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A Highly Sensitive Quantitative PCR for the Detection of *Bartonella bacilliformis* by Targeting a Multiple-Copy DNA Segment

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Abstract

Carrion's disease is a human disease caused by an infection with *Bartonella bacilliformis*. Sand fly is believed to be the transmitting vector. Acute infection without treatment is life-threatening with fatality rates as high as 88%. PCR based diagnostic assays have been developed for detecting *B. bacilliformis* DNA in clinical samples. Genome sequence analysis of *B. bacilliformis* had identified a segment of 1,162 bp which is present at three different locations. In this study we have developed a qPCR assay targeting this Multiple-copy DNA Segment (MTSeq) to reach a higher sensitivity. The assay sensitivity was evaluated by three different sets of primers. The best set of primers yielded the detection limit of 3.3 bacteria per reaction. DNA extracted from sand flies fed on blood containing *B. bacilliformis* was also tested. Flies fed on Day 1 and 3 were determined as positive for *B. bacilliformis*; the results were consistent with the earlier study targeting *pap31* gene. The consistency of the qPCR targeting the MTSeq was evaluated using samples containing 8.3 or 3.3 copies of genomic DNA. We demonstrated that 18 out of 36 reactions (50%) were positive for samples containing 8.3 copies of genome; similarly 12 out of 36 reactions (33%) were positive for samples containing 3.3 copies of genome. At the same time, only 8 (25%) and 2 (6%) reactions out of 36 reactions showed positive using primers targeting *pap31*, respectively. These results have demonstrated that qPCR targeting MTSeq is more sensitive for detecting *B. bacilliformis* than previous nucleic acid based method targeting *pap31*.

Keywords: Polymerase Chain Reaction (PCR); DNA segment; Carrion's disease

Introduction

Carrion's disease of human is caused by an infection with *Bartonella bacilliformis*, which is believed to be transmitted by *Lutzomyia verrucarum* sand flies [1,2]. Due to favorable environments for *L. verrucarum*, the disease has been confined to regions between 800 and 3000 m above sea level on the Western Andes Mountains in Peru, Columbia, and Ecuador. However, the disease has recently expanded to lower altitudes of the Andes and coastal regions due to increases in human migration and adaptations of sand flies to new geographic areas [3-5]. Also molecular detection of *B. bacilliformis* in *L. maranonensis* and ticks in Peru suggested the potential of spreading *B. bacilliformis* by these arthropods [6,7].

B. bacilliformis is known to penetrate erythrocytes and endothelial cells, where it can replicate and cause the biphasic Carrion's disease [1]. During the acute phase, or Oroya fever, symptoms such as severe acute-onset febrile illness and hemolytic anemia are developed soon after infection. As much as 80% of erythrocytes can be lysed [8]. Untreated cases of Oroya fever have fatality rates as high as 88% due to the high susceptibility to fatal secondary infections [9]. The chronic phase of infection, known as "verruga peruana", is associated with chronic skin eruptions which are self-limiting and can develop weeks or months after infection and persist for several years [5]. Moreover, the symptoms of Carrion's disease can widely vary from person to person. An epidemiology study has revealed that about 20% of patients infected with the bacteria remain asymptomatic and can act as reservoirs for infection [10].

In addition to the wide spectrum of symptoms, the low sensitivity of diagnostic tests has hindered the proper treatment of patients with effective antibiotics, such as ciprofloxacin or doxycycline. Thin blood smears, often used for detection of acute bartonellosis, have very high specificity but are limited by low sensitivities [11]. Culturing of *B. bacilliformis* from clinical samples is time-consuming, and serological tests such as IFA or immunoblot cannot distinguish between different *Bartonella* species and require paired acute and convalescent samples for confirmation [12]. A highly sensitive test for early detection of *B. bacilliformis* in patient samples would improve proper treatment and a better knowledge of the true disease burden.

Polymerase Chain Reaction (PCR)-based approaches have been used for the diagnosis of infection caused by *Bartonella* spp. and Carrion's disease [13-18]. PCR amplification of DNA shows promising sensitivity and specificity but requires a

specialized equipment and extensive end-user training, it is impractical for diagnosing the disease in remote areas. It was able to diagnose patients in acute phase previously classified as negatives by thin blood smear [19]. Nonetheless, a critical issue is the detection limit of these techniques, raising doubts about its usefulness in the detection of low-bacteremia carriers. However, most of PCR-based assays to detect *B. bacilliformis* use single-copy genes. Amplification targeting gene segment of multiple copies in the genome have been used to improve the detection limit of DNA amplification method [20,21]. In this study, we identified a segment of 1,162 bp (MTSeq) which is present at three different locations of the *B. bacilliformis* genome as the amplification target. PCR primers were designed and evaluated for sensitivity and specificity. Our results demonstrated a higher sensitivity for bacterial DNA detection.

Materials and Methods

Design of primers

Oligonucleotide primers used for PCR and qPCR assays were designed based on the KC 583 strain of *B. bacilliformis*. The primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and are described in **Table 1**.

Table 1 List of primer sequences for MTSeq cloning and qPCR.

Set	Primer Name	5' to 3'
MTSeq[a]	Bb-F	GCATGTTAATATTGGCACGATTG
	Bb-R	CCTAAACAATTATTCTAATTACTCG
A[b]	Bb-3031	TATTTTGTTAGCGCGTCAGGTT
	Bb-3196	GCTGACCCCTTAACAATCGG
B[b]	Bb-3230	AGGAGAGGATGCTGTTCGTC
	Bb-3392	TAACAACTCCACGCTCAACAC
C[b]	Bb-3177	CCGATTGTTAAGGGGTCAGC
	Bb-3352	TCCACGCCCTGAAATCGAAAA
[a]For cloning MTSeq to pCR-XL-TOPO vector		
[b]For qPCR reaction		

The Bb-F and Bb-R are forward and reverse primers for cloning the whole MTSeq, respectively. The 1,162 bp sequence is located at 3 different places in the *B. bacilliforms* genome between base pair 572720 to 573881, 683928 to 685089, and 710084 to 711245 **(Figure 1).** The qPCR primers for set A, B, and C were Bb-3031 and Bb-3196; Bb-3230 and Bb-3392; and Bb-3177 and Bb-3352, respectively.

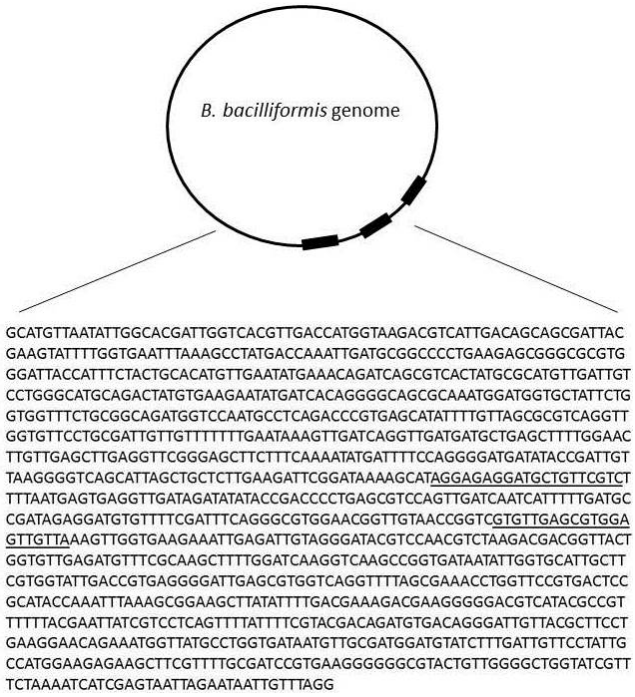

Figure 1 The locations of a multiple copy DNA segment in *B. bacilliformis* genome. Three copies of the 1162 bp DNA segment (black boxes) are present in strain KC 583 (1445021 bp). Their locations are from base pair 572720 to 573881, 683928 to 685089, and 710084 to 711245. The complete DNA sequences of the segment were listed below with location of set B primers underlined.

Plasmid and genomic DNA template

The MTSeq of *B. bacilliformis* KC 583 was cloned into pCR-XL-TOPO vector, and the closed circular plasmid (pCR-XL-TOPO-MTSeq) was purified using standard Qiagen plasmid mini kit (Qiagen, stockach, Germany) following the manufacturer's instruction. The pure pCR-XL-TOPO-MTSeq was quantified using a Nano-drop 2000 microsample spectrophotometer (Thermo Scientific, Wilmington, DE) and used as a standard for the selection of the best primer set to be used in the qPCR assay. The genomic DNA of *B. bacilliformis* KC 583 was used as the template in qPCR as described below. The genomic DNA from other bacteria (*O. tsutsugamushi, R. typhi, R. conorii, R. rickettsi, C. burnetii, T. cruzi*) transmitted by a variety of bloodsucking arthropod vectors capable of infecting humans were used to test the specificity of the qPCR assay. The copy number of each genomic DNA for different bacteria were determined similar to what was described above using a cloned plasmid containing a specific gene of each bacteria **(Figure 2)**.

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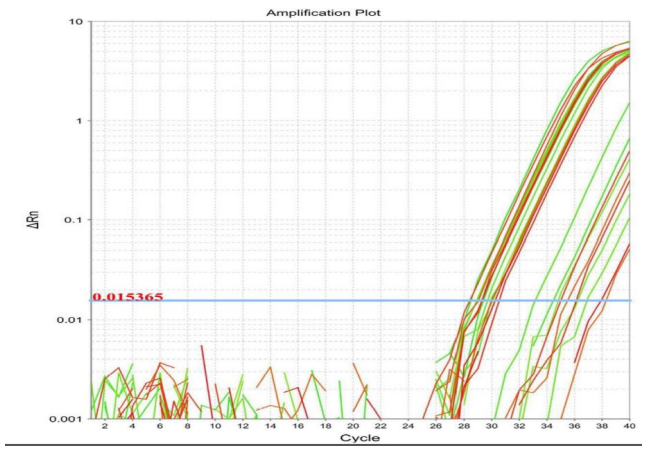

Figure 2 MTSeq PCR amplification plot.

Quantitative PCR

Conditions of quantitative PCR reactions were carried out as following. Each 20 µL reaction mixture included 0.5 µM of forward primer, 0.5 µM of reverse primer, 1×RT2 SYBR Green ROX qPCR Mastermix (Qiagen, Stockach, Germany), and DNA template. The qPCR was performed using the 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). Enzyme activation at 95°C for 3 min was followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was measured in duplicate and nuclease-free water was used as negative controls. Specificity of the product was determined by the melting curve **(Figure 3)**.

Detection of *B. bacilliformis* DNA targets in plasma samples

Normal human plasma was spiked with pCR-XL-TOPO-Bb-MTSeq for a final concentration of 25,000, 5,000, and 0 cp/mL. Total DNA in the spiked plasma samples was extracted using the QIAamp DNA Mini Kit (Qiagen, Stockach, Germany) according to the manufacturer's manual. A total of 200 µL plasma was used for extraction and eluted in 25 µL. The DNA extractions were performed independently three times.

Detection of *B. bacilliformis* in experimentally infected sand flies

Previously collected *L. longipalpis* female sand flies fed with blood containing *B. bacilliformis* were used to extract DNA [22]. DNA extraction of the whole genome of *B. bacilliformis* was performed using the DNeasy Blood & Tissue Kit (Qiagen, Stockach, Germany) following the manufacturer's protocol. Extracted DNA from six individual flies on Day 0, 1, 3, 5, 7, and 9 were used in duplicate for qPCR quantification. Ct values above 36 are considered negative.

Results

Selection of the best primer set

Ten-fold serial dilutions of plasmid DNA containing the MTSeq from 106 to 10 copies were used to evaluate the performance of each primer set. For all three primer sets, the limit of detection was 10 copies in the reaction. Table 2 shows slope, y-intercept, R2 value, and efficiency of the standard curve based on the average of two independent trials of ten-fold serial dilutions. Although these 3 primer sets performed similarly to detect 10 copies/reaction, their efficiency differed. Since high amplification efficiency will yield an increased PCR product and result in lower Ct values, primer set B with 99.96% efficiency was selected for the rest of this study.

Table 2 Comparison of qPCR results for 3 primer Sets[a].

Primer Set[a]	Slope	Y-intercept	R^2 value	Efficiency (%)
A	-3.61	39.54	0.997	89.56
B	-3.32	35.62	0.998	99.96
C	-3.46	39.47	0.996	94.39

[a]The qPCR was performed using the 7500 Fast Real-time PCR. Enzyme activation at 95°C for 3 min was followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Specificity of the qPCR

The specificity of the qPCR was evaluated by using the genomic DNA from other bacteria including *Orientia tsutsugamushi* (Karp, Kato, Gilliam, and TA763), Rickettsia species (*R. typhi, R. conorii, and R. rickettsii*), *Coxiella burnetii*, and *Trypanosoma cruzi*. About 106 copies of each genomic DNA was used as template and all tested negative with primer set B. The specificity of the primer set B was also analyzed *in silico* using the Primer-Blast program on the National Center for Biotechnology Information website. Another *B. bacilliformis* strain ATCC 35685D-5 had three identical segments. Two *Bartonella* species contained sequences very similar to the primer set B were also identified. *B. ancashensis* strain 20.00 had two segments with one mismatch to the forward primer sequences and two mismatches to the reverse primer sequences. B. sp. WD16.2 also had two DNA segments with two mismatches to the forward primer and two mismatches to the reverse primer.

Results for plasma samples spiked with MTSeq containing plasmid

Plasmid DNA (pCR-XL-TOPO-Bb-MTSeq) was spiked into normal human plasma to mimic patient samples, and qPCR was performed after DNA extraction using primer set B. The detection limit by qPCR was 79.5 copies of MTSeq (equals to 26.5 copies of organism) per reaction based on standard curves obtained from diluted plasmid **(Table 3)**.

Table 3 Detection limit of Bb-MTSeq in normal human plasma spiked with plasmid pCR-XL-TOPO-Bb-MTSeq.

Quantity of MTSeg at different steps of qPCR assay			
Starting material (cp/mL)	25000	5000	0
200 µL for extraction (cp)	5000	1000	0
Elute in 25 µL[a](cp/µL)	200	40	0
Add 5 µL in reaction (cp)	1000	200	0
qPCR quantification[b](cp)	501	79.5	N/A
Yield (%)	50.1	39.8	N/A
[a]Assuming 100% recovery for the extraction steps			
[b]Copy numbers are based on standard curves obtained from diluted MTSeq -XL-TOPO-Bb-MTSeq plasmid and averages of 3 independent trials.			

Results for sand flies fed with *B. bacilliformis* inoculated blood

L. longipalpis female sand flies fed on blood containing *B. bacilliformis* were subject to DNA extraction [19]. DNA from sandflies on Day 0, 1, 3, 5, 7, 9 was used in duplicate for qPCR. All 6 samples from Day 1 and 3 were positive for *B. bacilliformis*, while the 6 samples from day 0, 5, 7, and 9 were negative **(Table 4)**.

Table 4 Comparison of qPCR results for sand flies fed on *B. bacilliformis* inoculated blood.

	Days after feeding (No. positive / No. samples)					
Target	**Day 0**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 9**
MTSeq[a]	0% (0/6)	100% (6/6)	100% (6/6)	0% (0/6)	0% (0/6)	0% (0/6)
pap31[b]		86% (13/15)	63% (7/11)	0% (0/6)	0% (0/7)	0% (0/8)
[a]DNA extraction of the whole genome of *B. bacilliformis* was performed using the DNeasy Blood & Tissue Kit following the manufacturer's protocol. Extracted DNA from six individual flies on Day 0, 1, 3, 5, 7, and 9 were used in duplicate for qPCR quantification. Ct values above 36 are considered negative.						
[b]qPCR results from Angkasekwinai et al. [19].						

Sensitivity of qPCR targeting MTSeq as compared to that of *pap31* for genomic DNA detection

Three independent qPCR experiments were performed. Each experiment had 48 reactions. 24 reactions had genomic DNA of 25 copies of MTSeq (equivalent to 8.3 copies of the bacteria genome) and the other 24 reactions had 10 copies of MTSeq (equivalent to 3.3 copies of the bacteria genome). Our results clearly demonstrated that targeting MTSeq was more sensitive than targeting *Pap31* **(Table 5)**. At 8.3 copies of bacteria genome per reaction, 18 and 8 reactions out of 36 showed positive results with primers targeting MTSeq and *pap31*, respectively. But at 3.3 copies per reaction, 12 and 2 reactions out of 36 were tested positive with MTseq and *pap31* primers, respectively.

Table 5 Comparison of qPCR results for genomic DNA using primers targeting MTSeq and *pap31*.

Target	**8.3 cp[a]/rxn (No. positive / No. samples)**	**3.3 cp[a]/rxn (No. positive / No. samples)**
MTSeq	18/36[b]	12/36[b]
pap31	8/36[b]	2/36[b]
[a]genomic DNA copy numbers		
[b]Three independent qPCR runs were performed. Each run had total of 48 reactions. 24 qPCR reactions had 8.3 copies of genomic DNA, 12 were tested with MTseq primer set B and 12 were tested with *pap31* primers. The other 24 qPCR reactions were performed with the present of 3.3 copies of genomic DNA		

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Discussions

Diagnosis of Carrion's disease is currently limited by low sensitivity of thin blood smears, time-consuming cultures of *B. bacilliformis*, and unspecific serological tests [23]. PCR assays are the most sensitive techniques to diagnose *B. bacilliformis*. The purpose of this study was to develop a qPCR using a multiple-copy DNA segment to detect *B. bacilliformis* in clinical samples. The qPCR assay was found to detect 10 copies of plasmid containing the MTSeq equivalent of 3.3 organisms of *B. bacilliformis* per reaction. Angkasekwinai et al. [22]. reported that the limit of detection using a single-copy gene (*pap31*) for qPCR was 18 and 50 copies of bacteria per reaction in samples containing genomic DNA only and genomic DNA in presence of human DNA, respectively. Our assay was able to detect 3.3 copies of bacteria using genomic DNA only and 26.5 copies of bacteria (79.5 copies of MTSeq) using genomic DNA in presence of human DNA. Gomes et al. [24]. evaluated three different PCR approaches (16S rRNA, fla, and its genes) and found that targeting 16S rRNA performed the best with a similar detection limit. Therefore, the results in this study demonstrated a higher sensitivity by targeting this 1,162 bp multi-copy DNA sequence. Primer-Blast results showed potential non-specific DNA amplification with two other B. species. To further confirm the assay's specificity against highly homologous Bartonella species, additional assays will be performed using genomic DNA of B. ancashensis strain 20.00 and B. sp. WD16.2.

Bacterial DNA extracted from harvested *L. longipalpis* female sand flies fed on blood containing *B. bacilliformis* was also tested by this newly developed qPCR. Although there was some variation between the six sand flies from each day, all 6 samples were determined to be positive on Days 1 (Ct values between 29.34 to 32.76) and 3 (Ct values between 33.78 to 37.82) and negative on Days 0, 5, 7, and 9. Previously, Angkasekwinai et al. [22]. used qPCR targeting the single-copy *pap31* gene and found that only 86% (n = 15) or 63% (n = 11) of samples were determined positive for day 1 and 3, respectively. This study found that 100% (n = 6) of samples were positive for both day 1 and 3. The results demonstrated that this method can be used to detect the presence of *B. bacilliformis* in sand flies, making this qPCR a useful tool to monitor the migration of *B. bacilliformis* infected sand fly.

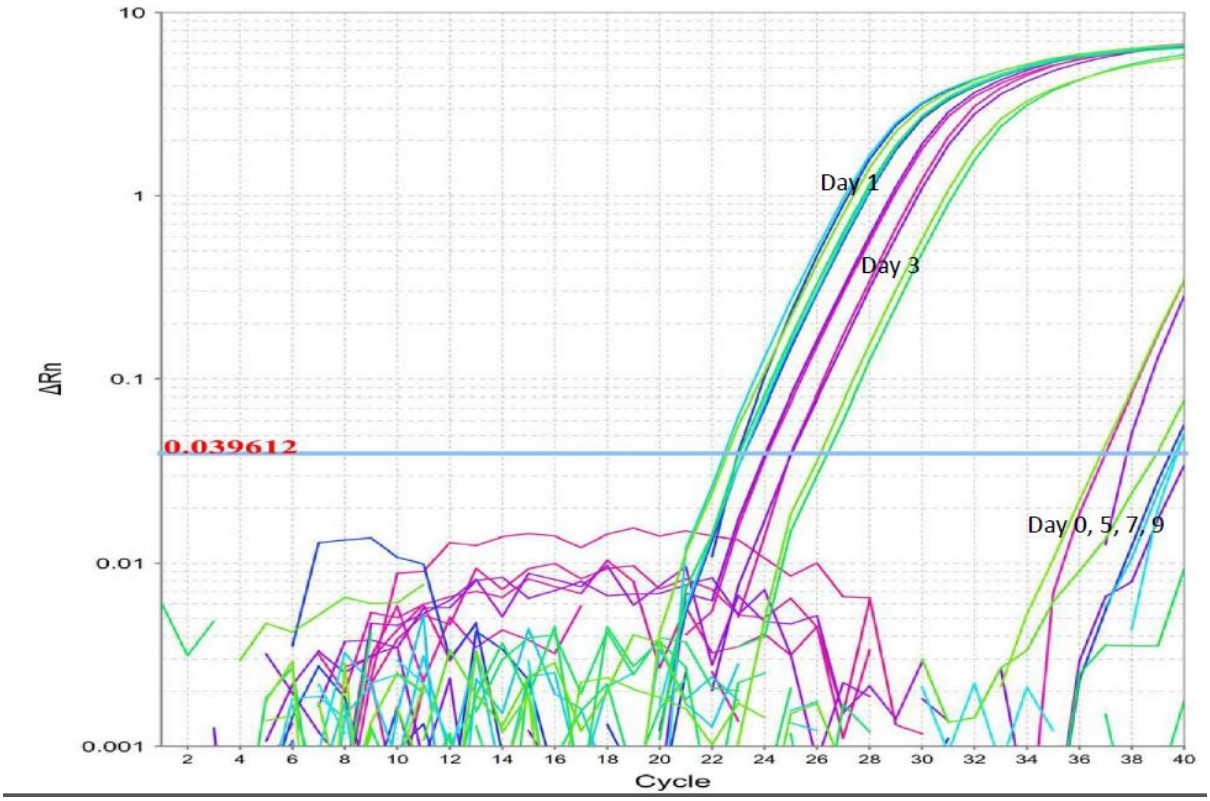

Figure 3 Sandfly PCR amplification plot.

In conclusion, this qPCR method using the multiple-copy DNA sequence has higher sensitivity as compared to previous assays. This will enhance our ability to diagnose an individual for *B. bacilliformis* infection and allow rapid and appropriate treatment of acute Carrion's disease. In the future, we would like to conduct qPCR assay on patient samples and vector samples to verify its utility. We will also develop loop-mediated isothermal amplification assay targeting this multi-copy DNA sequence for a robust and easy-to-perform assay suitable to be used in the endemic regions for asymptomatic surveillance studies.

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