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History and Results of the Two Inter-Laboratory Round Robin Endotoxin Assay Studies on Cotton Dust

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Background *In the US cotton industry, airborne cotton dust levels are regulated, and other countries are moving to specify safety limits for airborne endotoxins. There is concern about potential respiratory health hazards associated with agricultural and other organic dusts. In laboratories, ranking which samples have high and low levels of endotoxin is usually in good agreement between laboratories. When different laboratories assay identical samples, the levels differ. The objective of this research was to evaluate the intra- and inter-laboratory variability for 13 laboratories measuring endotoxin in cotton dust.*

Method *Two inter-laboratory round robin endotoxin assay studies were conducted using cotton dust. In the first round robin, each laboratory used their normal in-house assay method and then used a common extraction protocol. In the second round robin, a common extraction protocol and endotoxin assay kit was used.*

Results *The intra-laboratory results had small variations but inter-laboratory results had very high variations. The inter-laboratory results using a common extraction protocol showed reduced differences. Using the same extraction protocol and endotoxin assay kit, the intra-laboratory variation was small and inter-laboratory variation was reduced but not enough for inter-laboratory agreement. Most of the laboratories were able to discern between the high and low endotoxin concentration dusts.*

Conclusions *Standardization has reduced the differences in results between laboratories and possibly further standardization may bring closer inter-laboratory agreement.* Am. J. Ind. Med. 49:301–306, 2006. *Published 2006 Wiley-Liss, Inc.*†

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INTRODUCTION

One of the most serious health hazards associated with cotton is the occupational respiratory disease of byssinosis, whose victims first suffer acute and later chronic symptoms characterized by shortness of breath, coughing, wheezing, and decreased respiratory function. Historically, cotton dust and byssinosis are linked. This disease has been studied worldwide and has been of special interest to the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), at the Cotton Quality Research Station (CQRS) in Clemson, South Carolina, where the relationship between cotton dust and byssinosis has been studied for over two decades. Through the work done at other laboratories worldwide and the cooperative work done at CQRS, the most likely etiological agent within cotton dust became focused on gram-negative bacteria (GNB) and then later more specifically to the complex lipopolysaccharides (LPS) constituents of the external wall of GNB, called endotoxins [Castellan, 1997]. In the US cotton industry, airborne cotton dust levels are regulated, but other countries are moving to directly specifying safety limits for airborne endotoxin. More recently, other branches of agriculture and industry are becoming alarmed and concerned about potential respiratory health hazards associated with agricultural and other organic dusts. Thus, it stands to reason that endotoxin quantification is important not only in the study of respiratory dysfunctions but also in the realm of developing occupational health standards.

The sticking problem for researchers and others concerned with endotoxin levels, in cotton lint or dust and now in agricultural and other organic dusts, is exemplified by the experience at CQRS. During testing of cotton dusts from different sources, when identical samples were sent to different laboratories to be assayed for endotoxin, the results differed. Some small differences were to be expected since even though the majority of endotoxin assays are based on the limulus amoebocyte lysate (LAL) test for measuring LPS (endotoxins), many variations of the LAL assay are employed. Still, all the LAL assays refer back to a reference standard and should give approximately the same results for the same sample. However, the differences between the various laboratories often differed by orders of magnitude. While the quantitative levels returned to CQRS were often different, the rankings of the samples were nearly always the same between the different laboratories. This has permitted comparisons to be made for research purposes and accredits the various endotoxin assays for providing useful information. Still the difference in levels between identical samples has always been a bothersome concern. This has meant that endotoxin results of the same samples reported by different laboratories or read in the literature from different laboratories may or may not be directly comparable and must, therefore, be interpreted with caution with due consideration of the extraction and assay methods, the laboratory conducting the analysis, and possibly other variables.

The background behind efforts to resolve this conundrum began in the early 90s, when an inter-laboratory study using uniform cotton dust samples was proposed by a group of scientists involved with the relationship between byssinosis and cotton dust [Perkins et al., 1996]. Most notable among these were Henry H. Perkins, Jr., USDA, ARS, Clemson, SC (retired); Stephen A. Olenchock, NIOSH, Morgantown, WV; Ragnar Rylander, University of Gothenburg, Sweden; and Robert R. Jacobs, Eastern Virginia Medical School, Norfolk, VA. The study was originally envisioned to be a one-time study involving as many as the 4–6 laboratories, already involved with assaying cotton for endotoxin. However, except for planning and discussions, it was not until 1995 that the study was initiated beginning with collecting uniform cotton dust samples at CQRS [Perkins et al., 1996]. The study finally began in 1997 as a round robin study with two goals. The first objective was to determine the extent of variability among laboratories and the second objective was to determine if more commonality in the assay would make the results more comparable between laboratories. In both parts of the round robin, filter membranes with the same approximate amount and type of cotton dust were sent for analysis to research laboratories that ''routinely'' perform endotoxin analyses. In the first part of the study, 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 this first round robin were encouraging and prompted a follow-up round robin study [Chun et al., 1999]. In this second round robin study, a common extraction protocol as well as the same assay procedure and kit was adopted. Additionally, as a new supply of dust samples were created for this new round robin study, the dust samples consisted of both high endotoxin and low endotoxin content. This second round robin study is described in more detail elsewhere [Chun et al., 2002; Jacobs and Chun, 2004].

MATERIALS AND METHODS

Participating Laboratories

The first round robin study began with 14 laboratories, however 2 of the laboratories dropped out. An additional laboratory asked to take part in the study at a later date so 13 laboratories participated in the second part of the first round robin study. In the second round robin [Chun et al., 2002; Jacobs and Chun, 2004], 12 laboratories that 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 (Formerly BioWhittaker, Inc., Walkersville, MD, now Cambrex Bio Science Walkersville, Inc.: 50-650U, Kinetic-QCL 192 Test Kit) participated in the study. One of the laboratories also had the ability to run gas chromatographic-mass spectrometry (GC-MS) for total endotoxin (as purified LPS). This was a different laboratory than the one in the first round robin study.

Cotton Dust

In the first round robin study, the series of dust filters were collected in 1995 [Perkins et al., 1996]. Cotton dust was selected for the test material since CQRS has experience and an interest with cotton dust and its relation to byssinosis, as did the original organizers of the study. The dust collected was uniform, card generated, vertically elutriated cotton dust averaging from 0.3 to 0.7 mg per filter with a target of 0.5 mg per filter; and contained endotoxin levels that did not vary significantly. Each laboratory received a total of either seven or six filter samples in the first part and either seven or eight filter samples for analysis in the second part of the first round robin study. In the second round robin, a second set of dust-laden filters was collected in 1998 as described by Chun et al. [1999]. Each laboratory was given eight filter samples for analysis: four samples with dust of low endotoxin concentration and four samples with dust of high endotoxin concentration.

The General Approach

Each laboratory was randomly assigned an identification number for each of the three times they received samples for assay, except for the laboratory doing GC-MS analysis [Mårtensson et al., 1997; Saraf et al., 1999]. The first round robin study involved two parts. In the first part, each laboratory did the endotoxin analysis based on their in-house protocol. In the second part of the study, which was to see if variation between laboratories could be reduced, the ideal situation would have been conducted with all participating laboratories using the same extraction protocol, the same lot of the same assay kit, and all using the identical assay protocol including the same plate reader and analysis software. However, this study was not supported by external funding other than the generosity of the on-hand resources of the participating laboratories. More weight was placed on the methodology used by the laboratories whose assays yielded the higher levels of endotoxin. A common extraction protocol was adopted to attempt 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. These factors still remain as an unexplained systematic error. Again, the in-house protocol was used except that a common extraction protocol was used [Chun et al., 1999], except by the laboratory performing GC-MS analysis. Finally, in the second round robin, which is described in greater detail elsewhere [Chun et al., 2002; Jacobs and Chun, 2004], a common extraction protocol was used and a common endotoxin assay kit, Bio-Whittaker Kinetic QCL assay was used.

RESULTS

When the different laboratories analyzed ''identical'' dust samples, the results obtained were significantly different, even when the results from the laboratory using GC-MS were removed from the comparison (Table I). Variations within laboratories appear to be small (Fig. 1) so that results within a laboratory can be usefully employed to rank samples having different endotoxin contents. Later

TABLE I. Average Assay Results as EU/mg of the Participating Laboratories, From Part 1 of the Inter-Laboratory Endotoxin Assay Study

Laboratory[a] ID	Average EU/mg (Log_{10}EU/mg[b])	Average EU/mg (Log_{10}EU/mg[b,c])
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}
1	2.848^{H}	2.848^{G}
12	2.838^{H}	2.838^{G}
5[d]	0.840^{I}	0.840^{H}

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

[c]Average assay results as EU/mg of the participating laboratories, excluding Lab 14 (which used GC-MC).

[d]Assay was later redone on a second set of filters, which indicated that an error had probably occurred in the first assay resulting in the unusually low results reported here.

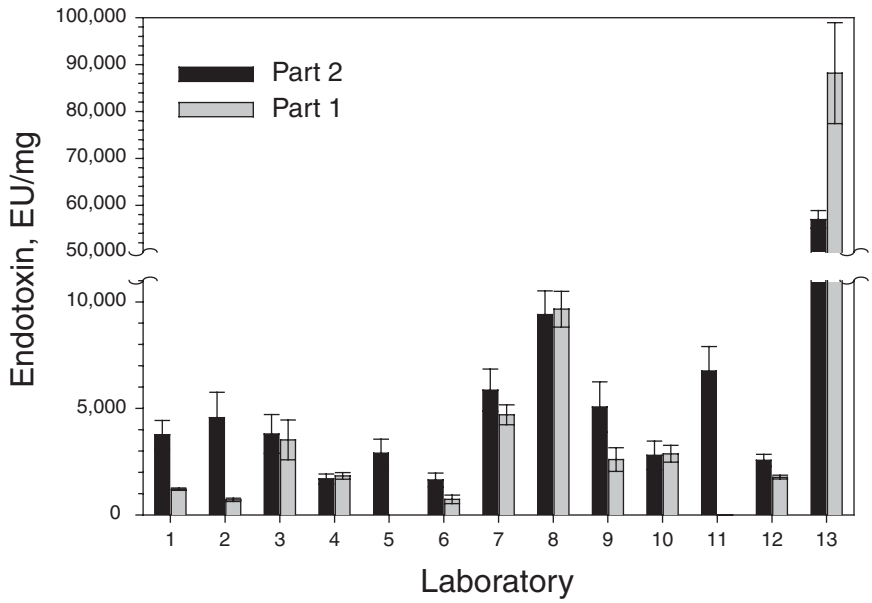

FIGURE 1. Average assay results from the same laboratories from part 2 and 1 of the first Round Robin Endotoxin Assay study, the laboratory ID number from part 2 of the study is used to identify the same laboratory in part 1 and 2 of the study; the axis break represents a change in scale to accommodate results from the laboratory doing GC-MS, EU/mg; each half bar represents 2 SE.

when a common extraction protocol with their own in-house assay was used, the results were significantly different and the variations found within the different laboratories were very small (Table II and Fig. 1) as before. While the differences among the laboratories were significantly different, the overlaps of the Duncan groupings were greater when a common extraction protocol was used (Tables I and II). The EU/mg (Log_{10}EU/mg) range was large, 0.84–3.98 when only the in-house assay was used; even when omitting the one anomalous low value, the range was still large, from 2.84 to 3.98. But in the second part of the study, where a common extraction protocol was adopted to the in-house assay, the range of the average Log_{10}EU/mg was much reduced and within the same order of magnitude, 3.20–3.97 (Table II). In addition, when a common extraction protocol was adopted, the overall concentration tended to be higher than reported in the first part of the study (Fig. 1), even though the samples were ''identical.''

When the average endotoxin concentration was sorted by endotoxin assay kit type used in the first round robin study, significant differences were observed between the assay kit type and that the in-house and Kinetic-QCL kit types tended to favor results with higher concentrations. The variation within assay kit type was small (Fig. 2). However, only a small number of laboratories used each of the endotoxin kit types. At this time, no conclusions could be drawn that specific endotoxin kit types favored lower or higher endotoxin results than others.

TABLE II. Average Assay Results as EU/mg and Endotoxin Assay Kit Type of the Participating Laboratories, From Part 2 of the Inter-Laboratory Endotoxin Assay Study

Laboratory ID	Average EU/mg (Log_{10}EU/mg[a])	Average EU/mg (Log_{10}EU/mg[a,b])	Endotoxin assay kit type[c]
13	4.755^{A}	—	
8	3.968^{B}	3.968^{A}	Kinetic-QCL
11	3.819^{C}	3.819^{B}	In-house
7	3.758^{CD}	3.758^{BC}	Kinetic-QCL
9	3.685^{DE}	3.685^{CD}	In-house
2	3.636^{DE}	3.636^{CD}	In-house
1	3.566^{EF}	3.566^{DE}	QCL-1000
3	3.558^{EF}	3.558^{DEF}	Kinetic-QCL
5	3.443^{FG}	3.443^{EFG}	Pyrogent-5000
10	3.429^{G}	3.429^{FG}	Kinetic-QCL
12	3.404^{G}	3.404^{G}	QCL-1000
4	3.223^{H}	3.223^{H}	Kinetic-QCL
6	3.202^{H}	3.202^{H}	QCL-1000

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

[b]Average assay results as EU/mg of the participating laboratories, excluding Lab 13 (which used GC-MC).

[c]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 LAL formulations not manufactured by BioWhittaker or using BioWhittaker kinetic LAL reagents used with conditions and standards independently referenced to EC6.

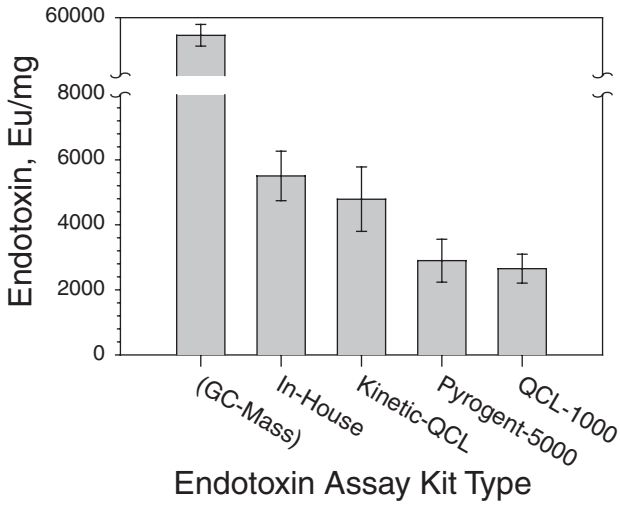

FIGURE 2. Average assay results from Part 2 of the study made with the method for total endotoxin content and by the four categories of kit types: three of the four kit types were endotoxin assay kits manufactured by BioWhittaker, Inc. (QCL-1000, Kinetic-QCL, and Pyrogent-5000) and the fourth kit type was a catch-all to include LAL formulations not manufactured by BioWhittaker or using BioWhittaker kinetic LAL reagents used with conditions and standards independently referenced to EC6.

The results of the second round robin where the same extraction procedure and endotoxin test kits were used are described in more detail elsewhere [Chun et al., 2002; Jacobs and Chun, 2004]. What was of interest was that the results on the low endotoxin concentration dust differed by about an order of magnitude, the EU/mg (Log_{10}EU/mg) ranged from 3.32 to 4.39; and the results of the high endotoxin concentration dust, with the exception of the results of one laboratory, were all within the same order of magnitude 4.25–4.92. Again while the intra-laboratory variation was small, the differences between laboratories gave results for the same dust sample that were significantly different. With the exception of one laboratory, all of the laboratories were able to discern between the high and low endotoxin concentration dusts.

DISCUSSION AND CONCLUSIONS

The results from this study came from research laboratories over a worldwide geographical and national range, and clearly indicate that significant differences in results can be expected when the same sample is assayed. These differences underscore the problem of comparing results made from one laboratory with those made by another laboratory and is important to point out since even some people engaged with endotoxin and its consequences are unaware of such inter-laboratory discrepancies. To circumvent such inter-laboratory discrepancies, the adoption of analysis for total endotoxin as the standard method of analysis has been suggested. However, the method (GC-MS) is not readily available to most laboratories currently involved with endotoxin analysis and requires greater resources to obtain and maintain. A more crucial question is whether total endotoxin, as pure LPS, relates best to the biological availability of endotoxin and hence its biological activity or health relevance, since under real-life conditions endotoxin includes bacterial proteins and other cell constituents as well as LPS. This is particularly more germane to cotton dust which is considerably more complex than swine or poultry dust.

By adopting a common extraction protocol, the results were encouraging since the gap between different laboratories was reduced considerably. Not only was the range of results smaller (2.84–3.98 vs. 3.20–3.97 Log_{10}EU/mg), but also the variation in the laboratory results was reduced (SD = 0.357 and 0.2317, respectively). Further standardization by adopting the same assay protocol and the same endotoxin assay kit from the same production lot provided results that were still significantly different between laboratories. However, as before, intra-laboratory variation is small so comparisons within laboratories would permit internal comparison of samples. It is encouraging to note that most of the laboratories were able to distinguish between samples having low and high endotoxin concentrations. Since some success for reducing the differences between different laboratories has been achieved by standardization, perhaps examination of the reasons for inter-laboratory differences and further standardization, such as training workshops or apprenticeship-training programs, use of identical equipment, etc., may eventually lead to inter-laboratory assays of identical samples that can be comparable. Alternatively, with the knowledge that inter-laboratory discrepancies exists, perhaps an entirely new assay method or refinement of the current endotoxin assays may be developed that will permit different laboratories to obtain comparable results when assaying the same sample.

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