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Evaluation of the *Limulus* Amebocyte Lysate and Recombinant Factor C Assays for Assessment of Airborne Endotoxin▽†

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As a potent inflammatory agent, endotoxin is a key analyte of interest for studies of lung ailments in domestic environments and occupational settings with organic dust. A relatively unexplored advance in endotoxin exposure assessment is the use of recombinant factor C (rFC) from the *Limulus* pathway in a fluorometric assay. In this study, we compared airborne endotoxin concentrations in laboratory- and field-collected parallel air samples using the kinetic *Limulus* amebocyte lysate (LAL) assay and the rFC assay. Air sampling was performed using paired Institute of Occupational Medicine (IOM) samplers, Button samplers, closed-face cassettes, and cyclone samplers. Field sampling was performed in 10 livestock production facilities, including those housing swine, chicken, turkey, dairy cows, cattle, and horses. Laboratory sampling was performed in exposure chambers using resuspended airborne dust collected in five livestock facilities. Paired samples were extracted in pyrogen-free water with 0.05% Tween 20 and analyzed using LAL and rFC assays. In 402 field sample pairs there was excellent agreement between endotoxin concentrations determined by LAL and rFC ($r = 0.93$; $P < 0.0001$). In 510 laboratory sample pairs there was also excellent agreement between the two assays ($r = 0.86$; $P < 0.0001$). Correlations for subgroups of facility or dust type ranged from 0.65 to 0.96. Mixed-model analysis of variance (ANOVA) for the field studies showed significant interactions of facility-sampler and facility-assay. rFC/LAL ratios of the geometric means were 0.9 to 1.14 for the samplers (not significantly different from 1.0). The data from this study demonstrate that the LAL assay and the rFC assay return similar estimates of exposure in livestock facilities. Both methods provided suitable lower limits of detection such that all but 19 of 1,824 samples were quantifiable.

Endotoxin, or lipopolysaccharide, is a pathogen-associated molecular pattern of Gram-negative bacteria that associates with MD-2 (lymphocyte antigen 96) to act as a ligand for Toll-like receptor 4 (10). Through this process, inhaled endotoxin induces lung inflammation and can both increase neutrophilic asthma and decrease allergy and atopic asthma (2, 8, 9, 17, 26, 33). Endotoxin is an inflammatory component of most organic dusts (6). Exposure to endotoxins in agricultural dusts, including swine, poultry, and grain, has been associated with asthma, chronic bronchitis, organic dust toxic syndrome (toxic pneumonitis), chronic obstructive pulmonary disease, and declines in pulmonary function (5, 18, 24, 25, 35, 36, 38). High endotoxin exposure also occurs in municipal composting (37), seed and bulb handling (27), and wastewater treatment plants (13, 28), to name a few. In addition to occupational environments, endotoxin exposure in domestic environments is a risk factor for asthma (33), with high occupancy, poverty, pets, pests, and household cleanliness being the major predictors of exposure (32).

The kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay is the most widely used assay for endotoxin measurement for environmental samples (6). This assay uses an endotoxin-triggered enzyme cascade from the Atlantic horseshoe crab (*Limulus polyphemus*) to cleave a colorimetric substrate. Although the LAL assay is exquisitely sensitive, variability can arise from interlot variations in the *Limulus* lysate and differences in laboratory methods for sample collection, sample handling and storage, sample extraction, and sample analysis (7, 12, 14, 15, 19, 21, 29, 30, 34). In addition, some implementations of the LAL assay may experience interference from other molecules, such as fungal (1→3)-β-D-glucans (3, 22). A recombinant factor C (rFC) assay that uses rFC reagent produced from the cDNA of the Mangrove horseshoe crab (*Cacinoscorpius rotundicauda*) was recently developed (4). Since the rFC assay uses a recombinant reagent, the reactivity to endotoxins should vary less between lots than for the *Limulus* lysate.

The goal of this study was to determine the comparability, across a wide range of endotoxin levels, of the kinetic chromogenic LAL and the fluorometric rFC assays for assessing airborne endotoxin. To accomplish this, we performed air sampling in 10 livestock environments and in chamber studies using resuspended dust collected from these livestock environments. Livestock environments are recognized as containing considerable amounts of airborne endotoxin arising from a variety of Gram-negative bacteria. Eight samples were collected simultaneously on a rotating mannequin using pairs of four types of commonly used samplers: two for inhalable dust (Institute of Occupational Medicine [IOM] and Button), one for total dust (closed-face cassette), and one for respirable dust (SKC aluminum cyclone). This resulted in 912 pairs of samples (total $n = 1{,}824$).

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MATERIALS AND METHODS

Collection of paired samples. Field samples (n = 402 pairs) were collected at 10 agricultural environments in Iowa and Colorado (two swine operations, two chicken houses, two turkey houses, two dairy barns, one cattle feedlot, and one horse stable) (16, 20). Samplers were centered within the buildings away from walls to the extent possible. In some cases, the locations of animals required setting the sampling station in an aisle or toward the end of the building. Sham personal samples were collected using eight commonly used personal samplers attached to vests and mounted on rotating mannequins (1 revolution/h): two IOM (Institute of Occupational Medicine, United Kingdom) inhalable dust samplers, two Button inhalable dust samplers, two 37-mm closed-face total dust cassettes, and two aluminum respirable dust cyclones (SKC Inc., Eighty Four, PA). Each collection of the set of eight samples constituted a trial. Sampler placement on the mannequin was varied systematically between trials. Preliminary studies with eight IOM samplers affixed to the mannequin showed there was less than a 10% variation among sampler locations. Excluding the closed-face cassettes, the selected samplers are designed to mimic accepted inhalable and respirable aerosol size deposition profiles for the human pulmonary system. Flow rates of 2.0, 4.0, 2.0, and 2.5 liters/min were set to within 5% for the IOM, Button, cassette, and cyclone samplers, respectively, and were calibrated before each trial with a Gilibrator flow meter (Sensidyne, Clearwater, FL). Sampling times ranged from 8 to 12 h. Polyvinyl chloride filters with a 5-μm pore size were pre- and postweighed using a microbalance (model MT5; Mettler-Toledo Inc., Columbus, OH) following equilibration to the gravimetrics lab conditions.

Settled dust was collected from five field sampling sites (swine, chicken, turkey, cattle feedlot, and horse) for generation of airborne dust in exposure chambers to evaluate assay performance under controlled conditions. Each dust sample was collected into a sterile HEPA sock fit into the nozzle of a vacuum cleaner hose with an attached clean crevice tool. Settled dust was vacuumed from horizontal surfaces such as railings and rafters. The dust was transferred on ice to the laboratory and then sieved (420 μm), dried in a desiccator, and stored at 4°C in sterile, pyrogen-free WhirlPak bags.

Laboratory samples (n = 1,020) were collected on mannequins in exposure chambers in the same fashion as described for field samples. Dust atmospheres were generated from sieved dust collected in the field sites using either a Wright dust feed operated at 12 liters/min and 0.6 rpm (BGI, Inc., Waltham, MA) or an NBS dust feeder operated at 50 liters/min (11). Exposure chambers included a 1-m^3 Hazleton-type vertical chamber (31) operated under negligible flow (<0.1 m/s) with injection from the Wright dust feed from above and a slight exhaust flow from below (settling) and a horizontal wind tunnel operated at 0.2-m/s and 1.0-m/s wind velocities with horizontal addition from the NBS dust feeder (flowing). Sampling times in the vertical chamber ranged from 3.5 to 4.0 h, while sampling in the wind tunnel lasted for 20 to 25 min.

Both the field and laboratory samplings followed written protocols, and samples were tracked with chain-of-custody forms and bar-coded labels. The sampling scheme is illustrated in Fig. 1. Between 9 and 11 trials were conducted, each with eight samplers of four types, for each of the 10 facilities in the field study. This generated a total of 402 sample pairs. In the laboratory study, 8 to 10 trials were conducted under each flow condition, each also with eight samplers, for each of the five dust types. This generated a total of 510 sample pairs. Thus, 912 matched pairs were available, with one sample quantified using the LAL assay and the other using the rFC assay.

Endotoxin analysis. In both the rFC and LAL assays, collected air samples were extracted in 10 ml certified pyrogen-free (PF) water (Lonza, Inc., Walkersville, MD) containing 0.05% Tween 20 for 1 h at 22°C with continuous shaking. Endotoxin concentrations in samples were calculated according to the standard curve. Four assay reagent blank wells served as reference and control for the PF status of the reagent water, centrifuge tubes, pipette tips, and microplates. Quality assurance spiking assays were performed to assess matrix interference or enhancement (31). Mean field blank values for field and laboratory studies are shown in Table S1 in the supplemental material. They illustrate that mean blank values were all less than 0.8% of mean sample values. Therefore, sample values were not blank corrected.

For the kinetic chromogenic LAL assay, extracts were centrifuged (15 min at 600 × g), and supernatants were transferred into PF cryotubes. Twofold serial dilutions of endotoxin standards (*Escherichia coli* O111:B4; Lonza, Inc.) and sample extracts were prepared using sterile, PF water with Tween 20 in borosilicate glass tubes that had been heated for 4 h at 200°C to remove endotoxin activity. A 12-point calibration curve and a 4-point endotoxin determination of samples were performed. Samples were assayed at dilution levels that were selected based upon the typical endotoxin content of the environment and the loading of the filters and ranged from 1:20 to 1:5,120. Higher dilutions were needed for total and inhalable dust samples from swine, chicken, and turkey barns, while lower dilutions were used for the respirable dust samples and dairy barns. The selected dilutions used varied depending upon the field site or dust type. The standard curve ranged from 0.025 to 50 endotoxin units (EU)/ml of standard endotoxin (r^2 > 0.995). Aliquots (100 μl) of the serial dilutions of endotoxin standards and extracts were pipetted into a PF polystyrene microplate and assayed via the addition of the LAL reagent and substrate. The absorbance in each well was measured at 405 nm every 30 s for 90 min (SpectraMax 340; Molecular Devices, Inc., Sunnyvale, CA). Endotoxin determinations were based upon the maximum slope of the absorbance-versus-time plot for each well. The endotoxin value for a sample was calculated from the arithmetic mean of those dilutions that fell in the middle two-thirds of the standard curve. By design, five different lots of *Limulus* amebocyte lysate (Lonza, Inc.) were used for the 912 samples.

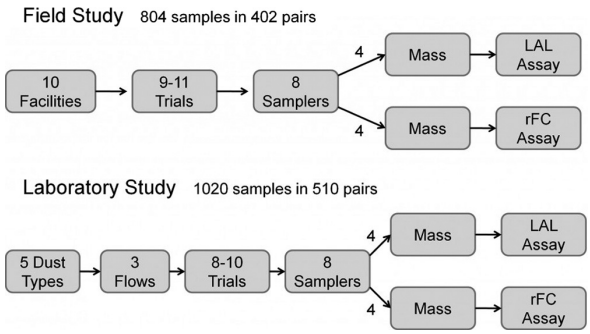

FIG. 1. Experimental design for the field and laboratory phases of the study, which generated 912 pairs of samples. Field studies were conducted in 10 facilities: two swine operations, two chicken houses, two turkey houses, two dairy operations, a cattle feedlot, and a horse stable. Laboratory studies were conducted using particulate matter collected from the swine, chicken, turkey, cattle, and horse field sites and resuspended in exposure chambers under well-controlled conditions. Chamber studies were conducted with near-stagnant air and settling dust or with air flowing horizontally at 0.2 or 1.0 m/s. Samplers used for both field and laboratory studies were the IOM, Button, cassette, and cyclone samplers. Settling-air studies used swine, chicken, and turkey dust, while horizontal-airflow studies used these three plus cattle feedlot and horse dust.

For the rFC assay, extracted samples were analyzed as previously described (23) according to the manufacturer's instructions (Lonza, Inc.). Twofold serial dilutions of endotoxin standards (*Escherichia coli* O55:B5; Lonza, Inc.) and sample extracts were prepared using sterile, PF water with Tween 20. The samples were added to a 96-well plate, followed by 100 μl of a mixture of enzyme, buffer, and fluorogenic substrate (Lonza, Inc.). The plates were incubated at 37°C for 1 h and read in a fluorescence microplate reader (FLX800TBIE; Biotek Instruments, Winooski, VT) at excitation and emission wavelengths of 380 and 440 nm, respectively. Background fluorescence was subtracted, and log change in fluorescence was plotted against log endotoxin concentration over the range of 0.01 to 10 EU/ml (r^2 > 0.995). The endotoxin value for a sample was calculated from the arithmetic mean of those dilutions that fell in the middle of the standard curve. Seven different lots of rFC were used for the analyses.

Statistical analysis. Dust and endotoxin concentration data were log-normally distributed. Thus, the geometric mean (GM) and geometric standard deviation (GSD) were used to express central tendency and variability. Samples with endotoxin or dust concentrations below the limit of detection (LOD) were assigned the value LOD ÷ $\sqrt{2}$. This was the case for only 4 of 912 rFC and 2 of 912 LAL dust concentrations (mg/m^3) and for only 6 of 912 rFC and 13 of 912 LAL endotoxin concentrations (EU/m^3). All statistical analyses were performed using SAS version 9.2 (SAS, Inc., Cary, NC). Agreement between paired samples assayed by the two methods was assessed by testing the significance of the Pearson correlation coefficient on log-transformed values. P values of less than 0.05 were considered significant, and those of less than 0.01 were considered highly significant. Analysis of variance (ANOVA) using mixed models was performed with trial treated as a random effect to assess differences by assay type, sampling device, and sampling location (field study) or dust type (laboratory study) and to assess interactions between these main effects. Reduced models

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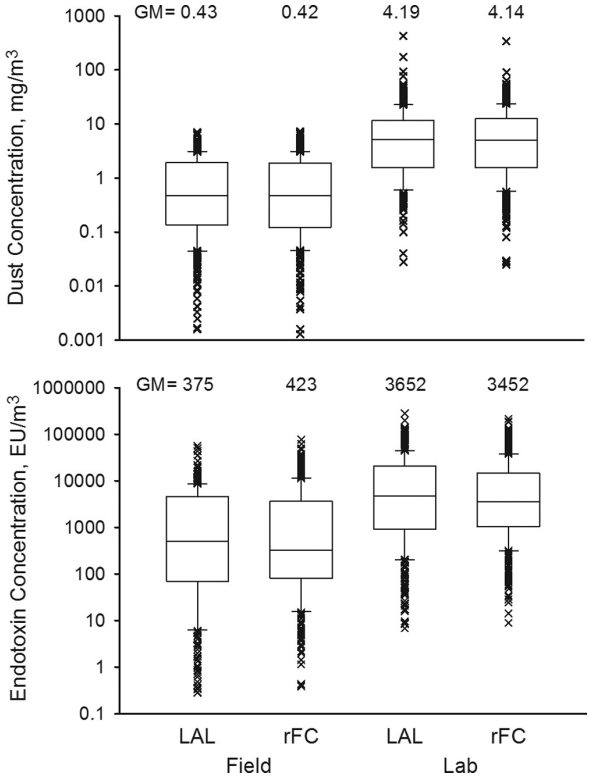

FIG. 2. Box plot graphs of all data, including dust concentration (mg/m^3) for air samples collected in the field and laboratory studies and submitted for LAL or rFC analysis of endotoxin (top) and field and laboratory study endotoxin concentration (EU/m^3) determined by LAL or rFC assay (bottom). Box plots indicate the median (center line), the 25th and 75th percentiles (box), the 10th and 90th percentiles (error bars), and extreme values (×). Listed above each box is the geometric mean (GM). All data are shown, including the cyclone data and data that fell below the limit of detection.

were developed based on initial full models that included all possible interaction terms. Highest-order interaction terms with a P value of ≥0.1 were dropped in each successive model reduction. Thus, final models included only primary variables and interaction terms with P values of <0.1. To evaluate most remaining interactions, interaction plots were created by graphing the proportional difference between modeled endotoxin values as a function of the interacting variable where the proportional difference has the following form: assay proportional difference = (log rFC endotoxin − log LAL endotoxin)/[1/2(log rFC endotoxin + log LAL endotoxin)]; airflow proportional difference = [log (0.2 m/s endotoxin) − log (1.0 m/s endotoxin)]/{1/2[log (0.2 m/s endotoxin) + log (1.0 m/s endotoxin)]}. Creation of this dimensionless parameter allowed us to consider the magnitude of the effect as a proportion of the overall geometric mean.

RESULTS

The livestock environments sampled in the field study displayed a wide range of dust and endotoxin concentrations with which to compare the LAL and rFC assays. These environments also ranged from hot and dry conditions (e.g., a horse stable in Colorado) to cooler, damp conditions (e.g., a swine confinement facility in Iowa). The medians and 10 to 90% ranges of dust concentration values are shown in the box plots in Fig. 2, top, for the field samples and the laboratory samples collected for endotoxin assay by the two methods. The GM concentration for the field samples used for LAL assessment was 0.43 mg/m^3 (GSD, 5.5), while the GM for the rFC assessment filters was 0.42 mg/m^3 (GSD, 5.4). Laboratory chamber studies using dust collected from field sites yielded dust concentrations that were 10-fold higher, with GMs of 4.19 (GSD, 4.1) and 4.14 (GSD, 4.3) mg/m^3 for the LAL and rFC samples, respectively. GM endotoxin concentrations were 375 and 423 EU/m^3 for the field LAL and rFC samples and 3,652 and 3,452 EU/m^3 for the LAL and rFC laboratory samples (Fig. 2, bottom). The 10th to 90th percentile field endotoxin values spanned 3 orders of magnitude, providing a wide range of values for this method comparison study.

Table 1 lists the endotoxin correlation coefficients by facility type and dust type for field and laboratory data. The assays were well correlated for field samples from most of the livestock facilities studied, with correlations ranging from 0.69 to 0.96 ($P < 0.0001$ for all), despite the lower number and narrower range of values for these subgroups compared with aggregated data. The correlations between the LAL and rFC assays by facility and dust type were higher in the field study than in the laboratory study. As seen from the endotoxin correlation coefficients by sampler type, the LAL assay and the rFC assay were more highly correlated for field sampling than for the laboratory studies (Table 2). Correlation coefficients ranged from 0.91 to 0.93 for the field study and from 0.76 to 0.88 for the laboratory study. In addition, the correlation coefficients were similar among the four samplers, and the assays showed reasonable agreement for inhalable dust, total dust, and respirable dust fractions.

Initial ANOVA mixed-model analyses of the field study data found significant second-order interactions for sampler, facility, and assay. This interaction appeared to be an artifact due to the majority of field cyclone values having very small

TABLE 1. Pearson correlation between LAL assay and rFC assay endotoxin concentration (EU/m^3) for field and laboratory studies with all data included

Study	Facility or dust type	No. of pairs	Correlation[a]
Field	Swine		
	IA	39	0.71
	CO	40	0.86
	Chicken		
	IA	44	0.69
	CO	40	0.96
	Turkey		
	IA	36	0.82
	CO	40	0.88
	Dairy		
	IA	44	0.95
	CO	39	0.85
	Cattle feedlot, CO	40	0.92
	Horse, CO	40	0.81
Laboratory	Swine	119	0.65
	Chicken	116	0.90
	Turkey	116	0.79
	Dairy		
	Cattle feedlot	80	0.79
	Horse	79	0.75

[a] All were highly significant, with P values of less than 0.0001.

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TABLE 2. Pearson correlation coefficients by sampler type for LAL endotoxin versus rFC endotoxin (EU/m^3) for paired samples with all data included

Sampler	Field study		Laboratory study	
	No. of pairs	Correlation[a]	No. of pairs	Correlation[a]
Overall	402	0.93	510	0.86
Inhalable dust				
IOM	101	0.91	129	0.78
Button	98	0.91	127	0.82
Total dust (cassette)	101	0.93	125	0.88
Respirable dust (cyclone)	102	0.93	129	0.76

[a] All were highly significant, with P values of less than 0.0001.

amounts of sampled dust, resulting in endotoxin values that were at or below the lower limit of detection. As a consequence, we excluded the cyclone data from subsequent ANOVA as well as from the scatter plots in Fig. 3 and Fig. 4.

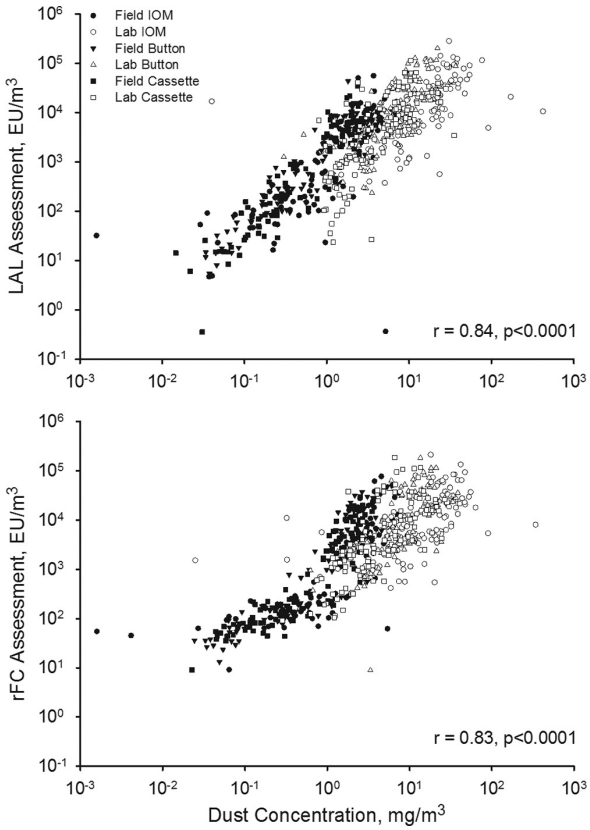

FIG. 3. Relationships between sampled dust concentration and endotoxin concentration as determined using the LAL assay or the rFC assay for the IOM, Button, and cassette samplers (cyclone data not shown). (Top) LAL endotoxin versus dust concentration. Regression equation: log(LAL endotoxin concentration) = 2.97 + 1.13 log(dust concentration). (Bottom) rFC endotoxin versus dust concentration. Regression equation: log(rFC endotoxin concentration) = 2.98 + 1.02 log(dust concentration).

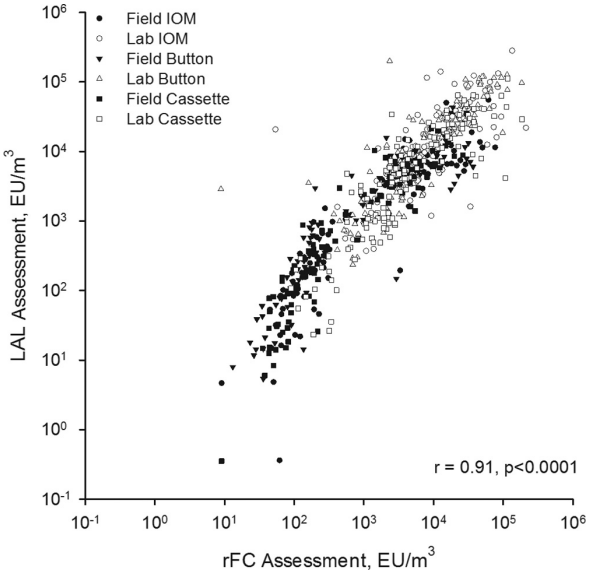

FIG. 4. Comparison of airborne endotoxin concentrations for paired samples analyzed using the LAL assay and the rFC assay for the IOM, Button, and cassette samplers. Regression equation: log(LAL endotoxin concentration) = −0.117 + 0.979 log(rFC endotoxin concentration).

However, plots including the cyclone data can be found in Fig. S1 and S2 in the supplemental material. The relationships between the dust and endotoxin concentrations for the field data (filled symbols) and laboratory data (open symbols) are shown in Fig. 3, top, for the LAL assay and in Fig. 3, bottom, for the rFC assay. These plots illustrate a high degree of correlation between the dust concentration and both the LAL assay and the rFC assay, with Pearson correlation coefficients of 0.84 and 0.83, respectively ($P < 0.0001$ for each). Figure 4 shows the relationship between the paired samples analyzed using the LAL assay and the rFC assay. The correlation coefficient of 0.91 for this relationship was highly significant ($P < 0.0001$). As seen from Fig. 4, this relationship held for both the field and laboratory samplings (filled and open symbols, respectively) and for total and inhalable dust fractions as indicated by the differently shaped symbols. The regression equation for these data, provided in the figure legend, indicates a slope (± standard error) of 0.979 ± 0.017. Five of the 912 data points deviated from the line of identity; three had higher LAL values, and two had higher rFC values.

Variance testing was performed using mixed models separately for the field and laboratory endotoxin concentration data. Model results for the field study are summarized in Tables 3 and 4. Significant interactions were observed for facility and sampler ($P = 0.0002$) and facility and assay ($P < 0.0001$); hence, main effects were difficult to interpret. Figure 5, which illustrates the first-order interaction of facility and assay, shows that for most facilities the difference between the two assays is small. However, there was a larger assay effect for the Colorado chicken, dairy, cattle feedlot, and horse facilities, for which proportional differences exceeded 0.1 (>10% from zero). Table 4 lists the parameter estimates of the modeled

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TABLE 3. Mixed-model ANOVA results for log-transformed endotoxin concentration (EU/m^3) from field studies, demonstrating relationships between parameters in the model (total of 300 pairs of samples)[a]

Effect	df		F	P value
	Numerator	Denominator		
Facility	9	82	160	<0.0001
Sampler	2	466	36.4	<0.0001
Assay	1	466	0.40	0.528
Assay-sampler	2	466	2.33	0.098
Facility-sampler	18	466	2.75	0.0002
Assay-facility	9	466	13.1	<0.0001

[a] Akaike information criterion = 279.1.

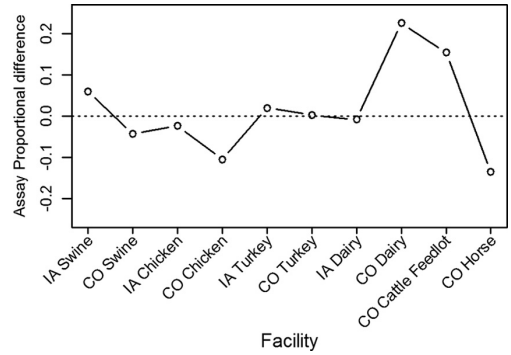

FIG. 5. Interaction plot for facility and assay, showing the proportional difference between assays for each facility visited in the field study. The formula for calculation of proportional difference is defined in Materials and Methods. Points above the zero line indicate rFC > LAL. Four of the 10 sampling sites demonstrated differences exceeding 10%, and one exceeded 20%. Connecting lines were added for clarity but do not signify a relationship between points.

GM, along with 95% confidence intervals (CI), for the rFC and LAL analyses and their ratio (rFC/LAL). The magnitudes of the differences in GM appear to be large because these were back transformed from the log domain. The rFC/LAL ratios in Table 4 show 14% higher and 6% higher endotoxin determinations for samples collected using the IOM or cassette, respectively, but 10% lower endotoxin determinations for the Button sampler. None of these rFC/LAL ratios were significantly different than 1.0. Assay ratios by facility ranged from 0.47 to 2.10. Five of the six ratios that were significant were for livestock facilities in Colorado.

Mixed-model ANOVA for the laboratory study was conducted separately for the settling chamber work done at the University of Iowa (Tables 5 and 6) and the wind tunnel studies done at Colorado State University (Table 7). For near-stagnant air conditions and settling dust, the interactions between assay and dust, as well as between dust and sampler, were nonsignificant ($P > 0.10$) in the initial ANOVA and thus were left out of the final model. Significant determinants of endotoxin concentration were the dust type ($P < 0.0001$), the sampler type ($P < 0.0001$), and the assay ($P = 0.009$); the interaction of sampler and assay, as depicted graphically in Fig. 6, was not significant ($P = 0.054$). For these samples, the LAL assay yielded endotoxin estimates that were 32% higher than those from the rFC assay ($P = 0.0105$). Modeled parameter estimates (Table 6) indicate that there was a nonsignificant 9% difference between the LAL and rFC determinations for the IOM sampler, whereas endotoxin determinations from the Button and cassette samplers were significantly different, with a greater value obtained with the LAL.

The results of the statistical analysis of the wind tunnel data were more complex than those for the stagnant sampling data in that there were more significant interaction terms, thereby making interpretation of main effects difficult. First-order interactions and two of the second-order interactions for dust type, airflow rate, sampler, and assay were significant (Table 7). Modeled parameter estimates for this ANOVA are pro-

TABLE 4. Modeled parameter estimates for the results shown in Table 3

Main effect	rFC		LAL		GM ratio (rFC/LAL)	P value
	Modeled GM	95% CI	Modeled GM	95% CI		
Sampler						
IOM	1,111	960, 1,300	978	840, 1,100	1.14	0.101
Button	741	640, 860	822	710, 950	0.90	0.187
Cassette	676	580, 780	636	550, 740	1.06	0.422
Facility						
Swine						
IA	12,452	8,500, 18,000	7,223	4,900, 11,000	1.72	<0.001
CO	2,197	1,500, 3,200	3,066	2,100, 4,500	0.72	0.019
Chicken						
IA	3,356	2,300, 4,800	4,079	2,800, 5,800	0.82	0.150
CO	175	120, 260	310	210, 450	0.57	<0.001
Turkey						
IA	17,150	12,000, 25,000	14,134	9,600, 21,000	1.21	0.205
CO	5559	3,800, 8,100	5,448	3,700, 7,900	1.02	0.887
Dairy						
IA	98.5	69, 140	102.2	71, 150	0.96	0.784
CO	39.6	27, 58	18.8	13, 27	2.10	<0.001
Cattle feedlot, CO	118.8	82, 170	60.1	41, 88	1.98	<0.001
Horse, CO	199.8	140, 290	428.7	290, 620	0.47	<0.001

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TABLE 5. Mixed-model ANOVA results for laboratory studies in settling air (<0.1 m/s) for log-transformed endotoxin concentration (EU/m^3), demonstrating relationships between parameters in the model[a]

Effect	df		F	P value
	Numerator	Denominator		
Dust	2	27	61.3	<0.0001
Sampler	2	143	18.6	<0.0001
Assay	1	143	6.99	0.009
Assay-sampler	2	143	2.98	0.054

[a] Akaike information criterion = 139.1.

TABLE 7. Mixed-model ANOVA results for the laboratory studies in the wind tunnel (horizontal air flow at 0.2 or 1.0 m/s) for log-transformed endotoxin concentration (EU/m^3), demonstrating relationships between parameters in the model[a]

Effect	dF		F	P value
	Numerator	Denominator		
Dust	4	90	242.1	<0.0001
Airflow	1	90	8.83	0.0038
Sampler	2	444	163.8	<0.0001
Assay	1	444	14.5	0.0002
Dust-airflow	4	90	7.91	<0.0001
Dust-sampler	8	444	12.85	<0.0001
Dust-assay	4	444	7.33	<0.0001
Airflow-sampler	2	444	4.70	0.009
Airflow-assay	1	444	5.78	0.017
Sampler-assay	2	444	15.1	<0.0001
Dust-airflow-sampler	8	444	2.08	0.036
Dust-airflow-assay	4	444	2.61	0.035
Dust-sampler-assay	8	444	1.73	0.088

[a] Akaike information criterion = 287.7.

vided in Table S2 in the supplemental material. Proportional differences for the two assays are shown in Fig. 7 for the interactions of dust type, sampler, and airflow (top) and assay, dust, and airflow (bottom). These suggest that the primary driver for the significance of these interactions may have been the experiments using the dust from the cattle feedlot. When the mixed-model ANOVA was rerun with the cattle feedlot dust experiment removed (data not shown), we found that the interactions of dust, airflow, and assay as well as dust, sampler, and assay were eliminated from the model. The interaction of dust, airflow, and sampler was still significant ($P = 0.029$).

Our study was not designed to assess the potential role of LAL or rFC lot variation in the determination of endotoxin. However, there were seven lots of rFC and six lots of *Limulus* lysate used. Because the sample analyses were not randomly distributed across lots, we could not analyze lot as a random effect in a mixed-model ANOVA. To explore a possible effect of lot, we ran a simple ANOVA model replacing the variable "assay" with "lot." This change resulted in only a small change in the model parameters. For the field study, the adjusted r^2 increased from 0.891 to 0.906, and for the laboratory study, the adjusted r^2 increased from 0.774 to 0.787. Thus, it appears that little of the assay variance was attributable to lot-to-lot variation. In addition, the Pearson correlation coefficient relating LAL-assessed values to rFC-assessed values of 0.92, despite the use of multiple lots of lysate and rFC, suggests this was not a large source of variance.

DISCUSSION

The kinetic chromogenic LAL assay has emerged as the principal assay used for the quantitation of endotoxin in epidemiologic and exposure assessment studies in occupational settings and domestic settings and for studies of ambient particulate matter. The collection, storage, transport, extraction, and assay methods have been extensively studied, leading to recent optimization of the assay (29, 30). Despite these efforts, a remaining potential source of assay variance is the *Limulus* lysate itself. Since the lysate is derived from the blending of hemolymph extracted from living organisms, *Limulus polyphemus*, there can be interlot variations. Recombinant factor C is potentially less variant in composition but may have interlot variation associated with production, purification, and storage. However, in this study, the LAL and rFC methods yielded comparable correlations with dust concentration (Fig. 3), were well correlated with each other (Fig. 4), and yielded a slope very close to unity (Fig. 4). This study found that the assays are similar for air samples drawn from a variety of agricultural environments and over a wide range of concentration. Further, both methods yielded few nondetectable values and few aberrant determinations.

Mixed-model ANOVA was performed to further evaluate significant determinants of endotoxin concentration and to determine modeled parameter estimates. For the field study, this revealed rFC/LAL ratios of close to 1.0 for the samplers (0.90 to 1.14, not significant). For the 10 facilities sampled in the field study the ratio ranged from as low as 0.47 (in a Colorado horse barn) to 2.1 (in a Colorado dairy barn). Thus, there were instances of significant differences between the assays where the rFC assay delivered a higher estimate than the LAL assay and some where the rFC was lower. This is best observed from Fig. 5, which reveals that field sites yielding proportional differences of greater than 10% included two sites where the rFC values were higher than the LAL values (a Colorado dairy and a Colorado cattle feedlot) and two where they were lower (Colorado chicken and Colorado horse facilities). This effect

TABLE 6. Modeled parameter estimates for the results shown in Table 5

Main effect (sampler)	rFC		LAL		GM ratio (rFC/LAL)	P value
	Modeled GM	95% CI	Modeled GM	95% CI		
IOM	11,227	8,400, 15,000	10,331	7,700, 14,000	1.09	0.646
Button	12,327	9,200, 16,000	19,856	15,000, 27,000	0.62	0.009
Cassette	5,713	4,300, 7,700	8,864	6,600, 12,000	0.64	0.018

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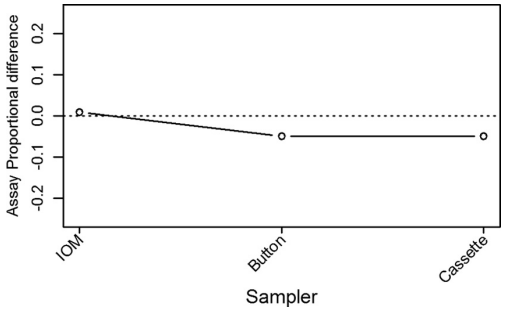

FIG. 6. Interaction plot for sampler and assay, showing the proportional difference between assays for the laboratory study under settling-air conditions. This nonsignificant interaction illustrates 5% lower endotoxin values for the rFC assay for the Button and cassette samplers but not the IOM sampler. Connecting lines were added for clarity.

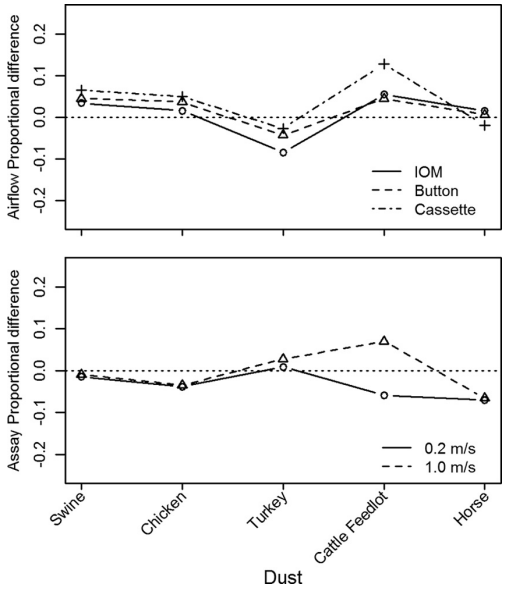

FIG. 7. Second-order interaction plots for the dust, sampler, and airflow (top) and assay, dust, and airflow (bottom), showing the proportional difference between airflows (top) and assays (bottom) for the wind tunnel laboratory study. The plots illustrate that the relationships were distinct for cattle feedlot dust experiments compared to other dust types generated. Connecting lines were added for clarity.

was not associated with a particular type of livestock dust, since the Iowa chicken and Iowa dairy sites did not demonstrate assay differences.

This is the second published study of which we are aware that compares the performance of the kinetic chromogenic LAL assay with that of the recombinant factor C assay. The other study was an evaluation of 60 house dust samples collected in homes in Connecticut (1). The range of concentrations in the samples in that study was 10 to 200 EU/mg. Although the LAL and rFC assays were well correlated (Spearman $r = 0.86$), the geometric mean of the LAL determinations was 1.9-fold higher than that of the rFC measurements. In our study, a large sample size allowed us to determine the effect of the assay independent of the facility or dust type, sampler type, and airflow. This analysis yielded overall LAL/rFC GM ratios of 0.89 for the field study and 1.06 for the laboratory study (Fig. 2). Further, the slope of the regression line relating LAL-assessed values to rFC-assessed values was very close to 1 (0.979) and the correlation coefficient between the two endotoxin assays was 0.92, showing that, on average, the assays returned similar values. Alwis and Milton (1) performed extractions and assays in a solution of 0.01% triethylamine in 0.05 M potassium phosphate buffer at pH 7.5, while we used pyrogen-free water with 0.05% Tween 20. Spaan and colleagues (29) showed that, in their hands, the LAL assay in this triethylamine-phosphate buffer solution yielded a 10-fold-lower estimate of endotoxin than when the assay is run in pyrogen-free water with or without Tween 20. However, since Alwis and Milton conducted both the rFC and the LAL assays in the triethylamine-phosphate buffer solution and the LAL results were higher, this cannot explain the observed difference. There can potentially be important differences in matrix effects and bacterial species between house dust samples and agricultural dust samples. These differences may have contributed to the higher LAL values than rFC values reported by Alwis and Milton but not observed by us.

There was one aspect of the study that can be viewed as either a strength or a weakness. Since the LAL assay was performed at the University of Iowa and the rFC was performed at Colorado State University, the variation between assays includes variance attributable to laboratory-dependent factors. A test of assay differences alone would require that the assays be carried out in one laboratory by the same analyst using the same analytical devices. The fact that the assays were similar even though the variance included an interlaboratory component in addition to the interassay component could be explained by the interlaboratory variation being in a different direction than the interassay variation, resulting in no apparent difference between the assays.

In conclusion, a very strong positive correlation was found between the LAL and the rFC endotoxin assays over a very wide endotoxin concentration range resulting from sampling in 10 livestock facilities for five types of agricultural organic dusts and with four types of samplers, three of which represent differing size fractions of airborne dust. Ratios of LAL to rFC were 0.89 for air samples from the field study and 1.06 for the laboratory-generated air samples, and the slope of the regression line relating LAL-assessed values to rFC-assessed values was close to unity with a very high correlation, showing that, on average, the assays returned similar values. ANOVA also demonstrated similar estimates of endotoxin concentration for the two assay methods in field studies. This indicates that neither method surpasses the other for endotoxin exposure assessment in livestock facilities.

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