

MULTIPLEX SYBR® GREEN-REAL TIME PCR (qPCR) ASSAY FOR THE DETECTION AND DIFFERENTIATION OF *Bartonella henselae* AND *Bartonella clarridgeiae* IN CATS

Rodrigo STAGGEMEIER(1), Diogo André PILGER(2), Fernando Rosado SPILKI(1) & Vlademir Vicente CANTARELLI(1,3)

SUMMARY

A novel SYBR® green-real time polymerase chain reaction (qPCR) was developed to detect two *Bartonella* species, *B. henselae* and *B. clarridgeiae*, directly from blood samples. The test was used in blood samples obtained from cats living in animal shelters in Southern Brazil. Results were compared with those obtained by conventional PCR targeting *Bartonella* spp. Among the 47 samples analyzed, eight were positive using the conventional PCR and 12 were positive using qPCR. Importantly, the new qPCR detected the presence of both *B. henselae* and *B. clarridgeiae* in two samples. The results show that the qPCR described here may be a reliable tool for the screening and differentiation of two important *Bartonella* species.

KEYWORDS: *Bartonella*; Cat Scratch Disease; qPCR.

INTRODUCTION

Bartonella spp. are emerging infectious zoonotic agents^{13,23} endemic in some South American countries¹⁹. *Bartonella* can infect humans as well as domestic and wild mammals². There are more than 23 described species within this genus^{6,8}, among which, 13 are related to human diseases¹⁵. The most commonly reported species are *B. bacilliformis*, *B. quintana*, *B. henselae*¹ and *B. clarridgeiae*¹⁸.

The main vectors associated with human infections are fleas (*Ctenocephalides felis*), lice (*Pediculus humanus*), sandflies (*Lutzomyia verrucarum*, *Lutzomyia peruensis*)¹⁵ and ticks (*Ixodes pacificus*)¹⁰. The main animal reservoirs are cats, dogs and rodents¹⁵. The infection is generally more severe in immunocompromised hosts, but may also occur in healthy subjects¹⁵. *Bartonella bacilliformis* is an important cause of severe illness and death among immunocompetent adults and children¹⁹.

B. henselae and *B. clarridgeiae* are known as the causative agents of cat-scratch disease (CSD), which is characterized by chronic lymphadenopathy¹¹. Furthermore, in some cases, these bacteria may result in Parinaud oculoglandular syndrome, encephalopathy, convulsions, endocarditis, hepatosplenomegaly, glomerulonephritis, pleurisy, mediastinal adenopathy, nodules in the head of the pancreas, bacillary angiomatosis, osteomyelitis, atypical pneumonia, mammary tumours, haemolytic anaemia and eosinophilic purpura⁷.

More than 4,000 cases of CSD are registered each year in the United States, resulting in over 2,000 hospitalizations in the same

period, with a rate of 0.77 to 0.88 / 100,000 hospitalizations, with 55% of cases occurring in patients younger than 18 years old¹⁴. In the Netherlands, an estimated 2,000 cases occur per year with a rate of 12.5 cases/100 thousand inhabitants³. In Asian countries, reports show that the seroprevalence of *B. henselae* among the cat population ranges from 9.1% to 15.1% in Japan, 68% in the Philippines, 48% in Singapore and 54% in Indonesia⁷.

Bartonella spp are difficult-to-culture bacteria, hampering laboratory diagnosis of these infections. Other diagnostic alternatives, such as Giemsa-stained blood smears⁴ and serology⁷, are available with limited sensitivity, which may further delay proper diagnosis.

Giemsa stained blood smears can be useful in screening blood samples, however, the presence of artifacts might be a confusing factor²⁴. Serology has only a retrospective value, suggesting that the patient might have been infected during some period of their life, and hence is also of limited value. To date, no gold standard method for the detection of *Bartonella* species is available⁹.

Molecular techniques are powerful tools that may be used to screen for the presence of these pathogens in clinical samples¹⁶. Conventional polymerase chain reaction (PCR) has been used for the detection of several *Bartonella* species, and most of those assays use gene targets located on the 16S rRNA gene⁵, *ribC* gene¹⁷, *rpo* B gene²², 16S-23S intergenic spacer region (ITS)²⁰ and *gltA* gene²¹. However, real-time PCR is substituting conventional PCR in many laboratories as it is not only quicker but also more sensitive and specific than its predecessor¹².

(1) Laboratório de Microbiologia Molecular, Universidade Feevale, RS 239 n° 2755, 93352-000 Novo Hamburgo, RS, Brazil.

(2) Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Brazil.

(3) Laboratório Qualidade, Novo Hamburgo, RS, Brazil

Correspondence to: Rodrigo STAGGEMEIER, E-mail: rstaggemeier@gmail.com

The aim of this study was to evaluate a SYBR® green qPCR assay to detect and identify the presence of two *Bartonella* species directly from feline blood samples.

METHODS

Blood samples from 47 cats were collected during 2009 in two municipal animal shelters (Novo Hamburgo and São Leopoldo, south of Brazil), and have been described previously²⁴. All procedures were performed under veterinary supervision and approved by the Animal Ethics Committee from Universidade Feevale, under the protocol number 2.12.03.09.1391.

DNA was extracted from blood samples with the QIAmpDNA blood mini Kit (Qiagen). Two new primer pairs were especially designed from consensus genome regions obtained from the DNA Data Bank of Japan (DDBJ). Primers targeting fragments of the Citrate synthase gene were: one generic forward primer (BART-LC-GEN-F: 5' – ATGGGTTTTGGTCATCGAGT – 3'); one specie-specific reverse *B. henselae* primer (BART-LC-HEN-R: 5' –AA ATCGACATTAGGGTAAAGTTTTT – 3'); and one specie-specific reverse *B. clarridgeiae* primer (BART-LC-CLA-R: 5'-CAAGAAGTGGATCATCTTGG – 3'). Specificity of the assay was assessed by testing known positive samples (*B. henselae*, *B. clarridgeiae* and *B. bacilliformis*, confirmed by DNA sequencing²⁴), and several other clinical and ATCC-derived bacterial species. No false-positive reaction was noted with these tests. Apart from that, the specificity of the assay was also confirmed by *in silico* analysis of the primers, which demonstrated no cross-reaction with other *Bartonella* species. All positive results were confirmed by DNA sequencing reaction to make sure that these are true positive results and no false-positive samples were present. The results were analyzed by the DNA Data Bank of Japan (DDBJ Blast).

The reaction mix contained 2 µL of extracted DNA, 10 pM each primer, 1µL of BSA, 10 µL of SYBR green qPCR Supermix (Invitrogen) for a final volume of 20 µL. The multiplex qPCR was performed using the LightCycler®1.5 Real-Time PCR System (Roche Diagnostics) under the following conditions: 96 °C for two min (DNA denaturation), followed by 40 cycles of 96 °C for two sec, 60 °C for five sec and 72 °C for eight sec. A melting curve analysis was performed at the end of the amplification cycles. Positive reactions were recognized by typical melting temperatures (T_m ~ 82 °C). Since both species showed the same T_m, identification of the species among positive samples was performed by a second qPCR using species-specific primers (Singleplex) under the same conditions.

RESULTS

The multiplex SYBR® green qPCR allowed the detection of *Bartonella* DNA in 25.5% (12/47) of the blood samples. In a previous study, conventional PCR analysis targeting *Bartonella* spp in the same blood specimens resulted in a detection rate of 17.02% (8/47)²⁴. DNA sequencing analysis of the PCR products obtained by conventional PCR revealed the presence of only two *Bartonella* species among those samples, which consisted of *B. henselae*, present in 10.63% (5/47) of the samples, and *B. clarridgeiae* in 6.38% (3/47)²⁴. When using the multiplex qPCR, additional positive samples were observed with *B. henselae* and *B. clarridgeiae* detected in 17.02% (8/47) and 12.76% (6/47) of the samples,

respectively (Table 1). Moreover, this assay allowed us to observe these species co-infecting two samples, this was not previously detected using conventional PCR.

Table 1
Results by qPCR and Conventional PCR for the diagnosis of *Bartonella* infection

Samples	qPCR	PCR
1	<i>B. c.</i>	<i>B. c.</i>
2	<i>B. h.</i>	<i>B. h.</i>
3	<i>B. c.</i>	Neg.
4	<i>B. h.</i>	Neg.
5	<i>B. h.</i>	Neg.
6	<i>B. h. / B. c.</i>	Neg.
7	<i>B. h.</i>	<i>B. h.</i>
8	<i>B. c.</i>	<i>B. c.</i>
9	<i>B. c.</i>	<i>B. c.</i>
10	<i>B. h.</i>	<i>B. h.</i>
11	<i>B. h. / B. c.</i>	<i>B. h.</i>
12	<i>B. h.</i>	<i>B. h.</i>
13-47	Neg. by both methods	

B.h. (*Bartonella henselae*), B.c. (*Bartonella clarridgeiae*), Neg. (Negative).

DISCUSSION

Using multiplex qPCR, an additional four positive samples were found, which may represent an increase in sensitivity in comparison with the conventional PCR method. Moreover, the whole technique, including DNA extraction, could be performed within 2-3 hours, without any need of subsequent DNA sequencing for species identification. Conventional PCR, targeting consensus regions of the Citrate synthase gene may not amplify all *Bartonella* species with the same efficiency, thus explaining the differences in sensitivities obtained by our qPCR assay. Moreover, the identification of the species in positive samples depends on subsequent DNA sequencing. The advantage of the conventional PCR followed by DNA sequencing analysis would be the opportunity to identify the other or newer species that might be present in the blood samples. In this case, in a direct comparison, using the new specific primers for the detection of the two species identified by DNA sequencing, the qPCR assay was able to detect 100% of these species.

An advantage for the qPCR is the possibility of identifying the most prevalent *Bartonella* species in cat's blood samples without the need of subsequent DNA sequencing, which is laborious and, in our case, was not able to detect the presence of co-infection in two samples.

In conclusion, our results suggest that the multiplex SYBR® green qPCR may be a useful technique to detect and differentiate the two most common species of *Bartonella* directly from blood samples. With the lack of a defined gold standard method to detect the presence of different *Bartonella* species, there is a need for improved, clinically

useful methods⁹ for diagnosis of these infections, and the method developed here may be a useful diagnostic tool. Furthermore, compared with conventional PCR, the multiplex SYBR® green qPCR seems to be more sensitive; however, this hypothesis needs to be further investigated using a more robust number of specimens. The real time PCR described here is faster than conventional PCR and can be adapted to detect other *Bartonella* species if necessary. The species found here are those associated with CSD, which, therefore, may pose some risk to public health, mainly for HIV-positive individuals.

RESUMO

PCR em Tempo Real (qPCR) multiplex utilizando SYBR® Green para a detecção e diferenciação de *Bartonella henselae* e *Bartonella clarridgeiae* em gatos

Um novo teste baseado na reação em cadeia da polimerase em tempo real (qPCR) com SYBR® Green foi desenvolvido para detectar duas espécies de *Bartonella*, *B. henselae* e *B. clarridgeiae*, diretamente em amostras de sangue. Este teste foi utilizado em amostras de sangue obtidas de gatos que vivem em abrigos de animais do sul do Brasil. Os resultados foram comparados aos obtidos pelo PCR convencional utilizado para a detecção de *Bartonella* spp. Das 47 amostras analisadas, oito foram positivas no PCR convencional e 12 foram positivas para qPCR. A reação de qPCR, permitiu a detecção da presença simultânea de *B. henselae* e *B. clarridgeiae* em duas destas amostras. Os resultados mostram que a qPCR aqui descrita pode ser uma ferramenta confiável para a detecção e diferenciação de duas espécies importantes de *Bartonella* spp.

ACKNOWLEDGEMENTS

The authors thank the employers and volunteers of municipal shelters for providing the animals for this study. This work was supported by Universidade Feevale and Laboratório Qualidade.

REFERENCES

- Anderson BE, Neuman MA. *Bartonella* spp. as emerging human pathogens. *Clin Microbiol Rev*. 1997;10:203-19.
- Angulo FJ, Glaser CA, Juraneck DD, Lappin MR, Regnery RL. Caring for pets of immunocompromised persons. *J Am Vet Med Assoc*. 1994;205:1711-8.
- Bergmans AM de, Jong CM, van Amerongen G, Schot CS, Schouls LM. Prevalence of *Bartonella* species in domestic cats in The Netherlands. *J Clin Microbiol*. 1997;35:2256-61.
- Billeter SA, Levy MG, Chomel BB, Breitschwerdt EB. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Med Vet Entomol*. 2008;22:1-15.
- Birtles RJ. Differentiation of *Bartonella* species using restriction endonuclease analysis of PCR-amplified 16S rRNA genes. *FEMS Microbiol Lett*. 1995;129:261-5.
- Birtles RJ, Harrison TG, Saunders NA, Molyneux DH. Proposal to unify the genera *Granhamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb.nov., *Bartonella peromysci* comb.nov., and three newspecies, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov. and *Bartonella doshia* esp. nov. *Int J Syst Bacteriol*. 1995;45:1-8.
- Boulouis HJ, Chang CC, Henn JB, Kasten RW, Chomel BB. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. *Vet Res*. 2005;36:383-410.
- Brenner DJ, O'Connor SP, Winkler HH, Steigerwalt AG. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb.nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family Bartonellaceae from the order Rickettsiales. *Int J Syst Bacteriol*. 1993;43:777-86.
- Caponetti GC, Pantanowitz L, Marconi S, Havens JM, Lamps LW, Otis CN. Evaluation of Immunohistochemistry in identifying *Bartonella henselae* in cat-scratch disease. *Am J Clin Pathol*. 2009;131:250-6.
- Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. *J Clin Microbiol*. 2001;39:1221-6.
- Chomel BB. Cat-scratch disease. *Rev Sci Tech*. 2000;19:136-50.
- Ciervo A, Ciceroni L. Rapid detection and differentiation of *Bartonella* spp by a single-run real-time PCR. *Mol Cell Probes*. 2004;18:307-12.
- Dehio C, Sander A. *Bartonella* as emerging pathogens. *Trends Microbiol*. 1999;7:226-8.
- Huarcaya E, Maguina C, Merello J, Cok J, Birtles R, Infante B, et al. A prospective study of cat-scratch disease in Lima-Peru. *Rev Inst Med Trop Sao Paulo*. 2002;44:325-30.
- Jacomo V, Kelly PJ, Raoult D. Natural history of *Bartonella* infections (an exception to Koch's postulate). *Clin Diagn Lab Immunol*. 2002;9:8-18.
- Jensen WA, Fall MZ, Rooney J, Kordick DL, Breitschwerdt EB. Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. *J Clin Microbiol*. 2000;38:1717-22.
- Johnson G, Ayers M, McClure SCC, Richardson SE, Tellier R. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (ribC). *J Clin Microbiol*. 2003;41:1069-72.
- Kordick DL, Hilyard EJ, Hadfield TL, Wilson KH, Steigerwalt AG, Brenner DJ, et al. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). *J Clin Microbiol*. 1997;35:1813-8.
- Maco V, Maguina C, Tirado A, Maco C V, Vidal JE. Carrion's disease (*Bartonellosis bacilliformis*) confirmed by histopathology in the High Forest of Peru. *Rev Inst Med Trop Sao Paulo*. 2004;46:171-4.
- Matar GM, Swaminathan B, Hunter SB, Slater LN, Welch DF. Polymerase chain reaction-based restriction fragment length polymorphism analysis of a fragment of the ribosomal operon from *Rochalimaea* species for subtyping. *J Clin Microbiol*. 1993;11:1730-4.
- Norman AF, Regnery R, Jameson P, Greene C, Krause DC. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the cytrate synthase gene. *J Clin Microbiol*. 1995;33:1797-803.
- Renesto P, Gouvetmet J, Drancourt M, Roux V, Raoult D. Use of rpoB gene analysis for detection and identification of *Bartonella* species. *J Clin Microbiol*. 2001;39:430-7.
- Spach DH, Koehler JE. *Bartonella*-associated infections. *Infect Dis Clin North Am*. 1998;12:137-55.
- Staggemeier R, Venker CA, Klein DH, Petry M, Spilki FR, Cantarelli VV. Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats in the south of Brazil: a molecular study. *Mem Inst Oswaldo Cruz*. 2010;105:873-8.

Received: 27 November 2012

Accepted: 31 July 2013