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Comparison of two matrix-assisted laser desorption ionization –time of flight (MALDI-TOF) mass spectrometry methods and API 20AN for identification of clinically relevant anaerobic bacteria

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Abstract

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is suitable for high-throughput and rapid microbial diagnosis at relatively low costs and can be considered an alternative for conventional biochemical and molecular identification systems in a Clinical Microbiological Laboratory including anaerobe laboratory. Two commercially available MALDI-TOF MS systems, Bruker Microflex MS™ and bioMerieux VITEK MS™, were evaluated for the identification of consecutive 274 clinically significant anaerobic bacteria recovered from routine cultures of clinical specimens in parallel with blinded comparison with conventional biochemical (API 20AN) or molecular methods. All were recovered cultures obtained from patients attending Mubarak Al Kabir Hospital during 6 months period. Discrepant results after 2 attempts at direct colony testing have failed to provide acceptable MALDI-TOF identification were resolved by gold standard 16S gene sequencing. VITEK MS™ gave high confidence identification of the 274 isolates of which all were correctly identified. Bruker Microflex MS system also gave high confidence identification for 272 of the 274. After discrepancy testing, the Bruker MS results agreed with biochemical or molecular method for 89.1% of the isolates at species level, 10.2% at genus level (0.72% were misidentified). In our hands, the level of agreement with VITEK MS was 100% species, 100% genus and none were misidentified. Our data suggest that implementation of MALDI-TOF MS as first step for identification will shorten the turnaround time and reduce the cost in Anaerobe Microbiology Laboratory.

Introduction

Anaerobes are important cause of several infections in the brain, lung, pelvis and abdomen. However, their isolation in culture and identification in routine diagnostic microbiology laboratory is difficult and time consuming, (Jousimies-Somer *et al*., 2002). Phenotypic and biochemical methods need time, commitment for several days and sometimes they do not distinguish closely related species or may give incorrect or inconclusive results especially with uncommon or fastidious organisms. Molecular methods e.g. PCR based amplification methods and sequencing (Drancourt & Raoult, 2005; Song, 2005) are expensive, not practical for routine use, and need technical expertise. In addition, they are not available to many clinical laboratories.

Recent development of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to be useful method for identification of different microorganisms. Several studies have reported the advantage and performance of MALDI-TOF system compared with the commercially available systems (Fedorko *et al.*, 2012; Nagy *et al.*, 2009; **Seng *et al*., 2009**). There are several commercially available MALDI-TOF MS systems with software and database for identification of microorganisms isolated from clinical specimens e.g. Bruker MS (Microflex^{TM}; Bruker Daltoniks, Bremen, Germany), VITEK MS^{TM} (bioMerieux, Marcy l'Etoile, France), and Shimadzu MS (AXIMA; Shimadzu Corporation, Kyoto, Japan). They are used to identify aerobic and anaerobic bacteria (Fedorko *et al*., 2012; Nagy *et al*., 2009; Nagy *et al*., 2012; van Veen *et al*., 2010; **Veloo *et al.*, 2011b**), mycobacteria (Saleeb *et al*., 2011), Nocardia (Verroken *et al*., 2010) and yeasts (van Veen *et al*., 2010) isolated on solid media from clinical specimens. MALDI-TOF MS has also been recently used for the identification of bacteria and yeast directly from positive blood culture bottles

(Ferroni *et al*., 2010; Stevenson *et al*., 2010). MALDI-TOF MS system appears to be associated with rapid turnaround time, low sample volume requirements and modest reagent costs. The present study was undertaken to determine the ability of two MALDI-TOF MS systems (Bruker Microflex™ MS and bioMerieux VITEK MS™), to identify clinically significant anaerobic bacteria in comparison with conventional API 20AN **(**bioMerieux).

Materials and methods

Setting: The evaluation of Bruker Microflex™ MS and VITEK MS™ was done in the routine Clinical Microbiology Laboratory, Mubarak Al Kabir Hospital, Kuwait over a period of 6 months, June - December 2011. Identification with API 20AN and 16S RNA sequencing were carried out in the Anaerobe Reference Laboratory, Mubarak Al Kabir Hospital, Kuwait. Our hospital is a 500-bed tertiary hospital with 9 satellite clinics.

Bacterial isolates: A total of 274 isolates, were recovered from routine examination of clinical specimens submitted to Mubarak Al Kabir Hospital, Kuwait during the study period. They were from various sources primarily derived from pus, blood cultures, tissues, intra-abdominal samples and wounds. A total of 5 genera and 14 species were encountered. The isolates were cultured on Brucella agar (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood, 5 µg/ml haemin and 1µgm/ml vitamin K1 and incubated at 37°C in anaerobic atmosphere of H_2 80%, CO_2 10% using Anoxomat™ Anaerobic System (AN2CTS model, Mart Microbiology B.V., Drachten, the Netherlands) for 48 h prior to procedure. Isolates were processed within 2 h after removal from the incubator. One dedicated laboratory technologist operated each system.

Routine identification: Initial bacterial identification in the laboratory was carried out using the API 20AN (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instruction. Each batch of Gram-negative isolates was run in parallel with control strains of *Bacteroides fragilis* ATCC 25285 and Gram-positive isolates with *Clostridium perfringens* type A strain, ATCC 13124 and *C. difficile* ATCC 700057.

Measurements with Bruker Microflex MALDI-TOF MS: All isolates were tested in duplicates. The system was operated as previously described by Cherkaoui *et al*. (2010). The colonies were picked up from the anaerobic Brucella blood agar and inoculated onto MALDI target plate. This system (compromising a Microflex MALDI-TOF mass spectrometry with flexControl software and the MALDI BioTyper DB Update-V3.3) was operated with 1µl matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrite-2.5% trifluoroacetic acid (Bruker α-cyano; Bruker Daltonics, Bremen, Germany). The target slide was then loaded into the Microflex MS machine and the sample submitted to multiple laser shots inside the Bruker MS machine.

Measurements with VITEK MALDI-TOF MS: All isolates were tested in duplicates. It was operated as previously described by Cherkaoui *et al*., 2010. Briefly, a portion of a colony was picked up from the anaerobic Brucella blood agar and inoculated onto a spot on the plate target slide. A ready-made matrix solution (consisting of a solution of 3.10g α-cyano-4-hydroxycinnamic acid in 25.44g acetonitrite, 25.57g ethanol in 100ml solvent) was applied to the spot on the target slide and allowed to dry. Then, the slide was loaded into the VITEK MS machine. As with the Microflex MS system, the sample was submitted to multiple laser shots inside the VITEK MS machine. The matrix absorbs the laser light and vaporizes along with the

sample in process of ionization. A VITEK mass spectrometer was used to generate spectra from the bacterial suspension and the Biotyper software (version 2.00) was used to analyze the results. Both systems were calibrated immediately before the analysis according to the manufacturer's instructions.

Quality control: *Escherichia coli* ATCC 8739, *B. fragilis* ATCC 25285 and *C. difficile* ATCC 700057 were included as positive quality controls in each run with both systems and matrices. A negative control consisting of matrix alone with no organism was also included in each run.

Data analysis: The identification criteria were chosen according to the cutoffs proposed by the manufacturers. For the Bruker Microflex MS identification interpretation was as follows: 0.00-1.699 represent no reliable identification, a score of ≥1.700 and ≤1.999 was considered as probable identification at the genus level, a score of ≥ 2.000 – 2.999 was considered as secure genus identification and probable species identification; and a score of ≥ 2.300 - 3.000 was considered as highly proper identification at species level. In other words, the manufacturer's recommended score cutoff were used to determine the genus (1.700-1.999) or species (≥2.000) level identification. For VITEK MS, the results were evaluated according to a coloured index: green for percentages ≥ 90%, yellow for those between 85 – 89.9% and white for those below 85%. All of the identifications to the genus or species level fell into the green zone, with a score above 90% considered reliable. Scores between 85 and 90% were also considered for acceptable identifications. A cutoff of 90% was chosen for VITEK MS.

Discrepancy: The first response to a discrepancy was to repeat the analysis using both Bruker Microflex MS and VITEK MS to eliminate the possibility of contamination. The remaining discrepancies were resolved by performing 16S rRNA gene amplification and sequencing.

16S rRNA gene amplification, sequencing and interpretation: DNA of the strain was isolated as described previously by Boom *et al*. (1990) and the 16S rRNA genes were amplified and sequenced using universal 16S rRNA–specific primers (Hiraishi, 1992). The sequences obtained were compared with sequences present in GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov).

Results

Table 1 depicts the 274 clinical isolates belonging to 5 genera and 14 species that were tested by both the Bruker MS and VITEK MS systems compared to API 20AN. The VITK MS identified all isolates to the genus and species level in agreement with the API 20AN, while the Bruker MS identified 99.2% of all isolates to the genus and species level compared to API 20AN.

Both systems correctly identified all species of *Clostridium* and *Peptostreptococcus* comparable with the conventional system. VITEK MS and API 20AN were in agreement in the identification of all *Bacteroides* spp., including *B. fragilis* and *B. thetaiotaomicron* isolates.

Bruker MS could identify 85.5% of the anaerobes to genus level including probable genus identification (48.5%), and highly probable genus identification (40%). Log (scores) of 6 isolates (1 *B. vulgatus*, **1.68;** 1 *P. bivia*, **1.179;** 1 *B. ovatus*, **1.154;** 1 *C. difficile*, **1.687;** 1 *C. sporogenes*, **1.629;** and 1 *P. asaccharolytica*, **1.461**) were <1.7 in the Bruker MS i.e. unreliable identification. However, they were correctly identified at the genus and species level according to the identification by 16S rRNA sequencing. Another 22 (8%) isolates [3 *Pr. bivia*, 1 *B. ovatus*, 2 *C. perfringens*, 2 *C. histolyticum*, 10 *C. difficile*, 2 *C. vulgatus*, 2 *B. fragilis*] gave scores between

1.7 and <2.00; meaning they could be validated only to genus level but with sequencing, their identification was correct at the genus and species levels.

Table 2 shows the sequencing results of those isolates that gave discrepant results between the Bruker MS, VITEK MS and API 20AN. Bruker MS misidentified one *B. fragilis* and one *B. thetaiotaomicron* isolates as *Malika spinosa* (score 1.393) and *Propionibacterium acne* (score 1.464), respectively. The 2 discrepant results were resolved by 16S rRNA sequencing in favor of bioMerieux VITEK MS and API 20AN.

Bruker identified 244 (89.1%) and 28 (10.2%) to the species and genus level, respectively. VITEK MS identified 247 (100%) isolates to species level. The Bruker MS misidentified only 2/274 (0.72%) of` the isolates compared to none among the VITEK MS.

Discussion

Conventional identification methods for anaerobes are cumbersome, time consuming and need specific anaerobic environment. MALDI-TOF MS has now been used and implemented in some laboratories for efficient, rapid and cost-effective identification of different classes of bacteria including anaerobes. The correct identification of an organism is dependent on the presence of the reference strains in the database because the species of the reference strain will give the closest match for the identification of the tested strain. In our study, more isolates could be identified to the species level with the VITEK MS system: 100% versus 89.1% by Bruker MS. **This is similar to a recent report by Veloo *et al*. (2011b) where the corresponding numbers were 61% with Shimadzu/SARAMIS system (old name for VITEK MS) and 51% with Bruker MS system.** However, this is in contrast to a recent paper by Justesen *et al*. (2011),

where the corresponding number was 43.8 - 49% for the Shimadzu/SARAMIS system and 67% for Bruker MS system. Although, the Bruker system gave unreliable score (i.e. <1.7) for 6/247 (2.1%), the final identification was accurate compared to the conventional system and sequencing. Likewise, a score between 1.7 and <2.0 was given to 22/274 (8%) isolates i.e. Bruker gave the correct identification to both genus and species level but according to the manufacturer's instructions, we can depend on their identification to the genus level only. This is in contrast to a recent report by Nagy *et al.* (2012), who could identify 218/283 (77%) isolates to the species level (with score ≥ 2.0) and 31/283 of isolates (10.95%) to genus level (with score 1.7-2.0) and 34 isolates (12%) gave none reliable identification (score <1.7). **Other group (Schmitt *et al.*, 2012) found that correct genus identification could be achieved for 57% (120/209) of anaerobes with score >2.0 and correct species identification was achieved for 80 % (168/209) of the isolates.**

The identification of anaerobes by MS offers several advantages in comparison with the conventional routine method. Shortening the time period required to identify an organism from days to a few minutes will improve the clinical outcome of the patients (Cherkaoui *et al.*, 2010). **There is a great and significant impact on observed in time-to-identification of biochemically inert, fastidious and slow-growing anaerobic cocci (Tan *et al.*, 2012). It is justified to use MALDI TOF for the identification of anaerobes in our laboratory where almost more than one third (113/274; 41%) of our isolates are *Bacteroides* species. This is related to the fact that MALDI TOF protocol can reduce the reagent use (from $4,068.84 to $161.41) and labor cost (from 36:04h to 5:48h) significantly (Tan *et al.*, 2012).** The strength of our study is the implementation of the MALDI-TOF MS in the routine setting with comparison of the conventional system on the clinical isolates and the use of 16S rRNA

sequencing for analysis of discrepancies. However, one limitation of our study is the small number of genera and the species that were isolated and tested **and certain species e.g. *Fingoldia magna* and *Parabacteroides* species were not tested** because of the small number of mixed anaerobes isolated during the study period, most of which were considered as part of the mixed normal flora and where thus not identified further.

One of the draw backs of MALDI-TOF is that it requires cultured organisms rather than directly clinical specimen. **In addition, the available database of the Bruker MS need to be optimized for routine identification of anaerobes as some organisms could not be identified by the Bruker MS (Veloo *et al.*, 2011a; Veloo *et al.*, 2011c).** Bruker MS has been evaluated for identification of organisms directly from blood culture but currently does not provide data about antimicrobial susceptibility pattern. In conclusion, MALDI-TOF is a rapid, simple, inexpensive technique, user friendly (VITEK MS > Bruker MS) and relatively small size machine (Bruker) that can be incorporated into the routine diagnostic laboratory and used for the identification of anaerobes. It can easily be implemented in the routine conventional laboratory.

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Table 1: API 20AN and MALDI-TOF MS results for 274 anaerobes compared to API 20AN

Genus & species ID by API 20AN	No (%) isolates	Bruker MS				VITEK MS	
	274	No with score <1.7	No with score 1.7-1.999	No with score 2-2.299	No with score ≥3.00	No with Score <85	No with Score 85-90
B. fragilis	113 (41.2)	1	2	34	76	0	113
B. ovatus	8 (2.9)	1	1	4	2	0	8
B. thetaiotamicron	15 (5.5)	1	0	13	1	0	15
B. uniformis	5 (1.8)	0	0	3	2	0	5
B. vulgatus	10 (3.6)	1	2	7	0	0	10
C. butyricum	1 (0.4)	0	0	1	0	0	1
C. difficile	70 (25.5)	1	10	51	8	0	70
C. histolyticum	2 (0.7)	0	2	0	0	0	2
C. perfringens	14 (5.1)	0	2	1	11	0	2
C. sporogenes	1 (0.4)	1	0	0	0	0	1
Prevotella bivia	31 (11.3)	1	3	16	11	0	31
Pr disiens	1 (0.4)	0	0	1	0	0	1
Peptostreptococcus assacchrolyticus	2 (0.7)	1	0	1	0	0	2
Veillonella parvula	1 (0.4)	0	0	1	0	0	1
Total No (%)	274	8(2.9)	22(8)	133(48.5)	111(40.5)	0	274(100)

Table 2: API 20AN identification, VITEK MS, and 16SrRNA sequencing data of isolates for which mismatched and no reliable identification was obtained by Bruker MS (i.e. score <1.7)

Species ID and score obtained by API	Species ID and score obtained by Bruker	Species ID and score obtained by VITEK MS	16S rRNA sequencing result
B. fragilis (99.9)	*Malika spinosa* (1.393)	*B. fragilis* (99.9)	*B. fragilis*
B. ovatus/ thetaiotamicron (99.9)	*Propionibacterium acnes* (1.464)	*B. thetaiotamicron* (99.9)	*B. thetaiotamicron*