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Short communication

Development of a real-time PCR for *Bartonella* spp. detection, a current emerging microorganism

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ABSTRACT

A real-time PCR assay using SYBR Green was optimized to detect those *Bartonella* that are most frequently described as pathogens. The assay was genus-specific. Sequencing allowed to distinguish species. Assay sensitivity was determined using 10-fold serial dilutions of genomic DNA. Dynamic range was 100 ng–100 fg and sensitivity was 50 copies/reaction.

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1. Study

Until early 1990s, a few *Bartonella* species were known. However, more than 30 species and subspecies are currently described. Likewise, both the range of animals in which *Bartonella* is detected and the number of *Bartonella* species described as pathogens is rising [1–5]. Nowadays, it is known that one specie can cause a wide variety of symptoms as well as different species can cause similar symptoms [1,2,6]. Moreover, it has been observed that drug susceptibilities can vary depending on species. *Bartonella* are currently considered emerging pathogens because of the increasing number of clinical cases and new identified species [7]. Therefore, this genus is arousing great interest on medicine and veterinary. Consequently, it is necessary to have more accurate detection methods to reach a proper diagnosis and to obtain new and accurate data on their epidemiology.

Bartonella are facultative intracellular microorganisms whose culture is time-consuming and shows low sensitivity [1,2,7]. Serology is the most used method of diagnosis because of its simplicity. However, it has some limitations: (1) cross-reactivity, even with members of other genera [1]; (2) it cannot be applied during the earliest stage of infection; (3) commercial kits are restricted to a few species. Molecular methods based on real-time PCR opened new perspectives. Different real-time PCR assays have been developed for *Bartonella* detection. They show differences related to sensitivity, use of probes or SYBR Green, and range of detected species [8–17]. Since *Bartonella* genus shows genetic diversity [8,18], a universal assay for the detection of most *Bartonella* species is hardly impossible. Many real-time PCR assays have been designed for detecting a small number of species [9–13], even one or a few ones [14–17]. To our knowledge, there is only one assay that covers a wide range of species [8].

The aim of this study was to develop a real-time PCR for the identification of the most frequent *Bartonella* pathogens [1,2]. This assay was designed using SYBR Green. The main reasons were: (1) Bartonellosis are described over the world, thus, SYBR Green, which is cheaper than probes, could be used worldwide; (2) considering *Bartonella* genome variability [8,18], probes may not match depending on the strain and PCR assay may yield false negative results; (3) SYBR Green versatility may allow to identify species/strains not described yet. Therefore, SYBR Green, together with primers designed in highly conserved regions, would provide flexibility to our PCR for detecting more species, even those not introduced in the GenBank or not described yet.

In order to find a highly conserved region, *Bartonella* genomes were analysed using VISTA browser. This is a suite of databases and programs that allows to compare and analyse whole genomes [19]. Genomes of *Bartonella henselae* Houston-1 (NC_005956), *Bartonella*

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Table 1
Design and optimization of the assay.

Primers 5′ – 3′	Optimal concentration	Thermocycler conditions	*In silico* analysis[a]
FOR: CAAGGCATCCACCAAATG REV: GTGAAGAGAAGATATATTCAGA	800 nM 500 nM	∘ 95 °C-10 min. ∘ 40 cycles: • 95 °C-5 sec. • 60 °C-1 min. ∘ Melting program.	100%: *B. bacilliformis, B. henselae, B. vinsonii, B. quintana, B. schoenbuchensis, B. australis, B. grahamii, B. tribocorum, B. washoensis, B. doshiae,* Candidatus *B. ancashi.* 1 mismatch/primer: *B. taylorii*

[a] 100%: both primers are 100% homologues to sequences from those species.

quintana Toulouse (NC_005955), *Bartonella bacilliformis* KC583 (NC_008783), *Bartonella tribocorum* CIP 105473, and *Bartonella grahamii* as4aup (NC_012846) were analysed. Three sequences showed the highest homologies. First sequence targeted the transcription termination factor Rho whose sequence was highly conserved in many genera, so it was discarded. The second and third sequences were 16S and 23S regions, respectively. 16S was highly conserved among *Bartonella* species and hardly matched against any other genera. 23S was homologue to not only *Bartonella* species, but other microorganisms. Due to high similarity of 16S and 23S sequences among *Bartonella* species, intergenic region was included in our analysis. Sequences of different sizes, which contained 16S, intergenic, and/or 23S regions, were analysed. Finally, *B. bacilliformis* region NC_008783:1111442–1111732 (Vista Browser) was selected. It corresponded to 254350–254060 region of *B. bacilliformis* genome CP000524 of GenBank. It included 74 nucleotides of 23S region and 217 nucleotides of intergenic region. This sequence was chosen because it was hardly homologue to other genera. Primers were designed using Primer BLAST program (NCBI) (Table 1). Forward primer was designed on 23S region whereas reverse primer was designed on intergenic region. The latter was forced to be within a zone exclusively homologue to *Bartonella* species using the Primer BLAST program. In order to confirm its specificity, this primer was re-analysed comparing it with sequences of GenBank using blastn program, which considers all somewhat similar sequences. This primer was *Bartonella*-specific. *In silico* analysis showed that primers matched against 12 species (Table 1). Primers were synthesized by STAB VIDA (Lisbon, Portugal).

Optimization of PCR was performed using DNA of *B. henselae* H1, kindly provided by Dr. Chomel (University of California, USA). DNA was obtained using Masterpure DNA purification kit (Epicentre, Madison, Wisconsin) according to the manufacturer's instructions. Symmetric and asymmetric primers concentrations (150–900 nM) as well as different annealing temperatures (56°C–60 °C) were tested. Negative controls (DNA-free water as template) were simultaneously tested for each condition. Table 1 shows optimal PCR conditions, defined as the temperature and the lowest primers

Table 2
Specificity assay.

Microorganisms	Source	Real-time PCR
Bartonella henselae	University of California[a]	+
Bartonella elizabethae	CCUG - 30455[b]	+
Bartonella vinsonii sp. *vinsonii*	CCUG – 30453[b]	+
Bartonella grahamii	CCUG – 50771[b]	+
Bartonella bacilliformis	CCUG – 50771[b]	+
Bartonella quintana	DNA provided by Vircell	+
Rickettsia felis	Unité des rickettsies, France[c]	–
Rickettsia typhi	Unité des rickettsies, France[c]	–
Rickettsia slovaca	University of Alcala, Spain[d]	–
Rickettsia massiliae - Bar29	*Rhipicephalus sanguineus*[e]	–
Rickettsia conorii - Malish 7	1 isolate from a patient (CSPT[f])	–
Proteus mirabilis	1 isolate from a patient (CSPT)	–
Staphylococcus aureus MSSA	3 isolates from 3 patients (CSPT)	–
Staphylococcus aureus MRSA	2 isolates from 2 patients (CSPT)	–
Pseudomonas aeruginosa	2 isolates from 2 patients (CSPT)	–
Enterococcus faecium	1 isolate from a patient (CSPT)	–
Streptococcus epidermis	2 isolates from 2 patients (CSPT)	–
Klebsiella pneumoniae	2 isolates from 2 patients (CSPT)	–
Escherichia coli	2 isolates from 2 patients (CSPT)	–
Enterococcus faecalis	1 isolate from a patient (CSPT)	–
Streptococcus pneumoniae	2 isolates from 2 patients (CSPT)	–
Clostridium difficile	1 isolate from a patient (CSPT)	–
Corynebacterium	1 isolate from a patient (CSPT)	–
Serum	patient (CSPT)[g]	–
Blood	patient (CSPT)[g]	–
Blood	2 Cats seronegative (UAB)[h]	–
Blood	2 Dogs seronegative (UAB)[h]	–

[a] School of Veterinary Medicine, University of California, USA.
[b] CCUG: Culture collection of Göteborg, University of Sweden.
[c] Kindly provided by the Unité de Rickettsies (Marseille, France).
[d] Kindly provided by Dr. Gegundez from Alcala University (Spain).
[e] Isolated from *Rhipicephalus sanguineus.*
[f] CSPT: Corporació Sanitària del Parc Taulí.
[g] DNA from blood and serum of a human patient without Bartonellosis diagnosis at CSPT.
[h] Healthy seronegative cats and dogs from Veterinary Faculty of Autonomous University of Barcelona.

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