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Comparison of Methods for Extraction of Nucleic Acid from Hemolytic Serum for PCR Amplification of Hepatitis B Virus DNA Sequences

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The sensitivity of PCR for the amplification of target nucleic acid sequences in clinical diagnostics may often be reduced due to the presence of inhibitory factors. Hemolytic serum contains a number of PCR inhibitors, one of which is hemin. In this study we have found that conventional methods of DNA extraction were not sufficient for the removal of PCR-inhibitory compounds in hemolytic serum. We have therefore compared the efficiency of several commercial and noncommercial methods of nucleic acid purification from hemolytic serum samples prior to PCR amplification. Separation with the QIAamp HCV kit, dialysis with Millipore filters, and bovine serum albumin absorption were all found to be suitable extraction methods for eliminating inhibitors from hemolytic serum for PCR amplification. Using these methods we were able to detect very low levels of hepatitis B virus DNA in hemolytic serum.

PCR is a powerful tool for nucleic acid analysis, theoretically enabling the detection of a single copy sequence (12), and is currently applied for diagnostic purposes. Indeed, detection of hepatitis B virus (HBV) DNA sequences in serum for diagnostic purposes is currently performed by PCR in many laboratories (8, 14). One of the major limitations of PCR-based diagnostic tests is the inhibition of *Taq* polymerase by substances present in clinical specimens such as blood, mucus, urine, sperm, and other body fluids. As little as 1% (vol/vol) blood completely inhibits *Taq* polymerase activity (13). Hemin, the prosthetic group of hemoglobin, reversibly binds to *Taq* polymerase and is a potent inhibitor of the enzyme (1). Hemin is released from erythrocytes following hemolysis and is frequently associated with the withdrawing of blood. In addition, hemin inhibits viral reverse transcriptase activity (11, 17).

The obstacle in performing PCR analysis in hemolytic mouse serum was encountered by us during the development of a small animal model for HBV and hepatitis C virus (HCV) infection (4, 5). To address this problem we have compared different protocols for DNA extraction from hemolyzed and nonhemolyzed human and mouse sera in order to test their efficacy in obtaining DNA suitable for PCR amplification analysis.

Hemolytic serum was generated by freezing and thawing of whole blood four times, resulting in 100% hemolysis. In order to compare the purity of DNA prepared with the listed protocols, human and mouse sera were spiked with 1 pg of plasmid HBV DNA and subjected to the extraction process according to each protocol. Altogether, six methods for DNA extraction from sera were compared.

(i) Proteinase K-phenol. A total of 0.2 ml of serum was treated with 0.5 mg of proteinase K (Sigma, St. Louis, Mo.)/ml in the presence of 25 mM Tris-HCl (pH 7.8)–2.5 mM EDTA–0.5% sodium dodecyl sulfate for 2 h at 60°C. The DNA was extracted once with phenol-chloroform and once with chloroform and was then precipitated with ethanol. The DNA pellet was washed with 70% ethanol, dried, and dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (TE).

(ii) Dialysis on Millipore filter. Following proteinase K-phenol extraction, DNA solutions were placed on a 0.025-μm-pore-size mixed cellulose ester filter (VSWP; Millipore, Bedford, Mass.), and floated on a pool of double-distilled water for 20 to 30 min.

(iii) DNAzol. A total of 0.1 ml of serum was mixed with 1 ml of DNAzol (Life Technologies, Gaithersburg, Md.). Ethanol (0.55 ml) was then added, the solution was mixed, and samples were centrifuged at 10,000 × *g* at 4°C. The pellet was washed twice with 95% ethanol, dried, and dissolved in TE.

(iv) QIAamp HCV kit. The QIAamp HCV kit was used according to the manufacturer's (QIAGEN, Hilden, Germany) protocol. In brief, a 150-μl serum sample was treated with the denaturing solution, which was followed by addition of ethanol. The mixture was loaded on the QIAamp spin column and washed twice, and the DNA was eluted with 50 μl of 80°C TE.

TABLE 1. Comparison of nucleic acid detection by PCR following different extraction methods

Extraction protocol	Nucleic acid detection in type of serum			
	Fetal calf serum	Nonhemolytic human serum	Hemolytic human serum	Mouse serum (mild hemolysis)
Proteinase K-phenol	+	+	−[a]	−[b]
DNAzol	+	+	+	−[b]
QIAamp HCV kit	+	+	+	+
Amplicor detection kit	+	+	−	−
Millipore filter	+	+	+	+
Nonextracted serum		−	−	ND[c]

[a] Positive at a 1:10 dilution.
[b] Positive at a 1:100 dilution.
[c] ND, not determined.

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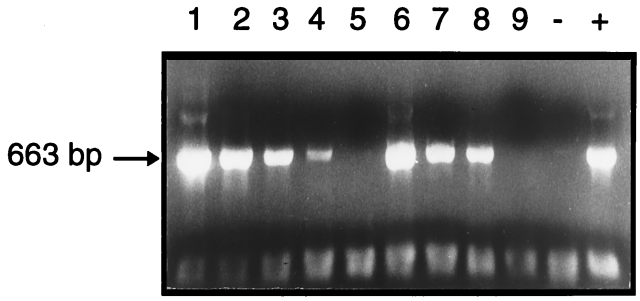

FIG. 1. Detection of HBV DNA in hemolytic human serum processed with the QIAamp kit for nucleic acid extraction. Hemolytic serum (lanes 1 to 5) or nonhemolytic serum (lanes 6 to 9) was spiked with HBV DNA and then subjected to nucleic acid extraction by using the QIAamp kit followed by PCR amplification. The amount of HBV DNA added to each reaction was as follows: 10 pg (lanes 1 and 6), 1 pg (lanes 2 and 7), 100 fg (lanes 3 and 8), 10 fg (lanes 4 and 9), or 1 fg (lane 5). Lanes containing negative (−) and positive (+) controls are indicated. The expected size of the HBV-specific PCR product is 663 bp. PCR products were analyzed by electrophoresis on 2% agarose gels.

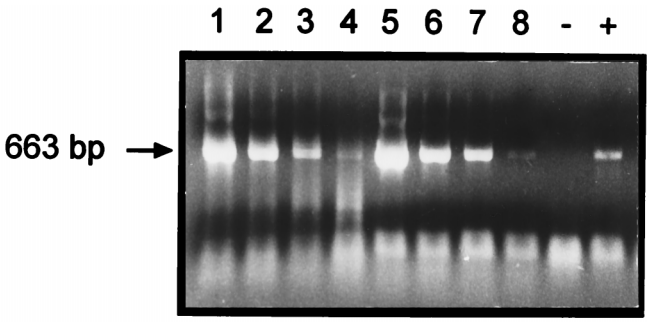

FIG. 2. Detection of plasmid HBV DNA by PCR in mouse serum (lanes 1 to 4) compared to PBS (lanes 5 to 8). DNA was added to the mouse serum or PBS and then subjected to nucleic acid extraction by applying the QIAamp kit. The amount of HBV DNA added was as follows: 10 pg (lanes 1 and 5), 1 pg (lanes 2 and 6), 100 fg (lanes 3 and 7), or 10 fg (lanes 4 and 8). Lanes containing negative (−) and positive (+) controls are indicated. The expected size of the HBV-specific PCR product is 663 bp. PCR products were analyzed by electrophoresis on 2% agarose gels.

(v) Amplicor HCV detection kit. This kit was used according to the manufacturer's (Roche Diagnostic Systems, Branchburg, N.J.) protocol.

(vi) BSA addition. Initial steps were as described for the proteinase K-phenol method. Following a 2-h incubation at 60°C, bovine serum albumin (BSA; Sigma, St. Louis, Mo.) was added to the mixture at a final concentration of 1 mg/ml. DNA was then extracted by two phenol-chloroform steps and one chloroform steps and was then precipitated by ethanol.

The HBV core sequences were detected by PCR amplification by the following protocol. Briefly, PCR mixtures (50 μl) containing 10 pmol of each oligonucleotide primer in reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin, 500 μM [each] dATP, dGTP, dCTP, and dTTP) and 0.5 U of *Taq* polymerase were overlaid with 30 μl of mineral oil and amplified by using the following program: 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. PCR products (10 μl) were analyzed on a 2% agarose gel. The oligonucleotide primers used for the precore-core PCR amplification (sequences are from subtype *adw*) were oligonucleotide 1, sense (nt 1778 to 1806), 5′ GGA-GGC-TGT-AGG-CAT-AAA-TTG-GTC-TGC-GC-3′; and oligonucleotide 2, antisense (nt 2444 to 2406), 5′ CCC-GAG-ATT-GAG-ATC-TTC-TGC-GAC-GCG-GCG-ATT-GAG-ACC-3′. The numbering starts from the *Eco*RI site. The expected size of the PCR DNA product is 663 bp.

The results of this analysis, as shown in Table 1, revealed that HBV DNA could not be detected when extracted from hemolytic human serum or mouse serum by either the proteinase K-phenol or the Amplicor method. However, dilution of serum samples prior to the extraction step improved the detection of target sequences by the proteinase K-phenol and DNAzol methods, indicating the presence of inhibitory compounds. Mouse sera had a much stronger inhibitory effect on the PCR amplification than human sera. In contrast, HBV DNA sequences were successfully detected in both mouse and human hemolytic sera following preparation with either the QIAamp HCV kit or the Millipore dialysis method or by the addition of BSA prior to phenol extraction with the proteinase K protocol. Identical results were observed using 1% (vol/vol) HBV-positive human sera with a viral titer of 10^9 particles per ml instead of plasmid DNA (data not shown).

To assess the sensitivity of HBV-DNA from hemolytic serum prepared with the QIAamp HCV kit, various amounts of plasmid HBV DNA (1 ng to 1 fg) were added to the hemolytic serum prior to the extraction step and then amplified by PCR. Agarose gel electrophoresis of the PCR products revealed that this process enabled the detection of as little as 10 fg of HBV DNA in hemolytic serum (Fig. 1). Similar results were also obtained by employing the Millipore dialysis method following proteinase K-phenol extraction.

Nonhemolytic mouse serum was observed to produce an inhibitory effect on PCR (Table 1). However, using the same extraction methods (proteinase K-BSA, proteinase K-Millipore, and QIAamp), we were able to detect HBV DNA sequences in mouse serum (Table 1). The sensitivity of HBV

TABLE 2. Summary of reported methods to overcome specific PCR inhibitors

PCR inhibitor	Modification of PCR to overcome inhibition	Reference
Phenol	Use of *Thermus thermophilus* (*Tth*) polymerase (*Tth* is unaffected by up to 15% phenol-saturated PBS)	9
Heparin	Incubation of nucleic acid sample in the presence of heparinase I	7
Denaturated albumin	NaOH denaturation or octanoate thermoprotection as pretreatment	18
Sewage and fecal wastes	Single-step guanidinium isothiocyanate extraction	16
Vitreous fluid	Dilution and chloroform extraction of nucleic acid	20
Urine	Ultrafiltration with Millipore cups	10
Ca alginate swabs	Applying Dacron nasopharyngeal swabs	19
Unknown	Prolonged heat soaking at 94°C	15
Acidic polysaccharides	Addition of Tween 20, dimethyl sulfoxide, or polyethylene glycol	3
Glove powder	Use of powderfree gloves or glove washing prior to handling of tubes	2
UV-treated mineral oil	Addition of 8-hydroxyquinoline	6

detection by PCR as assessed with the QIAamp extraction protocol on mouse serum was similar to that of DNA extraction from phosphate-buffered saline (PBS) (Fig. 2). We have observed that the addition of BSA to the proteinase K mixture enables us to detect quantities as low as 1 fg of HBV DNA in mouse sera (data not shown).

Over the last few years, since PCR amplification technology has been applied for diagnostics, a number of practical modifications have been suggested to overcome PCR inhibitors. In Table 2 we list reported PCR inhibitors and summarize the corresponding methods recommended for improving amplification reaction results. In addition to inhibition of the PCR, improper handling and storage conditions could also reduce the PCR signal.

The results of this study indicate that the occasional failure to detect HBV sequences in serum samples may be due to the presence of inhibitory factors of PCR amplification and that it is necessary to remove such factors from hemolytic sera prior to PCR amplification. This can be achieved by simple absorption on BSA, by dialysis on a Millipore membrane, or by separation on a QIAamp column.

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