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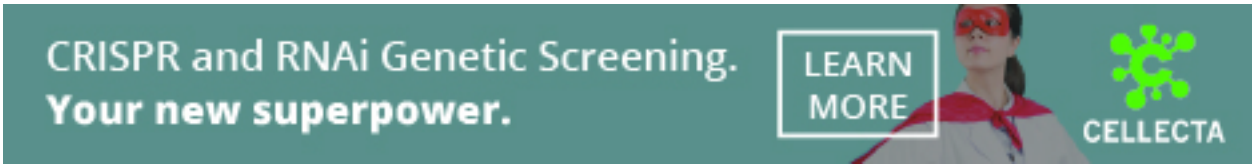

Different Methods of Sample Preparation Influence Sensitivity of *Mycobacterium tuberculosis* and *Borrelia burgdorferi* PCR

Wolfgang Liedtke,[1,4] Bertram Opalka,[2] Christoph W. Zimmermann,[1] and Ernst Schmid[3]

[1]Department of Neurology, [2]Department of Cancer Research, [3]Department of Microbiology, University of Essen, 45122 Essen, Germany

Pre-PCR sample preparation significantly influences the subsequent PCR assay. Although PCR diagnosis of diseases caused by mycobacteria and borreliae, especially nervous system infections is becoming increasingly important, few investigations deal with pre-PCR sample preparation. Five different methods (centrifugation, heating, sonication, glass powder, and guanidine) were compared in detail. For *Mycobacterium tuberculosis*, mere centrifugation and subsequent sonication of the sample is the method of choice. For *Borrelia burgdorferi*, centrifugation of the sample and a novel PCR based on the method of Goodman et al.[1] are recommended.

The PCR is gaining in significance in the diagnosis of diseases caused by *Mycobacterium tuberculosis* (Mtb) or *Borrelia burgdorferi* (Bb).[2–7] This is true especially for potentially lethal or irreversibly damaging nervous system infections.[8–13] Surprisingly, pre-PCR treatment of samples, particularly cerebrospinal fluid (CSF), received comparably little interest.[14–16]

We present a comparison of five methods of sample preparation: Centrifugation, heating, sonication, glass powder–,[17] and guanidine–DNA extraction.[18] The pathogens were diluted in water and in CSF containing inflammatory cells from a patient. Subsequent PCRs to detect Mtb[19,20] and Bb[1] were applied. We report an improved, nested Bb PCR assay based on the latter method.

MATERIAL AND METHODS

Mtb was a strain derived from ATCC (Rockville, MD, ATCC strain code 27294). An inoculum of 100 µl resulted in growth of 6×10^7 colony-forming units on Löwenstein–Jenssen medium. Mycobacteria were heat inactivated at 70°C for 12 hr. DNA content was <0.1 µg/µl.

Bb originated from subcultivation of a skin isolate of Bb strain PkO. Organisms (5×10^4/ml) could be detected by dark-field microscopy. The borreliae were centrifuged for 5 min at 5000*g* and washed three times in phosphate-buffered saline without magnesium chloride. The pellet was resuspended in this buffer leading to a final concentration of 10^3 Bb/µl. DNA content was <0.1 µg/µl.

Sample Preparation

For sonication, samples in microcentrifuge tubes were placed in the water reservoir of a sonicator (Bandelin electronic TK 52, Berlin, Germany), operating at a frequency of 35 kHz with 60 W performance. Sonication time was 2, 4, 6, 8, 10, 12, and 15 min.

Screw-capped microcentrifuge tubes containing the samples were placed in boiling water for 10 min.

Glass powder DNA isolation was performed according to Yamada et al.[17] using the "clean a gene" kit (Renner GmbH, Dannstadt, Germany). The pellets of the samples were covered with 50 µl of guanidine lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% lauryl sarcosyl, 0.7% mercaptoethanol) for at least 1 hr (Bb) or 24 hr (Mtb). Five microliters of glass powder suspension and 150 µl of 4 M sodium chloride were added. The nucleic acids were recovered from the glass powder by heating to 55°C.

The guanidine lysis buffer method was performed according to Sambrook et al.[18] Incubation with 1 ml of lysis buffer at 37°C was performed for at least 1 hr (Bb) or 24 hr (Mtb). Table 1 summarizes the sample preparation.

Bb and Mtb (processed as described above) were diluted in sterile, nuclease-free water in 10-fold dilutions. At least four, and regularly eight, replicate aliquots per dilution step were processed in PCR assays as described.

Mtb was assayed according to Eisenach et al.[19] (referred to as PCR method I) and to Pierre et al.[20] (PCR method II). Bb was assayed according to Goodman et al.[1] (PCR method III) and with a nested PCR applying the outer primers of Goodman et al. (PCR method IV). Details about PCR methods I–IV are given in Table 2, and examples of PCR products in Figure 1.

The influence of human DNA and hemoglobin on the PCRs was investigated by adding DNA, extracted from normal human bone marrow (10 ng, 100 ng, and 1 µg), and hemoglobin, obtained from osmotically lysed erythrocytes (750 ng, 7.5 µg, 75 µg, and 750 µg). The influence of hot-start PCR[21] was also tested.

Finally, the pathogens were diluted in CSF containing inflammatory cells originating from the transcranial ventricular drainage of a 20-year-old patient suffering from hydrocephalus caused by a brain tumor. The CSF contained 100 inflammatory cells/µl, 60 mg/dl of protein, and 1000 erythrocytes/µl (equivalent to

[4]Present address: Department of Neuropathology, Albert Einstein College of Medicine, Bronx, New York 10461-1602 USA.

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TABLE 1 Flow Sheet of Sample Preparations and PCR

Borrelia burgdorferi; Mycobacterium tuberculosis

Five methods of sample preparation

Borreliae	Mycobacteria
*1. centrifugation (c)	*1. centrifugation (c)
2. c + heating	*2. c + sonication (s)
3. c + sonication	3. s + heating
*4. c + glass powder	4. s + glass powder
*5. c + guanidine	*5. s + guanidine

Dilutions in aqua bidest
Dilutions in inflammatory CSF*

PCR to detect *Borrelia burgdorferi* or *Mycobacterium tuberculosis*

For dilution in inflammatory CSF (*), only methods 1, 2, and 5 were applied to *Mycobacterium tuberculosis* and 1, 4, and 5 to *Borrelia burgdorferi*. Interference of human DNA and hemoglobin was systemetically investigated in aqueous dilutional series.

1 ng of hemoglobin). This patient's CSF was sterile, and the inflammation resulted from chronic mechanical irritation. For details of the sample preparation, see Table 1. Standard PCR safety precautions were applied as described.[22,23] Controls with water, reaction mixture, and unspiked CSF were included in every PCR run.

As a means of expressing the highest dilution of samples leading to a specific amplification, we used the formula $T = 10^y$, $y = x_o - d/2 + d\Sigma n/k$. This formula originates from virology. T designates the "titer" analogous to viral titers; x_o is the $-\log_{10}$ of the highest dilution with 100% amplification in replicate assays; d is the $\log_{10}$ of the dilutional step; Σ includes all dilutional steps from 100–0% positive amplification; n is the number of replicate assays with positive amplification; and k is the number of replicate assays. As unit of the "titer," we propose ACN (amplifiable copy number).

RESULTS AND DISCUSSION

Here, we present a systematic investigation of pre-PCR sample preparation for Mtb or Bb focusing particularly on CSF containing inflammatory cells with Mtb or Bb in low concentrations. Results are shown in Table 3.

For the detection of Mtb, we recommend that the sample be processed by heating at 70°C for 12 hr, then centrifuged for 5 min at 15,800*g*, and finally, sonication for 12 min with 60 W/35,000 Hz. Subsequent PCR I[19] was more sensitive than PCR II,[20] as already mentioned.[24]

For Bb the pre-PCR method of choice is centrifugation for 5 min at 15,800*g*. The samples should be subjected to PCR immediately thereafter. Three weeks "incubation" at 4°C in CSF containing inflammatory cells reduced the ACN from $10^{6.5}$ to 10^3. Presumably, this was attributable to nucleases. Heating also reduces the titer by $10^{3.5}$ ACN. This may be attributable to DNA digestion at high temperatures.

Subsequent PCR IV for Bb as described here is an improved method: Its specificity is given through the outer primers,[1] and its sensitivity is increased by at least 10^1 ACN because of its nested design. PCR products can be visualized much better in gels than those of the non-nested PCR. The interference of CSF containing inflammatory cells or human DNA with PCR IV is reduced to a minimum.

For Mtb, smaller amounts of hemoglobin (750 ng) did not reduce the sensitivity of PCR I, but >7.5 μg of hemoglobin did so by 10^2. For Bb, hemoglobin efficiently inhibited PCR III and IV: 750 ng reduced the ACN by $10^{2.5}$ (PCR IV) or even 10^4 (PCR III). Hemoglobin as a strong PCR inhibitor interferes differen-

TABLE 2 Different PCRs

Mycobacterium tuberculosis

I.[18] IS 6110 insertion sequence (multiple copies in Mtb genome)

primer	IS1	5′–CCTGCGAGCGTAGGCGTCGG
primer	IS2	5′–CTCGTCCAGCGCCGCTTCGG
probe	IS3	5′–CTGCCCAGGTCACACAT [a]

II.[19] 65-kD mycobacterial heat shock antigen (single copy gene)

outer primer	Tb 1	5′–GAGATCGAGCTGGAGGATCC
outer primer	Tb 2	5′–AGCTGCAGCCCAAAGGTGTT
inner primer	Tb 28	5′–CCATCGATCCGAGACCCTGCTCAAGGGC
Inner primer	Tb 29	5′–TGCTGTAGACTCCTCGACGGTGATGACG
probe	Tb 4	5′–CGAAATCGCTGCGGTGGCCG [a]

Borrelia burgdorferi

III.[20] Ly1 chromosomal sequence

primer	679	5′–GAAATGGCTAAAGTAAGCGGAAT
primer	680	5′–TCTGTAAACTAATCCCACCTAAAA
probe	583	5′–TATTTTAGATGAGTATGGGGTT [a]

IV. nested PCR, outer primers, and probe[a] from III

inner primer	BorI1	5′–CAATTCAAAAAGGTAAAAGGC
inner primer	BorI2	5′–TTCAAGCACATCATGAGGAT

amplification parameters: temperatures 94/50/72°C
times 60/120/120 sec
cycling: 25/40
$MgCl_2$: 1.5 mmole
primer 679 and 680: 4.4 pmoles each,
BorI1, BorI2, and 583: 25 pmoles each

size of amplified fragments: 163 bp (BorI1 and BorI2)
135 bp (BorI2 and 583)

Experimental details are only listed if different from those in the references.
[a] Confirmation of PCR products was performed according to Kai.[26]

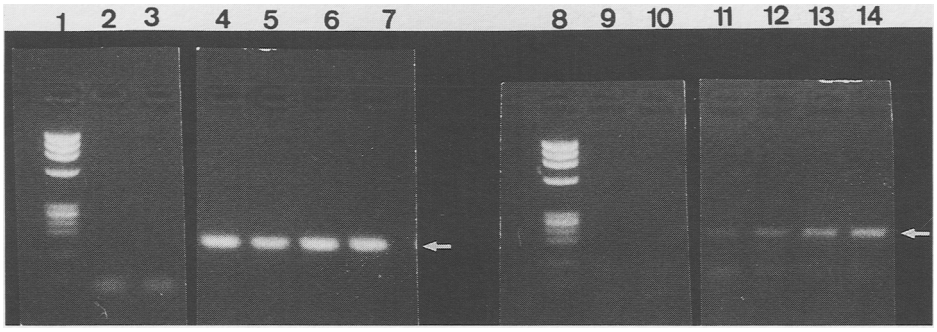

FIGURE 1 *Borrelia burgdorferi* PCR products (PCR III and IV) in 3% agarose gels stained with 500 ng/ml of ethidium bromide. (Lanes *1–7*) PCR products of nested PCR IV; (lanes *8–14*) PCR products of PCR III;[1] (lanes *1,8*) molecular weight standard $\phi\chi$ 174 *Hae*III digested (lanes *2,3,9,10*) negative controls; (lanes *4–7*) positive controls, diluted 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}, respectively, by PCR IV; (lanes *11–14*) positive controls, diluted 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}, respectively, by PCR III.

tially with amplification processes rather than showing a dose–effect relationship regardless of the PCR method.

Another important result of our investigation is the ACN per microliter exceeding the number of organisms by $10^{3.5}$ for Bb, and by $10^{1.7}$ for Mtb. This reflects the ability of PCR to amplify nucleic acid sequences of nonviable organisms as already mentioned.[25] Furthermore, the tendency of mycobacteria to form clusters, when inoculated on growth media, has to be considered. The difference between Mtb and Bb is most probably attributable to different sensitivity of dark-field microscopy (Bb) as compared with culture techniques (Mtb).

Different sample preparations show different efficiency depending on whether mycobacteria or borreliae were processed. Because of different cell wall compositions, mycobacteria are processed less efficiently by classical methods, such as guanidine– or glass powder–DNA extraction, whereas sonication was superior to other methods. More or less inadvertant degradation of Bb DNA decreased the efficiency of subsequent Bb PCR.

Further studies comparing systematically different methods of sample preparation from clinical samples are desirable. Unfortunately, we could not conduct our investigation with clinical samples: Because of the replicate assays and the comparative design of our study the necessary volume of positive samples could not be provided.

For Mtb, our findings are in accordance with Buck et al.[14], who applied slightly different, less favorable sonication methods. He did not vary sonication times, nor did he perform replicate assays of dilution series. He could detect 10^1–10^2 colony-forming units, whereas our ACNs figured higher than the colony-forming units. With regard to pre-PCR treatment of Mtb-containing sputum samples, Victor et al.[15] treated the samples with sucrose gradient centrifugation. Bose et al.[16] treated mycobacteria with cesium chloride to extract DNA. Neither investigator evaluated other methods of sample preparation comparatively, particularly sonication. As mentioned previously, there exists no such study for Bb.

TABLE 3 Amplifiable Copy Number of *Mycobacterium tuberculosis* or *Borrelia burgdorferi*

	Mycobacterium tuberculosis		*Borrelia burgdorferi*	
	($10^{5.8}$ cfu/μl)		(10^3 organisms/μl)	
PCR method	I	II	III	IV
Dilution in sterile water				
Centrifugation	$10^{6.5}$	$10^{4.5}$	10^5	$10^{6.5}$
Sonication[a]	$10^{7.5}$	10^6	10^4	$10^{5.5}$
Heating	$10^{6.5}$	10^5	10^2	10^3
Glass powder	$10^{6.5}$	10^5	10^4	$10^{6.5}$
Guanidine	$10^{6.5}$	10^5	$10^{5.5}$	$10^{6.5}$
Guanidine + human DNA	10^6 (100 ng)	10^5 (100 ng)	10^2 (1 ng)	$10^{6.5}$ (100 ng)
Guanidine + hemoglobin	$10^{5.5}$ (7.5 μg)	10^4 (7.5 μg)	$10^{1.5}$ (0.75 μg)	10^4 (0.75 μg)
Guanidine without "hot start"	10^6	$10^{4.5}$	$10^{4.5}$	$10^{5.5}$
Dilution in inflammatory CSF				
Centrifugation	10^5	$10^{4.5}$	10^5	$10^{6.5b}$
Glass powder	N.D.	N.D.	10^3	$10^{6.5}$
Guanidin	10^6	10^5	$10^{4.5}$	$10^{6.5}$
Sonication[a]	$10^{7.5}$	10^5	N.D.	N.D.
Guanidine without hot start	10^5	$10^{4.5}$	$10^{3.5}$	$10^{5.5}$

Copy number per μl depending on the method of sample preparation.
[a] Optimum duration of sonication 12 min.
[b] Titer 10^3 after 20 days/4°C.

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