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SHORT COMMUNICATION

Peptidoglycans in cutting fluids – a good indicator of bacterial contamination?

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Abstract

Objective. The aim of this study was to estimate the content of peptidoglycans in cutting fluids (CFs) and to assess the possibility of using them as a marker of bacterial contamination in this type of occupational environment.

Materials and methods. A total of 11 samples of CFs were collected: 8 were taken from the working machine systems and 3 were unused CF samples. The peptidoglycans were determinated with the kinetic version of the Silkworm Larvae Plasma (SLP) test.

Results. The average concentration of bacteria was 5.58×10^5 CFU/mL, and peptidoglycans – 28.2 ng/mL. The variability for peptidoglycans concentration was less pronounced than that for bacteria (GSD 6 and 13.3, respectively). Taking into consideration the National Research and Safety Institute (INRS – *Institut National de Recherche et de Sécurité*) limit value the concentrations of bacteria and peptidoglycans, as well as the usage of the fluids, the analysis showed that peptidoglycans reflect the differences between the studied factors much more accurately than bacteria. The correlation analysis, however, showed that the levels of peptidoglycans in the examined CFs strongly correlated with the concentrations of viable bacteria ($R^2 = 0.50$, $p<0.05$).

Conclusions. The study confirmed that the CFs may contain immunologically active substances of bacterial origin even though they did not show any bacterial growth. Moreover, it showed that the concentrations of peptidoglycans in CFs precisely reflect the exposure to bacteria, and as a structural component of the cell wall can be treated as their marker.

Key words

bacterial contamination, cutting fluids, peptidoglycans, SLP test

INTRODUCTION

The presence of water and organic compounds in cutting fluids (CFs) results in their substantial bacterial and fungal contamination. The microbial concentrations in overworked CFs may reach up to 1×10^6 colony forming units (CFU) in 1 mL [1, 2]. The control of microbial contamination of CFs in machining systems is a process of high economical and health importance. To date, the most popular control measures are based on a few methods (e.g. measurement of the adenosine-5'-triphosphate (ATP) content, real-time polymerase chain reaction, fluorescent *in situ* hybridization) that examine the presence of viable and/or non-viable microorganisms, disregarding their immunological potential which can also affect human health [3, 4, 5].

Several immunologically reactive, constituents of bacterial cell walls, such as endotoxins and peptidoglycans, can be mobilized and released into the environment in high quantities after destruction and/or death of the cells. Endotoxins present in CFs have been analyzed quite widely and for some time have been considered a good indicator of their contamination with Gram-negative bacteria [2, 6, 7]. An equally important, but still inestimable part of exposure to bacteria is the presence of peptidoglycans in CFs. It is estimated that peptidoglycans constitute of about 70% and 25% of the whole cell wall of Gram-positive and Gram-negative bacteria, respectively [8]. Knowledge about the adverse health effects caused by peptidoglycans is still very limited. According to available scientific data, they (such as endotoxins) are able to induce pro-inflammatory markers [9]. It is also suggested that peptidoglycans play a significant role in the pathogenesis of complex bacterial infections by reinforcing the biological effects of endotoxins [10]. In extreme cases, they may also induce sepsis [9]. Analysis of peptidoglycans in the workplace has not so far been widely used in environmental studies [11, 12, 13]. The work environment, where the use of CFs takes place, has never been tested in this regard.

Objective. The objective of the presented study was to estimate the content of peptidoglycans in CFs, and to assess the possibility of using them as a marker of bacterial contamination in this type of occupational environment.

MATERIALS AND METHODS

Sampling site characteristics. The study was carried out in a metal-working plant which manufactures machinery and equipment for the food industry. The plant processes metals, mainly acid-proof steel, with the use of a variety of machine tools, such as cylindrical grinders, lathes (including centre and numerically controlled lathes), metal cutting saws and drills. The above-mentioned production processes require the use of water-soluble CFs, which are replaced in the machines with new ones approximately every two months. At the time of the study, however, the variable of the fluid usage was not taken into account, as a tracking of the changes in the levels of fluid contamination was not the main goal of the study.

Collection and analysis of cutting fluid samples. A total of 11 samples of CFs were collected, out of which 8 were taken

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from the working machine systems (C01-C04 and C7-C10) and 3 were unused CF samples (C05, C06, C11). All samples were collected into sterile containers with the capacity of 50 mL.

In order to perform the quantitative assessment of bacteria, the samples were processed using the plate dilution method. For this purpose, each sample was serially diluted in 0.9% sodium chloride solution, and then 0.1 mL of each diluted sample was plated (in triplicate) onto Petri dishes containing trypticase soy agar (bioMerieux, France) with 5% additive of defibrinated sheep blood. The samples were incubated according to the following scheme: 1 day (37 °C) + 3 days (22 °C) + 3 days (4 °C). Prolonged incubation of the samples aimed at facilitating a slow growth of strains at lower temperatures. After incubation, all growing colonies were counted and the concentrations of bacteria in CFs were expressed as CFU/mL.

The remaining part of the collected fluids was used to determine the level of peptidoglycans. For this purpose, CF samples were eluted in 10 mL of pyrogen-free water (Lonza, USA) with an additive of 0.05% Tween 20 (Sigma, Poland), then shaken for 15 min and centrifuged at 1,000×g for 15 min. In the final step of the analysis, 1.8 mL of the eluate was sampled from the obtained supernatant, and the peptidoglycan concentrations (in ng/mL) were spectrophotometrically determined using the kinetic version of the Silkworm Larvae Plasma (SLP) test according to the manufacturer's recommendations (Wako Pure Chemical Industries, Ltd., Japan).

Statistical analysis. The raw data were used to calculate the geometric means (GM) and geometric standard deviations (GSD). In order to use the Student's *t*-test and Pearson's correlation analysis, these data were subsequently log-transformed. All calculations were performed using the package STATISTICA data analysis software system, version 7.1. (StatSoft, Inc., Tulsa, OK, USA, 2006), assuming a statistically significant value of $p<0.05$.

RESULTS AND DISCUSSION

Analysis of CFs revealed the presence of bacterial contamination in the majority of the samples (Tab. 1). The average bacterial concentration was 5.58×10^5 CFU/mL. The highest concentration (1.85×10^7 CFU/mL) was found in fluid C09, while in C03 and C05 fluids no bacterial growth was recorded. It should be noted that two analyzed samples of the unused CFs were already contaminated, even up to the level of 10^4 CFU/mL.

The average concentration of peptidoglycans in analyzed CFs was 28.2 ng/mL. The highest concentration was found in sample C10 (427 ng/mL) and the lowest in C05 (3.40 ng/mL). In contrast to viable bacteria, the presence of peptidoglycans was demonstrated in all of the collected CF samples, and their concentration variability was less pronounced than that for bacteria (GSD 6 and 13.3, respectively).

The bacterial contamination of analyzed CFs was on the same level (10^4 to 10^7 CFU/mL), as observed in other studies [2, 14]. On the world scale, the permissible level of CFs' contamination is not normatively regulated, but a few reference value proposals are available in the scientific literature. For instance, the guidelines proposed by the National Research and Safety Institute for the Prevention of Occupational Accidents and Diseases (INRS, France) [15] suggests that the unacceptable microbiological contamination of CFs occurs after exceeding the value of 1×10^6 CFU/mL. Taking this into account, in the case of 50% of the tested CF samples in the presented study, the levels of microbial contamination were higher than the suggested limit value. A striking fact was that two of the unused CF samples were significantly contaminated with bacteria, which may indicate irregularities during the storage and/or production processes. Regarding peptidoglycans, so far there are no threshold limit value proposals, and the possibility to compare the obtained results is rather limited.

Taking into consideration the INRS limit value, the concentrations of bacteria and peptidoglycans in CFs were analyzed, and new fluids were compared to those that were used (Tab. 2). Analyses revealed that peptidoglycans reflect the differences between the studied factors much more accurately than bacteria. The correlation analysis (Fig. 1), however, showed that the levels of peptidoglycans in the examined CFs strongly correlated with the concentrations of viable bacteria ($R^2 = 0.50$, $p<0.05$).

The presented study shows that the CFs may contain immunologically active substances of bacterial origin, even

Table 1. Concentrations of bacteria and peptidoglycans in the cutting fluids

Fluid sample	Concentration	
	Viable bacteria	Peptidoglycans
	[×10⁴ CFU/mL]	[ng/mL]
C01	75.0	16.0
C02	41.7	79.2
C03	0	8.00
C04	146	65.1
C05[a]	0	3.40
C06[a]	3.75	3.54
C07	26.5	30.0
C08	89.6	415
C09	1850	62.6
C10	160	427
C11[a]	0.43	3.47
GM[b]	**55.8**	**28.2**
GSD[c]	**13.3**	**6.00**

[a]unused cutting fluids; [b]GM – geometric mean; [c]GSD – geometric standard deviation

Table 2. Comparison of bacteria and peptidoglycan concentrations in cutting fluids, considering the INRS guidelines and usage of the fluids

Type of factor		N	Concentration					
			Viable bacteria			Peptidoglycans		
			[×10⁴ CFU/mL]			[ng/mL]		
			GM[a]	GSD[b]	p[c]	GM	GSD	p
INRS guideline	> 10⁶ CFU/mL	4	444	3.54	**<0.05**	163	2.97	**<0.01**
	< 10⁶ CFU/mL	7	10.6	8.26		10.3	3.43	
Usage of fluids	Used	8	164	4.75	ns[d]	62.0	4.10	**<0.01**
	Unused	3	1.27	4.59		3.46	1.02	

[a]GM – geometric mean; [b]GSD – geometric standard deviation; [c]p – probability; [d]ns – not significant

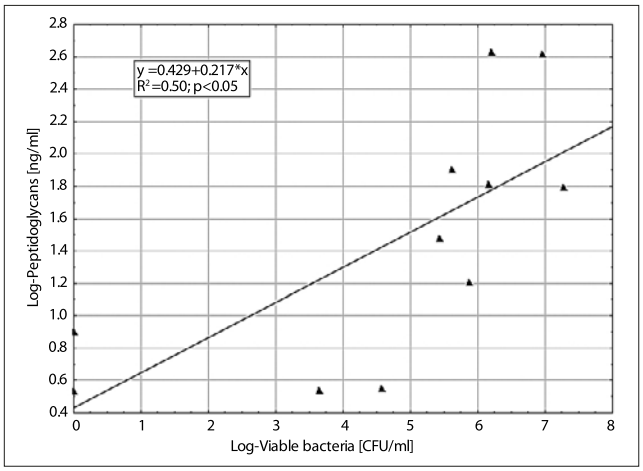

Figure 1. Relationship between the number of viable bacteria and peptidoglycans in examined cutting fluids

though they did not show any bacterial growth. Moreover, it shows that the concentrations of peptidoglycans in CFs precisely reflect the exposure to bacteria, and as a structural component of the cell wall can be treated as their marker. Despite these promising results, however, it is too early to postulate the analyses of this factor as a replacement for the traditional quantitative methods of bacteria evaluation. Before this happens, an explanation and/or clarification will be needed as to whether this type of dependence also occurs in the case of other types of CFs, and how strong is the impact on the levels of peptidoglycans exerted by the qualitative composition of bacterial CF community. The study reveals that the SLP test can be successfully used for determination of peptidoglycans in CFs. Such a procedure, however, should undergo validation, as it has been carried out with the LAL test for the determination of endotoxins [7]. In addition, there are no data on the health effects that may be caused by peptidoglycans, as well as on the possible interaction between this factor and endotoxins, or (1→3)-β-D-glucans of fungal origin. Thus, all these issues should be investigated in details in the future.

CONCLUSIONS

This is the first report of peptidoglycan analysis in cutting fluids (CFs) determined with Silkworm Larvae Plasma (SLP) test. The presented study shows that the CFs may contain immunologically active substances of bacterial origin, even though they did not show any bacterial growth. Moreover, it shows that the concentrations of peptidoglycans in CFs precisely reflect the exposure to bacteria, and as a structural component of the cell wall can be treated as their marker.

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