Conference Paper

ENDOTOXIN MEASUREMENT IN HOUSE DUST USING THE END-POINT LIMULUS AMOEBOCYTE LYSATE METHOD

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Endotoxin is a lipopolysaccharide, a part of Gram-negative bacteria cell membrane commonly present in general and many occupational environments. This paper describes sample preparation and endotoxin measurement in 16 samples of house dust from urban homes (Zagreb, Croatia) using end-point chromogenic Limulus amoebocyte lysate (LAL) bioassay. House dust was collected on cellulose filters by vacuuming bedroom and living room floors, and was kept frozen until assayed. Samples were extracted from filters with a 0.05 % solution of Tween-20 in endotoxin-free water. Serial dilutions of samples were measured in duplicates. The linearity of the standard curve was satisfying (r=0.983), as well as the recovery (92 and 110 %) and repeatability (coefficient of variation from 0 to 8.5 %). The endotoxin levels found in the house dust samples ranged from 4.8 to 200 EU/mg, with the arithmetic mean of 49.5 EU/mg (standard error of the mean of 12.1 EU/mg), and were in the range of house dust endotoxin values obtained by other authors.

KEY WORDS: *endotoxin, LAL assay, lipopolysaccharide, Limulus polyphemus*

Endotoxin is a lipopolysaccharide which comprises most of the cell membrane outer layer in all pathogenic and non-pathogenic Gram-negative bacteria (1). It is liberated into environment after bacterial death and during its multiplication (2).

Since Gram-negative bacteria are present all over the environment, endotoxin can be found in the soil, on vegetation, in water, as well as in the gastrointestinal tract and the upper airways (oral and nasal cavities) in humans and animals. It is, therefore, common in general environment, particularly in house dust (3, 4) and in occupational settings such as agricultural and in a number of industries such as cotton mills, sewage, waste collecting and composting (3, 5).

Endotoxin has strong inflammatory properties which are mostly attributed to the lipid component, lipid A (6). Inhaled endotoxin induces dose-related clinical symptoms (flu-like syndrome), changes in the lung function (decrease in FEV_1), and both bronchial and systemic inflammatory responses (4). In asthmatic subjects, endotoxin exposure aggravates the severity of asthma, acts in synergy with allergens, and increases susceptibility to rhinovirus infections (7-9). Endotoxin is also proposed to be one of the causative agents in byssinosis (10), and organic dust toxic syndrome (11).

Endotoxin can be measured using chemical methods such as the determination of fatty acids using gas chromatography or using biological methods, such as Limulus amoebocyte lysate (LAL) bioassay. Although chemical methods have high specificity, their sensitivity is much lower than that of LAL bioassay (3). This is why LAL test has become the gold standard for endotoxin determination. It is well standardised by the US Food and Drug Administration and is widely used for the detection of endotoxin contamination of water, food, parenteral drugs, dialysis water, blood products and immunological materials (1, 3). Amoebocytes are

the single type of cells circulating in the horseshoe crab hemolymph. They are nucleated, granulated, aggregating cells, forming a clot at the site of injury (3). Amoebocyte granules contain a clotting factor called coagulogen, which is sensitive to picogram quantities of bacterial endotoxins, specifically to the lipid A component (7, 12, 13). It was shown that clotting reaction is enzymatic, and Factor C is recognized as the endotoxin-sensitive serine protease that initiates the coagulation cascade (14) (Figure 1). Currently, four LAL techniques for endotoxin testing are commercially available and approved by the United States Pharmacopeia for the evaluation of end-product injectable drugs, medical devices, and raw materials: (1) gel-clot, (2) chromogenic assay, (3) end-point-turbidimetric, and (4) kinetic-turbidimetric (15, 16). In the most common chromogenic assay, bacterial endotoxin initiates the activation of a proenzyme in Limulus amoebocyte lysate that cleaves a synthetic chromogenic substrate, releasing p-nitroanilin (pNA) which is yellow and linearly correlates with the endotoxin concentration (Figure 1). Chromogenic assay has two variations: end-point and kinetic (15, 17, 18).

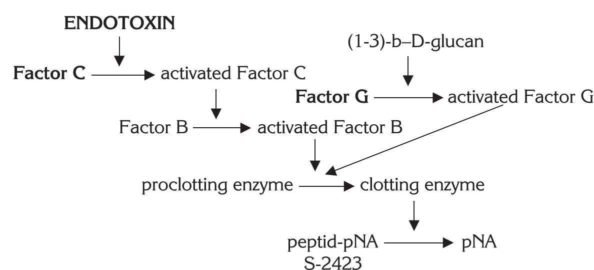

Figure 1 *Schematic representation of the coagulation cascade reaction in the horseshoe crab amoebocyte lysate initiated by endotoxin or (1-3)-β–D-glucan, where clotting enzyme cleaves a synthetic chromogenic substrate (S-2423), releasing p-nitroanilin (pNA) which produces a yellow colour that is measured spectrophotometrically. According to Ding and Ho (14).*

The Occupational Health and Environmental Medicine Unit (Unit) of the Institute for Medical Research and Occupational Health, Zagreb, Croatia, has introduced the end-point chromogenic LAL method to determine endotoxin levels in different home and occupational environments. This paper describes a method of sample preparation and endotoxin measurement in house dust from urban homes in Croatia. It also brings the preliminary results for endotoxin levels in samples of house dust. So far, endotoxin analysis in house dust samples, as well as in other environmental samples, have not been performed in Croatia.

MATERIALS AND METHODS

Reagents and materials for LAL-test

- LAL reagent: lyophilised ENDOCHROME^{tm} reagent (Charles River Endosafe, USA) containing buffered Limulus amoebocyte lysate stabilised by monovalent and divalent cations (LAL lot No. R3531CT);
- S-2423 substrate: 10 mg lyophilised chromogenic substrate with mannitol filler (Charles River Endosafe, USA; lot No. ET11372);
- Buffer: endotoxin-free, 0.05 mol/L Tris buffer, pH 9.0 (Charles River Endosafe, USA);
- *E. coli* endotoxin standard: EC-6 (Charles River Endosafe, USA), containing 14 EU/ng (Endotoxin Units, according to United States Pharmacopeia), Charles River Endosafe Certificate of Analysis;
- LAL reagent water: steam sterilised, non-LAL reactive (Charles River Laboratories, USA);
- 20% acetic acid (Kemika, Zagreb);
- Endotoxin-free microplates (Greiner Labortechnik, GmbH);
- Endotoxin-free glass dilution tubes (IASON Laboratory Medicine, Graz, Austria);
- Single-channel repeating pipettor with endotoxin-free tips (HandyStep, Brand GmbH + Co. KG, Germany)

Sample collection and preparation

House dust was collected on cellulose filters originally developed for collecting house dust samples for the Dustscreen test, that is, for the measurement of dust mite allergens (Heska, AG, Switzerland). The samples were collected by vacuuming bedroom and living room floors. In this preliminary study, 16 house dust samples were collected in the urban area of Zagreb, Croatia.

Dust samples were kept in filters in original plastic airtight tubes (Heska, AG, Switzerland) at –20 °C until assayed. Two hours before LAL assay, samples were defrosted at 24 °C. Approximately 50 mg of house dust was extracted from filters by adding 10 mL of LAL reagent water (endotoxin-free water) and 5 μL of Tween-20 (Merck-Schuchardt, Germany) in the tube and then vortexed 4 times during 60 minutes: 1 minute of vortexing every 15 minutes. After extraction, 2 mL of particle-free extracts were put into the endotoxin-free

glass tubes and heated at 75 °C for 15 minutes. Serial dilutions of samples were made with LAL reagent water, ranging from 1:10 to 1:10000.

To examine possible endotoxin contamination of dust filters, two clean filters with corresponding plastic tubes were rinsed with LAL reagent water and then processed exactly the same way as the dust samples (including extraction procedure) and analysed.

Interference testing was performed by spiking two diluted samples of house dust with standard solution according to the Guideline of the US FDA (16). The percentage of recovered endotoxin from two diluted samples was calculated using the following formula:

$$\text{recovery} = (\text{Endotoxin}_{spiked} - \text{Endotoxin}_{sample}) \times 100\% / 4\lambda$$

where $\text{Endotoxin}_{spiked}$ is an endotoxin concentration (EU/ml) of the sample spiked with standard solution containing 4 λ, $\text{Endotoxin}_{sample}$ is an endotoxin concentration of the unspiked sample, and λ is equal to the lowest endotoxin concentration used to generate the standard curve. Recoveries between 75 and 125 % are considered satisfying.

Procedure

The ENDOCHROME™ (Charles River Endosafe, USA; U.S. license No. 1197) commercial kit was used for the quantitative detection of bacterial endotoxin in aqeous solutions using the chromogenic end-point Limulus amoebocyte lysate method.

Preparation of standard solutions. Endotoxin standard was reconstituted with 2.3 mL of LAL reagent water. This stock solution was then diluted to obtain nine standard solutions with concentrations from 0.03 to 1.2 EU/mL.

Preparation of reagents. S-2423 Substrate was rehydrated with 7.2 mL of LAL reagent water. Then one volume of substrate solution was mixed with one volume of buffer (substrate-buffer solution). Ten minutes before use, LAL reagent was reconstituted with 1.4 mL of LAL reagent water.

Standards, samples, spiked samples and blanks were pre-incubated in a microplate at 37 °C for 5 minutes. LAL reagent was added to samples and microplate was incubated at 37 °C for 7 minutes. After the addition of substrate-buffer solution, the second incubation was performed at 37 °C for 5 minutes (as specified on the Certificate of Analysis). The yellow colour produced during the second incubation was measured spectrophotometrically as the absorbance at 405 nm, after stopping the reaction with acetic acid. Standard curve was constructed, and endotoxin concentrations of the samples determined. The incubation, absorbance measurement, standard curve construction and determination of sample endotoxin concentrations were performed by a fully automated analyser (IASON Libertas, IASON Laboratory Medicine, Graz, Austria). Endotoxin was analysed in duplicates. House dust endotoxin values were expressed as endotoxin units per millilitre of diluted extract and then calculated to endotoxin units per milligram of dust.

Within-assay repeatability and the evaluation of possible error due to time difference in pipetting with single-channel repeating pipettor were assessed by measuring five standard solutions three times in duplicates. The coefficient of variation lower that 10 % was considered adequate (ENDOCHROME™ Charles River Endosafe, USA). The first series of 5 standards (first measurement) was used to construct standard curve and was positioned on the left side of the microplate. The second series (second measurement) was placed in the middle of the microplate, and the third (third measurement) on the right side. Table 1 shows the position of the standards on the microplate.

Table 1 *Position of standard solutions on a microplate.*

	left columns				middle columns					right columns		
	1	2	3	4	5	6	7	8	9	10	11	12
A	↓	S-2 ↓	↓				S-4				S-2	
B		S-3				S-1	S-5				S-3	
C		S-3				S-1	S-5				S-3	
D		S-4				S-2					S-4	
E		S-4				S-2					S-4	
F	S-1	S-5				S-3				S-1	S-5	
G	S-1	S-5				S-3				S-1	S-5	
H	S-2					S-4				S-2		

Every cell in the table represents one well on a microplate.
Letters with numbers represent standard solutions. Other wells (empty cells in this table) were filled with blanks, samples or spiked samples. Arrows designate the direction of pipetting on a microplate.

RESULTS

We obtained a standard curve with satisfying linearity (r = 0.983) with the range from 0.03 to 0.42 EU/mL. Table 2 shows the repeated measurements of five standard solutions (with concentrations from

0.12 to 0.42 EU/mL). The coefficient of variation (CV) of three measurements for each solution was lower than 10 %, ranging from 0 to 8.5 %. The correlation coefficients (Pearson r) between these three series of data were above 0.99, with P<0.001.

Table 2 *Repeatability of endotoxin analysis by comparing three measurements of five different standard dilutions.*

Standards	Endotoxin concentration (EU/mL) Series 1 (left)	Series 2 (middle)	Series 3 (right)	Avg	SD	CV (%)
Standard 1	0.12	0.11	0.12	0.12	0.006	**4.9**
Standard 2	0.15	0.15	0.15	0.15	0	**0**
Standard 3	0.21	0.21	0.22	0.21	0.006	**2.7**
Standard 4	0.30	0.29	0.34	0.31	0.026	**8.5**
Standard 5	0.42	0.38	0.42	0.41	0.023	**5.7**

The values of three measurements (series) of 5 standard dilutions and their position on the microplate (left, middle and right) are presented as endotoxin concentrations (EU/mL), together with arithmetic means (Avg), standard deviations (SD) and coefficient of variation (CV) for each standard dilution.

Endotoxin values for 16 samples of house dust ranged from 4.8 to 200 EU/mg of dust, with the arithmetic mean of 49.5 EU/mg and standard error of the mean of 12.1 EU/mg. The median was 38.8 EU/mg. The coefficient of correlation (Pearson r) between these duplicate sample measurements was 0.995 with P<0.001 (Figure 2). We found that sample extract dilutions of 1:400, 1:800 or 1:2000 best fitted into the linear portion of the standard curve.

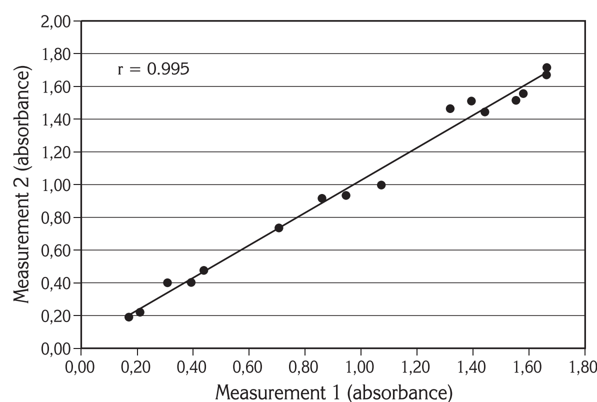

Figure 2 *Correlation (Pearson r) between repeated measurements of the same samples. Measurements are presented as absorbances.*

By spiking two diluted samples adequate recoveries of 92 and 110 % were obtained.

Endotoxin levels in two samples of endotoxin-free water that was used to rinse dust-collecting cellulose filters and plastic tubes, were far below the lowest standard concentration, and their absorbances were not higher than those of pure endotoxin-free water.

DISCUSSION

Standard chromogenic LAL assays are extremely sensitive (detecting picograms of endotoxin in various samples), but are not just as specific (14). Since they are biological methods, the batch of LAL reagent can also have a significant effect on endotoxin estimates (19). However, standardised procedures including sample storing, preparation and measurement should insure highly reproducible results of endotoxin analysis within one laboratory (3).

This preliminary endotoxin analysis whose aim was to introduce the chromogenic end-point Limulus amebocyte lysate method to our Unit, showed satisfying linearity of the standard curve, as well as adequate recovery and repeatability.

There is room for error when a single-channel repeating pipettor is used for pipetting LAL reagent and substrate-buffer solution into the wells on microplate due to time difference between the addition of reagents to standards and samples (17). This time difference may lead to different onset time of the reaction between LAL and endotoxin present in the standards and samples. However, good repeatability found in our assay, assured us that the duration of pipetting did not influence our measurements.

Recovery testing served to see whether there was an interference in the LAL assay. We measured the recovery of endotoxin concentration in a sample to which a known amount of endotoxin standard is added. If less endotoxin is detected than is known to be present, sample inhibits the assay, and if more endotoxin is detected than is present, sample enhances the assay (16). It is known that some substances may interfere with LAL-assay by inhibiting or enhancing the reaction between LAL and endotoxin. For example, sources of interference are endotoxin aggregation or adsorption, presence of chelating agents (e.g. EDTA), different proteases or protease inhibitors in the sample, and non-specific LAL activation (20). Thrombin, thromboplastin, certain synthetic polynucleotides, peptidoglycan from Gram-positive bacteria, exotoxins from Streptococci, yeast mannans, bacterial dextrans,

dithiols and LAL-reactive materials such as cellulose, and some sorts of plastic material can activate LAL, yielding false positive results (14). A contaminant of fungal origin, (1-3)-β–D-glucan, is also capable of initiating the coagulation cascade in horseshoe crab amoebocytes via the activation of Factor G (Figure 1) (21). To override these interfering factors different measures can be introduced. Thorough vortexing prevents endotoxin aggregation; heating the sample extracts at 75 °C for 15 minutes is expected to destroy proteases and protease inhibitors, and great number of other sources of interference can be resolved by diluting the sample (20). Since we adhered to these standard procedures for neutralising the interference, and since we obtained good recoveries, there is reason to believe that no significant interference occurred in our analysis. However, the presence of (1-3)-β–D-glucan and other sources of nonspecific LAL activation (especially of microbial origin) in our samples cannot be excluded, since the standard LAL techniques lack that sort of specificity (14).

Since endotoxin is a compound widely present in our environment, certain measures must be taken to avoid contamination. Endotoxin is rather termoresistant; for example, it cannot be destroyed by autoclaving (3). Its immune stimulatory capacity can be neutralised by prolonged heating at high temperature, e.g. 160 °C for 4 hours (1). To prevent contamination, all materials that come into contact with samples and reagents must be endotoxin-free. In our case, only plastic tubes with cellulose filters for collecting dust samples were not declared endotoxin-free by the producer (Heska, AG, Switzerland). Therefore, we tested them for endotoxin contamination and found them acceptably free of endotoxin.

Our analysis showed a wide range of endotoxin levels in house dust samples from urban homes in Croatia: from 4.8 to 200 EU/mg of dust. These results are in the range of values obtained by other authors (Table 3) (22-26). However, it is difficult to compare endotoxin values obtained from different laboratories due to inter-laboratory variations in results which are common. It is believed that even small and uncontrolled variations in the extraction and collection, storing and handling of the samples could cause differences in results (3). For example, a comparison of extraction methods showed that sonication in a phosphate-triethylamine buffer produced greater endotoxin activity than extraction in buffer with the addition of saponin or sodium dodecyl sulfate (19). A high variability in potency between different batches of LAL reagent also contributes to inter-laboratory variability (19).

Table 3 *Endotoxin levels in the house dust from our study compared to values obtained by other authors.*

Country	Number of samples	EU/mg house dust		Reference
		Mean	Range	
Belgium	28	25.9†	1.2 – 200	22
Germany	405	22.7‡	1.0 – 1200	23
Switzerland	493	22.8‡	8.2 – 63	24
Estonia	108	29.0§	0.3 – 280	25
Sweden	111	14.0§	0.3 – 99	25
New Zealand	77	22.7‡	2.9 – 414	26
Croatia	16	49.5† 32.1‡ 38.8§	4.8 – 200	

†Arithmetic mean; ‡Geometric mean; §Median.
All house dust level measurements, including ours, were performed using the chromogenic Limulus amoebocyte lysate method.

In conclusion, the chromogenic end-point Limulus amoebocyte lysate proved a reliable method for endotoxin measurement in house dust: samples were not contaminated; the linearity of the standard curve was good and so were the recovery and the repeatability of the method. Interferences can be avoided if the standardised procedure is observed. Our preliminary results of endotoxin levels in the house dust samples from urban homes in Croatia were in the range of house dust endotoxin values obtained by other authors.

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Sažetak

MJERENJE ENDOTOKSINA U KUĆNOJ PRAŠINI METODOM *END-POINT LIMULUS* AMEBOCITNOG LIZATA

Endotoxin je dio stanične stijenke gram negativnih bakterija, a po sastavu je lipopolisaharid. Široko je raspostranjen u okolišu, kako općem tako i radnom. U članku se opisuje priprema uzoraka i mjerenje endotoksina u 16 uzoraka gradske kućne prašine (Zagreb, Hrvatska) metodom *end-point* kromogeni *Limulus* amebocitni lizat test (LAL-test). Kućna prašina skupljana je u celulozne filtere usisavanjem podova spavaćih i dnevnih soba. Uzorci su do dana mjerenja čuvani smrznuti u hladnjaku na –20 °C. Ekstrakcija je provedena pomoću 0,05 % otopine Tween-20 u vodi bez endotoksina (*endotoxin-free water*). Serijska razrijeđenja uzoraka mjerena su u duplikatu pomoću analizatora IASON Libertas (IASON Laboratory Medicine, Austria). Postignuta je zadovoljavajuća linearnost standardne krivulje (r = 0,983), odsutnost interferencije i adekvatna ponovljivost (koeficijent varijabilnosti od 0 to 8,5 %). U uzorcima prašine izmjerene su vrijednosti endotoksina u rasponu od 4,8 do 200 EU/mg, sa srednjom vrijednošću (aritmetička sredina) od 49,5 EU/mg i standardnom pogreškom od 12,1 EU/mg. Ovo su prvi opisani rezultati mjerenja endotoksina u uzorcima kućne prašine u Hrvatskoj, a u skladu su s rasponima vrijednosti izmjerenih u kućnoj prašini u drugim zemljama.

KLJUČNE RIJEČI: *endotoksin, LAL test, lipopolisaharid, Limulus polyphemus*

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