ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2024;10(1):11-20 doi 10.52547/iem.10.1.11

Species diversity of non-fermenting gram-negative bacteria selected from wound drainage of patients of a multidisciplinary hospital

ARTICLE INFO

Article Type
Original Article

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How to cite this article
Kozlov A.V., Lyamin A.V., Neilenko A.A., Ereshchenko A.A., Kokorev D.A. Species diversity of non-fermenting gram-negative bacteria selected from wound drainage of patients of a multidisciplinary hospital. Infection Epidemiology and Microbiology. 2024;10(1): 11-20.

Article History
Received: January 12, 2024
Accepted: April 04, 2024
Published: April 12, 2024

ABSTRACT

Background: Non-fermenting gram-negative bacteria (NFGNB) pose a threat to the healthcare system. Thus, the purpose of this study was to determine the species diversity of this group isolated from the wound.

Materials & Methods: For species identification during the research period, the MALDI-TOF method of mass spectrometry using the Microflex LT mass spectrometer was applied. As a result, from 2018 to 2022, 7610 microbiological studies were conducted, no microflora growth was detected in 2039 cultures, 1797 strains were isolated and identified in 1523 cultures.

Findings: 261 cultures were found in monospecies; 34 cultures were represented by two or more strains of NFGNB; in 189 cultures, two or more genera of NFGNB were found together with another microflora; in 1039 cultures there was only one NFGNB representative as a part of a mixed culture containing another microflora. The following genera of NFGNB were most common (number of strains): *Acinetobacter spp*. (1002), *Pseudomonas spp*. (699), *Stenotrophomonas spp*. (52), *Alcaligenes spp*. (27), *Achromobacter spp*. (13), *Burkholderia spp*. (4). Within 5 years, an increase in the share of *Acinetobacter spp*. by 6.01% was noted; the share of *Pseudomonas spp*. decreased by 8.39%.

Conclusion: Many rare species have been found, so it is obligatory to ascertain whether penetration into the wound was an accident or the consequence of acquiring new pathogenic properties previously not typical for these microorganisms. No microflora growth was detected in more than 26% of cultures, which requires measures to improve the efficiency of microbiological diagnostics.

Keywords: MALDI-TOF, microbial diagnosis, non-fermenting gram-negative bacteria, surgical infection, wound drainage.

CITATION LINKS

[1] Sattler CA,Nonrespiratory Stenotrophomonas maltophilia Infection... [2] Tsay RW, et al., Alcaligenes xylosoxidans bacteremia: clinical... [3] Kumari M. Pathogen burden & associated antibiogram of... [4] Ayoub Moubareck C, Hammoudi Halat D. Insights into Acinetobacter baumannii: A Review of... [5] Ronin E, Derancourt C, Cabié A, Marion-Sanchez K. Achromobacter spp. Surgical Site... [6] Sheck E, et al., Acinetobacter Non-baumannii... [7] Catalano A, et al., Antibiotic-Resistant... [8] Cain AK, Uncovering resistance mechanisms and global... [9] Oliveira J, Reygaert WC. Gram-Negative Bacteria. In: StatPearls... [10] European Centre for Disease Prevention and... [11] Munita JM, Arias CA. Mechanisms of Antibiotic Resistance... [12] Yaghoubi S, et al., Tigecycline antibacterial... [13] Falagas ME, Kastoris AC, Karageorgopoulos DE, Rafailidis PI. Fosfomycin for... [14] Gómez-Garcés JL, Aracil B, Gil Y, Burillo A. Susceptibility of... [15] Greene C, Vadlamudi G, Newton D, Foxman B, Xi C. The influence of biofilm... [16] Khuntayaporn P, Thirapanmethee K, Chomnawang MT. An Update of Mobile... [17] Lepe JA, Martínez-Martínez L. Resistance mechanisms in Gram... [18] Shenkutie AM, Yao MZ, Siu GKH, Wong BKC, Leung PHM. Biofilm-Induced... [19] Kumari M, Bhattarai NR, Rai K, Pandit TK, Khanal B. Multidrug-Resistant... [20] Botelho J, Grosso F, Peixe L. Antibiotic resistance in Pseudomonas... [21] Chumbita M, New treatments for... [22] Guerrero DM, et al., Acinetobacter... [23] Chopra T, Marchaim D, et al., Epidemiology of Bloodstream... [24] Salehi M, et al., Ventilator-associated... [25] Tingsgård S, et al., Short course antibiotic... [26] Park JH, Jang Y, Yoon I, Kim TS, Park H. Comparison of Autof... [27] Malini A, Deepa E, Gokul B, Prasad S. Nonfermenting... [28] Yadav SK, Bhujel R, Mishra SK, Sharma S, Sherchand JB. Emergence of... [29] Kumar S, Kumar J, Juyal D, Prakash R, Pal S. Characterization and... [30] Buzilă ER, Năstase EV, Luncă C, Bădescu A, Miftode E, Iancu LS. Antibiotic resistance... [31] Haghighifar E, Kamali Dolatabadi R. Bacterial Infections...

Introduction

Non-fermenting gram-negative bacteria (NFGNB) are a heterogeneous group of microorganisms. They are characterized by several common properties: they do not form spores, exhibit reduced enzyme activity against carbohydrates; therefore, they are used through the oxidative pathway. *Acinetobacter baumannii*, *Pseudomonas aeruginosa , Stenotrophomonas maltophilia* are most often found in wounds; *Alcaligenes* spp. and *Achromobacter* spp., as well as *Acinetobacter* spp. and *Pseudomonas* spp., are found much less frequently; *Burkholderia* spp. is detected sporadically.[1–6]

The problem of NFGNB is very relevant nowadays; for example, *A. baumannii* and *P. aeruginosa* are included in the ESKAPE list – a list of the most significant pathogens that account for most of the morbidity and mortality due to multiple drug resistance (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* spp.).[7] This fact requires increased monitoring of the epidemiological situation and the prevalence of diseases associated with these pathogens by World Health Organization and local health authorities. NFGNB are characterized by various mechanisms of natural and acquired drug resistance, which undoubtedly reduce the effectiveness of empirical treatment initiated before the results of a bacteriological study are obtained.[7–10] Natural resistance manifests itself before the start of antibiotic treatment.[11] As a result of incorrectly selected empirical treatment, as well as the use of various combinations of antibiotic therapy, bacteria develop acquired resistance, which leads to a vicious circle.[11–16] Natural and acquired antibiotic resistance is the consequence of the production of enzyme systems, efflux pumps, the creation of a metabolic shunt, modification of the target, as well as changes in membrane permeability. [11,17] For example, *A. baumannii* produces AmpC-type cephalosporinase and OXA-51-type oxacillinase, efflux pumps (AdeIJK, AdeABC, ABEM), and may also contain plasmid beta-lactamases, mainly types PER, VEB and GES, as well as TEM-type and SHV-1-type ESBL (extended-spectrum β-lactamase). [17] The above-mentioned mechanisms may explain the presence of multidrug resistance in 36.8% of *Acinetobacter* spp. strains in the European population.[10] The ability of *A. baumannii* to form biofilms was also noted, which reduces the effectiveness of antibiotics and also allows long-term survival in hospital setting.[18, 19] As a result, a number of negative effects emerges. First of all, complications may happen, such as sepsis, nosocomial pneumonia, which, in addition to a serious condition, significantly worsens the prognosis, increasing the mortality of patients.[20, 21] As much as 28-43% of patients die from infections caused by *A. baumannii*; this number includes soft tissue infections mortality, which reaches 30%, and ventilator-associated pneumonia, which mortality rate is up to 65.8%.[22–24] Second of all, irrational long-term use of antibiotic therapy increases the likelihood of developing side effects. It leads to the deterioration in the quality of life or the need for additional treatment. [25]

Objectives: To analyze the structure of non-fermenting gram-negative bacteria isolated from wound drainage of patients of a multidisciplinary hospital from 2018 to 2022.

Material and methods

Samples: The study was conducted on the basis of the multidisciplinary hospital (the hospital has a capacity of 1100 beds). The study included adult patients who were

admitted to surgical departments (maxillofacial surgery, abdominal surgery, purulent surgery, general surgery, resuscitation and intensive care, traumatology and orthopedics, otorhinolaryngology) and underwent surgery. The research material was pus, smear from the wound surface, wound discharge, drainage fluid taken during surgery or dressings.

Sample collection: The sample was taken into a transport medium (Amies) and was delivered to the microbiological laboratory within one hour for study.

Cultivation of microorganisms: The sample was inoculated in quadrants on the following solid growth media: 5% blood agar with ram's blood (Himedia, India), commercial chromogenic differential diagnostic medium UriSelect (Himedia, India), special selective agar for *Burkholderia spp*. (Himedia, India). Sabouraud agar (Himedia, India) was used for the cultivation of yeast-like and filamentous fungi.

The cultures were placed in a thermostat and cultivated at a temperature of 37°C for 48 hours. When visible growth of microorganisms' colonies was detected, species identification of each type of microorganisms' colony was carried out.

Species identification: The identification of the grown colonies was carried out using the MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) mass spectrometry method on a Microflex LT device (Bruker Daltonik GmbH, Germany). All types of grown colonies were put on the target of the device, followed by coating with a matrix in the form of α-cyano-4-hydroxycinnamic acid. Next, the target was placed in a device where bacterial cells were repeatedly exposed to laser pulses. This process led to the release of ionization of ribosomal proteins of microorganisms that entered the time-of-flight analyzer and moved to the detector. The time of flight of the particles was estimated using the software of the device, which converted the information obtained into a molecular mass spectrum corresponding to the ratio of mass and charge of particles in the range from 2000 Da to 20000 Da. The mass spectra obtained during the identification of microorganisms were compared with a library, which is a database with reference spectra. Based on the similarity of proteins and their mass, the species identification of the microorganism took place. The reliability of identification was evaluated simultaneously using the MALDI Biotyper RTC software by determining the coefficient of coincidence (Score): if the obtained Score corresponds to the range from 0.000 to 1.699, the identification result is considered as low-confidence; A score from 1.700 to 1.999 indicates a highly reliable identification to the genus; a score from 2.000 to 2.999 is considered as a highly reliable identification to the species.

Statistics: Statistical Analysis. Statistical processing of the obtained data was carried out using Microsoft Office Excel 2013 (Office 365; Microsoft Corporation Washington, USA). Assessment of the microflora structure included calculation of the frequency of occurrence of the taxon (the proportion of genera/species of microorganisms in the studied bacterial population). Numeric results are given as derivatives (in percentages) and also as the absolute numbers from which the derivatives were calculated. The results have been elaborated in tables and charts.

Findings

Place of NFGNB in the structure of isolated microorganisms: From 2018 to 2022, 7610 studies of wound drainage were carried out; in 2039 cultures of which (26.79%) there was not found any microflora growth; some of NFGNB were found in 1523 cultures (20.05%), among them monospecies were found in 261 (17.14%) cultures; the mix

of NFGNB representatives only were found in 34 (2.23%) cultures, in 1039 (68.26%) cultures there were found mixes consisting of one genus of NFGNB and microorganisms not belonging to the group of NFGNB; there were also 189 (12.41%) multicomponent communities consisting of 2 or more genera/species of NFGNB and microflora not belonging to this group.

In addition, 1797 strains of NFGNB were detected: 261 (14.52%) strains were isolated in monospecies; 76 (4.22%) strains were isolated in mixes with NFGNB only, 1039 (57.81%) strains were found in mixes with other microflora, and 421 (23.42%) strains were found in complex mixes with both the NFGNB group and other microflora.

NFGNB cultures were distributed among departments as follows: maxillofacial surgery (73), otorhinolaryngology (59), traumatology and orthopedics (46), purulent surgery (660), general surgery (389), resuscitation and intensive care (296).

NFGNB strains were distributed among departments as follows: maxillofacial surgery (91), otorhinolaryngology (70), traumatology and orthopedics (55), purulent surgery (736), general surgery (478), resuscitation and intensive care (367).

As a result of the frequency analysis of occurrence of genera, the following data were obtained: the leading genus are *Acinetobacter* and *Pseudomonas.* The remaining genera are represented in the following: *Stenotrophomonas*, *Alcaligenes*, *Achromobacter* and *Burkholderia* (Figure 1).

Dominant species of isolated NFGNB: The frequency of species occurrence was analyzed; as a result, the following data were obtained: the most common types of NFGNB were *A. baumannii* and *P. aeruginosa.* Representatives of the species of *S. maltophilia*, *Acinetobacter haemolyticus* and *Acinetobacter nosocomialis* were also encountered. The remaining organisms in the amount of approximately 7% strains appeared sporadically (Figure 2).

When assessing the prevalence of NFGNB strains in different departments, the following data were obtained. *A. baumannii*

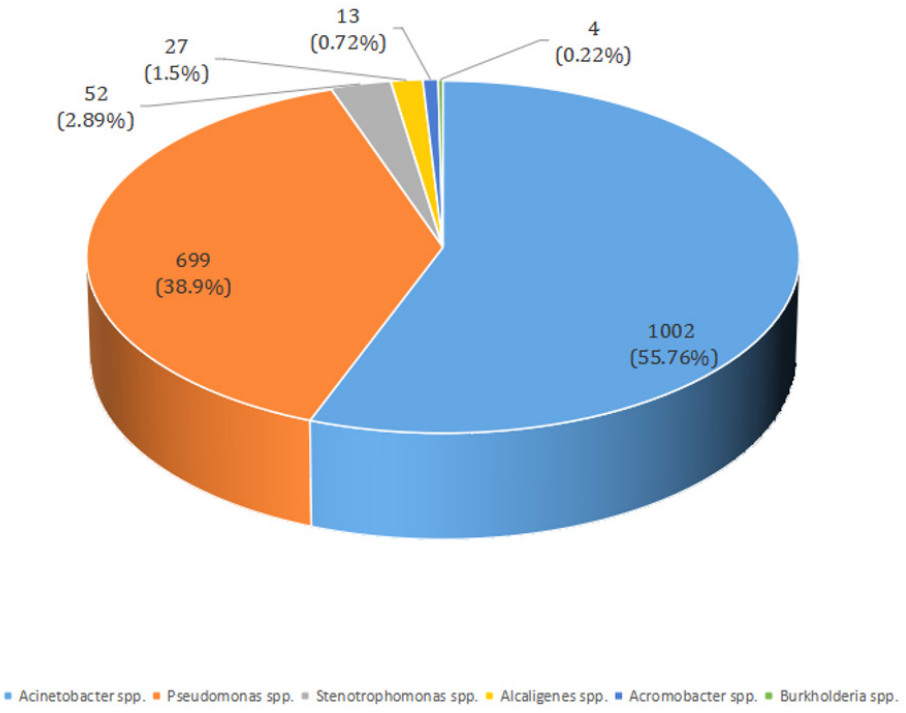

Figure 1) Structure of non-fermenting gram-negative bacteria genera, isolated from wound drainage in -2018 2022 in multidisciplinary hospital

strains were found in the department of maxillofacial surgery 44 species (48.36%), otorhinolaryngology department 37 species (52.85%), department of traumatology and orthopedics 15 species (27.27%), department of purulent surgery 390 species (52.98 %), department of general surgery 233 types (48.74%), department of resuscitation and intensive care 165 types (44.95%). *P. aeruginosa* strains were found in the department of maxillofacial surgery 32 species (35.16%), otorhinolaryngology department 20 species (28.57%), department of traumatology and orthopedics 28 species (50.9%), department of purulent surgery 353 species (47.96%), department of general surgery 143 types (29.9%), department of resuscitation and intensive care 92 types (25.06%). *S. maltophilia* strains were found in the department of maxillofacial surgery 4 types (4.39%), otorhinolaryngology department 2 types (2.85%), department of traumatology and orthopedics 1 type(1.81%), department of purulent surgery 25 types (3.39%), department of general surgery 12 types (2.51%), department of resuscitation and intensive care 7 types (1.9%). Other species are not listed due to their small numbers.

The dynamics of changes in the frequency of occurrence of the dominant genera of NFGNB in 2018-2022: The dynamics of changes in the frequency of occurrence of the dominant genera of NFGNB by year was also analyzed; as a result, the following data were obtained: the leading genus is *Acinetobacter* spp., which share in the wound drainage increased from 2018 to 2022 by 6.01% - from 53.25% to 59.26% or from 198 strains to 220 strains; the share of *Pseudomonas* spp. decreased by 8.39% - from 40.24% to 31.85% or from 155 to 122 strains. These data correspond to the statistics of WHO.[10] Representatives of *Stenotrophomonas* spp. occurred with the frequency of 2.03-3.88% (Figure 3).

Analysis of the species structure of the identified NFGNB genera: The species

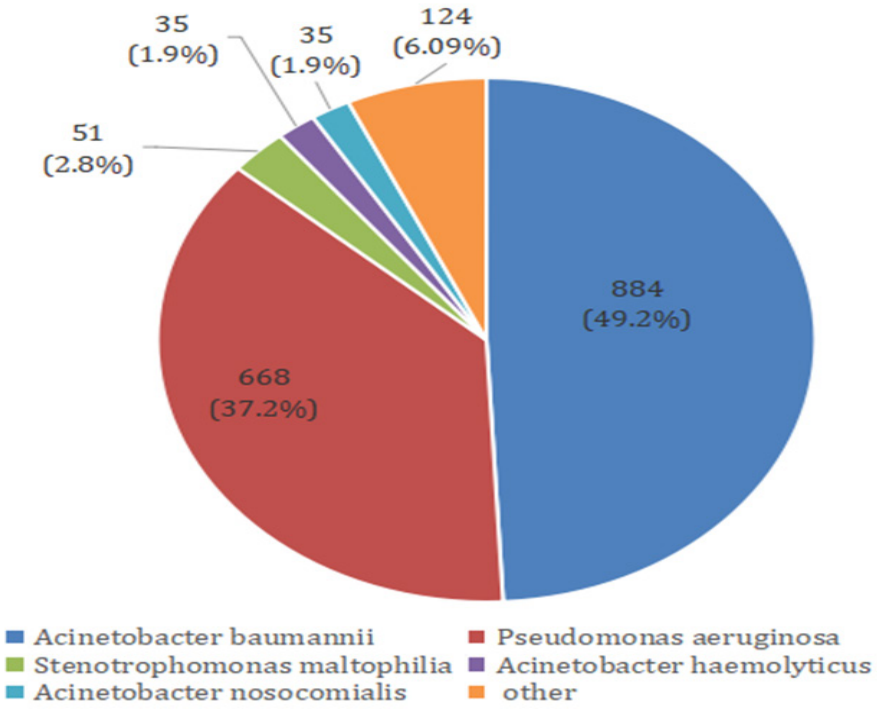

Figure 2) Dominant non-fermenting gram-negative bacteria species identified as a study result of wound drainage in 2022-2018 in multidisciplinary hospital.

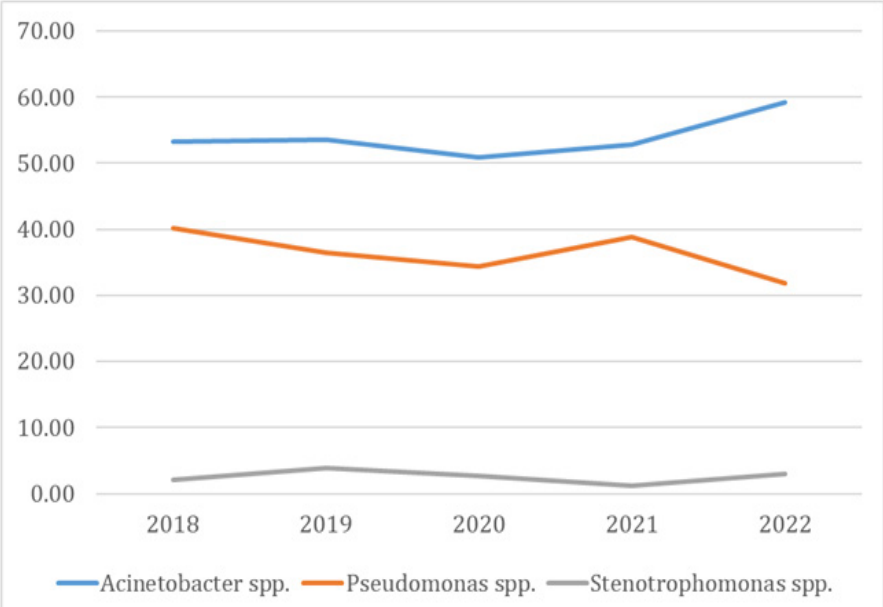

Figure 3) The dynamics of changes in the frequency of occurrence of the dominant genera of non-fermenting gram-negative bacteria in 2022-2018 in multidisciplinary hospital.

structure of the genera of microorganisms was analyzed; as a result, the following data were obtained: *Acinetobacter* spp. is represented by 14 species.

The most common was *A. baumannii*, which was isolated in 88% or in the amount of 884 strains. The second most common were *A. nosocomialis* and *A. haemolyticus* (35 strains each or 3.49%), *Acinetobacter pitti* (20 strains or 2%), *Acinetobacter lwoffii* (9 strains or 0.9%), *Acinetobacter bereziniae* (5 strains or 0.5%), *Acinetobacter johnsonii* and *Acinetobacter junii* (3 strains or 0.3%), *Acinetobacter calcoaceticus* and *Acinetobacter schindleri* (2 strains or 0.2%); *Acinetobacter ursingii*, *Acinetobacter radioresistens*, *Acinetobacter dijkshoorniae*, *Acinetobacter lactuca* were isolated once (0.1%). As a result, 1002 strains of *Acinetobacter* spp. were identified, among them 122 strains or 12.2% in monospecies, 27 strains or 2.7% in mixes with NFGNB only, 853 strains (85.1%) in mixes with other microflora, of which 679 strains (67.75%) contained only the genus *Acinetobacter*, as opposed to 174 strains (17.35%), where other representatives of the NFGNB were also present. *Pseudomonas* spp. included 11 types: *P. aeruginosa* (672 strains or 95.57%), *Pseudomonas stutzeri* (9 strains or 1.29%), *Pseudomonas putida* (5 strains or 0.72%), *Pseudomonas monte* (3 strains or 0.43%); *Pseudomonas koreensis*, *Pseudomonas luteola*, *Pseudomonas veronii* contained 2 strains each (0.29%); *Pseudomonas plecoglossicida*, *Pseudomonas thivervalensis*, *Pseudomonas nitroreducens*, *Pseudomonas lundensis* were isolated once (0.14%). A total of 699 strains of *Pseudomonas* spp. were identified; among them, 116 (16.59%) strains in monospecies, 34 (4.9%) strains in mixes with NFGNB only, 549 (85.03%) strains in mixes with another microflora, while 381 of them contained *Pseudomonas* spp. and another microflora. 167 strains contained 2 or more NFGNB genera, one of which was *Pseudomonas* spp., as well as another microflora. *Stenotrophomonas* spp. is represented by 2 species: *S. maltophilia* in the amount of 51 (98.08%) strains and *Stenotrophomonas acidaminiphila*, which was isolated once (1.92%). Among *Stenotrophomonas* spp. there were 3 strains (5.8%) in monospecies, in mixes with NFGNB only there were 7 strains (13.44%), in mixes with other microflora there were 42 strains (80.76%), while 13 (25%) of them contained *Stenotrophomonas* spp. only

Table 1) The structure of simple and complex bacteria mixes, isolated from wound drainage in 2018-2022.)

Genera in a mix	Number of mixes	Complex mixes with another microflora
Acinetobacter spp. + *Pseudomonas* spp.	24	143
Pseudomonas spp. + *Stenotrophomonas* spp.	5	5
Pseudomonas spp. + *Achromobacter* spp.	2	1
Pseudomonas spp. + *Alcaligenes* spp.	1	9
Acinetobacter spp. + *Pseudomonas* spp. + *Stenotrophomonas* spp.	1	7
Acinetobacter spp. + *Stenotrophomonas* spp.	1	15
Acinetobacter spp.+ *Alcaligenes* spp.	0	3
Acinetobacter spp. + *Achromobacter* spp.	0	2
Acinetobacter spp. + *Stenotrophomonas* spp. + *Achromobacter* spp.	0	1
Acinetobacter spp. + *Alcaligenes* spp. + *Pseudomonas* spp.	0	1
Acinetobacter spp. + *Pseudomonas* spp. + *Achromobacter* spp.	0	1
Acinetobacter spp. + *Achromobacter* spp. + *Stenotrophomonas* spp.	0	1

along with another microflora, in contrast to 29 (55.76%) strains, where, in addition, representatives of other genera were present. *Alcaligenes* spp. was represented by one species of *Alcaligenes faecalis* in the amount of 27 strains. 1 strain (3.7%) was found in mixes containing NFGNB only, 26 strains (96.3%) were found in mixes containing another microflora, 13 of them contained *A. faecalis* only without other genera of NFGNB, as opposed to 13 cultures in a mix with other representatives of NFGNB and another microflora.

Three species were found among *Achromobacter* spp.: 10 strains (of *Achromobacter xylosoxidans*, 2 strains (15.38%) of *Achromobacter spainus, Achromobacter mucicolens* was isolated once (7.69%). 13 strains were identified, of which 7 strains (53.84%) of *Achromobacter* spp. were in monoculture, 6 in a mix with another microflora. Representatives of *Burkholderia* spp. were also found in the amount of 4 strains. All representatives of *Burkholderia* spp. were found in communities containing other microflora. We have studied the structure of mixes containing NFGNB only, as well as mixes containing several genera of NFGNB and other microorganisms isolated from wound drainage (Table 1). Several strains of the same species/genus were present in some cultures.

Discussion

The MALDI-TOF mass spectrometry method is highly sensitive and highly informative. The accuracy of species identification using Microflex LT mass spectrometer (Bruker Daltonik GmbH, Germany) with flexanalysis 3.0 software (Bruker Daltonik GmbH, Germany) is 97.6%- 99.3%.[26]

As shown by the results the study, 2039 cultures (26.79% of all cultures of wound

drainage) did not contained any microflora growth; therefore, some measures should be taken to increase the possibility of the cultivation and the quality of the identification of pathogens, such as the cultivation under anaerobic conditions and prolonged incubation of bacterial cultures, since in some cases false negative results are possible. In 1523 cultures, NFGNB were identified, the total number of which was 1797 strains. From 2018 to 2022, the share of *Acinetobacter* spp. increased by 6.01% - from 53.25% to 59.26%, or from 198 strains to 220 strains; the share of *Pseudomonas* spp. decreased by 8.39% - from 40.24% to 31.85%, or from 155 to 122 strains; nevertheless, *Acinetobacter* spp. still remains the dominant genus.

Taking into account the possibility of persistence of many representatives of NFGNB in hospital settings, careful compliance with the measures of asepsis and antisepsis is necessary.

The most common species was *A. baumannii* (884 strains or 49.2%), the second most common one was *P. aeruginosa* (668 strains or 37.2%), the third one was *S. maltophilia* (51 strains or 2.8%), the fourth ones were *A. haemolyticus* and *A. nosocomialis* in the amount of 35 strains or 1.9% each. The remaining microorganisms in the amount of 126 strains or 6.90% appeared sporadically (a percentage of all strains).

In similar studies, the following figures are given: *A. baumannii* was found with a frequency of 19.74%-53.95%, P. aeruginosa - with a frequency of 36.36-66.38%. [27-31] Summarizing the data from these articles, 1784 strains of NFGNB are mentioned with the share of P. aeruginosa being 1107 strains or 62.05% and the share of *A. baumannii* being 545 strains or 30.55%. [27-31] When analyzing research data, it is necessary to take into account samples frequently being small (not exceeding 200 strains), the emphasis on common species with the increase of the clinical significance of rare species of NFGNB; therefore, we consider it to be important to assess the entire profile of pathogens found in wounds.

The obtained dynamics may be the consequence of the acquisition of various mechanisms of antibiotic resistance in different microorganisms, as well as the result of interspecific competition of bacteria in the intrahospital environment. Noteworthy is the fact of the decrease in the number of isolated *Pseudomonas* spp. with a simultaneous increase in cases of *Acinetobacter* spp.

excretion. *A. baumannii* has become a widespread hospital strain, which raiese the problem of targeted antibiotic therapy and the effectiveness of disinfectants in medical institutions.

Limitations: Not all commercially available universal chromogenic culture media were used in our study. For species identification, only MALDI-TOF method of mass spectrometry was used.

Conclusion

Many rare species have been discovered, so it is obligatory to ascertain whether their entrance into the wound was an accident or the consequence of acquiring new pathogenic properties previously not typical for these microorganisms. Further research is also required to clarify the role of interspecific interactions in the pathogenesis of wound infections. In general, the problem of the spread of NFGNB as the main pathogens causing wound infections of various genesis persists everywhere and is an urgent health problem.

Acknowledgements

We would like to express our gratitude to the Rector and Vice-Rector for Research of Samara State Medical University for their support.

Ethics Committee Approval: The study

was approved by Ethics Committee of the Samara State Medical University, Samara, Russian Federation (date: 31/10/2018).

Conflict of interest: The authors declare that there is no conflict of interests.

Funding source: This work was not funded by any source.

Authors' Contributions: Study design: AVL and AVK Data collection and analysis: AAN and DAK, Manuscript preparation: AAE.

Informed Consent: Retrospective study.

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