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NEW OPPORTUNITIES TO IDENTIFY AND TYPE *STAPHYLOCOCCUS* spp. BY USING MALDI-TOF MASS SPECTROMETRY

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Abstract. Mass spectrometry profiles of microorganisms obtained by time-of-flight matrix-associated laser desorption/ionization (MALDI-TOF) mass spectrometry are a source of information about peptide profiles can be used for microbial identification and typing. A variety of technical and bioinformational solutions complicate developing of a united mass-spectro-profile database. *Staphylococcus* spp. strains are good studied objects for identification by MALDI-TOF mass spectrometry, frequently resulting in nosocomial infections, especially in immunocompromised patients. Rapid differentiation of nosocomial, multiresistant and highly virulent isolates of *Staphylococcus* spp. allows to reduce the lethality in weakened and immunocompromised patients. The study was aimed at assessing comparability and reproducibility of identification and typing results for Staphylococcus spp by MALDI-TOF mass spectrometry. Comparing 292 *Staphylococcus* spp. isolates in clinical specimens obtained fron the multidisciplinary hospital at the NWSMU im. I.I. Mechnikov was carried out by using MALDI-TOF mass spectrometer BactoSCREEN ID (Litech, Russia) and Bruker Biotyper 3.1 (Bruker GmbH, Germany). Comparability of *Staphylococcus* spp. identification showed that 95.9%; 12 isolates (4.1%) by "Bruker Biotyper 3.1" and 3 isolates (1.1%) by using "BactoSCREEN ID" were incorrectly identified. Repeated identification leveled the differences between the systems used. In addition, it was shown that the method of protein extraction did not affect reliability of *Staphylococcus* spp. species identification by using databases (χ^2, $p > 0.05$) compared to intraspecific typing (χ^2, $p < 0.0001$). Using different extraction protocols showed that *Staphylococcus* spp. mass-spectra differed by peak intensity level within the mass range up to 4000 m/z, 5300±600 m/z and 6500±500 m/z, as well as higher than 7000 m/z. Peaks of low-molecular weight peptides were detected under full protein extraction compared to sample preparation on plate extraction. To develop a unified protocol for mass-spectrometry profile processing, a reliability of the basic statistical variables (mode, median, maximum, minimum and arithmetic mean) was evaluated. Analysis of the median mass spectrometry profiles is recommended for *Staphylococcus* spp. intraspecific typing by using MALDI-TOF mass spectrometry as the most reproducible and consistent approach. As a result, two systems for MALDI-TOF mass spectrometry reliably identify *Staphylococcus* spp., but standardization of sample preparation and bioinformation analysis is required for *Staphylococcus* spp. typing.

Key words: *Staphylococcus spp., MALDI-TOF mass spectrometry, typing, mass-spectra, cluster analysis, identification.*

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НОВЫЕ ВОЗМОЖНОСТИ ДЛЯ ИДЕНТИФИКАЦИИ И ТИПИРОВАНИЯ *STAPHYLOCOCCUS* spp. МЕТОДОМ MALDI-TOF МАСС-СПЕКТРОМЕТРИИ

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Резюме. Масс-спектро-профили микроорганизмов, получаемые с помощью времяпролетной матрице-ассоциированной лазерной десорбции/ионизациии (MALDI-TOF) масс-спектрометрии являются источником информации о пептидных профилях, которая может быть использована для идентификации и типирования. Разнообразие технических и биоинформационных решений затрудняет формирование единой базы масс-спектро-профилей. Бактерии рода *Staphylococcus* являются одними из наиболее изученных объектов для идентификации с помощью MALDI-TOF масс-спектрометрии, а также являются частыми возбудителями внутрибольничных инфекций, в особенности среди иммунокомпрометрированных пациентов. Методы быстрой дифференцировки нозокомиальных, полирезистентных и высоковирулентных изолятов *Staphylococcus* spp. позволят снизить летальность среди ослабленных и иммунокомпрометированных пациентов. Целью исследования была оценка сопоставимости и воспроизводимости результатов идентификации и типирования *Staphylococcus* spp. с помощью MALDI-TOF масс-спектрометрии. Сравнительные исследования 292 изолятов *Staphylococcus* spp., выделенных из клинических образцов на базе многопрофильного стационара СЗГМУ им. И.И. Мечникова проводили с помощью MALDI-TOF масс-спектрометрометров «BactoSCREEN ID» (ООО «Литех», Россия) и Bruker Biotyper 3.1 (Bruker GmBH, Германия). Сопоставимость результатов видовой идентификации *Staphylococcus* spp. составляла 95,9%; причем среди неправильно идентифицированных изолятов изолятов 12 (4,1%) составляли идентифцированные с помощью Bruker Biotyper 3.1 и 3 изолята (1,1%) *Staphylococcus* spp. с помощью BactoSCREEN ID. Повторная идентифкация нивелировала различия между используемыми системами. Выявили, что способ экстракции белков не влиял на надежность видовой идентификации *Staphylococcus* spp. с использованием сравниваемых библиотек данных (χ^2, $p > 0,05$) в отличие от внутривидового типирования (χ^2, $p < 0,0001$). Масс-спектры *Staphylococcus* spp. при использовании различных протоколов экстракции различались по уровню интенсивности пиков диапазонов масс до 4000 m/z, 5300±600 m/z и 6500±500 m/z и более 7000 m/z. Пики низкомолекулярных пептидов выявляли при полной экстракции белка в отличии от пробоподготовки на поверхности мишени. Для формирования унифицированного протокола обработки масс-спектро-профилей проводили оценку надежности базовых статистических величин (мода, медиана, максимум, минимум и среднее арифметическое). Анализ медианы масс-спектро-профилей рекомендуется использовать для воспроизводимости и стабильности результатов внутривидового типирования *Staphylococcus* spp. с помощью MALDI-TOF масс-спектрометрии. В результате сравнительных исследований выявили, что две системы для MALDI-TOF масс-спектрометрия надежно идентифицируют *Staphylococcus* spp., а для типирования требуется унификация пробоподготовки и биоинформационного анализа.

Ключевые слова: *Staphylococcus spp., MALDI-TOF масс-спектрометрия, типирование, масс-спектр, кластерный анализ, идентификация.*

Introduction

Staphylococcus spp. are important causative agents of human infections especially in intensive care units (ICU) [2]. The spread of methicillin-resistant isolates of *Staphylococcus* spp. is the cause of the patients condition burden in hospital media [1, 6].

MALDI-TOF mass spectrometry is an effective method for identifying *Staphylococcus* spp. Nevertheless, increasing of the number of nosocomial infections associated with *Staphylococcus* spp. requires rapid and reliable identification and obtaining additional information about resistance potential and epidemiological significance [10, 18]. The problem solution is impossible without unified typing protocol development and a multicentral comparison of a MALDI-TOF mass spectrometry results, but to the date MALDI-TOF mass spectrometers are represented by several independent technical solutions with independent mass spectrometry libraries [18]. Methods of sample preparation, technological features of devices and approaches to bioinformatic analysis among manufacturing companies are different, which interfere obtaining of a multicentral data analysis. Moreover, the proposed methods for typing *Staphylococcus* spp., in particular MRSA, do not have united approaches [14, 19]. Data about reproducibility of MALDI-TOF mass spectrometry as a method of typing are contradictory [3, 5, 17]. Thus, to form a unified system for identification and typing of *Staphylococcus* spp. where is necessary to evaluate the reliability, reproducibility and comparability of the results of identification and

typing conducted using various technical solutions and data libraries.

At present, MALDI-TOF mass spectrometers are presented as technical solutions from manufacturers: "Bruker" (Germany), "bioMerieux" (France), "Shimadzu" (Japan), "Litech" (Russia). "Bruker", "bioMerieux" and "Shimadzu" use mass-spectra databases "Saramis" and "MS RUO" for identification each are very close [11], but "Litech" uses an original database, different from previously noted. For the identification of microorganisms cultures, manufacturers sugest different methods of sample preparation, which is due to the features of obtaining mass-spectro-profiles included in the data libraries [11]. Due to the libraries of MALDI-TOF mass spectrometers are separated, where is difficult to compare the effectiveness of MALDI-TOF mass spectrometry for the typing of *Staphylococcus* spp. and screening for methicillin resistance [11].

Thus, the study purpose was a comparative assessment of the comparability of the results of species identification and typing of *Staphylococcus* spp. using various libraries, sample preparation protocols and technical solutions in the field of MALDI-TOF mass spectrometry.

Materials and Methods

Staphylococcus spp. isolates (n = 292) were obtained from samples of patients from a multidisciplinary hospital in St. Petersburg. First inoculation was carried out on a set of media: Colombia agar with 5% sheep erythrocytes (Biomedia, Russia), yolk-salt agar (NITF, Russia). Plates were incubated for 24 hours at 37°C.

MALDI-TOF mass spectrometry was performed using Bruker AutoFleX Speed mass spectrometers (Bruker GmBH, Germany) and BactoSCREEN ID (Litech, Russia). Incubation of *Staphylococcus* spp. on Colombia agar with 5% sheep erythrocytes for 24 hours at 37°C. Extraction of peptides was carried out by the following methods:

1. Direct extraction on the target using a 70% solution of formic acid, drying in air for 2 minutes [4].
2. Total extraction by alcohol-acid method [15] with purification by acetonitrile solution.
3. Short protocol of alcohol-acid extraction without precipitation of cells components and acetonitrile purification [13].

After this, α-cyano-4-hydroxycinnamic acid was added and dried in air.

Using a Bruker AutoFleX Speed mass spectrometer, mass spectra were collected using a linear TOF protocol with a laser frequency of 20 Hz and an estimate in the mass range of 2000 to 20 000 m/z. The voltage on the acceleration was 20 kV, the voltage IS2 was 18.6 kV. For total spectrum, 1200 separate spectra were collected from the entire target area. The identification of cultures was carried out according to the protocol "Bruker Taxonomy", included in the software package "Bruker Real Time Classification 3.1". In the case of "Score" above 2.0, the identification was considered good, for isolates that had "Score" from 1.4 to 1.9 were re-identified. To increase the reliability, this procedure was repeated twice for each isolate.

Sampling and analysis of mass spectra obtained with the MALDI-TOF mass spectrometer "Bruker AutoFleX Speed" was carried out using the "R 3.3.3" software package with the MaldiQuantForeign extension package [7]. The Savitzky-Golay protocol was used to smoothing, and the baseline level was performed using a statistically-dependent non-linear cross-referenced algorithm (SNIP) [12]. Noises were excluded using the "Friedman's Super Smoother" method included in the R 3.3.2 MaldiQuantForeign software package. Analysis of variance was used to estimate the variability of peak intensities on the obtained mass spectra in the R-commander software package.

Using the BactoSCREEN ID mass spectrometer, identification of *Staphylococcus* spp. provided accordance with manufacturer recomendations. Identification was carried out twice for each culture in order to improve reliability. ATCC 29213 *S. aureus* strain was used as a control sample. A preliminary grouping of the results of MALDI-TOF mass spectrometry using the mass spectrometer data was carried out using software package R 3.3.3 by the original program code.

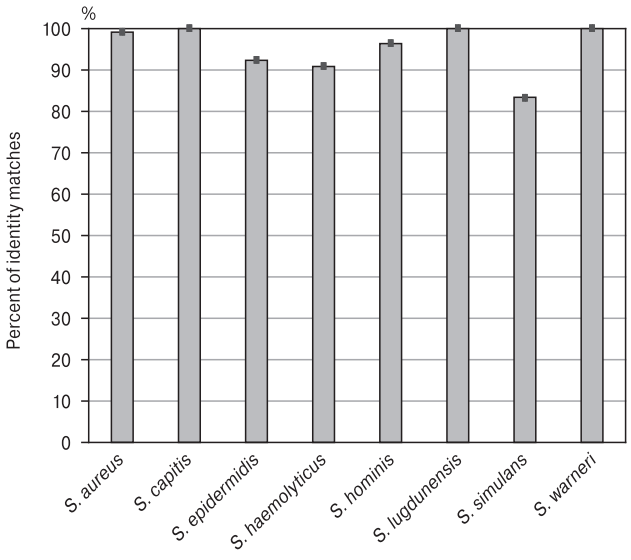

Figure 1. *Staphylococcus* spp. identification results using Bruker Autoflex Speed and BactoSCREEN ID (Litech Ltd.)

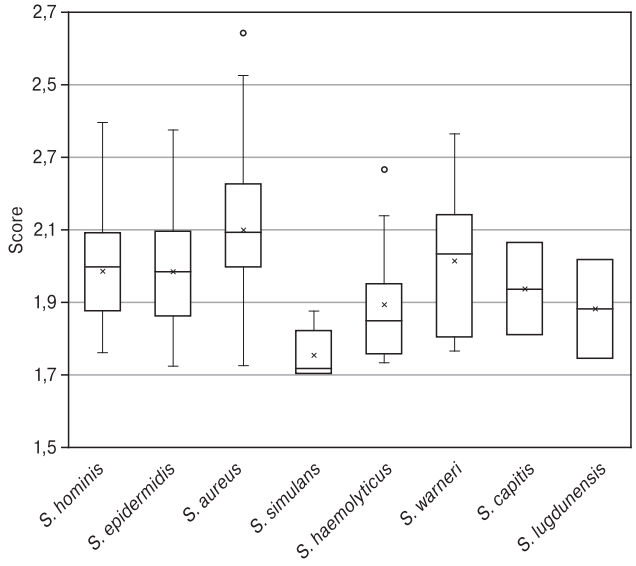

Figure 2. Reliability of *Staphylococcus* spp. identification using "Bruker AutofleX Speed" (Biotyper 3.1) library

Results

Identification of *Staphylococcus* spp. 292 isolates using MALDI-TOF mass spectrometer "BactoSCREEN ID" and "Bruker AutofleX Speed" have revealed a high level of comparability (fig. 1).

Identification of 12 *Staphylococcus* spp. isolates (4.1%) was unreliable using Bruker AutofleX Speed (Biotyper 3.1): five *S. epidermidis*, one *S. aureus*, two *S. auricularis*, one *S. haemolythicus*, three *S. simulans*, but the differences have been eliminated by repeated identification.

Using the database of the "BactoSCREEN ID" device, 3 isolates (1.1%) were not identified: two *S. simulans* and one *S. epidermidis*, which were reliably identified by Bruker Biotyper 3.1. The isolates were identified correctly after second round of identification.

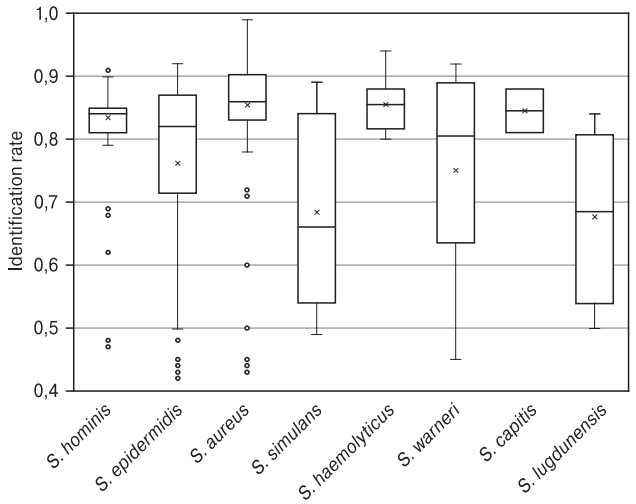

Figure 3. Reliability of *Staphylococcus* spp. identification using the "BactoSCREEN ID" library

When using "Bruker AutofleX Speed" (Biotyper 3.1), the lowest reliability of identification was revealed for *S. simulans* and *S. haemolythicus* (fig. 2).

Analyzing the stock of *Staphylococcus* spp. using the database of the "BactoSCREEN ID", the low reliability of the *S. simulans* species identification and atypical spectra of *S. hominis*, *S. epidermidis* and *S. aureus* was revealed, which were lay outside the general population of values (fig. 3).

Evaluating the effect of extraction protocols on the identification reliability, where was estimated the level of reliability of identification using databases "Bruker Biotyper 3.1" and "BactoSCREEN ID" (fig. 4) did not depend on the extraction protocol (Fisher criterion, $p > 0.05$). Statistically significant differences in the reliability of identification were revealed between the databases during the on-plate extraction (Fisher criterion, $p < 0.001$).

To assess the differences in mass spectra profiles obtained with different extraction protocols, the differences in the structure of the mass spectrometry profiles of *S. epidermidis* (n = 82) were compared; linear and diagonal discriminatory analysis were used. Total extraction is characterized by a rather low intensity of peaks in the regions: 5300±600 m/z and 6500±500 m/z and higher than 7000 m/z. On the other hand, the intensity of signals of low molecular weight peptides (3500±100 m/z) was higher at complete extraction (fig. 5). The combined extraction method was characterized as an intermediate, including the positive sides of both full extraction and direct application.

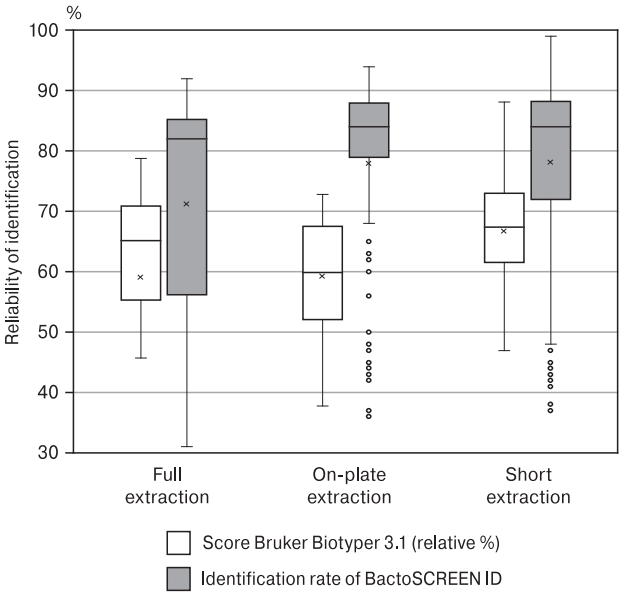

Figure 4. Dependence of the "Score" BrukerBiotyper 3.1 and identification reliability using the database of the device "BactoSCREEN ID" from the extraction protocol

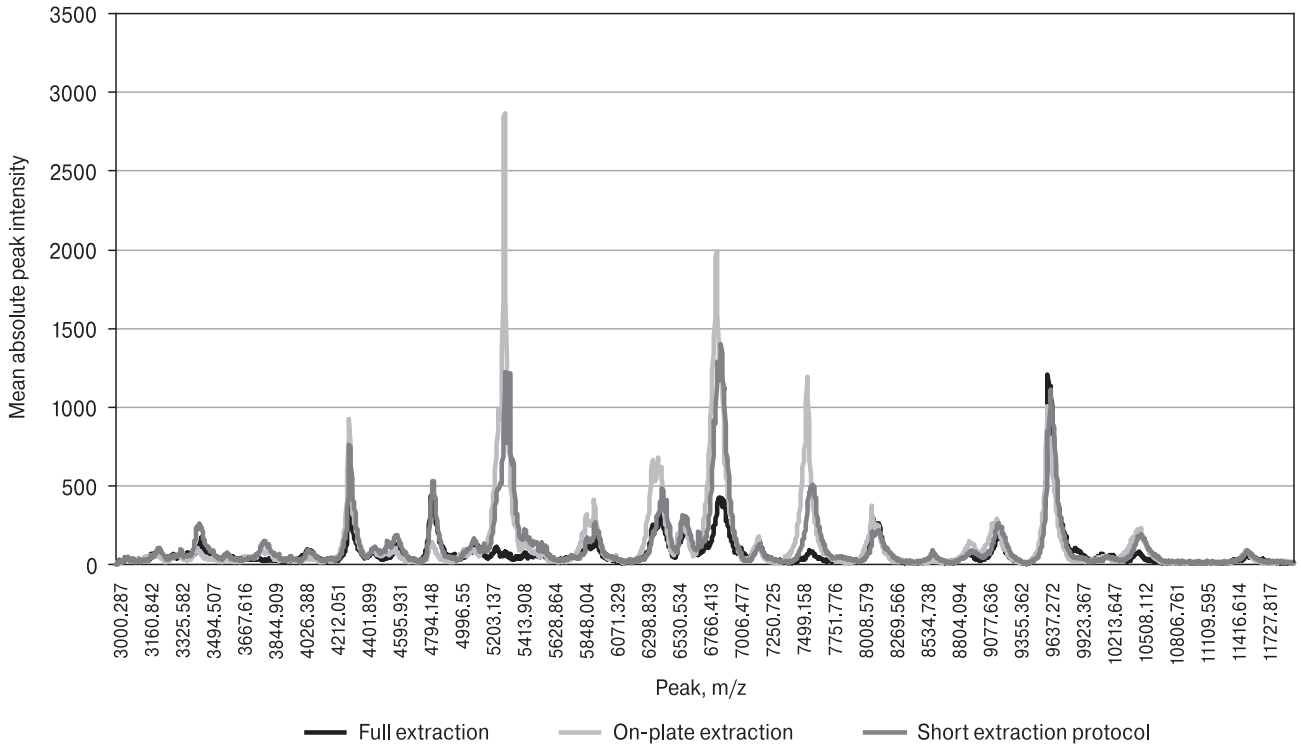

Figure 5. Comparison of mass spectrometry profiles of *S. epidermidis*, depending on the method of extraction of peptides

The structure of the mass spectrometry profiles of other *Staphylococcus* spp. species also differed depending on the method of protein extraction: the intensity of the median mass range peak peptides was the most intensive at direct application and extraction on the target, while the use of a complete extraction protocol allows more effective detection of peaks of low molecular weight proteins (up to 4000 m/z) .

To estimate the effect of peptide extraction on the identification result, the possibilities of harmonizing the sample of mass spectra based on main statistical parameters of the mass spectrometer profile were evaluated. In contrast to the previously proposed harmonization methods for high-intensity stable sites, median harmonization was used [18]. Since the scatter of the peaks in the 6800–9500 m/z ranges was significant and the harmonization was not universal for all species (fig. 6).

Comparing the structure of the *S. epidermidis* population by analysis of harmonized and unharmonized mass-spectrometry profiles, revealed statistically significant differences in the intraspecific clusters structure (fig. 7).

The discrimination of *S. epidermidis* into clusters was characterized by high stability: only 10% of the isolates were assigned to different clusters at the re-identification and mass spectrometer profile evaluation.

Intraspecific structure of other *Staphylococcus* spp. also differed depending on using of mass spectra harmonization protocols. Based on bootstrap analysis, where was obtained the harmonization allow to discriminate only two general clusters in all *Staphylococcus* spp. with stapility during 100-fold repetitions (alpha > 0.95). Nevertheless, the protein extraction method had a statistically significant effect on the intraspecific *Staphylococcus* spp. differentiation despite the harmonization using (χ^2, $p < 0.0001$).

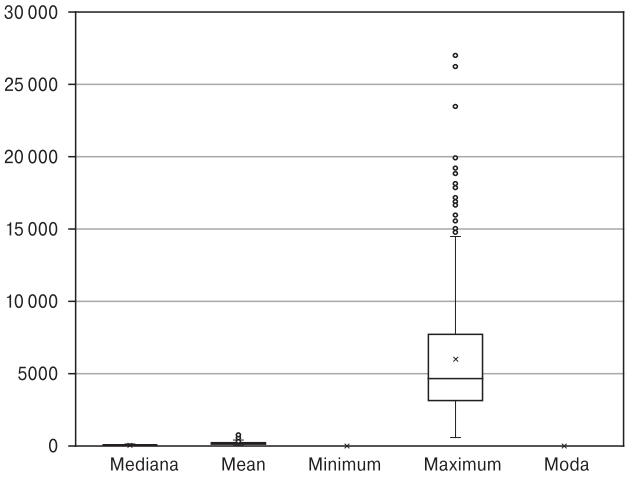

Figure 6. Evaluation of the stability *Staphylococcus* spp. mass-spectra profiles

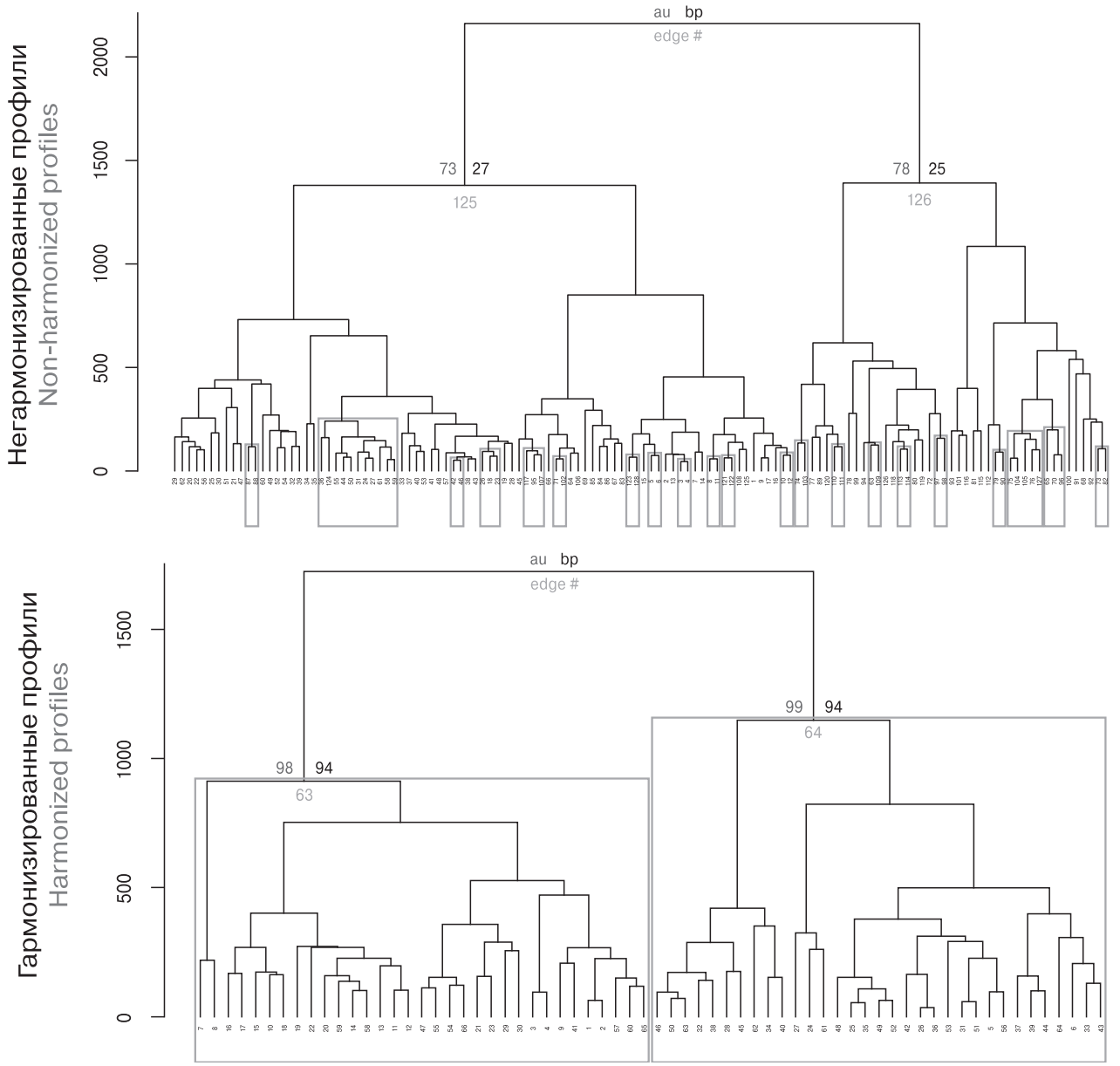

Figure 7. Subspecies structure of *S. epidermidis*

Discussion

The comparison of the identification results 292 *Staphylococcus* spp. isolates revealed 95.9% of the coincidence of species identification using both the Bruker "Biotyper 3.1" databases and the "BactoSCREEN ID" devices. The lowest rating of identification reliability was revealed for *S. simulans* using both instrument solutions.

Reliability of species identification, expressed as "Score" (Bruker Biotyper 3.1) and "Identity Reliability" (BactoSCREEN ID), did not differ significantly depending on the protocol of protein extraction, but statistically significant differences in the intensity of individual peaks of mass spectra were found. Moreover, when using extraction on the target, some peaks of low molecular weight proteins were not detected, which can be used for typing or evaluating resistance [16]. Similar results of bacterial identification were obtained by comparing the instruments of Vitek MS (bioMerieux, France) and Bruker Microflex: for gram-positive bacteria, the comparability of identification results between instruments was 97.4% [9], for Gram-negative bacteria — 99.4% [8]. Thus, despite the differences in bioinformation and technical solutions, the species identification using MALDI-TOF mass spectrometry is reliable, and the results are comparable. The differences obtained by comparing the identification results can be explained by the library structure features of mass-spectrometer, which are formed on the basis of the complete extraction protocols of the protein (Bruker Biotyper 3.1) and extraction on-plate extraction (BactoSCREEN ID).

The results of our research show that to compare the results of typing on different systems for MALDI-TOF mass spectrometry, where is necessary to carry out mass-spectrometry harmonization based on the mass-spectrum median estimate similary to the Savitzky–Golay protocols [7]. Using of previously proposed calibrations of peaks intensity on the range 6800±300 m/z [14] is less reliable due to the high variability of the absolute intensity of these peaks.

The structure of the obtained clusters in the case of *S. epidermidis* was stable for 90% isolates during MALDI-TOF mass-spectrometry repitition. However, statistically significant effect of used extraction protocol on the results of intraspecific typing of all *Staphylococcus* spp. was observed.

Thus, the use of MALDI-TOF mass spectrometry for *Staphylococcus* spp. identification was characterized by high reproducibility of results using various technical solutions in the field of mass spectrometry, extraction protocols and various data libraries. However, quantitative analysis of peaks for intraspecific typing requires not only a single bioinformational approach, but also unification of sample preparation protocols.

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