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Preliminary Report on the Results of the Second Phase of a Round-Robin Endotoxin Assay Study Using Cotton Dust

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In an on-going endotoxin assay study, a two-part interlaboratory endotoxin assay study has been completed. The purpose of the study was to compare the variation in assay results between different laboratories, and, if the variation was high, to see if a common protocol would reduce the variation. In both parts of the study, membrane filters laden with the same approximate amount and type of cotton dust were sent for analysis to laboratories that "routinely" perform endotoxin analyses. First, each of these laboratories performed the analysis using the methodology common to its laboratory. In the second part of the study, membrane filters with cotton dust were again sent to the same laboratories where the analyses were performed as before but with a common extraction protocol. The preliminary results from the first phase of the study have been collected and showed that intra-laboratory variations were small, but large and significant interlaboratory variation was observed. The results were reported elsewhere. The preliminary results from the second part of the study consisting of the data currently collected are presented here. Again, intra-laboratory variations were small, but, also again, large and significant interlaboratory variation was observed. However, in this part of the study, the range between the highest and lowest average results was narrower than in the first part of the study. Influence of the assay kit type was examined. The variation within assay kit type was small but significant differences in results were observed between assay kit types. The findings suggest that endotoxin concentration in samples can be ranked within laboratories, but not necessarily between laboratories. However, some of the variation between laboratories has been reduced by a common extraction protocol which suggests the possibility of further standardization that may lead to better comparability between laboratories.

Keywords Round-Robin Endotoxin Assay Study, Endotoxin Assay, Limulus Amoebocyte Lysate Test (LAL), Lipopolysaccharide (LPS), Cotton Dust

At the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Cotton Quality Research Station (CQRS) in Clemson, South Carolina, research on cotton

dust and byssinosis has been underway for over two decades. Cooperative work done at this laboratory and other laboratories worldwide has implicated endotoxins (lipopolysaccharides, LPS) in cotton dust as the most likely etiological agent of byssinosis.[1–5] Thus, accurate assay of endotoxin is an essential component in the study of respiratory dysfunctions.[6–11]

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, 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. Although the quantitative levels returned were different, the ranking of the samples was nearly always the same between the different laboratories. Although this has permitted comparisons to be made and accredits the endotoxin assay for providing useful information, the differences in levels has always been a nagging concern because it 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.[12–16]

For this reason, about five or six years ago, the need for and the possibility of conducting an inter-laboratory test on uniform dust was discussed among scientists, most notably Henry H. Perkins, Jr., USDA, ARS, Clemson, South Carolina (retired); Stephen A. Olenchock, National Institute for Occupational Safety and Health (NIOSH), Morgantown, West Virginia; Ragnar Rylander, University of Gothenburg, Sweden; and Robert R. Jacobs, University of Alabama, Birmingham. Even so, actual activity was slow and delayed until 1995 when a study involving 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 inter-laboratory 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. The results from the first phase of the study showed that intra-laboratory variations were small, but large and significant inter-laboratory variation was observed.[17] 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 all but two of the participating laboratories have been collected and are reported here.

METHODS AND MATERIALS

Participating Laboratories

Participants in the round-robin endotoxin assay study are listed in Table I. In the first part of the study, 14 laboratories

TABLE I
Principal laboratory investigators participating in the two-part round-robin endotoxin assay study[A]

Principal participant/ contact person	Affiliation	Location
Bartlett, Karen[B]	University of British Columbia, Occupational Hygiene Program	Vancouver, Canada
Chew, Victor[C]	USDA, ARS, South Atlantic Area (SAA) Biometrical Services	Gainesville, FL
Chun, David T.W.	USDA, ARS, CQRS	Clemson, SC
Gordon, Terry	New York University Medical Center, Nelson Institute of Environmental Medicine	Tuxedo, NY
Jacobs, Robert R.	University of Alabama-Birmingham, Environmental Health Sciences	Birmingham, AL
Larsson, Britt-Marie	National Institute for Working Life, Department of Occupational Medicine	Sweden
Larsson, Lennart	Department of Medical Microbiology	Sweden
Lewis, Daniel M.	NIOSH, Division of Respiratory Disease Studies (DRDS)	Morgantown, WV
Liesivuori, Jyrki	Kuopio Regional Institute of Occupational Health, Occupational Hygiene and Toxicology Section	Finland
Michel, Olivier	Hôpital Universitaire Saint-Pierre, Clinique de Pneumologie et D'Allergologie	Belgium
Milton, Donald K.	Harvard School of Public Health, Department of Environmental Health	Boston, MA
Rylander, Ragnar	University of Gothenburg, Department of Environmental Health	Gothenburg, Sweden
Thorne, Peter S.	University of Iowa, Department of Preventive Medicine and Environmental Health	Iowa City, IA
White, Eugene M.	NIOSH, Division of Physical Sciences and Engineering Methods Research	Cincinnati, OH
Brown, Mary E.	Branch (DPSEMRB)	

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

had originally planned to participate but two of the interested parties dropped out (they are not listed) and an additional laboratory asked to take part. In the second part of the study, all 13 laboratories were involved and results from these laboratories have been collected. Two of the laboratories have not sent in their results or the results were sent too late to be included.

Cotton Dust

Cotton dust was collected in 1995, as described by Perkins et al.,[18] on polyvinyl chloride filters[19] using CQRS's model card room.[20] This was 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 containing endotoxin levels that 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 part 1 of the study and the remaining half were used in part 2. 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) and stored in the dark at room temperature (~ 22° ± 1°C) until used.

General Protocol

The 13 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, lab 13. A randomized incomplete block design with VE lot 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 seven or eight 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. Unlike the first part of the study, where each laboratory performed sample extraction and endotoxin analysis based on its in-house protocol, each laboratory performed a common extraction protocol but did the endotoxin analysis based on its in-house protocol.

The common protocol sent to each laboratory is as follows:

General Protocol:

1. Assay should be done same day as extraction.
2. Use pyrogen free water (PFW) for extraction.
   Use conditioned borosilicate tubes and PFW for making up dilution series. Condition borosilicate tubes to refer to clean or new tubes which 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).
3. Add 20 ml PFW to tubes containing membranes (extract in the shipping centrifuge tubes).
4. Place on rotary/wrist shaker and shake at fastest practical rate for 60 minutes at room temperature.
5. After extraction, assay.
6. Please report results as EU/mg and give details on whole assay (If reporting as ng/mg dust, please indicate recommended conversion factor to convert to EU, otherwise 10 EU/ng will be used):
   a. problems with extraction etc.
   b. Endotoxin assay method/kit e.g. Kinetic QCL or QCL 1000 or other.

The dust samples were mailed July 29, 1997, to the participating labs. Results were received from them by facsimile transmission, regular mail, or by e-mail. Approximate dates of receipt of the data are given in Table II. 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 (L. Larsson, personal communication). Four categories of kit types were used. Three of the four kit types were endotoxin assay kits manufactured by BioWhittaker, Inc. (Walkersville, Maryland): QCL-1000 (endpoint chromogenic limulus amoebocyte lysate assay), Kinetic-QCL (chromogenic kinetic assay), and Pyrogent-5000 (kinetic turbidimetric assay). The fourth kit type was used as a catch-all to include assay kits not manufactured by BioWhittaker, Inc. or a BioWhittaker assay kit used differently from the kit instructions.

TABLE II

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

Laboratory ID	Approx. arrival date of results
1	September 15, 1997
2[A]	
3	August 23, 1997
4	December 22, 1997
5	August 15, 1997
6	November 12, 1997
7	December 12, 1997
8	August 6, 1997
9	November 18, 1997
10	January 26, 1998
11	February 9, 1998
12	November 10, 1997
13[A]	

[A]Results not received or sent too late to be included in presentation. Results are expected from these laboratories.

Statistical Analysis

Data were analyzed using release 6.12 or earlier releases of SAS (Statistical Analysis System; SAS system for Windows version 4.0950; SAS Institute Inc., Cary, North Carolina) for making mean comparisons. Otherwise, data manipulation was done with Microsoft EXCEL for Windows 95 version 8.0 (Microsoft Corporation, Redmond, Washington) and plotted using DeltaGraph 4.0 (SPSS, Inc.,).

RESULTS AND DISCUSSION

The time period for results to be returned from the participating laboratories ranged from less than a month to seven months, with results from two laboratories still to be received (Table II). Previously, the time period ranged from less than a month to four months in the first part of this round-robin study.[17] Even though the time period was longer, no significant differences in results due to delays in assay between the laboratories were expected and so no correction was taken into account.

As shown in Table III, the results from the different laboratories were significantly different from one another. Again, the variations found within the different laboratories were very small (Table III and Figure 1). Compared to the averages observed in the first part of the round-robin,[17] there were fewer Duncan grouping differences—seven groupings in Part 1 of study versus only four groupings in Part II of the study (Table III). The range of average EU/mg (Log_{10}EU/mg) was large and ranged from 0.84 to 3.98 in the first part of the study;[17] even when omitting the one anomalous low value, the range of average Log_{10}EU/mg was still large, ranging from 2.85 to 3.98. In this second part of the study, the range of the average Log_{10}EU/mg was much reduced and ranged within the same order of magnitude, 3.30 to 3.97 (Table III). No strong distribution pattern was observed between the average Log_{10}EU/mg and the endotoxin assay kit type used (Table III). Even so, when the average Log_{10}EU/mg obtained was sorted by endotoxin assay kit type, significant differences were observed between the assay kit types (Table IV and Figure 2) and that the in-house and Kinetic-QCL kit types favored higher concentrations. The variation within assay kit type was small. The range of concentration was from 3.39 to 3.85. Still, the number of laboratories using some of the endotoxin

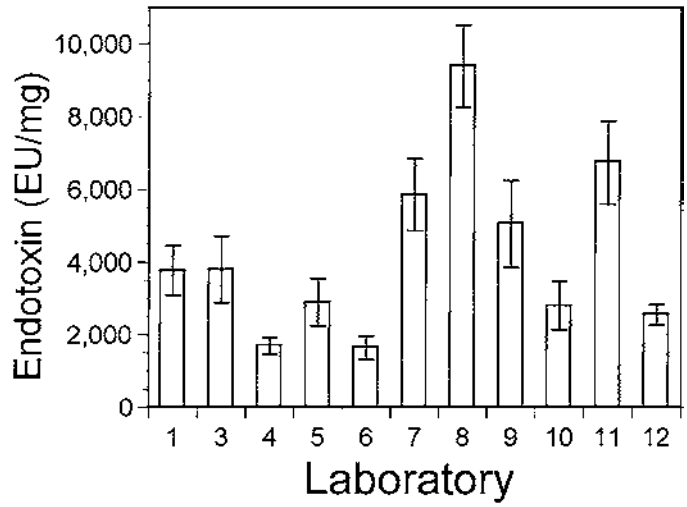

FIGURE 1
Average assay results of the participating laboratories (EU/mg; each half bar represents 2 s.e.).

TABLE III
Average assay results as Log_{10}EU/mg of the participating laboratories

Laboratory ID	Average Log_{10}EU/mg	Duncan grouping[A]	Standard error, Log_{10}EU/mg	Endotoxin assay kit[B]
8	3.968	A	0.0248	Kinetic-QCL
11	3.819	B	0.0361	in-house
7	3.758	BC	0.0375	Kinetic-QCL
9	3.685	C	0.0480	in-house
1	3.566	D	0.0373	QCL-1000
3	3.558	D	0.0585	Kinetic-QCL
5	3.443	DE	0.0542	Pyrogent-5000
10	3.429	E	0.0475	Kinetic-QCL
12	3.404	E	0.0250	QCL-1000
4	3.223	F	0.0288	Kinetic-QCL
6	3.202	F	0.0447	QCL-1000

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

[B]Four categories of kit types: three of the four kit types were endotoxin assay kits manufactured by BioWhittaker, Inc. (QCL-1000, Kinetic-QCL, and Pyrogent-5000) and the fourth kit type was a catch-all to include assay kits not manufactured by BioWhittaker, Inc. or a BioWhittaker assay kit used differently from the kit instructions.

TABLE IV
Average results based on endotoxin assay kit type

Assay kit type[A]	Average Log_{10}EU/mg	Duncan grouping[B]	Standard error. (Log_{10}EU/mg)
in-house	3.752	A	0.0337
Kinetic-QCL	3.593	B	0.0466
Pyrogent-5000	3.443	C	0.0542
QCL-1000	3.391	C	0.0603

[A]Four categories of kit types: three of the four kit types were endotoxin assay kits manufactured by BioWhittaker, Inc. (QCL-1000, Kinetic-QCL, and Pyrogent-5000) and the fourth kit type was a catch-all to include assay kits not manufactured by BioWhittaker, Inc. or a BioWhittaker assay kit used differently from the kit instructions.

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

kit types were small and no strong conclusions should be made at this time that one endotoxin kit type favored production of higher results over others (Table III).

This study is far from the most comprehensive because many factors are not addressed. Some of the factors have been explored elsewhere and dealt mostly with extraction, bioaerosol source, and filter media[11–13,16,21]; among those factors not addressed here is that possibly an important source of variability in results had derived from differences between lots of LAL used in the analyses (Milton, personal communication). However, one of the goals of this study was to see how wide the gap was between results from different laboratories; results from the two parts of this study have shown that although intra-laboratory differences were small, inter-laboratory results can vary widely. However, the results from the second part of this study were encouraging because, by just adopting a common extraction protocol, this gap was reduced considerably. This suggests strongly that further standardization might reduce the differences even more to the point that inter-laboratory results might become directly comparable.

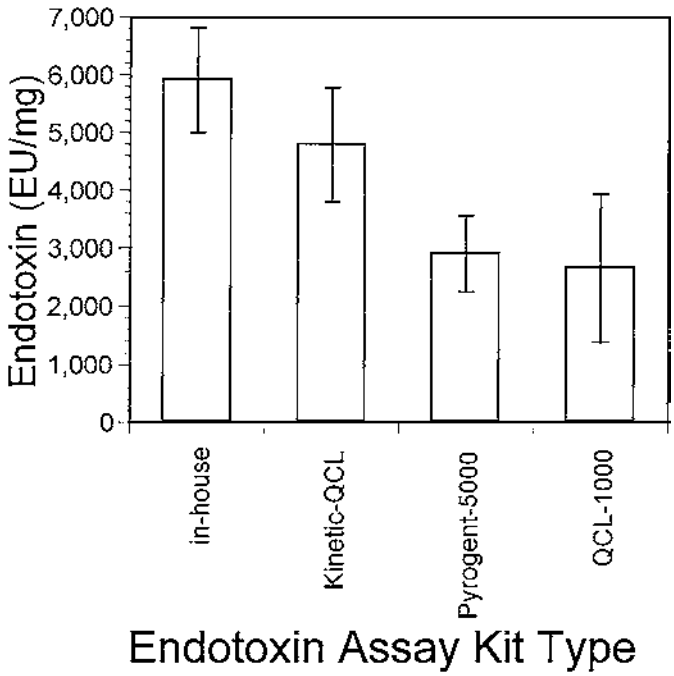

FIGURE 2
Average assay results made with the four categories of kit types: three of the four kit types were endotoxin assay kits manufactured by BioWhittaker, Inc. (QCL-1000, Kinetic-QCL, and Pyrogent-5000) and the fourth kit type was a catch-all to include assay kits not manufactured by BioWhittaker, Inc. or a BioWhittaker assay kit used differently from the kit instructions (EU/mg; each half bar represents 2 s.e.).

DISCLAIMER

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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