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APPLICATION OF THE CLASSIC *LIMULUS* TEST AND THE QUANTITATIVE KINETIC CHROMOGENIC LAL METHOD FOR EVALUATION OF ENDOTOXIN CONCENTRATION IN INDOOR AIR

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Abstract: The classic (gel-clot procedure) *Limulus* test (CLT) and the quantitative kinetic chromogenic LAL method (KQCL) used for the evaluation of bacterial endotoxin concentration in the indoor air of dwellings were compared. The scientific procedure included analyses of 40 air samples supplemented by the analysis of 20 sample duplicates (selected at random) which were taken during the fall season from 10 flats located in 3 towns of the Upper Silesian region (southern Poland). The particulate aerosol probes were sampled by Harvard impactor and Casella sampler. The same samples were analyzed in the Netherlands using the quantitative kinetic chromogenic LAL method, and in Poland using the classic *Limulus* test. Comparison of both methods revealed that the quantitative kinetic chromogenic LAL method was more precise, with better reproducibility (the coefficient of variation between analyses of the main probe and its duplicate was over two times smaller in the KQCL method than in the CLT method), fully automated in the phase of analysis and data reading, and faster and more effective than the classic *Limulus* test. Nevertheless, on the basis of the obtained results, the usefulness of the classic *Limulus* method for assessment of the degree of pollution of indoor air with bacterial endotoxin seems to be confirmed as in the majority of examined samples (21 out 40) the results obtained by both methods were of the same order of magnitude, and in the remaining 19 samples did exceed one order of magnitude. Thus, the data received by means of the classic *Limulus* test may be regarded as acceptable.

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Key words: endotoxin, classic *Limulus* test, quantitative kinetic chromogenic LAL method, indoor air, dwellings.

INTRODUCTION

The *Limulus* test is the most widely used method for the evaluation of the bacterial endotoxin concentration in airborne and settled dust. This test is based on the phenomenon discovered by Bang and thereafter described scientifically in detail by Levin and Bang [10] in which blood of the horseshoe crab *Limulus polyphemus*, strictly the lysate of its amoebocytes (LAL), undergoes coagulation at the presence of picomole quantity of endotoxins. The mechanism of this reaction consists of activating serine protease by lipopolysaccharides (at the part of Ca^{2+} ions) and carrying the restricted proteolysis of the coagulogen by active enzyme. The conversion of protein lysate into gel stadium is the final result of this reaction (Figure 1).

At present, the *Limulus* test is carried out in many modifications. The gel-clot procedure (determination of the coagulation end point) referred to further as "the

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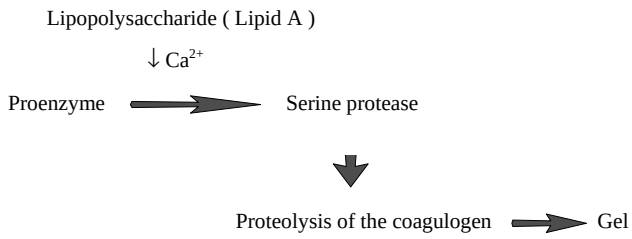

Figure 1. The classic *Limulus* test - principle of the method.

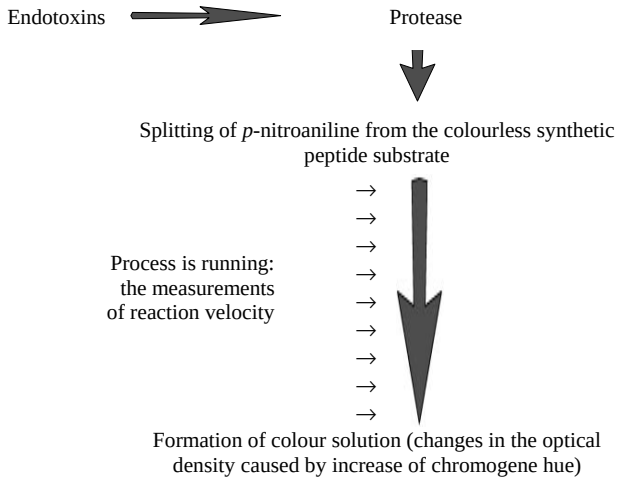

Figure 2. Kinetic chromogenic LAL test - principle of the method.

classic *Limulus* test" (CLT) is its simplest form. Later modifications utilize spectrophotometric measurements using coloured substrates and special analytical instruments. The most sophisticated modification of the *Limulus* test is its kinetic variant. The principle of this method is presented in Figure 2. The protease activated by the endotoxin detaches *p*-nitroaniline from the synthetic (colourless) peptide substrate. The effect of this reaction is the formation of the colour solution in which intensity of the chromogene hue is proportional to the quantity of endotoxin in the examined extract of the dust. In the kinetic modification of the *Limulus* test, the photometric measurements are carried out during the whole reaction (incubation) process. On the basis of the measurements of speed changes in the optical density of examined solution, the calculation of the endotoxin content in the sample is carried out.

The aim of this study was to compare the classic and kinetic methods and to estimate their usefulness for the evaluation of the concentration of environmental endotoxin in indoor air of dwellings. While the usefulness of different modifications of *Limulus* test has been compared in the work environments polluted with organic dusts, there are only few data on this subject from living environments (dwellings, offices) which are much less polluted with dust and endotoxin.

MATERIALS AND METHODS

During the autumn season, in 10 flats located in 3 towns (Sosnowiec, Katowice and Bytom) in the Upper Silesian region (southern Poland), the bacterial endotoxin contents in the particulate aerosol samples were examined. Taking into consideration the important influence of smoking on the pollution of indoor air [2, 6, 7, 9], the following two types of human dwellings were sampled: a) flats polluted with tobacco smoke by resident person(s) who smoke at least one packet of cigarettes per day (5 flats); b) flats not polluted with tobacco smoke (5 flats). In a living room of each flat, at the height of 1.4 m above ground level (responding to the human breathing zone), the particulate aerosol was sampled in 4 fractions: particles with aerodynamic diameter up to 2.5 µm (PM 2.5) and up to 10 µm (PM 10) - using Harvard impactors and up to 5 µm (PM 5) and total suspended particles (TSP) - using Casella air samplers. Thus, a total of 40 (2 × 20) samples were taken. The sampling times were: 5 hours for Harvard impactors (at the flow rate of 10 l/min) and 24 hours for Casella samplers (at the flow rate of 1.9 l/min and 2.0 l/min for PM 5 and TSP, respectively). The particulate aerosol was collected on sterile 37-mm teflon filters [3], which were used for the determination of endotoxin levels by classic *Limulus* test (CLT) using the technique applied by Clark *et al*. [3, 5] and by the quantitative kinetic chromogenic LAL method (KQCL) [1].

The filters were extracted by vigorous rocking in 5 ml of pyrogen-free water (NPBI, Emmer-Compascuum, The Netherlands) for 1 hour at the room temperature. After extraction, the suspensions were centrifuged at 1000 g for 10 min. The clear supernatant of each sample was divided into 4 parts (3 × 0.250 ml as analytical samples and a single 2 ml as a reserve sample), poured into 4 disposable pyrogen-free tubes and freezed at -20°C (since the analysis was not performed on the same day) [4]. As Olenchock *et al.* [12] and Douwes *et al.* [4] showed, the influence of freezing process on the endotoxin activity seems to be unimportant.

In the KQCL method, the endotoxin was assayed using automated microtiter plate reader (Kinetic QCL-reader Whittaker Bioproducts) and microtiter 96 well flatbottom plates (Costar Corp.; Cat. No. 3596). The test was incubated for 50 minutes at 37°C. The photometrical measurements (at λ = 405 nm) took place continuously at 30 second intervals during the whole sample incubation process. The LAL substrate reagent (BioWhittaker; Lot No. 6L029Y) was reconstituted before the use with pyrogen-free water and standard endotoxin (CSE) *Escherichia coli* 055:B5 (BioWhittaker; Lot No. 5L2110) was used as a positive control. Before the main analysis of the samples, the inhibition/enhancement test for the LAL activity checking [8] was performed. In this test, the serial dilutions of several samples selected at random were analysed according to the normal KQCL procedure. The results of the analysis of particular samples in the form

Table 1. Characteristics of the particulate aerosol samples used for endotoxin analysis.

No. of measurement	No. of sample	Town	Area of flat (m^2)	No. of inhabitants	Aerosol fraction	Sampler	Particulate aerosol concentration ($\mu g/m^3$)
				Flats without tobacco smokers			
1	6	Sosnowiec	56	2	PM 2.5	Harvard	30
	14				PM 10	impactor	53
	5				PM 5	Casella	33
	13				TSP	sampler	59
2	12	Sosnowiec	54	3	PM 2.5	Harvard	83
	35				PM 10	impactor	127
	36				PM 5	Casella	121
	34				TSP	sampler	160
3	1	Katowice	72	6	PM 2.5	Harvard	102
	2				PM 10	impactor	148
	16				PM 5	Casella	118
	15				TSP	sampler	150
4	26	Katowice	25	1	PM 2.5	Harvard	30
	27				PM 10	impactor	67
	28				PM 5	Casella	56
	29				TSP	sampler	110
5	38	Sosnowiec	51	1	PM 2.5	Harvard	30
	40				PM 10	impactor	51
	39				PM 5	Casella	44
	37				TSP	sampler	111
				Flats with tobacco smokers			
6	4	Sosnowiec	27	4/2*	PM 2.5	Harvard	183
	18				PM 10	impactor	240
	19				PM 5	Casella	184
	17				TSP	sampler	258
7	9	Bytom	37	1/1	PM 2.5	Harvard	57
	11				PM 10	impactor	84
	8				PM 5	Casella	80
	7				TSP	sampler	122
8	33	Katowice	50	3/2	PM 2.5	Harvard	88
	32				PM 10	impactor	154
	31				PM 5	Casella	154
	30				TSP	sampler	201
9	10	Sosnowiec	46	2/1	PM 2.5	Harvard	209
	25				PM 10	impactor	261
	23				PM 5	Casella	241
	24				TSP	sampler	274
10	22	Sosnowiec	54	4/2	PM 2.5	Harvard	77
	3				PM 10	impactor	100
	20				PM 5	Casella	98
	21				TSP	sampler	267

* number of inhibitants/smokers

Table 2. Comparison of results of endotoxin concentration analysis carried out using the classic *Limulus* test (CLT) and kinetic chromogenic LAL method (KQCL).

Fraction	No. of sample	CLT (concentration in ng/m^3)			KQCL (concentration in ng/m^3)		
		Main probe	Duplicate	CV (%)	Main probe	Duplicate	CV (%)
Flats without tobacco smokers							
PM 2.5	1	0.077	0.115	28.0	0.372	nd	nd
PM 2.5	6	0.041	0.029	24.2	0.018	0.020	7.9
PM 2.5	12	0.416	0.312	20.2	0.435	0.432	0.5
PM 2.5	26	0.083	nd	nd	0.026	0.021	14.7
PM 2.5	38	0.266	0.319	12.8	0.254	nd	nd
PM 5	5	0.457	0.343	20.2	5.417	nd	nd
PM 5	16	0.038	0.055	25.9	0.044	0.038	9.5
PM 5	28	0.087	nd	nd	0.003	0.005	28.3
PM 5	36	0.046	0.069	28.3	0.078	nd	nd
PM 5	39	0.091	0.114	15.9	0.007	nd	nd
PM 10	2	0.192	nd	nd	3.197	2.994	4.6
PM 10	14	0.029	0.041	24.2	0.080	0.079	1.2
PM 10	27	0.833	0.917	6.8	1.534	1.763	9.8
PM 10	35	0.416	nd	nd	2.127	nd	nd
PM 10	40	0.133	0.106	16.0	0.834	nd	nd
TSP	13	0.030	nd	nd	0.077	nd	nd
TSP	15	0.260	nd	nd	0.571	nd	nd
TSP	29	0.083	nd	nd	0.048	nd	nd
TSP	34	0.434	0.347	15.8	0.300	0.262	9.5
TSP	37	0.087	nd	nd	0.076	nd	nd
Mean ± SD		0.205 ± 0.210			0.774 ± 1.376		
Median		0.089			0.167		
Flats with tobacco smokers							
PM 2.5	4	0.083	nd	nd	0.331	0.310	4.6
PM 2.5	9	0.413	nd	nd	0.223	nd	nd
PM 2.5	10	0.080	0.064	15.7	0.078	0.083	4.5
PM 2.5	22	0.081	nd	nd	0.008	0.006	19.7
PM 2.5	33	0.079	0.118	28.0	0.406	0.410	0.6
PM 5	8	0.457	0.366	15.6	0.968	0.800	13.4
PM 5	19	0.110	0.082	20.6	0.010	nd	nd
PM 5	20	0.044	nd	nd	0.156	0.142	6.7
PM 5	23	0.091	nd	nd	0.226	nd	nd
PM 5	31	0.046	nd	nd	0.128	nd	nd
PM 10	3	0.028	nd	nd	0.026	0.028	4.9
PM 10	11	0.028	nd	nd	0.098	nd	nd
PM 10	18	0.083	nd	nd	0.004	0.004	6.1
PM 10	25	0.402	0.724	40.4	0.527	nd	nd
PM 10	32	0.394	0.472	12.7	0.227	0.233	1.9
TSP	7	0.868	0.651	20.2	1.740	nd	nd
TSP	17	0.052	nd	nd	0.062	nd	nd
TSP	21	0.042	nd	nd	0.168	nd	nd
TSP	24	0.087	0.078	7.7	0.151	0.122	15.2
TSP	30	0.087	nd	nd	0.211	0.265	15.9
Mean ± SD		0.178 ± 0.217			0.287 ± 0.409		
Median		0.083			0.162		

CV - coefficient of variation; nd - not done; SD - standard deviation

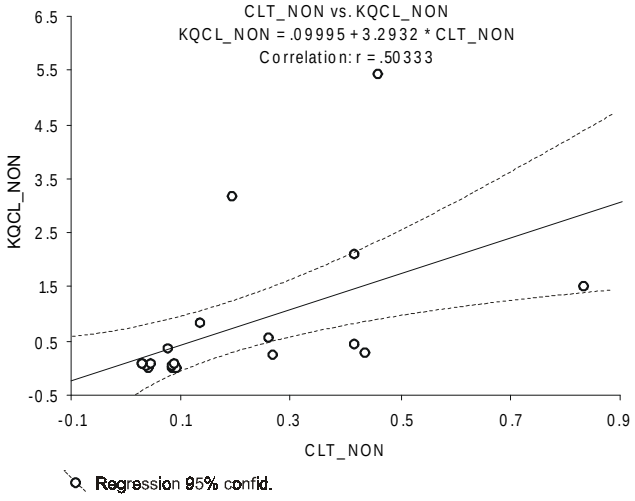

Figure 3. Correlation between endotoxin concentrations determined by the classic *Limulus* method (CLT_NON) and kinetic method (KQCL_NON) in nonsmokers' flats.

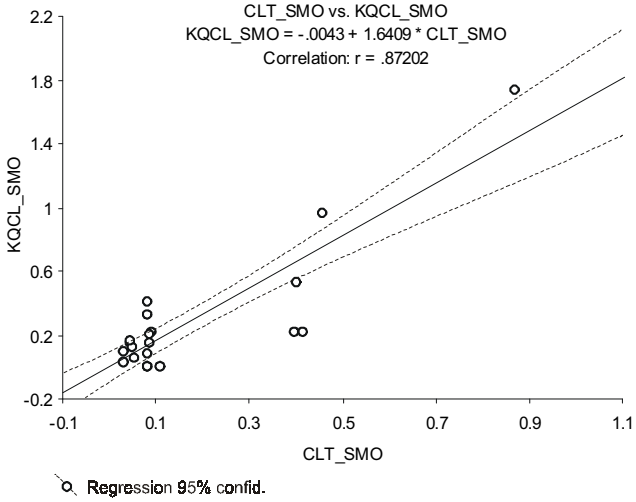

Figure 4. Correlation between endotoxin concentrations determined by the classic *Limulus* method (CLT_SMO) and kinetic method (KQCL_SMO) in smokers' flats.

of curves (where the maximal speeds of the reaction were plotted against the values of dilution coefficient) were compared with standard curve. The examined samples did not show inhibition or enhancement of the *Limulus* assay.

In the CLT method, the serial dilutions of each sample were mixed with equal volumes of the *Limulus* reagent (Pyroquant Diagnostik GmbH; Lot No. 27-21-712). The test was incubated for 1 hour in a water bath at 37°C. As a positive control of this test, the standard endotoxin (CSE) *Escherichia coli* O113:H10 (Associates of Cape Cod Inc., Woods Hole, USA, Lot No. 63) was used.

In both cases, the pyrogen-free water (NPBI, Emmer-Compascuum, The Netherlands) was utilized as a negative control of the tests.

The results were reported as weight equivalents of the standard endotoxin *Escherichia coli* O55:B5 in nanograms per cubic meter of air. One nanogram was equal to 9.5 Endotoxin Units (EU).

The main analysis, including examinations of 40 samples with both methods was supplemented by the analysis of 20 sample duplicates (selected at random).

The results were subjected to statistical analysis using Wilcoxon test for matched pairs and Spearman correlation tests (with the aid of software package: STATISTICA for Windows, release 4.5, StatSoft©, Inc. 1993).

RESULTS AND DISCUSSION

The characteristics of the samples of particulate aerosol used for endotoxin analysis are given in Table 1. The bacterial endotoxin concentrations in the particulate aerosol fractions obtained with the use of the classic *Limulus* test and the kinetic chromogenic LAL method are presented in Table 2. As can be seen, the differences between endotoxin concentrations obtained by both methods did not exceed one order of magnitude. The higher values were mostly noted in the kinetic LAL method. The obtained endotoxin concentration median values for all fractions were as follows. In the classic *Limulus* test: for flats without smokers 0.089 ng/m^3, for flats with smokers 0.083 ng/m^3 and for total flats 0.087 ng/m^3 (range: 0.028-0.868 ng/m^3). In the kinetic chromogenic LAL method: for flats without smokers 0.167 ng/m^3, for flats with smokers 0.162 ng/m^3 and for total flats 0.162 ng/m^3 (range: 0.003-5.417 ng/m^3).

In particular aerosol fractions, the mean endotoxin concentrations estimated by the kinetic LAL method were 1.33 (for PM 2.5), 5.21 (for PM 5), 3.14 (for PM 10) and 1.68 (for TSP) times higher than the concentrations obtained by the classic *Limulus* test. This tendency was observed in both analyzed groups of dwellings, i.e. in flats with and without tobacco smokers. The only exception to this rule was the endotoxin concentration in PM 10 fraction originated from flats with tobacco smokers. In this instance, the concentration value received with the use of classic method was 1.06 times higher than in the kinetic method.

The comparison of the measurement reproducibility made on the grounds of variation coefficients (CV) for the main probes and their duplicates (Table 2) showed higher CV values in the group of samples analyzed by the classic *Limulus* test than in the group analyzed by the kinetic LAL method. For all investigated flats of the Upper Silesian region, the mean CV value for determinations obtained by the CLT test was 2.22 times higher than the value obtained by the KQCL test. This tendency was observed in both investigated groups of flats. The differences in measuring accuracy of endotoxin concentrations in these two groups of flats expressed as coefficients of variation were: 19.9% and 20.1% for the CLT method and 9.6% and 8.5% for the KQCL method, in nonsmokers and smokers flats respectively.

Table 3. Correlation between endotoxin concentrations (obtained by the classic *Limulus* test and kinetic chromogenic LAL method) and other investigated parameters (particulate aerosol concentration, area of flat and number of inhabitants).

Studied dependence	R	p
MBC_CLT vs PAC	0.15	ns
MBC_KQCL vs PAC	0.30	ns
MPP_CLT vs PAC	-0.02	ns
MPP_KQCL vs PAC	-0.09	ns
MRE_CLT vs PAC	-0.01	ns
MRE_KQCL vs PAC	0.06	ns
MBC_CLT vs POWM_MBC	-0.24	ns
MBC_CLT vs LOS_MBC	-0.06	ns
MBC_KQCL vs POWM_MBC	0.32	ns
MBC_KQCL vs LOS_MBC	0.38	ns
MPP_CLT vs POWM_MPP	-0.41	ns
MPP_CLT vs LOSP_MPP	-0.46	<0.05
MPP_CLT vs LOS_MPP	-0.50	<0.05
MPP_KQCL vs POWM_MPP	-0.02	ns
MPP_KQCL vs LOSP_MPP	-0.39	ns
MPP_KQCL vs LOS_MPP	-0.55	<0.05
MRE_CLT vs POWM	-0.22	ns
MRE_CLT vs LOS	-0.31	ns
MRE_KQCL vs POWM	0.19	ns
MRE_KQCL vs LOS	-0.01	ns

vs – versus, R - Spearman correlation coefficient, p – probability, ns - not significant, PAC - particulate aerosol concentration, POWM - area of the flat, LOS - number of inhabitants, LOSP - number of inhabitants who smoke, MRE - endotoxin concentrations in the particulate aerosol samples originated from both kinds of investigated flats, MBC - endotoxin concentrations in the particulate aerosol samples originated from flats without tobacco smokers, MPP - endotoxin concentrations in the particulate aerosol samples originated from flats with tobacco smokers, CLT - endotoxin concentrations estimated by classic *Limulus* test, KQCL - endotoxin concentrations estimated by kinetic chromogenic *Limulus* test.

The comparison of the results of endotoxin concentrations in particulate aerosol samples obtained by both methods was performed on the basis of the regression analysis and by the Wilcoxon matched pair test. The analysis of regression (Figures 3 and 4) showed a moderate correlation of both methods for nonsmokers' dwellings (Fig. 3) and a very high correlation for smokers' dwellings (Fig. 4). The comparison of the CLT and the KQCL methods for all investigated flats in the Wilcoxon test showed that the difference between them was statistically significant ($p < 0.05$). However, the same analysis made for two subgroups, e.g. for the flats with and without tobacco smokers, did not show the statistically significant differences between the results obtained with both methods ($p > 0.05$).

The correlations between endotoxin concentrations obtained by the classic *Limulus* test and the kinetic chromogenic LAL method and other investigated parameters (particulate aerosol concentration, area of flat and number of inhabitants) are presented in Table 3. No statistically significant correlations were observed in the group of all investigated flats and in the group of flats inhabited by nonsmokers. In the group of smokers' flats three relations were statistically significant ($p < 0.05$): the endotoxin concentrations determinated by the CLT method *versus* the number of inhabitants and the number of smokers as well as the endotoxin concentrations determinated by the KQCL method *versus* the number of inhabitants. Nevertheless, all of these correlations were negative with the Spearman correlation coefficient R equal to -0.46, -0.50 and -0.55, respectively.

CONCLUSIONS

The comparative analysis of the classic *Limulus* test and the quantitative kinetic chromogenic LAL method for the environmental air samples taken in dwellings showed that the quantitative kinetic chromogenic LAL method was more precise, with better reproducibility (the coefficient of variation between analyses of the main probe and its duplicate was over two times smaller in the KQCL method than in the CLT method), fully automated in the phase of analysis and data reading (fewer possibilities for sample contamination in the laboratory), faster (during one day's work, it is possible to determine several hundred samples) and more effective (the *Limulus* lysate losses for the preparation of serial dilutions do not exist) than the classic *Limulus* test. Thus, the kinetic method is fully recommended for all laboratories which can cover the cost of the system.

For all its faults, on the basis of the above mentioned results, the usefulness of the classic *Limulus* method (less analytically complicated) for the assessment of the concentration of environmental endotoxin in indoor air of dwellings seems to be confirmed, as in the majority of examined samples (21 out of 40) the results obtained by both methods were of the same order of magnitude, and in the remaining 19 samples did not exceed one order of magnitude. A very high correlation of both methods found in the smokers' flats supports also the usefulness of the classic method in particular cases. Thus, from the scientific point of view, though the CLT method is distinctly inferior to KQCL method, the data received by means of the classic *Limulus* test may be regarded as acceptable for the general assessment of the risk of exposure to bacterial endotoxin in the human living environment.

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