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Evaluation of lot-to-lot repeatability and effect of assay media choice in the recombinant Factor C assay

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Abstract

Measurement of environmental endotoxin exposures is complicated by variability encountered using current biological assay methods arising in part from lot-to-lot variability of the limulus-amebocyte lysate (LAL) reagents. Therefore, we investigated the lot-to-lot repeatability of commercially available recombinant Factor C (rFC) kits as an alternative to LAL. Specifically, we compared endotoxin estimates obtained from rFC assay of twenty indoor dust samples, using four different extraction and assay media, to endotoxin estimates previously obtained by Limulus amebocyte lysate (LAL) assay and amounts of 3-hydroxy fatty acids (3-OHFA) in lipopolysaccharide (LPS) using gas-chromatography mass spectroscopy (GC-MS). We found that lot-to-lot variability of the rFC assay kits does not significantly alter endotoxin estimates in house dust samples when performed using three of the four assay media tested and that choice of assay media significantly altered endotoxin estimates obtained by rFC assay of house dust samples. Our findings demonstrate lot-to-lot reproducibility of rFC assay of environmental samples and suggest that use of rFC assay performed with Tris buffer or water as the extraction and assay medium for measurement of endotoxin in dust samples may be a suitable choice for developing a standardized methodology.

Graphical Abstract

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Introduction

Endotoxins are lipopolysaccharides (LPS) that comprise the outer membrane of Gram-negative bacteria¹. As biologically active compounds, endotoxins are diverse mediators of the immune system causing fever, inflammation, respiratory distress, shock or even death^{1–3}. It is well known that airborne endotoxin is ubiquitous and airborne endotoxin exposure levels can vary significantly, over several orders of magnitude, depending on environmental conditions². Exposure to indoor environmental endotoxin has been associated with airway inflammation⁴ and increase risk of wheeze in children⁵. By contrast, other studies have shown that exposure to environmental endotoxin may be protective against the development of asthma^{6,7} and allergic diseases⁸. Although the underlying mechanisms of how endotoxin exerts these disparate effects are not fully understood, current research proposes explanations related to differential stimulation of innate immune responses by endotoxin exposures at different exposure levels, frequencies, duration and age at exposure^{4–8}.

The commercialization of the *Limulus* amoebocyte lysate (LAL) assay for endotoxin began with the discovery of Bang et al that the blood of *Limulus polyphemus* forms a gel clot upon exposure to Gram negative bacterial endotoxin resulting from a coagulation cascade involving the Factor C protein^{9,10}. Subsequent efforts made by Levin led to the development of the standard LAL assay that is currently used for detection of bacterial endotoxin contamination by the pharmaceutical industry^{11–13}. The LAL assay became widely used for quantitative measurement of endotoxin in environmental samples. However, many studies have demonstrated difficulties with LAL assay reproducibility when used for quantification as compared with detection of endotoxin in environmental samples, due to various methodological issues^{14–26}. One major limitation of the LAL assay with environmental samples is that the lot of LAL reagent can significantly alter endotoxin estimates¹⁹. Since the LAL reagent is obtained from wild *Limulus polyphemus* populations, it is understandable that lot-to-lot variability of the LAL reagent can be high. This variability limits the utility of the LAL assay as a tool for measurement of environmental endotoxin exposures and presents significant challenges to longitudinal epidemiological studies aimed at understanding the health effects of endotoxin exposure.

A newer methodology, the recombinant Factor C (rFC) assay, has since been developed for measurement of environmental endotoxin^{10,27,28}. Through genetic engineering, a cloned version of the Factor C protein was developed for endotoxin assay that displays high

enzymatic sensitivity to endotoxin¹⁰. Unlike the Factor C protein present in blood samples obtained from *Limulus polyphemus*, recombinant Factor C can be manufactured under controlled conditions and offers the potential for improved lot-to-lot consistency and reproducibility.

Despite these advantages, the utility of the rFC assay is hampered by the lack of standardized methods. Many studies have demonstrated the difficulties of inter-lab endotoxin assay reproducibility and highlighted the importance of standardization of assay methodology^{14–26}. Additionally, it has been shown that correlation of endotoxin measurements made by biological assays such as rFC, LAL and chemical endotoxin analyses using gas chromatography-mass spectrometry (GC-MS) for quantitation of 3-hydroxy fatty acids (3-OHFA) in LPS are highly dependent on the specific aspects of the biological assay methods used^{22, 29–34}. Standardization of the rFC assay would greatly improve inter-lab reproducibility and accuracy of endotoxin estimates.

Many studies have examined the effect of sample extraction and assay media on endotoxin estimates for environmental samples assayed by LAL^{16, 19, 20, 24, 25}. Several studies have compared the use of pyrogen-free water and pyrogen-free water containing Tween-20 for use as extraction and assay medium^{16, 24, 25}, while others have evaluated the use of triethylamine phosphate (TAP) buffer^{17, 19} for use as extraction and assay medium when assaying environmental samples by LAL. According to one manufacturer of LAL and rFC assay kits, reagents used in their endotoxin detection assays have been optimized for samples at a pH range of 6 – 8 and recommend using 50mM Tris buffer.

In this study, we aim to evaluate the lot-to-lot repeatability of the rFC assay performed in four commonly used endotoxin assay media, pyrogen-free water, pyrogen-free water containing Tween-20, TAP buffer and Tris buffer. Demonstration of rFC lot-to-lot repeatability would address a significant problem encountered by researchers attempting to measure environmental endotoxin. Additionally, we investigate the effect of assay and extraction media choice on endotoxin measurements and assay reproducibility. Taken together, the findings of this study will contribute to methodological improvements in measurement of environmental endotoxin that may improve the quality of epidemiological studies and allow researchers to better understand the respiratory health effects of environmental endotoxin exposure and how they relate to chronic respiratory disease.

Materials and methods

House dust samples were obtained from a continuing birth cohort study of childhood asthma and stored at -80°C ³⁵. A subset of twenty dust samples was selected that included previously analyzed samples with low, mid-range, and high levels of endotoxin activity and 3-OHFA, a chemical marker for LPS²⁹. Endotoxin activity was previously determined using the *Limulus* Amoebocyte Lysate (LAL) assay performed in 0.01% triethylamine in 0.05M potassium phosphate buffer (TAP; pH 7.5) and 3-OHFA content was determined using gas chromatography-mass spectroscopy (Fig. 1)^{29, 31}. In this work, we reassayed selected dust samples using rFC and compared rFC endotoxin estimates with LAL and 3-OHFA results obtained in earlier studies (Fig. 1)^{29, 31}.

Five mg aliquots of dust samples were extracted in 1 ml of each of the following four assay media: (a) pyrogen-free water (PFW; Associates of Cape Cod, Falmouth, MA); (b) 0.01% triethylamine in 0.05M potassium phosphate buffer (TAP; pH 7.5); (c) pyrogen-free water with 0.05% (v/v) Tween-20 (Tween; polysorbate 20; Sigma-Aldrich, St. Louis, MO); and (d) 50mM Tris buffer (Lonza Biosciences, Walkersville, MD). Extraction was performed in baked ($270^{\circ}\text{C} \times 30 \text{ min}$) borosilicate glassware using bath sonication (Branson Cleaning Equipment Co., Shelton, CT) for 1 hr with vortexing every 15 min³⁶. Immediately after sonication, three dilutions (1:6) of each dust sample suspension were made following an initial dilution of 1:25, covering a concentration range of 0.9 – 200 μg dust sample/mL extraction media³⁶. Two replicate sample wells were included for each dilution for a total of 8 microtiter plate wells per dust sample (4 dilutions, 2 wells/dilution). Five dilutions of reference standard endotoxin (20 – 0.015 EU/mL range, RSE; U.S. Pharmacopeia, Rockville, MD) were prepared and assayed in duplicate for each plate for a total of 10 standard wells (5 dilutions, 2 wells/dilution). In all experiments, a single assay media type was used for both extraction and sample and RSE dilution steps. Dust extracts and RSE standards were assayed for endotoxin activity immediately after dilution using the Pyrogene recombinant factor c (rFC) endotoxin detection system (Lonza Bioscience, Walkersville, MD) and pyrogen-free microtiter plates³⁶. Three different lots of Pyrogene rFC kits were tested in this study. Thus, each dust sample was assayed once in four different assay media in each of three different kit lots. Kinetic readings were taken using a fluorescent plate reader (BioTek FLx800IU, Winooski, VT) with incubation at 37°C (excitation 380/20 nm; emission 440/30 nm). The gain or sensitivity setting of the plate reader was adjusted for each lot of RSE and each assay media type in order to maintain a consistent dynamic range of 3.5 log relative fluorescence units (RFU) as recommended by the assay manufacturer³⁶. Endotoxin activity of each dust sample was computed using the parallel-line estimation method with ordinary least squares regression¹⁷. The analysis software used for endotoxin activity determination was developed in R (R Development Core Team 2004) and is available from the author.

Total number of samples requiring repeat analysis and total number of invalid samples after repeat analysis from all three lots tested were tabulated and represented as a percent of total samples assayed for each assay media. Invalid samples were defined as those samples where the dose-response slope was not parallel to that of the standard based on an analysis of covariance as previously described^{17, 19}. Any sample displaying invalid endotoxin estimates was repeated once using the same lot of reagents. Samples with invalid endotoxin estimates after repeat analysis were noted and the estimates with the larger p value for the test of parallel slopes (standard vs. sample curve) were reported.

Summary statistical analyses were performed using SAS statistical software (version 9, SAS Institute, Cary, NC). The Proc MIXED procedure was used to generate regression models and compute least squares mean values for endotoxin estimates (dependent variable) using the saturated model which included all effects. The fixed effects were sample number, assay kit lot, and assay media type. The random effect was assay number. Analysis of variance tests were used to examine the significance of the fixed effects and any possible interactions. Comparisons of rFC endotoxin estimates with LAL endotoxin measurements made in TAP buffer³⁶ and the amount of mid-chain 3-OHFA ($\text{C}_{10:0}$, $\text{C}_{12:0}$, and $\text{C}_{14:0}$) determined using

gas chromatography – mass spectrometry (GC-MS)^{29, 31} were made using Spearman correlation. Coefficients of variation (CV) were calculated for measurements from all three lots for each dust sample assayed in each of four assay media.

Results

Frequency of Repeat Analyses and Invalid Endotoxin Estimates

Table 1 shows the number of dust samples that produced invalid endotoxin estimates and required repeat rFC analysis expressed as a % of total number of samples tested by each assay media using three lots of rFC kits. Tween produced the highest number of samples requiring repeat analysis (8.3%) followed by TAP buffer (3.3%) and then Tris buffer (1.7%). The use of water as the assay media did not produce any samples requiring repeat rFC analysis. Additionally, Table 1 shows the number of dust samples that continued to produce invalid endotoxin estimates after a single repeat rFC analysis. Tween produced the highest number of invalid sample endotoxin estimates after one repeat assay (5.0%) followed by TAP buffer (1.7%). Tris buffer and water displayed no invalid endotoxin estimates after one repeat rFC analysis.

rFC Assay Variability

Coefficient of variation (CV) values for samples assayed in each assay media for all three assay kit lots tested were used to calculate a Mean CV value for each assay media type (n=60). Mean CV values for dust samples assayed by three lots of rFC assay kits in Tris buffer, TAP buffer, Tween and water were 35.0%, 47.8%, 31.2% and 37.3%, respectively (Table 1).

Effect of Assay Media and rFC Assay Kit Lot on rFC Endotoxin Estimates

Comparison of mean endotoxin estimates from dust samples (95% CI, n=60) assayed by rFC in Tris buffer, TAP buffer, Tween and water (74.0, 48.7, 100.0, and 120.7 EU/mg, respectively, Fig. 2) indicated a significant effect of assay buffer on endotoxin estimates ($p < 0.001$).

Figures 3 A, B, C, and D display mean endotoxin estimates from dust samples (95% CI, n=20) by rFC assay kit lot for each of the four extraction media tested (Tris buffer, TAP buffer, Tween and water). Within media, there were no significant lot-to-lot differences in mean endotoxin estimates for Tris buffer, Tween, or water (Figures 3 A, C, and D, $p > 0.3$). Lot-to-lot differences in mean endotoxin estimates for samples assayed in TAP buffer were borderline significant (Fig. 3 B, $0.1 > p > 0.05$), a suggestion that lot 1 produced higher endotoxin estimates.

Lot-to-lot differences between rFC assay kits displayed borderline significance when all four assay media were included in the model ($p = 0.09$) and appeared to be driven by results from rFC assay kit lot 1 in TAP buffer. When results from samples assayed in TAP buffer were removed from the analysis, no significant effect of assay kit lot was observed among the remaining three assay media ($p = 0.44$). No significant interaction was observed between lot and assay media ($p = 0.68$).

Correlation of Endotoxin Estimates and Amounts of 3-OHFAs

Strong and statistically significant correlations in mean of endotoxin estimates were found (i) between the LAL assay performed in TAP buffer and rFC assay performed in either Tris buffer, TAP buffer, Tween or water (Table 1, Spearman correlation 0.94–0.95, $p < 0.001$), (ii) between the rFC assay performed in either Tris buffer, TAP buffer, Tween or water and the amount of mid-chain ($C_{14:0}$) 3-hydroxy-fatty acids (pmol/mg) (Spearman correlation 0.90 – 0.93, $p < 0.001$, Table 1) and (iii) between the rFC assay performed in either Tris buffer, TAP buffer, Tween or water and the amount of other mid-chain ($C_{10:0}$, $C_{12:0}$, and total $C_{10:0} + C_{12:0} + C_{14:0}$) 3-hydroxy-fatty acids (pmol/mg) (Spearman correlation 0.76 – 0.93, $p < 0.001$, Supplemental Table 1). Additional Spearman correlation values for endotoxin estimates obtained by LAL performed in TAP buffer and the amount of other mid-chain ($C_{10:0}$, $C_{12:0}$, and total $C_{10:0} + C_{12:0} + C_{14:0}$) 3-hydroxy-fatty acids ranged from 0.80 to 0.92 and were significant (Supplemental Table 1, $p < 0.001$).

Discussion

Environmental endotoxin, depending on exposure history and levels, is associated with both adverse and protective health effects ^{4–8}. Attempts to fully understand the underlying mechanisms of how endotoxin exposures produce such disparate health effects has relied primarily on the LAL assay to measure endotoxin exposure levels in environmental samples ^{9, 10, 27, 28}.

There are several sources of potential differences between Limulus-based endotoxin bioassay methods that have made standardization difficult: interference, lot-to-lot differences in reagents, and effects of extraction and dilution media.

The problem of interference by inhibition and enhancement has been described in a number of studies ^{17, 19, 37, 38}. Here, we employed a parallel-line bioassay method that used data from multiple dilutions for each sample, identified optimal dilution, and provided internal validation of assay results for every sample. This method does not rely on an assumption that each sample from a given environment will behave identically and have the same optimal dilution factor.

Lot-to-lot variability of the LAL assay reagent is a significant problem when using LAL for measurement of environmental endotoxin in longitudinal studies, inter-lab comparison of results and reproducibility of study findings ¹⁹. A prospective birth cohort study on home microbial exposures and childhood asthma reported a substantial drop in average home endotoxin levels over a 7 year time period, even after adjustment for LAL reagent lot ³⁹. Although this difference may be real, degradation of the stored LAL reagent lot used for the earlier measures may have decreased the validity of the lot-to-lot comparison conducted for endotoxin measurement several years later. Therefore, LAL reagent lot could still be a source of variability in longitudinal endotoxin exposure assessment, even if a previous LAL lot is available for comparison. In addition to complicating longitudinal studies, inconsistencies in endotoxin measurement techniques across studies have also made comparison of epidemiological findings difficult. Lastly, exposure misclassification, as a

result of imprecise measurement of endotoxin, may bias or obscure the true effects of this bacterial component in population studies of environmental exposures and health.

The rFC assay, utilizing a recombinant Factor C protein, offers the advantage of much improved lot-to-lot reproducibility^{10, 27, 28}. However, currently no standardized assays exist for analysis of endotoxin present in environmental samples and many studies have demonstrated problems with assay reproducibility and poor correlation of results^{14–26}. In this study we examined the effect of rFC assay kit lot on endotoxin estimates. Additionally, we assessed the significance of assay media choice on rFC endotoxin estimates and evaluated the performance of four commonly used assay media for potential recommendation of a single assay media choice for future rFC assay of environmental dust samples.

Lot of rFC assay kit used was not a significant effect in the model and no significant interaction effect of assay kit lot and assay media was observed. There were no significant lot-to-lot differences between assay kits in three of the media (Tris buffer, Tween, and water) we tested, and only borderline differences were observed with the fourth media (TAP buffer) (Fig. 3). The improved lot-to-lot reproducibility of endotoxin estimates made with rFC assay as compared to LAL assay¹⁹, is most likely attributable to the fact that the key reagent in the rFC assay is a cloned protein that is manufactured whereas the LAL reagent is taken from a variety of wild specimens of *Limulus polyphemus*. The observed improved repeatability of rFC endotoxin estimates across lots of assay kits as compared to LAL assay represents a significant finding for future studies employing biological assays for measurement of environmental endotoxin.

Our results provide strong evidence that assay media choice significantly alters rFC endotoxin estimates (Fig. 2). Differences in mean potency estimates for dust samples assayed by rFC in Tris buffer, TAP buffer, Tween or water were significantly different ($p < 0.0001$) with estimates for samples assayed in water approximately two-fold higher than those assayed in Tris buffer (Fig. 2). Variations in the number of invalid endotoxin estimates obtained from rFC assay were also observed across assay media. Of the four assay media tested, Tween produced the highest number of samples producing invalid endotoxin estimates requiring repeat analysis (8.3%, Table 1). After repeat analysis of all samples producing initial invalid endotoxin estimates, Tween continued to produce the highest number of invalid samples (5.0%, Table 1). Only 1.7% of those samples assayed in TAP buffer remained invalid after repeat assay. All of the samples assayed in Tris produced valid endotoxin estimates after the repeat assay. Water produced no samples requiring repeat analysis. Lack of a standardized, appropriate assay media for measurement of environmental endotoxin exposure could be one important factor decreasing reproducibility of environmental endotoxin estimates^{14–16, 22}. Observation of significant differences in endotoxin estimates obtained from the same assay using different extraction and assay media highlights the need for developing and adopting standardized methods for environmental endotoxin assays such as LAL or rFC so that meaningful studies of health exposures and effects can be conducted.

Previous work by Alwis et al. found high correlations between rFC and LAL endotoxin estimates performed in TAP buffer³⁶. Our findings (Table 1) indicate strong correlation between rFC and LAL endotoxin estimates and agree with the conclusions of the Alwis et al. study. Our findings also provide further evidence that rFC estimates correlate as well as LAL assay performed in TAP buffer and with 3-OHFA levels, a chemical marker of endotoxin measured using GC-MS²⁹.

It has been reported that use of Tween-20, a surfactant, in the extraction medium results in higher measured endotoxin bioactivity levels when assaying dust samples with the LAL assay^{16, 24, 40} that may be attributable to improved extraction efficiency of endotoxin. However, it has also been reported that use of detergents in the extraction medium when assaying dust samples by LAL did not extract or yield higher levels of LPS than use of water alone¹⁹. Comparison of LPS content in dust extracts made with various media did not demonstrate greater LPS extraction with Tween compared with other extraction media, including water²². This study did not find any evidence that use of Tween-20 in the extraction medium yields higher levels of endotoxin biological activity than water when assayed by rFC or that Tween provided any additional benefits over the other three media tested.

Reviewing the performance of the four assay media tested in this study, it is clear that TAP buffer should not be recommended for use with rFC assay due to lot-to-lot variability, and frequency of repeated and invalid samples (Table 1, Fig. 2) observed when dust samples are assayed by rFC in TAP buffer. Use of Tween should also not be recommended for assay of dust samples by rFC because it produced the highest number of samples requiring repeat analysis, persistent invalid endotoxin estimates, and can thus be considered an inferior choice when compared to the use of Tris buffer or water with rFC assay (Table 1).

Use of water as the assay media for rFC analysis of dust samples produces higher mean potencies than use of Tris buffer (Fig. 2). However, mean potency is a measure of the relative biological activity of sample endotoxin activity as compared to that of reference standard endotoxin under particular assay conditions (because endotoxin assays are comparative bioassays, not analytical assays⁴¹), and is not a measure of absolute recovery. Thus, correlation of mean potency with amounts of LPS, the biologically active component of endotoxin, may be more appropriate. In our study, correlations of mean potency estimates from rFC assay performed in Tris or water with amount of C_{14:0} 3-OHFA in LPS were similar (0.90 vs. 0.93, Table 1). Mean CV values for endotoxin estimates from rFC performed in Tris buffer were slightly lower than those from rFC performed in water (35% vs 37%, Table 1).

The manufacturer of the rFC assay kit used in this study (Lonza Biosciences, Walkersville, MD) states that rFC assay reagents are optimized to work with samples with a pH range of 6–8 and recommends use of buffer for samples falling outside of this pH range. Previous work by Milton et al indicated that pure LPS produced different dose-response curves when exposed to two different types of glassware used for sample preparation, borosilicate and flint glass and highlighted the importance of using buffer to decrease the chance of introducing significant changes in endotoxin estimates due to interference from various

environmental factors¹⁷. This knowledge, along with our current findings, suggest that Tris buffer, which is readily available in endotoxin free formulations from the kit manufacturer, is the best choice for rFC assay media. However, water may be a cheaper, more readily available option for rFC analysis of environmental samples. Additional studies to more fully evaluate the use of Tris buffer compared to water for the analysis of dust samples by rFC assay is warranted.

Conclusions

Our findings demonstrate lot-to-lot reproducibility of rFC assay that is superior to that previously reported for LAL assay of environmental endotoxin in house dust samples. This study highlights the need for standardization of assay media choice in order to obtain meaningful inter-lab comparisons and reproducibility of results obtained from endotoxin detection assays. Our findings suggest that use of rFC assay performed with Tris buffer or water as the extraction and assay medium for measurement of endotoxin in dust samples may be a suitable choice for developing a standardized methodology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Summary of experimental design and methods.

Fig. 2. Effect of assay media on mean endotoxin levels reported by recombinant Factor C assay. Mean endotoxin levels (95% CI) of dust samples (n=60) were determined by rFC assay using Tris buffer, TAP buffer, Tween or water across three lots of rFC assay kits. Differences in mean endotoxin levels were significant ($p < 0.001$).

Fig. 3. Effect of lot by assay media type on mean endotoxin levels reported by recombinant Factor C assay. Mean endotoxin levels (95% CI) of dust samples (n=20) reported by each of three lots of rFC assay performed in Tris buffer (A), TAP buffer (B), Tween (C) or water (D).

Table 1

Summary of rFC Assay Performance by assay medium.

	Spearman Correlation for rFC Estimates			
	%Repeats ^a	%Invalid ^b	Mean CV ^c	LAL ^d C _{14:0} ^{e,f}
Tris	1.7%	0.0%	35.0%	0.95
TAP	3.3%	1.7%	47.8%	0.94
Tween	8.3%	5.0%	31.2%	0.94
Water	0.0%	0.0%	37.3%	0.93

^{a,b} % Repeats and %Invalid denote total number of samples requiring repeat rFC assay and total number of samples with invalid endotoxin estimates after repeat rFC assay, respectively, for each assay medium expressed as % of total samples assayed in three lots of assay kits (n=60).

^c Mean coefficient of variation (CV) values were calculated for each assay media type (n=60).

^d Spearman correlation values of mean endotoxin estimates (EU/mg) for dust samples (n=60) assayed by three rFC assay kit lots in Tris buffer, TAP buffer, Tween, or water with results obtained from LAL assay in TAP buffer. All Spearman correlation values were significant (p < 0.001).

^e Spearman correlation values for mean rFC endotoxin estimates (EU/mg, n=60) by assay medium with amounts of C14:0 3-hydroxy-fatty acids (pmol/mg) as determined by GC-MS. All Spearman correlation values were significant (p < 0.001).

^f Spearman correlation of mean endotoxin estimates (EU/mg) for dust samples assayed by LAL (n=20) with amounts of C14:0 3-hydroxy-fatty acids (pmol/mg) as determined by GC-MS C14:0 was 0.94 and significant (p<0.0001).