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Preparation of Bacterial DNA Template by Boiling and Effect of Immunoglobulin G as an Inhibitor in Real-Time PCR for Serum Samples from Patients with Brucellosis▽

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Real-time PCR is a widely used tool for the diagnosis of many infectious diseases. However, little information exists about the influences of the different factors involved in PCR on the amplification efficiency. The aim of this study was to analyze the effect of boiling as the DNA preparation method on the efficiency of the amplification process of real-time PCR for the diagnosis of human brucellosis with serum samples. Serum samples from 10 brucellosis patients were analyzed by a SYBR green I LightCycler-based real-time PCR and by using boiling to obtain the DNA. DNA prepared by boiling lysis of the bacteria isolated from serum did not prevent the presence of inhibitors, such as immunoglobulin G (IgG), which were extracted with the template DNA. To identify and confirm the presence of IgG, serum was precipitated to separate and concentrate the IgG and was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. The use of serum volumes above 0.6 ml completely inhibited the amplification process. The inhibitory effect of IgG in serum samples was not concentration dependent, and it could be eliminated by diluting the samples 1/10 and 1/20 in water. Despite the lack of the complete elimination of the IgG from the template DNA, boiling does not require any special equipment and it provides a rapid, reproducible, and cost-effective method for the preparation of DNA from serum samples for the diagnosis of brucellosis.

Brucella, one of the world's major zoonotic pathogens, is responsible for enormous economic losses, as well as considerable human disease in areas of endemicity (7). The detection of *Brucella* DNA by real-time PCR (RT-PCR) in serum samples simplifies the technique and shortens the turnaround time compared with that for conventional PCR techniques. While much attention has been directed toward minimizing false-positive reactions resulting from specimen contamination or amplicon carryover, relatively little attention has been given to the causes of false-negative PCR results.

Our group has recently developed a LightCycler-based RT-PCR assay for serum samples for the diagnosis of human brucellosis; this test is more sensitive than blood cultures and more specific than the serologic tests commonly used (8, 10). We chose boiling as the DNA preparation method for the diagnosis of brucellosis because the technique is simple, is reproducible, can be performed rapidly, and is effective with other clinical samples, such as urine and cerebrospinal fluid (4, 9), and because no sophisticated equipment is necessary. The most important reason, however, is because the number of circulating bacterial cells in serum samples from patients with brucellosis is probably very small, and moreover, the nucleic acids from the pathogen are likely released into the circulation as breakdown products during bacteremia (11). Although Al-Soud and colleagues (1, 2) did not recommend the use of this method, De Medici et al. (6) selected boiling as their preferred extraction method for the detection of *Salmonella enterica* by RT-PCR in poultry samples.

Immunoglobulin G (IgG) is considered an inhibitor of *Taq* polymerase, and because boiling is simply a DNA preparation process, it is unable to remove the IgG. This may be important in the amplification process with samples which have very low DNA concentrations. In this study we evaluated the effects of the sample volume, boiling as the bacterial DNA preparation method, and the role of IgG on the efficiency of the amplification process for RT-PCR for the diagnosis of brucellosis with serum samples.

MATERIALS AND METHODS

Clinical specimens. Serum samples from 10 patients with brucellosis and 10 controls (healthy blood donors with no history of brucellosis or exposure to *Brucella* spp.) were drawn before any antibiotic treatment. The diagnosis of brucellosis was established by the isolation of *Brucella* spp. in cultures of blood from all 10 patients (8). Written informed consent was obtained from each patient, according to institutional procedures.

Preparation of DNA by boiling lysis of bacteria isolated from serum. DNA from serum was prepared by boiling. The samples were centrifuged at 15,000 × *g* for 15 min. The supernatant was eliminated, and the pellet was resuspended in molecular biology-grade water (Eppendorf, Hamburg, Germany) and centrifuged at 15,000 × *g* for 10 min. The supernatant was eliminated, and the pellet was resuspended in 40 μl of molecular biology-grade water, subjected to boiling at 100°C in a water bath for 10 min, cooled on ice, and centrifuged at 15,000 × *g* for 10 s before it was stored at −20°C. Aliquots of 2 μl of template DNA were used for PCR.

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TABLE 1. *Cp* values of SYBR green I amplification products from serum samples spiked with cells of *Brucella abortus* B-19

No. of cells spiked[a]	Amt of DNA[b] (fg)	*Cp* of amplification products extracted from serum samples with the following vol (ml) by boiling[c]:				
		0.2	0.4	0.6	0.8	1
2×10^7	10^8	21.38 ± 0.05	21.37 ± 0.03	22.11 ± 0.06	23.47 ± 0.04	24.31 ± 0.04
2×10^6	10^7	26.13 ± 0.03	26.29 ± 0.04	28.21 ± 0.03		
2×10^5	10^6	28.50 ± 0.06	28.31 ± 0.04	29.15 ± 0.02		
2×10^4	10^5	30.37 ± 0.02	30.19 ± 0.06	31.24 ± 0.05		
2×10^3	10^4	31.71 ± 0.03	31.92 ± 0.03	32.98 ± 0.02		
2×10^2	10^3	34.09 ± 0.04	33.89 ± 0.04	35.44 ± 0.04		
20	10^2	37.79 ± 0.08	38.43 ± 0.05	39.88 ± 0.06		
2	10	39.77 ± 0.16	39.92 ± 0.09	30.35 ± 0.14		

[a] Number of cells added in different serum volumes assayed.
[b] Amount of DNA in the different serum volumes assayed.
[c] The results are the means ± standard deviations of three experiments.

RT-PCR with SYBR green I. LightCycler-based RT-PCR amplifications were performed in capillary tubes with a LightCycler instrument (Roche Diagnostic, S.L., San Cugat del Valles, Spain) and primers B_4 and B_5 (Tib Molbiol, Berlin, Germany), described by Baily et al. (3). Briefly, 2 μl of template DNA was added to a final volume of 20 μl of PCR mixture consisting of 2 μl of 10× LightCycler-FastStart DNA master mixture for SYBR green I (Roche Diagnostic, S.L.), 0.5 μM each primer, and 4 mM $MgCl_2$. All capillaries were sealed and then centrifuged at 600 × *g* for 5 s before amplification. Following polymerase activation (95°C for 10 min), 45 cycles were run, with each cycle consisting of 10 s of denaturation at 95°C, 10 s of annealing at 60°C, and 9 s of extension at 72°C. The temperature transition rate was 20°C/s for all steps. The double-stranded PCR product was measured during the 72°C extension step. Fluorescence curves were analyzed with LightCycler software (version 3.5). Melting-curve analysis was performed immediately after the amplification protocol: 0 s at 95°C (the hold time on reaching the temperature), 15 s at 71°C, and 0 s at 95°C. The temperature change rates were 20°C/s for all steps except the final step, which was 0.1°C/s. The crossing point (*Cp*; or the threshold cycle) was defined as the maximum of the second derivative from the fluorescence curve. *Cp* values over 40 are considered RT-PCR negative because the fluorescent signal and the melting curve correspond to primer-dimers. Each assay was performed by using a standard curve with a range of concentrations (10^5 to 10 fg) of DNA from a *Brucella abortus* B-19 culture and negative controls (water).

Concentration of IgG and Western blotting procedures. The separation and concentration of IgG were done with a commercial immunoprecipitation starter pack kit (Amersham Biosciences, Buckinghamshire, England) and protein G Sepharose 4 fast flow medium. Forty microliters obtained from 0.2 ml of serum treated by boiling were supplemented with 160 μl 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). An aliquot of polyclonal antibody (anti-human peroxisome proliferator-activated receptor alpha; Santa Cruz Technology) at a final concentration of 1 mg/ml was used as a positive control to verify the correct operation of this commercial kit (data not shown). All the samples were gently mixed for 1 h at 4°C with rotation. Twenty-five microliters of the protein G Sepharose 4 fast flow medium suspension (50% slurry) was added to the tubes, the contents were gently mixed for 1 h at 4°C with rotation, the tubes were centrifuged at 12,000 × *g* for 20 s, and the pellets were saved. The pellets were washed three times with 1 ml of 1× PBS and centrifuged at 12,000 × *g* for 20 s between each wash. The final pellets were resuspended in 40 μl loading buffer (8% sodium dodecyl sulfate [SDS], 8 mM EDTA, 40% glycerol, 1 M β-mercaptoethanol, and 0.1% bromphenol blue in 0.25 M Tris-HCl, pH 7.5), heated to 95°C for 3 min, and centrifuged at 12,000 × *g* for 20 s. Aliquots of 20 μl of the supernatants obtained were heated at 100°C for 3 min and electrophoresed in 12% analytical SDS-polyacrylamide gels by employing a Mini-Protean III apparatus (Bio-Rad Laboratories, Inc., Spain), and the gels were stained with Coomassie brilliant blue R-250. Coomassie brillant blue-stained gels were scanned with a GS-800 densitometer (Bio-Rad Laboratories), and the files generated were analyzed with Quantity One one-dimensional analysis software. For Western blotting, the proteins from duplicate gels were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.) by applying a constant current of 250 mA for 1 h. A piece of the membrane was stained with Ponceau red to verify protein transfer. The blots were blocked in PBS plus 0.1% Tween 20 containing 5% fat-free milk powder for 1 h at room temperature. An anti-human IgG peroxidase conjugate (diluted 1:5,000; Promega, Madison, WI) was used as the second antibody. Finally, immunoreactive bands were visualized by using an Immun-Star horseradish peroxidase kit for the detection of horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Inc.). The specific protein pattern was visualized by using an Auto-Chemi system (UVP; Bio-Imaging Systems) and the image acquisition analysis software Labworks (version 4.6).

RESULTS AND DISCUSSION

To determine whether the inhibition of the RT-PCR employed for the detection of *Brucella* spp. in serum samples was dependent upon the sample volume used in the extraction process, different volumes of serum (0.2 to 1 ml) from healthy subjects artificially spiked in a titration experiment with decreasing inocula of *B. abortus* B-19 (2×10^7 to 2 cells) were submitted to boiling. Serum volumes of 0.2 to 0.6 ml produced the correct amplification at all dilutions used, with a detection threshold of 10 fg DNA, equivalent to two bacterial cells. However, serum volumes above 0.6 ml resulted in total inhibition of the amplification except at the highest concentration of 10^8 fg DNA (2×10^7 cells), possibly due to the presence of Igs, which can interact with the single-stranded DNA during activation of the FastStart polymerase and block the amplification process by inhibiting the polymerase (Table 1) (1, 5).

By using the boiled lysis-prepared DNA, 10 samples of 0.2 ml of serum from patients with brucellosis were processed for the detection of *Brucella* DNA. Six patients showed the correct amplification, and samples from four patients (samples 2, 3, 5, and 9) were falsely negative. In an attempt to detect the pres-

TABLE 2. *Cp* values obtained by LightCycler RT-PCR in samples from 10 patients with brucellosis

Patients	*Cp* value obtained by boiling and with the following dilution[a]:		
	Undiluted	1/10	1/20
1	34.70 ± 0.10	33.41 ± 0.10	37.52 ± 0.07
2	Neg	Neg	Neg
3	44.94 ± 0.06	33.24 ± 0.12	35.08 ± 0.14
4	31.72 ± 0.15	29.71 ± 0.16	29.57 ± 0.08
5	44.46 ± 0.14	Neg	Neg
6	28.53 ± 0.13	27.20 ± 0.14	29.76 ± 0.07
7	29.83 ± 0.14	33.55 ± 0.02	30.94 ± 0.02
8	28.06 ± 0.06	30.79 ± 0.05	30.98 ± 0.04
9	44.95 ± 0.05	31.69 ± 0.16	33.01 ± 0.06
10	29.63 ± 0.09	31.50 ± 0.07	32.53 ± 0.10

[a] The results are the means ± standard deviations of two experiments. *Cp* values over 40 are considered real-time PCR negative (Neg).

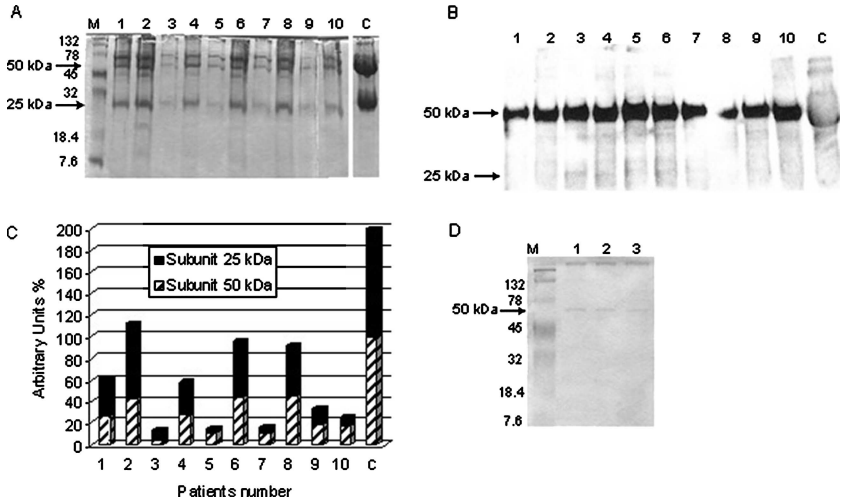

FIG. 1. (A) SDS-PAGE analysis of IgG concentrated by precipitation in 40 μl of template DNA obtained by boiling from 0.2 ml of serum and 0.2 ml of crude serum from patients with brucellosis (control). Lane M, molecular size marker (Kaleidoscope prestained standards; Bio-Rad Laboratories, Inc.) used to determine the sizes of the protein bands; lanes 1 to 10, subunits of 50 and 25 kDa of IgG from 10 patients with brucellosis, respectively; lane 11, crude serum from a patient with brucellosis; lane C, control. (B) Western blotting of the protein patterns for the same 10 patients described for panel A and the crude serum analyzed by SDS-PAGE. (C) Data generated by densitometry and Quantity One analysis software of the SDS-polyacrylamide gel shown in panel A. The concentrations of both subunits (25 and 50 kDa) were calculated by comparison with the concentrations of these subunits in crude serum, which was used as a control. (D) Protein pattern obtained when serum was precipitated and analyzed by SDS-PAGE. Lane M, molecular size marker (Kaleidoscope prestained standards); lanes 1 to 2, dilution of template DNA 1/10; lane 3, dilution of template DNA 1/20.

ence of inhibitors, the samples were diluted 1/10 and 1/20, with 8 of the 10 samples amplifying correctly (samples 2 and 5 remained negative) (Table 2). None of the control samples showed any amplification signal.

In order to identify the presence of IgG, the major inhibitor in human serum, the serum samples that had been treated by boiling were precipitated to separate and concentrate the IgG and were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). That analysis showed two protein bands with approximate molecular masses of 25 and 50 kDa in the samples from all patients (Fig. 1A). These band patterns in the 10 patients were confirmed to be IgG by Western blotting (Fig. 1B). For all samples, these two bands were measured with a densitometer. The intensities of these bands were different for all the patients, and except for sample 2, no correlation with the false-negative results in the RT-PCR was found; sample 2 showed the most intense band pattern (Fig. 1C). A weak band of 50 kDa appeared when the sample was diluted 1/10. This band decreased until it became almost inappreciable as the dilution increased to 1/20 (Fig. 1D).

This study, together with our other recently published work (4, 8, 9), indicates that boiling seems to be a promising and cheap method for the preparation of serum samples with volumes between 0.2 to 0.4 ml, as it significantly reduces the total processing time and the risk of carryover contamination. The possible inhibitory effects associated with the presence of IgG can be eliminated by diluting the boiled lysis-prepared DNA samples 1/10 and 1/20 in water. However, while specimen dilution is an inexpensive method for the elimination of PCR inhibition, dilution procedures can also contribute to false-negative results.

As the amount of IgG in the clinical samples detected by SDS-PAGE and confirmed by Western blotting changed considerably and was not related to a positive or a negative RT-PCR result, the inhibitory effects of IgG in serum samples were not concentration dependent. The persistence of two patients with negative results is not surprising if we consider the small amount of the circulating bacterial inoculum in patients with this disease and the fact that the sample was diluted, which could explain the absence of target DNA.

In conclusion, the preparation of a bacterial DNA template from serum by boiling did not prevent the presence of PCR inhibitors such as IgG, and the use of serum volumes above 0.6 ml yielded total inhibition of the amplification process. The inhibitory effect of IgG was not concentration dependent, and it could be eliminated by diluting the serum samples 1/10 and 1/20 in water.

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REFERENCES

1. **Al-Soud, W. A., L. J. Jonsson, and P. Radstrom.** 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. J. Clin. Microbiol. **38:**345–350.
2. **Al-Soud, W. A., and P. Radstrom.** 2001. Purification and characterization of PCR-inhibitory components in blood cells. J. Clin. Microbiol. **39:**485–493.
3. **Baily, G. G., J. B. Krahn, B. S. Drasar, and N. G. Stoker.** 1992. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J. Trop. Med. Hyg. **95:**271–275.
4. **Colmenero, J. D., M. I. Queipo-Ortuño, J. M. Reguera, G. Baeza, J. A. Salazar, and P. Morata.** 2005. Real time polymerase chain reaction: a powerful tool for the diagnosis of neurobrucellosis. J. Neurol. Neurosurg. Psychiatry **76:**1025–1027.
5. **De Franchis, R., N. C. P. Cross, N. S. Foulkes, and T. M. Cox.** 1988. A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. Nucleic Acids Res. **16:**10355.
6. **De Medici, D., L. Croci, E. Delibato, S. Di Pasquale, E. Filetici, and L. Toti.**

2003. Evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. Appl. Environ. Microbiol. **69:**3456–3461.
7. **Pappas G., N. Akritidis, M. Bosilkovski, and E. Tsianos.** 2005. Brucellosis. N. Engl. J. Med. **352:**2325–2336.
8. **Queipo-Ortuño, M. I., J. D. Colmenero, J. M. Reguera, M. A. Garcia-Ordoñez, M. E. Pachon, M. Gonzalez, and P. Morata.** 2005. Rapid diagnosis of human brucellosis by SYBR green I-based real-time PCR assay and melting curve analysis in serum samples. Clin. Microbiol. Infect. **11:**713–718.
9. **Queipo-Ortuño, M. I., J. D. Colmenero, N. Muñoz, G. Baeza, E. Clavijo, and P. Morata.** 2006. Rapid diagnosis of *Brucella* epididymo-orchitis by real-time PCR assay in urine samples. J. Urol. **176:**2290–2293.
10. **Queipo-Ortuño, M. I., J. D. Colmenero, G. Baeza, and P. Morata.** 2005. Comparison between LightCycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. Clin. Infect. Dis. **40:**260–264.
11. **Zerva, L., K. Bourantas, S. Mitka, A. Kansouzidou, and N. J. Legakis.** 2001. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. J. Clin. Microbiol. **39:**1661–1664.