



Detection of *Bartonella quintana* DNA in the presence of human and feline whole blood by single-tube PCR without DNA extraction

Bridget Ann Morton and Pedro Paulo Vissotto de Paiva Diniz*

*Correspondence: pdiniz@westernu.edu

Western University of Health Sciences, College of Veterinary Medicine, 309 East Second Street, Pomona, California, 91766, USA.

Abstract

Background: Rapid and cost-effective detection of emerging zoonotic blood-borne pathogens, such as *Bartonella* spp., is important for diagnostic and surveillance purposes. DNA extraction is a tedious process that increases the risk of cross-contamination, decreases the amount of target nucleic acids, and increases costs. The concept of using a direct PCR protocol for the detection of *Bartonella* in the presence of whole feline or human blood without DNA extraction was evaluated.

Findings: An optimized, single-tube, direct PCR for the detection of *B. quintana* in the presence of 20% of EDTA whole blood was optimized and validated using a DNA polymerase resistant to common PCR inhibitors. Ten genome equivalents of *B. quintana*/μl of blood were detected 100% of the time, and 2 genome equivalents/μl were detected 91% of the time.

Conclusions: The direct PCR for the detection of *Bartonella quintana* in whole blood is a viable, quick, highly sensitive, and cost-effective alternative that deserves further exploration.

Keywords: 16S-23S ribosomal RNA intergenic transcribed spacer, Alphaproteobacteria, Bartonellosis, Diagnosis, Direct PCR

Introduction

Nucleic acid amplification via polymerase chain reaction (PCR) is a well-established and highly sensitive tool for the molecular detection of pathogenic organisms from blood, tissues, and other clinical samples in humans and animals. Typically, highly purified template DNA is required for adequate gene amplification. However, a wide range of proteins, ionic detergents, organic solvents, alcohols, and salts can decrease or even inhibit polymerase activity [1,2]. Clinical samples such as whole blood and other specimens contain various PCR inhibitors. In whole blood, these inhibitors may be natural components such as heme, immunoglobulins, and leukocyte DNA, or added anticoagulants such as EDTA and heparin [1-3].

In order to decrease the presence of PCR inhibitors, multiple DNA extraction techniques have been developed and extensively tested. Although these methods have various advantages, they also present many disadvantages such as being labor intensive, time consuming, sample specific, and sometimes costly. In addition, they may decrease the detectable amount of target nucleic acids, increase the risk of cross-contamination between samples, and even introduce exogenous DNA from contaminated reagents [1-3]. Therefore, several protocols for nucleic acid amplification without previous purification of nucleic acids (direct PCR) have been published for the detection of virus, bacteria, protozoan, fungi, animal, or human genetic material [4-6]. However, to the knowledge of the authors, no direct PCR has been designed and validated for the detection of intraerythrocytic tick-borne diseases in humans, due to the presence of PCR inhibitors in whole blood.

Bacteria of the genus *Bartonella* are fastidious, gram-negative,

aerobic bacilli that are mainly transmitted by insect bites, such as fleas, ticks, and sand flies. *Bartonella* causes a long-lasting intraerythrocytic bacteremia in humans and other mammals and is considered an emerging zoonotic pathogen [7]. In humans, organisms from this genus are the causative agents of bacillary angiomatosis, endocarditis, myocarditis, cat scratch disease, intraocular inflammation, and recently have been associated with progressive neurological disease [7-9]. Domestic cats can be infected with several species of *Bartonella*, including *B. henselae*, *B. clarridgeiae*, *B. koehlerae*, *B. quintana*, and *B. bovis*. Cats are able to directly transmit the infection to humans by bites and scratches [7,10].

Diagnosis of intracellular pathogens that maintain chronic infections are typically detected via one or more diagnostic procedures, such as bacterial isolation, enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), or PCR amplification [7,11]. However, antibody detection has limited diagnostic value in humans and animals because infected subjects frequently lack antibody response to *Bartonella* spp. [12,13]. Culture remains the gold-standard for laboratory diagnosis of *Bartonella*; however, over two months of culture may be required, due to the slow-growth characteristic of these organisms [14]. Among numerous PCR assays described for *Bartonella* species, very few have used direct PCR amplification, and none has directly targeted this organism in blood samples without DNA extraction steps. *Bartonella* direct PCR was only reported twice, from heart valve vegetations [15] and from synovial fluid [4]. In addition, *Bartonella* spp. have been detected in healthy blood donors [16], and can survive in stored blood for over 35 days [17], suggesting a potential for transfusion-

associated infection. However, the use of direct PCR for the detection of *Bartonella* spp. from blood samples from humans and animals has not yet been explored.

Due to the diagnostic challenges faced when attempting to detect *Bartonella* spp. from EDTA-blood and the disadvantages of the DNA extraction process, this study aimed to test the concept of using direct PCR for the detection of *Bartonella quintana* from human and feline blood as a rapid molecular diagnosis method of Bartonellosis.

Materials and methods

Bartonella quintana isolate and negative controls

B. quintana strain ND1 (GenBank accession number DQ648598) isolated from a woman after putative cat bite transmission [18] was used as positive control in order to mimic the natural infection in both species. Purified and quantified DNA of this strain was serially diluted to concentrations of 500 genome equivalents (GE)/ μ l, 50 GE/ μ l, 10 GE/ μ l, 5 GE/ μ l, and 1 GE/ μ l, aliquoted in multiple vials and kept at -30°C . Each dilution was tested 11 times in the presence of feline whole blood or human whole blood. No single vial of each dilution was frozen and thawed more than five times to prevent DNA degradation. EDTA-blood from a healthy human and asymptomatic cat were used as negative controls.

Conventional 16S-23S ribosomal RNA intergenic transcribed spacer (ITS) PCR

A previously published conventional PCR assay designed to amplify the 16S-23S ribosomal RNA (rRNA) intergenic transcribed spacer (ITS) of *Bartonella* species [11] was used to rule-out natural *Bartonella* infection in both human and feline blood negative controls prior to artificial inoculation with quantified amounts of *Bartonella* DNA. In order to prevent contamination, DNA extraction, reaction setup, PCR amplification, and amplicon detection were performed in separate areas. This conventional PCR assay was able to detect 50, 25, 10, and 5 GE of *B. quintana* per reaction tube 100% of the time.

Blood direct PCR conditions and variables tested

A commercially available kit (Phusion[®] Blood Direct PCR Kit, Finnzymes, Finland) was used. The following variables were tested in combination to determine the most sensitive and reproducible results: total PCR reaction volumes of 20 μ l, 25 μ l, and 50 μ l; whole blood concentrations of 5%, 10%, 20%, 25%, 30%, and 40%; primer concentration from 7.5 pmol to 25.5 pmol; MgCl_2 gradients of 3 μM , 4 μM , and 4.5 μM (50 mM of MgCl_2 solution); and 2.5 μ l of 100% DMSO (in 50 μ l total reaction volume). Primers targeting the 16S-23S rRNA ITS described above [11] were used for blood direct PCR. Amplifications were performed under the following conditions: one cell lysis cycle at 98°C for 5 minutes; followed by 55 cycles of denaturing at 98°C for 5 seconds; annealing for 20 seconds; and extension at 72°C for 20 seconds. The

Table 1. Optimal conditions and analytical sensitivity of direct PCR amplification of *Bartonella quintana* DNA in whole blood from humans and cats.

Component	25 μ l reaction
H_2O	5.8 μ l
2x PCR buffer*	12.5 μ l
Primer ITS 438s (30 mM)	0.35 μ l
Primer ITS 1000as (30 mM)	0.35 μ l
DNA polymerase†	0.35 μ l
MgCl_2 (50 mM)	1 μ l
Whole blood	5 μ l (20%)
Primer annealing temperature	
Human blood	68°C
Feline blood	67°C
Analytical sensitivity	
50 GE/reaction [‡] - 10 GE/ μ l of human or feline blood [§]	100% (11/11)
10 GE/reaction (2 GE/ μ l of human or feline blood [§])	90.9% (10/11)
5 GE/reaction (1 GE/ μ l of feline blood)	45.5% (5/11)
5 GE/reaction (1 GE/ μ l of human blood)	36.4% (4/11)

*Phusion[®] Blood PCR Buffer (includes dNTPs and 3 mM MgCl_2)

†Phusion[®] Blood II DNA Polymerase

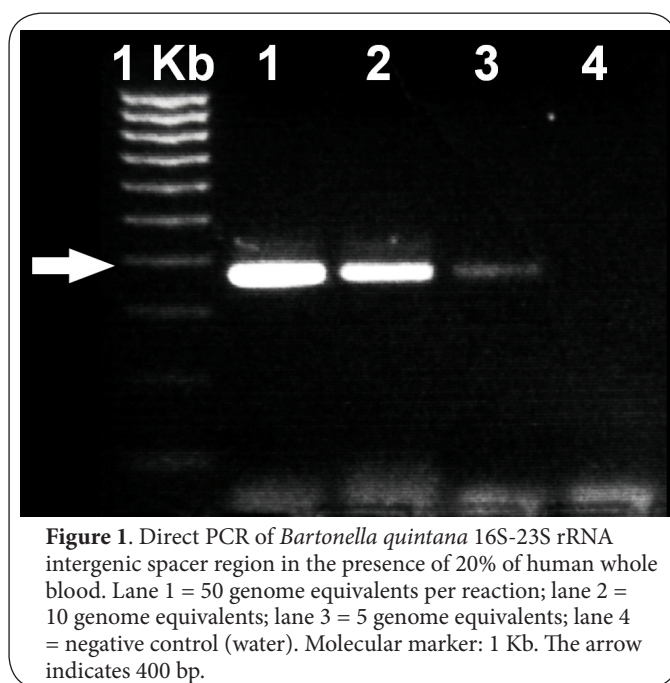
‡ Genome equivalents of *B. quintana* per PCR reaction tube

§ Eleven replicates were tested for each host blood (human or feline)

annealing temperature was tested at 66°C , 67°C , and 68°C in a thermocycler with a maximum sample ramp rate of 3.35 $^{\circ}\text{C}$ per second (Verity[®] Thermal Cycler, Applied Biosystems, Life Technologies Corp. Carlsbad, CA). One microliter of each quantified dilution of *B. quintana* DNA was used as positive control. Molecular grade water was used as negative control. *B. quintana* amplicons were identified with approximately 377 bp in size by 2% agarose gel electrophoresis under UV exposure. Non-spiked human and feline blood were used as negative controls.

Results

Optimized conditions with the best analytical sensitivity results for blood direct PCR are provided (Table 1). *Bartonella* DNA was amplified in the presence of 5%, 10%, and 20% of whole blood, but not in the presence of 40%. Therefore, 20% was established as the targeted concentration of blood for the optimization of sensitivity. Serial dilutions of *B. quintana* in EDTA-blood were detected at concentrations of 50 GE per reaction 100% of the time in 11 replicates of human whole blood, as well as 11 replicates of feline whole blood; 10 GE per reaction were detected 90.9% (10 out of 11 replicates) of the time in both human and feline blood; 5 GE per reaction were detected 45.5% (5 out of 11 replicates) of the time in feline blood, and 36.4% (4 out of 11 replicates) of the time in human blood. Since 5 μ l of whole blood were added in each reaction tube, the analytical sensitivity for a naturally-infected blood sample was 10 GE/ μ l 100% of the time, with the limit of detection of 1 GE/ μ l (Figure 1). No amplifications were obtained from negative controls in any condition tested.



Discussion

This study demonstrates the ability of direct PCR to detect *Bartonella* DNA in the presence of human or feline blood without DNA extraction and purification. The commercial kit used in this study employs a modified hot-start, high-fidelity DNA polymerase that exhibits extremely high resistance to inhibitors found in blood. A wide range of blood concentrations, from 1% to 40%, can be used with this kit. Since the detection of low numbers of targeted DNA per reaction is directly related with the volume of whole blood added in a direct PCR reaction, the optimization of this assay was designed to use the maximum amount of blood that would not significantly interfere with analytical sensitivity, determined to be 20% for this direct PCR assay. The analytical sensitivity achieved with this direct PCR assay was surprisingly close to that obtained from conventional PCR using the same ITS primers used in this study [11]. The natural infection of *Bartonella* spp. in immunocompetent humans causes an average bacteremia level of 1 to 10 GE/ μ l of whole blood, while in infected cats it is estimated to be between 10^5 to 10^6 GE/ μ l [7]. Therefore, the analytical sensitivity of 2 to 10 copies/ μ l of blood obtained with the direct PCR assay in this study supports the use of this diagnostic test for both species. Other conventional *Bartonella* PCR assays have reported detection limits between 2 and 50 genome equivalents [13,14,19,20]. Using the same direct PCR kit from this study, Fuehrer *et al.*, (2011) described the detection of 3 to 5 GE of *Plasmodium* spp. from dried blood samples in filter paper [5]. Therefore, the results of this study are in line with the analytical sensitivity obtained by conventional PCR assays, suggesting that the direct PCR can be a quick and cost-effective alternative for the diagnosis of *Bartonella* infection.

The cost effectiveness of blood direct PCR has been recently described [5]; however, in that report the direct PCR was used only in the first step of a nested PCR using 50 μ l of reaction volume in each step. Because nested PCR has an intrinsically higher risk of cross-contamination [19] and uses twice as many reagents per tested sample, this approach was not used in our study. Our study achieved successful amplifications by using only a single step direct PCR with only 25 μ l of reaction volume, resulting in consequent economy of reagents. The extended number of cycles used in this study provided sufficient analytical sensitivity without interfering with the specificity of the amplification, as previously reported with other DNA polymerases [11,13].

Bartonella species may be present in blood or other clinical samples in concentrations below the limit of detection of molecular techniques [12,18]. A *Bartonella* Alpha Proteobacteria Growth Medium (BAPGM) was designed to multiply the number of targeted organisms prior to PCR amplification and/or isolation in culture plates [14]. However, liquid culture samples with high concentration of *Bartonella* organisms need to be subjected to laborious DNA extraction, increasing the chances of cross-contamination. The direct PCR assay described in this study may also be used for fast screening of liquid cultures after the enrichment process without the need for DNA extraction.

Despite proving the concept of detecting very low numbers of *Bartonella* DNA in the presence of whole blood, this study has several limitations. Red blood cells with intracellular *Bartonella* spp. were not used due to the technical challenges of obtaining erythrocytes infected with these organisms *in vitro*, and precisely quantifying the number of genome equivalents in whole blood without DNA extraction. The quantification of colony-forming units (CFU) from blood spiked with a *Bartonella* isolate was not an option in this study due to the fastidious nature of these organisms and their sub-optimal growth in solid media cultures. The use of quantified DNA based on the molecular weight of the fully sequenced *B. quintana* genome provided an accurate quantification of genome equivalents used in this study, as previously reported [11,13]. Because ITS primers used in this study were originally designed to amplify multiple *Bartonella* species, it is expected that the direct PCR assay reported here would be able to accurately detect other species of *Bartonella*. However, additional studies are needed to evaluate the efficacy of the direct PCR in detecting other *Bartonella* species, not only in human and feline blood, but also other mammalian species.

The results of this study indicate that direct PCR from blood is a viable, rapid, highly sensitive, and cost effective alternative for the diagnosis of *Bartonella* spp. without a DNA extraction step. The use of highly-resistant polymerases for the detection of *Bartonella* and other intracellular organisms, not only in blood but also in other diagnostic specimens, deserves further investigation.

List of abbreviations

BAPGM: Bartonella Alpha Proteobacteria Growth Medium
CFU: colony-forming unit
GE: genome equivalent
IFA: immunofluorescence assay
ITS: intergenic transcribed spacer
Kb: kilobase
PCR: polymerase chain reaction
rRNA: ribosomal RNA
UV: ultraviolet light

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PPVPD conceived and designed the study, and helped to draft the manuscript. BAM carried out laboratory experiments and wrote the manuscript. Final manuscript was read and approved by both authors.

Acknowledgement

DNA from *B. quintana* was kindly provided by Edward B. Breitschwerdt and Ricardo G. Maggi, from the Intracellular Pathogens Research Laboratory at North Carolina State University. This study was supported by Western University of Health Sciences (WesternU). Bridget Morton was supported by a summer fellowship from WesternU. Blood direct PCR kits were kindly provided by New England BioLabs Inc., Ipswich, MA, which did not participate in the study design, data collection, analysis, conclusions, or generation of this manuscript. The authors thank John R. Greenwood for critical review of the manuscript and for administrative support. The authors also thank Maryam Tngrian, Dominique Griffon and Steven Henriksen for support at various levels at WesternU.

Publication history

Received: 21-Feb-2013 Revised: 22-Mar-2013
Accepted: 10-Apr-2013 Published: 03-May-2013

References

- Al-Soud WA and Radstrom P: **Purification and characterization of PCR-inhibitory components in blood cells.** *J Clin Microbiol* 2001, **39**:485-93. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Radstrom P, Knutsson R, Wolffs P, Lovenklev M and Lofstrom C: **Pre-PCR processing: strategies to generate PCR-compatible samples.** *Mol Biotechnol* 2004, **26**:133-46. | [Article](#) | [PubMed](#)
- Wilson IG: **Inhibition and facilitation of nucleic acid amplification.** *Appl Environ Microbiol* 1997, **63**:3741-51. | [Pdf](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Dillon B, Cagney M, Manolios N and Iredell JR: **Failure to detect Bartonella henselae infection in synovial fluid from sufferers of chronic arthritis.** *Rheumatol Int* 2000, **19**:219-22. | [Article](#) | [PubMed](#)
- Fuehrer HP, Fally MA, Habler VE, Starzengruber P, Swoboda P and Noedl H: **Novel nested direct PCR technique for malaria diagnosis using filter paper samples.** *J Clin Microbiol* 2011, **49**:1628-30. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Li H, Xu H, Zhao C, Sulaiman Y and Wu C: **A PCR amplification method without DNA extraction.** *Electrophoresis* 2011, **32**:394-397. | [Article](#)
- Breitschwerdt EB, Maggi RG, Chomel BB and Lappin MR: **Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings.** *J Vet Emerg Crit Care* 2010, **20**:8-30. | [Article](#) | [PubMed](#)
- Breitschwerdt EB, Mascarelli PE, Schweickert LA, Maggi RG, Hegarty BC, Bradley JM and Woods CW: **Hallucinations, sensory neuropathy, and peripheral visual deficits in a young woman infected with Bartonella koehlerae.** *J Clin Microbiol* 2011, **49**:3415-7. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Jacomo V, Kelly PJ and Raoult D: **Natural history of Bartonella infections (an exception to Koch's postulate).** *Clin Diagn Lab Immunol* 2002, **9**:8-18. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Chomel BB, Boulouis HJ, Maruyama S and Breitschwerdt EB: **Bartonella spp. in pets and effect on human health.** *Emerg Infect Dis* 2006, **12**:389-94. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Cherry NA, Maggi RG, Rossmesl JH, Hegarty BC and Breitschwerdt EB: **Ecological diversity of Bartonella species infection among dogs and their owner in Virginia.** *Vector Borne Zoonotic Dis* 2011, **11**:1425-32. | [Article](#) | [PubMed](#)
- Breitschwerdt EB, Maggi RG, Robert Mozayani B, Hegarty BC, Bradley JM and Mascarelli PE: **PCR amplification of Bartonella koehlerae from human blood and enrichment blood cultures.** *Parasit Vectors* 2010, **3**:76. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Diniz PP, Maggi RG, Schwartz DS, Cadenas MB, Bradley JM, Hegarty B and Breitschwerdt EB: **Canine bartonellosis: serological and molecular prevalence in Brazil and evidence of co-infection with Bartonella henselae and Bartonella vinsonii subsp. berkhoffii.** *Vet Res* 2007, **38**:697-710. | [Article](#) | [PubMed](#)
- Maggi RG, Duncan AW and Breitschwerdt EB: **Novel chemically modified liquid medium that will support the growth of seven bartonella species.** *J Clin Microbiol* 2005, **43**:2651-5. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Qin X and Urdahl KB: **PCR and sequencing of independent genetic targets for the diagnosis of culture negative bacterial endocarditis.** *Diagn Microbiol Infect Dis* 2001, **40**:145-9. | [Article](#) | [PubMed](#)
- Magalhaes RF, Cintra ML, Barjas-Castro ML, Del Negro GM, Okay TS and Velho PE: **Blood donor infected with Bartonella henselae.** *Transfus Med* 2010, **20**:280-2. | [Article](#) | [PubMed](#)
- Magalhaes RF, Pitassi LH, Salvadego M, de Moraes AM, Barjas-Castro ML and Velho PE: **Bartonella henselae survives after the storage period of red blood cell units: is it transmissible by transfusion?** *Transfus Med* 2008, **18**:287-91. | [Article](#) | [PubMed](#)
- Breitschwerdt EB, Maggi RG, Sigmon B and Nicholson WL: **Isolation of Bartonella quintana from a woman and a cat following putative bite transmission.** *J Clin Microbiol* 2007, **45**:270-2. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Apfalter P, Reischl U and Hammerschlag MR: **In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results?** *J Clin Microbiol* 2005, **43**:5835-41. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
- Hagege AA, Caudron E, Damy T, Roudaut R, Millaire A, Etchecopar-Chevreuil C, Tran TC, Jabbour F, Boucly C, Prognon P, Charron P and Germain DP: **Screening patients with hypertrophic cardiomyopathy for Fabry disease using a filter-paper test: the FOCUS study.** *Heart* 2011, **97**:131-6. | [Article](#) | [PubMed](#)

Citation:

Morton B and Diniz PP: **Detection of Bartonella quintana DNA in the presence of human and feline whole blood by single-tube PCR without DNA extraction.** *Microbiology Discovery* 2013, **1**:7.
http://dx.doi.org/10.7243/2052-6180-1-7