ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2007, 14, 291-298

LEVELS OF BACTERIAL ENDOTOXIN IN AIR OF ANIMAL HOUSES DETERMINED WITH THE USE OF GAS CHROMATOGRAPHY – MASS SPECTROMETRY AND *LIMULUS* TEST

Dorota Pomorska[1], Lennart Larsson[2], Czesława Skórska[3], Jolanta Sitkowska[3], Jacek Dutkiewicz[3]

[1]Department and Clinic of Internal Medicine, Faculty of Veterinary Medicine, Agricultural University of Lublin, Poland
[2]Department of Laboratory Medicine, Division of Medical Microbiology, Lund University, Lund, Sweden
[3]Department of Occupational Biohazards, Institute of Agricultural Medicine, Lublin, Poland

Pomorska D, Larsson L, Skórska Cz, Sitkowska J, Dutkiewicz J: Levels of bacterial endotoxin in air of animal houses determined with the use of gas chromatography – mass spectrometry and *Limulus* test. *Ann Agric Environ Med* 2007, **14**, 291-298.

Abstract: Air samples were collected on glass fibre filters in 22 animal houses and 3 hay storage barns and examined for the presence of bacterial endotoxin with the *Limulus* (LAL) test and the gas chromatography – tandem mass spectrometry (GC-MSMS) technique, based on detection of 3-hydroxy fatty acids (3-OH-FAs) as chemical markers of the endotoxin lipopolysaccharide. The median concentrations of airborne endotoxin determined with LAL test in poultry houses, sheep sheds, piggeries, cow barns, and horse stables were respectively 62.49 μg/m^3, 26.2 μg/m^3, 3.8 μg/m^3, 1.65 μg/m^3, and 1.14 μg/m^3, while those determined with the GC-MSMS technique were respectively 1.06 μg/m^3, 7.91 μg/m^3, 0.2 μg/m^3, 0.31 μg/m^3, and 1.42 μg/m^3. The median concentrations of airborne endotoxin determined with LAL test and GC-MSMS technique in hay storage barns were much smaller, 0.09 μg/m^3 and 0.03 μg/m^3, respectively. The concentrations of airborne endotoxin (LPS) detected with GC-MSMS method in the air of sheep sheds were significantly greater than in all other examined facilities, while those detected in hay storage barns were significantly smaller than in all other examined facilities ($p<0.05$). The concentrations of airborne endotoxin determined with LAL test and GC-MSMS analysis exceeded in most of animal houses examined (91% by each method) the threshold limit value for airborne endotoxin of 0.1 μg/m^3 proposed by various authors. A significant correlation ($p<0.05$) between the concentrations of endotoxin determined with the LAL and GC-MSMS techniques was found in the air samples collected in poultry houses and sheep sheds, but not in other examined facilities. 3-OH FAs with C_{14}-C_{18} chains were predominant in the air of the facilities under study. A significant correlation ($p<0.05$) was found between the concentrations of endotoxin determined with LAL test and the amounts of 3-OH FAs with C_{14}-C_{16} chains. In conclusion, endotoxin in the concentrations detected in this study may present a respiratory hazard to both humans and livestock animals.

Address for correspondence: Dr Dorota Pomorska, Department and Clinic of Internal Medicine, Faculty of Veterinary Medicine, Agricultural University, Głęboka 30, 20-612 Lublin, Poland. E-mail: pomorska@list.pl

Key words: endotoxin, organic dust, air, animal houses, gas chromatography, mass spectrometry, 3-hydroxy fatty acids, *Limulus* test.

INTRODUCTION

The air in animal farms is contaminated with large amounts of biological agents, including allergens of plant and animal origin, bacteria, moulds, and microbial products. Among the latter, a serious health risk is posed by endotoxin, a major constituent of the outer membrane of Gram-negative bacteria composed mainly of lipopolysaccharide (LPS) [37, 38, 41, 51]. Bacterial endotoxin is widespread in nature and occurs in different habitats: plants,

Received: 29 September 2007
Accepted: 14 November 2007

animals, raw plant and animal materials, dust, air, water, sewage, waste materials, bedding, and soil [38]. Within on-farm facilities for livestock, endotoxin occurs abundantly in airborne organic dust which contains faeces and plant material of fodder origin contaminated with Gram-negative bacteria.

Endotoxin present in airborne organic dust has been identified as a cause of respiratory disease in humans and animals [37, 41]. Inhaled endotoxin interacts primarily with macrophages through CD-14 and TLR-4 receptors which initiate numerous cell-mediated and humoral responses. These reactions are triggered by mediators released by activated macrophages, such as cytokines (interleukins IL-1, IL-6, IL-8, TNFα), lysosomal enzymes, serotonin, and arachidonic acid metabolites (prostaglandins, leukotrienes, PAF). The outcome may be an inflammatory response or other detrimental reactions in the lung. Severe bronchospasms may develop due to the action of leukotrienes, prostaglandins and platelet-activating factor (PAF). In addition, endotoxin increases neutrophil and platelet viscosity, platelet aggregation and release of free radicals from neutrophils which may lead to acute inflammatory conditions and disrupted gaseous exchange in the peripheral lung regions [10, 26, 37, 38]. As a result of this, many respiratory disorders may develop. In humans, these conditions include organic dust toxic syndrome (toxic pneumonitis), byssinosis, chronic obstructive pulmonary diseases (COPD), asthma-like syndrome, and airway hyperreactivity [35, 41, 51]. On the other hand, exposure to endotoxin in early life might reduce atopy and prevent the development of allergic diseases [38, 41].

In contrast to human medicine, the effects of bacterial endotoxins in livestock animals are underestimated by veterinary medicine professionals and scarcely discussed in literature, although respiratory diseases associated with exposure to organic dusts are common in these animals. While referring to these conditions, different authors use extremely diverse clinical terminology. In the case of the horse, the term COPD (chronic obstructive pulmonary disease) is adopted by the analogy with human medicine [5, 28]. With reference to cattle, allergic alveolitis is the most common term used nowadays [52]. Clinical entities in pigs which present as respiratory signs difficult to treat and prevent are referred to as Porcine Respiratory Disease Complex (PRDC) [17].

Endotoxin is commonly detected with the *Limulus* test (LAL) which is based on an enzymatic coagulation of blood of a primitive marine arthropod, horseshoe crab (*Limulus polyphemus*), in the presence of a minimal amount of the endotoxin. This technique, detecting a biologically active endotoxin module is considered as useful but often non-specific and thus not sufficiently precise. It was found that a measurement technique based on a detection of specific LPS molecule compounds, such as 3-hydroxy fatty acids, may present a more precise alternative to the LAL test. This technique features the detection of chemical markers specific to particular bacteria by the means of gas chromatography and mass spectrometry (GC-MSMS) [20, 21, 39, 44, 45, 51]. In contrast to LAL test, it measures a total concentration of LPS (total bacterial endotoxin).

So far, there are no officially approved threshold limit values for allowable endotoxin content in the air. The LAL-based proposals of such values destined for humans are within a broad range of 0.005-0.2 μg/m^3 [3, 6, 9, 13, 19, 25, 34], most often between 0.1-0.2 μg/m^3 [3, 13, 25, 34]. The only proposal for such a value destined for farm animals is 0.15 μg/m^3, raised by Donham *et al.* [6].

The concentrations of endotoxin in the air of animal houses, determined by various authors with the use of LAL test, are usually high and in many cases exceed the proposed threshold limit values. The concentrations of airborne endotoxin found in cow barns varied between 0.00125-0.157 μg/m^3 [1, 11, 18, 27, 41, 46, 50], in horse stables between 0.006-3.44 μg/m^3 [11], in piggeries between 0.014-75.0 μg/m^3 [2, 4, 7, 11, 15, 16, 23, 29, 30, 32, 33, 40, 41, 46, 48, 50, 54, 55], and in poultry houses between 0.022-12.0 μg/m^3 [8, 27, 32, 36, 41, 46, 47, 48, 49, 50, 53]. A significant relationship has been found between the level of airborne endotoxin in animal houses and the decline of lung function and occurrence of respiratory symptoms in exposed workers [8, 15, 33, 43].

The aim of the present study was to determine the concentrations of airborne bacterial endotoxin in farm facilities harbouring different animal species with the use of two complementary methods: LAL (*Limulus*) test for determining biologically active endotoxin, and detection of chemical markers by gas chromatography – tandem mass spectrometry (GC-MSMS) for determining the total endotoxin lipopolysaccharide (LPS). The study was also aimed at determining the correlation between these two methods as well as their usefulness.

MATERIALS AND METHODS

Examined farm facilities. The study of endotoxin content in the air was carried out in 22 animal houses (4 cow barns, 4 piggeries, 4 sheep sheds, 4 poultry houses, and 6 horse stables), and in 3 buildings for storage of hay. All buildings were located on the territory of the Lublin province (eastern Poland). The characteristics of examined buildings is presented in Table 1. The air samples were collected from February until September 2001 during day-to-day routine activities.

Air sampling. The air samples were collected with the use of portable single-unit aspirator AP-2A (TWO-MET, Zgierz, Poland) for 30 min at flow rate 2 l/min on pre-weighed glass fibre filters of the diameter 37 mm and pore size 1.0 μm (SKC Inc., Eighty Four, PA, USA). The samples were taken at the central point of the facilities at the height of 145 cm. In each facility, two samples were collected: one for LAL test, and the second for analysis with GC-MSMS. All samples were stored at -15°C.

Table 1. Characteristics of examined farm facilities.

Type of facility	No.	Animals kept	Number of animals	Surface (m^2)	Ventilation	Activity during sampling
Cow barns	C-1	Dairy cattle, calves	15	130	natural v.	None
	C-2	Dairy cattle, bulls	20	160	natural v.	None
	C-3	Dairy cattle	30	240	natural v.	None
	C-4	Dairy cattle	18	150	natural v.	None
Piggeries	PI-1	Piglets (1-5 months old)	1100	825	natural v.	Feeding
	PI-2	Sows	320	300	natural v.	Feeding
	PI-3	Sows and piglets	54	80	natural v.	Feeding
	PI-4	Fattening pigs	68	100	natural v.	Feeding
Sheep sheds	S-1	Adult sheep	150	300	natural v.	Straw bedding
	S-2	Yeanlings, kids	40	150	natural v.	Straw bedding
	S-3	Adult sheep	96	200	natural v.	Straw bedding
	S-4	Yeanlings (3-7 months old)	30	150	natural v.	Straw bedding
Poultry houses	PO-1	Chickens (6-7 weeks old)	3000	200	low pressure v.	None
	PO-2	Chickens (6-7 weeks old)	3000	200	low pressure v.	None
	PO-3	Chickens (5 weeks old)	5000	250	low pressure v.	None
	PO-4	Chickens (7 weeks old)	4000	250	low pressure v.	None
Horse stables	HO-1	Mares	19	200	natural v.	Empty stable
	HO-2	Mares and foals (<7 months old)	21	220	natural v.	Fodder storing
	HO-3	Stallions	15	180	natural v.	Tending animals
	HO-4	Foals (7-9 months old)	20	200	natural v.	Tending animals
	HO-5	Mares and foals	20	250	natural v.	Tending animals
	HO-6	Stallions (Arabian)	14	170	natural v.	None
Hay storage barns	HA-1	-	-	150	natural v.	Hay unloading
	HA-2	-	-	75	natural v.	Hay unloading
	HA-3	-	-	90	natural v.	Hay unloading

Determination of biologically active endotoxin with LAL test. The concentration of biologically active bacterial endotoxin in the airborne dust was determined by the *Limulus* amebocyte lysate gel tube test (LAL) [22]. The filters were extracted for 1 hour in 10 ml of pyrogen-free water at room temperature, heated to 100°C in a Koch apparatus for 15 min (for better dissolving of endotoxin and inactivation of interfering substances), and after cooling, serial dilutions were prepared. The 0.1 ml dilutions were mixed equally with the "Pyrotell" *Limulus* reagent (Associates of Cape Cod, Falmouth, MA, USA). The test was incubated for 1 hour in a water bath at 37°C, using pyrogen-free water as a negative control and the standard lipopolysaccharide (endotoxin) of *Escherichia coli* 0113: H10 (Difco) as positive control. The formation of a stable clot was regarded as a positive result. The estimated concentration of endotoxin in dust (ng/mg) was multiplied per estimated concentration of dust in the air (mg/m^3) and the results were reported as micrograms of the equivalents of the *E. coli* 0113:H10 endotoxin per 1 m^3 of air. To convert to Endotoxin Units (EU), the value in nanograms was multiplied by 10.

Determination of LPS with GC-MSMS test. The concentration of the total LPS (often referred to as total endotoxin) in the air was determined by the detection of specific chemical markers – 3-hydroxy fatty acids (3-OH FAs) with the use of gas chromatography – tandem mass spectrometry (GC-MSMS) technique [51]. Before GC-MSMS analysis, all samples were chemically processed.

Samples in Teflon-lined glass test tubes were heated in 1 ml of 2 M methanolic HCl at 85°C overnight. Subsequently, 30 µl of a methanolysate of 13 C-labelled cyanobacterial cells (corresponding to 30 µl of cyanobacteria) was added, and the mixture was extracted with 1.5 ml of water-*n*-heptane (1:2, vol/vol). The heptane (upper) layer was used for analysis of FAs. 3-OH $C_{16:0}$ was used as internal standard. To determine the FAs, the heptane (upper) layer was evaporated under the stream of nitrogen at room temperature, redissolved in 1 ml of heptane- dichloromethane (1:1 vol/vol), and purified using a disposable silica gel column (100 mg). Prior to use, the silica gel column was washed twice with 1 ml of diethylether and twice with 1 ml of heptane-dichloromethane; the methyl ester-containing mixture was then added. Diethyl ether (2 ml) was then added to the

column to elute the hydroxyl FAs; the eluate was evaporated in room temperature. Trimethylsilyl (TMS) derivatives of the hydroxyl FAs were prepared by adding 50 µl BSTFA (*N*,*O*-bis-(trimethylsilyl) trifluoroacetamide) and pyridine (5 µl) followed by heating for 20 min at 80°C. Heptane (10 µl) was then added. The preparations were analyzed following storage at 4°C overnight.

The analysis was carried out by using an autosampler-equipped gas chromatography-mass spectrometry instrument (Saturn 2000 ion trap, Varian, Palo Alto, CA, USA). The chromatographic separation was carried out on a fused silica capillary column 30 m long and 0.25 mm in diameter. Helium was used as the carrier gas (69 kPa). The temperature of the column was set to change from 90°C to 280°C at 20°C per minute. The temperature of the injector and the interface between the chromatograph and mass spectrometer was maintained at 290ºC.

The ion trap temperature was 180ºC. All analyses were made in the electron impact (EI) mode. Mass spectra of the methyl ester/TMS 3-OH FA derivatives show abundant ions of *m/z* (M-15), due to loss of a CH_3 group and *m/z* 175, due to cleavage of the C-3/C-4 linkage. The derivatized acids were measured by monitoring *m/z* 131 (a product of *m/z* 175) in GC-MSMS. The number of moles of LPS in each sample was calculated by dividing the number of moles of the 3-OH C_{10} to C_{18} FAs by four. To give an estimate of the weight amount of LPS, the determined number of LPS moles was multiplied by 8,000 (assumed as an average molecular weight of environmental LPS) [55].

Statistical analysis. The data were analyzed by Shapiro-Wilk test for distribution, by Mann-Whitney test for determining the differences between particular environments, and by Spearman's test for determining a correlation between the two methods, using Statistica for Windows v. 5.0 package (Statsoft©, Inc., Tulsa, Oklahoma, USA).

RESULTS

Concentration of the biologically active airborne endotoxin determined with LAL test. The concentration of biologically active bacterial endotoxin in the air measured with *Limulus* test was the greatest in poultry houses ranging from 0.42-104.22 µg/m³ (median: 62.49 µg/m³). In sheep sheds it ranged from 0.21-104.06 µg/m³ (median: 26.2 µg/m³), in piggeries from 2.22-19.44 µg/m³ (median: 3.8 µg/m³), and in cow barns from 0.15-2.81 µg/m3 (median: 1.65 µg/m³). In horse stables, the median concentration of endotoxin was the smallest, ranging between 0.1-208.36 µg/m³ (median: 1.14 µg/m³) (Tab. 2).

The concentration of airborne endotoxin measured in the barns during unloading of hay was much smaller than in animal houses, ranging between 0.06-0.48 µg/m³ (median: 0.09 µg/m³).

The results of LAL test showed a non-parametric distribution characterized by large variation. Hence, the only significant differences between the facilities concerned the endotoxin concentration in piggeries which proved to be significantly greater than in cow barns and hay storage barns ($p<0.05$).

Concentration of the airborne LPS determined with GC-MSMS analysis. The concentration of LPS in the air measured with GC-MSMS method was the greatest in sheep sheds, varying from 2.72-10.03 µg/m³ (median: 7.91 µg/m³). This concentration was significantly greater compared to all other examined farming facilities ($p<0.05$) (Tab. 2). In horse stables, the concentration of airborne LPS was between 0.43-8.13 µg/m³ (median: 1.42 µg/m³), in poultry houses between 0.19-1.67 µg/m³ (median: 1.06 µg/m³), and in cow barns between 0.034-1.35 µg/m³ (median: 0.31 µg/m³). The concentration of airborne LPS was smallest in piggeries, ranging from 0.09-0.27 µg/m³ (median: 0.2 µg/m³) (Tab. 2).

During unloading of hay in the barns, the LPS concentration was, similar to the LAL test, much smaller than in livestock facilities and ranged between 0.012-0.032 µg/m³ (median: 0.03 µg/m³). The concentration was significantly smaller compared to all examined livestock facilities ($p<0.05$) (Tab. 2).

Concentration of LPS determined with GC-MSMS for individual types of 3-hydroxy fatty acids (C_{10}-C_{18}). As LPSs of different Gram-negative bacteria species contain 3-OH-FAs of diverse chain length, quantitative and distribution analyses of fatty acids, 3-OH-FAs, with 10-18 carbon chains were performed. This provides a basis for developing a profile of Gram-negative bacteria detected in a sample.

The following types of fatty acids were predominant in particular farming environments: – 3-OH-C_{16} and 3-OH-C_{18} in sheep sheds, poultry houses, and horse stables; – 3-OH-C_{14} and 3-OH-C_{16} in piggeries and cow sheds; – and 3-OH-C_{14} and 3-OH-C_{18} during unloading of hay. Summarizing, fatty acids with 14-18 carbon chains were predominant in the air of the facilities under study. Fatty acids with shorter carbon chains (10-12 C) were less abundant and did not prevail in any environment.

Comparison of the results yielded with two methods (LAL vs. GC-MSMS). In the air samples collected in poultry houses and sheep sheds, a statistically significant correlation was found between the concentration of biologically active endotoxin determined with LAL test and the concentration of total LPS determined with GC-MSMS (correlation coefficients were respectively $r=1.0$ and $r=0.95$, $p<0.05$). In these samples, a significant correlation was also found between the concentration of endotoxin determined with LAL test and the following concentrations of 3-hydroxy fatty acids (3-OH FAs): 3-OH-C_{12}, 3-OH-C_{14}, 3-OH-C_{16}, and total sum of 3-OH FAs determined in nanomoles with GC-MSMS ($p<0.05$) (Tab. 3). No significant

Table 2. Endotoxin (LPS) concentration in air of animal houses assessed by LAL and GC-MSMS.

Farm facilities	Sample	LAL	GC-MSMS										
			3-hydroxy fatty acids (nanomoles)								LPS		
		Endotoxin (BAE) in the air (μg/m³)	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{10-14}	C_{16-18}	Total	Total (nmol)	In dust (nmol/mg)	In air (μg/m³)
Sheep sheds (N = 4)	S-1	104.060	0.201	0.144	0.501	0.674	0.609	0.846	1.283	2.129	0.076	0.380	10.029
	S-2	52.190	0.026	0.047	0.132	0.200	0.199	0.205	0.400	0.605	0.071	0.101	9.491
	S-3	0.210	0.026	0.003	0.095	0.078	0.055	0.124	0.133	0.258	0.030	0.051	2.717
	S-4	0.210	0.082	0.042	0.119	0.101	0.074	0.243	0.175	0.419	0.027	0.068	6.337
	Median	26.20	0.05	0.05	0.12	0.15	0.14	0.22	0.29	0.51	0.05	0.08	7.91**
Poultry houses (N = 4)	PO-1	104.220	0.070	0.099	0.263	0.403	0.340	0.431	0.743	1.15	0.083	0.209	1.669
	PO-2	104.170	0.070	0.058	0.203	0.285	0.168	0.330	0.453	0.783	0.108	0.154	1.230
	PO-3	20.810	0.039	0.053	0.139	0.218	0.285	0.232	0.503	0.735	0.079	0.112	0.899
	PO-4	0.420	0.019	0.022	0.039	0.016	0.016	0.080	0.032	0.112	0.017	0.024	0.193
	Median	62.49	0.05	0.05	0.17	0.25	0.23	0.28	0.48	0.76	0.08	0.13	1.06
Horse stables (N = 6)	HO-1	10.420	0.267	0.408	1.111	2.278	1.927	1.786	4.205	5.991	0.102	1.016	8.128
	HO-2	0.210	0.132	0.106	0.306	0.495	0.437	0.544	0.933	1.477	0.104	0.260	2.080
	HO-3	0.100	0.138	0.087	0.342	0.529	0.394	0.567	0.922	1.489	0.109	0.274	2.192
	HO-4	0.100	0.033	0.037	0.109	0.205	0.189	0.179	0.394	0.573	0.077	0.096	0.768
	HO-5	2.080	0.023	0.020	0.083	0.139	0.093	0.125	0.232	0.357	0.086	0.066	0.528
	HO-6	208.360	0.013	0.021	0.067	0.115	0.092	0.101	0.208	0.309	0.081	0.054	0.433
	Median	1.14	0.08	0.06	0.21	0.35	0.29	0.36	0.66	1.02	0.09	0.18	1.42
Piggeries (N = 4)	PI-1	19.440	0.068	0.178	0.433	0.572	0.197	0.680	0.769	1.449	0.313	0.034	0.272
	PI-2	4.460	0.106	0.273	0.3934	0.200	0.119	0.772	0.319	1.091	0.243	0.011	0.089
	PI-3	3.140	0.125	0.345	0.598	0.725	0.121	1.068	0.846	1.914	0.449	0.022	0.178
	PI-4	2.220	0.106	0.395	0.912	1.304	0.259	1.413	1.563	2.977	0.340	0.028	0.225
	Median	3.80*	0.11	0.31	0.51	0.65	0.16	0.92	0.81	1.68	0.33	0.02	0.20
Cow barns (N = 4)	C-1	2.810	0.045	0.096	0.184	0.176	0.118	0.325	0.294	0.619	0.055	0.004	0.034
	C-2	2.170	0.022	0.087	0.162	0.163	0.201	0.271	0.363	0.634	0.125	0.048	0.385
	C-3	1.130	0.010	0.029	0.048	0.049	0.034	0.087	0.083	0.170	0.119	0.031	0.244
	C-4	0.150	0.051	0.081	0.132	0.104	0.125	0.264	0.230	0.494	0.339	0.169	1.354
	Median	1.65	0.03	0.08	0.15	0.13	0.12	0.27	0.26	0.56	0.12	0.04	0.31
Hay storage barns (N = 3)	HA-1	0.060	0.009	0.022	0.021	0.009	0.037	0.052	0.046	0.098	0.092	0.004	0.032
	HA-2	0.090	0.039	0.043	0.111	0.114	0.172	0.193	0.286	0.479	0.014	0.002	0.012
	HA-3	0.480	0.018	0.028	0.061	0.049	0.036	0.106	0.084	0.191	0.086	0.004	0.030
	Median	0.09	0.02	0.03	0.06	0.05	0.04	0.11	0.08	0.19	0.09	0.004	0.03***

BAE = biologically active endotoxin; *significantly greater than in cow barns and hay storage barns (Mann-Whitney test, $p<0.05$); **significantly greater than in all other facilities (Mann-Whitney test, $p<0.05$); ***significantly smaller than in all other facilities (Mann-Whitney test, $p<0.05$); N – number of samples.

correlation could be found between the concentration of biologically active endotoxin and the concentrations of 3-OH FAs and LPS in the air samples collected in the remaining animal houses (piggeries, cow barns, horse stables) and in hay storage barns.

For total samples collected in all examined facilities, a significant correlation was found between the concentration of biologically active endotoxin determined with LAL test and the following concentrations of 3-hydroxy fatty acids (3-OH FAs): 3-OH-C_{14}, 3-OH-C_{16}, sum of 3-OH-C_{16}-3-OH-C_{18}, and total sum of 3-OH FAs determined in nanomoles with GC-MSMS ($p<0.05$) (Tab. 3). Nevertheless, no significant correlation could be found for total samples between the concentration of airborne endotoxin determined with LAL test and the concentration of LPS in the air determined with GC-MSMS ($p>0.2$).

The concentrations of airborne endotoxin determined with *Limulus* test were, in most cases (19 out of 25, 76%), greater than the concentrations of airborne LPS determined with GC-MSMS method.

Table 3. Correlation between concentrations of 3-hydroxy fatty acids (3-OH FAs) and LPS determined with GC-MSMS in air of farm facilities and concentrations of biologically active endotoxin (BAE) determined with LAL in air of these facilities, as assessed by Spearman's rank order test.

Farm facility		3-OH-FAs with 10-18 carbon chains (nanomoles) vs. BAE (LAL)								LPS vs. BAE (LAL)	
		C_{10}	C_{12}	C_{14}	C_{10-14}	C_{16}	C_{18}	C_{16-18}	Total	nmol/m^3	µg/m^3
Piggeries	r	-0.67	-0.11	-0.02	-0.05	0.02	0.34	0.06	0.01	-0.24	0.61
	p	0.91	0.86	0.98	0.93	0.97	0.58	0.92	0.99	0.76	0.39
Cow barns	r	-0.11	0.42	0.57	0.43	0.72	0.32	0.53	0.50	-0.89	-0.85
	p	0.89	0.58	0.43	0.57	0.28	0.68	0.46	0.51	0.11	0.15
Sheep sheds	r	0.63	0.95	0.95	0.63	0.95	0.95	0.95	0.95	0.95	0.95
	p	0.37	0.05*	0.05*	0.37	0.05*	0.05*	0.05*	0.05*	0.05*	0.05*
Horse stables	r	-0.32	-0.12	-0.32	-0.32	-0.32	-0.23	-0.23	-0.23	-0.23	-0.32
	p	0.54	0.83	0.54	0.54	0.66	0.66	0.66	0.54	0.66	0.54
Poultry houses	r	0.95	1.0	1.0	1.0	1.0	0.8	0.8	1.0	0.8	1.0
	p	0.05*	0.05*	0.05*	0.05*	0.05*	0.2	0.2	0.05*	0.2	0.05*
Hay storage barns	r	-0.16	-0.22	0.003	-0.066	-0.067	-0.45	-0.304	-0.221	0.37	0.38
	p	0.90	0.86	0.99	0.96	0.96	0.70	0.80	0.86	0.75	0.75
Total facilities	r	0.2	0.35	0.41	0.37	0.47	0.3	0.4	0.41	0.11	0.24
	p	0.34	0.08	0.042*	0.07	0.018*	0.15	0.047*	0.041*	0.6	0.25

BAE = biologically active endotoxin; r = correlation coefficient; p = probability of correlation coefficient; *correlation statistically significant ($p \leq 0.05$).

DISCUSSION

In all five types of examined livestock buildings, the degree of inhalation exposure to bacterial endotoxin was large, creating a potential health risk to farm workers and housed animals. The concentrations of biologically active airborne endotoxin determined with LAL method and those of total airborne LPS determined with GC-MSMS method exceeded in most of animal houses examined (91% by each method) the threshold limit value for airborne endotoxin of 0.1 µg/m^3 proposed by various authors [3, 25, 34]. In 15 out of 22 samples examined by LAL (68.2%) and in 10 out of 22 samples examined by GC-MSMS (45.5%), airborne endotoxin occurred in large quantities of the order 10^0-10^2 µg/m^3, posing a risk of respiratory disease in exposed workers and animals [35]. By contrast, in 3 air samples collected in hay storage barns, exceeding the threshold limit value of 0.1 µg/m^3 was stated in only 1 case by LAL, and in no case by GC-MSMS. The restrictive threshold limit value of 0.005 µg/m^3 proposed by DECOS [9] was exceeded in all samples under study, examined either by LAL or GC-MSMS.

In most cases, the levels of airborne endotoxin found in this work are greater compared to earlier studies performed in various countries. It should be mentioned that these comparisons are not always accurate because of the marked differences between the methods used and environmental conditions. The median concentrations of airborne endotoxin determined in the present study in cow barns, either by LAL or GC-MSMS, are greater compared to those reported hitherto from Germany, Poland, USA and the Netherlands [1, 11, 18, 27, 41, 46, 50]. The median concentration of airborne endotoxin determined in piggeries by LAL is greater compared to those reported from Korea, Canada, USA, the Netherlands, Denmark, Germany, and UK [2, 4, 7, 15, 16, 29, 30, 32, 33, 41, 46, 48, 50], similar to those reported from Canada [54] and Sweden [55], and smaller compared to those reported earlier from Poland [11, 23]. The median concentration of airborne LPS determined in piggeries by GC-MSMS is greater compared to those reported from Korea [2], Canada [4], USA [16, 33], Denmark [32], and Germany [32, 41], similar to those reported from the USA [7], the Netherlands [15, 29, 30, 50], and Germany [46], and smaller compared to those reported from the UK [48], Canada [54], Sweden [55], and earlier from Poland [11, 23]. The median concentration of airborne endotoxin determined in poultry houses by LAL is greater compared to those reported from the USA, Switzerland, Sweden, Germany, the UK, and the Netherlands [8, 27, 32, 36, 41, 46, 47, 48, 49, 50, 53]. The median concentration of airborne LPS determined in poultry houses by GC-MSMS is greater compared to those reported from the USA [8, 27], Switzerland [32], Sweden [36], Germany [41], the Netherlands [50], and the UK [53], similar to those reported from Northern Europe [46], and Sweden [49] and smaller compared to those reported from Germany [47] and the UK [48]. The median concentrations of airborne endotoxin determined in horse stables by LAL and GC-MSMS are within a range reported by an earlier Polish study [11].

To the best of our knowledge, no studies on the exposure to airborne endotoxin have been conducted so far in sheep houses. The present study shows that the quantity of airborne endotoxin in sheep sheds is extremely large, in the case of the LPS (total endotoxin) detected by GC-MSMS even significantly greater than in all other types of animal

houses. This result is in accordance with the results of questionnaire studies performed by Magarolas *et al.* [24], Radon & Winter [31], and Hashemi *et al.* [14] who found that sheep breeders are at high risk for the development of work-related respiratory symptoms. Most probably, bacterial endotoxin could be one of the factors causing these symptoms.

A significant correlation was found between the concentrations of airborne endotoxin determined with the LAL (*Limulus*) and GC-MSMS methods in sheep sheds and poultry houses but not in other animal houses and hay storage barns. The concentrations of biologically active endotoxin determined with the LAL test in the course of the present work were usually greater than those of the LPS (total endotoxin) determined with the GC-MSMS method. This is not consistent with the results of the earlier studies on comparison of the LAL and GC-MSMS tests [19, 40, 49, 55] and may be due, at least in part, to the presence of non-specific LAL reactions which hinder the correlation of two methods. The results obtained in this study with GC-MSMS method showed less variation compared to those obtained with LAL test and seem to be more specific. Thus, the GC-MSMS analysis based on detection of chemical markers could be recommended as a reliable method of endotoxin determination.

The results of the GC-MSMS analyses indicate that endotoxin present in the air of animal houses contained 3-hydroxy fatty acids with 14-18 carbon chains (3-OH C_{14}-C_{18}). 3-OH C_{14} is characteristic for LPSs produced by Gram-negative bacteria belonging to Enterobacteriaceae family [40, 51]. This was previously reported as a dominant fatty acid in swine dust [51] and related to the occurrence of respiratory symptoms in the workers exposed to organic dusts [19]. It is noteworthy that in the present work a distinct and significant correlation was found between the concentrations of biologically active endotoxin determined with LAL test and the amounts of 3-OH-C_{14} and 3-OH-C_{16} determined with GC-MSMS analysis. This suggests that endotoxin characterized by the prevalence of 3-OH-FAs with 14-16 carbon chains exerts most biological effects in animal houses. The detection of 3-OH-C_{18} might be less specific as this chain could be abundantly present also in some common species of Gram-positive Actinobacteria [44].

CONCLUSIONS

The concentrations of bacterial endotoxin found in the air of five types of livestock facilities with the use of two analytical methods (LAL test and GC-MSMS) were high, and in circa 90% of the samples exceeded the levels regarded as allowable for humans and animals. It is noteworthy that particularly large endotoxin concentrations were detected in the air of sheep sheds which until recently have not been examined in this respect. During unloading of hay in the barns, the endotoxin concentration was much smaller than in livestock facilities.

A significant correlation between the concentration of biologically active endotoxin determined with LAL test and the concentration of LPS (total endotoxin) determined with GC-MSMS was found in the air samples collected in poultry houses and sheep sheds, but not in other examined facilities.

The analysis of the length of 3-hydroxy fatty acids chains contained in the endotoxin molecule has shown that 3-hydroxy fatty acids comprising C_{14}-C_{18} chains were most prevalent in the facilities included in the study. This may suggest that the Gram-negative coliform bacteria of Enterobacteriaceae family were the source of airborne endotoxin. A distinct and significant correlation was found between the concentrations of endotoxin determined with LAL test and the amounts of fatty acids with C_{14}-C_{16} chains.

Summarizing, endotoxin in the concentrations determined in the study may present a respiratory hazard to both humans and livestock animals. In the latter case, it may cause inflammatory respiratory disorders. Thus, prevention measures aiming to lower the content of bacterial endotoxin in the air of livestock facilities are highly desirable.

Acknowledgements

The skillful assistance of Ms. Wiesława Lisowska and Ms. Halina Wójtowicz in performing the study is gratefully acknowledged.

REFERENCES

1. Berger I, Schierl R, Ochmann U, Egger U, Scharrer E, Nowak D: Concentrations of dust, allergens and endotoxin in stables, living rooms and mattresses from cattle farmers in southern Bavaria. *Ann Agric Environ Med* 2005, **12**, 101-107.
2. Chang CW, Chung H, Huang CF, Su HJ: Exposure assessment to airborne endotoxin, dust, ammonia, hydrogen sulfide and carbon dioxide in open style swine houses. *Ann Occup Hyg* 2001, **45**, 457-465.
3. Clark CS: Report on prevention and control. **In:** Rylander R, Peterson Y, Donham KJ (Eds): Health Effects of Organic Dusts in the Farm Environment. Proceedings of an International Workshop held in Skokloster, Sweden, April 23-25, 1985. *Am J Ind Med* 1986, **10**, 267-273.
4. Cormier Y, Israël-Assayag E, Racine G, Duchaine C: Farming practices and the respiratory health risks of swine confinement buildings. *Eur Respir J* 2000, **15**, 560-565.
5. Derksen FJ: Chronic obstructive pulmonary disease. **In:** Robinson NE (Ed): *Current Therapy in Equine Medicine*, 448-520. W. B. Saunders Company, Philadelphia 1987.
6. Donham KJ, Haglind P, Peterson Y, Rylander R, Belin L: Environmental and health studies of farm workers in Swedish swine confinement buildings. *Brit J Ind Med* 1989, **46**, 31-37.
7. Donham KJ: Association of environmental air contaminants with disease and productivity in swine. *Am J Vet Res* 1991, **52**, 1723-1730.
8. Donham KJ, Cumro D, Reynolds SJ, Merchant JM: Dose-response relationships between occupational aerosols exposures and cross-shift declines of lung function in poultry workers. Recommendations for exposure limits. *J Occup Environ Med* 2000, **42**, 260-269.
9. Dutch Expert Committee on Occupational Standards (DECOS): *Endotoxins, Health-based Recommended Occupational Exposure Limit*. Gezondheidsraad, The Netherlands 1998.
10. Dutkiewicz J, Jabłoński L: *Occupational Biohazards*. PZWL, Warsaw 1989 (in Polish).

11. Dutkiewicz J, Pomorski ZJH, Sitkowska J, Krysińska-Traczyk E, Skórska C, Prażmo Z, Cholewa G, Wójtowicz H: Airborne microorganisms and endotoxin in animal houses. *Grana* 1994, **33**, 85-90.

12. Dutkiewicz J, Skórska C, Burrell R, Szuster-Ciesielska A, Sitkowska J: Immunostimulative effects of repeated inhalation exposure to microvesicle-bound endotoxin of *Pantoea agglomerans*. *Ann Agric Environ Med* 2005, **12**, 289-294.

13. Górny RL, Dutkiewicz J: Bacterial and fungal aerosols in indoor environment in Central and Eastern European countries. *Ann Agric Environ Med* 2002, **9**, 17-23.

14. Hashemi N, Mirsadraee M, Shakeri MT, Varasteh AR: Prevalence of work-related respiratory symptoms in Iranian farmers. *Can Respir J* 2006, **13**, 198-202.

15. Heederik D, Brouwer R, Biersteker K, Boleij JSM: Relationship of airborne endotoxin and bacteria levels in pig farms with the lung function and respiratory symptoms in farmers. *Int Arch Occup Environ Health* 1991, **62**, 595-601.

16. Jolie R, Bäckström L, Gunderson P: Airborne contaminants and farmers health in swine farms with high and low prevalence of respiratory diseases in pigs. *Ann Agric Environ Med* 1998, **5**, 87-92.

17. Kołodziejczyk P, Karbowiak S, Arh J, Mokrzycka A: Efficacy of Floron® inj. in treatment of the Porcine Respiratory Disease Complex (PRDC) on an industrial farm. *Mag Wet* 2003, **5**, 69-71 (in Polish).

18. Kullman GJ, Thorne PS, Waldron PF, Marx JJ, Ault B, Lewis DM, Siegel PD, Olenchock SA, Merchant JA: Organic dust exposures from work in dairy barns. *Am Ind Hyg Assoc J* 1998, **59**, 403-413.

19. Laitinen S, Kangas J, Husman K, Susitaival P: Evaluation of exposure to airborne bacterial endotoxins and peptidoglycans in selected work environments. *Ann Agric Environ Med* 2001, **8**, 213-219.

20. Larsson L: Determination of microbial chemical markers by gas chromatography – mass spectrometry – potential for diagnosis and studies on metabolism in situ. *APMIS* 1994, **102**, 161-169.

21. Larsson L: Determination of air-borne microorganisms by gas chromatography – mass spectrometry. **In:** Agashe SN (Ed): *Aerobiology. 5th International Conference, Bangalore 1994*, 527-536. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi 1997.

22. Levin J, Bang FB: The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull Johns Hopkins Hosp* 1964, **115**, 265-274.

23. Mackiewicz B: Study on exposure of pig farm workers to bioaerosols, immunologic reactivity and health effects. *Ann Agric Environ Med* 1998, **5**, 169-175.

24. Magarolas R, Monsó E, Aguilar X, Radon K, Nowak D, Martínez C, Morera J: Prevalence and risk factors of respiratory symptoms in farmers. *Med Clin* (*Barc*) 2000, **114**, 685-689 (in Spanish).

25. Malmros P, Sigsgaard T, Bach B: Occupational health problems due to garbage sorting. *Waste Manag Res* 1992, **10**, 227-234.

26. Milanowski J: Noxious effects of organic dust on respiratory tract. *Pneumonol Alergol Pol* 1996, **64**, 118-129 (in Polish).

27. Nieuwenhuijsen MJ, Noderer KS, Schenker MB, Vallyathan V, Olenchock S: Personal exposure to dust, endotoxin and crystalline silica in California agriculture. *Ann Occup Hyg* 1999, **43**, 35-42.

28. Olszewski M, Kluciński W: Chronic disease of lower airways in horses (COPD). *Med Wet* 1992, **49**, 70-73 (in Polish).

29. Preller L, Heederik D, Kromhout H, Boleij JSM, Tielen MJM: Determinants of dust and endotoxin exposure of pig farmers: Development of a control strategy using empirical modeling. *Ann Occup Hyg* 1995, **39**, 545-557.

30. Preller L, Heederik D, Boleij JSM, Vogelzang PFJ, Tielen MJM: Lung function and chronic respiratory symptoms in pig farmers: focus on exposure to endotoxins and ammonia and use of disinfectants. *Occup Environ Med* 1995, **52**, 654-660.

31. Radon K, Winter C: Prevalence of respiratory symptoms in sheep breeders. *Occup Environ Med* 2003, **60**, 770-773.

32. Radon K, Danuser B, Iversen M, Monso E, Weber C, Hartung J, Donham KJ, Palmgren U, Nowak D: Air contaminants in different European farming environments. *Ann Agric Environ Med* 2002, **9**, 41-48.

33. Reynolds SJ, Donham KJ, Whitten P, Merchant JM, Burmeister LF, Popendorf WJ: Longitudinal evaluation of dose-response relationships for environmental exposures and pulmonary function in swine production workers. *Am J Ind Med* 1996, **29**, 33-40.

34. Rylander R: The role of endotoxin for reactions after exposure to cotton dust. *Am J Ind Med* 1987, **12**, 687-697.

35. Rylander R: Organic dusts – from knowledge to prevention. *Scand J Work Environ Health* 1994, **20**, 116-122.

36. Rylander R, Carvalheiro MF: Airways inflammation among workers in poultry houses. *Int Arch Occup Environ Health* 2005, **79**, 487-490.

37. Rylander R: Endotoxin and occupational airway disease. *Curr Opin Allergy Clin Immunol* 2006, **6**, 62-66.

38. Rylander R: Endotoxin in the air. Good or bad for you? *Clin Pulm Med* 2007, **14**, 140-147.

39. Saraf A, Larsson L: Use of gas chromatography/ion-trap tandem mass spectrometry for the determination of chemical markers of microorganisms in organic dust. *J Mass Spectrometry* 1996, **31**, 389-396.

40. Saraf A, Larsson L, Burge H, Milton D: Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a *Limulus* amebocyte lysate assay. *Appl Environ Microbiol* 1997, **63**, 2554-2559.

41. Schierl R, Heise A, Egger U, Schneider F, Eichelser R, Neser S, Nowak D: Endotoxin concentrations in modern animal houses in southern Bavaria. *Ann Agric Environ Med* 2007, **14**, 129-136.

42. Schulze A, van Strien R, Ehrenstein V, Schierl R, Küchenhoff H, Radon K: Ambient endotoxin level in an area with intensive livestock production. *Ann Agric Environ Med* 2006, **13**, 87-91.

43. Schwartz DA, Donham KJ, Olenchock SA, Popendorf WJ, Van Fossen DS, Burmeister LF, Merchant JA: Determinants of longitudinal changes in spirometric function among swine confinement operators and farmers. *Am J Respir Crit Care Med* 1995, **151**, 47-53.

44. Sebastian A, Szponar B, Larsson L: Characterization of the microbial community in indoor environments by chemical marker analysis: an update and critical evaluation. *Indoor Air* 2005, **15** (**Suppl. 9**), 20-26.

45. Sebastian A, Madsen AM, Mårtensson L, Pomorska D, Larsson L: Assessment of microbial exposure risks from handling of biofuel wood chips and straw – effect of outdoor storage. *Ann Agric Environ Med* 2006, **13**, 139-145.

46. Seedorf J, Hartung J, Schröder M, Linkert KH, Phillips VR, Holden MR, Sneath RW, Short JL, White RP, Pedersen R, Takai H, Johnsen JO, Metz JHM, Groot Kroerkamp PWG, Uenk GH, Wathes CM: Concentrations and emissions of airborne endotoxin and microorganisms in livestock buildings in Northern Europe. *J Agric Eng Res* 1998, **70**, 97-109.

47. Seedorf J, Schröder M, Hartung J: Emissions and immisions of bio-aerosols from a duck fattening unit. *Zentralbl Hyg Umweltmed* 1998, **201**, 387-403.

48. Simpson JC, Niven RM, Pickering CA, Oldham LA, Fletcher AM, Francis HC: Comparative personal exposures to organic dusts and endotoxin. *Ann Occup Hyg* 1999, **43**, 107-115.

49. Sonesson A, Larsson L, Schütz A, Hagmar L, Hallberg T: Comparison of the *Limulus amebocyte* lysate test and gas chromatography-mass spectrometry for measuring lipopolysaccharides (endotoxins) in airborne dust from poultry-processing industries. *Appl Environ Microbiol* 1990, **56**, 1271-1278.

50. Spaan S, Wouters IM, Oosting I, Doekes G, Heederik D: Exposure to inhalable dust and endotoxins in agricultural industries. *J Environ Monit* 2006, **8**, 63-72.

51. Szponar B, Larsson L: Use of mass spectrometry for characterising microbial communities in bioaerosols. *Ann Agric Environ Med* 2001, **8**, 111-117.

52. Taszkun I: *Studies on the role of Erwinia herbicola in the etiopathogenesis of natural cases of allergic alveolitis in cattle*. DVM Thesis. Agricultural University, Lublin 1995 (in Polish).

53. Wathes CM, Holden MR, Sneath RW, White RP, Phillips VR: Concentrations and emission rates of aerial ammonia, nitrous oxide, methane, carbon dioxide, dust and endotoxin in UK broiler and layer houses. *Br Poult Sci* 1997, **38**, 14-28.

54. Zejda JE, Barber E, Dosman JA, Olenchock SA, McDuffie HH, Rodes C, Hurst T: Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. *J Occup Med* 1994, **36**, 49-56.

55. Zhiping W, Malmberg P, Larsson BM, Larsson K, Larsson L, Saraf A: Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. *Am J Respir Crit Care Med* 1996, **154**, 1261-1266.