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Identification of *Bacillus* spp. colonizing the nasal mucosa of healthy adults living in the suburban area using the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) system

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ABSTRACT

Bacillus spp. can be regarded as a rare component of the nasal mucosa microflora.
The aim of this study was to identify *Bacillus* spp. from the nasal mucosa of healthy adults living in the suburban area near Lublin using the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) system.
A total of 11 bacterial isolates from the nasal specimens were cultured. The following species were identified using the routine microbiological methods: *Staphylococcus aureus* (3 isolates), *S. epidermidis* (1 isolate), *S. intermedius* (1 isolate) and *Staphylococcus* spp. (1 isolate). Moreover, 2 strains of *Escherichia coli* were isolated. Besides, 3 isolates of *Bacillus* spp. were found. These isolates were characterized by means of MALDI-TOF MS, resulting in highly specific mass spectral fingerprints and these were identified as *B. pumilus*, *B. safenis* and *B. licheniformis*. It was observed that all studied *Bacillus* spp. isolates only had the masses in common at 3864 ± 2, 7727 ± 2, and 14301 ± 4. The spectra of *B. safensis* and *B. pumilus* showed peaks at m/z 4914 ± 3, 6621 ± 3 and 14291 ± 2, which were absent in the spectrum of *B. licheniformis*. For *B. safenis* and *B. pumilus*, other potential biomarkers could be found at *m/z* 12620 and 16668, respectively.

INTRODUCTION

The microbiota of the upper respiratory tract mucosa composition is rich and extremely diverse. A typical nasal microflora is composed of staphylococci, including *S. aureus* and coagulase-negative staphylococci, mainly *S. epidermidis* [13]. Besides, other bacteria like *Bacillus* spp. or Gram-negative rods, e.g. *E. coli* may be a part of this microflora. People living in suburban areas are often exposed to the presence of many opportunistic environmental pathogens, being then the colonizers of the human body. Routine methods of microbiological diagnostics together with commercial tests allow identifying only the most frequently isolated microorganisms [8,15,20,22]. Nowadays a growing incidence of *Bacillus* spp. is observed in several clinical specimens where they are not the contamination [9, 25]. This may cause diagnostic problems and require the use of more sophisticated methods not available in medical laboratories. Here we describe the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform for the rapid discrimination of *Bacillus* spp. isolates and other selected bacteria isolated from the nasal mucosa of healthy adults living in the suburban area near Lublin.

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MATERIALS AND METHODS

In the present study conducted between June and July 2014, bacterial isolates from the nasal mucosa of 7 healthy adults living in the neighborhood in the suburban area near Lublin, Poland (the Ethics Committee Medical University

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of Lublin approved the study protocol, KE-0254/75/2011) were included. A total of 14 swabs were collected from the nasal mucosa of both nostrils of each person. The specimens were cultured on routinely used media (agar, blood agar, Chapman and McConkey media) at 35°C for 24-48 h. The bacterial isolates were initially identified based on the morphology of their colonies, Gram stain morphology and/or biochemical methods (API 20E and ID 32 Staph systems, bioMerieux, France). Eleven isolates were included in the analysis by MALDI-TOF MS system (Daltonik Bruker, Germany). The reference species of *Escherichia coli* ATCC 25922 and *Staphylococcus epidermidis* ATCC 12228 were chosen as positive controls for the MALDI-TOF MS evaluation because they are representatives of the microflora of the nasal mucosa.

All samples for mass spectrometry were prepared as recommended by the manufacturer [15,35]. The identification was preceded by preliminary extraction of proteins with ethanol and formic acid. For this purpose, a single colony of a fresh 18-24 hour culture growing on agar medium at 35°C was suspended in 300 µl of sterile deionized water, after which 900 ml of pure ethanol (POCH) was added. Then each sample was mixed thoroughly by vortexing. The resulting sample was then centrifuged for 2 minutes at 13 000 rpm/min. After the supernatant had been discarded, 50 ml of 70% aqueous formic acid and then 50 ml of acetonitrile (Fluka Analytical, Switzerland) were added to the precipitate and the sample was thoroughly mixed by vortexing. After centrifugation (13 000 rpm/min for 2 min), 1 µl of the supernatant was collected, applied on a metal plate and allowed to dry at room temperature. Then 1 µl of matrix solution was applied and the sample was left to dry at room temperature. The metal plate with the samples was placed in a MALDI Biotyper chamber for analysis. An automatic measurement of the spectrum and a comparative analysis with reference spectra of bacteria were performed using the Ultraflextreme mass spectrometer and MALDI-Biotyper 3.0 software (Bruker Daltonik, Germany), including 3672 spectra. The reliability of identification in the MALDI-Biotyper 3.0 software was expressed in points. A score ≥1.7 and <2.0 indicated identification to the genus level, while scores ≥2.0 indicated identification to the species level. The similarity values required to separate *Bacillus* spp. species on the basis of the protein profile were according to MALDI Biotyper 3.0 software spectra.

RESULTS

A total of 11 bacterial species were isolated from the nasal mucosa membrane of healthy adults living in the suburban area near Lublin (Table 1). In the diagnostic workflow of the isolates, the majority of bacterial species were analysed and identified by routine diagnostic methods and all of them by MALDI-TOF MS system. Routine diagnostic methods revealed the occurrence of 6 isolates of staphylococci: *S. aureus* (3 isolates), *S. epidermidis* (1 isolate), *S. intermedius* (1 isolate), *Staphylococcus* spp. (1 isolate). In addition, 2 isolates of *E. coli* were found. Besides, 3 isolates of *Bacillus* spp. were identified using colony morphology and Gram-staining (Figure 1)

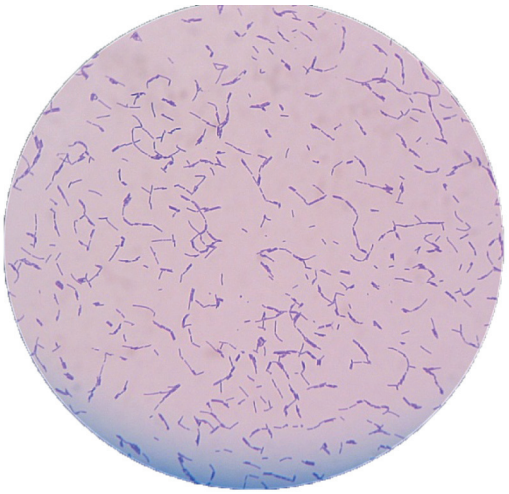

Figure 1. A microscopic image of *Bacillus* spp. isolate from the nasal mucosa of healthy adults staining by the Gram method

All *Bacillus* spp. isolates were analysed after formic acid extraction by MALDI-TOF MS (Table 1). Among these isolates *B. licheniformis* (1 isolate), *B. pumilus* (1 isolate) and *B. safensis* (1 isolate) were identified. Besides, 1 isolate of *Staphylococcus* spp. was identified as *S. pseudintermedius.*

Table 1. Prevalence of bacterial isolated from nasal mucosa of healthy adults living in the suburban area

No.	Sex	Identification of isolates by using	
		routine diagnostic methods	MALDI-TOF MS
1	F	*Bacillus spp.*	*Bacillus licheniformis*
2	F	*Bacillus spp.*	*Bacillus pumilus*
3	F	*Staphylococcus aureus*	*Staphylococcus aureus*
4	M	*Staphylococcus aureus*	*Staphylococcus aureus*
6	M	*Staphylococcus intermedius*	*Staphylococcus intermedius*
		Staphylococcus spp.	*Staphylococcus pseudintermedius*
5	M	*Bacillus spp.*	*Bacillus safensis*
		Staphylococcus epidermidis	*Staphylococcus epidermidis*
7	M	*Staphylococcus aureus*	*Staphylococcus aureus*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*

Note: F – female, M – male

The results of MALDI-TOF MS spectral profiles indicate that 3 strains isolated from the nasal mucosa of healthy adults represent a bacterial species within the genus *Bacillus* (Figure 2). Striking differences in the peaks of these bacteria were demonstrated. All mass lists were analyzed and compared over the mass interval from 2000 to 20 000 Da because of good reproducibility. All final strain-specific peak mass lists were then compared to each other with the application of flexAnalysis (version 3.3) to determine characteristic peak masses.

It should be noted that the peak with the masses at *m/z* 3864 ± 2, 7727 ± 2, and 14301 ± 4 were exclusively present in the studied strains of *Bacillus* spp., suggesting that these peaks could represent a genus-specific biomarker. Furthermore, a number of peaks were found in common among the spectral profiles of *B. pumilus* and *B. safensis* (4914 ± 3, 6621 ± 3 and 14291 ± 2), which has no counterpart

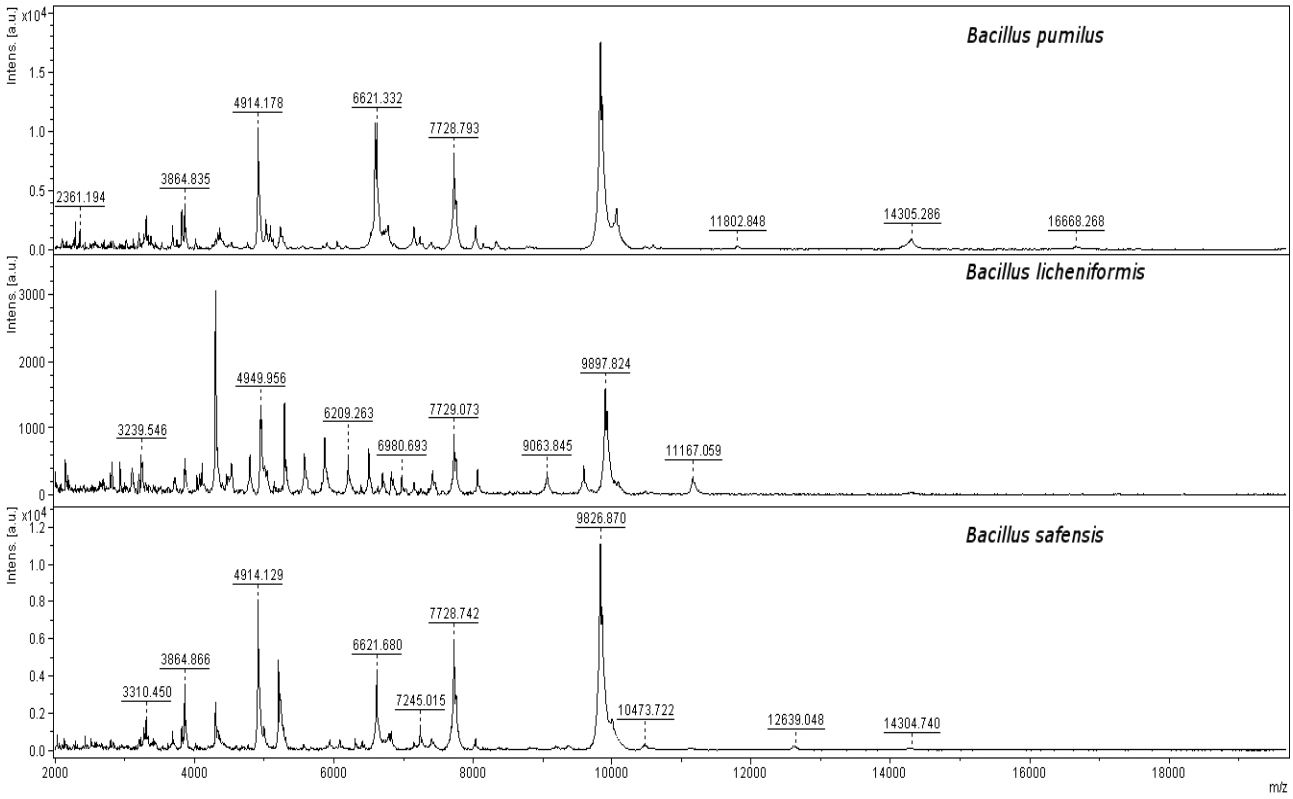

Figure 2. MALDI-TOF mass spectral profiles of *B. pumilus*, *B. licheniformis*, and *B. safensis* tested isolates

in the spectrum of *B. licheniformis*. The presence and/or absence of some peaks and differences in the peak masses indicated intra-genus and intra-species variability, allowing the discrimination between the different species. A number of different peaks were found in the studied *Bacillus* spp. (*B. pumilus* – 96, *B. licheniformis* – 86*, B. safensis* – 37). For *B. safensis*, other potential biomarkers can be found at *m/z* 12620 and for *B. pumilus* at *m/z* 16668.

DISCUSSION

The upper respiratory tract mucosa membranes provide many different habitats and are colonized by a wide range of microorganisms. The environment in which people live may influence the composition of microbiota. The environment and the animals, e.g. domestic or wild birds, cats, dogs or other organisms, may pose a risk factor as well as be a source of different pathogens or opportunistic bacteria. They can reside in their nasal or throat cavities for a long time without ever causing any clinical signs of disease. For healthy people the risk of catching opportunistic bacteria from domestic birds or other animals and then developing a disease is rare but possible.

Three different *Bacillus* spp. strains (*B. pumilus, B. licheniformis, B. safenis*) were identified during our studies with MALDI-TOF MS as microflora colonizing the nasal mucosa of healthy adults living in the suburban area. Log(score) values of the isolated *B. pumilus*, *B. safensis* and *B. licheniformis* reached 1.78, 2.093 and 2.101, respectively. It means that *B. pumilus* represents the correct identification at the genus level. In contrast to other isolates, a log(score) value represents a good identification at the species level (≥ 2). Sometimes unique mass profiles are yielded and the results of these bacteria do not match in the database [1].

Bacillus species, e.g. *B. marinus, B. subtilis, B. pumilus, B. licheniformis, B. cereus,* and *B. mycoides*, are saprophytic aerobic endospore formers widely distributed in the natural environment [9]. These Gram-positive or Gram-variable rods are usually found in soils, vegetable, water, dust, air and deep subterranean sources [19]. *B. licheniformis, B. pumilus* and *B. safensis* have recently been isolated from marine or other environments, in addition to other species such as *B. subtilis* or *B. cereus* [14,23,26,30].

Bacillus spp. may contaminate the hospital environment (hospital linen and dialysis equipment), ventilator equipment and disinfectants [3,7,11,17]. Some of these species have been troublesome to food producers and they were also isolated from foodborne infections. Except for a few species, a large majority have no pathogenic potential and have rarely been associated with opportunistic diseases in man or animals.

Some *Bacillus* spp. are part of the human microbiota. "Nonpathogenic" *Bacillus* spp. are rarely implicated with human infections but they are among the most widespread microorganisms in the environment and are more frequently isolated as contaminants (in soil, water, air or food) [14,23,26]. They have been troublesome to immunocompromised people on account of their environmental presence and resistant endospores or toxins production [10,25,27,32,33]. They have been increasingly recognized as opportunistic pathogens, especially in hospitalized patients. *Bacillus* spp. implicated in opportunistic infections include *B. cereus*, *B. subtilis*, *B. sphaericus*, *B. alvei*, *B. laterosporus*, *B. licheniformis*, *B. megaterium* and *B. pumilus* [2-3,5,7,10-11,14,18,21].

Identification of bacteria in a clinical microbiology laboratory by using conventional methods are still the gold standard in many laboratories but it is often difficult and takes a long time. Thus, reliable but less complex and modern diagnostic methods of microorganisms isolated from various sources are sought [6,15,20,22,35]. Nevertheless, a new identification method using Bruker MALDI-TOF MS system with prior formic acid extraction provided the best diagnostic results and reduced the identification time (often lower than 1 minute) [4,8,13,15,20,24,28-29,31]. This kind of method was used previously for the identification and classification e.g. *B. pumilus* or *B. safenis* spores [12,16,30]. MALDI-TOF MS technique makes high diagnostic accuracy for a simple, rapid technique for the identification of various types of Gram-positive or Gram-negative bacteria or other organisms. Compared to conventional biochemical and molecular techniques for microorganism identification, MALDI-TOF MS requires minimal sample preparation and achieves more than 92% correct species identification [34]. Besides, these methods are still expensive and accordingly not available for use under the routine laboratory [4,13,15,29].

CONCLUSIONS

Using MALDI-TOF MS, a rapid and modern diagnostic technique, the identification of *Bacillus* spp. and other bacterial isolates (staphylococci and *E. coli*) from the nasal mucosa of healthy adults living in the suburban areas near Lublin was easy to perform. The results of MALDI-TOF MS spectral profiles enabled us to identify *B. pumilus*, *B. safensis* and *B. licheniformis*, which are difficult for routine diagnostics.

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