

SECOND INTER-LABORATORY STUDY COMPARING ENDOTOXIN ASSAY RESULTS
FROM COTTON DUST

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Abstract: Previously, a large two-part inter-laboratory round robin endotoxin assay study was completed. This first study showed that when cotton dust samples, which are practically identical, are assayed for endotoxin that the intra-laboratory results had a very small variation while intra-laboratory results of the sample had a very high variation. In the first part of the study, each laboratory followed its own in-house assay protocol; but in the second part of the study, when the extraction protocol was standardized, the inter-laboratory results showed a lower variation, which suggested that with further standardization, further reduction of differences between laboratories might be achieved in order that results between laboratories would become more comparable. The results stimulated interest in extending the study to include cotton dust with two levels of endotoxin, standardization of the extraction protocol, and using the same assay kit from the same production lot. The results of this second round robin endotoxin assay study indicate that differences between laboratories are still high, but most of the laboratories could discern the cotton dusts with the different levels of endotoxin.

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INTRODUCTION

The connection between cotton dust and byssinosis is found in the substance called endotoxin that is produced

in the cell walls of Gram-negative bacteria [14, 16]. After decades of research, most researches now believe that the causal agent of byssinosis, an occupational respiratory disease caused by the long-term inhalation of cotton, flax,

or hemp dust and characterized by shortness of breath, coughing and wheezing, is endotoxin [4, 5, 6, 16]. The cotton industry in the US is regulated by a stringent set of regulations in the Cotton Dust Standard [1] to protect its workforce. Recently, other segments of agriculture and industry and many other countries are just now awakening to the hazards of air quality safety and the possibility that endotoxin may also be a problem [2, 3, 10, 11, 15]. Currently, there are no regulations that limit the amount of endotoxin in the air. However, some movement in that direction is being suggested [12, 13]. This makes the quantification of endotoxin important not only in the study of respiratory dysfunctions but also important for imposing regulatory health standards.

This makes the measurement of endotoxin all the more important. A problem that exists and not widely recognized is that often, results obtained by one laboratory on the same sample made by another laboratory are not comparable. This was established when a round robin endotoxin assay was conducted with a large number of laboratories, both nationally and internationally [7]. When a common extraction protocol was adopted, the differences in results was reduced, suggesting that perhaps by further standardization, differences would be reduced further. A second round robin endotoxin assay was therefore conducted using the same assay procedure, including using the Bio-Whittaker Kinetic-QCL assay kits from the same batch. The results of this study are presented here.

MATERIALS AND METHODS

Participants in the Inter-laboratory Endotoxin Assay Study. Participants in the round robin endotoxin assay study are listed in Table 1. Twelve laboratories were involved in the study. These participating laboratories were familiar with assaying for endotoxin and had the necessary equipment to perform the kinetic endotoxin type of assay as used in the Bio-Whittaker Kinetic QCL assay (BioWhittaker, Inc., Walkersville, MD: 50-650U, Kinetic-QCL 192 Test Kit). One of the laboratories had the ability to run gas chromatographic-mass spectrometry (GC-MS) for total endotoxin (as purified lipopolysaccharide, LPS). This was a different laboratory than the one in the first round robin study [7]. The results from the GC-MS assay will be presented separately and will be described as the results from a thirteenth laboratory.

Cotton Dust. Cotton dust was collected in 1998 as described by Chun, *et al.* [8]. Thoroughly blended cotton was carded and the dust collected on filters using vertical elutriators (VE) in the model cardroom at the Cotton Quality Research Station (CQRS) in Clemson, SC [9]. The dust was collected on both polyvinyl chloride (PVC) and glass filters (Pall Gelman Sciences, Ann Arbor, MI: GLA-5000 Membrane, 5- μ m 37 mm, PVC membrane filter; Type A/E Glass Fiber Filter 1- μ m 37 mm, respectively). The dust was uniform, card generated and vertically elutriated cotton dust. Dusts of 3 different

Table 1. Principal Participants in the Second Inter-Laboratory Endotoxin Assay Study.

Principal Participant/ Contact Person	Affiliation	Location
Bartlett, Karen	University of British Columbia, School of Occupational and Environmental Hygiene	Vancouver, Canada
Chew, Victor ¹	USDA, ARS, South Atlantic Area (SAA) Biometrical Services	Gainesville, FL, USA
Chun, David T.W.	USDA, ARS, CQRS	Clemson, SC, USA
Gordon, Terry	New York University School of Medicine, Nelson Institute of Environmental Medicine	Tuxedo, NY, USA
Jacobs, Robert R. ²	Graduate Program in Public Health, Eastern Virginia Medical School	Norfolk, VA, USA
Larsson, Britt-Marie	Program for Respiratory Health and Climate, Dept. of Occupational Medicine	Solna, Sweden
Lewis, Daniel M.	NIOSH, Division of Respiratory Disease Studies (DRDS)	Morgantown, WV, USA
Liesivuori, Jyrki	Kuopio Regional Institute of Occupational Health, Occupational Hygiene and Toxicology Section	Kuopio, Finland
Michel, Olivier	Clinic of Allergies and Respiratory Disease, Saint-Pierre University Hospital	Brussels, Belgium
Rylander, Ragnar	University of Gothenburg, Dept. of Environmental Health	Gothenburg, Sweden
Thorne, Peter S.	University of Iowa, College of Public Health	Iowa City, IA, USA
White, Eugene M. ³ & Gunn, Varina C.	Milacron, Inc., and NIOSH, Division of Applied Research and Technology, respectively	Cincinnati, OH, USA
Würtz, Helle	National Institute of Occupational Health	Copenhagen, Denmark

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concentrations of endotoxin, low, medium and high, were obtained by carding cotton from 'three sources of cotton' which had different cotton dust and endotoxin potential and then collecting the dust on 2 types of filters using vertical elutriators in the model cardroom at the Cotton Quality Research Station in Clemson, SC. The source of cottons was from the 1997 harvest year grown in the Mississippi Delta region. Since better grade (whiter and less trash) cottons tend to have lower dust content and the dust is of lower endotoxin concentrations than those of lower grade cottons, bales of 'strict low middling' (grade 41, a relatively clean grade of cotton) and bales of 'low

Table 2. Approximate date results from participating laboratories were received by facsimile transmission, mail, or E-mail.¹

Lab ID	Approx. Date
1	27-Dec-1999
2	21-Dec-1999
3	25-Oct-1999
4	4-Oct-1999
5	3-Feb-2000
6	24-Mar-2000
7	18-Apr-2000
8	31-Mar-2000
9	20-Apr-2000
10	12-Oct-1999
11	1-Dec-1999
12	8-Jan-2000
13	14-Oct-1999

¹Dust samples were mailed to the participating laboratories on 21 September 1999.

middling light spotted' (grade 53, a grayer and higher trash content cotton) were used. To obtain an intermediate grade of cotton, a 1:1 mixture of the strict low middling and the low middling light spotted cotton was blended. These cottons were blended in a card room and the dust released during carding was ducted to a remote room where 30 vertical elutriators (Model GMW-4000; General Metal Works, Cleaves, OH) were distributed in 3 rows, 16, 8 and 6 per row, to collect the cotton dust (8 and 9). Each weighed dust-laden membrane was transferred to a 50-ml screw-top polypropylene conical tube (Falcon[®] 2998; Becton Dickinson and Co., 2 Bridgewater Lane, Lincoln Park, New Jersey 07035) and stored in the dark at room temperature ($\sim 22^{\circ}\pm 1^{\circ}\text{C}$). Over 3,000 filter samples, each with 0.3 to 0.8-mg cotton dust with a target weight of 0.5 mg dust, were collected to satisfy the current needs of this test and for anticipated future endotoxin assay studies. For this study, only the cotton dust on PVC filters and of the low and high endotoxin concentration dust was used - from low endotoxin containing cotton, 'A', and high endotoxin containing cotton, 'B', respectively.

A randomized complete block design with VE run/lot as blocks was used. Only VE runs/lots with 13 or more PVC filters containing 0.3 and 0.8 mg dust/filters were used; and 13 filters from each of these lots were assigned to the laboratories for testing. The 12 laboratories and the laboratory conducting GC-MC analysis were randomly assigned a laboratory identification number. The laboratory conducting the GC-MC was Lab #12. Each laboratory was given 8 filters samples for analysis: 4 samples with dust of low endotoxin concentration and 4 samples with dust of high endotoxin concentration. The dust weight was provided along with the dust samples. Control or blank filters were not sent unless requested by the investigator.

Assay Protocol. Each laboratory (except Lab #12) was sent the following sample extraction and endotoxin assay procedure as described below:

Sample Extraction, dilution and analysis

A. Samples should be extracted and analyzed within a month of receipt. The assay should be carried out on the same day as extraction! (LAL reagents age, even under proper storage conditions.)

B. For extraction:

1. Use room temperature pyrogen free water (PFW) and extract directly in the 50-ml centrifuge tubes used to send the samples.

2. For 37 mm diameter filters, extract with 20 ml of PFW.

3. Place on a rotary/wrist shaker and shake at the highest possible setting for 60 minutes at room temperature.

4. After the extraction period, centrifuge at a minimum of 2,000 rpm for 10 minutes.

C. Sample dilution

1. After centrifugation, dilute the supernatant for analysis.

2. Prepare 10-fold serial dilutions in borosilicate tubes that have been heated to render them pyrogen free. Conditioned borosilicate tubes to refer to clean or new tubes that had been heat-treated to render the tubes pyrogen free (heat treatment as normally conducted in individual's lab. For example, methods used by some labs include heating tubes in an oven at 200°C for 8 hours or more; or 180°C for 3 hours or more; or heating at 250°C for 30 minutes.)

3. Use PFW for dilution preparations.

D. Sample assay

1. Assay appropriate dilutions using LAL reagents and protocol provided by Bio-Whittaker.

2. Report results as EU/mg dust.

Twelve Kinetic-QCL endotoxin assay kits were purchased by CQRS; and arrangements were made with BioWhittaker that all of the kits would be from the same production batch and would be mailed at about the same time that the dust samples were mailed to the participating laboratories. All of the dust samples, each in its own 50 mL screw-top polypropylene conical tube, in cardboard boxes and protected by packing material, were sent to the participating Laboratories in September 1999 (Tab. 2) using FedEx Express mailing. The participating laboratories were responsible for storing the samples from the time of receipt to returning results. The results were returned for analysis to CQRS as endotoxin units per milligram dust (EU/mg) or were converted to EU/mg by conversion factors provided by the researcher or by an assumed conversion factor (such as, 10 EU = 1 ng endotoxin).

Table 3. Low Endotoxin Content Dust, Endotoxin Concentration Determined by Different Laboratories.

Laboratory ID	Average Endotoxin Concentration, Log ₁₀ EU/mg	Duncan Grouping ¹
6	4.394	A
1	4.262	A
3	4.151	AB
13	4.004	BC
2	3.780	CD
7	3.745	D
9	3.726	D
4	3.673	D
5	3.645	D
11	3.642	D
8	3.623	D
10	3.316	E

¹Mean separation within columns by Duncan's multiple range test, 5% level. Means with the same letter are not significantly different.

Table 4. High Endotoxin Content Dust, Endotoxin Concentration Determined by Different Laboratories.

Laboratory ID	Average Endotoxin Concentration, Log ₁₀ EU/mg	Duncan Grouping ¹
1	4.919	A
3	4.877	AB
13	4.794	AB
6	4.681	BC
9	4.523	CD
5	4.501	CD
7	4.498	CD
11	4.399	DE
4	4.394	DE
2	4.330	DE
10	4.248	E
8	3.488	F

¹Mean separation within columns by Duncan's multiple range test, 5% level. Means with the same letter are not significantly different.

Statistical Analysis. Data were analyzed using release 6.12 or earlier releases of SAS (SAS, Statistical Analysis System; SAS system for Windows version 4.0950; SAS Institute Inc., Cary, NC, USA) for making mean comparisons. Otherwise, additional testing and data manipulation was done with Microsoft EXCEL 2000 or earlier releases of EXCEL (Microsoft Corporation, USA) and plotted using SigmaPlot for Windows version 05 (SPSS, Inc., USA).

RESULTS AND DISCUSSION

The time period for results to be returned from the participating laboratories ranged from less than a month to just over 6 months after the samples were mailed (Tab. 2).

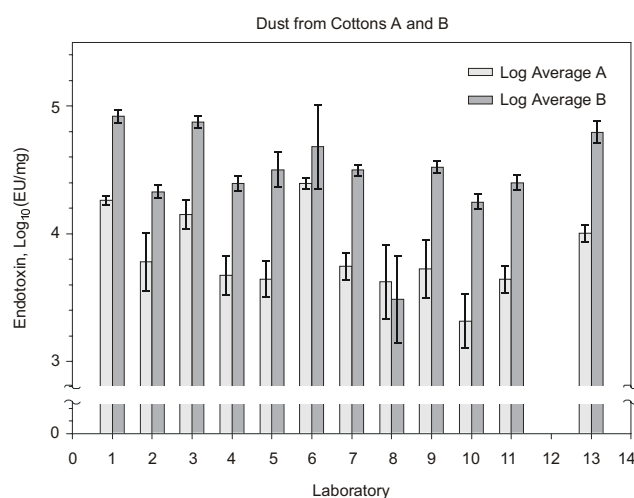


Figure 1. Average assay results of cotton dust with a low and high endotoxin concentration by the participating laboratories, Log₁₀(EU/mg) each half bar represents 2 S.E.

This was in keeping with the time frame observed from the first inter-laboratory study [7]. As before, the time periods to return the results did not seem unusually long and no significant differences in results due to delays in assay from aging of the samples or of the endotoxin kits between the laboratories were expected. No correction was taken into account; since at CQRS, cotton dust endotoxin content for samples did not change after 5 years of storage in the dark at room temperature (Henry H. Perkins, Jr., personal communiqué), these shorter storage times were therefore not expected to affect results, except as possibly due to the individual laboratory's storage conditions. Also, all of the results were returned long before the expiration date of the endotoxin assay kits and no effects of aging would be expected.*

Since total endotoxin is measured by GC-MS, the GC-MS results were expectedly high for both the low and high endotoxin concentration dusts. The comparison between laboratories did not include the GC-MS results since the results were so high as to be significantly different from the other results for endotoxin, as was also the case in the first inter-laboratory study [7]. The average Log₁₀(EU/mg) results returned from the GC-MS analyses were 7.857 and 8.280 (s.d. = 0.111) for the low and high endotoxin dusts, respectively. The low endotoxin dust average had 3 missing data and was therefore made from only a single observation. This makes comparing the GC-MS results from the low and high endotoxin concentration dusts difficult.

* The time for results to be returned is included here since it has value in representing real world 'wait' time and should give the uninitiated a feel for how long it can take to get results back from research orientated laboratories that have voluntarily contributed their time and resources. Possibly shorter intervals can be expected from commercial laboratories.

When the results from the laboratories carrying out the limulus type assays (Fig. 1, Tab. 3) on the low endotoxin concentration dust are compared, the results differ by about an order of magnitude (Tab. 3), ranging from 3.316–4.394. Still, most of the laboratories have results that are significantly different from one another. The same can be said for the high endotoxin dust results (Fig. 1, Tab. 4). However, here the differences actually seem smaller in that with the exception of the results from Lab #8, the laboratories are all within the same order of magnitude, the results ranging from 4.248–4.919. Still, enough interlaboratory differences exist that results between laboratories of the same dust sample are significantly different, but, with the exception of Lab #8, all of the laboratories were able to discern between the high and low endotoxin concentration dusts (Fig. 1). Interestingly, most of the laboratories showed lower intra-laboratory variation for the high endotoxin samples than for the low endotoxin concentration samples (Fig. 1). As in the first round robin study, intra-laboratory variations are small so that ranking comparisons of samples within laboratories are well grounded. Inter-laboratory results are still not directly comparable for all laboratories. This is important, since some people engaged with endotoxin and their consequences are unaware of such inter-laboratory discrepancies.

The results from the first inter-laboratory study indicated that the variation between laboratories was reduced by following a common extraction protocol. Extrapolation of this observation was that further reduction in variation might be further achieved by having the different laboratories adopt the same assay protocol. In this study, each laboratory used the same assay protocol, the same endotoxin assay kit and the kits were all from the same production batch. Still the results between laboratories were different enough to be statistically different so again comparisons of results from the same samples made by different laboratories must be considered carefully. Since intra-laboratory differences are small, some area of commonality should be achievable between laboratories that will some day make direct comparisons between laboratories possible. Since so many common approaches have already been adopted, reasons for the differences between laboratories should be examined further. Perhaps an apprenticeship-training program, use of identical equipment, etc. may lead to results that are more common between different laboratories assaying identical samples.

CONCLUSIONS

Further standardization by adopting the same assay protocol, the same endotoxin assay kit and using assay kits from the same production lot, did not sufficiently reduce variation in results between laboratories that results between laboratories were statistically similar. However, intra-laboratory variation is small so that comparisons within laboratories would permit internal

comparison of samples; and most of the laboratories were able to distinguish between samples having low and high endotoxin concentrations.

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