Immunopathologia Persa
DOI:10.34172/ipp.2023.39481
http immunopathol.com

The comparative survey of phenotypic methods and the 16S rRNA gene sequencing method for detecting genus and species of non-fermented gram-negative bacteria isolated from blood samples in Isfahan, Iran

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Received 22 Jan. 2023
Accepted 15 July 2023
Published online 29 July 2023

Keywords: Nonfermented, Gram-negative, 16 RNA sequence, Phenotypic method

Abstract

Introduction: This study compared phenotypic methods and the 16S rRNA gene sequencing method to identify the genus and species of non-fermenting gram-negative bacilli isolated from blood culture samples.
Objectives: The aim of this study was to evaluate the effectiveness of using the 16S rRNA gene sequencing method in detecting the genus and species of non-fermented gram-negative bacteria isolated from blood samples.
Materials and Methods: A cross-sectional study was conducted from April 2019 to April 2020, including all patients who required sterile culture at AL Zahra and Kashani hospitals. Specimens were cultured in BACTEC and subjected to standard phenotypic methods. Subsequently, 16S rRNA gene sequencing was performed to detect gram-negative non-fermenting *Bacillus* bacteria at the genus and species level. A comparative evaluation was then conducted.
Results: The study identified 30 bacteria. The 16S rRNA gene sequencing method observed that 83.3% of the diagnoses were *Acinetobacter baumannii*, while the phenotypic approach identified 86.7% as *Acinetobacter*.
Conclusion: The results indicate a significant difference between the phenotypic method and 16S rRNA sequencing in detecting non-fermenting gram-negative bacilli (NFGNB).

Citation: Eskandari S, Shabani N, Baradaran A, Mobasherizadeh S, Rostami S, Derakhshan M. The comparative survey of phenotypic methods and the 16S rRNA gene sequencing method for detecting genus and species of non-fermented Gram-negative bacteria isolated from blood samples in Isfahan, Iran. Immunopathol Persa. 2023;x(x):e39481. DOI:10.34172/ipp.2023.39481.

Introduction

The "non-fermenting" group comprises gram-negative bacilli that do not ferment glucose and other sugars. They represent approximately 15% of gram-negative bacilli isolated from hospitalized patients. While several non-fermenting genera exist, *Pseudomonas aeruginosa* accounts for 75% of clinically relevant cases, while *Acinetobacter*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia* make up the majority of the remaining 25%. These bacteria are primarily found in the environment and are not considered normal flora of the human body, except as colonizers in hospitalized patients. Gram-negative bacilli, including Enterobacteriaceae, are commonly part of the normal flora in the digestive tract. Gram-negative non-fermenting bacilli, such as *P. aeruginosa* and *Acinetobacter baumannii*, can cause infections when host defenses are compromised. Diagnosing gram-negative non-fermentative disorders in the laboratory can be challenging and time-consuming, leading to treatment failures and adverse outcomes (1).

Key point

A comparative study was conducted to identify the genus and species of non-fermenting gram-negative bacilli isolated from blood culture samples. The results highlight a significant difference between the phenotypic method and 16S rRNA sequencing in identifying the bacteria.

Usually, bacterial identification involves morphological and biochemical tests, while additional tests to determine antibiotic susceptibility and resistance patterns on solid culture media are necessary. These conventional methods include microscopic observation (such as gram staining), phenotypic examination of bacterial characteristics through culture on artificial media, serological methods to detect antibodies against bacterial structures, and antimicrobial sensitivity determination.

However, these methods can be time-consuming, and may require more precise identification, especially at the species level (2).

In the 16S rRNA genotyping method, genetic diagnosis of bacteria involves utilizing conserved sequences in genetic targets related to phylogenetic information, such as the small subunit (16S) of the rRNA gene. The 16S rRNA gene micro-sequencing kit enables bacterial identification based on their 16S rRNA gene sequence. The 16S rRNA gene sequencing has been crucial identifying bacteria for research and diagnostic purposes (3).

Given the significance of non-fermenting gram-negative bacilli (NFGNB) and the associated risks, as well as the limitations of conventional methods in detecting low concentrations of microorganisms in blood samples (2), a comparative study was conducted to compare the phenotypic methods with the genotypic method of 16S rRNA gene sequencing in identifying the genus and species of NFGNB isolated from blood samples.

Objectives

The aim of this study was to evaluate the effectiveness of using the 16S rRNA gene sequencing method in detecting the genus and species of non-fermented gram-negative bacteria isolated from blood samples.

Materials and Methods

Study design

This descriptive-analytical cross-sectional study evaluated 30 samples collected from April 2019 to 2020 from all patients at AL Zahra and Kashani hospitals who required blood cultures. The samples were cultured in Castaneda and BACTEC™ culture medium, followed by Eosin-methylene blue (EMB) culture medium, blood agar, and chocolate agar. Daily checks were conducted in the laboratory for seven days to monitor turbidity, hemolysis, and colony formation. Subculture was prepared, and the characteristics of the microorganism and the time of positivity for each method were recorded separately. Each positive result was compared with the patient's history and clinical symptoms, considering it valuable if it matched and considering as contamination if it did not match. Grown colonies were coded after preliminary investigations, including Gram staining, movement, and differential tests such as examination of triple sugar iron (TSI) medium, squalene test, and oxidation of sugars (glucose, lactose, maltose, mannitol, dextrose, and sucrose). Additional tests, including nitrate reduction, gelatin test, and citrate test, were performed in the oxidative-fermentative (OF) medium. If the surface and depth of the TSI medium exhibited a red color (alkaline/alkaline), further differential tests such as oxidase, OF, lysine, and DNase were conducted at a temperature of 44 degrees Celsius to determine the genus and species. Moreover, AP120NE biochemical tests were conducted to confirm the identity of some isolates. Additionally, in addition to the conventional phenotypic method, the samples were analyzed using the genetic process of 16S rRNA gene sequencing to detect non-fermenting gram-negative bacilli at the genus and species level. These methods were then compared.

Statistical analysis

The chi-square test was employed to compare and determine the relationship between the two methods. All analyses were performed using SPSS version 27. A significance level of $P < 0.05$ was considered.

Results

In this study, 30 bacteria were identified. The number of each bacteria is shown separately in the following diagrams. In the sequence method, it is observed that 83.3% of the diagnoses were *A. baumannii*, while in the phenotypic process, 86.7% of the diagnosis were *Acinetobacter*. We found a significant difference in the phenotypic and sequence methods in detecting the type of bacteria existed since the value is P <0.002 was detected, which means that the detection of these two methods is different in the type of bacteria (Figures 1, 2 and Table 1).

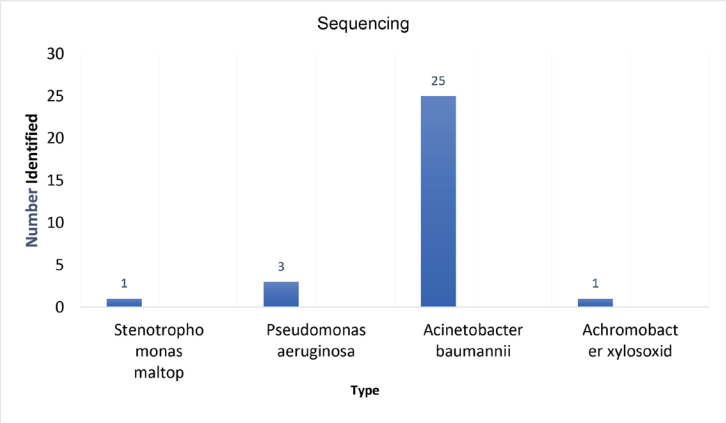
Discussion

This study aimed to evaluate the use of 16S rRNA gene sequencing for identifying NFGNB in diagnostic laboratories. The objective was to compare phenotypic identification with molecular identification using sequencing. The results obtained from phenotypic identification were compared with the sequencing process. To our knowledge, this is the first study in Iran that compares sequence and phenotypic methods.

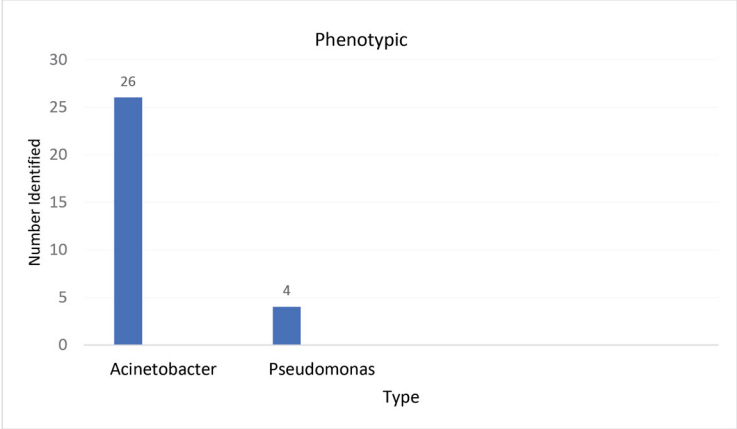
The 16S rDNA sequencing method is conducted to identify pathogens in sterile clinical specimens or species that cannot be cultured. Other researchers have also utilized this molecular identification tool to identify bacteria, often

Table 1. Comparison of phenotypic and sequence methods

		Phenotypic		*P* value*
		Acinetobacter	*Pseudomonas*	
Sequence	*Achromobacter xylosoxidans*	1	0	<0.002
	Acinetobacter baumannii	24	1	
	Pseudomonas aeruginosa	0	3	
	Stenotrophomonas maltophilia	1	0	



Figures 1. Frequency and types of microorganisms detected by the sequence methods.



Figures 2. Frequency and types of microorganisms detected by the phenotypic method.

comparing it with the phenotypic method (3-5).

Numerous studies have compared the utility of 16S rDNA sequencing with conventional or commercial methods for identifying various groups of essential bacteria. In general, 16S rDNA sequencing yields the identification of a more significant number of species compared to traditional or commercial methods, as indicated by several studies.

A previous study comparing sequencing and phenotyping methods showed that 27 microorganisms were not represented in the MicroSeq database compared to two undefined microorganisms in Smart Gene that affected the final identification. We did not check this in our study. This study also showed that sequencing is much more potent than phenotyping in studying gram-negative bacteria, similar to our research (4).

In a 2006 study comparing sequencing and phenotyping methods for gram-negative bacteria, 98 isolates were assigned to the species level and nine to the genus level using 16S rRNA gene sequencing. One isolate exhibited 100.0% identity to *Pseudomonas fluorescens* and *Pseudomonas jessenii* strains, while API 20 NE identified only 58 out of 107 isolates to the species level. Several isolates showed high similarity to sequences of *Achromobacter xylosoxidans* and *Achromobacter piechaudii*. Our study showed that 83.3% of *A. baumannii* isolates were identified using the sequencing method, while 86.6% were identified using phenotyping. This study also indicated a significant difference between the sequencing and phenotyping methods regarding bacterial identification, which is similar to our research. Although *P. aeruginosa* was excluded from their study, we investigated it in our research. Therefore, it is suggested that when NFGNB cannot be identified using the phenotypic method, they should be subjected to 16S rRNA gene sequencing (5).

In other studies, 16S rDNA sequencing has been explored for bacterial identification, the discovery of new disease genera and species, the detection of uncultivable bacteria, and the identification of harmful agents (6). Accurate gram-negative non-fermenting bacilli (NFB) identification is crucial for effective patient management.

In a study comparing the phenotypic identification of 96 clinical NFB isolates with identifications from 5′ 16S rRNA gene sequencing, 88 isolates (91.7%) with more than 99% similarity were assigned. Around 61.5% of the sequencing results were consistent with the phenotypic effects, indicating the feasibility of using sequencing to

identify NFB (7).

Another study conducted in Hong Kong in 2011 demonstrated the high effectiveness of the sequencing method in identifying gram-negative bacteria (8), which aligns with the findings of our study. However, there is a limited number of studies comparing only phenotypic and sequencing techniques in gram-negative bacteria, indicating the need for further studies with larger sample sizes.

Conclusion

The results of this study highlight a significant difference between the phenotypic method and 16S rRNA sequencing in the identification of NFGNB.

Limitations of the study

The limitation of the study was limited study population. We also suggest that more studies with extended follow-up periods should be performed.

Authors' contribution

Conceptualization: AB, SE, NS.
Methodology: All authors.
Validation: AB, SE.
Formal Analysis: NS.
Investigation: All author.
Resources: AB, SE, NS.
Data Curation: AB, SE, NS.
Visualization: AB, SE, NS.
Supervision: AB, SE.
Project administration: AB, SE, NS.
Funding acquisition: AB, SE, NS.
Writing–original draft: AB, SE, NS.
Writing–review and editing: AB, SE.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of Isfahan University of Medical Sciences approved this study (Ethical code#IR.MUI.MED.REC.1398.551). Accordingly, written informed consent was taken from all participants before any intervention. This study was extracted from the M.D., thesis of Nasim Shabani (Thesis#398791) at this university. The authors have completely observed ethical issues (including plagiarism, data fabrication, and double publication).

Funding/Support

This study was supported by the Isfahan University of Medical Sciences, Isfahan, Iran (Grant #398791).

Acknowledgments

We thank all the patients for their participation in the study. In addition, we thank Nazila Kasaian and Samere Nouri for their assistance.

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