Evaluation of MALDI-TOF mass spectrometry (VITEK-MS) compared to the ANC card (VITEK 2) for the identification of clinically significant anaerobes

Avaliação da espectrometria de massas MALDI-TOF (VITEK-MS) diante do cartão ANC (VITEK 2) na identificação de anaeróbios clinicamente significantes

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ABSTRACT

Introduction: The identification of anaerobic bacteria by conventional methods employed in clinical laboratories requires a lot of work and a long response time [turnaround time (TAT)]. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an accurate, rapid and inexpensive technique with promising results for bacterial identification. **Objective:** To evaluate the MALDI-TOF mass spectrometry (VITEK-MS, bioMérieux, France) compared to the ANC card (VITEK 2, bioMérieux, France) for the identification of anaerobes, and also veriying the cost variation between both methodologies. **Methods:** 421 anaerobes were concomitantly identified by ANC (VITEK 2) and MALDI-TOF (VITEK MS). The conflicting results or those presenting low differentiation of the species were subjected to the 16S ribosomal ribonucleic acid (rRNA) sequencing. **Results:** Thirty-five strains were not identified by ANC (VITEK 2), and only one isolate was not identified by MALDI-TOF (VITEK MS). From the 386 anaerobes identified by the two methodologies, 97% agreement was observed on the identification of genus and species between the methodologies. Thirteen (3%) isolates were submitted to 16S sequencing. The agreement observed was 70% using ANC (VITEK 2) using 92% by MALDI-TOF (VITEK MS). **Conclusion:** Both methodologies showed an excellent performance for the identification of the strains tested with great differences in relation to cost-benefit. MALDI-TOF MS allowed 35 additional identifications and a saving of BRL$ 7,786 with the release of culture positive result five days ahead of the ANC (VITEK 2). TAT reduction may contribute to a successful clinical resolution.

Key words: matrix assisted laser desorption ionization-time of flight mass spectrometry; clinical laboratory techniques; bacteroides infections; anaerobic bacteria; *Clostridium* infections.

INTRODUCTION

Anaerobes are closely related to the human microbiota and behave as opportunistic pathogens capable of causing devastating infections; they are less recovered in pathologies of exogenous origin. Generally, they are associated with infections of skin, soft tissues, head and neck, pleuropulmonary, intra-abdominal, pelvic regions, bone and blood, commonly with mortality up to 40%[1-3].

These microorganisms require special procedures for isolation, and their identification are classically performed by phenotypic methods such as Gram staining, colony morphology and biochemical profile; however, some anaerobes have morphologically variable colonies and are described as bright or opaque, flat or elevated, circular or irregular and are presented as pleomorphic, gram-variable or gram-negative, even belonging to a genus known as Gram positive, such as *Clostridium tertium* and *Clostridium clostridiiforme*, requiring a great deal of expertise in morpho-staining analysis. Several species are biochemically inactive and require a high inoculum, difficult to obtain by the nature of these bacteria that present poor growth in solid media[4]. Phenotypic identification of anaerobes in clinical laboratories requires a lot of work and a long response time [turnaround time (TAT)] of seven to 14 days, which makes this culture more confirmatory than essential for choosing an appropriate therapy, a factor that often contributes to an unsuccessful clinical resolution[1, 3].

In the last decades, the increasing use of sequencing techniques has brought great changes in anaerobes taxonomy, including the description of new genera and species, however it is still technically complex methodology that is scarcely approachable and expensive. However, proteomic analysis has revolutionized bacterial identification, and Raman spectroscopy and matrix assisted laser desorption and the ionization time-of-fight mass spectrometry (MALDI-TOF MS) are consolidating as the most promising methods. MALDI-TOF MS is fast, reliable and low-costing, with correct percentages of satisfactory identifications using only a few colonies, a factor that reinforces the importance of studies that evaluate the application of this new methodology for anaerobic identification[5, 6].

OBJECTIVE

To evaluate the performance of MALDI-TOF VITEK MS method (bioMérieux, France) for the identification of the anaerobes involved in infections, compared to ANC card (VITEK 2) Compact System (bioMérieux, France), and also verifying the cost variation between both methodologies.

METHODS

During a six-month period, all anaerobic microorganisms isolated from several clinical materials were identified by the ANC card (VITEK 2) biochemical and enzymatic tests method and by MALDI-TOF mass spectrometry (VITEK MS database 2.0), following the manufacturer recommendations (bioMérieux, France).

The ANC card comprises a set of miniaturized biochemical and enzymatic tests. This method requires a high bacterial inoculum between 2.7 and 3.3 on the McFarland scale in saline solution (0.45% NaCl), which corresponds to approximately $8\text{-}10 \times 10^8$ colony-forming units (CFU/ml). The results of identification by VITEK 2 are obtained in 24 hours after the correlation with the morpho-staining characteristics visualized by Gram staining, and with the result of the aerotolerance testing manually informed by the microbiologist. The VITEK 2 equipment database is managed by AES software and allows the classification of the results into four groups: correct identification (excellent, very good, good or acceptable), low differentiation, identification error and not identified[5-7].

To perform MALDI-TOF in the VITEK MS equipment (BioMérieux, França) a bacterial spot of 1 to 3 colonies is placed on the stainless steel plate (which accommodates up to 48 simultaneous tests) with the addition of the matrix for the ionization of proteins [consisted of α-cyano-4-hydroxycyanic acid (CHCA)]. In the mass spectrometer, beams with pre-established wavelengths laser by the manufacturer are emitted for each analyte. The matrix absorbs the energy of the laser, and desorption of the sample occurs with formation of ions of different mass. These ions travel through the equipment vacuum tube and the time of flight is measured in mass. The mass spectra (MS) obtained represent the bacterial digital printing and, when compared to the reference library of Myla 2.0 software (recommended use for routine laboratories) or RUO SARAMIS (used in research laboratories) – Spectral Archive and Microbial Identification System (BioMérieux, France), they allow the identification of the genus and species in 10 minutes. These results are detailed into three categories: "good identification", when the generated MS finds perfect correlation (99.9% probability or confidence value), "low differentiation" (> 60%) or "not identified"[4].

Conflicting or low differentiation results in species identification were submitted to 16S sequencing of ribosomal ribonucleic acid (rRNA).

As quality control, *Clostridium septicum* ATCC 12464 and *Bacteroides ovatus* ATCC 1304 strains were used for both methodologies, and *Escherichia coli* ATCC 8739 for calibration of the VITEK MS system and qualification of bacterial spots.

Genomic DNA extraction was performed using the QIAamp DNA Mini® kit (Qiagen). For the strains that presented conflicting results between the methodologies, a region of 1380 pb of the gene encoding the 16S rRNA, was amplified. The products obtained by PCR were visualized on electrophoresis gel composed of 1.5% agarose and ethidium bromide. Subsequently, the amplified regions were sequenced by ABI Prism 3130® equipment (Applied Biosystems) and the sequences analyzed manually in BLAST and compared to the GenBank database.

The direct costs of the inputs involved in the analytical phase of the anaerobic culture were verified according to the standard operating procedure adopted at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC/FMUSP), and the anaerobes identification was performed by ANC (VITEK 2) and MALDI-TOF (VITEK MS). The description of the items evaluated and their respective prices are shown in **Table 1**. The culture for positive anaerobes with one identification was defined. In this analysis, the equipment used was not included.

TABLE 1 – Direct cost of the inputs for the performance of culture of the anaerobes used in the study

Inputs		Identification	
		ANC	MALDI-TOF
		(VITEK 2)	(VITEK MS)
		Value (BRL$)	
Chocolate Agar	1 plate	1.90	-
Slide	1 slide	0.07	-
Disposable strap	1 handle	0.12	-
Swab	1 swab	0.18	-
ANC Card (Vitek 2)	1 card	17.3	-
Slide MALDI TOF (Vitek MS)	1 spot	-	1.4
CHCA Matrix (Vitek MS)	1 µl	-	0.04
Disposable stick	1 unit	-	0.02
Total		20	1.5

CHCA: α-Cyano-4-hydroxycinnamic acid.

RESULTS

During the study period, 5.952 clinical samples and 421 anaerobes from different isolation sites were received for culture of anaerobes.

ANC (VITEK 2) and MALDI-TOF (VITEK MS) performance for the identification of anaerobic microorganisms are shown in **Table 2**.

Thirty-five strains were not identified by ANC (VITEK 2)and only one isolate was not identified by MALDI-TOF (VITEK MS).

From the 386 strains that were identified by the two methodologies, agreement of genus and species was observed in 97% (373) of strains.

In thirteen (3%) isolates, there was agreement of genus and conflicting or low differentiation of the species. These strains were then submitted to 16S sequencing, and agreement was 70% for ANC (VITEK 2) and 92% for MALDI-TOF (VITEK MS), as described in **Table 3**.

MALDI-TOF (VITEK MS) allowed final release of the positive culture five days before ANC (VITEK 2), since the average time to obtain the inoculum needed to perform ANC (VITEK 2) is seven days and for MALDI-TOF (VITEK MS) is 48 hours. The survey of inputs using ANC (VITEK 2) revealed that the cost of one identification, from the isolated colony, is BRL$ 20, since this methodology requires, besides the ANC card, an additional aerotolerance testing and a bacterioscopic examination, which results must be informed by the microbiologist so that the equipment completes the analysis and releases the identification of the bacterium.

The cost of identification using MALDI-TOF is BRL$ 1.50, which represents about 13 times less than the amount spent with ANC (BRL$ 20).

DISCUSSION

Studies carried out with MALDI-TOF MS demonstrate that this methodology is superior to conventional methods of identification, however less studied groups, such as anaerobes, present more heterogeneous results[8-13].

The data presented in this study showed that the agreement of genus and species between ANC (VITEK 2) and MALDI-TOF (VITEK MS) was observed in 97% of the identified 386 strains. From the thirteen (3%) strains that presented conflicting results or low differentiation of the species, 16S sequencing showed greater agreement of 92% with MALDI-TOF (VITEK MS).

MALDI-TOF (VITEK MS) allowed additional identification of 35 strains that were not identified by ANC (VITEK 2), and only one *Clostridium innocuum* isolate remained unidentified by MALDI-TOF (VITEK MS). For this isolate, ANC (VITEK 2) found low differentiation between *Clostridium subterminale* and *C. sporogenes*. In both methodologies, the composition of the database for *Clostridium* spp. was composed mainly by Grosse-Herrenthey *et al.* (2008)[14], and some studies show good identification for this genus, however, the database AES (ANC card), MYLA and SARAMIS (MALDI-TOF MS database 2.0) do not contemplate *C. innocuum*, justifying the failure to identify this isolate that, despite the low pathogenicity and the inability to produce toxins, it presents intrinsic resistance to vancomycin and causes severe infections in immunocompromised patients[14-16].

The results found in our study are consistent with other studies involving the identification of anaerobic bacteria using MALDI- TOF MS. Yang Li *et al.* (2014)[17] analyzed the performance of MALDI-TOF (VITEK MS database 2.0) compared to ANC (VITEK 2) and concluded that the agreement of genus and species of the 50 strains tested was 86%, revealing that MALDI-TOF (VITEK MS) was superior for determining the species and identifying 92% of them. Lee *et al.* (2015)[13], when analyzing the performance of MALDI-TOF (VITEK MS) and the conventional API Rapid ID 32 A and ANC (VITEK 2) methods compared to 16S sequencing to identify 274 anaerobes, verified that MALDI-TOF (VITEK MS database 1.1) correctly identified 83.9% (209) with a 100% performance for *Bacteroides fragilis*,

TABLE 2 – Performance of ANC (Vitek 2) and MALDI-TOF (Vitek MS) for the identification of 421 isolated anaerobes

Organisms	Quantity of isolates n	Vitek 2 ANC Card					Vitek MS				
		Identification		Low differentiation of the species	Not identified	Identification error of the species	Identification		Low differentiation of the species	Not identified	Identification error of the species
		Genus	Species				Genus	Species			
Bacteroides fragilis	113	113	113	0	0	0	113	109	4	0	0
Bacteroides caccae	7	7	3	4	0	0	7	3	4	0	0
Bacteroides thetaiotaomicron	45	45	42	4	0	0	45	45	0	0	0
Bacteroides ovatus	35	35	35	0	0	0	35	35	0	0	0
Bacteroides uniformis	7	7	7	0	0	0	7	7	0	0	0
Bacteroides vulgatus	4	4	4	0	0	0	4	4	0	0	0
Parabacteroides distasonis	12	12	12	0	0	0	12	12	0	0	0
Fusobacterium nucleatum	21	17	17	0	4	0	21	21	0	0	0
Fusobacterium mortiferum	3	1	1	0	2	0	3	3	0	0	0
Fusobacterium necrophorum	5	4	4	0	1	0	5	5	0	0	0
Fusobacterium varium	2	2	2	0	0	0	2	2	0	0	0
Prevotella bivia	9	9	9	0	0	0	9	9	0	0	0
Prevotella buccae	6	6	6	0	0	0	6	6	0	0	0
Prevotella denticola	5	5	5	0	0	0	5	5	0	0	0
Prevotella intermedia	2	1	1	0	1	0	2	2	0	0	0
Prevotella melaninogenica	7	7	6	1	0	0	7	7	0	0	0
Prevotella oralis	0	0	0	1	0	1	0	0	0	0	0
Actinomyces viscosus	1	0	0	0	1	0	1	1	0	0	0
Clostridium clostridiiforme	11	11	9	0	0	2	11	11	0	0	0
Clostridium difficile	2	1	1	0	1	0	2	2	0	0	0
Clostridium ramosum	7	7	5	0	0	0	7	7	0	0	0
Clostridium septicum	2	2	2	0	0	0	2	2	0	0	0
Clostridium sordellii	1	1	1	0	0	0	1	1	0	0	0
Clostridium sporogenes	1	1	1	1	0	0	1	1	0	0	0
Clostridium subterminale	0	0	0	1	0	0	0	0	0	0	0
Clostridium innocuum	1	0	0	0	0	0	0	0	0	1	0
Clostridium tertium	1	1	1	0	0	0	1	1	0	0	0
Clostridium perfringens	19	19	19	0	0	0	19	19	0	0	0
Eubacterium lentum	3	3	2	0	1	0	3	3	0	0	0
Propionibacterium acnes	19	19	19	0	0	0	19	19	0	0	0
Finegoldia magna	14	14	14	0	0	0	14	14	0	0	0
Helcococcus kunzii	1	0	0	0	1	0	1	1	0	0	0
Parvimonas micra	7	5	5	0	2	0	7	7	0	0	0
Peptoniphilus asaccharolyticus	9	9	9	0	0	0	9	9	0	0	0
Peptostreptococcus anaerobius	16	16	16	0	0	0	16	16	0	0	0
Veillonella parvula	23	2	2	0	21	0	23	23	0	0	0
Cumulative total (%)	421	386 (92)	373 (89)	6 (1)	35 (8)	3 (1)	420 (100)	412 (98)	4 (1)	1 (0)	0

TABLE 3 – Conflicting results or with low differentiation of the species obtained by ANC (VITEK 2) and MALDI-TOF (VITEK MS), submitted to the 16S rRNA sequencing

Number of isolates	Identified microorganisms	Identification	
(n = 13)	by 16S sequencing	ANC (VITEK 2)	MALDI-TOF (VITEK MS)
1	*Prevotella bivia*	*Prevotella oralis*	*Prevotella bivia*
2	*Clostridium ramosum*	*Clostridium clostridiiforme*	*Clostridium ramosum*
4	*Bacteroides fragilis*	*Bacteroides fragilis*	*B. fragilis/B.caccae*
4	*Bacteroides thetaiotaomicron*	*B. thetaiotaomicron/B. caccae*	*Bacteroides thetaiotaomicron*
1	*Prevotella melaninogenica*	*P. oralis/P. melaninogenica*	*Prevotella melaninogenica*
1	*Clostridium innocuum*	*C. sporogenes/C. subterminale*	Not identified

rRNA: ribosomal ribonucleic acid.

Bacteroides thetaiotaomicron and *Clostridium perfringens*, not identifying six of the 26 strains of *Eggerthella lenta* and four of nine strains of *Peptoniphilus asaccharolyticus*. MALDI-TOF (VITEK MS) allowed 36% additional identifications compared to conventional methodologies that identified only the genus, mistakenly identified or did not identify the microorganisms – of which 26% were *Veillonella parvula*. Twenty-five isolates were not present in the VITEK MS database and could not be identified by mass spectrometry[13, 17].

Garner *et al.* (2014)[18] found 91.2% of correct identifications (genus and species), evaluating 651 anaerobes submitted to identification by16S sequencing and by MALDI-TOF (VITEK MS database 2.0), which presented an excellent performance in the identification of *Bacteroides fragilis* and a greater difficulty in the identification of the *Fusobacterium* spp. and *Propionibacterium* spp. species.

A study by La Scola *et al.* (2011)[11] analyzed the identification of 544 anaerobes isolated from clinical samples using MALDI-TOF (Bruker Biotyper – Becton Dickinson) and 16S sequencing, demonstrating that MALDI-TOF correctly identified 61% of strains, 100% of *Bacteroides* spp. and of *Clostridium perfringens*, but only 50% of *Finegoldia magna*, *Fusobacterium* spp. and *Prevotella* spp. strains. Justesen *et al.* (2011)[12] evaluated MALDI-TOF Bruker Biotyper and VITEK MS (database 2.0) for the identification of anaerobic microorganisms, revealing that these methodologies were able to correctly identify 67.2% and 49% of 290 isolates, respectively.

Many authors believe that these percentage differences reflect the quality and quantity of the bacterial inoculum, since the lower percentages of identification are related to the more fastidious strains[8-10, 12, 13].

The common sense evidenced in all studies cited is that MALDI-TOF allows the direct identification of the first plaque performed, without the need for new isolation, since it requires few colonies for microbiological analysis, neutralizing a great hurdle of anaerobic culture: the late and weak growth in solid media. This characteristic is determinant for the microbiological diagnosis, especially for fastidious anaerobes that often lose their viability during the isolation stages, which disables the confirmation of the anaerobic etiology[17-19].

The accuracy in the identification of the *Bacteroides fragilis* group is also constant and very relevant, since MALDI-TOF mass spectrometry has allowed a number of taxonomic discoveries that, associated with molecular studies, have been establishing the pathogenicity of anaerobic microorganisms and increasingly evidencing the *B. fragilis* group as the most virulent. Its polysaccharide capsule is known to be related to the formation of abscesses, and the anaerobic microorganism is more related to the growing reports of antimicrobial resistance, including carbapenems and metronidazole[19, 20].

The data found in our study showed that MALDI-TOF (VITEK MS) correctly identified 98.2% of the strains belonging to the *Bacteroides fragilis* group. These species have very close peaks, which may justify the low differentiation of the mass spectrum generated in the four isolates found[19].

ANC (VITEK 2) has satisfactory performance endorsed by the literature for the identification of most anaerobes of medical importance, however it may present difficulties of identification with the species that have an asaccharolytic profile, as some strains belonging to the *Clostridium* spp. genus[5, 10, 17].

The results with low differentiation of species using ANC (VITEK 2), found in this study, are expected by the manufacturer that proposes the use of additional tests, such as the production of lipase, lecithinase and indole to complete the identification, mainly for *Prevotella* spp. and *Clostridium* spp., however these tests require specific media such as EYA agar (Egg Yolk Agar), not commercially available in Brazil. Some anaerobes present variable results to the indole test, as some species of the *Bacteroides fragilis* group, that is, even if available, these additional tests are not always determinant for the bacterial identification, as in the cases found in this sampling[5, 10, 17].

It is worth emphasizing that the quality and reliability of the identification in both methodologies used are closely related to the availability of a complete and updated database. With regard to conventional methodologies, MALDI-TOF (VITEK MS) offers an open library, that is, with the possibility of including new spectra that can enlarge the database, allowing for future identifications[13].

The *Clostridium innoccum* strain identified only by 16S can be included in the VITEK MS database[21-24].

The introduction of the MALDI-TOF system in microbiology laboratories is initially expensive, as is the inclusion of any other diagnostic platform, due to the high cost of the equipment, around BRL$ 600,000 to BRL$ 800,000. However, this expense is quickly recovered by the number of examinations carried out, the discontinuation of traditional methodologies, the reduction of bacterial identification time, the improvement of workflows and the benefits generated by patients, such as the reduction of morbidity and mortality, reported in several studies due to the better management of antimicrobial therapy[22, 24].

Galliot *et al.* (2011)[24] found a reduction in costs of 89.3% with identifications performed by the laboratory during the first year of the MALDI-TOF implementation and a significant reduction (about 32 times) in the waste generated during that period.

The analysis of the identification cost, from the isolated colony in solid medium carried out in this study, revealed a value of BRL$ 1.50 when using MALDI-TOF (VITEK MS), and BRL$ 20 when using ANC (VITEK 2). Using this technology, it was possible to reduce the TAT of the positive result in five days, since the average time to obtain the inoculum needed for ANC (VITEK 2) performance was seven days and for MALDI-TOF (VITEK MS) was 48 hours and a saving of BRL$ 7,786 in the sampling of this study. In addition, the identification process by ANC (VITEK 2) takes up to 24 hours, and for the same strain by MALDI-TOF (VITEK MS), 10 minutes.

MALDI-TOF for anaerobes routine presents a great advantage over conventional automated methods, as it does not require a large amount of inoculum and performs the identification by proteomic analysis independent of the phenotypic variants, such as the expression of characteristics and performance compared to the biochemical tests, since these bacteria are mostly fastidious and biochemically asaccharolytic[5, 22-24]. For these microorganisms, the recognition of the morpho-staining (Gram) and morphological characteristics of the colony remain critical for the correct and effective identification, therefore the investment and the training of specialized professionals must proceed concomitantly with the new Technologies[4, 5].

CONCLUSION

MALDI-TOF (VITEK MS) showed excelent porformance in anaerobe identification, reducing workload and positive TAT reduction in five days with a better use of resources

CONFLICTS OF INTEREST

There are no conflicts of interest.

ETHICS COMMITTEE

The study was approved by the Ethics Committee of the School of Medicine of the Universidade de São Paulo and is enrolled in the Brazil Platform (CAAE 39682314.5.0000.0068).

RESUMO

Introdução: *A identificação das bactérias anaeróbias por métodos convencionais empregados nos laboratórios clínicos demanda muito trabalho e um longo tempo de resposta (TAT). A espectrometria de massa por ionização e dessorção a laser assistida por matriz (MALDI-TOF MS) é uma técnica precisa, rápida e barata, com resultados promissores para a identificação bacteriana.* ***Objetivo:*** *Avaliar a espectrometria de massas MALDI-TOF (VITEK MS, bioMérieux, France) diante do cartão ANC (VITEK 2, bioMérieux, France) para a identificação de anaeróbios, bem como verificar a variação de custos entre as metodologias.* ***Métodos:*** *Foram identificados 421 anaeróbios concomitantemente pelo ANC (VITEK 2) e pelo MALDI-TOF (VITEK MS). Os resultados discordantes ou que apresentaram baixa discriminação das espécies foram submetidos ao sequenciamento do 16S do ácido ribonucleico ribossonal (rRNA).* ***Resultados:*** *Trinta e cinco cepas não foram identificadas pelo ANC (VITEK 2), e somente um isolado ficou sem identificação pelo MALDI-TOF (VITEK MS). Dos 386 anaeróbios identificados pelas duas metodologias, a concordância na identificação de gênero e espécie foi observada em 97%. Treze (3%) isolados foram submetidos ao sequenciamento do 16S; a concordância observada foi de 70% com o ANC (VITEK 2) e 92% com MALDI-TOF (VITEK MS).* ***Conclusão:*** *Ambas as metodologias demonstraram ótimo desempenho para identificação das cepas testadas com grandes diferenças em relação ao custo-benefício. O MALDI-TOF MS permitiu 35 identificações adicionais e uma economia de R$ 7.786,00 com a liberação do resultado positivo da cultura cinco dias à frente do ANC (VITEK 2). A redução do TAT pode contribuir para uma resolução clínica bem-sucedida.*

Unitermos: *espectrometria de massas por ionização e dessorção a* laser *assistida por matriz; técnicas de laboratório clínico; infecções por bacteroides; infecções por* Clostridium*; bactérias anaeróbias.*

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