ENDOTOXIN IN THE OCCUPATIONAL ENVIRONMENT OF BAKERS: METHOD OF DETECTION*

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Key words: Endotoxin, Limulus test, Flour dust, Gram-negative bacteria, Bakery work sites, Area sampling

Abstract. The present study was conducted in a large mechanical city bakery. The AS-50 aspirators equipped with cellulose membrane filters were used for dust sampling. Airborne microbial content was assessed by means of sedimentation and aspiration using an Andersen sampler on Petri plates containing McConkey's medium. The following Gram-negative rods were detected in the bakery atmosphere: Erwinia herbicola, Acinetobacter Lwofi and Klebsiella oxytoca, in concentrations ranging from 1.4 $10^4$ to 3.5 $10^5$ colony forming units per cubic meter (cfu/m$^3$). Endotoxin concentration in flour dust sampled in selected work areas of the bakery ranged from 6.7 micrograms of endotoxin per gram of dust (µg/g) to 20.3 µg/g. Endotoxin level in the air was 0.04—0.05 micrograms of endotoxin per cubic meter (µg/m$^3$) The results of our study show that aspiration sampling is necessary for evaluation of airborne bacterial content and demonstrate the efficacy of the Limulus test of varying sensitivity to assay endotoxin level in the airborne dust. The advantage of this method is the possibility of assessing endotoxin in crude dust extracts.

INTRODUCTION

Endotoxin, a biologically active lipopolysaccharide (LPS) (20) present in the cell wall of Gram-negative bacteria is a well-known inflammatory agent in the induction of so-called toxic pyrexia (ODTS) and an aggravating stimulus in the course of several allergic diseases such as bronchial asthma, a common work-related condition among bakers (1, 2, 6, 17, 31). It is responsible for impaired respiratory function (lowered ventilation index FEV$_1$) (3) and circulatory disorders in occupationally exposed persons (8).

*The presented study was conducted and financed as a part of the research programme IMP 19.4. "Introduction of endotoxin assessment by means of LAL tube test-efficacy and sensitivity of the assay".

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The pathogenic role of endotoxin consists in the activation of alveolar macrophages to release pyrogens such as interleukin 1, 6, 8 and other mediators of inflammation such as cachectin, prostaglandins, leukotrienes and others (9, 32). Occupational exposure to endotoxin occurs most frequently in the following branches of industry: textile (12, 15, 22, 23, 27), food processing (16), timber (7, 25), agriculture (21) and agricultural processing (13, 14, 24, 29, 33), including the baking industry (6, 31).

Studies of environmental endotoxin content have been introduced only recently. Endotoxin concentrations in the atmosphere of different work areas vary over a wide range of values. For example, 0.2—1870 μg/m³ in the animal feed industry in Holland (29); 54.93 μg/m³ in grain mills in Poland (8). In the poultry slaughter house endotoxin concentration in the air was 0.02—1.5 μg/m³ (14). In the house dust endotoxin concentration measured with the Limulus test was found to be in the range of 0.12—20 μg/m³ (19). In the presence of domestic air humidification systems, the level of endotoxin varies between 0.13 and 0.39 μg/m³ (11, 26).

Flour dust is the main source of endotoxin in bakeries. Surveys conducted during the 1980s in the Polish baking industry showed that the concentrations of flour dust frequently exceeded admissible levels. Respiratory and skin diseases attributed to flour dust allergens were found to occur in 8—44% of all currently employed bakers. According to what was then a generally accepted point of view (6), the respiratory disorder in bakers was essentially of allergic nature and the characteristic disease syndrome was called “bakers’ asthma”. The presumed causal agents of the disease were allergens present in flour dust, mainly bacteria, spores of Actinomycetes and molds, as well as storage mites (e.g. Tyroglyphus and others) (6).

The investigations conducted in Britain in 1992 confirmed that the increase in immunological reactivity and airway responsiveness in bakers correlate with increased concentrations of flour dust (31).

The pathogenic properties of flour dust can be, at least, partly explained, by the presence of Gram-negative endotoxic bacteria often found in grain from which flour is obtained. It has been shown recently that plant enzymes such as alpha-amylase can act as pathogens, thus contributing to the allergenic properties of flour dust. These enzymes proved to be an important factor in the pathogenesis of allergy in bakers (1, 2).

Some countries have adopted a hygienic standard of exposure to endotoxin-containing organic dust (the Occupational Exposure Limit = OEL). For example, in Denmark, OEL is equal to 5 mg/m³ (33), in the USA and Canada 3 mg/m³ and in Holland 4 mg/m³ (29); in Poland, according to the updated OEL documentation for dust of plant and animal origin (12), the proposed limit is 4 mg/m³ for total dust and 2.0 mg/m³ for respirable dust containing less than 10% of free silica.

Gram-negative bacteria found on the surface of vegetal raw materials such as grain, herbs, or cotton are the sources of endotoxin in organic dust (such as flour dust) (5). The most commonly identified bacterial species occurring in organic dust are: Erwinia herbicola and Alcaligenes faecalis.

Erwinia herbicola or (synonymously), Enterobacter agglomerans, occurs in its natural environment on the surface of fruit, seed, and leaves of various plants, particularly on cereal leaves and stipules of cotton bushes. In grain mills and storehouses, the total number of these bacteria in airborne dust varies from
Endotoxin in environment of bakers

100 – 5000 thousand/m³ air. In grain dust, the concentration of *Erwinia herbicola* is about $3 \times 10^8$ cfu/1g dust, and in flour dust — $1.4 \times 10^5$ cfu/1 g dust (8).

Environmental determination of bacterial content based on culture methods is insufficient in evaluating exposure, because only live forms of microorganisms, capable of growing, are detected. Although non-viable forms preserve some of their endogenic properties, it is useful to introduce endotoxin concentration as a new parameter for a more accurate estimation of exposure to that particular pathogen in the environment.

The Limulus Amoebocyte Lysate (LAL) Endotoxin Test is commonly used to assay endotoxin concentration (18, 32). Airborne endotoxin concentration can also be determined by means of gas chromatography, but the correlation between these two methods is rather poor (28). According to Swedish investigators, the Limulus test detects only the fraction of lipopolysaccharides dissociating through the cell wall. Gas chromatography allows to measure the total lipopolysaccharide concentration, including the fraction that remains inactive in the Limulus method, as well as other non-endotoxic bacteria and various cellular artefacts — e.g. polynucleotides, proteins (10).

The purpose of the present study was to examine the usefulness of the Limulus test of varying sensitivity for the estimation of endotoxin level in airborne flour dust in selected work areas in the bakery. Air contamination with Gram-negative bacteria was evaluated in parallel to the measurement of endotoxin level.

**MATERIALS AND METHODS**

1. **Air sampling**

Air samples were collected in the main facilities of a large mechanical bakery at the following work sites:

a. at the kneading trough covering the process of adding flour and kneading

b. at the set of machines where bread or rolls are formed, during the process of dough dividing and portioning.

Air samples were collected to estimate the content of Gram-negative bacteria in the bakery atmosphere and as a source of flour dust in which endotoxin concentration was measured. Bacterial concentration was determined using Koch’s sedimentation method of passive air sampling (exposure of Petri plates containing Mc Conkey’s medium for 20 minutes), and by aspiration using a 6-level Andersen sampler with the following pore sizes:

<table>
<thead>
<tr>
<th>Level</th>
<th>Pore Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1.81 mm</td>
</tr>
<tr>
<td>2nd</td>
<td>0.91 mm</td>
</tr>
<tr>
<td>3rd</td>
<td>0.71 mm</td>
</tr>
<tr>
<td>4th*</td>
<td>0.53 mm</td>
</tr>
<tr>
<td>5th*</td>
<td>0.34 mm</td>
</tr>
<tr>
<td>6th*</td>
<td>0.25 mm</td>
</tr>
</tbody>
</table>

*These are levels at which the respirable fraction of the aerosol is collected only.*
Air samples were collected by an AS-50 aspirator at a flow rate of 28.3 l/min for 20 minutes on Mc Conkey's medium (Diagnostic Merc) (4); two plates were provided for each type of measurement. The plates were then incubated for three days at the temperature of 27°C followed by one day in the refrigerator (optimal growth conditions for saprophytic bacteria).

Airborne dust was assessed in the same work areas in which samples were taken for microbiological analysis. Air was aspirated with an AS-50 aspirator for 30 minutes through cellulose membrane filters of 0.8 μm pore size. Dust content was measured by weighing the filters before and after aspiration; the difference in weight was equal to the mass of the aspirated dust.

2. Bacterial concentration and identification of species

Air samples containing bacteria were collected on Mc Conkey's medium (Diagnostic Merc). Species identification was based on the analysis of biochemical properties of the cultured strains, using the Epl 21 HTL Warszawa diagnostic test for Gram-negative rod identification. Following incubation, the number of colonies on the plates was counted and bacterial concentration was calculated from the number of colonies and the volume of air passing through the impactor according to the formula quoted by Dutkiewicz (8).

a) in the case of sedimentation

\[ x = \frac{a \cdot 10000}{\pi r^2 \cdot K} \]

where: 
- \( x \) — number of bacteria in 1 m³ of air, expressed as (cfu)
- \( a \) — average number of colonies on 1 plate
- \( r \) — plate radius (cm)
- \( \pi r^2 \) — plate area (cm²)
- \( K \) — exposure time in minutes/5

b) in the case of aspiration:

\[ x = \frac{a \cdot 1000}{V} \]

where: 
- \( x \) — number of bacteria in 1 m³ of air, expressed as (cfu)
- \( a \) — average number of colonies on 1 plate
- \( V \) — volume of air sampled during time \( t \) (in m³)

3. Obtaining dust extract and endotoxin assay

Dust containing filters were placed in 50 ml sterile Erlenmayer flasks and immersed in 10 ml of sterile Coca's solution (30) at pH 8.2 to obtain endotoxin extract. Thus prepared, the samples were frozen and defrozen twice according to the procedure described by Dutkiewicz (8). Endotoxin was assayed in crude dust extract (at dilutions 1:10, 1:100, 1:1000) by means of the Limulus — Endotoxin-Test (LAL) manufactured by \( \alpha \)-DiaLab of sensitivity 0.125 EU/ml and 0.06 EU/ml containing reference \( E. coli \) 055:B5 at titre 100 ng = 1000 EU (Endotoxin Units) and calculated from the formula presented in the updated OEL documentation (9). Endotoxin content in 1 g of flour dust according to the formula (1):
where: $B$ — concentration of endotoxin in studied dust, expressed as $(\mu g/g)$

$ASE$ — minimal dose of standard endotoxin capable of producing coagulation of test reagent

$ORP$ — inverse of the highest sample dilution capable of producing coagulation of test reagent

$SPW$ — initial concentration of dust sample in distilled water (g/ml).

The concentration of endotoxin in the air samples collected through a filter was calculated according to the formula (2):

$$Y = \frac{B \cdot A}{1000}$$  \hspace{1cm} (2)

where: $Y$ — concentration of airborne endotoxin, expressed as $(\mu g/g)$

$B$ — concentration of endotoxin in airborne dust, expressed as $(\mu g/g)$

$A$ — concentration of airborne dust, expressed as $(mg/m^3)$ (see: next formula (3)).

Concentration of airborne dust was calculated according to (3):

$$A = \frac{G}{V \cdot 1000}$$

where: $A$ — concentration of dust in air, expressed as $(mg/m^3)$

$G$ — mass of dust collected on a filter, expressed as (mg)

$V$ — volume of air passing through a filter, expressed as (liters).

Dust extract collected on membrane filters was diluted in the following proportions: 1:10, 1:100, 1:1000, using nonpyrogenic water and nonpyrogenic test tubes included in the Limulus reagent set (α-DiaLab). A parallel dilution series of $E.\ coli$ B5:055 reference endotoxin was prepared to assess the reactivity of the Limulus reagent of varying sensitivity.

### Table 1. Dilution series of standard $E.\ coli$ — 055:B5 endotoxin used as a positive control in the Limulus test (1 EU = 0.1 ng)

<table>
<thead>
<tr>
<th>Concentration of reference endotoxin in EU/ml (in endotoxic units EU/ml)</th>
<th>Reactivity to the Limulus reagent of varying sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>1</td>
<td>500 000</td>
</tr>
<tr>
<td>2</td>
<td>10 000</td>
</tr>
<tr>
<td>3</td>
<td>5 500</td>
</tr>
<tr>
<td>4</td>
<td>2 500</td>
</tr>
<tr>
<td>5</td>
<td>1.250</td>
</tr>
<tr>
<td>6</td>
<td>0.620</td>
</tr>
<tr>
<td>7</td>
<td>0.310</td>
</tr>
<tr>
<td>8</td>
<td>0.150</td>
</tr>
<tr>
<td>9</td>
<td>0.075</td>
</tr>
<tr>
<td>10</td>
<td>0.037</td>
</tr>
<tr>
<td>11</td>
<td>0.016</td>
</tr>
</tbody>
</table>
assay of varying sensitivity. The values of reactivity for the reagents used throughout the test are shown in Table 1. Reactivity assessment is recommended before the use of each new lot. In the case of Limulus reagent of sensitivity 0.62 EU/ml maximum dilution of dust extract, still producing coagulation, was 1:100 (with negative result for 1:1000 dilution). Coagulation was obtained in the 1:1000 dilution after addition of the Limulus reagent of sensitivity 0.075 EU/ml to a similar dilution series of dust extract. The more sensitive reagent produced coagulation in all the dilutions of the series, i.e., 1:10, 1:100, 1:1000.

RESULTS

a) Bacteria

The content of Gram-negative bacteria in the atmosphere of the bakery facilities is shown in Table 2. The content of Gram-negative bacteria in the studied work areas was measured in parallel to the estimation of dustiness. The results show considerable variation dependent on the method of measurement, work shift and the fraction of airborne dust. In Koch's sedimentation method, the levels of airborne bacterial concentration were significantly lower in comparison with those obtained by aspiration sampling. Possible explanations of this are: a smaller volume of air in close contact with the culture medium in the case of Koch's method and the growth of only those particles which can freely settle on the surface of the medium. In the case where Andersen sampler cannot be used (no available electric power supply), Koch's method allows only an approximate estimation of the presence of bacteria in the studied environment. The discrepancy between the results obtained by Koch's method and those obtained by aspiration sampling (Andersen's) can reach two ranges of magnitude.

In the air samples collected during the night shift, bacterial content was found to be higher in comparison with the day shift. This was true for both sampling methods.

Table 2. Gram-negative bacterial content in the atmosphere of the bakery facilities measured by: a) Koch's sedimentation method, and b) Andersen's aspiration method

<table>
<thead>
<tr>
<th>Work site</th>
<th>Work shift</th>
<th>Total no. of</th>
<th>Bacterial Content in dusty air contaminants in cfu/m³</th>
<th>Respirable fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Kneading trough</td>
<td>night</td>
<td>280 000(b)</td>
<td>88 000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250 (a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>day</td>
<td>14 000 (b)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 (a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Dough dividing</td>
<td>night</td>
<td>350 000 (b)</td>
<td>304 000</td>
<td></td>
</tr>
<tr>
<td>machine</td>
<td></td>
<td>4 400 (a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>day</td>
<td>14 000 (b)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 (a)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*— arithmetic mean of 9 measurements
In the dough portioning area, where several persons work in constant movement at sliding production chains, airborne bacterial content assessed by Koch’s sedimentation method was higher than in the proximity of the kneading trough where only one worker was employed. Aspiration sampling by means of the Andersen sampler showed no such difference in bacterial content between the two investigated work areas. This may be due to a relative proximity of the two sites, and because the Andersen sampler collects large quantities of air over wide production spaces, the two environments were assessed as a whole. The use of the Andersen sampler permitted to evaluate bacterial content in respirable dust, i.e. the fraction of the aerosol reaching pulmonary alveoli of particle size less than 3.5 µm.

As shown in Table 2, bacterial content in the respirable fraction, sampled at the kneading trough during the night shift, was $8 \times 10^4$ cfu, i.e. 28.5% of total bacterial content in total dust, whereas in the dough portioning area the respirable fraction constituted as much as 86% of total bacterial content.

During the day shift total bacterial content, at both locations, was lower than that of the night shift, which was equal to $1.4 \times 10^4$ cfu. Also, no respirable fraction was detected in the day shift samples. The discrepancy might be due to a lower production output, especially of small assortment, as well as to variable micro-climatic conditions in the production facilities.

Examination of colonies and biochemical analysis permitted to identify the species of Gram-negative bacteria detected in the bakery atmosphere. The following species of Gram-negative bacteria were found:

- *Erwinia herbicola* — Gram-negative endotoxic rods originating (= *Enterobacter agglomerans*) mainly from grain.
- *Actinetobacter Lwoffi* — Gram-negative endotoxic bacteria, widely distributed in the environment, occurring in soil, water, on the surface of skin and mucosa of humans and animals, on plant and animal products. Present in the atmosphere of farming facilities.
- *Klebsiella oxytoca* — Gram-negative rod occurring in the intestinal tract of humans and animals.

b) Airborne endotoxin

Endotoxin concentrations in the bakery atmosphere measured at the studied work locations are shown in Table 3. At the kneading trough endotoxin concentration in airborne dust, measured in reference to standard endotoxin of sensitivity 0.62 EU/ml. was 6.7 µg/g, and 8.1 µg/g if using the standard of sensitivity 0.075 EU/ml.

In the area of dough portioning, assessed similarly endotoxin concentrations were 16.7 µg/g and 20.3 µg/g, respectively. Higher endotoxin content in airborne dust in the environment of dough portioning machine was due to higher concentration of Gram-negative bacteria in work area, which may be due to larger number of persons employed as well as to the type of flour used (in this case, wheat flour). However, because airborne dust concentration in the environment of dough was much lower than in the proximity of the kneading trough (Table 4), endotoxin concentration at both locations was in the same range of values (0.04—0.05 µg/m$^3$).
Table 3. Endotoxin concentration in dust (µg/g) and endotoxin concentration in air (µg/m³) in the studied work areas of the bakery

<table>
<thead>
<tr>
<th>Work site</th>
<th>Mass of dust collected on a filter (mg)</th>
<th>Endotoxin concentration in dust (µg/g)</th>
<th>Endotoxin concentration in air (µg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kneading trough—preparation of dough</td>
<td>9.2</td>
<td>6.7 (a)</td>
<td>0.04 (a)</td>
</tr>
<tr>
<td>2. Dough dividing machine—dividing and forming bread and rolls</td>
<td>3.7</td>
<td>16.7 (a)</td>
<td>0.04 (a)</td>
</tr>
</tbody>
</table>

The assessed endotoxin concentrations in dust and air are lower than corresponding levels in grain elevators and storehouses due to high degree of dustiness from fresh raw material.

Table 4. Total dust concentration in the studied work areas of the bakery (determined by stationary air sampling using the AS-50 aspirators)

<table>
<thead>
<tr>
<th>Work site</th>
<th>Sampling time (min.)</th>
<th>Air filtration rate (1/min.)</th>
<th>Total dust concentration (mg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kneading trough—preparation of dough</td>
<td>30</td>
<td>50</td>
<td>27.5; 16.3; 11.6; 6.1</td>
</tr>
<tr>
<td>2. Dough dividing machine—dividing and forming bread and rolls</td>
<td>30</td>
<td>50</td>
<td>2.1; 1.9; 2.5; 2.0</td>
</tr>
</tbody>
</table>

CONCLUSIONS

1. In the atmosphere of a large mechanical bakery we detected Gram-negative endotoxic bacteria of the following species:
   - *Erwinia herbicola*
   - *Acinetobacter Lwoffi*
   - *Klebsiella oxytoca*

2. The concentrations of Gram-negative bacteria in the investigated work areas were higher than the proposed hygienic standard value of OEL = 20 000 cfu/m³ (9).

3. Endotoxin was detected in airborne dust and air in the selected work areas of the bakery.

4. Endotoxin concentrations measured in the work areas were lower than the proposed standard value of OEL = 0.2 µg/m³ (9).
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Received for publication: March 30, 1994
Approved for publication: April 28, 1994