Inhibition Controls for Qualitative Real-Time PCR Assays: Are They Necessary for All Specimen Matrices?

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A retrospective analysis of 386,706 specimens representing a variety of matrix types used in qualitative real-time PCR assays determined the overall inhibition rate to be 0.87% when the inhibition control was added preextraction to 5,613 specimens and 0.01% when the inhibition control was added postextraction but preamplification in 381,093 specimens. Inhibition rates of $\leq 1\%$ were found for all specimen matrix types except urine and formalin-fixed, paraffin-embedded tissue.

Inhibitors can be found in various specimen matrices, and these substances can interfere with PCRs by interacting directly with DNA and blocking the activity of the polymerase or other PCR mixture components (e.g., $MgCl_2$), thereby preventing target amplification. Examples of PCR inhibitors include bile salts in feces, heme in blood, and urea in urine. In addition, some components of common laboratory collection devices (e.g., viral transport medium, heparin, formalin, or swabs containing gel or charcoal) are known inhibitors of PCR. Inhibition (or internal) controls added directly to the specimen are often used in order to detect inhibition associated with the specimen matrix or the processing method (1, 2). Clinical and Laboratory Standards Institute document MM3-A2 recommends that the addition of an inhibition control be determined on a case-by-case basis by specimen type and with consideration of the potential consequences of a false-negative result (3). The College of American Pathologists recommends spiking an aliquot of the clinical specimen with target nucleic acid but indicates that “the practice can be discontinued once the laboratory accumulates sufficient data that the inhibition rate falls within acceptable limits” (MIC 63278; http://www.cap.org/apps/docs/laboratory_accreditation/checklists/new/microbiology_checklist.pdf). Some clinical laboratories are licensed by the New York State Department of Health, which indicates that an inhibition control is needed unless the inhibition rate is $<1\%$ for a specimen matrix (4). However, addition of inhibition controls by laboratory staff is not without issues, including added cost and labor.

In order to determine whether inhibition controls are necessary for each specimen matrix type, we performed a retrospective evaluation of 386,706 specimens used in validation studies or submitted to our laboratory for real-time PCR analysis across a variety of analytes in 28 qualitative real-time PCR assays using the LightCycler 1.2 and 2.0 platforms (Roche Applied Sciences, Indianapolis, IN). These assays consisted of 28 qualitative laboratory-developed tests (LDTs) that employ standardized specimen processing, extraction, and amplification methods. For each specimen matrix, we calculated the rate of inhibition observed when target DNA or a whole organism was used to spike an aliquot of a specimen prior to extraction and the rate of observed inhibition when a recovery template, target DNA, or a whole organism was added postextraction but before PCR amplification.

MATERIALS AND METHODS

Specimens. Inhibition rates were determined for specimens submitted for analysis or included in validation studies from 2004 to 2012 for LDTs. The specimen matrices examined were grouped according to similar physical or anatomic characteristics and included swabs (nasopharyngeal, nasal, throat, dermal/genital, anogenital, ocular, and perianal), EDTA-preserved whole blood and blood components, respiratory specimens (i.e., bronchoalveolar lavage fluid, bronchial wash samples, sputum samples, tracheal secretions, swabs submitted from respiratory sources), fresh tissue (organ tissue, bone, muscle, and connective tissue), body fluids (i.e., peritoneal/abdominal, pleural, abscess, and synovial fluids), cerebrospinal fluid (CSF), ocular fluid, stool samples, and urine. In addition, inhibition was determined by using formalin-fixed, paraffin-embedded (FFPE) tissue blocks.

Specimen processing. Standardized specimen processing protocols were used for all assays. A brief description of the preextraction processing steps used for each specimen matrix type is provided below; details can be found in the respective publications listed in Table 1. Specimens were extracted on the MagNA Pure LC platform and included whole blood, plasma, serum, ocular fluid, CSF, respiratory specimens, other body fluids (e.g., amniotic and synovial fluids), specimens collected on swabs (with the exceptions described below), stool samples, and fresh and FFPE tissue samples.

Swab processing. Specimens collected on swabs, with the exception of swab specimens for group A *Streptococcus*, *Bordetella pertussis*/*Bordetella parapertussis*, *Staphylococcus aureus*, and *vanA/vanB* assays, were extracted with the MagNA Pure LC instrument. Twenty-five percent of the swabs tested were supplied in M5 viral transport medium. These assays used a simple lysis procedure in which a stainless steel wire cutter with a flat bottom was used to cut the swab shaft. The swab was then placed into a methicillin-resistant *Staphylococcus aureus* lysis tube (Roche Molecular Diagnostics, Indianapolis, IN). The capped tube was placed on a Thermomixer R (Eppendorf AG) for 6 min at 99°C and 1,400 rpm and then centrifuged at $20,800 \times g$ for 20 s. Five microliters of the supernatant was used in the assay.

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TABLE 1 Real-time PCR assays compared in this study

Test	Gene target	Internal control target	ASR ^a or TIB MolBiol primer-probe set	Other master mixture ^b components: water/MgCl ₂ (μl) added to primer/probe sets	Reference(s) (if applicable)
Adenovirus	Penton	Plasmid, whole virus	261	1,140/160	7
<i>Babesia</i> species	18S rRNA	Plasmid, genomic DNA	142	1,060/240	NA
<i>Bartonella</i> species	Citrate synthase	Plasmid, whole organism	141	980/320	8
<i>B. dermatitidis</i> / <i>H. capsulatum</i>	DRK1/GAPDH	Plasmid	535	1,140/160	9
<i>B. pertussis</i> / <i>B. paraptussis</i>	IS481/IS1001	Plasmid	ASR	NA ^c	10
<i>Borrelia</i> species	Plasminogen-binding protein	Plasmid, whole organism	124	1,060/240	11
BK virus	Large T antigen	Plasmid	288	1,140/160	12
<i>Campylobacter</i> / <i>Salmonella</i> / <i>Yersinia</i> / <i>Shigella</i> species	<i>cadF</i> / <i>invA</i> / <i>lysP</i> / <i>ipaH</i>	Genomic DNA	602, 604, 664, 663	860/240	13
CMV	UL54	Plasmid, whole virus	ASR	NA	12
<i>Coccidioides</i> species	Internal transcribed spacer	Plasmid	258	1,140/160	6
<i>C. difficile</i> toxin	<i>tcdC</i>	Whole organism	296	1,060/240	5
EBV	Latent membrane protein	Plasmid, whole virus	ASR	NA	12
Group A <i>Streptococcus</i>	Heat shock protein	Plasmid	ASR	NA	14
HHV6	Immediate-early protein	Plasmid	210	1,140/160	NA
Herpes simplex virus	DNA polymerase	Plasmid	ASR	NA	15, 16
JC virus	Large T antigen	Plasmid	289	1,140/160	NA
<i>Legionella</i> species	5S rRNA	Plasmid, whole organism	404	1,060/240	17, 18
<i>Microsporidium</i> species	18S rRNA	Plasmid, whole organism, genomic DNA	1669		19
<i>Mycobacterium</i> genus screen	Internal transcribed spacer	Plasmid, genomic DNA	202	1,060/240	20
<i>M. tuberculosis</i> complex	<i>katG</i>	Genomic DNA	257	1,060/240	21
Parvovirus	Nonstructural protein	Plasmid, whole virus	162		NA
<i>Pneumocystis jirovecii</i>	<i>cdc2</i>	Plasmid	140	1,060/240	22
<i>Plasmodium</i> species	18S rRNA	Plasmid	268	980/320	23, 24
<i>S. aureus</i>	Thermonuclease	Plasmid	327	1,060/240	25, 26
Shiga toxin	<i>stx1/stx2</i>	Whole organism	315	980/320	27
<i>T. whipplei</i>	<i>hsp65</i>	Plasmid	121	1,060/240	28
<i>vanA/vanB</i> (<i>Enterococcus</i>)	<i>vanA, vanB</i>	Plasmid	ASR	NA	29
VZV	Gene 29	Plasmid	ASR	NA	30

^a ASR was from Roche Molecular Diagnostics.^b A 200-μl volume of FastStart (LightCycler FastStart DNA Master HybProbe) was added to each master mixture.^c NA, not applicable.

Stool sample processing. Stool samples were processed by transferring a pea-sized amount of material with a sterile cotton swab into a 2-ml microcentrifuge tube containing 50% stool transport and recovery buffer (stool sample dilution of approximately 1:10; Roche Applied Sciences). The suspension was vortexed and allowed to settle for 1 min. Two hundred microliters of the supernatant was placed into a specimen cartridge for extraction with a total nucleic acid isolation kit on the MagNA Pure system (Roche) (5). For *Mycobacterium tuberculosis* complex and *Coccidioides* species assays, specimens were placed into equal volumes of sterile water, heated at 95 to 100°C for 5 min, and then subjected to a bead-beating step for 2 min on the Disruptor Genie (Scientific Industries, Bohemia, NY). These modifications were performed in order to kill any organism present prior to the extraction step. Two hundred microliters of the supernatant was placed into a specimen cartridge for extraction with a total nucleic acid isolation kit on the MagNA Pure LC system.

Respiratory specimen processing. Respiratory specimens (bronchoalveolar lavage fluid, bronchial washings, sputum, and tracheal secretions) were processed as previously described (6) by pipetting 500 μl of raw specimen and 100 μl of proteinase K (Roche Applied Sciences) into a

1.5-ml tube containing 0.1-mm silica glass beads and 2.4-mm zirconia beads (BioSpec Products, Bartlesville, OK). Specimens were incubated at 55°C for 15 min on a Thermomixer R (Eppendorf) at 1,400 rpm and subsequently placed on a 95°C heat block for 5 min. To facilitate complete lysis and nucleic acid liberation, specimens were placed on a Disruptor Genie for 2 min and then centrifuged briefly at 20,800 × g to collect the sediment at the bottom of the tube (6). Two hundred microliters of solution was placed into the MagNA Pure LC cartridge for extraction.

Tissue processing. Fresh tissue specimens were processed for PCR analysis by placing a small piece of tissue, approximately 0.5 cm³, into a sterile 1.5-ml tube containing 400 μl of 1× Tris-EDTA (TE; Sigma-Aldrich, St. Louis, MO), 100 μl of proteinase K (Roche Applied Sciences), and 50 μl of 10% sodium dodecyl sulfate (Sigma-Aldrich). The specimens were vortexed briefly and placed on a Thermomixer R overnight at 55°C at a mixing speed of 500 rpm (6). The solution was mixed gently, and 200 μl was placed into the MagNA Pure cartridge for extraction.

MagNA Pure extraction. Two hundred microliters of processed specimen was added directly to a MagNA Pure cartridge. The specimen was extracted with the MagNA Pure LC instrument (Roche Applied Sciences)

TABLE 2 Inhibition percentages grouped according to specimen matrix

Sample(s)	Inhibition control added preextraction		Inhibition control added postextraction, preamplification	
	No. of specimens tested	No. (%) of specimens inhibited	No. of specimens tested	No. (%) of specimens inhibited
Amniotic fluid	63	0 (0.00)	646	0 (0.00)
Body fluids	319	3 (0.94)	475	0 (0.00)
Synovial fluid	547	1 (0.18)	83	1 (1.20)
Blood and components				
Bone marrow	482	1 (0.21)	134	0 (0.00)
Plasma	149	0 (0.00)	43,606	0 (0.00)
Serum	ND ^a	ND	722	0 (0.00)
Whole blood (EDTA)	936	9 (0.96)	26,244	0 (0.00)
CSF/ocular fluid	603	4 (0.66)	69,904	0 (0.00)
Upper respiratory tract swab				
Throat	NA ^b	NA	62,455	6 (0.01)
Nasal	NA	NA	543	1 (0.18)
Nasopharyngeal	NA	NA	66,949	6 (0.01)
Perianal swab	NA	NA	38,430	23 (0.06)
Anogenital swab	NA	NA	54,957	0 (0.00)
Respiratory specimens (nonswab) ^c	581	6 (1.03)	2952	3 (0.10)
Tissue				
Fresh	790	4 (0.51)	1373	0 (0.00)
FFPE	210	14 (6.67)	232	4 (1.72)
Stool	551	3 (0.54)	544	1 (0.18)
Urine	382	4 (1.05)	10,844	1 (0.01)
Total	5,613	49 (0.87)	381,093	46 (0.01)

^a ND, not determined.^b NA, not applicable; nonextracted specimen matrix.^c Includes sputum, induced sputum, bronchial washes, tracheal secretion, and bronchoalveolar lavage fluid.

with the MagNA Pure total nucleic acid isolation kit (small volume) and the Blood, Plasma, Serum program. All specimens were eluted into a final elution volume of 100 μ l.

LightCycler PCR conditions. Fifteen microliters of the “hot start” reaction mixture containing the LightCycler FastStart DNA master mixture (including *Taq* polymerase, PCR buffer, a deoxynucleoside triphosphate mixture with dUTP, and 10 mM $MgCl_2$), additional $MgCl_2$, primer pairs, fluorescence resonance energy transfer hybridization probes, and a recovery template (if applicable) was added to the LightCycler cuvette. Five microliters of lysed or extracted specimen was added, and the reaction mixture was placed into the LightCycler. Two cycling/melting profiles were used on the LightCycler. The principal profile was as follows: 95°C for 10 min; 45 amplification cycles of 10 s at 95°C, 15 s at 55°C (single acquisition), and 15 s at 72°C; melting curve analysis for 0 s at 95°C, 20 s at 59°C, 20 s at 45°C (ramp rate of 0.2°C/s), and 0 s at 85°C (ramp rate of 0.2°C/s and continuous acquisition); and finally cooling for 30 s at 40°C. The *M. tuberculosis* complex, *Mycobacterium* genus screen, *Coccidioides* species, and *Histoplasma capsulatum*/*Blastomyces dermatitidis* assays used the same cycling parameters except that a 15-s annealing step at 60°C rather than 55°C was used to improve the specificity of the assays.

PCR assay information. The LDT assays used for this study targeted adenovirus, *Babesia* species, *Bartonella* species, BK virus, *Bordetella pertussis*/*Bordetella parapertussis*, *Borrelia* species, *Campylobacter jejuni*/*Campylobacter coli*, *Clostridium difficile*, *Coccidioides* species, *Coxiella burnetii*, cytomegalovirus (CMV), *Ehrlichia* species, *Enterococcus vanA/vanB*, Epstein-Barr virus (EBV), group A *Streptococcus*, herpes simplex virus 1/2, *Histoplasma capsulatum*/*Blastomyces dermatitidis*, JC virus, human herpesvirus 6 (HHV6), *Legionella* species, *Microsporidia* species, the genus

Mycobacterium, the *M. tuberculosis* complex, parvovirus, *Plasmodium* species, *Pneumocystis jirovecii*, *Salmonella* species, *Shigella* species, *Staphylococcus aureus*, Shiga toxin 1/2, *Tropheryma whipplei*, varicella-zoster virus (VZV), and *Yersinia* species. For many of the assays listed above, primers and probes are commercially available from TIB MolBiol (Adelphia, NJ) or as analyte-specific reagents (ASR) from Roche Molecular Diagnostics (Table 1). The majority of the assays (24/28) have been published or presented as abstracts at national meetings. References are provided in Table 1.

Determination of inhibition. Inhibition was determined at two points in the testing process. Inhibition controls were added preextraction by spiking an aliquot of the specimen matrix with a whole organism or target DNA incorporated into a plasmid at a level of 100 target copies/ μ l (approximately 10 times the limit of detection) and then subjecting it to processing and extraction as described above. In addition, inhibition controls were added postextraction but preamplification by incorporation of an ASR recovery template into the PCR master mixture or by spiking an aliquot of specimen extract with a whole organism or a target plasmid when recovery templates were unavailable. The recovery template, whole organism, or plasmid containing the target was for spiking at a level of 100 target copies/ μ l. Inhibition rates were calculated on the basis of lack of detection of the recovery template, whole organism, or target DNA-containing plasmid. Confidence intervals for the inhibition rates were determined by the modified Wald method.

RESULTS

Of 386,706 specimens examined, 95 (0.02%) had evidence of inhibitors present (Table 2). The inhibition rate when inhibition

controls were added preextraction was 49/5,613 specimens, for an overall rate of 0.87% (95% confidence interval, 0.69 to 1.1%). FFPE tissue had the highest rate of inhibition of any individual specimen source, at 6.67%, with urine having the second highest rate (1.05%). When the inhibition control was added postextraction but preamplification, inhibition was observed in 46/381,093 specimens, for an overall rate of 0.01%. FFPE tissue (1.72%) was the only matrix with an inhibition rate above 1%.

DISCUSSION

On the basis of the data presented in Table 2, PCR inhibition controls are not necessary for the majority of the specimen matrices and assays listed when the extraction method (MagNA Pure LC total nucleic acid kit) and PCR platform (LightCycler) are used as described above. Inhibition in real-time PCR assays occurred in $\leq 1\%$ of the specimens tested, regardless of the specimen matrix, except with urine and FFPE tissues. The urine inhibition rate was only slightly above 1%, at 1.05%.

Given the low rates of amplification inhibition identified in this study, spiking an aliquot of specimen with target DNA to act as an inhibition control or addition of recovery templates to the assays described appears to add little value while adding to the cost and complexity of the assay for most specimen matrices. The obvious exception where an inhibition control is necessary is FFPE tissue, where inhibition rates are well above 1%. The cost of adding an inhibition control or a recovery template depends upon the source of the control, with laboratory-developed controls adding only a few cents per sample and commercially acquired recovery templates adding \$1.00 to \$1.50 per test reaction. In addition to the cost of the added control itself, an additional 1 to 2 min per sample of technologist postanalysis time is required to evaluate the inhibition control result. Ultimately, the laboratory director must weigh the risks of specimen matrix-associated inhibition against the associated cost and labor in order to set policy for the laboratory while maintaining compliance with regulatory agency directives.

The main limitation of our study is the use of a single extraction platform type (MagNA Pure LC) and a single real-time PCR instrument (LightCycler); extension of these studies to other extraction and PCR platforms is needed. As shown in Table 1, the majority of the internal controls used were added postextraction and 1.5% were added preextraction. These differences could potentially bias the results, but when the data were analyzed separately between pre- and postextraction, the conclusions were consistent with urine and FFPE being the sources, with inhibition rates above 1.0%, regardless of when the internal controls were added. The single exception was synovial fluid, which had an inhibition rate of 1.2% when controls were added postextraction and 0.18% when they were added preextraction. However, the number of synovial fluid specimens tested with postextraction addition of controls was small ($n = 83$), with a single inhibited specimen leading to the 1.2% inhibition rate. Another limitation is that our study presents data from qualitative real-time PCR assays, and therefore, the target analyte was not quantitated to determine whether any was lost during the extraction process. However, for many assays, we spiked with target material at a level of approximately 100 target copies/ μ l, suggesting that any inhibitors present caused a loss of < 100 targets/ μ l. Inhibition controls remain necessary in quantitative assays to normalize for potential analyte concentration losses during extraction.

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