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Real time micro-organisms PCR in 104 patients with polymorphic signs and symptoms that may be related to a tick bite

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ORIGINAL RESEARCH PAPER

ABSTRACT

Introduction: Ticks are frequently polyinfected and can thus transmit numerous microorganisms. A large number of bacteria, parasites and viruses are transmitted by tick bites and could cause different signs and symptoms in patients. The main goal of this study was to search for these numerous microorganisms in patients presenting with persistent polymorphic syndrome possibly due to a tick bite (SPPT). *Patients and methods:* The following microorganisms were searched for in saliva, urine, venous and capillary blood by using real time PCR: *Borrelia burgdorferi sensu lato, Borrelia miyamotoi, Borrelia hermsii, Bartonella* spp., *Bartonella quintana, Bartonella henselae, Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., *Coxiella burnetii, Brucella* spp., *Francisella tularensis, Mycoplasma* spp., *Chlamydia* spp., *Babesia* spp., *Theileria* spp. *Results:* 104 patients were included. 48% of the patients were poly-infected, and 25% harboured at least three different microorganisms. *Borrelia* spp. were not the most frequent bacteria observed, observed far behind *Mycoplasma* spp., *Rickettsia* spp. and *Ehrlichia* spp. which were the most frequent microorganisms observed. Piroplasms were found in a significant number of patients. The most sensitive matrix was saliva, followed by urine, capillary blood and venous blood. *Conclusion:* Our prospective study has shown that patients with SPPT, a syndrome close to fibromyalgia, could harbour several tick borne microorganisms.

KEYWORDS

lyme, *Borrelia, Babesia,* PCR, PTLDS, SPPT, capillary blood, saliva

INTRODUCTION

Lyme disease is a tick-borne infectious disease caused by *Borrelia burgdorferi sensu lato* (including *B. burgdorferi sensu stricto, Borrelia afzelii* and *B. Garinii*). The prevalence seems to be increasing in many countries around the world, particularly in France. In addition, some experts make the assumption that Lyme disease is not the only factor to explain the persistent polymorphic syndrome possibly due to a tick bite (SPPT), a syndrome close to the post-treatment Lyme disease syndrome (PTLDS) [1]. A large number of bacteria (other than *Borrelia*), parasites and viruses are transmitted by tick bites and could cause different signs and symptoms in patients, the so-called co-infections. In addition, the efficiency of Lyme serology is controversial with a tough scientific debate and it seems that the recommended

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two-tier testing for antibody detection (ELISA test followed, if positive, by a Western blot test) lacks sensitivity [2–8]. Above all, this serology only looks for some species of *Borrelia*. Another controversial issue is the persistence of the bacterium after antibiotic treatment.

Indeed ticks are frequently polyinfected and can transmit numerous microorganisms [9–12], whether bacteria (other species of *Borrelia, Bartonella* spp., *Ehrlichia* spp., *Rickettsia* spp.. . .), viruses, or parasites, first and foremost piroplasms (*Babesia*). Lyme disease is due to *B. burgdorferi sensu lato*, other species of *Borrelia* are responsible for relapsing fevers (transmitted by ticks or lice according to the species. *Borrelia miyamotoi* is now found in symptomatic patients, either with acute or chronic disease [13, 14]. The best known species of *Bartonella* are *Bartonella henselae,* responsible for cat scratch disease and *Bartonella quintana*, transmitted by lice and the agent of trench fever. They are usually investigated by serology [15–17]. In fact, there are many other species of *Bartonella* which may be transmitted by ticks and may cause neurological damage: radiculitis, myelitis, neurocognitive disorders for example. *Bartonella* have also been identified from patients with psychiatric diseases [17].

Other species of *Borrelia*, known in veterinary medicine has been found in the blood of patients with chronic syndromes [18]. *Ehrlichia* are responsible for ehrlichiosis, a tick-borne disease, which may be a Lyme disease co-infection [19]. *Rickettsia* species are numerous and are responsible for different groups of diseases, such as spotted fevers and typhus. Piroplasms are tick-borne monocellular parasites infecting red blood cells. Piroplasms include *Babesia* and *Theileria* species. Babesiosis is mainly known as an acute severe disease in splenectomised or deeply immunosuppressed persons, but the chronic form of the disease has not been much studied and is not well known. At the present time, *Theileria* have been only identified in animals, mainly horses. In the literature, no formal correlation between the isolation of such various micro-organisms and persistent signs and symptoms could be established.

Thus, the main goal of this study was to look for the different microorganisms transmitted or not by ticks in patients suffering from polymorphic signs and symptoms (SPPT/PTLDS), using real time qPCR, which is a direct diagnostic method amplifying the DNA of the microorganisms sought. As the yield of PCRs looking for Borrelia or co-infections in the venous blood is low [20, 21], another aim was to compare the results obtained by quantitative real time polymerase chain reaction (real time qPCR) from several matrices (venous blood, urine, saliva and capillary blood). The last aim was to evaluate the relevance to draw two samples, at Day 0 and Day 2.

PATIENTS AND METHODS

This is a prospective multi-centre observational study associating 8 clinical centres and one laboratory performing PCR analyses (AdNucleis) including patients with persistent polymorphic syndrome possibly due to a tick bite (SPPT). SPPT is a syndrome officially recognized by the French High Authority for Health (HAS) (a governmental institution https://www.has-sante.fr). SPPT includes post-treatment Lyme disease syndrome (PTLDS), but also addresses other hidden infections (crypto-infections).

SPPT is defined by a clinical triad associating several times a week, for more than 6 months: a polyalgic syndrome (musculoskeletal pain and/or neuropathic pain and/or headaches); persistent fatigue with reduced physical capacities; cognitive complaints. The difference between SPPT and PTLDS is that a diagnosis of Lyme disease has not to be proven and patients may have not been treated [22, 23].

Patients

The criteria for inclusion in this prospective study were: SPPT patients of at least 18 years of age, of either sex, presenting with the following signs or symptoms evolving for more than 6 months:

1. Neurocognitive disorders.
2. More than 2 of the following chronic sign or symptom categories:
   – Musculoskeletal: muscle pain, arthritis or arthralgia;
   – Neurological: facial paralysis, central or peripheral involvement, myelitis, root pain, paresthesias, dysesthesias, radiculopathy.
3. Abnormal asthenia.
4. Lack of etiology.
5. Have given their signed informed consent.

The following patients were excluded from the study:

1. Patients under 18 years of age, a context of acute intercurrent infectious pathology.
2. Patients who had received prior systemic antibiotic or antiparasitic treatment within two months prior to blood collection.

The protocol was approved by the ethical committee (Comité de protection des personnes CPP SUD EST VI Clermont Ferrand, France; document number: Ref. CPP AU 1396 Ref. ID-RCB 2017-A02705-48). All patients and control persons signed an informed consent in accordance with the Declaration of Helsinki.

Microorganisms searched

The following microorganisms were searched for by using real time PCR (Tables 1 and 2): *B. burgdorferi sensu lato (s.l.), B. afzelii, B. miyamotoi, Borrelia hermsii, Bartonella* spp., *B. quintana, B. henselae, Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., *Coxiella burnetii, Brucella* spp., *Francisella tularensis, Mycoplasma* spp., *Chlamydia* spp., *Babesia* spp., *Theileria* spp. *Theileria* spp. was investigated only at the end of the study for 33 patients.

Sample collection and nucleic acid sample preparation. Five ml of blood were collected by venous puncture in tubes with EDTA as anti-coagulant, capillary blood from the tip

Table 1. Real Time Multiplex polymerase chain reaction (PCR)

Samples	Urine and saliva were collected in dry bottles, five milliliters of blood were collected by venous puncture and around 500 µl of capillary blood were collected by finger prick in tubes with EDTA as anti-coagulant, before any antibiotic treatment and were sent in Vacutainer® K2 tubes. Samples (venous blood, urine, saliva, capillary blood) were drawn twice at Day 0 (D0) and Day 2 (D2).
Selection of Primers	To allow the detection of bacteria and parasites, primers targeting specific genes of each microorganism were used to amplify DNA by qPCR. Details of qPCR kits used is listed in Table 2.
Robustness of PCR Mixes	The portion of target genes were synthesized and introduced into a plasmid to obtain a control DNA and facilitate its multiplication. This control DNA was used to validate the amplification mixes. Serial dilution of the plasmid was performed and amplified to determine the robustness parameters of each PCR kit: the limit of detection (LOD), the limit of quantification (LOQ), the repeatability and the reproducibility.
DNA Extraction and Purification	The DNA was extracted without any prior treatment using 300 µl of whole blood with an equal volume of ADNucleis extraction buffer (5 M guanidium thiocyanate, 500 mM TrisHCL, 50 mM EDTA, 20% Tween 20, 20% Triton X-100, 750 µg proteinase K). After incubation for 20 min at 56°C and 15 min at 80°C, the extracted DNA was purified by means of silica magnetic beads and eluted in 250 µl of elution buffer (10 mM TrisHCl, pH 8.5).
Control of the Extraction	Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was used as a housekeeping gene as an internal control for PCR extraction and inhibition. The extracted samples were first checked with a PCR targeting the GAPDH gene. If the results of this PCR were consistent (Ct of GAPDH below 32), the samples were then analyzed for the other pathogens. The sequence of interest of GAPDH was inserted into a plasmid and this plasmid was used as a positive DNA for the validation of GAPDH primers and PCR mix as well as a positive control for subsequent PCRs. The primers used for GAPDH are described in Table 2.
Real-Time PCR (real time PCR)	Real-time PCR was carried out in a total volume of 50 µl with a PCR mix containing ADNucleis PCR buffer (20 mM Tris-HCl, 10 mM NH_4SO_4, 10 mM KCl, 2 mM Mg2+, 0.1% TritonX-100, pH 8.8), 2 mM of each dNTP, 600 nM of each primer, 1 µl of Evagreen and 5 units of Taq polymerase ADNucleis. Twelve µl of extracted samples were amplified. An initial denaturation step of 5 min at 95°C was followed by 42 cycles of 15 s at 95°C and 40 s at 60°C (hybridization-elongation). The dissociation curves were generated by a last step of 10 min with temperature increments from 75 to 95°C for qPCR kits using Sybr green technology.
Quantification	Positive samples were quantified using a standard curve obtained by amplifying known and calibrated concentrations of control DNA of the desired targets. Quantification was obtained using the standard curve equation (Ct = a (Log10 [DNA]) + b) where "a" is the slope and "b" the intercept of the curve. The results were expressed in genome units (UG) per ml of sample.

LOD: limit of detection.
LOQ: limit of quantification.
UG: Genome Units.
Ct: Cycle threshold.
LOD limit of detection and LOQ limit of quantification.
The LOD is calculated by comparing the response of the PCR kit with respect to a reference method, which is most often a method for cultivating the microbial population.
Once this microbial population has been cultured, it is stopped when the population is most abundant (eg 10E9); a count is carried out and the microbial population is subjected to successive dilutions in order to be able to have samples from 10E9 to 0, passing through all the intermediates (10E8, 10E7, etc.); these samples constitute the reference and the Borrelia analysis kit is evaluated for each dilution; we look for the sensitivities of the PCR kits making it possible to detect at least 10E2 DNA copies/PCR reaction volume, at best 10 copies/PCR reaction volume or less.
LOD is therefore expressed in DNA copies (or RNA for most viruses) detected per PCR reaction volume; and when we evaluate the regression of the response of the kit (in Ct with respect to each dilution) we must obtain a straight line of which we evaluate the linearity (equation) and the slope (a of the equation $y = ax + b$, y being the log value of the concentration of the bacterial population, x the value of the response of the kit in Ct); most often, this linearity is not complete, in particular for low concentrations of the microbial population; Then comes the LOQ (limit of quantification) which is the lowest detection value evaluated on the linear part of the regression line. The LOQ is therefore always greater than the LOD, if the latter is of the order of 5 copies/PCR reaction volume, the LOQ can be between 40 and 100 copies/PCR reaction volume; these values are always carefully assessed by the manufacturer before placing the PCR reaction kit on the market.

of the fingers was collected in tubes with EDTA, urine was collected in sterile bottle and saliva was collected using swab. 300 µl of venous and capillary blood were used to extract nucleic acid. 10 ml of urine were first centrifuged 10 min at 4,500 rpm in swinging bucket rotor, then the supernatant was discarded, and the pellet was resuspended in 300 µl of Rnase Dnase free water. Saliva swab sample was resuspended in 1 ml of Tris HCl 10 mM EDTA 1 mM buffer. 300 µl of the resuspension was used to extract nucleic acid.

Table 2. List of desired targets and details of PCR kits

Microorganisms	Species	Genes/name	Technology	Primers F	Primers R	Probe	Taqman Dye/Tm °C Sybr	LOD (UG/PCR)	LOQ (UG/PCR)
Borrelia burgdorferi	*sensu lato*	Flagelline	Tqm	CAAAYCAAGATGAAGCDATTGCWGTA	CTTCYTSTTGARCWCCYTCTTGAA	TGCAGYCTGAGCAGYYTGAGCT	FAM	5.7	2280
Borrelia	*miyamotoi*	glpQ	Sybr	TGCACAATTATTTCCCAATCGA	TTCACTGAGACTTAGTGATTTAAGTTCAGTT		80°C	12.5	18.8
Borrelia	*hermsii*	flaB	Sybr	AGCTGGATCACAAGCTTCATGGACA	CCCTCTATCTTTGCAAGTGACA		87°C	12.5	125
Borrelia	*afzelii*	CP009058.1	Sybr	AATTGCTTGTAGAGTTAA	AAGTTGCTGTTAGTATAG			63.6	636.2
Bartonella	spp.	rpoB	Sybr	CARGATTTRATTAAYGCRAA	ACRTCRCGMACTTCAAAR		87°C	2.57	12.8
Bartonella	*henselae*	ribC	Sybr	GATATCGGTTGTGTTGAAGA	AATAAAAGGTATAAAACGCT		84°C	19	125
Bartonella	*quintana*	ribE	Sybr	GATATCGGTTGTGTTGAAGA	AAAGGGCGTGAATTTTG		84°C	2.5	125
Babesia	spp.	18S	Sybr	ACCTGCTAACTAGTDBCC	CACAGACCTGTTATTGCC		84°C	5.7	5.7
Rickettsia	spp.	ARN 23S NR_076610.1	Sybr	ACCGATAGTGAACAAGTA	GGGTCTAATTYATCTAACTAAA		85°C	35.6	1780
Ehrlichia	spp.	16S	Sybr	GAGGATTTTATCTTTGTATTGTAGCTAAC	TGTAAGGTCCAGCCGAACTGACT		85°C	6	6
Anaplasma	spp.	Gène MSP4	Sybr	TTGTTTACAGGGGGCCTGTC	CTTGCCTAGCCTCTAACGTATGAG		85°C	25	25
Coxiella	*burnetii*	is111a	Tqm	AATTTCATCGTTCCCGGCAG	GCCGCGTTTACTAATCCCCA	TGTCGGCGTTTATTGGGTTGGTCCC	FAM	2.28	114
Mycoplasma	spp.	ARN 16S	Tqm	CACACTGGGACTGAGATA	TTCGCCCATTGTGGAATA	CCCTACTGCTGCCTCCCGTA	FAM	5.65	283
Brucella	spp.	IS711	Sybr	CAATCTCGGAACTGGCCATCTCGAACGGTAT	ATGTTATAGATGAGGTCGTCCGGCTGCTTGG		88°C	48.4	48.4
Francisella	*tularensis*	fopA	Tqm	AACAATGGCACCTAGTAATATTTCTGG	CCACCAAAGAACCATGTTAAACC	TGGCAGAGCGGGTACTAACATGATTGGT	FAM	11.4	114
Theileria	spp.	ARN 18S	Tqm	ACCTCTTCCAGAGTATCA	GCAGAAATTCAACTACGAG	CAAGTCTGGTGCCAGCAGCC	FAM	11.7	1170
Chlamydia	spp.	16S	Tqm	TGGCTCTCATGCAAAAGGCA	GATGCCTGGCATTGATAGGCGAWGAAGGA	TGGTTTCAGGTTCTATTTCACTCCC	FAM	48.4	484
hGAPDH		hGAPDH	Tqm	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC	FAM	22.8	227.9

Tqm: Taqman.

Sybr: Syber Green fluorophore.

The choice of the PCR technique (Sybr green or Taqman) is essentially linked to the sensitivity of each of the techniques; contrary to what is usually said, the two techniques are roughly equivalent, one being better than the other for certain targets and vice versa. And since we are looking for the best sensitivity in all cases, the laboratory has kept both techniques.

FAM: 6-carboxyfluorescéine (fluorophore).

LOD: limit of detection.

LOQ: limit of quantification.

UG: Genome Units.

The sensitivity of each kit, in particular the limit of detection (LOD) and the limit of quantification (LOQ), are the subject of an analytical evaluation calculated according to the recommendations of Regulation (EU) 2017/746.

LOD limit of detection and LOQ limit of quantification.

The LOD is calculated by comparing the response of the PCR kit with respect to a reference method, which is most often a method for cultivating the microbial population.

Once this microbial population has been cultured, it is stopped when the population is most abundant (eg 10E9); a count is carried out and the microbial population is subjected to successive dilutions in order to be able to have samples from 10E9 to 0, passing through all the intermediates (10E8, 10E7, etc.); these samples constitute the reference and the analysis kit is evaluated for each dilution; we look for the sensitivities of the PCR kits making it possible to detect at least 10E2 DNA copies or genome unit (UG)/PCR reaction volume, at best 10 copies/PCR reaction volume or less.

LOD is therefore expressed in DNA copies (Genome Units UG) detected per PCR reaction volume; and when we evaluate the regression of the response of the kit (in Ct with respect to each dilution) we must obtain a straight line of which we evaluate the linearity (equation) and the slope (a of the equation $y = ax + b$, y being the log value of the concentration of the bacterial population, x the value of the response of the kit in Ct); most often, this linearity is not complete, in particular for low concentrations of the microbial population; Then comes the LOQ (limit of quantification) which is the lowest detection value evaluated on the linear part of the regression line. The LOQ is therefore always greater than the LOD, if the latter is of the order of 5 copies/PCR reaction volume, the LOQ can be between 40 and 100 copies/PCR reaction volume; these values are always carefully assessed by the manufacturer before placing the PCR reaction kit on the market.

The ADNucleis DNA extraction-purification bead kit (ref ADNPVG300+BM) was used to extract DNA directly from 300 µl of blood samples or from processed sample as described above (urine and saliva).

Real time PCR method. Control DNA plasmids containing the amplified fragment was constructed to validate the amplification mixes, to be used as positive control. Serial dilution of the plasmid from 1–10 copies/PCR to 10^8 copies/PCR was made and used to determine the limit of detection (LOD), the limit of quantification (LOQ) of each target, Tm for the Sybr mixes (listed in Table 2). The primer and probes are listed in Table 2. Primes and probes were from the literature or designed for this study. Each target was tested as individual monoplex. The real time PCR was performed using TaqMan or 'SYBR' technologies. Each mixes were validated with specific PCR components used and developed by ADNucleis (including the Taq polymerase). The thermal cycling conditions of rt PCR were as following: 95 °C 5 min, 42 cycles of 95 °C 10s and 60 °C 40s for the TaqMan rt PCR. The EvaGreen PCR thermal cycling condition were the same the TaqMan one, expect the addition of the melting curve step (95 °C 15s, 75 °C 15s increasing temperature to 95 °C and 95 °C 15s). The reaction volume was 40 and 12 µl of DNA samples. A Ct cycle thermic (Ct) higher than 38 was considered as negative for the TaqMan mixes. A Tm within the range TM +/-1 °C was considered positive for the EvaGreen mixes. Each mixes were validated with specific PCR components used and developed by AD Nucleis (including the Taq polymerase).

Ethics statement

The study has been approved by an appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. National laws have been observed. The protocol was approved by the ethical committee (Comité de protection des personnes CPP SUD EST VI Clermont Ferrand, France; document number: Ref. CPP AU 1396 Ref. ID-RCB 2017-A02705-48).

RESULTS

Lack of detection of micro-organisms in healthy persons using venous blood qPCR

In a group of 24 healthy asymptomatic students, *B. burgdorferi* s.l., *B. miyamotoi*, *Ehrlichia* spp., *Babesia* spp., *B. hermsii, B. henselae, B. quintana* were searched by PCR in venous blood. All the PCR results were negative (Table 3).

Table 3. Lack of detection of micro-organisms in the healthy persons in venous blood qPCR

Borrelia burgdorferi s.l., *Borrelia miyamotoi*, *Ehrlichia* spp., *Babesia* spp., *Borrelia hermsii, Bartonella henselae, Bartonella quintana*, were searched by qPCR on a control group of 24 healthy asymptomatic students. For all extracted blood samples, a Ct of less than 32 was detected for the GAPDH extraction control. All the PCR results were negative.

	PCR Inhibition	Ct GAPDH values	Detection
FDC071	No	29.44	Not Detected
MCM072	No	24.46	Not Detected
MGA073	No	28.57	Not Detected
MFA074	No	28.47	Not Detected
FBF075	No	27.7	Not Detected
MDW076	No	27.76	Not Detected
FDT077	No	30.97	Not Detected
MAJ078	No	28.29	Not Detected
MMC079	No	28.81	Not Detected
FMS081	No	28.08	Not Detected
MSL082	No	31.28	Not Detected
MMD085	No	31.55	Not Detected
MPA088	No	30.57	Not Detected
FVA089	No	29.98	Not Detected
MGW092	No	31.17	Not Detected
FDN093	No	29.15	Not Detected
MBA094	No	31.87	Not Detected
FFS095	No	28.32	Not Detected
FBA096	No	28.7	Not Detected
FGA098	No	28.89	Not Detected
MACA101	No	26.54	Not Detected
MLS103	No	30.83	Not Detected
FLH105	No	31.77	Not Detected
FLL106	No	28.44	Not Detected
Positive control	No	22.83	Detected
Negative control	No	0	Not Detected

PCR and patients

In this study, 109 patients were included. For five patients, samples were analyzed at D0 only, and for 104 patients, samples were analyzed at D0 and D2. Venous blood was explored in 97 patients, urine and saliva in 104 patients. In addition to these three matrices, capillary blood was explored in 65 patients. A total of 730 samples were analyzed. Among them, 366 PCRs (50.1%) were positive.

Theileria spp., a parasite close to *Babesia* spp., was investigated in only 33 patients, because this research having been added late in the course of the study. Thus, the results are presented separately.

Real time Qpcr at day 0 (D0) and day 2 (D2)

No microorganisms were found in 5 (4.8%) patients. Ninety-nine patients out of 104 (95.2%) were positive for a least one microorganism (Figs 1–5) (Table 4), 47 (45.2%) patients were positive for at least one microorganism excluding *Mycoplasma* spp. The most frequent bacteria found were *Mycoplasma* spp., following by *Rickettsia* spp. and *Ehrlichia* spp. For two bacterial genera, *Borrelia* and *Bartonella*, several species of the same genus were identified. Five PCRs (1 at D0 and 4 at D2) in 4 patients (3.85%) were positive for *Borrelia*: 2 (1 at D0 and 1 at D2) in 1 patient (0.96%) for *B. afzelii*, 1 (1 at D0 and 0 at D2) in 1 patient (0.96%) for *B. miyamotoi*, 2 (0 to D0 and 2 to D2) in 2 patients (1.92%) for *B. hermsii*.

Twenty-three PCRs (14 at D0 and 9 at D2) in 16 patients (15.38%) were positive for *Bartonella*, 5 (4 at D0 and 1 at D2)

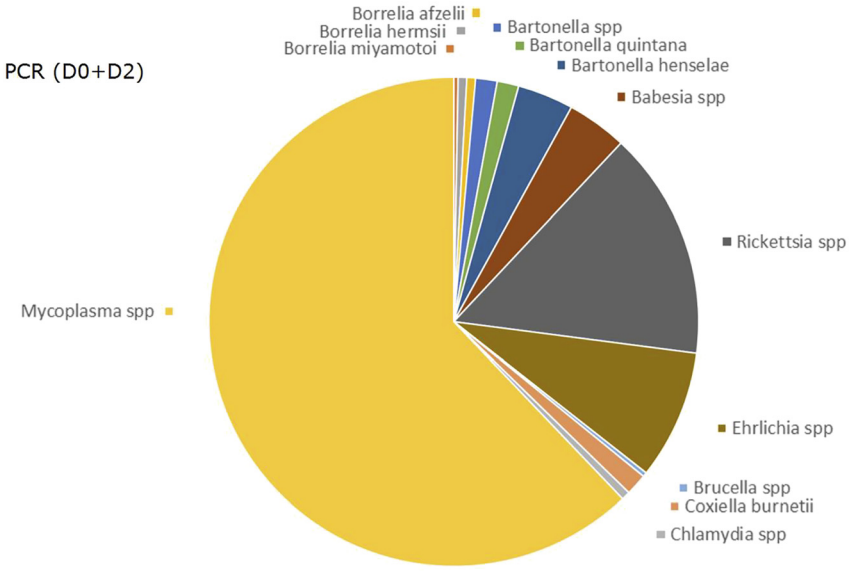

Fig. 1. PCR results at D0 + D2

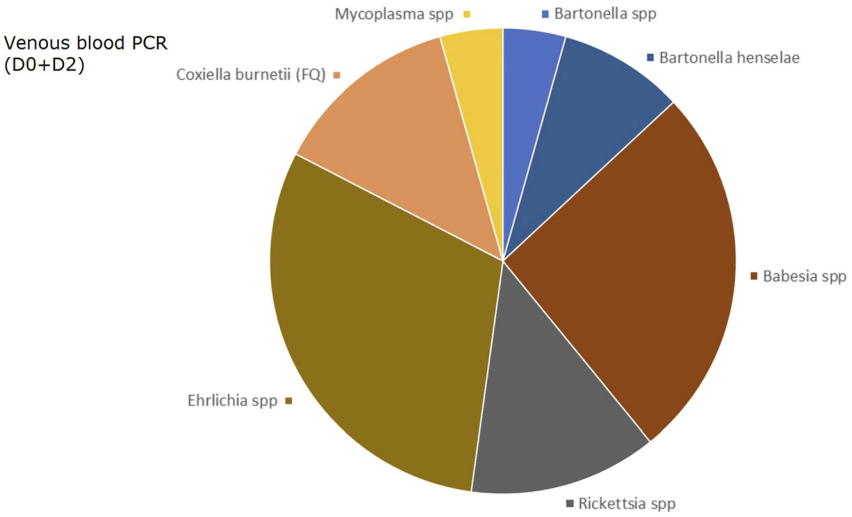

Fig. 2. Venous blood PCR results at D0 + D2

in 4 patients (3.85%) for *B. quintana*, 13 (7 at D0 and 6 at D2) in 4 patients (3.85%) for *B. henselae*, and 5 (3 at D0 and 2 at D2) in 8 patients (7.7%) for other *Bartonella* spp.

14 PCRs (6 at D0 and 8 at D2) in 9 patients (8.65%) were positive for *Babesia* spp.

53 PCRs (30 at D0 and 23 at D2), in 31 patients (29.8%) were positive for *Rickettsia* spp.

5 PCRs (3 at D0 and 2 at D2), 3 patients (2.88%) were positive for *Coxiella burnetii*.

30 PCRs (15 at D0 and 15 at D2), 15 patients (14.42%) were positive for *Ehrlichia* spp.

1 PCR (0 to D0 and 1 at D2), in 1 patient (0.96%) was positive for *Brucella* spp.

2 PCRs (0 to D0 and 2 at D2), 2 patients (1.92%) were positive for *Chlamydia* spp.

218 PCR (113 at D0 and 105 at D2), 91 patients (87.5%) were positive for *Mycoplasma* spp.

Anaplasma spp. and *F. tularensis* were not detected in this study.

17 PCRs, in 11 patients out of 33 tested (33%) were positive for *Theileria* spp.

Day 0 (D0) versus day 2 (D2). Globally the results are similar between D0, D2 and D0 + D2. Individually, in 7 cases where real time PCR had isolated no micro-organisms at D0, the second analysis found microorganisms. In some cases, the second analysis showed different microorganisms (Fig. 6) (Table 4).

Matrices

Venous blood was explored in 97 patients (97 at D0, 97 at D2), urine in 104 patients (104 at D0, 104 at D2), saliva in 104 patients (102 at D0, 101 at D2), and capillary blood in

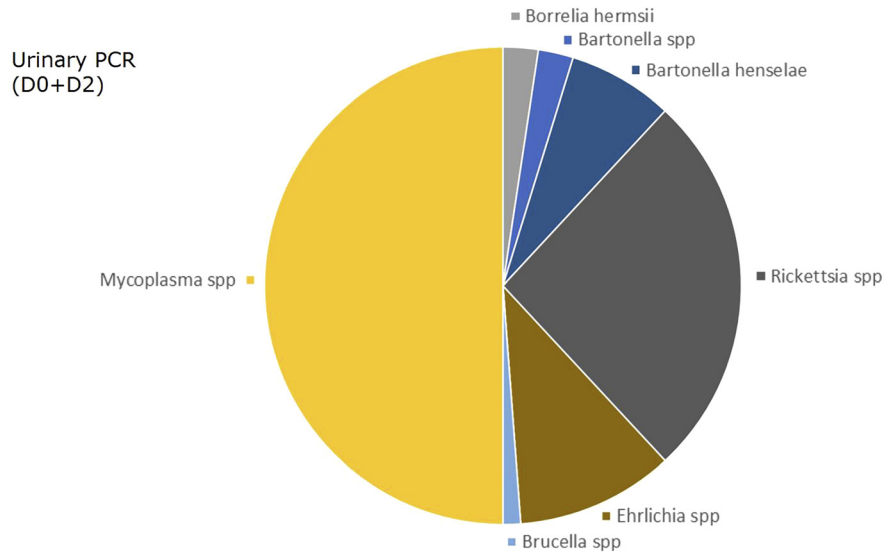

Fig. 3. Urinary PCR results at D0 + D2

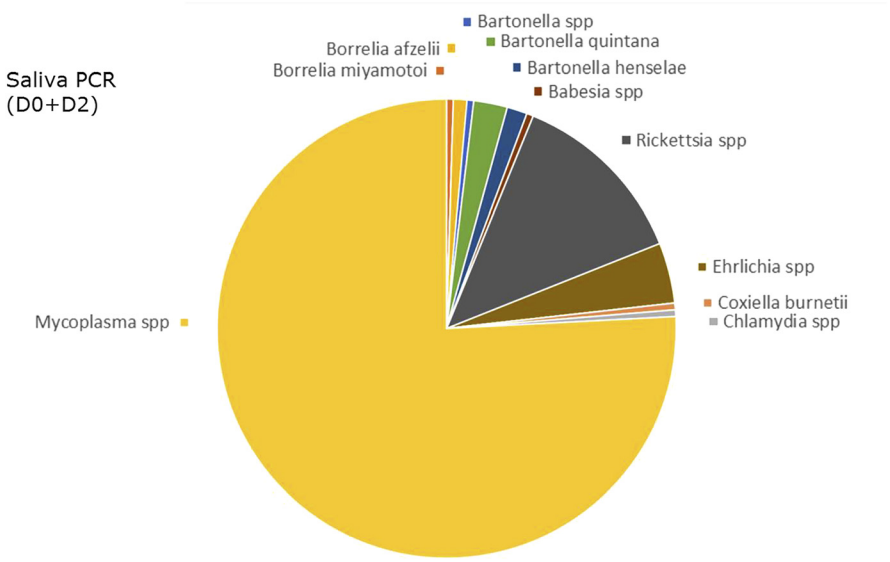

Fig. 4. Saliva PCR results at D0 + D2

only 65 patients (63 at D0, 62 at D2). For most patients, PCRs were studied twice, at D1 and D2: 97 of 97 from venous blood, 104 of 104 from urine, 100 of 104 from saliva and 62 of 65 from capillary blood.

Microorganisms found by matrix. In venous blood, the main microorganism found was *Ehrlichia* spp. (found 7 times out of a total of 23 positive results, 30.4%) followed by *Babesia* spp. (found 6 times out of a total of 23 positive results, 26.1%) (Fig. 2).

In urine, the main microorganism found was *Mycoplasma* spp. (found 42 times out of a total of 85 positive results, 49.4%) followed by *Rickettsia* spp. (found 22 times out of a total of 85 positive results, 25.9%) (Fig. 3).

In saliva, the main microorganism found was *Mycoplasma* spp. (found 160 times out of a total of 222 positive results, 72.7%) followed by *Rickettsia* spp. (found 27 times out of a total of 222 positive results, 12.3%) (Fig. 4).

In capillary blood, the main microorganism found was *Mycoplasma* spp. (found 15 times out of a total of 38 positive results, 39.5%) followed by *Babesia* spp. (found 7 times out of a total of 38 positive results, 18.4%) (Fig. 5).

Matrix performance - overall results. Percentage analyses were performed by weighting the results according to the number of analyses performed for each matrix.

Overall, the most informative matrix was saliva (42.08%), followed by urine (27.95%), capillary blood (18.23%), and venous blood (11.73%) (Fig. 7).

Matrix performance - results by microorganism. The results per microorganism (pathogen-specific PCR sequences)

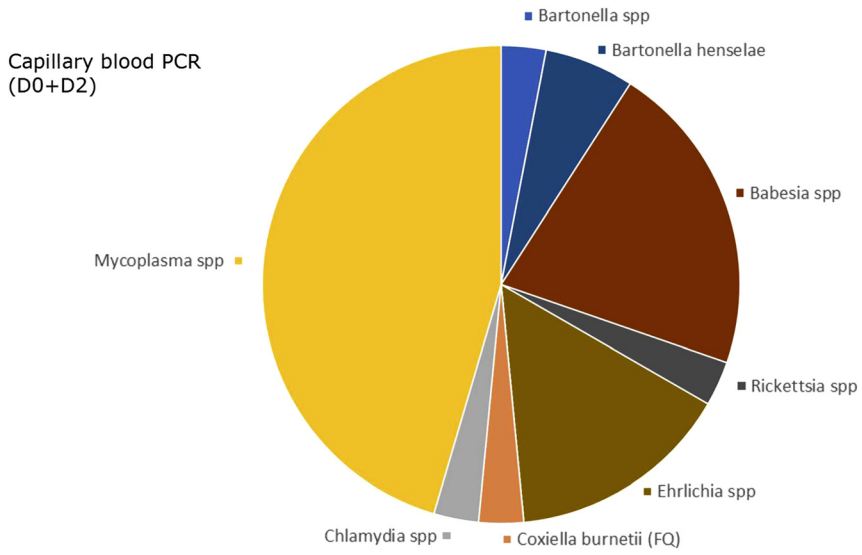

Fig. 5. Capillary blood PCR results at D0 + D2

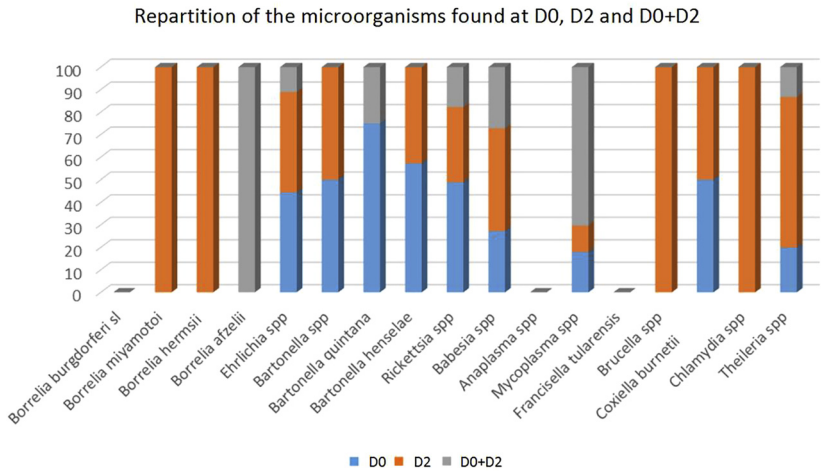

Fig. 6. Distribution of the microorganisms at D0, D2 and D0+D2

are weighted in relation to the number of analyses performed for each matrix.

Mycoplasma spp. were the most frequently identified micro-organisms. *Mycoplasma* spp. were found mainly in saliva. In this "open" environment, it is possible to hypothesize commensal microorganisms. *Mycoplasma* spp. were found once in venous blood (0.5%), 15 times in capillary blood (10.6%), 42 times in urine (18.5%) and 160 times in saliva (70.5%).

For *Borrelia*, the two informative matrices were urine (50%) and saliva (50%).

Bartonella spp. were found in all 4 matrices: *B. quintana* was found 5 times in saliva (100%). *B. henselae* was found twice in venous blood (14.9%), twice in capillary blood (22.3%), 6 times in urine (41.8%) and thrice in saliva (20.9%).

Bartonella spp. were found once in venous blood (18.9%), once in capillary blood (28.2%), twice in urine (35.3%) and once in saliva (17.6%).

Babesia spp. were found 6 times in venous blood (34.5%), 7 times in capillary blood (60.1%), 0 time in urine and once in saliva (5.4%).

Rickettsia spp. were found thrice in venous blood (6%), once in capillary blood (3%), 22 times in urine (40.9%) and 27 times in saliva (50.2%).

C. burnetii was found thrice in venous blood (55.3%), once in capillary blood (27.5%), 0 time in urine, and once in saliva (17.2%).

Ehrlichia spp. was found 7 times in venous blood (22.4%), 5 times in capillary blood (23.9%), 9 times in urine (26.9%), and 9 times in saliva (26.9%).

Brucella was found once in urine.

Chlamydia spp. was found once in capillary blood (61.5%), once in saliva (38.5%).

Theileria spp. were searched for in only 33 patients and found 17 times in total, 5 times in capillary blood (29.4%), once in urine (5.9%) and 11 times in saliva (64.7%).

Table 4. PCRs results

	D0	D2	D0 + D2	D0 + D2				Patients
				Blood	Urine	Saliva	Capillary blood	
Borrelia burgdorferi s.l.	0	0	0	0	0	0	0	0/104 (0%)
Borrelia miyamotoi	0	1	1	0	0	1	0	1/104 (0.96%)
Borrelia afzelii	1	1	2	0	0	2	0	1/104 (0.96%)
Borrelia hermsii	0	2	2	0	2	0	0	2/104 (1.92%)
Ehrlichia spp.	15	15	30	7	9	9	5	15/104 (14.42%)
Bartonella spp.	3	2	5	1	2	1	1	4/104 (3.85%)
Bartonella quintana	4	1	5	0	0	5	0	4/104 (3.85%)
Bartonella henselae	7	6	13	2	6	3	2	8/104 (7.7%)
Rickettsia spp.	30	23	53	3	22	27	1	31/104 (29.8%)
Babesia spp.	6	8	14	6	0	1	7	9/104 (8.65%)
Anaplasma spp.	0	0	0	0	0	0	0	0/104 (0%)
Francisella tularensis	0	0	0	0	0	0	0	0/104 (0%)
Brucella spp.	0	1	1	0	1	0	0	1/104 (0.96%)
Coxiella burnetii	3	2	5	3	0	1	1	3/104 (2.88%)
Chlamydia spp.	0	2	2	0	0	1	1	2/104 (1.92%)
Theileria spp.	5	12	17	0	1	11	5	11/33 (33.3%)
Mycoplasma spp.	113	105	218	1	42	160	15	91/104 (87.5%)

Spp.: species plurimae (all species in the genus).

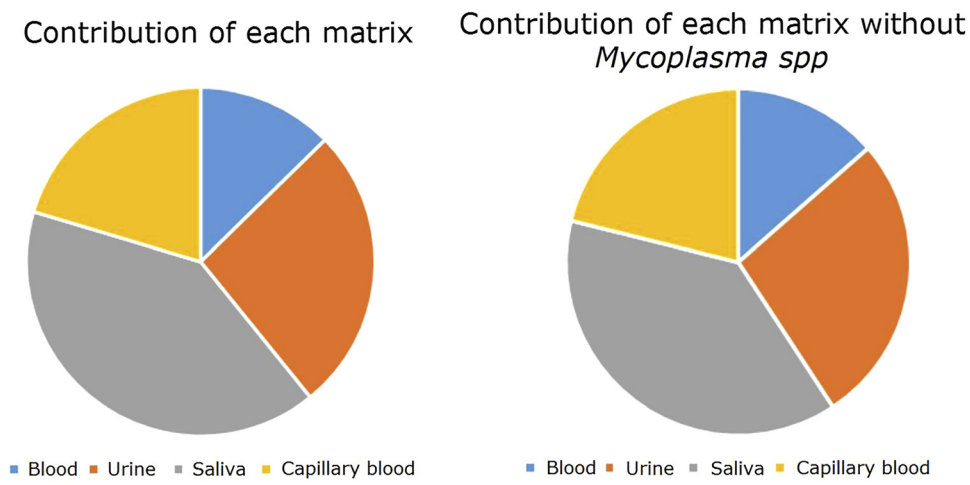

Fig. 7. Overall contribution of matrices with and without *Mycoplasma* spp.

For a given patient, microorganisms were most often found in one matrix only (67.4%), then in two matrices (25%), three matrices (6%), and finally four matrices (1.6%).

Ehrlihia spp. and *B. henselae* were the only microorganisms observed in all 4 matrices, once and twice, respectively (Table 5).

Poly-infection

Among the 104 patients who had a complete analysis (at D0 and D2), no microorganisms were found in 5 patients (4.81%), only one microorganism in 49 patients (47.12%), two different microorganisms in 25 patients (24.04%), three or more microorganisms in 25 patients (24.04%) (Figs 8 and 9) (Table 6). Thus, 48% of patients were poly-infected. The same analysis was performed without taking into account *Mycoplasma* spp., that could be considered as commensal bacteria. In this analysis, no microorganisms were found in 48 patients (46.15%), one single microorganism in 30 patients (28.85%), two different microorganisms in 16 patients (15.38%), three or more microorganisms in 10 patients (9.62%) Excluding *Mycoplasma* spp., 26% of patients were poly-infected.

DISCUSSION

This study showed that most patients with a combination of signs and symptoms consistent with the diagnosis of SPPT/PTLDS harbour microorganisms that are well detected using real time qPCR. As a matter of fact, *Borrelia* spp. were not the most frequent bacteria, observed far behind *Mycoplasma* spp., *Rickettsia* spp. and *Ehrlichia* spp. which were the most frequent microorganisms identified (Fig. 1). It could be hypothesized that the frequency of *Borrelia* is lower than expected or that this bacterium is particularly difficult to detect.

Table 5. Number of positive matrices per microorganism for a given patient

	Detected in 1 matrix	Detected in 2 matrices	Detected in 3 matrices	Detected in 4 matrices	Number of patients +
Borrelia burgdorferi s.l.					0
Borrelia miyamotoi	1				1
Borrelia afzelii	1				1
Borrelia hermsii	2				2
Ehrlichia **spp.**	9	3	1	2	15
Bartonella **spp.**	4				4
Bartonella quintana	4				4
Bartonella henselae	4	1	1	1	8
Rickettsia **spp.**	22	10			31
Babesia **spp.**	9	1			9
Anaplasma **spp.**					0
Francisella tularensis					0
Brucella **spp.**	1				1
Coxiella burnetii	2	1			3
Chlamydia **spp.**	2				2
Theileria **spp.**	7	4			11
Mycoplasma **spp.**	56	26	9		91

Spp.: species plurimae.

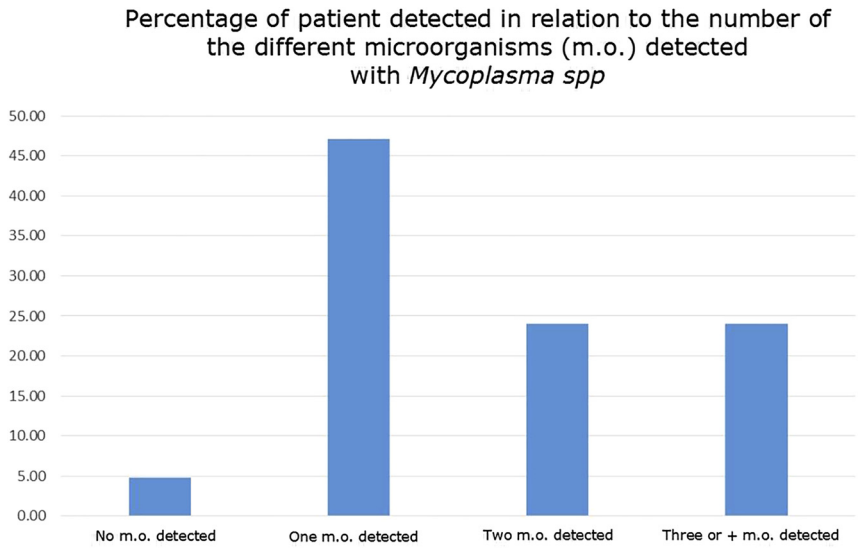

Fig. 8. Percentage of patients detected in relation to the number of the different microorganisms detected with *Mycoplasma* spp.

Borrelia could be present in deeper locations in a wide range of organs (brain for example) and thus could not be detected in this study, despite the use of several matrices [24].

In our study, piroplasms were found in a significant number of patients. Before this study, *Theileria* pathogen-specific PCR sequences, well known in veterinary medicine as it usually infests horses [25], were never isolated from humans. There is a very great genetic proximity between *Babesia microti* and *Theileria microti* and our PCR primers are studied to specifically isolate the genus *Babesia* from the genus *Theileria*. The fact that only *Babesia* species were known in human medicine is not an argument to say that theileriasis is not a human disease. A surprising result is that we isolated much more *Theileria* spp. than *Babesia* spp. These preliminary results suggest that *Theileria* could be a significant pathogen for humans. These results should be confirmed by additional studies. *Babesia* are most commonly known to cause severe infection with shock, mainly in patients who have undergone splenectomy. However, some articles describe some authentic *Babesia* spp. infections in immunocompetent patients, sometimes recurrent, with a torpid, chronic presentation [26–28]. Babesiosis is in fact poorly known, and it is probable that the frequency of infection by these parasites is underestimated [29]. As previously suggested by Muriel Vayssier Taussat et al. [18], various species of *Bartonella,* including species which were only known in veterinary medicine, could be responsible for various persistent signs and symptoms in humans. In further

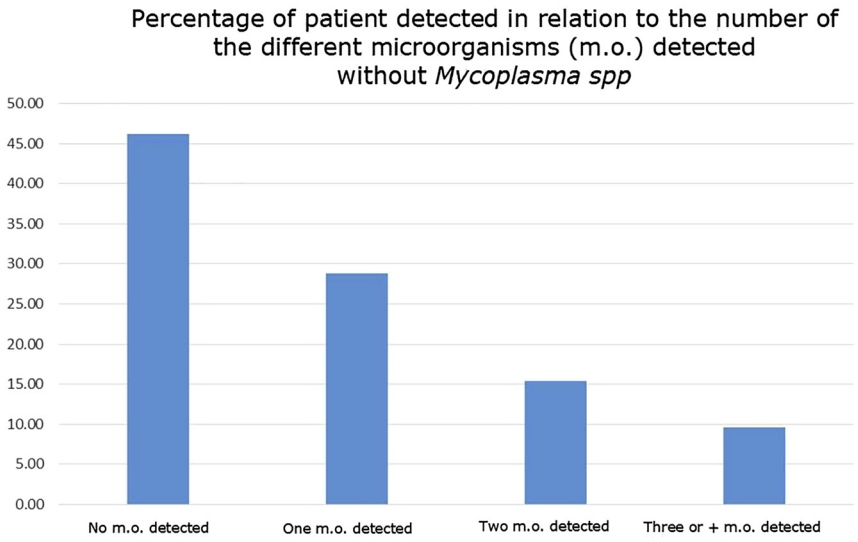

Fig. 9. Percentage of patients detected in relation to the number of the different microorganisms detected without *Mycoplasma* spp.

Table 6. Poly-infection: number of microorganisms detected per patient

	Percentage of patients based on the number of different microorganisms detected				
Number of microorganism(s) detected per patient	0	1	2	3 and +	
With *Mycoplasma* **spp.**	4.81	47.12	24.04	24.04	
Without *Mycoplasma* **spp.**	46.15	28.85	15.38	9.62	
	Number of patients				
Number of microorganism(s) detected per patient	0	1	2	3 and +	Total
With *Mycoplasma* **spp.**	5	49	25	25	104
Without *Mycoplasma* **spp.**	48	30	16	10	104

Spp.: species plurimae.

studies, the identification of the *Bartonella* species should be an important goal. Some bacteria such as *Mycoplasma* spp. are not constantly transmitted by ticks and could be commensal bacteria. However, some mycoplasmas such as *Mycoplasma fermentans* can be transmitted by ticks and be pathogenic [30]. Furthermore, Lyme disease can lead to a depression of the immune system [31], resulting in the occurrence of opportunistic infections, which could include *Mycoplasma* infections. These microorganisms, in particular *Mycoplasma*, could be transmitted by other sources than the tick bite, for example by sexual contamination. It might be thus more accurate to change the paradigm, and consider the term "crypto-infections" rather than exclusively "tick-borne infections" [32].

The study showed that nearly half of the patients were poly-infected, and a quarter of them harboured at least three different microorganisms. Indeed, at this preliminary stage of the research, it is not possible to distinguish between latent carriage and active infection.

The isolation of micro-organisms in saliva may be difficult to interpret. As the mouth is an open area, it could be a contamination from the outside. However, salivary secretion of micro-organisms exists. Some micro-organisms have a tropism for salivary glands, and the role of saliva for transmission of some infections, such as rabies or Epstein Barr virus, is well known. Salivary glands are holomerocrine. Secretion needs disruption of the apex of the acini cells. Thus, these acini cells must multiply rapidly. This could enhance the tropism of some micro-organisms. The possible secretion of micro-organisms by saliva should be further investigated. The results obtained from the different matrices provide interesting data.

For *Mycoplasma*, a high level of isolation from saliva and urine was observed. There was a trend for a superiority of capillary blood samples. For the *Mycoplasma* urine results, a possible contamination from the genital tract cannot be excluded. A precise characterization of the *Mycoplasma species* should be further studied. For *Bartonella*, urine samples seemed interesting. For *Ehrlichia*, the four matrices seemed interesting. For *Rickettsia*, a high level of isolation from saliva and urine was observed, without benefit from the capillary blood samples. For *Coxiella*, venous blood

samples provided the best results with a small additional benefit when combining with capillary blood samples. The most astonishing results were the very low sensitivity for *Borrelia*. For *Babesia* isolation, it was better to combine venous and capillary blood samples. Thus, this study showed that venous blood was the less sensitive matrix, far behind saliva, urine and capillary blood. In our study, *Borrelia* was found a few times in urine and saliva, but not in the blood. This confirms data from previous studies that showed a low interest of venous blood PCR for the detection of *Borrelia*. In this setting, PCR sensitivities and specificities are heterogeneous [33]. Some studies used PCR to identify *B. burgdorferi* in early Lyme disease, while we studied it in a chronic stage [34, 35]. In Eshoo et al.'s study, the sensitivity at an early stage was 62% and the specificity 100% [35]. Liveris et al. reported a sensitivity of 40.6% [34]. In these studies on early Lyme disease, the direct detection sensitivity was lower than that of the two-tier serology. However, in Bil-Lula's study, 3% of patients with negative ELISA IgM test, 2.8% with negative Line blot IgM test, 3.1% and 2.7% with negative ELISA IgG and Line blot IgG tests, respectively, were positive in real time PCR [20]. Few studies looked by PCR for *Borrelia* in urine. In one study, detection rate was 91% in patients with Lyme disease skin lesions [36]. In another study, results were disappointing [37].

Our study showed the advantage of a double sample for PCR at Day 0 and Day 2: individually, in 7 cases where real time PCR had isolated no micro-organisms at D0, the second analysis found microorganisms. In some cases, the second analysis showed different microorganisms. Therefore performing the PCR assays several times (in this case at D0 and D2), could increase the sensitivity of detection and improve the diagnosis of patient infections.

The clinical signs observed in SPPT/PTLDS may thus have an infectious origin (even if dysimmunitary phenomenons can play a role), and not a psychiatric origin as it has been previously hypothetised [38]. In our experience, the detection of bacteria and the presence of multiple co-infections often induce a more severe disease. However, our study was not designed to evaluate this specific point, which needs to be proven in future clinical studies. Most of our patients had a wide variety of clinical signs as follows: joint, muscle and tendon pain, neurological pain, tingling, sweats, hot flashes, fatigue, sleep difficulty, focusing/concentrating, neck pain, headache, difficulty finding words, irritability, low back pain, joint swelling, double vision, drooping facial muscle, drooping eyelid, tinnitus, heart palpitations. To confirm our results, subsequent studies showing that tick-exposed, healthy individuals do not exhibit a similar infection rate are necessary. The evaluation of appropriate anti-infective treatments in these patients, and real time qPCR tests after treatment, is also needed.

SPPT/PTLDS is very similar and difficult to dissociate from fibromyalgia syndromes [22, 23, 39, 40]. These syndromes associate three major signs: disabling fatigue, neuropsychic disorders (memory, sleep, concentration disorders) and various somatic signs; pain remains a predominant symptom [22, 23].

As all patients, in opposition to the persons in the control group, suffered from signs and symptoms, it is possible that the isolated microorganisms were actually responsible for the disease, or at least a cofactor. However, further studies on large populations, including healthy control persons, should look at the possibility of asymptomatic carriage.

In conclusion, our prospective study has shown that patients with persistent polymorphic syndrome possibly due to a tick bite (SPPT)/post-treatment Lyme disease syndrome (PTLDS), a syndrome close to fibromyalgia, could harbour several tick borne microorganisms. Microbiologic analyses should not be limited to *Borrelia*'s research alone. Taking several successive samples on different days could improve the detection of these microorganisms. Venous blood seems to be the less sensitive matrix, far behind saliva, urine and capillary blood. For some microorganisms, capillary blood seems to be more informative than venous blood. Thus, in the case of SPPT/PTLDS, qPCR analyses should be performed on different matrices to ensure optimal cost-effectiveness diagnostic. This study should be considered as a preliminary one. Due to the strong Lyme controversy, the absence of gold standards, the limited results published on co-infections identification, the low sensitivity of blood PCRs, it is difficult to rely on the literature to try to establish comparisons. Further studies are needed.

Key points: Our prospective real time qPCR (quantitative polymerase chain reaction) study has shown that patients with persistent polymorphic syndrome possibly due to a tick bite (SPPT)/post-treatment Lyme disease syndrome (PTLDS) could harbour several tick borne microorganisms.

Venous blood seems to be the less sensitive matrix, far behind saliva, urine and capillary blood.

The study showed that there was a benefit to performing the analyses twice, by increasing the detection of micro-organisms.

Conflict of interest: Michel Franck is CEO of ADNucleis.

The others authors do not declare any conflict of interest.

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Véronique Perronne: Writing original draft.

Yannick Lequette: Writing, review and editing.

Michel Franck: Investigation, Writing, review and editing, Resources, Validation.

Christian Perronne: Writing, review and editing; Supervision.

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