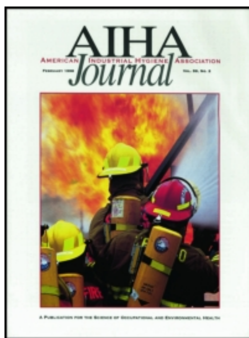

AIHA Journal

ISSN: 1542-8117 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/uaih20

Comparison of Endotoxin Assays Using Agricultural Dusts

Stephen J. Reynolds , Peter S. Thorne , Kelley J. Donham , Elizabeth A. Croteau , Kevin M. Kelly , Daniel Lewis , Mike Whitmer , D.J.J. Heederik , Jeroen Douwes , Ian Connaughton , Sharon Koch , Per Malmberg , Britt-Marie Larsson & Donald K. Milton

To cite this article: Stephen J. Reynolds , Peter S. Thorne , Kelley J. Donham , Elizabeth A. Croteau , Kevin M. Kelly , Daniel Lewis , Mike Whitmer , D.J.J. Heederik , Jeroen Douwes , Ian Connaughton , Sharon Koch , Per Malmberg , Britt-Marie Larsson & Donald K. Milton (2002) Comparison of Endotoxin Assays Using Agricultural Dusts, AIHA Journal, 63:4, 430-438, DOI: 10.1080/15428110208984731

To link to this article: https://doi.org/10.1080/15428110208984731

Published online: 04 Jun 2010.

Submit your article to this journal

Article views: 81

View related articles

Citing articles: 25 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=uoeh20

Comparison of Endotoxin Assays Using Agricultural Dusts

Authors
Stephen J. Reynolds[a,*]
Peter S. Thorne[a]
Kelley J. Donham[a]
Elizabeth A. Croteau[a]
Kevin M. Kelly[a]
Daniel Lewis[b]
Mike Whitmer[b]
D.J.J. Heederik[c]
Jeroen Douwes[c]
Ian Connaughton[d]
Sharon Koch[d]
Per Malmberg[e]
Britt-Marie Larsson[e]
Donald K. Milton[b]

[a]College of Public Health, Department of Occupational and Environmental Health, 140 IREH, University of Iowa, Iowa City, IA 52242;
[b]Division Respiratory Disease Studies/NIOSH, 1095 Willowdale Road, Morgantown, WV26505;
[c]Institute for Risk Assessment Sciences, Environmental and Occupational Health Group, University of Utrecht, P.O. Box 238, 6700 AE Wageningen, The Netherlands;
[d]Bendigo Agricultural Centre, Bendigo Mail Centre, Victoria 3554, Australia;
[e]National Institute for Working Life, S-112 79 Stockholm, Sweden;
[f]Dept. Environmental Health, Harvard School of Public Health, 665 Huntington Ave, Boston, MA 02115
*Current address: Department of Environmental and Radiological Health Sciences, Colorado State University, 154A EHB, Fort Collins, CO 80523-1676; E-mail: stephen.reynolds@colostate.edu

This work was funded by the Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health as a component of the University of Iowa Great Plains Center for Agricultural Health (NIOSH-U07 CCU 706145-05-01) and the Environmental Health Sciences Research Center (NIH-P30 E5056505-01).

Endotoxins from gram-negative bacteria pose a significant respiratory hazard. Establishing dose-response relationships is problematic because there are no standard procedures for sampling and analysis. The goal of this study was to compare endotoxin analyses in six laboratories using Limulus-based assays for analysis of organic dusts from three agricultural environments: chicken barns, swine barns, and corn processing facilities. For each dust generation experiment 14 side-by-side air samples were collected on 37-mm glass fiber filters at flows of 1.8 L/min. Each laboratory was randomly allocated two filters from each of seven experiments per dust type. Three laboratories used the QCL-1000 endpoint assay, and three used the kinetic-QCL method. To eliminate variability among different lots, a single lot of Limulus amebocyte lysate for endpoint assays and one similar lot for kinetic assays was provided. Precision of assays performed within labs was very good, with pooled coefficients of variation for replicate samples ranging from 1 to 11% over all labs and all dust types. There were significant differences between laboratories for all three dust types ($p<0.01$). The pattern of differences between labs varied by dust type. For chicken dust, labs using the endpoint method reported higher results than those using kinetic methods. For swine and corn dusts, labs using the kinetic method reported the highest endotoxin values. For chicken dust, results from all labs except A and B were highly correlated ($r=0.86$–1.00). For swine dust, only labs B and E, and C and D were correlated. For corn, A, B, and D were significantly correlated with most other labs. In conclusion, statistical differences in performance between laboratories were apparent and may be related to the extraction and analytical methods. The results of this study will be useful for standardization of sampling and analysis of airborne endotoxin in agriculture.

Keywords: endotoxin, lipopolysaccharide, method validation

In vitro studies, animal and human inhalation studies, and research in agricultural and industrial environments have shown that endotoxins, found in the cell walls of gram-negative bacteria, pose a significant respiratory hazard.(1–16) Illnesses attributed, at least in part, to endotoxin exposure include byssinosis, airway hyperreactivity, and organic dust toxic syndrome.(3,5–9,11–16) Inhalation of endotoxin induces a variety of biological responses such as cell activation (neutrophils, macrophages), with mediator release (interleukins), activation of complement, and damage to epithelial and endothelial cells.(8–10,14,16,17,18) Decrements in pulmonary function, especially FEV1, have also been demonstrated in response to endotoxin exposure.(1,2,6,7,12,14,15) Occupational exposure to endotoxin occurs in a variety of industries including agriculture, wastewater plants, refuse-derived fuel production, metals machining, snowmaking, and in the manufacture of pharmaceuticals and medical devices.(3,11,12,14,18,19–24) Although occupational exposure limits have been suggested for endotoxin, evaluation of dose-response relationships is problematic, because sampling and analytical methods have not been standardized.

The most common analytical methods currently used for endotoxin in environmental

 Copyright 2002, American Industrial Hygiene Association

samples are based on the chromogenic Limulus amebocyte lysate (LAL) assay. In these assays, endotoxin activates a proenzyme that cleaves a synthetic substrate. This releases a dye that absorbs light at 405–410 nm. Two methods of measuring the absorbance and calculating endotoxin concentration are (1) the endpoint method, in which acetic acid is used to stop the reaction before measuring absorbance; and (2) the kinetic method, which measures absorbance as the reaction proceeds over time.[26,27] In contrast to assay methods that measure biological activity, chemical analyses using either high-pressure liquid chromatography or gas chromatography-mass spectrometry (GC-MS) have also been developed. The chemical methods primarily focus on quantification of 3-hydroxy fatty acids in lipopolysaccharides (LPS).[28–30]

Variability in endotoxin evaluation methods reported to date include sample environment (dust type); collection of sample (pumps, flow rates, filter types); sample handling and storage (use of desiccant, storage temperature); analysis of sample (extraction media, extraction time, rocking, sonication, temperature, assay type, control standards); and reporting of results (units).[32–40] The composition of organic dust from different environments is variable, and thus could alter the outcome of analysis through differences in type of endotoxin, and inhibition or enhancement.[32,40,41] Researchers have investigated differences in collection filter material, extraction media, and extraction time.[29,32,39] Gordon et al. suggested that aerosol binding to filters can be affected by chemical and physical differences in the filters, thus causing variation in endotoxin extraction.[34] Using an endpoint chromogenic method, Gordon et al. found significantly higher recoveries of endotoxin from glass fiber and cellulose acetate filters compared with polycarbonate, polyvinyl chloride, polysulfone, and polytetrafluoroethylene filters. Olenchock et al. reported that an extraction time of 2 hours yielded optimal results using sterile pyrogen-free water.[35] Other investigators demonstrated that using surfactants such as Tween 20 and Saponin in the extraction media increased the amount of biological activity of detected endotoxin.[31,35] The use of a potassium phosphate/triethylamine buffer to counteract inhibition and enhancement of the assay was suggested by Milton et al.[26,27] Using samples from a potato processing plant, Douwes et al. investigated differences among four types of filters, two extraction media, two extraction temperatures, and vigorous versus quiet rocking during extraction.[31] They found that glass fiber filters yielded slightly but not significantly higher endotoxin biological activity than polycarbonate filters, with cellulose acetate filters showing the lowest recovery of activity. An extraction solution containing 0.05% Tween 20 also yielded higher endotoxin biological activity than pyrogen-free water. Extraction temperature and agitation method did not contribute significantly.

An important factor affecting the comparison of results from endotoxin assays is the units in which concentrations are reported. Results are often expressed as either nanograms per cubic meter (mass) or endotoxin units per cubic meter (EU/m^3; potency). Although there are conversion factors to relate these units, they do not express the same thing. The conversion factor depends on the manufacturer of the endotoxin standard, and it can be different between laboratories. Therefore, it is important that each laboratory report results in EU relative to one standard reference endotoxin so that results can be compared between laboratories.[36]

A few limited interlaboratory comparisons have been conducted. Reynolds and Milton compared an endpoint chromogenic LAL assay to a kinetic Limulus assay for samples from turkey barns.[36] Statistically significant differences were found between the two assays when results were calculated in nanograms of endotoxins, but not when results were calculated in EU relative to EC5 standard reference endotoxin (except for samples from one barn). Both methods detected a significant difference between barns and ranked them in the same order. This study found that the two LAL-based methods, despite general agreement, did not behave in the same manner although there was no evidence of interferent compounds. The differences may have resulted more from intrinsic differences in the sensitivity of the lots of lysate used than from differences inherent in the methods themselves—similar to the differential sensitivity to different forms of endotoxin described by Saraf et al. for kinetic lysate formulations from three manufacturers.[41]

Thorne, Reynolds, Milton and colleagues conducted an interlaboratory study evaluating relationships between different filter sampling materials, extraction procedures, and LAL-based analyses.[37] Replicate air samples were collected on polycarbonate and glass fiber filters in swine and chicken facilities. The filters were analyzed using a chromogenic endpoint LAL (QCL) method and a kinetic chromogenic LAL (KLARE) method with a buffer extraction. Extraction with heat (30 min at 68°C) and without heating (120 min at 22°C) was also performed for the endpoint method. Overall, the extraction methods were comparable. Glass fiber filters yielded higher levels of endotoxin biological activity than polycarbonate filters, and under some circumstances the QCL and KLARE yielded significantly different estimates of endotoxin activity. Correlations between the QCL and KLARE methods were high for polycarbonate filters ($r=0.90$) and moderate for glass fiber filters ($r=0.53$). Analysis of variance (ANOVA) demonstrated that assay method, filter type, barn type, and interactions between assay-filter type and assay-barn type were all important factors.

Recently a two-phase round-robin interlaboratory study was conducted using cotton dust.[38,39] Twelve laboratories participated in the first phase and 13 in the second phase. Cotton dust was collected on PVC filters in a model cardroom. During the first phase of the study each laboratory performed endotoxin assays using their usual methodology. In the second phase a common extraction procedure was used. In the first phase of the study significant differences between laboratories were found. However, intralaboratory variations were small, and the rankings were consistent. The use of a common extraction protocol in the second phase considerably reduced the differences between laboratories.

The goal of this study was to compare the performance of six laboratories using Limulus-based assays for analysis of organic dusts from three agricultural environments. Dusts from a chicken barn, a swine barn, and a corn processing facility were used to generate aerosols in the laboratory. For each dust type, each laboratory was randomly allocated seven pairs of duplicate dust samples from experiments generating different dust and endotoxin levels. Three laboratories used the QCL-1000 endpoint assay, and three used the kinetic-QCL method. To minimize variability due to the use of different LAL lots, a known and potentially major source of variability, a single lot of LAL for endpoint assays, and one similar lot for kinetic assays, were provided.

METHODS

Bulk Dust Samples

Bulk dust samples were collected from a chicken laying facility, a swine farrowing building, and an animal feed (corn) manufacturing company. The corn dust was sifted to remove kernels and large

debris. All three sets of samples were stored at 4°C. The bulk dust was characterized by quantification and classification of bacteria species and fungal genera. Three grams of each dust was added to 30 mL of pyrogen-free phosphate buffered saline (PBS) (University of Iowa Hybridoma Facility) and rocked for 1 hour at room temperature. Serial dilutions were plated on trypticase soy agar (TSA), eosin methylene blue (EMB) agar, and malt extract agar (MEA) (Difco, Detroit, Mich.). Cycloheximide (Sigma, St. Louis, Mo.) was added to the TSA and EMB to inhibit fungal growth. Chloramphenicol (Amresco, Solon, Ohio) was added to the MEA to inhibit bacterial growth. The TSA and EMB plates were incubated at 35°C for 5 days. MEA plates were incubated at room temperature for 5 days. Predominant colonies from each culture media were subcultured and sent to the University of Iowa Hygienic Laboratory for bacterial analysis. Fungal identification, at the genus level, was performed by collection and staining hyphae and spores with lactophenol cotton blue and characterization under light microscopy using standard reference keys.[42]

Aerosol Generation

Prior to aerosolization, bulk dust was ground using a Mixer Mill Type MM-2 (Brinkman, Westhaven, N.Y.) to achieve an aerosol particle size that was inspirable (<10 μm). The following speeds and total times were used: (1) chicken dust, speed setting=50%, time=10 min; (2) swine dust, speed setting=50%, time=15 min; (3) corn dust, speed setting=50%, time=20 min. Aerosols were generated using a modified version of the Pitt-3 aerosol generator[43] called the Acoustical Dry Aerosol Generator/Elutriator (ADAGE) previously described by Thorne.[44] Aerosols were carried from the ADAGE at a flow rate of 17±1 L/min into a 1 m³, Plexiglas™ laminar flow chamber previously described by Reynolds.[45] Air was exhausted from the bottom of the chamber, through a 0.45 μm filter (Gelman Sciences, Ann Arbor, Mich., Mini Capsule, Clearwater, Fla.), at a flow rate of 75±5 L/min.

A 12-port critical orifice manifold was used to collect most of the dust samples inside the chamber. The 12 critical orifices (Millipore) had flow rates of 1.8±0.3 L/min. Two Gilair (Gillian) personal pumps were used in addition to the critical orifice manifold when a seventh laboratory was added to the study (one of the seven labs did not report results, however). A total of 14 side-by-side samples were therefore collected for each experiment.

Preliminary work was conducted to characterize the gravimetric and particle size distributions, endotoxin and microbial content of the aerosols under differing experimental conditions. Gravimetric samples were collected on desiccated 37-mm glass fiber filters (Gelman Sciences), with cellulose support pads (Gelman Sciences). Gravimetric analyses were conducted using a Mettler MT-5 balance (Mettler Instruments, Westerville, Ohio). Mass median aerodynamic diameter (MMAD) and geometric standard deviation (σg) were determined using an eight-stage Marple Cascade Impactor (Grasby Andersen, Atlanta, Ga.) with 37-mm mylar filters at a flow rate of 2.0 L/min.

A subset of the gravimetric samples from the trial runs were analyzed for endotoxin using the endpoint assay method, LAL QCL-1000 (BioWhittaker, Walkersville, Md.). Samples were eluted for 1 hour in 30 mL pyrogen-free PBS at room temperature. Dilutions were assayed using a 96 well microplate reader (Fisher BioTech, Pittsburgh, Pa.).

Microbial Measurements

Two all glass impingers (AGI) in series were used to collect microbiological samples at a flow rate of 10.00±0.02 L/min. Each AGI contained 20 mL of 1% peptone water with 1% Antifoam A. All materials were sterilized prior to use. The peptone water was autoclaved and then filter sterilized (0.2 μm filter, Gelman Sciences). Sample times ranged from 15 to 30 min. Nine milliliters of each impinger solution were added to 1 mL of 37% formaldehyde (EM Science, Gibbstown, N.J.) to be used for fluorescent staining for total bacterial counts. The remaining solution was serially diluted and plated on TSA and EMB media with cycloheximide. These plates were incubated at 35°C and colonies were counted for up to 5 days, with results expressed in colony forming units per cubic meter of air (CFU/m³). Estimates of the amount of gram-positive and gram-negative bacteria in each dust type were determined from gram staining of colonies from the AGI plates (Fisher, Pittsburgh, Pa.). Slides were viewed at 1000×, oil immersion, with a light microscope. Fluorescent microscopy was used to determine the number of total bacteria (viable and non-viable).[45] Five milliliter aliquots of peptone water/formaldehyde dilutions were suctioned onto a 25-mm black polycarbonate filters, 0.4 μm (Poretics Corp., Livemore, Calif.). Two milliliters of acridine orange stain (AO) (Difco, Detroit, Mich.) were applied to the filters for 2 min. Fluorescent optical filters used for AO were a 455-nm supplementary excitation filter, a 490-nm excitation filter, a 500-nm dichroic mirror, and a 515-nm barrier field. The number of total bacteria per cubic meter of air (organisms/m³) is reported.

Dust Generation Experiments

A total of seven generation sessions, or experiments, were performed for each dust type. The sample times were varied from 10 to 50 min to produce a range of endotoxin loadings on the sample filters. Fourteen side-by-side samples were collected on 37-mm glass fiber filters as described above. After sample collection the backs of filter cassettes were attached to cassettes containing silica gel desiccant and stored at 4°C until shipped in insulated boxes with freeze packs. Each laboratory received 14 randomly selected samples (2 from each dust generation session) and 1 blank for each dust type. After each laboratory extracted the filters, a lyophilized portion of each extract was sent to Dr. Lennart Larsson, Lund, Sweden, for GC-MS analysis. Results of the GC-MS analysis are reported separately. Table I summarizes the extraction and assay methods used by each laboratory. BioWhittaker supplied endotoxin assay kits with standardized LAL from two comparable lots—one lot for endpoint assays and one lot for kinetic assays. The functional parameters (i.e., potency, linearity of response) of the LAL and LPS control standard endotoxin (CSE) were defined using both U.S. Reference Standard EC6 and the European Reference Standard BRP-2.

Statistical Analysis

Statistical analyses were performed using SAS version 6.04 (SAS Institute, Cary, N.C.). The PROC UNIVARIATE procedure was used to evaluate the distributions of data. Because data were log-normally distributed, they were log-transformed before proceeding with analysis. PROC UNIVARIATE confirmed that log-transformed data was normally distributed. Pooled coefficients of variation were calculated for each dust type to evaluate precision within each lab. PROC GLM (General Linear Models) multiway ANOVA was used to test for overall differences among laboratories, assay methods, and dust types. PROC GLM is considered to be fairly robust with regards to the assumption of equal variances, and inspection of residuals provides confirmation. Bonferroni (Dunn) t-tests were used to compare results from endpoint and

TABLE I. Endotoxin Extraction and Assay Methods by Laboratory

	Laboratory					
	A	B	C	D	E	F
Extraction[A]						
Tween	no	no	no	yes	no	no
Buffer	no	yes	no	no	no	no
Volume (mL)	30	5	10	5	10	10
Container[B]	PP	glass	PP	glass	glass	?
Temperature	22°C	20°C	room	room	room	room
Time (min)	60	60	60	60	60	60
Agitate/Sonicate	rock	sonic	rock	rock	rotate	rotate
Froze extract	no	no	no	yes	yes	yes
Assay						
Endpoint/Kinetic	endpt	kinet[A]	kinet	kinet[A]	endpt	endpt
Usual number of dilutions assayed	3	5	5–7	1–8	?	?
EU to ng conversion factor	10/1	calc each lot ~10/1	?	calc	12/1	12/1

Notes: Laboratory B used the same buffer for extraction and dilutions of samples and standard LPS (0.05 M KPO4, 0.01% triethylamine); however, LAL and standard LPS were suspended in water. Labs A, E, and F used BioWhittaker's QCL-1000 Lysate; and Labs B, C, and D used BioWhittaker's Kinetic QCL Lysate. Labs B and D used parallel line approaches to test for inhibition and enhancement. All laboratories used reagents and control standard endotoxin (*E. coli* 055B5) standardized in water against the reference standard E6 and supplied by BioWhittaker, except for Lab B which used the reference standard USP Lot G (EC6) in place of the control standard.
[A]All extractions were in sterile pyrogen free water, except as noted. Lab D used 0.05% Tween 20 in sterile pyrogen free water for extraction, dilution, and dissolving LAL and standard LPS.
[B]PP = polypropylene; glass = borosilicate glass.

kinetic assay methods for each dust type, and paired t-tests were used to test for differences between individual laboratories. Spearman correlations were used to estimate associations between the results from individual labs.

RESULTS

Aerosol Characteristics

In preliminary experiments the chicken and swine dust showed good homogeneity throughout the chamber under final conditions, with coefficients of variation (CVs) of 6 and 12%, respectively, for gravimetric measures, and 13 and 15% for endotoxins (n=4 in both cases). Corn appeared to be less homogenous (CV=58% for gravimetric), and the small number of samples (n=3) may have been a contributing factor. Mass median aerodynamic diameters remained very consistent from trial to trial and were 2.0 μm for chicken dust, 5.5 μm for swine dust, and 7.0 μm for corn dust.

Predominant bacterial species recovered from the bulk samples included *Pseudomonas fluorescens* (chicken); *Staphylococcus kloosii* (swine); and *S. xylosus*, *Bacillus subtilis*, and *Enterobacter agglomerans* (corn). Predominant fungal genera recovered from bulk samples included *Penicillium*, *Fusarium*, *Cladosporium* (chicken); *Penicillium*, *Cladosporium*, *Saccharomyces* (swine); and *Penicillium*, *Acremonium*, *Saccharomyces*, and *Mucor* (corn). In the trial aerosols, culturable bacteria concentrations ranged from 1.1×10^4 to 6.9×10^5 CFU/m³. Total (direct count) bacteria concentrations were higher ranging up to 7.9×10^9 CFU/m³.

Interlaboratory Endotoxin Assay Comparisons

Airborne endotoxin concentrations generated in the chamber spanned the ranges typically reported in field investigations of occupational exposure to organic dust. Over all experiments for chicken dust, endotoxin concentrations ranged from 764 to 37,190 EU/m³ (Figure 1). Labs using kinetic assay methods have shaded symbols, and labs using endpoint assay methods have unshaded symbols. For swine dust, endotoxin concentrations ranged from 434 to 40,650 EU/m³ (Figure 2). The range of endotoxin concentrations for corn dust was 287 to 16,240 EU/m³ (Figure 3). Pooled coefficients of variation $[(\Sigma\ CV^2/n)^{1/2}]$ for replicate samples showed that all labs demonstrated excellent within-lab precision, with all CVs less than 11%. (Table II).

A full ANOVA model (Table III) found all main effects (assay

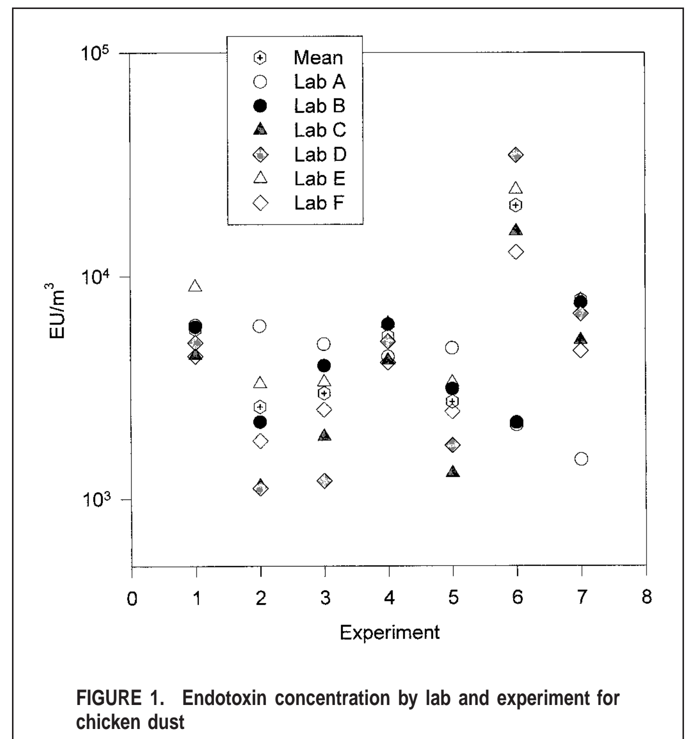

FIGURE 1. Endotoxin concentration by lab and experiment for chicken dust

APPLIED STUDIES

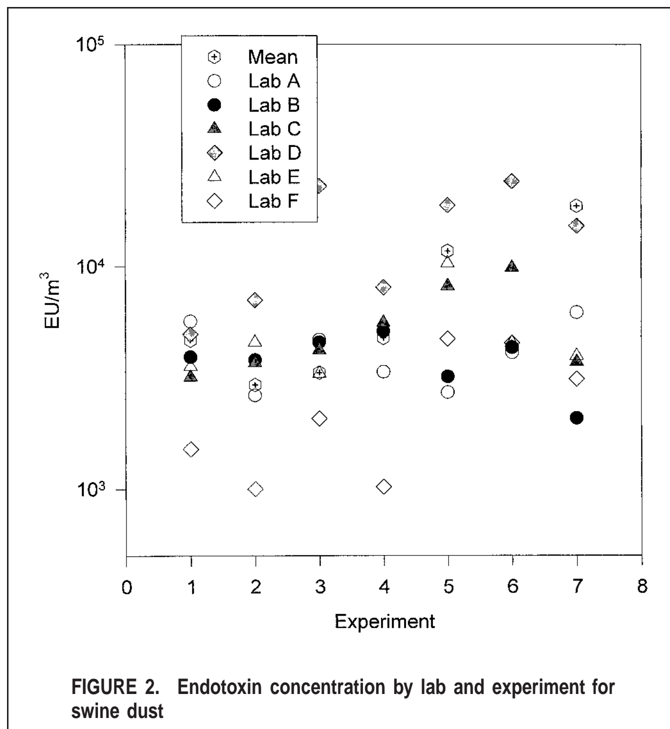

FIGURE 2. Endotoxin concentration by lab and experiment for swine dust

method, dust type, laboratory, experiment) and all interactions (not shown in table) to be statistically significant (n=252, r^2=0.947, p=0.0001). Using coefficients (β) to calculate the relative contribution of laboratory variance versus lab × dust type interaction (10^β Lab × 10^β Lab*Dust), the rank order of laboratories was different for each type of dust. Possibly due to the variability and number of parameters in the model, least square means for the main effects were not estimated by the statistical program.

Tables IV, V, and VI present results of ANOVA performed separately for each dust type. Main effects including assay method

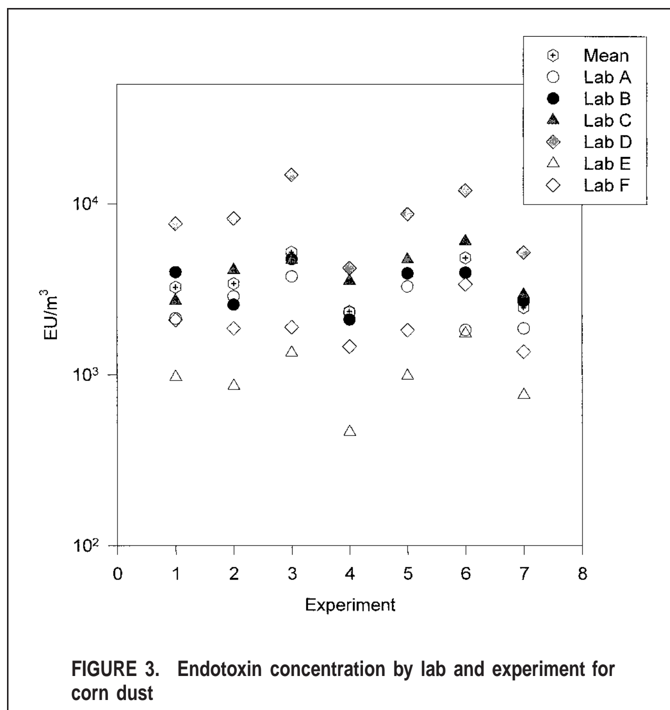

FIGURE 3. Endotoxin concentration by lab and experiment for corn dust

TABLE II. Pooled Coefficients of Variation for Replicate Samples by Lab and Dust Type (CV%)

	Dust Type		
Lab	Chicken	Swine	Corn
A	4	3	11
B	3	3	3
C	4	1	4
D	4	2	1
E	1	2	2
F	5	2	2

Pooled CV = $[(\Sigma\ CV^2/n)^{1/2}]$.

(endpoint versus kinetic), laboratory, experiment, and the interaction between laboratory and experiment were significant for all three dusts. Bonferroni (Dunn) t-tests showed that labs using endpoint assay methods reported higher endotoxin results than labs using kinetic assay methods for chicken dust. Labs using kinetic assay methods reported the highest results for swine and corn dust. Bonferroni (Dunn) t-tests were also calculated to compare endotoxin biological activity reported by laboratory. For chicken dust, Lab E was the only one significantly different (higher) from the others. For swine dust, Lab D was significantly higher than the others, and F was significantly lower. Results from Labs C and A were comparable, as were results from Labs B and E. For corn dust Lab D was again significantly higher. Labs F and E were different from each other, and significantly lower than the other labs. Labs C, B, and A all reported comparable results.

Spearman correlation coefficients among the six laboratories are presented in Table VII. For chicken dust, results from all of the labs except A and B were positively and highly correlated (r=0.85 to 1.00). Lab A was negatively correlated with Labs D and F. It should be noted, however, that these correlations for Lab A are very likely due to chance and that the probabilities (0.05) should be considered in regards to the multiple (15) comparisons for each dust type. For swine dust, only Labs B and E, and D and C were correlated. For corn dust, Labs A, B, and D were correlated with most other labs and (r=0.75 to 0.93).

DISCUSSION

In this interlaboratory comparison of endotoxin assays two important sources of variability were controlled for. Aerosolized samples were collected using one filter material (37-mm glass fiber) and all six laboratories used one of two standardized lots of reagents and LPS standard prepared by BioWhittaker. The lots of reagents and LPS prepared for endpoint and kinetic assays were selected for comparable performance. However, it is still possible that the two lots of LAL may have responded differently to the varying spectrum of LPS in the different dust types. In all other aspects the participating laboratories stored, extracted, and assayed the samples using their usual procedures. Review of Table I indicates the many remaining differences in the extraction procedures and in the assay procedures used by the participating laboratories. Laboratories A, C, E, and F used sterile pyrogen-free water for extraction and dilutions, whereas Laboratory D added a surfactant (0.05% Tween 20) and Laboratory B used a buffer (0.05 M KPO4, 0.01% triethylamine). Extraction volumes varied from 5 to 30 mL; extraction containers were either borosilicate glass or polypropylene; and samples were rocked, rotated, or sonicated during extraction. Extractions were carried out at room

TABLE III. Analysis of Variance (GLM), Full Model With Replicates for Log_{10} EU/M^3

N = 252 Model df = 125 Error df = 126		r^2 = 0.947 Model SS = 30.06 Error SS = 1.67		p = 0.0001 MS = 0.2405 Intercept ($10^β$) = 35,653	
Effect	**Partial r^2 (%)**	**p-value**	**Parameter**	**Ratio, $10^β$**	**(95% CI)**
Assay method	8.77	0.0001	endpoint	0.214	(0.128, 0.359)
			kinetic[A]	1.000	—
Dust type	3.71	0.0001	chicken	0.603	(0.359, 1.014)
			corn	0.178	(0.106, 0.299)
			swine[A]	1.000	—
Laboratory	8.43	0.0001	E	1.347	(0.801, 2.260)
			B	0.040	(0.030, 0.085)
			A	0.355	(0.211, 0.598)
			C	0.362	(0.215, 0.607)
			D	1.000	—[B]
			F+	1.000	—
Experiment	8.53	0.0001	1–7	—	—

[A]Selected as reference.
[B]No standard error of estimate generated by model.

temperature for all labs except A and B, which used controlled temperatures of 22±2°C, and 20±2°C respectively. Laboratories D, E, and F stored extractions at −20, −20, and −70°C until assays were performed. Although three laboratories used the same Kinetic-QCL assay kits, two (B and D) reported using parallel-line strategies for interpreting the assay. It is interesting to note that two of the laboratories reported determination of endotoxin activity (EU) to mass (ng) conversion factors on a case-by-case basis (determined using the specific lot), whereas others reported using a standard conversion factor that was not determined based on the specific LAL and CSE lots. To avoid problems created by using different conversion factors, results in this study are presented in terms of endotoxin activity relative to EC6 (EU/m^3) rather than in mass (ng/m^3).

ANOVA showed that dust type, assay method (endpoint, kinetic), laboratory, experiment, and their interactions were all significant determinants of the outcome variable—the logarithm of endotoxin concentration. It should be noted, however, that assay method may be correlated with the complete laboratory analyses (storage, extraction, assay, interpretation). The full ANOVA model (Table III) accounted for 94.7% of the overall variance. Interactions between dust type and the other main effects accounted for a large proportion (56.3%) of the variance in the full model. Differences in endotoxin content of these organic dusts may result from different chemical and microbiological makeup. Dusts from chicken and swine environments contain animal proteins as well as plant material, and livestock dusts contain fecal bacteria not found in the corn dust environment. The interaction between laboratory and dust type is noteworthy, indicating that comparison of laboratory performance should take into account the environmental source of the endotoxin. Variability in extraction efficiency resulting from physical-chemical effects of the sample matrix may be the most likely explanation for this observation.

ANOVAs conducted separately for each dust type confirmed that assay method, laboratory, and experiment were all important determinants of endotoxin concentrations (Tables IV, V, VI). Freezing of extracts before analysis was not found to be a significant determinant, and was not included in the final model. The greatest differences between labs are approximately one order of magnitude (Figures 1, 2, 3). Compared with the variability (three

TABLE IV. Analysis of Variance (GLM) for Chicken Dust, With Replicates for Log_{10} EU/M^3

N = 84 Model df = 41 Error df = 42		r^2 = 0.926 Model SS = 9.3538 Error SS = 0.7491		p = 0.0001 MS = 0.2281 Intercept ($10^β$) = 6,491	
Effect	**Partial r^2 (%)**	**p-value**	**Parameter**	**$10^β$**	**(95% CI)**
Assay method	1.22	0.0120	endpoint	0.709	(0.388, 1.296)
			kinetic[A]	1.000	—
Laboratory	5.68	0.0001	E	1.691	(0.925, 3.089)
			B	1.151	(0.630, 2.102)
			A	0.324	(0.178, 0.593)
			C	0.790	(0.433, 1.444)
			D	1.000	(0.546, 1.832)
			F	1.000	—
Experiment	39.51	0.0001	1–7	—	—
Lab × Exper	46.18	0.0001	—	—	—

[A]Selected as reference.

TABLE V. Analysis of Variance (GLM) for Swine Dust, With Replicates for Log_{10} EU/M³

N = 84 Model df = 41 Error df = 42	r^2 = 0.9505 Model SS = 11.1817 Error SS = 0.5822	p = 0.0001 MS = 0.2727 Intercept (10^β) = 33,274

Effect	Partial r^2 (%)	p-value	Parameter	10^β	(95% CI)
Assay method	14.44	0.0001	endpoint	0.214	(0.126, 0.364)
			kinetic[A]	1.000	—
Laboratory	21.16	0.0001	E	1.346	(0.791, 2.289)
			B	0.051	(0.030, 0.086)
			A	0.356	(0.209, 0.605)
			C	0.361	(0.212, 0.614)
			D	1.0000	(0.587, 1.702)
			F	1.0000	—
Experiment	16.91	0.0001	1–7	—	—
Lab × Exper	42.08	0.0001	—	—	—

[A]Selected as reference.

to four orders of magnitude) of endotoxin concentrations in typical work environments, these interlaboratory differences may be significant from a practical standpoint as well as statistically. However, the strong correlations between laboratories (Tables VII) indicate that these differences can be accounted for. The excellent intralaboratory agreement for replicate samples (Table II) also demonstrates consistent specificity in each lab's performance of its extraction and assay procedures.

In addition to this study using chicken, swine, and corn dusts, round-robin comparisons using other organic dusts (cotton, compost) recently were conducted, with some of the same laboratories participating in both this and the cotton dust project. In both phases of the cotton dust study, intralaboratory differences were small.[38,39] This finding is consistent with the current study. In Phase 1 of the cotton dust study, differences between laboratories were significant, exceeding an order of magnitude in some cases.[38,39] The somewhat smaller variability observed in this study may be due in large part to the use of standard lots of assay reagents. In Phase 2 of the cotton dust study the use of a standardized extraction protocol resulted in an important reduction in variability between labs. An attempt was made in this study to evaluate the relative contribution of variability in extraction procedures, by analyzing lyophilized extracts using a chemical method (GC-MS). The results of those analyses are presented in a separate article. In this study differences between labs using Kinetic-QCL and QCL-1000 assays were found and the order of differences depended on the dust type. Similarly, the cotton dust round-robin also found differences between labs using in-house, Kinetic-QCL, Pyrogent-5000, and QCL-1000 assays in that rank order.[38,39]

Establishment of occupational exposure limits for endotoxin depend on developing a consensus on standardized methods for sampling and analyzing endotoxin. Much work has been done evaluating the many factors, such as filter type, extraction procedures, and assay method that contribute to variability. The results of this interlaboratory study and other round-robin evaluations using different organic dusts provide data that can be used to reach this consensus. Given the potential contribution that varying lots of reagents and LPS can have on measurement error, it is important that endotoxin assay results be reported in activity units (EU) standardized to one reference endotoxin (e.g., EC6). If assay results are reported in mass units (ng), the conversion factor used should be calculated for each lot of lysate and reagents and also reported. Continued research to characterize the performance of extraction and assay solutions and of different lots and types of LAL when working with different sample matrices is needed.

TABLE VI. Analysis of Variance (GLM) for Corn Dust, With Replicates for Log_{10} EU/M³

N = 84 Model df = 41 Error df = 42	r^2 = 0.9612 Model SS = 8.3471 Error SS = 0.3377	p = 0.0001 MS = 0.2036 Intercept (10^β) = 5,169

Effect	Partial r^2 (%)	p-value	Parameter	10^β	(95% CI)
Assay method	43.16	0.0001	endpoint	0.263	(0.176, 0.394)
			kinetic[A]	1.000	—
Laboratory	34.81	0.0001	E	0.559	(0.373, 0.838)
			B	0.492	(0.329, 0.738)
			A	2.304	(1.537, 3.453)
			C	0.566	(0.377, 0.848)
			D	1.000	(0.667, 1.500)
			F	1.000	—
Experiment	13.10	0.0001	1–7	—	—
Lab × exper	5.04	0.0371	—	—	—

[A]Selected as reference.

TABLE VII. Pearson Correlation Coefficients and (Probabilities)—Log_{10} Transformed EU/m^3

Lab	Lab A	Lab B	Lab C	Lab D	Lab E	Lab F
Chicken Dust						
A	1.00	−0.21	−0.71	**−0.75**	−0.36	**−0.75**
		(0.64)	(0.07)	**(0.05)**	(0.43)	**(0.05)**
B		1.00	0.25	0.21	0.14	0.21
			(0.59)	(0.64)	(0.76	(0.64)
C			1.00	**0.96**	**0.89**	**0.96**
				(<0.01)	**(<0.01)**	**(<0.01)**
D				1.00	**0.86**	**1.00**
					(0.01)	**(<0.01)**
E					1.00	**0.86**
						(0.01)
Swine Dust						
A	1.00	0.57	−0.04	0.00	−0.64	−0.07
		(0.18)	(0.94)	(1.00)	(0.12)	(0.88)
B		1.00	−0.39	−0.5	**−0.86**	−0.71
			(0.38)	(0.25)	**(0.01)**	(0.07)
C			1.00	**0.96**	0.61	0.61
				(<0.01)	(0.15)	(0.15)
D				1.00	0.64	0.71
					(0.12)	(0.07)
E					1.00	0.54
						(0.22)
Corn Dust						
A	1.00	0.57	**0.86**	**0.79**	**0.75**	0.29
		(0.18)	**(0.01)**	**(0.04)**	**(0.05)**	(0.53)
B		1.00	0.46	**0.89**	**0.93**	**0.82**
			(0.29)	**(<0.01)**	**(<0.01)**	**(0.02)**
C			1.00	**0.75**	0.71	0.36
				(0.05)	(0.07)	(0.43)
D				1.00	**0.93**	0.64
					(<0.01)	(0.12)
E					1.00	**0.76**
						(0.04)

Note: Bold = statistically significant.

CONCLUSION

The goal of this study was to evaluate the performance of six laboratories using Limulus-based assays for analysis of organic dusts from three agricultural environments. Three laboratories used the QCL-1000 endpoint assay, and three used the Kinetic-QCL assay method. To eliminate variability among different lots, a single lot of LAL for endpoint assays and one similar lot for kinetic assays were provided. There were significant differences between laboratories for all three dust types ($p<0.01$). The pattern of differences between labs varied by dust type. For chicken dust, labs using the endpoint method reported higher results than those using kinetic methods. For swine and corn dusts, the labs using the kinetic method reported the highest endotoxin values. For chicken dust, results from all four labs were highly correlated ($r=0.85$ to 0.99). For swine dust, few correlations were found. For corn dust, four of the labs were significantly correlated. In conclusion, statistical differences in performance between laboratories were apparent and may be related to the extraction and analytical methods. The results of this study will be useful in developing a standardized sampling/analytical method for airborne endotoxin in agriculture.

ACKNOWLEDGMENTS

The authors thank the University of Iowa Hygienic Laboratory for identification of bacteria, and Marsha O'Neill and Jeanine DeKoster for assistance with dust generation and characterization. The authors also thank Steve Olenchock and Robert Woolson for their advice, and Ron Berzofsky and Bio Whittaker for providing standardized lots of LAL reagents and assay kits.

REFERENCES

1. **Castellan, R.M., S.A. Olenchock, and J.L. Hankinsen:** Acute bronchoconstriction induced by cotton dust: Dose-related responses to endotoxin and other dust factors. *Ann. Intern. Med. 101*:157–163 (1984).
2. **Castellan, R.M., S.A. Olenchock, K.B. Kinsley, and J.L. Hankinsen:** Inhaled endotoxin and decreased spirometric values, an exposure-response relation for cotton dust. *N.E. J. Med. 317*:605–610 (1987).
3. **Donham, K., P. Haglind, Y. Peterson, R. Rylander, and L. Belin:** Environmental and health studies of farm workers in Swedish swine confinement buildings. *Br. J. Ind. Med. 46*:31–37 (1989).
4. **Rylander R.:** Endotoxins in the Environment. *Prog. Clin. Biol. Res. 392*:79–90 (1995).
5. **Donnham, K.J., S.J. Reynolds, P. Whitten, J.A. Merchant, L. Burmeister, and W.J. Popendorf:** Respiratory dysfunction in swine production facility workers: dose-response relationships of environmental exposures and pulmonary function. *Am. J. Ind. Med. 27*:405–418 (1995).
6. **Haglind, P., and R. Rylander:** Exposure to cotton dust in an experimental card room. *Br. J. Ind. Med. 41*:340–345 (1984).
7. **Heederik, D., R. Brouwer, K. Biersteker, and J.S.M. Boleij:** Relationship of airborne endotoxin and bacteria levels in pig farms with the lung function and respiratory symptoms of farmers. *Int. Arch. Occup. Environ. Health 62*:595–601 (1991).
8. **Jacobs, R.R.:** Airborne endotoxins: An association with occupational lung disease. *Appl. Ind. Hyg. 4*:50–55 (1989).
9. **Olenchock, S.A.:** Health effects of biological agents: The role of endotoxins. *Appl. Occup. Environ. Hyg. 9*:62–64 (1994).
10. **Thorne, P.S., J.A. DeKoster, and P. Subramanian:** Pulmonary effects of machining fluids in guinea pigs and mice. *Am. Ind. Hyg. Assoc. J. 57*:1168–1172 (1996).
11. **Reynolds, S.J., K.J. Donham, P. Whitten, J.A. Merchant, L.E. Burmeister, and W.J. Popendorf:** Longitudinal evaluation of dose response relationships for environmental exposure and pulmonary function in swine production workers. *Am. J. Ind. Med. 29*:33–40 (1996).
12. **Reynolds, S.J., D. Parker, D. Vesley, D. Smith, and R. Woellner:** Cross-sectional epidemiological study of respiratory disease in turkey farmers. *Am. J. Ind. Med. 24*:713–722 (1993).
13. **Rylander, R., and R. Burrell:** Endotoxins in inhalation research: Summary of conclusions of a workshop held at Clearwater, Florida, USA. 28–30 Sept.1987. *Ann. Occup. Hyg. 32*:553–56 (1987).
14. **Schwartz, D.A., P.S. Thorne, S.J. Yagla, L.F. Burmeister, and S.A. Olenchock:** The role of endotoxin in grain dust-induced lung disease. *Am. J. Resp. Crit. Care Med. 152*:603–608 (1995).
15. **Smid, T., D. Heederik, R. Houba, and P.H. Quanjer:** Dust and endotoxin-related respiratory effects in the animal feed industry. *Am. Rev. Resp. Dis. 146*:1474–1479 (1992).
16. **Zejda, J.E., E. Barber, and J.A. Dosman:** Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. *JOM 36*:49–56 (1994).
17. **Helander, I.:** Pulmonary toxicity of endotoxins: Comparison of lipopolysaccharides from various bacterial species. *Infect. Immun. 35*: 528–532 (1982).
18. **Von Essen, S.G., R.A. Robbins, A.B. Thompson, R.F. Ertl, J. Linder, and S. Rennard:** Mechanisms of neutrophil recruitment to the lung by grain dust exposure. *Am. Rev. Respir. Dis. 138*:921–927 (1988).

19. **Dutkiewicz, J., S.A. Olenchock, and W.G. Sorenson:** Levels of bacteria, fungi, and endotoxin in bulk and aerosolized corn silage. *Appl. Environ. Microbiol. 55*:1093–1099 (1989).
20. **Laitinen, S., A. Nevalainen, M. Kotimaa, J. Liesivuori, and P. Martikainen:** Relationship between bacterial counts and endotoxin concentration in the air of wastewater treatment plants. *Appl. Envrion. Micorbiol. 58*:3774–3776 (1992).
21. **Lenharts, S.W., P.D. Morris, R.E. Akin, S.A. Olenchock, W.S. Service, and W.P. Boone:** Organic dust, endotoxin, and ammonia exposures in the North Carolina poultry processing industry. *Appl. Occup. Environ. Hyg. 5*:611–618 (1990).
22. **Palchak, R. B., R. Cohen, and M. Ainslic:** Airborne endotoxin associated with industrial scale production of protein products in gram-negative bacteria. *Am. Ind. Hyg. Assoc. J. 49*:420–421 (1988).
23. **Sigsgaard, T., P. Malmros, L. Niersting, and C. Petersen:** Respiratory disorders and atopy in Danish refuse workers. *Am. J. Resp. Crit. Care Med. 149*:1407–1412 (1994).
24. **Rask-Anderson, A., P. Malmberg, and M. Lundholm:** Endotoxin levels in farming: absence of symptoms despite high exposure levels. *Br. J. Ind. Med. 46*:412–416 (1989).
25. **Douwes, J., and D. Heederik:** *Endotoxins: Health Based Recommended Exposure Limit. A report of the Health Council of the Netherlands: Dutch Expert Committee on Occupational Standards (DECOS)* (Publication no.1998/03WGD). Rijswijk: Health Council of the Netherlands, 1998.
26. **Milton, D.K., R.J. Gere, H.A. Feldman, and I.A. Greaves:** Endotoxin measurement: Aerosol sampling and application of a new Limulus method. *Am. Ind. Hyg. Assoc. J. 51*:331–337 (1990).
27. **Milton, D.K., H.A. Feldman, D.S. Neuberg, R.J. Bruckner, and I.A. Greaves:** Environmental endotoxin measurements: The kinetic Limulus assay with resistant-parallel-line estimation. *Environ. Res. 57*: 212–230 (1992).
28. **Sonesson, A., L. Larsson, and A. Schultz:** Comparison of the Limulus amebocyte lysate test and gas chromatography—mass spectrometry for measuring lipopolysaccharides (endotoxins) in airborne dust from poultry-processing industries. *Appl. Environ. Microbiol. 56*: 1271–1278 (1990).
29. **Walters, M., D. Milton, L. Larson, and T. Ford:** Airborne environmental endotoxin: A cross-validation of sampling and analysis techniques. *Appl. Environ. Microbiol. 60*:996–1005 (1994.).
30. **Mielniczuk, Z., E. Mielniczuk, and L. Larsson:** Gas chromatography-mass spectrometry methods for analysis of 2- and 3- hydroxylated fatty acids: Application for endotoxin measurement. *J. Microbiol. Meth. 17*:91–102 (1993).
31. **Douwes, J., P. Versloot, A. Hollander, D. Heedrick, and G. Doekes:** Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. *Appl. Environ. Microbiol. 61*: 1763–1769 (1995).
32. **Hollander, A., D. Heedrick, P. Versloot, and J. Douwes:** Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. *Am. Ind. Hyg. Assoc. J. 54*:647–653 (1993).
33. **Case, M.J., and T.J. Novitsky:** Comparison of several endotoxin standards with a kinetic LAL test. In *Endotoxin and Their Detection With Limulus Amebocyte Lysate Test, Progress in Clinical and Biological Research,* vol. 93, pp. 161–171. New York: Liss, 1982.
34. **Gordon, T., K. Galdanes, and L. Brosseau:** Comparison of sampling media for endotoxin-contaminated aerosols. *Appl. Occup. Envrion. Hyg. 7*:472–477 (1992).
35. **Olenchock, S.A., D.M. Lewis, and J.C. Mull:** Effects of different extraction protocols on endotoxin analyses of airborne grain dusts. *Scand. J. Work. Environ. Health 15*:430–435 (1989).
36. **Reynolds, S. J., and D.K. Milton:** Comparison of methods for analysis of airborne endotoxin. *App. Environ. Occup. Hyg. 8*:761–767 (1993).
37. **Thorne, P.S., S.J. Reynolds, D.K. Milton, et al.:** Field evaluation of endotoxin air sampling assay methods. *Am. Ind. Hyg. Assoc. J. 58*: 792–799 (1997).
38. **Chun, D.T., V. Chew, K. Bartlett, et al.:** A quick summary of the highlights of parts 1 and 2 of the first round-robin endotoxin assay study and a brief preview of the next round. In P. Dugger and D.A. Richter, editorial coordinators, *1999 Proceedings of the Beltwide Cotton Conferences.* Memphis, Tenn.: National Cotton Council of America, 1999.
39. **Chun, D.T., V. Chew, K. Bartlett, et al.:** Preliminary report on the results of the first phase of a round robin endotoxin assay study using cotton dust. *Appl. Occup. Environ. Hyg. 15*:152–7 (2000).
40. **Elin, R.J., and S.M. Wolff:** Nonspecificity of the Limulus amebocyte lysate test: Positive reactions with polynucleotides and proteins. *J. Infect. Dis. 128*:349–352 (1973).
41. **Saraf, A., L. Larsson, H. Burge, and D. Milton:** Quantification of ergosterol and 3-hydroxyfatty acids in settled house dust by gas chromatography-mass spectrometry: Comparison with fungal culture and determination of endotoxin by a Limulus amebocyte lysate assay. *Appl. Environ. Microbiol. 63*:2554–2559 (1997).
42. **Campbell, M.C., and J.L. Stewart:** The Medical Mycology Handbook. New York: John Wiley & Sons, 1980.
43. **Weyel, D.A., M. Ellakkani, Y. Alarie, and M. Karol:** An aerosol generator for the resuspension of cotton dust. *Toxicol. Appl. Pharmacol. 76*:544–547 (1984).
44. **Thorne, P.S.:** Experimental grain dust atmospheres generated by wet and dry aerosolization techniques. *Am. J. Ind. Med. 25*:109–112 (1994).
45. **Reynolds, S.J., L. Etre, P.S. Thorne, P. Whitten, M. Selim, and W. Popendorf:** Laboratory comparison of vacuum, OSHA, and HUD sampling methods for lead in household dust. *Am. Ind. Hyg. Assoc. J. 58*:439–446 (1997).
46. **Lange, J.L., P.S. Thorne, and N. Lynch:** Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria. *Appl. Environ. Microbiol. 63*:1557–1563 (1997).