PRELIMINARY REPORT ON THE RESULTS OF THE FIRST PHASE OF A ROUND ROBIN ENDOTOXIN ASSAY STUDY USING COTTON DUST

D.T. Chun, Microbiologist, USDA, ARS
Cotton Quality Research Station
Clemson, SC
and the Endotoxin Assay Committee,
see Table 1 below

Abstract

A two part interlaboratory endotoxin assay study is underway. In both parts of the study, filter membranes with the same approximate amount and type of cotton dust were sent for analysis to laboratories that 'routinely' perform endotoxin analyses. Each of these laboratories performed the analysis using the methodology common to their laboratory. In the second part of the study, filter membranes with cotton dust were again sent to the same laboratories where the analyses were performed as before but with a common extraction protocol. The results from the first phase of the study have been collected and the first hand interpretation will be reported here. The intralaboratory variations were small; but large and significant interlaboratory variation was observed.

Introduction

At the USDA, ARS, Cotton Quality Research Station (CQRS) in Clemson, SC, research on cotton dust and byssinosis has been underway for over two decades. Cooperative work done at this laboratory and other laboratories world wide has implicated endotoxins (lipopolysaccharides, LPS) in cotton dust as the most likely etiological agent of byssinosis (Castellan, 1997; Castellan, et al. 1984, 1987; Rylander, et al., 1984; Rylander, et al., 1985). Thusly, the assay of endotoxin has garnered in importance in the study of respiratory dysfunctions (Jacobs, 1997; Godby, et al., 1995; Michel, et al., 1996; Rylander, 1997).

However, researchers and others concerned with endotoxin levels, in cotton lint or dust and now in agricultural and other organic dusts, have become concerned that when identical samples are assayed for endotoxin content that level differences, often in the orders of magnitude, may be reported between different laboratories. This has been frustratingly true; and so identical samples were often sent from CQRS to different laboratories for assay. While the quantitative levels returned were different, the ranking of the samples was nearly always the same between the different laboratories. This has permitted comparisons to be made and accredits the endotoxin assay for providing useful information; but the differences in levels has always been a nagging concern since this meant that results reported and read in the literature must be interpreted with caution with due consideration of the extraction methods and the laboratory conducting the analysis (Chun and Perkins, Jr. 1994; Jacobs and Pietrowski, 1995; Milton, et al., 1992; Walters, et al., 1994; Wood and Jacobs 1997).

For this reason, about 5 or 6 years ago, the need for and the possibility for conducting an interlaboratory test on uniform dust was discussed among scientists, most notably — Henry H. Perkins, Jr., USDA, ARS, Clemson, SC (retired); Stephen A. Olenchock, NIOSH, ALOSH, Morgantown, WV (now at National FarmMedicine Center, Marshfield, WI); Ragnar Rylander, University of Gothenburg, Sweden; and Robert R. Jacobs, University of Alabama, Birmingham, AL. Even so, actual activity was slow and delayed until 1995 when a study between 10 laboratories was planned and uniform cotton dust samples were collected. Further delays due to the make up of the interested parties occurred, but the study is being continued as a two part interlaboratory round robin endotoxin assay study. In both parts of the study, filter membranes with the same approximate amount and type of cotton dust were sent for analysis to laboratories that 'routinely' perform endotoxin analyses. Each of these laboratories performed the analysis using the methodology common to their laboratory. In the second part of the study, filter membranes with cotton dust were again sent to the same laboratories where the analyses were performed as before but with a common extraction protocol. The results from the first part of the study have been collected and will be presented.

Methods and Materials

Endotoxin Assay Committee

Participants in the round robin endotoxin assay study are listed in Table 1. Originally 14 laboratories were to participate in the first part of the study but two of the interested parties dropped out (not listed) and an additional laboratory asked to take part in the study. So in the second part of the study, 13 laboratories were still interested in participating and results from these laboratories are forthcoming.

Cotton Dust

Cotton dust was collected in 1995 as described by Perkins, et al. (1996) on polyvinyl chloride filters (Perkins, Jr., 1975) using CQRS's model card room (Chun and Perkins, 1997). These were uniform, card generated, vertically elutriated cotton dust averaging from 0.3-0.7 mg per filter with a target of 0.5 mg per filter; and contained endotoxin levels which did not vary significantly either between vertical elutriator (VE) locations or between positions within locations. Twelve dust laden filters were produced from each vertical elutriator run. Seventeen VE runs were made. However, complete sets of 12 filters were found for only 16 of the 17 VE runs. Half of the filters were used in

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part 1 of the study and the remaining half were used in part 2 of the study. 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 (~22°±1°C) until used.

General Protocol

Originally 14 laboratories were involved with part 1 of the study. These laboratories were randomly assigned a laboratory identification number except for the laboratory doing GC-mass spectrophotometric analysis for total endotoxin content. This laboratory was assigned the last laboratory identification number in both parts of the study. A randomized complete block design with VE run as blocks was used. The 12 filters in each VE lot run were randomly assigned to each laboratory so that each laboratory received a total of either 7 or 6 filter samples for analysis. The dust weight was provided along with the dust samples. Control or blank filters were not sent unless the investigator requested them. Each laboratory performed sample extraction and endotoxin analysis based on their in-house protocol.

The dust samples were mailed February 25, 1997 to the participating labs. Results were received from the participating laboratories by facsimile transmission, mail, or by e-mail. Approximate dates of receipt of the data are given in Table 2. 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). Where the data was provided in nanomoles, the MW (environmental LPS) = 8,000 was used for conversion to EU/mg (Larsson, personal communiqué) .

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 data manipulation was done with Microsoft EXCEL for Windows 95 version 8.0 (Microsoft Corporation, USA) and plotted using DeltaGraph 4.0 (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 almost four months after the samples were mailed (Table 2). The time period did not seem unusually long. No significant differences in results due to delays in assay between the laboratories were expected and so no correction was taken into account.

The significantly different results obtained from almost 'identical' dust samples did restate the problem of samples assayed by different laboratories (Table 3). Essentially, the results from different laboratories were significantly different from one another. However, the variation within a laboratory appears to be small so that results within a laboratory can be usefully employed to rank samples having different endotoxin contents (Figures 1 and 2).

Laboratory 14 used a GC-mass spectrophotometric analysis which measured total endotoxin in a sample. All other laboratories used an extraction protocol and one of the limulus amebocyte lysate (LAL) type assays which accounted for about a tenth to a hundredth of the total endotoxin present (Table 3 and Figure 1; Sonesson et al., 1990). Practically, the results can be separated into two groups, the result from laboratory 14 and the results obtained by the other laboratories. Argumentatively, one might suggest adopting analysis for total endotoxin as the standard method of analysis. However, the method is not readily available to most laboratories currently involved with endotoxin analysis and requires greater resources to obtain and to maintain. But more crucial is the question of whether total endotoxin relates best to the biological availability of endotoxin and hence its biological activity. Current feeling is that the limulus-type of assays which involves aqueous extraction better reflect the biological active endotoxin since total endotoxin may also include inactive and inaccessible endotoxin (Sonesson et. al, 1990). Whether this is true or not would have to be determined elsewhere. Still, total endotoxin may be very useful as an upper base line or upper limit for comparisons and in determining a practical extraction and assay protocol.

For this reason, in planning for part 2 of the study, more weight was placed on the methodology used by the laboratories whose assays yielded the higher levels of endotoxin (Figure 2). Most of the laboratories used one of the LAL assay kits or reagents manufactured by BioWhittaker, Inc. (8830 Biggs Ford Road, Walkersville, MD 21793, USA). A table of the different procedures or kit types used was not made because insufficient descriptive methodology was returned by some of the laboratories. For the second part of the study, a common extraction protocol seemed to be the best approach to reduce the variation between laboratories since changing to a common LAL assay kit, plate reader and analysis software, was an unrealistic request to be made of the participating laboratories; and these factors will remain as unexplained systematic error. A simplified extraction protocol along with the second set of filters was sent out to the participating labs and results should be forthcoming. Request for more details on the LAL assay kit was requested so hopefully the role of the assay kit might be determined.

One of the goals of this study was to see how wide the gap was between results from different laboratories and perhaps point to some ways this gap might be reduced. The study is far from the most comprehensive since many factors are not addressed. Some of the factors have been explored elsewhere and dealt mostly with extraction, bioaerosol

source and filter media (Chun and Perkins, 1994; Jacobs and Pietrowski, 1995; Thorne, et al., 1997; Wood and Jacobs, 1997); among those factors not addressed here is that possibly a major source of variability in results had derived from differences between lots of LAL used in the analyses (Milton, personal communiqué). One is reluctant to produce uniform dust laden membranes again because of the material and resources required (Perkins, et al., 1996), but dust laden membranes can be more easily collected in lots without regard to uniformity between lots and then ranked by the total endotoxin method to provide samples if further investigations are warranted such as having different laboratories perform the analysis on the same cotton dusts using LAL from the same manufactured lot.

Summary

A two part interlaboratory endotoxin assay study is underway. In both parts of the study, filter membranes with the same approximate amount and type of cotton dust were sent for analysis to laboratories that 'routinely' perform endotoxin analysis. Each of these laboratories performed the analysis using the methodology common to their laboratory. The results from the first phase of the study showed that when different laboratories assay almost identical samples for endotoxin that the results can vary by as much as one or more orders of magnitude. However, the intralaboratory variations were very small and ranking of samples to different endotoxin levels is valid. The LAL assays only measured soluble endotoxin and the concentrations reported were a tenth to a hundredth of the total sample endotoxin.

Disclaimer

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Table 1. Principal laboratory investigators participating in the two-part Round Robin Endotoxin Assay Study — 'Endotoxin Assay Committee'[1].

Principal Participant / Contact Person	Affiliation	Location
Bartlett, Karen[2]	University of British Columbia, Occupational Hygiene Programme	Vancouver, Canada
Chew, Victor[3]	USDA, REE-ARS, SAA-OD	Gainesville, FL, USA
Chun, David T.W.	USDA, ARS, CQRS	Clemson, SC, USA
Gordon, Terry	New York University Medical Center, Nelson Institute of Environmental Medicine	Tuxedo, NY, USA
Jacobs, Robert R.	University of Alabama-Birmingham, Environmental Health Sciences	Birmingham, AL, USA
Larsson, Britt-Marie	National Institute for Working Life, Dept. of Occupational Hygiene and Toxicology Section	Sweden
Larsson, Lennart	Dept. of Medical Microbiology	Sweden
Lewis, Daniel M.	NIOSH, DRDS	Morgantown, WV, USA
Liesivuori, Jyrki	Kuopio Regional Institute of Occupational Health, Occupational Hygiene and Toxicology Section	Finland
Michel, Olivier	Hopital Universitaire Saint-Pierre, Clinique de Pneumologie et D'Allergologie	Belgium
Milton, Donald K.	Harvard School of Public Health, Dept. of Environmental Health	Boston, MA, USA
Rylander, Ragnar	University of Gothenburg, Dept. of Environmental Health	Gothenburg, Sweden
Thorne, Peter S.	University of Iowa, Dept. of Preventive Medicine and Environmental Health	Iowa City, IA, USA
White, Eugene M. & Brown, Mary E.	NIOSH, DPSEMRB	Cincinnati, OH, USA

[1]Two laboratories dropped out of the first part of the study (not listed) and were not participants in the second part of the study.
[2]Joined the study too late to participate in the first part of the study.
[3]Biometrician.

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

Laboratory ID	Approx. Arrival Date of Results
1	March 27, 1997
2	March 20, 1997
3	May 30, 1997
4	April 21, 1997
5	May 28, 1997
6	April 1, 1997
7	March 12, 1997
8	May 5, 1997
9	Dropped out of study
10	March 21, 1997
11	June 2, 1997
12	April 30, 1997
13	Dropped out of study
14	June 5, 1997

Table 3. Average assay results as EU/mg of the participating laboratories.

Laboratory[1] ID	Average EU/mg, Log_{10}EU/mg[2]	Average EU/mg, Log_{10}EU/mg[2,3]
14	4.941^{A}	—
8	3.982^{B}	3.982^{A}
4	3.669^{C}	3.669^{B}
6	3.525^{D}	3.525^{C}
2	3.452^{DE}	3.452^{CD}
11	3.401^{E}	3.401^{D}
7	3.260^{F}	3.260^{E}
10	3.247^{F}	3.247^{E}
3	3.080^{G}	3.080^{F}
12	2.848^{H}	2.848^{G}
1	2.838^{H}	2.838^{G}
5	0.840^{I}	0.840^{H}

[1]Two laboratories dropped out of the first part of the study (not listed) and were not participants in the second part of the study

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

[3]Average assay results as EU/mg of the participating laboratories, excluding Lab 14 (which assayed for total Endotoxin).

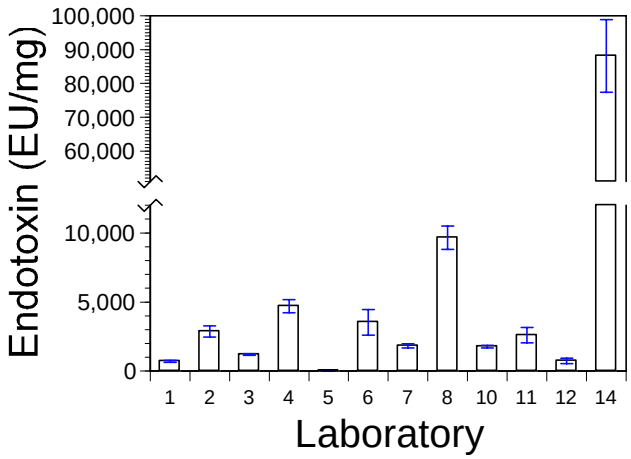

Figure 1. Average assay results of the participating laboratories; the axis break represents a change in scale to accommodate results from Laboratory 14 — total endotoxin content (EU/mg; each half bar represents 2 s.e.).

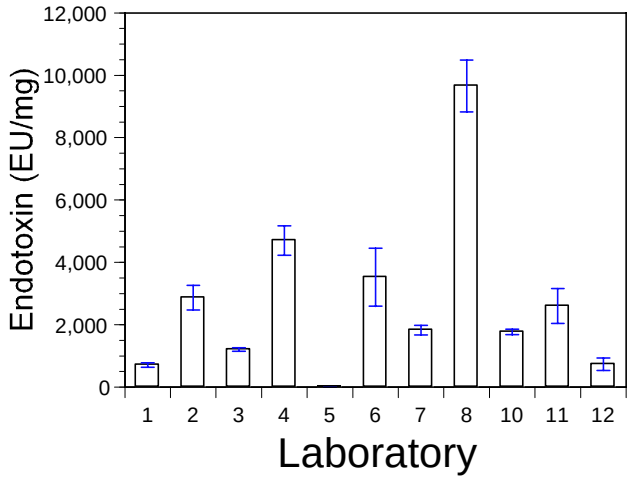

Figure 2. Average assay results of the participating laboratories. Results from Laboratory 14 which assayed for total endotoxin are not included (EU/mg; each half bar represents 2 s.e.).