Original Article

Comparison of PCR-Based Diagnosis with Centrifuged-Based Enrichment Method for Detection of *Borrelia persica* in Animal Blood Samples

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(Received 12 Oct 2010; accepted 24 May 2011)

Abstract

Background: The mainstay of diagnosis of relapsing fever (RF) is demonstration of the spirochetes in Giemsa-stained thick blood smears, but during non fever periods the bacteria are very scanty and rarely detected in blood smears by microscopy. This study is aimed to evaluate the sensitivity of different methods developed for detection of low-grade spirochetemia.

Methods: Animal blood samples with low degrees of spirochetemia were tested with two PCRs and a nested PCR targeting *fla*B, *GlpQ*, and *rrs* genes. Also, a centrifuged-based enrichment method and Giemsa staining were performed on blood samples with various degrees of spirochetemia.

Results: The *fla*B-PCR and nested *rrs*-PCR turned positive with various degrees of spirochetemia including the blood samples that turned negative with dark-field microscopy. The *GlpQ*-PCR was positive as far as at least one spirochete was seen in 5-10 microscopic fields. The sensitivity of *GlpQ*-PCR increased when DNA from Buffy Coat Layer (BCL) was used as template. The centrifuged-based enrichment method turned positive with as low concentration as 50 bacteria/ml blood, while Giemsa thick staining detected bacteria with concentrations ≥ 25000 bacteria/ml.

Conclusion: Centrifuged-based enrichment method appeared as much as 500-fold more sensitive than thick smears, which makes it even superior to some PCR assays. Due to simplicity and minimal laboratory requirements, this method can be considered a valuable tool for diagnosis of RF in rural health centers.

Keywords: *Borrelia persica*, Relapsing fever, Diagnosis, PCR, Enrichment method

Introduction

Relapsing fever (RF) is an infectious disease with a sudden onset of high fever; it is caused by several species of bacteria belonging to the genus *Borrelia* and, as its name indicates, is characterized by the occurrence of one or more spells of fever after the subsidence of the primary febrile attack (Burgdorfer 1976). The presence of massive amounts of spirochetes during fever peaks makes diagnosis of the infection an easy practice with dark-field microscopy or Giemsa staining method (Assous and Wilamowski 2009). However, between the peaks and in milder infections these methods are often negative due to a low number of bacteria in blood stream, making the infection under diagnosed. PCR assays that target different genes including 16S ribosomal RNA (*rrs*), Flag-ellin (*flaB*), and Glycerophosphodiester phosphodiesterase (*GlpQ*) were successfully used for detection of spirochetes in blood and *Ornithodoros* tick vectors (Ras et al. 1996, Assous et al. 2006, Halperin et al. 2006, Nordstrand et al. 2007, Oshaghi et al. 2010). Species-specific PCR and RCR-RFLP method were also developed for diagnosis of some *Borrelia* species (Assous and Wilamowski

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2009, Oshaghi et al. 2010). However, the method is costly, labor intensive and requires well-equipped laboratories. The ELISA based on product of GlpQ gene can discriminate between RF and Lyme borreliosis but cannot differentiate between an active and past RF infection (Schwan et al. 1996). Quantitative buffy coat (QBC) analysis of blood samples showed to be a reliable for diagnosis of RF when the spirochetes are low in numbers, but it relies on florescent microscopy, the equipment rarely available in rural health centers (van Dam et al. 1999, Cobey et al. 2001). Recently, a novel centrifugation-based method with minimal laboratory requirements showed very promising, detecting concentrations less than 10 bacteria/ml blood (Larsson and Bergstrom 2008). In this study we compare the sensitivity of several PCR assays that amplify DNA sequences of three different loci including *rrs*, *fla*B, and *GlpQ* with the centrifugation-based enrichment method and Giemsa staining to detect bacteria in animal blood samples.

Materials and methods

Borrelia

Borrelia persica was isolated from *Ornithodoros tholozani* ticks, collected from Ardebil Province, and maintained in guinea pigs through serial passages for seven successive years.

Blood samples

Adult guinea pigs were inoculated intraperitoneally with 0.5 ml of *B. persica* infected blood preserved in -70º C with 50% glycerol. From day three, daily amount of 500-600µl of blood was taken from animals' heart using insulin syringes and bacteria count was determined using dark-field microscopy. Blood collection continued for eight days, until no spirochete was detectable in the blood samples with dark-field microscopy. The intensity of infection in blood samples was obtained by counting the spirochetes using a Neubauer haemocytometer. When animals were negative for two successive days (days nine and ten) large amounts of blood was collected from their hearts and amounts of 10 ml were examined for presence of spirochetes using centrifuged-based enrichment method. Also, Buffy Coat Layer (BCL) was obtained from 3 ml of the same blood samples using the lymphocyte®-H kit (Cedarlane, Netherland). We also prepared fifteen serial dilutions from an infected guinea pig blood sample containing 25×10^4 spirochetes/ml blood, with sodium citrate-anticoagulated blood from healthy individuals (Table 3). Giemsa-stained thin and thick smears were prepared in triplicate from all dilutions and the rest of blood samples were examined for presence of spirochetes using centrifuged-based enrichment method.

Centrifuged-based enrichment method

The method basically comprised two centrifugation steps; the blood samples were first centrifuged at 500 x g for 5 min, the plasma were recovered to new tubes and second centrifugation was performed at 5000 x g for 10 min. The supernatants were decanted, and the pellets were resuspended in the few remaining microliters of plasma. The suspensions were smeared onto a glass slides and air dried for 10 min. The smears were fixed by heating over a flame followed by a 30 s dip in methanol. The slides were stained with Giemsa and examined for recovered spirochetes at 1000X magnification.

DNA extraction method and PCR

Amounts of 200µl of blood samples and 500 µl of buffy coats were subjected to DNA extraction, using the Miniprep DNA extraction Kit (Kiagen, Germany) according to manufacturer's recommendations.

Detection of *B. persica* by PCR was performed through amplification of three different genes including *rrs, flaB, and GlpQ*. The *fla*B and *GlpQ* genes were amplified using the primers and thermocycler programs outlined by other authors (Assous et al. 2006, Halperin et al. 2006). The 25µl reactions contained 20 pmol of each primer, 1.7mM $MgCl_2$, 10mM Tris-HCl,

50 mM KCl, 200M of dNTPs, 1U of *Taq* and 3µl of DNA. Amplification of *rrs* was performed using the nested PCR technique as described previously (Brahim et al. 2005, Nordstrand et al. 2007). The list of primers, target genes, and expected band sizes are reflected in Table 1.

Table 1. List of primers used in this study

Primer	Sequence (5' to 3')	Target gene		Expected band size
128F	cag aac ata cct tag aag ctc aag c	*GlpQ*		212 bp
340r	gtg att tga ttt ctg cta atg tg			
BOR1	taa tac gtc agc cat aaa tgc	*flaB*		750 bp
BOR2	gct ctt tga tca gttatc att c			
Fd3	aga gtt tga tcc tgg ctt ag	*rrs*	1st round	613 bp
595R	ctt gca tat ccg cct act ca			
Fd4	ggc tta gaa cta acg ctg gca g		2nd round	527 bp
500R	ctg ctg gca cgt aat tag cc			

Results

PCR

The *GlpQ*-PCR did not yield the expected 212bp band with low sprirochetemia blood samples i.e. those that were negative with dark-field microscopy (Table 1, Days 9 and 10). However, the DNA from BCL of the same blood samples was successfully amplified using the same reagents and PCR conditions. The *fla*B-PCR and nested *rrs*-PCR were positive with various degrees of spirochetemia including those that were negative by dark-field microscopy. The details of PCR assays are shown in Table 2.

Centrifugation-based enrichment method

Centrifugation-based enrichment recovered 890 and 357 spirochetes form two 10 ml of blood samples that were negative with dark-field microscopy examination (Table 2). We could recover 25 spirochetes on the glass slides by microscopy when as low as about 250 bacteria (a concentration equivalent to 50 bacteria/ml) were spiked into 5ml blood samples. No spirochete was recovered with concentration below 25 bacteria in ml of blood (Table 3).

Geimsa-stained smears analysis

Geimsa-stained thin smears were consistently positive with samples containing $\geq 10^5$ spirochetes/ml blood, but only two of the three smears at concentrations 6.26×10^4 spirochetes/ml and one of three smears at concentrations 5×10^4 spirochetes/ml were positive. The Giemsa-stained thick smears were all positive at $\geq 5\times10^4$ spirochetes/ml, but only two of the three smears at concentrations 4×10^4 and 3×10^3 spirochetes/ml, and one of the three smears at concentration 25×10^3 spirochetes/ml were positive (Table 2).

Table 2. Details of blood samples and other blood products used for PCR assays

Samples	Days after inoculation	Dark field microscopy	Spirochetes in µl of blood	Type and amount of sample	*GLPQ* PCR	*flaB* PCR	Nested *rrs*PCR	Spirochetes ecovered by CEM
1	3	10p *f*	2750	Blood/200 µl	+	+	+	NP
2	4	20p *f*	6750	Blood/200 µl	+	+	+	NP
3	5	1p5 *f*	375	Blood/200 µl	+	+	+	NP
4	6	1p *f*	1500	Blood/200 µl	+	+	+	NP
5	7	1p10 *f*	250	Blood/200 µl	+	+	+	NP
6	8	1p5*f*	250	Blood/200 µl	+	+	+	NP
7	9	Neg.	Not seen	Blood/200 µl	Neg.	+	+	NP
8	10	Neg.	Not seen	Blood/200 µl	Neg.	+	+	870 and 375
9	9	Neg.	Not seen	BCL/500 µl	+	NP	NP	NP
10	10	Neg.	Not seen	BCL/500 µl	+	NP	NP	NP

BCL = Buffy Coat Layer, NP = Not Performed, CBE = Centrifuged-based Enrichment Method
10p*f*=10 spirochetes in one microscopic field, 20p*f*= 20 spirochetes in one microscopic field, 1p5*f*= 1spirochete in 5 microscopic fields, 1p*f*= 1 spirochetes in one microscopic field, 1p10*f*=1spirochete in 10 microscopic fields, Neg.= negative, no spirochetes was seen in 30 microscopic fields.

Table 3. Details of blood samples used for Giemsa-stain analysis and centrifuged-based enrichment method

Sample code	Amounts of blood (ml)	Number of bacteria spiked into	Giemsa-stained thin smears	Giemsa-stained thick smears	Number of Recovered bacteria	Ratio of spirochetes (Recovered/spiked into)
1	5.00	250000	+ + +	+ + +	NP	NA
2	5.00	150000	+ + +	+ + +	NP	NA
3	6.00	125000	+ + +	+ + +	970	1/128
4	5.00	100000	+ + +	+ + +	NP	NA
5	5.00	62500	+ + −	+ + +	NP	NA
6	5.00	50000	+ − −	+ + +	NP	NA
7	5.00	40000	− − −	+ + −	NP	NA
8	5.00	30000	− − −	+ + −	NP	NA
9	5.20	25000	− − −	+ − −	255	1/98
10	5.00	20000	− − −	− − −	NP	NA
11	5.10	12500	− − −	− − −	132	1/94
12	5.00	6250	− − −	− − −	52	1/120
13	5.00	1250	− − −	− − −	31	1/40
14	5.00	625	− − −	− − −	25	1/25
15	5.00	250	− − −	− − −	25	1/10
16	5.00	125	− − −	− − −	0	0

NP= Not performed, NA= Non applicable

Discussion

Tick-borne Relapsing fever (TBRF) is one of the prevalent bacterial diseases in different parts of the world (Karimi 1981, Barmaki et al. 2010). The disease in Iran is caused primarily by *Borrelia persica*, which is transmitted by *Ornithodoros tholozani* ticks. Other *Borrelia* species including *B. microtii*, *B. latyschevii*, and *B. baltazardi* have also been reported from Iran (Karimi et al. 1979, Karimi 1981). From 1997 to 2006, a total of cases 1415 were reported from the entire country, some from areas out of *O. tholozani* distribution includ hormozgan and Fars Provinces (Masoumi Asl et al. 2009). Most of the RF cases in Hormozgan Province were detected during attempts for diagnosis of malaria parasite in Giemsa-stained blood smears from febrile patients. Since, thick smears commonly turn positive during fever peaks that are associated with massive spirochetemia; it is posssible that a large number of RF cases that refer to hospitals and health care centers during non-febrile periods remain underdiagnosed. The inabilities of microscopic analysis to detect spirochetes in blood have been demonstrated by some authors (Assous et al. 2006, Halperin et al. 2006, Nordstrand et al. 2007). PCR-based diagnosis offered a new approach to this problem. Our results showed that the *flaB*-PCR could detect *Borrelia* flagellin DNA in blood samples with low grade bacteremia that are commonly negative by dark-field microscopy (Table 2). The nested *rrs*-PCR also showed very sensitive and was positive with various degrees of bacteremia (Table 2); however it was very vulnerable to cross-contamination and led to false positive results when positive controls were included in assays. Dilution of first round products with distilled water with 1:10 ratio reduced the false positivity results by 90%. The *GlpQ*-PCR was not positive with low grade spirochetemia blood samples i.e. those that turned negative by dark-field microscopy. However, the DNA from BCL of the same blood samples yielded the expected band with the same PCR protocol (Table 2). Accumulation of spirochetes in BCL was already documented by other authors (van

Dam et al. 1999, Cobey et al. 2001). Thus, extraction of DNA from BCL increases the chance of detecting infection with PCR, particularly during non febrile periods. We could recover 870 and 357 spirochetes by centrifuged–based enrichment method from two 10 ml of guinea pig blood samples that were negative by dark-field microscopy and *GlpQ*-PCR. In our study, the sensitivity of the centrifuged–based enrichment method improved as number of spirochetes spiked into the blood samples decreased; the highest ratio (recovered/ spiked into) was obtained with 250 spirochetes/ 5ml of blood (Table 3). Since the sensitivity of thick smears, the routine method for detection of RF agents, was around 25000 spirochetes in ml of blood (Table 2), the centrifuged–based enrichment method could be as much as 500 times more sensitive than thick smear analysis. In conclusion, PCR particularly when DNA is extracted from BCL is a useful tool for diagnosis of RF cases that cannot be diagnoses by microscopic analysis. However, the method is commonly available in big hospitals and well-equipped laboratories. Centrifuged based enrichment method showed a high sensitivity and even appeared to be superior to *GlpQ*-PCR. Regarding the fact that it requires the equipments that are commonly available in small laboratories, this method is more feasible for RF diagnostics in underprivileged rural health centers.

Acknowledgements

We are very grateful to Dr N Piazak for his technical assistance and Dr M Assmar for his guidance throughout the project. This study was a M.Sc. thesis project founded by Pasteur Institute of Iran (Project No. 500).

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