribbean from Africa during the time of the Atlantic triangular trade, we may have detected bacteria probably characterized by an old introduction through infected ticks and subsequent local evolution within their vector over a long period (Barré and Uilenberg, 2012; Estrada-Peña *et al.,* 2018). *Borrelia* spp. and borreliosis cases reports in the Caribbean are scarce and still in debate. In Cuba, one study suggested the presence of antibodies to *Borrelia burgdorferi sensu stricto* in human serums associated with clinical cases of Lyme disease-like syndrome (Rodríguez *et al.,* 2004, 2012). However, the real specificity of these serum antibodies was questioned (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). Finally, Erythema Migrans–like skin lesions and illness have been reported in four Caribbean nationals (Sharma *et al.,* 2010). Regarding the importance of *Borrelia* spp. in human and animal health, the characterization of these potential new *Borrelia* species which seemed associated to tropical tick species require further investigations.

Lastly, *Leishmania* spp. were detected in *R. microplus* specimens from Martinique, and a sequence was identified as *Leishmania martiniquensis* Tick389 (accession number MK049850). Studies on *Leishmania* nomenclature highlighted the fact that isolates of "*L. siamensis"* have never been officially characterized and therefore, 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*. Parasites of the genus *Leishmania* are usually transmitted by female phlebotomine sand flies, (Diptera: Pshychodidae: Phlebotominae), and involve generally a wide variety of animal species, mostly dogs and canids in the epidemiological cycle. They are responsible for Leishmaniasis, a zoonosis widespread in tropical and sub-tropical areas (Steverding, 2017). *L. martiniquensis* belongs to the *L. enriettii* complex and has been described in Martinique and in Thailand, where it was responsible for both cutaneous 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 yet been described (Desbois *et al.*, 2014). Our study represents the first report of *L. martiniquensis* in *R. microplus* ticks from the French Antilles. Althought *Leishmania* spp. have been reported in ticks, such as *L. infantum* in *R. sanguineus*, or *L. guyanensis* in *R. microplus* ticks in Peru, the role of ticks in *Leishmania* transmission is still a debate and no evidence of vector capacity have been reported yet (Dantas-Torres, 2011; Rakhshanpour *et al.*, 2017; Rojas-Jaimes *et al.*, 2017). In addition, the finding of *Leishmania* spp. in a tick species that feed mainly on cattle also raises question on the potential role of cattle in the epidemiology of leishmaniasis (Alam *et al.*, 2018, Paixão-Marques *et al.*, 2019). The participation of ticks in *Leishmania* epidemiology deserve further investigation, especially since *R. microplus* ticks could parasitizing human (Lamattina and Nava, 2016).

Surprisingly, co-infections with two or more TBPs were found in more than 50% of the infected ticks, both for *A. variegatum* and *R. microplus* and in the two islands. In addition, we could not identify any association or exclusion of the infection by one pathogen to another one. These observations illustrate the efficiency of ticks as reservoir of multiple pathogens with apparently no significant effects on their life traits.

To conclude, although screening tools are useful for pathogen discovery in ticks, the epidemiological meaning of such results warrants further analyzes. 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, however they provide useful and complementary information to 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. A region-wide epidemiological survey on TBPs in ticks collected in different countries and territories of the Caribbean area to strengthen our results and organized in collaboration with the Caribbean Animal Health Network, CaribVET and the ticks and tick-borne diseases working group may be an interesting way to complete and strengthen some of the findings of this paper.

Conclusion

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 promising and could improve significantly TBPs surveillance and study, allowing a rapid screening of multiple especially in regions where few epidemiological data are available and TBD are numerous.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' 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 primers/probe sets constituting the Biomark system, with positive controls used for their validation.

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**
		Bor_ans_fla_R	TTGGAGAATTAACCCCACCTG		
		Bor_ans_fla_P	TGCAAGCAACTCCAGCTCCAGTAGCT		
Borrelia lonestari	*glpQ*	Bor_lon_glpQ_F	GATCCAGAACTTGATACAACCAC	99	

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
		Bor_lon_glpQ_R	TTCATCTAGTGAGAAGTCAGTAG		***Amblyomma americanum***
		Bor_lon_glpQ_P	AGTAATATCGTCCGTCTTCCCTAGCTCG		**naturally infected**
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**
		Fr_tu_fopA_R	CAACACTTGCTTGAACATTTCTAG		
		Fr_tu_fopA_P	AACAGGTGCTTGGGATGTGGGTGGTG		
***Anaplasma* spp.**		Ana_spp_16S_F	CTTAGGGTTGTAAAACTCTTTCAG	160	******

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
	16S rRNA	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		
Panola Mountain Ehrlichia	*gltA*	Eh_PME_gltA_F	GCTAGTTATGAGTTAGAATGTAAAC	121	***Amblyomma americanum* naturally infected**
		Eh_PME_gltA_R	TACTATAGGATAATCTTGAATCAGC		
		Eh_PME_gltA_P	TTGCTATCGCTAAAATTCCAAGTATGATTGCG		
	groEL	Neo_mik_groEL_F	AGAGACATCATTCGCATTTTGGA	96	**Blood of infected rodent**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
Neoehrlichia mikurensis		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**
		Bab_mi_CCTeta_R	GCGACATTTCGGCAACTTATATA		
		Bab_mi_CCTeta_P	TACTCTGGTGCAATGAGCGTATGGGTA		
Theileria parva		Th_pa_18S_F	GAGTATCAATTGGAGGGCAAG	173	**Culture**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
	18S rRNA	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.**	16S rRNA	Tick_spp_16S_F	AAATACTCTAGGGATAACAGCGT	99	******
		Tick_spp_16S_R	TCTTCATCAAACAAGTATCCTAATC		
		Tick_spp_16S_P	CAACATCGAGGTCGCAAACCATTTTGTCTA		
	ITS2	Amb_var_ITS2_F	GCCAGCCTCTGAAGTGACG	117	**Wild tick from Guadeloupe**

Microorganisms	Target	Design Name	Sequence (5' → 3')	Amplicon size (bp)	Positive controls
Amblyomma variegatum		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		

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.

Additional Files

Additional file 1.pdf: GPS coordinates of ticks collection sites.

A total of 578 adult ticks collected on bovine from Guadeloupe and Martinique were used for the screening of tick-borne pathogens with the newly implemented BioMark™ Real-Time PCR system.

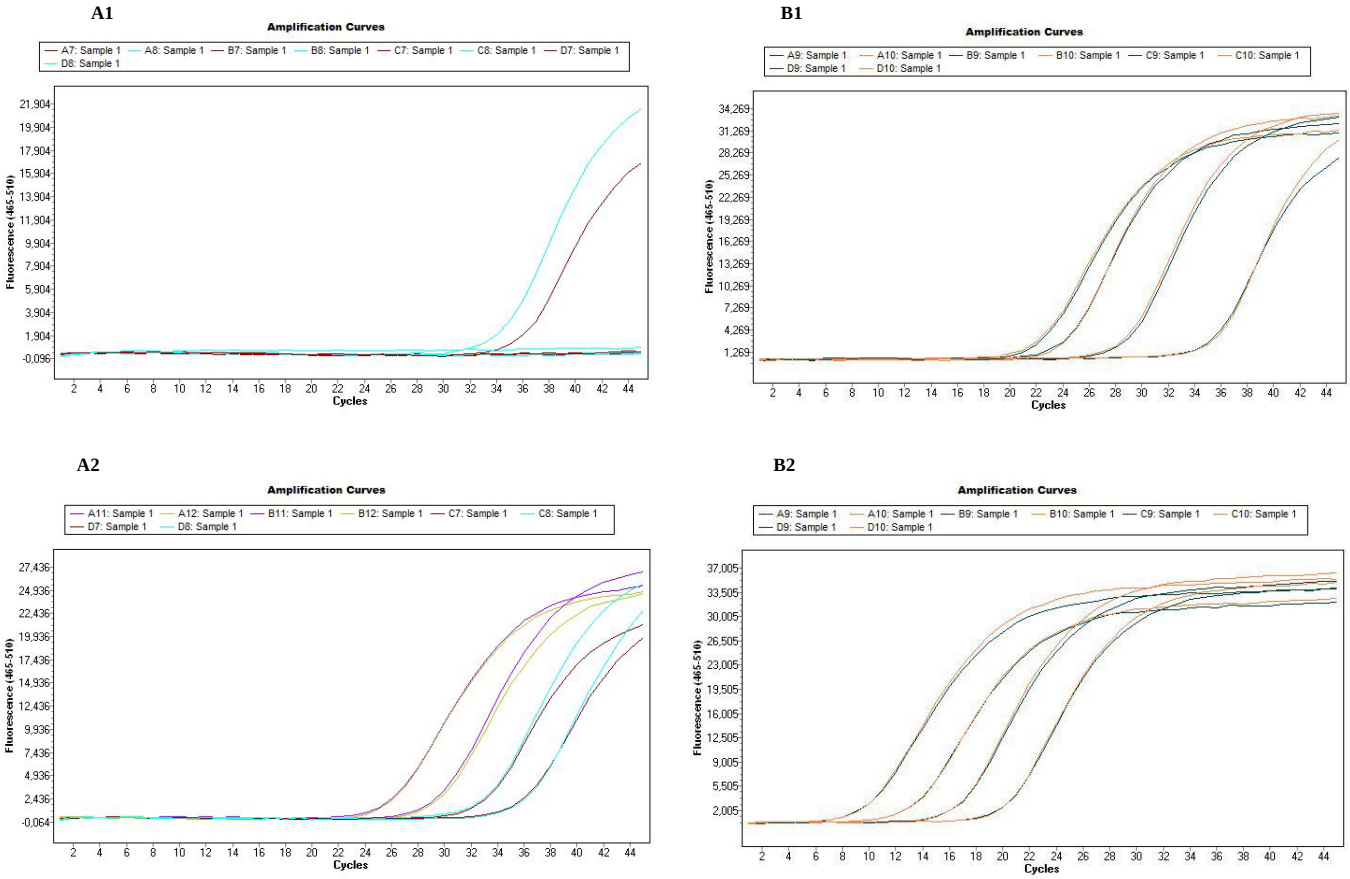
132 *Amblyomma variegatum* and 165 *Rhipicephalus microplus* adult specimens were collected on cattle in Guadeloupe in this study. Ticks were sampled between February 2014 and January 2015, on 40 bovines originating from 22 different herds including : four herds from Les Abymes (N 16° 16'; O 61° 31'), three from Sainte-Anne (N 16° 14'; O 61° 23'), Petit Bourg (N 16° 10'; O 61° 36'), Capesterre (N 16° 2'; O 61° 34'), Le Gosier (N 16° 13'; O 61° 28'), Morne-À-l'Eau (N 16° 19'; O 61° 28'), two from Pointe-Noire (N 16° 13'; O 61° 45'), and one from Bouillante (N 16° 7'; O 61° 46').

In Martinique, only *Rhipicephalus microplus* specimens were collected on bovine. Indeed, ticks were provided in the frame of the ResisT project, funded by the Regional Cooperation Fund of Guadeloupe and Martinique which aimed to analyze *Rhipicephalus microplus* acaricides resistance in the Caribbean. Thus, 281 *Rhipicephalus microplus* adult specimens were collected on cattle in Martinique between February and March 2015, on 29 bovines originating from 29 herds: eight from Le

Vauclin (N 14° 32'; O 60° 50'), four from Sainte-Anne (N 14° 26'; O 60° 50'), Le Robert (N 14° 40'; O 60° 56'), two from Le François (N 14° 36'; O 60° 53'), Saint-Joseph (N 14° 40'; O 61° 2'), one from Rivière-Pilote (N 14° 28', O 60° 54'), Le Diamant (N 14° 29'; O 61° 1'), Le Lamentin (N 14° 37'; O 60° 59'), Le Lorrain (N 14° 49'; O 61° 2'), Le Morne-Vert (N 14° 42'; O 61° 8'), Rivière-Salée (N 14° 31'; O 60° 57'), Saint-Esprit (N 14° 33'; O 60° 55'), Sainte-Luce (N 14° 29'; O 60° 56'), and Sainte-Marie (N 14° 46'; O 60° 59'). All the ticks were collected on cattle, partially engorged, and then conserved at -80°C. Ticks were morphologically identified at the species level (Walker *et al*., 2003).

Additional file 2.pdf: Improvement of detection signals by pre-amplification.

Test of primers/probe set sensitivity on a dilution range of positive controls by Taqman real-time PCRs using LightCycler 480, before and after pre-amplification. A1/A2: Test of sensitivity of the design *Leishmania infantum* using *Leishmania infantum* culture, before (A1) and after pre-amplification (A2); B1/B2: Test of sensitivity of the design *Rickettsia* spp. using *Rickettsia conorii* positive controls (extract from infected *Rhipicephalus sanguineus* tick), before (B1) and after pre-amplification (B2).



Additional file 3.pdf: Analysis of the specificity of the 61 designs constituting the Biomark system developed for Caribbean tick-borne pathogen screening.

Among the 61 design used in this study, 1 were removed because of absence of signals, 43 were specific for their target; 8 displayed cross-reactions with one to two closely related species, 2 were removed because of multiple cross-reactions and 7 displayed unexpected signals due to the potential presence of co-infections in complex control samples corresponding to DNA extracted from wild ticks or blood samples.

We explored the hypothesis of potential co-infection in controls corresponding to DNA from tick collected in the field or DNA from infected blood and we confirmed some results using conventional PCR and amplicon sequencing (Table S2). Both *Rickettsia* spp. and *Rickettsia felis* designs gave positive results when testing the *Borrelia lonestari*, *Panola Mountain Ehrlichia*, *Ehrlichia ewingii*, *Ehrlichia chaffensis* controls. All those samples corresponded to DNA extracted from infected *Amblyomma americanum* collected on the field. This result supports the fact that a *Rickettsia* species may be present in those samples, being *Rickettsia felis* or a closely related *Rickettsia* spp. infecting *Amblyomma americanum* ticks. When tested the *Panola Mountain Ehrlichia* sample for *Rickettsia* spp. by conventional PCR targeting the *gltA* gene (Regnery *et al.*, 1991), we obtained a 382 bp sequence (accession number MK049843) sharing 99% of sequence identity with *Rickettsia* spp. (MF511253.1) (Table S2). This result, in addition to natural co-infections documented in *Amblyomma americanum* ticks, suggest that the detection of *Rickettsia* spp. - within the four outgroup positive controls corresponding to *Amblyomma americanum* DNA – in this assay were not cross-reactions (Mixson *et al*., 2006; Trout Fryxell and DeBruyn, 2016). Moreover, the *Amblyomma variegatum* sample, corresponding to ticks collected from the field, was found positive for both *Rickettsia* spp. and *Rickettsia africae*. As this sample originated from Guadeloupe, where *Rickettsia africae* circulates, this result could be in agreement with a natural infection (Parola *et al*., 1999). As well, the *Rhipicephalus sanguineus* sample was positive with four designs targeting parasites – Apicomplexa, *Hepatozoon* spp., *Hepatozoon canis* and *Hepatozoon americanum.* As *Rhipicephalus sanguineus* ticks are involved in tick-borne parasites epidemiology including *Hepatozoon* spp., those results suggest strongly the occurrence of such parasite in the biological sample (Aktas and Özübek, 2017). Thus, those observations suggest that our

designs were actually capable of detecting pathogens present in naturally-infected ticks rather than giving an unsuspected cross-reaction. The *Neoehrlichia mikurensis* sample, corresponding to DNA extracted from rodent blood, was also found positive for both Apicomplexa and *Hepatozoon* spp.. Amplicon obtained with the *Hepatozoon* spp. design from this sample was sequenced. The 169 bp sequence obtained, (accession number MK071735) displayed 99% of sequence identity with *Hepatozoon* spp. sequences (AB771515.1) (Table S2). As rodents could be infected by *Hepatozoon* parasites, this result could reflect also a natural infection (Hamšíková *et al*., 2016). The *Anaplasma marginale* sample, corresponding to a blood sample of experimentally infected cow, was found also positive for both *Apicomplexa* and *Babesia bigemina*. Amplicon obtained with the Apicomplexa design from this sample was sequenced. The 104 bp sequence obtained (accession number MK071737) displayed 99% of sequence identity with Apicomplexan sequences, including *Babesia bigemina* (MG604302.1) (Table S2). As *Babesia bigemina* and *Anaplasma marginale* are two cattle pathogens which often evolved in the same region, and are transmitted by the same vector tick, co-infections by those two pathogens have already been reported (Hailemariam *et al.*, 2017). Thus, this cross-reaction could be again the reflection of parasites co-infections. Finally, Apicomplexa gave also positive results in *Borrelia lonestari* and the *Panola Mountain Ehrlichia* controls. As highlighted previously, those two controls corresponded to DNA extracted from *A. americanum* ticks. Amplicons obtained with the Apicomplexa design from those two samples were sequenced. The two sequences of 102 bp obtained were identical, (accession number MK071736) and displayed 98% of sequence identity with sequences of *Theileria cervi* (MH085203.1) (Table S2). As *Theileria cervi* is a common deer pathogen found in *Amblyomma americanum*, the occurrence of this parasite in those two control samples could explain the unexpected signals (Laird *et al.*, 1988; Mans *et al.*, 2015).

Table S1. List of the design and their specificity using the Biomark system. CR: Cross-reactions with closely related species samples; CI: Potential co-infections in control samples; *: designs removed from the screening analysis.

Design	Target detection	Specificity	Outgroup control samples
Rickettsia spp. gltA	*YES*	CI	*Borrelia lonestari* (Infected *A. americanum* ticks)
	YES	CI	*Ehrlichia chaffensis* (Infected *A. americanum* ticks)
	YES	CI	*Ehrlichia ewingii* (Infected *A. americanum* ticks)
	YES	CI	*Panola mountain Ehrlichia* (Infected *A. americanum* ticks)
	YES	CI	*Amblyomma variegatum* (Wild tick)
Rickettsia massiliae 23S-5S ITS	*YES*	*CR*	*Rickettsia slovaca* (Culture)
Rickettsia rickettsii 23S-5S ITS	*YES*	*CR**	*Rickettsia slovaca* (Culture)
	YES		*Rickettsia conorii* (Infected *R. sanguineus* ticks)
	YES		*Rickettsia africae* (Culture)
	YES		*Amblyomma variegatum* (Wild tick)
Rickettsia conorii sca1	*YES*	*CR*	*Rickettsia slovaca* (Culture)
Rickettsia africae sca1	*YES*	CI	*Amblyomma variegatum* (Wild tick)
Rickettsia felis orfB	*YES*	CI	*Borrelia lonestari* (Infected *A. americanum* ticks)
	YES	CI	*Ehrlichia chaffensis* (Infected *A. americanum* ticks)
	YES	CI	*Ehrlichia ewingii* (Infected *A. americanum* ticks)
	YES	CI	*Panola mountain Ehrlichia* (Infected *A. americanum* ticks)
Rickettsia typhi ompB	*YES*	*YES*	
Rickettsia prowazekii gltA	*YES*	*YES*	
Borrelia spp. 23S rRNA	*YES*	*YES*	
Borrelia burgdorferi sensu stricto glpA	*NO**		
Borrelia anserina fla	*YES*	*YES*	
Borrelia lonestari glpQ	*YES*	*YES*	
Borrelia parkeri gyrB	*YES*	*YES*	
Borrelia bissettii rpoB	*YES*	*YES*	
Borrelia theileri glpQ	*YES*	*YES*	
Bartonella spp. ssrA	*YES*	*YES*	
Bartonella bacilliformis rpoB	*YES*	*CR*	*Bartonella henselae* (Culture)
Bartonella henselae ribC	*YES*	*CR*	*Bartonella bacilliformis* (Culture)
Bartonella vinsonii subsp. berkhoffii ITS	*YES*	*YES*	
Coxiella burnetii icd	*YES*	*YES*	
Coxiella burnetii IS 1111	*YES*	*YES*	
Francisella tularensis tul4	*YES*	*YES*	
Francisella tularensis fopA	*YES*	*YES*	
Anaplasma spp. 16S rRNA	*YES*	*YES*	
Anaplasma marginale msp1b	*YES*	*YES*	
Anaplasma phagocytophilum msp2	*YES*	*YES*	
Anaplasma platys groEL	*YES*	*YES*	
Anaplasma bovis groEL	*YES*	*YES*	
Anaplasma ovis msp4	*YES*	*YES*	
Ehrlichia spp. 16S rRNA	*YES*	*YES*	
Ehrlichia canis gltA	*YES*	*YES*	
Ehrlichia chaffeensis dsb	*YES*	*YES*	
Ehrlichia ewingii dsb	*YES*	*YES*	
Ehrlichia ruminantium gltA	*YES*	*YES*	
Panola Mountain Ehrlichia gltA	*YES*	*YES*	
Neoehrlichia mikurensis groEL	*YES*	*YES*	
Aegyptianella pullorum groEL	*YES*	*YES*	
Apicomplexa 18S rRNA	*YES*	CI	*Borrelia lonestari* (Infected *A. americanum* ticks)
	YES	CI	*Anaplasma marginale* (Infected cow blood)
	YES	CI	*Panola mountain Ehrlichia* (Infected *A. americanum* ticks)
	YES	CI	*Neoehrlichia mikurensis* (Infected rodent blood)
	YES	CI	*Rhipicephalus sanguineus* (Wild tick)
Babesia canis vogeli hsp70	*YES*	*CR*	*Babesia canis canis* (Infected dog blood)

Design	Target detection	Specificity	Outgroup control samples
Babesia ovis 18S rRNA	*YES*	*CR**	*Rickettsia massiliae* (Culture)
	YES		*Borrelia lonestari* (Infected *A. americanum* ticks)
	YES		*Anaplasma marginale* (Infected cow blood)
	YES		*Ehrlichia chaffensis* (Infected *A. americanum* ticks)
	YES		*Ehrlichia ewingii* (Infected *A. americanum* ticks)
	YES		*Panola mountain Ehrlichia* (Infected *A. americanum* ticks)
	YES		*Neoehrlichia mikurensis* (Infected rodent blood)
	YES		*Babesia divergens* (Culture)
	YES		*Babesia canis rossi* (Infected dog blood)
	YES		*Babesia canis canis* (Infected dog blood)
	YES		*Babesia canis vogeli* (Infected dog blood)
	YES		*Babesia microti* (Culture)
	YES		*Theileria annulata* (Culture)
	YES		*Theileria Lestoquari* (Culture)
	YES		*Theileria parva* (Culture)
	YES		*Hepatozoon canis* (Infected dog blood)
	YES		*Rhipicephalus sanguineus* (Wild tick)
Babesia bigemina 18S rRNA	*YES*	CI	*Anaplasma marginale* (Infected cow blood)
Babesia gibsoni Rap1	*YES*	*YES*	
Babesia caballi Rap1	*YES*	*YES*	
Babesia bovis CCTeta	*YES*	*YES*	
Babesia duncani ITS2	*YES*	*YES*	
Babesia microti CCTeta	*YES*	*CR*	*Babesia canis canis* (Infected dog blood)
Theileria parva 18S rRNA	*YES*	*CR*	*Theileria annulata* (Culture)
	YES	*CR*	*Theileria Lestoquari* (Culture)
Theileria mutans ITS	*YES*	*YES*	
Theileria velifera 18S rRNA	*YES*	*YES*	
Theileria equi ema1	*YES*	*YES*	
Cytauxzoon felis ITS2	*YES*	*YES*	
Hepatozoon spp 18S rRNA	*YES*	CI	*Neoehrlichia mikurensis* (Infected rodent blood)
	YES	CI	*Rhipicephalus sanguineus* (Wild tick)
Hepatozoon canis 18S rRNA	*YES*	CI	*Rhipicephalus sanguineus* (Wild tick)
Hepatozoon americanum 18S rRNA	*YES*	CR	*Hepatozoon canis* (Infected dog blood)
	YES	CI	*Rhipicephalus sanguineus* (Wild tick)
Leishmania spp hsp70	*YES*	*YES*	
Leishmania infantum ITS	*YES*	*YES*	
Rangelia vitalii 18S rRNA	*YES*	*YES*	
Tick spp 16S rRNA	*YES*	*YES*[a]	
Amblyomma variegatum ITS2	*YES*	*YES*	
Rhipicephalus microplus ITS2	*YES*	*YES*	
Rhipicephalus sanguineus ITS2	*YES*	*YES*	

Note : [a] Tick spp. primers required to be part of the preamplification mix in order to achieve correct detection signals.

Table S2. Taxonomic assignation of the sequences obtained after sequencing of PCR products to confirm the presence of co-infections in complex control samples corresponding to DNA extracted from wild ticks or blood samples. AN: Accession number, % I: Percentage of identity, % C: Percentage of coverage.

Tested control sample	Tested for	Deposited sequence	Lenght (bp)	Closest homology	% I	% C	AN
***Panola mountain Ehrlichia* (Infected *A. americanum* ticks)**	*Rickettsia* spp.	MK049843	382	*Rickettsia* spp.	99	100	MF511253.1
***Neoehrlichia mikurensis* (Infected rodent blood)**	*Hepatozoon* spp.	MK071735	169	*Hepatozoon* spp.	99	100	AB771515.1
***Anaplasma marginale* (Infected cow blood)**	Apicomplexa	MK071737	104	*Babesia spp., Theileria spp. (including Babesia bigemina)*	100	99	MG604302.1
***Panola mountain Ehrlichia* (Infected *A. americanum* ticks)**	Apicomplexa	MK071736	102	*Theileria* spp. *(including T. cervi)*	98	100	MH085203.1
***Borrelia lonestari* (Infected *A. americanum* ticks)**	Apicomplexa						

Additional file 4.pdf: Co-infections found in ticks of Guadeloupe and Martinique.

	***A. variegatum* from Guadeloupe (n=132)**	***R. B. microplus* from Guadeloupe (n=165)**	***R. B. microplus* from Martinique (n=281)**
Non-infected	**0.7%**	**43.4 – 60.2%**	**18.5 - 20.6%**
Total Infected	**99.3%**	**39.8 – 56.6%**	**79.4 – 81.5%**
Infected by one pathogen	**51.1%**	**28.3 – 41.6%**	**35.2 – 35.9%**
Infected by two pathogens	**40.2%**	**9.6 – 13.3%**	**31.3 – 32.4%**
Infected by three pathogens	**7.3%**	**1.8%**	**9.3 – 9.6%**
Infected by four pathogens	**0.7%**	**0%**	**3.2%**
Infected by five pathogens	**0%**	**0%**	**0.4%**

Focus on *Rickettsia africae* and *Theileria velifera* in *A. variegatum* in Guadeloupe: 132 ticks were analyzed, the percentages of non-, single- and dual- infected ticks are presented in the table. There is no bias in the prevalence of *T. velifera* in ticks infected by *R. africae*.

***A. variegatum* (n=132)**	***Rickettsia africae* (+)**	***Rickettsia africae* (-)**
***Theileria velifera* (+)**	40.1%	2.2%
***Theileria velifera* (-)**	55.5%	2.2%

Focus on *Anaplasma marginale* and *Theileria velifera* in *R. B. microplus* in Martinique: 281 ticks were analyzed, the percentages of non-, single- and dual-infected ticks are presented in the table. There are no biases in the prevalence of the two pathogens in single versus dual-infected ticks.

***R. B. microplus* (n=281)**	***Anaplasma marginale* (+)**	***Anaplasma marginale* (-)**
***Theileria velifera* (+)**	12.1%	13.5 – 13.9%
***Theileria velifera* (-)**	27.4 – 29.2%	44.8 - 47%

Focus on *Ehrlichia spp.* and *Theileria velifera* in *R. B. microplus* in Martinique: 281 ticks were analyzed, the percentages of non-, single- and dual-infected ticks are presented in the table. There are no biases in the prevalence of the two pathogens in single versus dual-infected ticks.

***R. B. microplus* (n=281)**	***Ehrlichia spp.* (+)**	***Ehrlichia spp.* (-)**
***Theileria velifera* (+)**	11.4%	14.2 – 14.6%
***Theileria velifera* (-)**	36.3 – 37.7%	36.3 – 38.1%

Focus on *Ehrlichia spp.* and *Anaplasma marginale* in *R. B. microplus* in Martinique: 281 ticks were analyzed, the percentages of non-, single- and dual-infected ticks are presented in the table. There are no biases in the prevalence of the two pathogens in single versus dual-infected ticks.

***R. B. microplus* (n=281)**	***Ehrlichia spp.* (+)**	***Ehrlichia spp.* (-)**
***Anaplasma marginale* (+)**	24.9 – 26.3%	14.6 – 14.9%
***Anaplasma marginale* (-)**	22.8%	36 - 37.7%

1 Raw data

		Number of *Amblyomma variegatum* (percentage)	**Number of *Rhipicephalus microplus from Guadeloupe***	**Number of *Rhipicephalus microplus from Martinique***
			min - max (percentage)	**min - max (percentage)**
Total of ticks		**132 (100%)**	**165**	**281**
Total of Non-infected ticks		**1 (0.7)**	**72 (43.4%) - 100 (60.2%)**	**52 (18.5%) - 58 (20.6%)**
Total of infected ticks		**136 (99.3%)**	**66 (39.8%) - 94 (56.6%)**	**223 (79.4%) - 229 (81.5%)**
Infections by one microorganisms	**Total**	**70 (51.1%)**	**47 (28.3%) - 69 (41.6%)**	**99 (35.2%) - 101 (35.9%)**
	Rickettsia africae	67 (48.9%)	0	0
	Rickettsia spp.	2 (1.5%)	15 (9%) - 24 (14.5%)	0
	Theileria velifera	1 (0.7%)	25 (15.1%) - 36 (21.7%)	14 (5%) - 15 (5.3%)
	Anaplasma spp.	0	1 (0.6%)	10 (3.6%)
	Anaplasma marginale	0	2 (1.2%) - 3 (1.8%)	22 (7.8%) - 23 (8.2%)
	Ehrlichia spp.	0	3 (1.8%) - 4 (2.4%)	43 (15.3)
	Babesia bigemina	0	1 (0.6%) - 2 (1.2%)	7 (2.5%)
	Borrelia spp.	0	0	3 (1.1%)
Co-infections by two microorganisms	**Total**	**55 (40.2%)**	**16 (9.6%) - 22 (13.3%)**	**88 (31.3%) - 91 (32.4%)**
	Rickettsia spp. / *Theileria velifera*	2 (1.5%)	6 (3.6%) - 7 (4.2%)	0
	Rickettsia africae / Ehrlichia ruminantium	3 (2.2%)	0	0
	Rickettsia africae / Borrelia spp.	3 (2.2%)	0	0
	Rickettsia africae / Ehrlichia spp.	3 (2.2%)	0	0
	Rickettsia africae /	44 (32.1%)	0	0

	Theileria velifera			
	Anaplasma spp. / *Borrelia* spp.	0	1 (0.6%)	0
	Anaplasma spp. / *Theileria velifera*	0	2 (1.2%)	3 (1.1%)
	Anaplasma marginale / *Theileria velifera*	0	2 (1.2%) - 3 (1.8%)	10 (3.6%)
	Ehrlichia spp. / *Rickettsia* spp.	0	2 (1.2%) - 5 (3%)	0
	Ehrlichia spp. / *Theileria velifera*	0	1 (0.6%)	8 (2.8%)
	Theileria velifera / *Theileria mutans*	0	2 (1.2%) - 3 (1.8%)	1 (0.4%)
	Anaplasma marginale / *Borrelia* spp.	0	0	3 (1.7%)
	Anaplasma marginale / *Ehrlichia* spp.	0	0	39 (13.9%) - 42 (15)%
	Anaplasma marginale / *Babesia bigemina*	0	0	4 (1.4%)
	Anaplasma spp. / *Ehrlichia* spp.	0	0	6 (2.1%)
	Ehrlichia spp. / *Borrelia* spp.	0	0	3 (1.1%)
	Ehrlichia spp. / *Leishmania* spp.	0	0	1 (0.4%)
	Theileria velifera / *Babesia bigemina*	0	0	10 (3.6%)

	Total	**10 (7.3%)**	**3 (1.8%)**	**26 (9.3%) - 27 (9.6%)**
Co-infections by three microorganisms	*Rickettsia africae / Theileria velifera / Ehrlichia ruminantium*	3 (2.2%)	0	0
	Rickettsia africae / Theileria velifera / Ehrlichia spp.	2 (1.5%)	0	0
	Rickettsia africae / Theileria velifera / Borrelia spp.	3 (2.2%)	0	0
	Rickettsia africae / Theileria velifera / Theileria mutans	2 (1.5%)	0	0
	Anaplasma marginale / Ehrlichia spp. / *Rickettsia* spp.	0	1 (0.6%)	0
	Anaplasma marginale / Rickettsia spp. / *Theileria velifera*	0	1 (0.6%)	0
	Rickettsia spp. / *Theileria velifera / Theileria mutans*	0	1 (0.6%)	0
	Anaplasma marginale / Ehrlichia spp. / *Borrelia* spp.	0	0	1 (0.4%)
	Anaplasma marginale / Ehrlichia spp. /	0	0	8 (2.8%) 9 (3.2%)

	Babesia bigemina			
	Anaplasma marginale / Ehrlichia spp. / *Theileria velifera*	0	0	15 (5.3%)
	Anaplasma spp. / *Ehrlichia* spp. / *Babesia bigemina*	0	0	1 (0.4%)
	Anaplasma spp. / *Ehrlichia* spp. / *Theileria velifera*	0	0	1 (0.4%)
Co-infections by four microorganisms	**Total**	**1 (0.7%)**	**0**	**9 (3.2%)**
	Rickettsia africae / Theileria velifera / Borrelia spp. / *Ehrlichia ruminantium*	1 (0.7%)	0	0
	Anaplasma marginale / Borrelia spp. / *Theileria velifera / Theileria mutans*	0	0	1 (0.4%)
	Anaplasma marginale / Ehrlichia spp. / *Theileria velifera / Babesia bigemina*	0	0	5 (1.8%)
	Anaplasma marginale / Ehrlichia spp. / *Theileria velifera / Babesia bovis*	0	0	1 (0.4%)

	Anaplasma marginale / Leishmania spp. / *Theileria velifera / Theileria mutans*	0	0	1 (0.4%)
	Anaplasma spp. / *Ehrlichia* spp. / *Borrelia* spp. / *Theileria velifera*	0	0	1 (0.4%)
Co-infections by five microorganisms	**Total**	**0**	**0**	**1 (0.4%)**
	Anaplasma marginale / *Ehrlichia* spp. / *Borrelia* spp. / *Theileria velifera* / *Theileria mutans*	0	0	1 (0.4%)

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