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Effects of Various Treatments on the Quantitative Recovery of Endotoxin from Water-Soluble Metalworking Fluids

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Three extraction methods were compared for their effectiveness in the quantitative removal of endotoxin from unused and used bulk water-soluble metalworking fluid (MWF) samples. Soluble, synthetic, and semisynthetic fluids were studied. The three modes of extraction consisted of (1) pyrogen-free water (PFW); (2) PFW and Tween 20 (polyoxyethylene sorbitan monolaurate); and (3) PFW, Tween 20, and sonication. Results suggest that vigorous recovery methods yield higher amounts of endotoxin from MWF samples than mild recovery methods in PFW alone. Additional studies are required to aid in the understanding of the factors that significantly affect endotoxin extraction yields from these fluids.

Keywords: endotoxin, *Limulus* amoebocyte lysate (LAL) assay, metalworking fluids

Researchers at the National Institute for Occupational Safety and Health have initiated investigations in response to concerns about respiratory illnesses in workers who are exposed to aerosolized metalworking fluids (MWFs). An MWF constituent thought to cause health effects is endotoxin. Endotoxin, an ubiquitous constituent of water-based MWF aerosols and a potential etiological agent in MWF-inspired respiratory illnesses, is a biomolecule of the outer membrane of gram-negative bacteria. This lipopolysaccharide has been implicated as a cause of adverse respiratory effects in a variety of occupational environments.[1–6] One estimate suggests that more than 10 million workers are exposed to MWF mists.[7]

The most widely used analytical method for endotoxins is based on the *Limulus* amoebocyte lysate (LAL) assay, which measures the biologically available portion of endotoxin, i.e., Lipid A, in a given sample. Numerous variations of this bioassay have been used extensively to quantify endotoxin in a variety of sample matrices.[8–11] However, few methods are recognized by consensus standards or industrial hygiene organizations for the sampling, extraction, and analysis of environmental endotoxins. This lack of method standardization fosters inconsistency of results and makes interlaboratory comparisons of environmental endotoxin data questionable.[12] For instance, one study showed a 10-fold difference in endotoxin yields due to different extraction methods.[13] Another study in which various methods were used to extract endotoxin from house dust produced greater endotoxin activity with sonication of membrane filters in phosphate-triethylamine buffer, as opposed to extraction with addition of saponin or sodium dodecyl sulfate.[14] An investigation of airborne endotoxin concentrations in animal confinement buildings compared two aqueous filter methods in which extraction occurred for 120 min at 22°C with vigorous shaking, or 30 min at 68°C with gentle rocking.[15] Data from this study showed that endotoxin activities from the two methods were not significantly different and were highly correlated. Numerous microbiological studies involving bulk MWFs have placed much emphasis on the characterization of microbial populations and their health effects.[16–20] The research described in this article addresses a need to develop quantitative methods for the quantitative recovery of endotoxin from bulk water-soluble MWF sample matrices.

MATERIALS

Samples were analyzed for endotoxin by using Kinetic-QCL test kits (BioWhittaker, Inc., Walkersville, Md., Lot no. 6L5430). Each

Preliminary results of this study were presented at the 6th Annual National Institute for Occupational Safety and Health Interdivisional Aerosol Symposium at Ohio State University in Columbus, Ohio, September 24, 1997.
Mention of company names or products does not constitute endorsement by the National Institute for Occupational Safety and Health.
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BioWhittaker kit contained (1) lyophilized *E. coli* 055:B5 control standard endotoxin (50 endotoxin units per milliliter, EU/mL, Lot no. 6L2950); (2) pyrogen-free water (Lot no. 6L4870); and (3) lyophilized (LAL) (Lot no. 6L437W); all kits were stored at 2–8°C prior to use. Disposable borosilicate glass tubes (BioWhittaker, Cat. no. N201) were conditioned by dry-heat at 200°C for a minimum of 24 hours to destroy exogenous endotoxin. Solution manipulations were facilitated with Oxford BenchMate™ continuously adjustable pipettors; a Brinkmann eight-channel pipette; Eppendorf nonpyrogenic pipette tips (Cat. no. 2249 008-7); Fisher brand pyrogen-free pipette tips (Fisher Scientific, Pittsburgh, Pa., Cat. no. 21-197-8K) and various sizes of Pyrex® disposable sterile plugged borosilicate glass pipettes (dry-heated as described above); and Costar nonpyrogenic multipipet reagent reservoirs (Cat. no. 4870). Solutions were analyzed in Costar polystyrene 96-well, flat-bottomed nonpyrogenic microtiter plates (Cat. no. 3596). Mixing of solutions was performed with a Fisher Vortex Genie 2 vortexer (Cat. no. 12-812). Sonication was performed with a Fisher FS9H bath sonicator. A BioWhittaker K-QCL automated microtiter plate reader (Cat. no. 25-141B) with dedicated WinKQCL™ software was used for analysis of reaction data from test solutions.

MWF Samples

The study utilized 24 bulk water-soluble MWF samples: 12 unused (4 soluble, 4 semisynthetic, 4 synthetic), and 12 used (4 soluble, 4 semisynthetic, 4 synthetic). The unused samples were collected aseptically from newly opened containers at an MWF manufacturing facility and dispensed into 10 × 75 mm nonpyrogenic test tubes, where they were stored at 2–8°C, prior to analysis. Grab samples of used, i.e., spent, MWF samples, collected from a variety of plant locations with active operations involving MWFs, were dispensed and stored similarly.

METHODS

Sample Preparation

Each sample was vortexed in its original tube at full power (37 W) for 15 min at room temperature, approximately 25°C, before being subjected to the following treatments.

(1) Method 1 (normal treatment): In this method, an aliquot of 100 μL of a given MWF sample was pipetted into a nonpyrogenic borosilicate glass tube containing 900 μL of pyrogen-free water (PFW). This mixture was then vortexed at full power for 1 min.

(2) Method 2 (Tween-20 treatment): A volume of 100 μL of a given MWF sample was pipetted into a nonpyrogenic borosilicate glass tube containing 850 μL of PFW and 50 μL of Tween-20, and then the mixture was vortexed at full power for 1 min.

(3) Method 3 (Tween-20 + bath sonication treatment): A volume of 100 μL of a given MWF sample was pipetted into a nonpyrogenic borosilicate glass tube containing 850 μL of PFW and 50 μL of Tween-20, bath sonicated at full power for 15 min, and then vortexed at full power (103 W) for 1 min.

Preparation of Analyte Solutions and Positive Product Controls

A volume of 100 μL of each sample extract was plated in triplicate in the microtiter plate, according to a predetermined template pattern. Solution blanks of PFW were treated in the same manner. Lyophilized control standard endotoxin was reconstituted in its vial, as per kit directions, by adding 2.7 mL of pyrogen-free water, and vortexing this mixture for 15 min to yield an effective endotoxin concentration of 50 EU/mL. Serial dilutions of the 50 EU/mL solution were prepared in nonpyrogenic test tubes to form standard solutions with concentrations of 5.0, 0.5, 0.05, and 0.005 EU/mL. Each standard solution was dispensed into wells of a nonpyrogenic microtiter plate in triplicate volumes of 100 μL, according to the template pattern.

Positive product controls (PPCs) were prepared in parallel for each set of extracted samples by pipetting 100 μL of a given sample into an assigned well and then adding 10 μL of a 5 EU/mL control standard endotoxin solution. All PPCs were prepared in triplicate.

Subsequent to the dispensing of all analyte solutions in triplicate in the nonpyrogenic microtiter plate, the plate was positioned in the microplate reader chamber and pre-incubated at 37°C and shaken on a programmed basis for 12 min in the reader.

LAL Analysis

Kinetic-QCL reagent (colyophilized mixture of lysate and chromogenic substrate) was reconstituted in its vial with 2.6 mL of nonpyrogenic water by gentle swirling to avoid foaming. On manual ejection of the microtiter plate at the end of the preincubation period, a multipipettor was used to dispense 100 μL of the reconstituted Kinetic-QCL reagent into each analyte well of the microtiter plate. On reintroduction of the microplate within the reader, the computed-aided assay was performed on an automated basis with WinQCL software.

Brief Explanation of the Assay

The assay used in this study involves the activation of an enzyme which catalyzes the release of p-nitroaniline (p-NA) from a synthetic substrate, i.e., Ac-Ile-Glu-Ala-Arg-pNA:

$$\text{Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Enzyme} \quad (1)$$

$$\text{Substrate} + H_2O \xrightarrow{\text{Enzyme}} \text{Peptide} + \text{pNA} \quad (2)$$

The reaction imparts a yellow color to the analyte solution, which is then continuously measured photometrically at 405 nm during the assay.

Computer software (BioWhittaker K-QCL) associated with the endotoxin reader computes the endotoxin concentration of samples by performing log/log linear correlation calculations.

Reaction time is inversely proportional to the amount of endotoxin present; the more endotoxin present the shorter the reaction time. The color intensity of an analyte solution is proportional to the amount of endotoxin in the sample. A log/log correlation exits between the release of p-NA and endotoxin concentration and the relationship is linear from 0.005 to 50.0 EU/mL.

RESULTS

In accordance with Kinetic-QCL kit performance criteria, results were considered valid, i.e., neither inhibited or enhanced, if they met the following criteria.

(1) The linearity of the standard curve (log-log) was verified if the correlation coefficient of the absolute value (r)≥0.980.

(2) The coefficient of variation for the triplicate samples and PPCs ≤10%.

(3) Lack of inhibition or enhancement as demonstrated by accurate

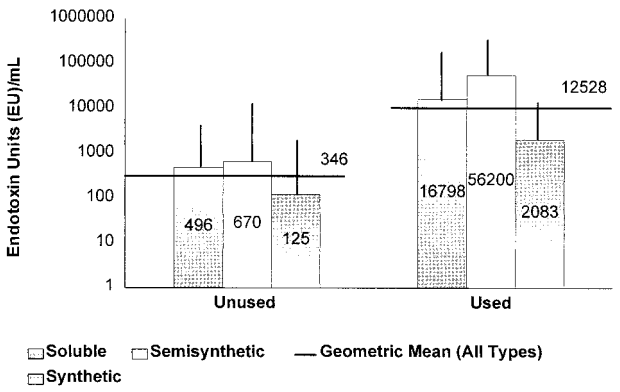

FIGURE 1. Comparison of endotoxin levels in used versus unused fluids

endotoxin recoveries (±50%) for the known concentration of the positive control spikes, according to the kit manufacturer criteria.

As part of a preliminary determination, the optimal dilution for each sample was selected, i.e., a value from a dilution that met the validity criteria described above. Values below the 0.005 EU/mL detection limit were assigned values equal to the detection limit divided by 2 (L/2), where L = the limit of detection.[21]

Statistical data analyses were facilitated by using the analysis of variance (ANOVA) procedure and Sheffe's multiple comparison test to examine differences in unused and used fluid samples (see Figure 1). Differences in the effects of the methodologies on the three classes of fluids are shown in Figure 2. Raw data generated by the WinKQCL software was transformed to a log scale for statistical analysis to meet the general assumptions of normality and homogeneity of variance required by the ANOVA procedure.[22–24] Predictably, all used fluid samples had significantly higher levels of endotoxin than unused samples regardless of the class of MWF or the type of extraction method, according to the probability level, i.e., p-value = 0.001. There were no significant differences among the classes of MWF for the unused samples as shown in Figure 1.

For the soluble fluids, the endotoxin concentrations resulting from the differing sample preparations (Methods 1, 2, and 3) were not significantly different from each other. A significant difference

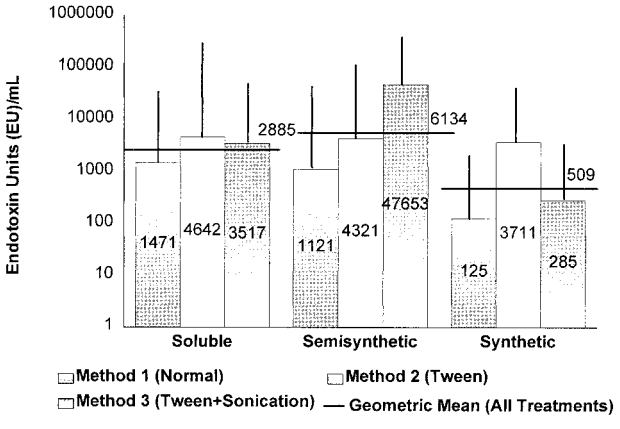

FIGURE 2. Comparison of endotoxin levels by type of MWF (used and unused)

TABLE I. Geometric Mean (GM) and Geometric Standard Deviation (GSD) of Endotoxin Levels (Units in EU/mL)

	Soluble		Semisynthetic		Synthetic	
	GM	GSD	GM	GSD	GM	GSD
Unused	496	8.8	670	15.6	125	18
Used	16,798	13.7	56,200	7	2,083	7.7

in endotoxin recovered from semisynthetic fluids was found (p = 0.002). When these fluids were treated with Method 3 (PFW + Tween + sonication), the most vigorous method, there were higher endotoxin yields than those treated with Method 1 (normal), the least vigorous method (47,700 and 1100 EU/mL, respectively). On analysis of used fluid samples, for synthetic fluids, a significant difference was found (p = 0.01); used and unused fluids subjected to Method 2 (Tween), i.e., moderate treatment, yielded higher recoveries than either Method 1 or Method 3, as shown in Figure 2.

Statistical analysis of the log-transformed data was performed to determine the homogeneity of variance. The geometric means and geometric standard deviations of the data sets corresponding to Figures 1 and 2 are shown in Tables I and II, respectively.

DISCUSSION

The results of this study demonstrate the influences that various methods have on the relative yields of endotoxin from bulk water-soluble MWFs. Though MWFs have numerous formulations, many of which are proprietary, the choice of aggressive extraction methods for the provision of quantitative endotoxin recovery data appears to be preferable to relatively mild treatments. Thus, the resultant data suggest modes of extraction rather than a specific method. Indeed, there may be numerous specific combinations of physical and chemical extraction procedures that will produce optimal endotoxin yields from certain water-soluble MWFs, as opposed to the usage of generalized methods for a wide range of these fluids. However, for purposes of promulgating standard methods, endotoxin methods and generalized performance criteria are needed for a wide variety of water-soluble fluids.

This and similar studies suggest that water extraction alone does not reflect an accurate measure of endotoxin in bulk MWFs.[25–27] As a way of facilitating endotoxin standard methods development, the careful selection of representative types of MWF in each major fluid category may be an approach to reduce data variability and facilitate interlaboratory comparisons of data.

Endotoxin recoveries for the PPCs in this study met prescribed assay criteria (±50%), which is indicative that endotoxin is not bound by the sample matrix. This result is interesting, because it

TABLE II. Geometric Mean (GM) and Geometric Standard Deviation (GSD) of Endotoxin Levels (Units in EU/mL)

	Method 1 (Normal)		Method 2 (Tween)		Method 3 (Tween + Sonication)	
	GM	GSD	GM	GSD	GM	GSD
Soluble	1471	25	4642	26	3517	14
Semisynthetic	1121	38	4321	24.5	47,653	5.6
Synthetic	125	16	3711	11	285	11

shows that PPC recoveries may not be good indicators of endotoxin recovery from bulk samples. However, further research is needed in this area.

The development of consensus and quantitative methods for extracting endotoxins from MWFs is requisite for providing reliable data that can be used by MWF users and industrial hygienists to make a variety of decisions to protect worker health. In this regard, a recent effort to provide standardization to environmental LAL methodology is exemplified in a recently approved ASTM method for the personal sampling and analysis of endotoxin in MWFs.[28]

Endotoxin concentrations in bulk MWFs are indicative of the content and proliferation of gram-negative bacteria species in a given fluid distribution system. Therefore, the development of effective methods to determine endotoxin concentrations in MWFs can provide important data relevant to MWF management and the maintenance of distribution systems. Furthermore, in machining facilities that utilize MWFs, there is a continuous production of endotoxin-laden aerosols, especially during high-energy processes. As the result of the latter conditions, the predisposition of workers to endotoxin-induced respiratory disorders by the inhalation of respirable aerosols may be correlated to the gram-negative bacteria concentration in the bulk fluids of the distributions systems. In a study involving guinea pigs, results demonstrated that used MWF aerosols produced airway obstruction and acute lung injury, whereas unused MWF aerosols produced minimal effects.[29] The observed effects in this study were attributed to microbial contamination. Studies are needed to definitively describe the nature of the latter relationship. In this regard, our research and other studies may prove helpful in providing answers to questions about the role that endotoxins have on the respiratory health of workers in MWF environments.

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