Validation of Methods for Producing Spiked Blood and Plasma Samples for Use to Evaluate Analytical Sensitivity and Reproducibility for Rare Pathogens

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Abstract

Introduction: Isolates of pathogens capable of being used as biological weapons are uncommon, but development and validation of diagnostic devices for these and other rare pathogens is needed. To mitigate this problem, standardized methods to generate mock clinical samples by spiking cultured pathogens into blood or plasma have been developed. The goal of this study was to assess the sensitivity and reproducibility when mock samples produced by standardized methods are used for validation of diagnostic devices for detection of potential biological threat pathogens.

Methods: Mock samples were prepared by the FDA by spiking *Bacillus anthracis*, *Staphylococcus aureus*, *Leishmania donovani*, *Yersinia pseudotuberculosis*, and Dengue virus into blood or plasma of healthy donors or donors with fever, signs and symptoms at high, medium, and low concentrations. The isolated nucleic acids (NAs) were quantified in PCR Detectable Units/mL (PDU/mL) using TaqMan® assays. The same NAs extracted from the mock samples were blinded and tested with Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR™). Amplicons were hybridized to probes coupled to barcoded magnetic beads and analyzed on an Applied BioCode-2000 reader.

Results: There was 100% correlation of TEM-PCR™ and TaqMan® Assays at the highest concentrations, with one exception. The TEM-PCR™ assay detected 90% of *B. anthracis* samples at 1e5 PDU/mL, compared to the FDA TaqMan® Assay, which detected 100% at the same concentration. Results of the two assays did not vary more than 15% at medium and low concentrations, with one exception. *L. donovani* was detected at 40% with the TEM-PCR™ assay and 70% with the TaqMan® Assay at the lowest concentration (1e3 PDU/mL). There was one false positive in the unspiked blood sample with TEM-PCR™. There were no false positives in unspiked samples with the TaqMan® Assay. There was no difference in results using spiked blood or plasma from symptomatic or healthy donors at high concentrations with a correlation of 100% between the two datasets. There was variation from 4% to 43% between the two datasets at medium and low concentrations of spiked blood, and 14% variation at the low concentration of spiked plasma.

Conclusions: Testing of blinded mock samples by end-point TEM-PCR™ correlated with results generated with TaqMan® Assays. This correlation strongly supports the validity of the methods used to prepare mock samples. Standardized methods for creating mock samples may allow a means for evaluating analytical sensitivity and reproducibility of diagnostic devices for detecting rare pathogens.

Introduction

A barrier to the development of diagnostic devices for rare pathogens has been the lack of isolates to demonstrate sensitivity and reproducibility required for FDA clearance. Standardized methods for spiking blood or plasma with quantified pathogens were developed and used to make mock specimens. Molecular assays for the following pathogens (see targets below) were used to demonstrate the reproducibility of mock specimens made by these standardized methods. The effect of using blood from healthy versus symptomatic donors in preparation of mock samples was also investigated.

- Dengue virus type 2 – polyprotein gene
- *Bacillus anthracis* – protective antigen gene on pXO1 plasmid & *racE* gene
- *Leishmania donovani* – kinetoplast minicircle & AAH gene
- *Staphylococcus aureus* – *nuc* gene
- *Yersinia pseudotuberculosis* - *gapA* gene & 16s rRNA

Materials & Methods

Mock Specimens Produced by CBER, FDA

- Blood was collected from healthy and symptomatic donors by BioreclamationIVT (Hicksville, NY). Symptomatic donors had fever and flu-like illness.
- Organisms, quantified by TaqMan® Assays in PCR Detectable Units (PDUs), were spiked into whole blood.
- Dengue virus was spiked into plasma.
- The concentrations included: high (aimed at 100% detection), medium (aimed at below 100% detection), and low.
- DNA was isolated with a Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA).
- Dengue RNA was isolated with a Qiagen QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA).
- Aliquots of NAs were provided to Diatherix (Huntsville, AL).
- NAs from defined mock specimens, including NAs from un-spiked blood and plasma, were used to set cutoff values, determining positive or negative targets.

Materials & Methods *(continued)*

PCR

- A TEM-PCR™ multiplex test was developed to simultaneously detect *B. anthracis, S. aureus, L. donovani* and *Y. pseudotuberculosis* from DNA samples.
- A separate TEM-PCR™ multiplex test was developed to detect Dengue virus type 2 from RNA samples.

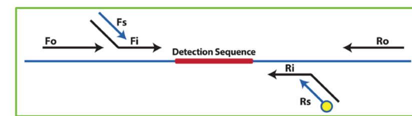

Figure 1. TEM-PCR™ Scheme.
A gene specific sequence is enriched with primers Fo/Ro. Primers Fi/Ri label the sequence with tags for the priming of universal primers, Fs/Rs. This allows for the multiplexed, gene specific primers to be present in very low concentrations and the two universal primers at high concentration. The universal reverse primer is labeled with biotin which binds fluorescent dye for the detection of the amplicons.

Detection of TEM-PCR™ amplicons

- Amplicons were hybridized to Barcoded Magnetic Beads (BMBs) from Applied BioCode Inc. (Santa Fe Springs, CA).

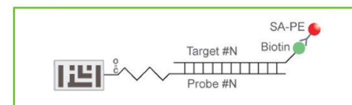

Figure 2. Probes are covalently bound to unique BMBs. Probes capture biotin labeled amplicons. The addition of of streptavidin phycoerythrin allows for fluorescent detection of the amplicons hybridized to specific probes.

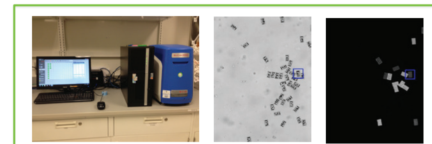

Figure 3. Applied BioCode Reader (left). Software associates images of BMB barcodes in bright-field (middle) with fluorescence intensity (right).

- Isolated NAs, supplied by the FDA, were used to demonstrate specificity of the assay and to assess the sensitivity.
- Blinded NAs from mock specimens produced with blood collected from healthy and symptomatic donors were tested with TEM-PCR™ by Diatherix and real-time PCR by the FDA.

Table 1. Numbers of blinded specimens tested with TEM-PCR and with real-time PCR. *Y. pseudotuberculosis, B. anthracis*, *L. donovani*, and *S. aureus* were spiked into whole blood. Dengue virus was spiked into plasma.

	Healthy Donors		Symptomatic Donors	
Spiked Pathogen, concentration PDU/mL	TEM-PCR	Real-Time PCR	TEM-PCR	Real-Time PCR
B. anthracis, 1E5	10	10	7	7
B. anthracis, 1E4	10	9	7	7
B. anthracis, 1E3	10	10	7	7
L. donovani, 1E5	10	10	7	7
L. donovani, 1E4	10	10	7	7
L. donovani, 1E3	10	10	7	7
S. aureus, 1E6	10	10	7	7
S. aureus, 1E5	10	10	7	7
S. aureus, 1E4	10	10	7	7
Y. pseudotuberculosis, 1E4	10	10	7	7
Y. pseudotuberculosis, 1E3	10	10	7	7
Y. pseudotuberculosis, 1E2	10	10	7	7
Blood NC	20	40	20	40
Dengue 1E3	10	10	7	7
Dengue 1E2	10	10	7	7
Dengue 1E1	10	10	7	7
Plasma NC	10	10	10	10

NC – Negative Control

Results

Testing of Known Samples

Table 2. Specificity of the TEM-PCR™ assay was tested with isolated DNA. The percentage detected is shown for each gene target used in the assay (n=10).

Sample	*B. anthracis*	*S. aureus*	*Y. pseudo-tuberculosis*	*L. donovani*
Genomic Equiv./test	7.10E+04	1.30E+05	7.70E+04	1.10E+04
Assay target				
B.a. protective antigen gene	100	10*	0	0
B.a. racE gene	100	0	10*	0
L.d. kinetoplast minicircle	0	0	0	100
L.d. 18s rRNA	0	0	0	100
L.d. AAH gene	0	0	0	100
*S.a.*nuc gene	0	100	0	0
Y. p. gapA gene	0	0	100	0
Y. p. 16s rRNA	0	0	100	0

* One false positive was observed for each of the *B. anthracis* targets.

Table 3. Whole blood samples spiked with pathogens at high, medium and low concentrations were tested with TEM-PCR™. The percentage positive at each concentration are shown for each gene target (n=4, Negative Control: n=43).

Sample	*B. anthracis*			*S. aureus*			*Y. pseudo-tuberculosis*		*L. donovani*			Neg. Cont.
PDU/mL	1E+05	1E+04	1E+03	1E+06	1E+05	1E+04	1E+04	1E+03	1E+05	1E+04	1E+03	0E+00
Assay Target												
B.a. protective antigen gene	**100**	**50**	**75**	0	0	0	0	0	0	0	0	4.65
B.a. racE gene	**75**	**75**	**75**	0	0	0	0	0	0	0	0	4.65
L.d. kinetoplast minicircle	0	0	25	0	0	0	0	0	**100**	**25**	0	0
L.d. 18s rRNA	0	0	0	0	0	0	0	0	**75**	0	0	0
L.d. AAH gene	0	0	0	0	0	0	0	0	**75**	0	0	0
*S.a.*nuc gene	0	0	0	**100**	**50**	0	0	0	0	0	0	4.65
Y. p. gapA gene	0	0	0	0	0	0	**75**	**25**	0	0	0	0
Y. p. 16s rRNA	0	0	25	0	0	0	**100**	**75**	0	0	0	2.33

True positives are shown in bold text.

Table 4. Plasma samples spiked with Dengue virus at high, medium and low concentrations (n=8, Negative Control: n=12)

Sample	Dengue type 2			Negative Control
PDU/mL	1.00E+06	1.00E+05	1.00E+04	1.00E+00
% Detected Dengue 2 polyprotein gene	**100**	**100**	**87.5**	0

Testing of Blinded Mock Specimens

- Results were similar for real-time PCR (performed by the FDA) and TEM-PCR™ (Diatherix).

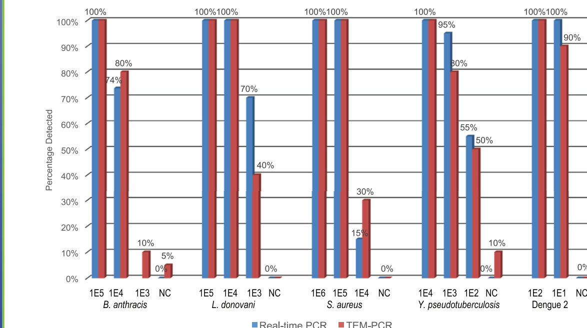

Figure 4. Comparison of percentage (shown above columns) of detected pathogens from real-time PCR and TEM-PCR™. Numbers of blinded mock specimens tested are shown in Table 1.

Results *(continued)*

- A decrease of sensitivity was observed for most pathogens with matrix from symptomatic donors used for mock specimens.

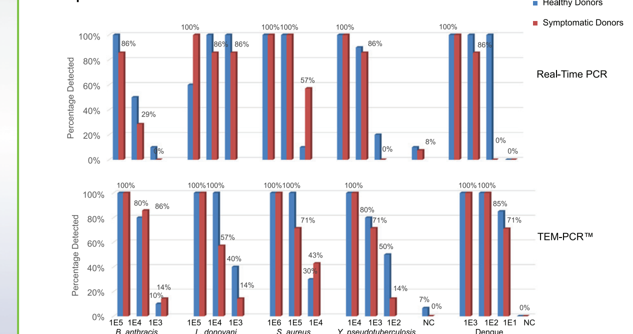

Figure 5. Comparison of percentage (shown above columns) of detected pathogens from healthy blood donors verses symptomatic blood donors tested with RT-PCR (upper) and TEM-PCR™ (lower). Numbers of blinded mock specimens tested are shown in Table 1.

Discussion

- Mock specimens of rare pathogens are needed so that molecular diagnostics manufacturers will have a means for performing clinical sensitivity studies to obtain FDA clearance.
- Standardization of the methods used to generate mock specimens benefits both the device developers and those that regulate them.
- This study demonstrates reproducibility by an alternative testing method, by an independent laboratory, of mock specimens generated by standardized methods.

Conclusions

- The choice of matrix is an important factor to consider when making mock specimens for sensitivity and specificity testing. We observed that blood from symptomatic donors significantly affected the assay sensitivity and specificity, but results may vary by specific target.
- Both test systems, real-time PCR-based and end-point PCR combined with hybridization provide highly correlated results for testing mock samples. This strongly supports the validity of the methods used to prepare mock samples.
- Standardized methods for creating mock samples may allow a means for evaluating analytical sensitivity and reproducibility of diagnostic devices for detecting rare pathogens.

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