JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1993, p. 2356–2360
0095-1137/93/092356-05$02.00/0

Vol. 31, No. 9

Preventing False Positives: Quantitative Evaluation of Three Protocols for Inactivation of Polymerase Chain Reaction Amplification Products

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Received 17 March 1993/Returned for modification 29 April 1993/Accepted 11 June 1993

False-positive results because of carryover contamination by previously amplified nucleic acids are currently the greatest impediment to routine implementation of nucleic acid amplification protocols. We evaluated three methods for inactivation of a 156-bp *Borrelia burgdorferi* polymerase chain reaction (PCR) product: (i) post-PCR cross-linking with isopsoralen (IP), (ii) pre-PCR treatment of a dU-containing PCR product with uracil *N*-glycosylase (UNG), and (iii) post-PCR alkaline hydrolysis (primer hydrolysis) of PCR products synthesized by using primers containing 3′ ribose residues. The sensitivities of the PCR performed under the conditions of each protocol were comparable. Inactivation of amplified DNA was highly efficient for all three protocols; the IP and UNG protocols eliminated at least to 3×10^9 copies of the product. The primer hydrolysis protocol varied in efficiency depending on the number and position of the 3′ ribose residues, but inactivation ranged from 10^4 to 10^9 copies. We conclude that with some modifications, all three systems are effective for eliminating amplified DNA products. Routine implementation of at least one method should help to avoid false-positive results because of carryover contamination.

The very feature that makes nucleic acid amplification systems so powerful, that is, their high degree of sensitivity, also makes them prone to false-positive results because of inadvertent contamination by nucleic acids. Such contamination occurs primarily from the following three sources: (i) cloned target molecules in plasmid vectors that are initially used for isolation and characterization of the target sequence; (ii) DNA carried over from clinical specimens containing large numbers of organisms, from cultures used to grow the organism, or within reagents used for amplification (17, 22); and (iii) the products of the amplification reactions themselves (12–15). The first two types of contamination can be avoided by avoiding areas in which cloning and sequencing of target DNA has been carried out and by using careful laboratory technique. However, contamination by previously amplified nucleic acids may occur in any laboratory and is thus the most likely form of contamination to be encountered in the course of routine testing. Although careful laboratory technique, including physical separation of pre- and postamplification steps (14), can delay the onset of contamination, such problems still occur and may be extremely difficult to overcome.

The ultimate long-term success or failure of all enzyme-catalyzed nucleic acid amplification methods likely depends on how well the products of amplification are contained or inactivated. Fortunately, in the last few years, inactivation protocols that use chemical, photochemical, and enzymatic methods for reducing the problems associated with amplicon contamination have been developed (1–11, 16, 18–21, 23, 25–27). It is presumed that the routine implementation of one or more of these protocols, coupled with careful laboratory technique and strict quality control procedures, will help to avoid carryover contamination in the clinical laboratory setting. However, no independent studies comparing the effectiveness of these methods have been published to date. In the study described here, we compared three different protocols for inactivation of known numbers of a *Borrelia burgdorferi* amplification product (24), and in this report we comment on the efficiency and usefulness of these systems for preventing false-positive polymerase chain reaction (PCR) results.

MATERIALS AND METHODS

Accurate quantitation of PCR products by determining the A_{260} of gel-purified DNA is extremely difficult because of interference from even small quantities of oligonucleotides and deoxyribonucleoside triphosphates (5, 11). Thus, in order to standardize the DNA input amounts for each inactivation protocol, we performed quantitative dot blot hybridization. A dot blot was prepared on a Hybond N nylon membrane (Amersham Corp.) by using a Minifold I Microsample Filtration Manifold (Schleicher & Schuell). Tenfold serial dilutions of amplicon obtained by each method were prepared. A total of 5 μl of each dilution was added to 195 μl of cold TE (10 mM Tris-HCl, 1 mM EDTA), 20 μl of 3 M NaOH was added, and samples were incubated at 65°C for 60 min to denature the DNA. Tubes were cooled on ice, briefly centrifuged to remove condensation, 180 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was added, and the samples were stored on ice until they were blotted. Samples were blotted and probed with a ^{32}P-end-labeled probe as described previously (24). A dilution of a known concentration of a plasmid DNA (p197-OspAB-N40) containing the target sequence was used to estimate the copy number of the various amplified DNA products. The radioactivity in each well was quantitated by using an AMBIS scanner (AMBIS, Inc.) after hybridization. Beta emissions, measured in counts per minute, were obtained to determine the relative dilution factors required for standardization of amplicon concentrations.

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A standard PCR master mixture was modified as appropriate for each inactivation protocol as follows: 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% bovine serum albumin); 200 μM (each) dATP, dCTP, dGTP, and dTTP (200 μM dUTP substituted for dTTP in the uracil *N*-glycosylase [UNG] protocol only); 50 pmol of oligonucleotide primers OSPA2 and OSPA4 (the corresponding 3′-ribo primer pairs were substituted as described [Integrated DNA Technologies, Inc., Coralville, Iowa]); 10% glycerol; 1.25 U of AmpliTaq; 100 μg of isopsoralen compound 10 (IP-10) per ml in the IP protocol only, and 0.5 U of UNG (Perkin-Elmer Corp.) in the UNG protocol only. Water was added to yield a 50-μl reaction volume. Target input (5 μl per 50-μl reaction) consisted of the plasmid dilution, the treated or untreated amplicon dilution, or water for the negative controls.

Unmodified primer sequences were as follows: OSPA2, GTT TTG TAA TTT CAA CTG CTG ACC; OSPA4, CTG CAG CTT GGA ATT CAG GCA CTT C; OSPA3 (probe), GCC ATT TGA GTC GTA TTG TTG TAC TG.

All reactions were performed in a DNA thermal cycler (model 480; Perkin-Elmer Corp.). The same thermal cycler profile was used in all experiments to facilitate comparison among methods. The components were incubated at room temperature for 10 min and were denatured at 95°C for 10 min prior to 45 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; this was followed by a final extension at 72°C for 7 min and a 72°C hold at the completion of the profile. The initial incubation at room temperature and the incubation at 95°C were included to accommodate the UNG protocol and were adopted in all subsequent experiments to control for the possible loss of AmpliTaq enzyme activity. A minimum annealing temperature of 55°C was used since UNG has enzymatic activity below 55°C (27), which may decrease amplicon yield. A 72°C hold file prevents possible degradation of the amplicon prior to analysis because of residual UNG activity. Reactions were cooled rapidly to 4°C before removal from the thermal cycler.

In order to determine the inactivation efficiency of each protocol, 10-fold dilutions of the quantitated amplification reactions were prepared as described above and 5-μl aliquots from parallel dilution series of treated and untreated amplicons were reamplified by using the conditions described. The inactivation efficiency was determined by comparing the number of positive reamplification reactions in each series prepared from treated reactions relative to those of a parallel control series prepared from untreated amplification products. The last positive PCR in a dilution series defined the inactivation efficiency.

The 156-bp products were visualized on ethidium bromide-stained 3% NuSieve–1% SeaKem agarose gels (FMC Bioproducts). Amplification products were denatured in the gel and were transferred to a nylon membrane (Hybond N; Amersham Corp.) by Southern blotting as described previously (24), except that the membranes were cross-linked by using a Hoefer UV cross-linker. The ^{32}P-end-labeled OSPA3 internal oligonucleotide probe was prepared as described previously (24), and hybridizations were performed in glass bottles in a Hybaid Mini Hybridization Oven (National Labnet). After 1 h of prehybridization in 10 ml of hybridization solution (5× Denhardt's solution, 5× SSPE [20× SSPE is 2.98 M NaCl, 0.2 M $NaPO_4$, and 0.02 M EDTA; pH 7.4], 0.5% sodium dodecyl sulfate [SDS], 100 μg of denatured salmon sperm DNA per ml), the labeled probe was added (180 ng per hybridization) and hybridized for 3 h at 55°C. Filters were washed twice for 10 min with 150 ml of wash buffer 1 (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) and once for 30 min at 55°C with 150 ml of prewarmed wash buffer 2 (1× SSC, 0.1% SDS). Autoradiograms were prepared by exposing Kodak X-Omat film overnight to 48 h at −70°C.

IP protocol. Isopsoralen inactivation was performed essentially as described by Cimino et al. (5) and Issacs et al. (11). A volume of untreated amplicon containing 1.2×10^{10} copies of the 156-bp product was divided into four 50-μl aliquots. An equal volume of water or IP-10 (final concentration, 100 μg/ml; HRI Associates) was added when appropriate, resulting in the following four test samples (3×10^9 copies in each): I (−IP, −UV), II (−IP, +UV), III (+IP, −UV), IV (+IP, +UV). Samples II and IV were exposed to UV light (365 nm) for 15 min at 4°C in an HRI 100 UV Photochemical Reaction Chamber (HRI Associates). After treatment, 10-fold dilution series were prepared as described above and 5-μl aliquots were reamplified and detected after dot blotting or Southern blotting.

UNG protocol. The UNG protocol functions by incorporating dUTP into amplification products, which are selectively degraded by UNG (16, 20). A standardized dU-containing amplicon preparation was prepared by amplification of template DNA in the presence of dATP, dCTP, dGTP, and dUTP. The quantity of the dUTP-containing product was then determined by quantitative hybridization and was adjusted to a final concentration of 3×10^{10} copies per 50-μl reaction mixture. A 10-fold dilution series was made starting from 5 μl (3×10^9 copies), and two sets of reamplification reactions, with and without UNG (Perkin-Elmer Corp.), were prepared. After amplification, the products were dot blotted and detected as described above.

PH protocol. The primer hydrolysis (PH) protocol was performed essentially as described in the package insert (Integrated DNA Technologies, Inc.). Modified primers containing one or two ribose residues at or near the 3′ end were synthesized. The 3′-ribo primer sequences (kindly provided by Integrated DNA Technologies, Inc.) were as follows: OSPA2r1, GTT TTG TAA TTT CAA CTG CTG ACr(C); OSPA4r1, CTG CAG CTT GGA ATT CAG GCA CTTr(C) (set A); OSPA2r2, GTT TTG TAA TTT CAA CTG CTG Ar(C)r(C); OSPA4r2, CTG CAG CTT GGA ATT CAG GCA CTr(U)r(C) (set B); OSPA2r2 spaced, GTT TTG TAA TTT CAA CTG CTG r(A)Cr(C); OSPA4r2 spaced, CTG CAG CTT GGA ATT CAG GCA Cr(U)TrC (set C) (r indicates ribose linkage). Amplicon preparations were made according to the conditions described above. After amplification, 100-μl aliquots were treated with the reagents supplied by the manufacturer, as follows. A total of 25 μl of solution 1 (5 M NaOH) (for hydrolysis) was added and tubes were incubated for 30 min at 95°C in a thermal cycler and cooled to room temperature (time delay file linked to soak file), and then 25 μl of solution 2 (5 M HCl) (for neutralization) was added. Untreated 100-μl samples were processed in parallel, substituting water for the NaOH and HCl solutions. Tenfold dilution series were prepared from treated and untreated samples, and 5-μl aliquots of each dilution were subjected to reamplification and detection of resulting products.

RESULTS

Effects of inactivation on product detection. The photocross-linking of DNA by isopsoralens results in covalent modification of one strand of the DNA molecule. We hypothesized that this covalent modification would have an

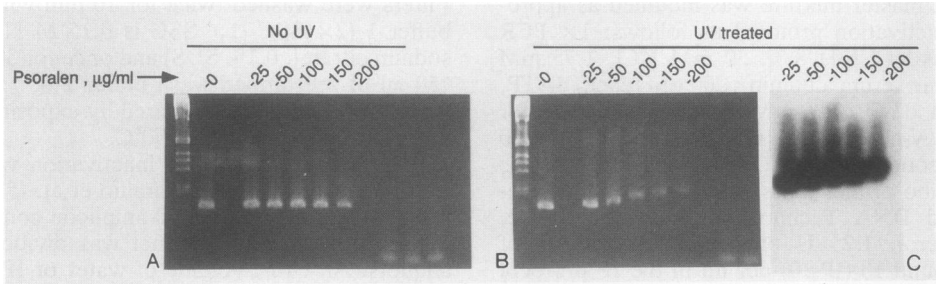

FIG. 1. Effects of IP inactivation on gel mobility and hybridization of PCR products. Concentrations of IP-10 (HRI Associates) ranging from 0 to 200 μg/ml were added to PCR mixtures prior to amplification. Without UV treatment (A) there was a slight dose-dependent decrease in amplification efficiency at 200 μg of IP per ml. After UV exposure (B and C), there was a concentration-dependent increase in the apparent molecular mass of the PCR product and a decrease in ethidium bromide staining intensity. A slight dose-dependent decrease in hybridization of the internal oligonucleotide probe is also observed at the highest IP-10 concentrations (100 μg of IP-10 per ml was selected for further inactivation experiments).

effect on the electrophoretic mobilities of the PCR products or their staining by ethidium bromide. Accordingly, for the IP protocol, we observed a stepwise increase in the apparent molecular mass of the amplification products and a concomitant decrease in ethidium bromide staining intensity that was directly dependent on the IP concentration (Fig. 1A and B). However, this did not result in a significant loss of sensitivity after hybridization with an internal oligonucleotide probe except at the highest IP concentrations (Fig. 1C). Consequently, we chose 100 μg of the IP compound per ml for further inactivation experiments. Amplification products that were prepared according to the conditions of the PH and UNG protocols had no apparent effect on electrophoretic mobility, ethidium bromide staining, or hybridization efficiency (data not shown).

Effects of the inactivation protocols on product yield and reaction sensitivity. Two of the inactivation protocols required modification of conditions in order to overcome decreases in reaction sensitivity. For the IP protocol, to counteract a slight inhibitory effect of the IP on PCR at high IP concentrations, 10% glycerol was added to the reactions. This resulted in sensitivity that was equal to that of the unmodified reactions (Fig. 2A, top two rows). For the UNG protocol, a substantial loss of reaction sensitivity was initially observed when the enzyme was added to the reaction mixture prior to amplification because of the breakdown of amplification products via residual UNG activity during or shortly after amplification (data not shown) (27). In order to overcome the latter problem, reactions were held at 72 or 4°C (instead of room temperature) prior to analysis. The PH method required no alteration of existing protocols except for substitution of the modified primers.

With appropriate modifications in place, the sensitivities of the amplification reactions and the apparent yield of amplification products obtained for each protocol were comparable; for the IP, UNG, and PH protocols, untreated dilution series produced detectable amplification products in the 10^{-8} dilutions (Fig. 2A and B; Fig. 3). Reactions in the 10^{-9} dilution were observed in many experiments, which was consistent with the Poisson distribution of single-molecule detection by PCR (Fig. 2 and 3).

Efficiency of inactivation of previously amplified DNA. For inactivation of the 156-bp *B. burgdorferi*-specific amplification product, all three protocols were of comparable efficiency. The IP and UNG protocols resulted in inactivation of all input copies (corresponding to ca. 3×10^9 copies) of the amplification product (Fig. 2). Interestingly, whereas UV light alone seemed to produce some inactivation of the amplified products (Fig. 2A, second row from the bottom), inactivation was incomplete; at least low levels of active template were detected in all but the last dilution. The combination of UV light plus IP treatment eliminated contaminating templates up to the limit of the study (3×10^9 copies). Preincubation of dU-containing templates with UNG also resulted in elimination of reamplifiable templates at all of the tested concentrations (Fig. 2B).

For the PH protocol, the efficiency of inactivation varied with the primers used for amplification. The primers containing single 3′ ribose linkages yielded a product that was completely inactivated at all of the tested concentrations by base hydrolysis (Fig. 3A). Unexpectedly, the primers containing two ribose linkages yielded incompletely inactivated products (Figs. 3B and C). For these latter primers, inactivation of only 10^4 and 10^5 copies was observed following base hydrolysis. This incomplete inactivation may have been due to the loss of the ribose residues during primer synthesis or storage, resulting in products that were resistant to alkaline hydrolysis.

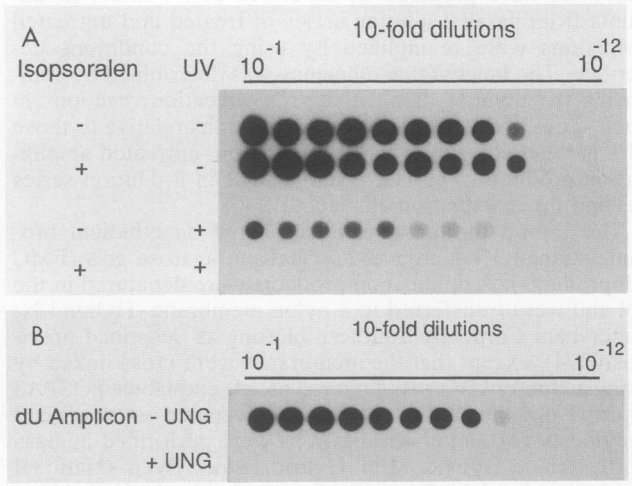

FIG. 2. Inactivation efficiency by the IP and UNG protocols. (A) Inactivation by isopsoralen. Tenfold serial dilutions of the amplified products were reamplified after treatment according to the conditions indicated on the left. (B) Inactivation by UNG. A 10-fold dilution series of dU-containing product was reamplified with (bottom row) or without (top row) UNG pretreatment.

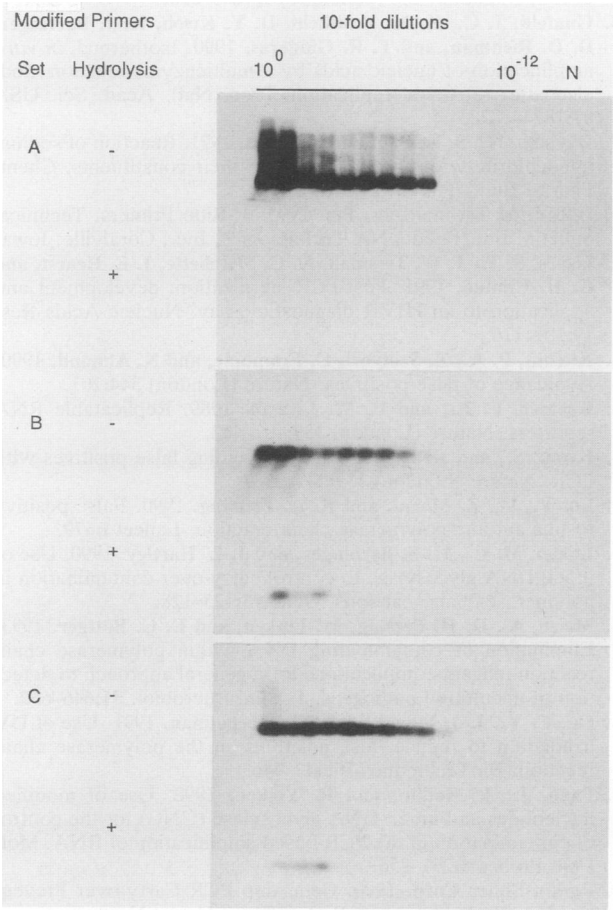

FIG. 3. Inactivation by primer hydrolysis. PCR products were generated by amplification with 3'-ribo primer set A (A), set B (B), and set C (C). A 10-fold dilution series was made from products with and without alkaline posttreatment. Untreated reamplification series are shown at the top of each panel.

DISCUSSION

Despite the description in the literature and the commercial availability of reagents and procedures for the control of PCR product contamination (5, 10, 11, 20), these methods have infrequently been used in published PCR applications to date. This may be due in part to the lack of formal evaluations of these methods by an independent laboratory. In the present study we challenged three such protocols with the same amplification product under conditions that were determined to minimize artifactual differences in inactivation efficiency (i.e., introduction of unmodified templates into the UNG protocol). The photochemical procedure (IP protocol) incorporates a photochemically reactive isopsoralen into the amplification mixtures prior to amplification. After amplification, but before the reaction tubes are opened, the tubes are exposed to UV light, which produces photochemically induced single-stranded DNA adducts. Once this reaction occurs, the likelihood of reamplifying a contaminating template is greatly reduced because DNA polymerases are unable to extend past the photochemical modifications of the template. The enzymatic method (UNG protocol) exploits the ability of UNG to selectively degrade DNA templates containing deoxyuracil. If routine PCR amplification is carried out by using deoxyuracil triphosphates in place of thymidine triphosphates, laboratory-generated dU-containing contaminants are degraded prior to amplification by a brief incubation of the reaction mixtures in the presence of UNG. The third method (PH protocol) uses modified primers containing one or two ribose residues near their 3' ends. After amplification, the tubes are opened and NaOH is added. The ribose linkages (but not the DNA linkages) are susceptible to base hydrolysis, and a cleavage that greatly reduces the efficiency of reamplification of contaminating templates is produced in each strand (28).

In theory, all enzyme-catalyzed nucleic acid amplification methods are susceptible to false-positive reactions because of amplified substrate accumulation. A typical PCR amplification reaction may contain up to 10^{12} copies of an amplified template; the inactivation protocols evaluated in the present study are thus capable of eliminating all but a few hundred copies of a contaminating template. The IP protocol theoretically inactivates the native template in addition to the amplified product; thus, a net loss in the number of active templates may actually be possible, since many reactions will contain more than 100 copies of the target nucleic acid at the start. This may become an important consideration for clinical laboratories that perform amplifications with clinical samples containing thousands or even millions of copies of the target organism, as is the case for bacterial cultures and for viruses such as hepatitis B virus.

The effectiveness of the IP and UNG inactivation protocols is theoretically influenced by the percentage of G+C residues present in the product and by the length of the product (5, 11). In our study, both protocols were challenged with a relatively short (156-bp) product, but they were nonetheless effective. This is probably a function of the low G+C content of the product tested. The photochemical reactivities of isopsoralens are enhanced for DNA sequences rich in A+T residues, since these compounds are more likely to form cross-links with A+T-rich regions of the helix. Likewise, the UNG protocol is probably more effective on products with low G+C contents because of the high concentration of deoxyuridine target residues within the product. In the accompanying manuscript (5a), the efficiencies of the IP and UNG protocols are shown to vary widely depending on the length and G+C content of the product. Thus, the choice of a system for inactivation of a PCR product must take into consideration the size of the product and its nucleic acid composition. The conditions for using a protocol should be established and evaluated for each target system.

Each of the systems that we evaluated in the present study presented certain drawbacks. The IP protocol uses potentially hazardous isopsoralen compounds that, like ethidium bromide, must be handled with care in the laboratory. Although the reagent itself is inexpensive, a dedicated UV transilluminator must be purchased for maximum effectiveness. The UNG protocol is expensive because of the higher cost of dUTP compared with that of TTP and the requirement for an extra enzyme in the reaction mixtures. Furthermore, care must be taken to prevent residual UNG activity from destroying the amplification product prior to analysis. Our experience has been that most reactions tolerate substitution of dUTP for dTTP (5a), but some do not (20). Poor reaction efficiency after dUTP substitution is probably due to the lower incorporation efficiency of dUTP by *Taq* polymerase or to changes in primer annealing on dUTP-substituted templates. The primer hydrolysis method appears to be highly effective and is cost competitive, but as it is currently performed, tubes containing amplified DNA must be opened

to allow the addition of NaOH. Until reaction vessels that allow unidirectional reagent addition after amplification are developed, the opening of the tubes may provide opportunities for contamination by aerosolized amplification products.

None of the methods described here is mutually exclusive. Isopsoralens can be used to inactivate dU-containing DNA, and the amplifications themselves can be performed with ribose-modified primers. Indeed, our general approach to implementation of an inactivation protocol is to routinely replace dUTP for TTP in amplification mixtures, but to use IP inactivation as the frontline prophylactic measure. Thus, if contamination occurs despite the use of the IP protocol, UNG can be added on an interim basis to control the problem until the source of contamination is identified.

Contamination control in laboratories that regularly perform PCR should incorporate an integrated approach to the prevention of amplicon carryover. Strict separation of pre- and postamplification steps and topical application of 10% sodium hypochlorite (bleach) to working surfaces and to spilled amplified product should be implemented (25). Despite these precautions, however, false-positive reactions may still occur because of low levels of contaminating amplification products that are carried over on the skin, hair, and clothing of the laboratory workers within various laboratories (12). Careful consideration of contamination control in the design of an amplification protocol may provide the final "missing link" in the evolution of practical and useful tests based on nucleic acid amplification.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI30548, AI32403, and AR41497 (to D.H.P.).

We thank Doug Hauschild for excellent manuscript preparation.

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