SECOND INTERLABORATORY STUDY COMPARING ENDOTOXIN ASSAY RESULTS FROM COTTON DUST

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Abstract

Previously, a two-part interlaboratory round robin endotoxin assay study was completed. This first study showed that intralaboratory results had small variation but that intra-laboratory results had very high variation. This held true for both parts of the study; 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 so that results between laboratories would become comparable. The results stimulated interest in extending the study to include cotton dust with two levels of endotoxin, standardization of the extraction protocol and assay with the assay kits all from the same production lot. The results of a second round robin endotoxin assay study where a common assay protocol using the same endotoxin assay kit is reported.

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 (Jacobs, 1997; Rylander, 1997). 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 (Castellan, 1997; Castellan, et al., 1984, 1987; Rylander, et al., 1985). The cotton industry has a stringent set of regulations in the Cotton Dust Standard (Anonymous, 1978) to protect its workforce; and recently other areas of agriculture and industry are just now awakening to the hazards of air quality safety and the possibility that endotoxin may also be a problem.

Currently, there are no regulations that limit the amount of endotoxin in the air. However, some movement in that direction is being suggested (Heederik, 1997; Anonymous 1998). This makes the measurement of endotoxin all that more important. A problem that exists 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 (Chun et. al., 1999). When a common extraction protocol was adopted, the differences in results was reduced suggesting that perhaps by further standardization, differences would be reduced further. So a second round robin endotoxin assay was conducted using the same assay procedure, including using the Bio-Whittaker Kinetic-QCL assay kits from the same lot. The results of this study will be presented here.

Methods and Materials

Endotoxin Assay Committee

Participants in the round robin endotoxin assay study are listed in Table 1. Twelve laboratories were involved in the study; and they consisted of laboratories 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. One of the laboratories also 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. The results from this thirteenth lab will be presented also.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1303-1307 (2001)
National Cotton Council, Memphis TN

Cotton Dust

Cotton dust was collected in 1998 as described by Chun, et al. (1999). The dust was collected on both polyvinyl chloride (PVC) and glass filters using CQRS's model card room (Chun and Perkins, 1997). These were uniform, card generated, and vertically elutriated cotton dust. Dusts of three different concentrations of endotoxin, low, medium and high, was obtained by carding cotton from 'three sources of cotton' and then collecting the dust on two types of filters using vertical elutriators in the model cardroom at the Cotton Quality Research Station in Clemson, SC. 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 were used.

General Protocol

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 doing GC-MC analysis were randomly assigned a laboratory identification number. The laboratory doing the GC-MC was Lab #12. Each laboratory was given 8 filter samples for analysis: 4 samples from dust with low endotoxin concentration and 4 samples from dust with high endotoxin concentration. The dust weight was provided along with the dust samples. Control or blank filters were not sent unless the investigator requested them. 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 done on the same day as extraction! (LAL reagents do age, even under proper storage conditions)
b. For extraction:
    i. Use room temperature **pyrogen free water** (PFW) and extract directly in the 50-ml centrifuge tubes used to send the samples.
    ii. For 37 mm diameter filters, **extract with 20 ml** of PFW
    iii. **Place on a rotary/wrist shaker and shake at the highest possible setting for 60 minutes at room temperature**
    iv. After the extraction period, **centrifuge at a minimum of 2000 rpm for 10 minutes.**
c. Sample dilution
    i. After centrifugation, dilute the supernatant for analysis.
    ii. 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 done 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.)
    iii. Use PFW for dilution preparations
d. Sample assay
    i. Assay appropriate dilutions using LAL reagents and protocol provided by Bio-Whittaker.
    ii. Report results as EU/mg dust

All of the dust samples were sent to the participating Laboratories in September 1999 at about the same time that Bio-Whittaker sent out the Kinetic-QCL assay kits (44 50-650U, Kinetic-QCL (192 Test Kit), Bio-Whittaker, Walkersville MD). The assay kits were all from the same production lot and all kits were sent out at the same time directly by Bio-Whittaker. Results were provided as endotoxin units per milligram (EU/mg) or were converted to EU/mg by conversion factors provided by the researcher or by assumed conversion factors (such as, 10 EU = 1 ng endotoxin).

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 six months after the samples were mailed (Table 2). As in the previous round robin study, the time period 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 and so no correction was taken into account. 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 rather than from commercial laboratories.

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. The average Log_{10}(EU/mg) results returned from the GC-MS analyses were 7.8573 and 8.2802 (s.d. = 0.1113) for the low and high endotoxin concentration dusts, respectively. The low concentration average had three missing data and so was made from only a single observation. This makes comparing the GC-MS results from the low and high endotoxin concentration dusts difficult.

In comparing the results from the laboratories doing the limulus type assays (Figure 1 & Table 3), we find that with the low endotoxin concentration dust that most of the results differ by about an order of magnitude (Table 3), ranging from 3.3162 to 4.3943. Still most of the laboratories have results that are significantly different from one another.

The same can be said for the high endotoxin concentration dust results (Figure 2 & Table 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 4.2476 to 4.9187. There is still not enough interlaboratory agreement for the same samples. But what is encouraging is that all of the laboratories, with the exception of Lab #8, were able to discern between the high and low endotoxin concentration dusts (Figure 3). Interestingly, many of the laboratories showed lower intra-laboratory variation for the high endotoxin samples than for the low endotoxin concentration samples. As in the first round robin study, intra-laboratory variations are small and comparisons of samples within laboratories could be made. Inter-laboratory results are still not directly comparable for all laboratories. Would an apprenticeship training program, identical equipment, etc. lead to more common results from different laboratories assaying identical samples? Since so many common approaches have already been adopted, reasons for the differences between laboratories should be examined further.

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Table 1. 'Endotoxin Assay Committee' — Principal Participants in the Second Round Robin Endotoxin Assay Study

Principal Participant/ Contact Person	Affiliation	Location
Bartlett, Karen	University of British Columbia, Occupational Hygiene Programme	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	Hôpital Universitaire Saint-Pierre, Clinique de Pneumologie et D'Allergologie	Bruxelles, 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., Brown, Mary E.	NIOSH, Division of Applied Research and Technology, and US Environmental Protection Agency, respectively	Cincinnati, OH, USA
Würtz, Helle	National Institute of Occupational Health	Copenhagen, Denmark

[1]Biometrician, retired.
[2]Previously at University of Alabama-Birmingham, Environmental Health Sciences, Birmingham, AL, USA

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

[1]Dust samples were mailed September 21, 1999 to the participating labs

Table 3. Endotoxin Concentration Determined by Different Laboratories of Dust from Cotton A.

Laboratory ID	Average Endotoxin Concentration, Log_{10}EU/m	Duncan Grouping[1]
6	4.3943	A
1	4.2624	A
3	4.1509	AB
13	4.0039	BC
2	3.7793	CD
7	3.7447	D
9	3.7257	D
4	3.6727	D
5	3.6446	D
11	3.6419	D
8	3.6226	D
10	3.3162	E

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

Table 4. Endotoxin Concentration Determined by Different Laboratories of Dust from Cotton B.

Laboratory ID	Average Endotoxin Concentration, Log10EU/m	Duncan Grouping[1]
1	4.9187	A
3	4.8767	AB
13	4.7943	AB
6	4.6811	BC
9	4.5226	CD
5	4.501	CD
7	4.4983	CD
11	4.3993	DE
4	4.3936	DE
2	4.3297	DE
10	4.2476	E
8	3.4876	F

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

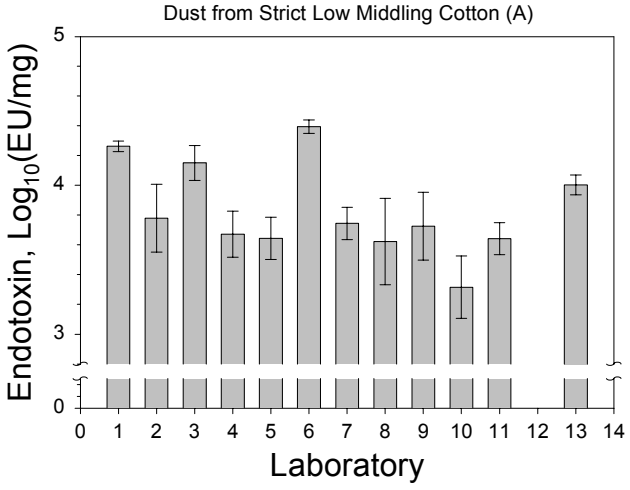

Figure 1. Average assay results of cotton dust with a low endotoxin concentration by the participating laboratories, Log_{10}(EU/mg) each half bar represents 2 s.e.

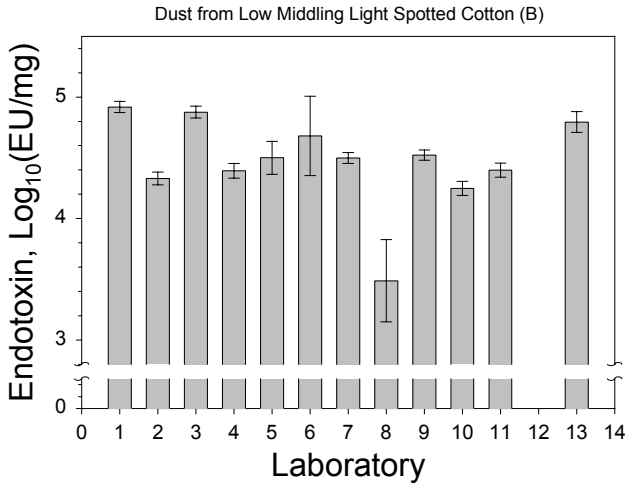

Figure 2. Average assay results of cotton dust with a high endotoxin concentration by the participating laboratories, Log_{10}(EU/mg) each half bar represents 2 s.e.

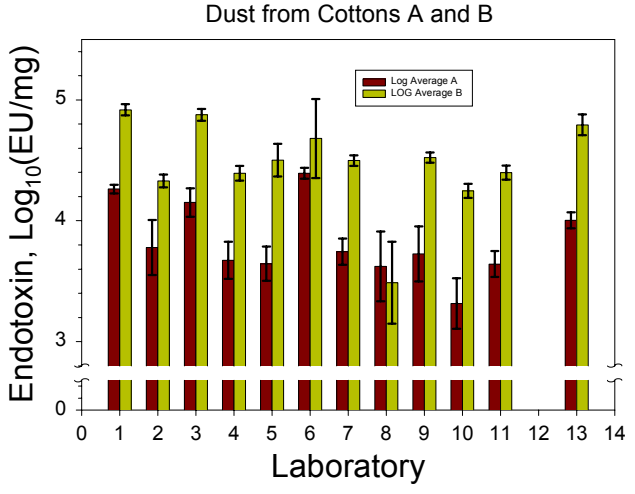

Figure 3. Average assay results of cotton dust with a low and high endotoxin concentration by the participating laboratories, Log_{10}(EU/mg) each half bar represents 2 s.e.