Bull. Environ. Contam. Toxicol. (1999) 62:25-29

Bulletin of Environmental Contamination and Toxicology

Interlaboratory Comparison of Endotoxin Analyses in Occupational Exposure

R. Sarantila,[1] J. Kangas,[2] H. Savolainen[3]

[1] Service of Occupational Health, City of Kokkola, Central Administrative Office, Post Office Box 43, FIN-67101 Kokkola, Finland
[2] Regional Institute of Occupational Health, Post Office Box 93, FIN-70701 Kuopio, Finland
[3] Institute of Occupational Health Sciences, Lausanne, Switzerland

Received: 26August 1998/Accepted: 17 November 1998

Bacterial endotoxins are very potent biological contaminants of many organic or otherwise perishable dusts. Furthermore, they show also structure-related potency in their toxic and inflammatory effects. This has a direct impact on the measuring methods, as the endotoxin levels are conventionally analyzed with the so-called Limulus lysate test which makes use of their toxicity. This study shows that the extraction method is the most critical aspect in the analysis, while the reference toxin has a lesser impact on the standard curves, provided that the samples are appropriately diluted to the linear section of the graph. For quality assurance, it is necessary to participate frequently in a control scheme.

Endotoxins are an integral part of Gram-negative bacteria. They are complex lipopolysaccharides that have important toxic and inflammatory effects already at very small concentrations. Different bacteria and different strains of the same species may show molecular heterogeneity which may affect their biological effects (Rietschel et al. 1994). This is important from the practical point of view as the endotoxin concentrations are mostly assayed for practical purposes by the so-called Limulus lysate test (Milton et al. 1990).

The established no-effect levels of inhaled endotoxin are quite low in healthy control subjects (Michel et al. 1997) and even lower concentrations can be tolerated by asthmatic subjects (Michel et al. 1996). This sets obvious limits to the analytical laboratories as to their method and proficiency. Therefore, in order to control the accuracy of ordinary service analyses, we carried out a blinded evaluation of endotoxin analyses in three well-established laboratories.

MATERIALS AND METHODS

Air samples were collected on glass fiber filters with a tested pore size of 0.3 µm (Macherey-Nagel Corp., NN 85/220, diameter 37 mm). They were autoclaved 20 min before use at 121°C. For reference, samples were also collected on cellulose filters (pore size 0.45 µm, Millipore HA). For actual sampling, the filters were aseptically placed in plastic filter holders (Millipore MAWG 037 AO). Air samples were then collected by pumping air through the filters for 60 min at a velocity of

Correspondence to: R. Sarantila

2.5 1/mm. All samples were then stored at -20°C until analysis. In the standard procedure, the filters were transferred aseptically to vials containing 10 ml of pyrogen-free water which were then shaken for 1 h at room temperature. An aliquot of the water was taken and centrifuged at 1,000 r.p.m. for 20 min. The supernatant was then diluted with the pyrogen-free water to 1:10-1:10^6 to achieve the linear working concentrations for the Limulus lysate test (0.02-0.1 ng/ml).

Limulus lysate (Associates of Capecod, Inc., MA, USA) was dissolved in ice-cold 0.4 M Tris-$MgCl_2$ buffer (10 ml, pH 8.0) which was then divided into 10 one-ml tubes. Chromogenic substrate (S-2423) was purchased from Kabi-Vitrum (Mölndal, Sweden) and the reference lipopolysaccharide (LPS) standard was the E.coli 111:B4 obtained from KabiVitrum (Mölndal, Sweden). Pyrogen-free water was used throughout the work and the glassware was autoclaved at 121°C for 20 min before use.

100 µl of substrate were pipetted in ice cold test tubes, then 100 µl of Limulus lysate solution were added followed by the diluted samples or standards. The tubes were capped and shaken for one min and then incubated at 37°C for 15 min. The reaction was ended by adding 600 µl of 20% acetic acid in each tube. The samples were' then transferred onto microtiter plates and the absorbance in each well was measured at 405 nm (Multiskan MCC/340, Labsystems, Helsinki, Finland). Standard E.Coli 111:B4 endotoxins gave identical results with the EC-5 standard in this procedure, and the filter blanks were all negative.

For interlaboratory comparison water samples containing 1 to 49 000 ng endotoxin/ml were exchanged with another laboratory which used E.Coli 026:B6 as a standard endotoxin. At a later stage, grain dust samples were shared with three laboratories which used their standard methods for the extraction technique and endotoxin.

RESULTS AND DISCUSSION

The different batches furnished KabiVitrum or by Bioproducts, Inc. (MD, USA) obtained consistently somewhat differing results (Table 1).

It seems that this systematic difference was due to minor differences in the composition of the lyophilized endotoxin powder. It would therefore, be important to know the origin of the standards when comparing results even in the same laboratory.

When comparing the results of the water samples in the two laboratories, we found the results to be in agreement (Table 2).

Table 1. Comparison of two E.Coli 111:B4 standards

Sample	KabiVitrum	Bioproducts	Recovery[a]
1	4.9	3.5	71
2	4.9	3.6	74
3	7.5	5.3	71
4	12	9.0	75
5	14	10	71
6	174	123	71

Each figure (ng/ml) represents the mean of triplicate analyses.
[a]compared to KabiVitrum batch

Table 2. Comparison of endotoxin water samples

Sample	Endotoxin concentration (ng/ml)	
	Laboratory I	Laboratory II
1	1	1.1
2	1.3	2.4
3	2.2	0.9
4	3.1	3.8
5	33.5	2.8
6	7600	5400
7	46000	49000

Table 3. Comparison of endotoxin analyses in grain dust samples

	Laboratory				
	I	**II**		**III**	
Endotoxin	111:B4	111:B4	026:B6	111:B4	026:B6
Extraction liquid	1% saponin in PFW	0.01% albumin in PFW	PFW	PFW	PFW
Rye dust	845	830	108	107	122
Wheat dust	5,169	3,400	n.d.	229	123
Wheat dust	900	700	3	1	2
Mixed oats and barley dust	6,736	8,900	55	55	123

PFW =pyrogen-free water; n.d. = not determined. The figures (ng/mg) are means of triplicate analyses.

Laboratory I used E.Coli 111:B4 endotoxin standard and laboratory II E.Coli 026:B6 endotoxin. The results were mostly comparable, especially at high concentrations, despite the fact that the standard endotoxins were different. This meant that for practical purposes, the utilized method is acceptable.

However, the extraction technique appeared to be crucial, as it affected profoundly the endotoxin levels as extracted from the grain matrices (Table 3).

It is clear that the pyrogen-free water could extract only a minor part of the endotoxins in the dust samples. This observation has practical consequences, because the use of only one extraction method could lead to missing important endotoxin exposure. It is known that endotoxins have an affinity for cationic proteins (Anspach and Hilbeck 1995) with the histidine residue serving as the preferential binding center (Minobe et al. 1994). Consequently, the extraction of endotoxin from biological matrices becomes more complete after a proteolytic treatment of the samples (Petsch et al. 1998). Experimental protein degradation by proteinases is a lengthy procedure so that it can be applied only in specific research situations. Furthermore, it seems that the utilization of a chaotrope (saponin) or a competetive ligand (albumin) liberates most of the bound endotoxins in samples for their more quantitative assay in routine analyses.

In conclusion, current routine endotoxin analyses using the Limulus lysate test are reasonably reliable. In view of the low no-effect levels, specific chemical methods are, however, highly desirable for their analysis (Johannsen 1993).

Acknowledgment. This work was financially supported by the Finnish Work Environment Fund.

REFERENCES

Anspach FB, Hilbeck O (1995) Removal of endotoxins by affinity sorbents. J Chromatogr A 711: 81-92

Johannsen L (1993) Biological properties of bacterial peptidoglycan. Acta Path. Microbiol. Serol.101: 337-334

Michel 0, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R, Sergysels R (1996) Severity of asthma is related to endotoxin in house dust. Am J Respir Crit Care Med 154: 1614-1646

Michel 0, Nagy A-M, Schroeven M, Duchateau J, Nève J, Fondu P, Sergysels R (1997) Dose-response relationship to inhaled endotoxin in normal subjects. Am J Respir Crit Care Med 156: 1157-1164

Milton DK, Gere RJ, Feldman HA, Greaces IA (1990) Endotoxin measurement: Aerosol sampling and application of a new Limulus method. Am Ind Hyg Assoc J 51: 331-337

Minobe S, Nawata M, Shigemori N, Watanabe T (1994) Assay of endotoxin in human plasma using immobilized histidine, Limulus amoebocyte lysate and chromogenic substrate. Eur J Clin Chem Clin Biochem 32: 797-803

Petsch D, Deckwer W-D, Anspach FB (1998) Proteinase K digestion of proteins improves detection of bacterial endotoxins by the Limulus ambocyte lysate assay: Application for endotoxin removal from cationiic proteins. Analyt Biochem 259: 42-47

Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Lopnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F, Schreier M, Brade H (1994) Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J 8: 217-225