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Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry for identification of *Clostridium* species isolated from Saudi Arabia

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ABSTRACT

The aim of this study was to identify different *Clostridium* spp. isolated from currency notes from the Ha'il region of Saudi Arabia in September 2014 using MALDI–TOF-MS. *Clostridium* spp. were identified by Bruker MALDI–TOF-MS and compared with VITEK 2. The confirmation of the presence of different *Clostridium* spp. was performed by determining the sequence of the 16S ribosomal RNA gene. In this study, 144 *Clostridium* spp. were isolated. Among these specimens, MALDI–TOF-MS could identify 88.8% (128/144) of the isolates to the species level and 92.3% (133/144) to the genus level, whereas, VITEK 2 identified 77.7% of the (112/144) isolates. The correct identification of the 144 isolates was performed by sequence analysis of the 500 bp 16S rRNA gene. The most common *Clostridium* spp. identified were *Clostridium perfringens* (67.36%), *Clostridium subterminale* (14.58%), *Clostridium sordellii* (9%) and *Clostridium sporogenes* (9%). The results of this study demonstrate that MALDI–TOF-MS is a rapid, accurate and user friendly technique for the identification of *Clostridium* spp. Additionally, MALDI–TOF-MS has advantages over VITEK 2 in the identification of fastidious micro-organisms, such as *Clostridium* spp. Incorporating this technique into routine microbiology would lead to more successful and rapid identification of pathogenic and difficult to identify micro-organisms.

Introduction

Many *Clostridium* spp. are capable of causing invasive infections in humans, some of which could be serious and life threatening, such as myonecrosis and bacteremia. The ability of *Clostridium* spp. to cause serious infections results predominantly from the production of harmful toxins.[1] Production of potent toxins by *Clostridium* spp., particularly by *C. botulinum*, *C. perfringens*, *C. tetani* and *C. difficile*, leads to severe diseases such as botulism, gas gangrene, tetanus and pseudomembranous colitis.[2–4] *Clostridium* spp. are fastidious in nature, and their isolation, culture and identification in a routine diagnostic microbiology laboratory are complicated and

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time consuming. There are several reasons that the identification of *Clostridium* spp. in a routine microbiology laboratory is difficult, including the requirement for a specific anaerobic system, such as an anaerobic jar for the culture, an extended incubation period, and an occasional loss of isolates during subculture because of oxygen sensitivity. Phenotypic and biochemical methods require time because the procedures are lengthy, and at times, they fail to distinguish between closely related spp. PCR-based molecular methods and sequencing are expensive and difficult to use for routine diagnostic procedures, and they require committed technical expertise.[5]

Recently, many technological improvements to methods for the identification of micro-organisms, such as MALDI–TOF-MS, have successfully been incorporated in microbiology laboratories globally. Compared with conventional methods, MALDI–TOF-MS is a useful, rapid, accurate and simple technique for the correct identification of micro-organisms.[6] Several studies have highlighted the advantages and performance of MALDI–TOF-MS including, rapidity, low sample volume requirements and low reagent costs compared with currently available methods. Many studies using this technology, which is predominantly used for the identification of aerobic bacteria, have led to this technology being used in many clinical laboratories worldwide. Very few studies have been conducted on the use of MALDI–TOF-MS to identify anaerobic bacteria. The aim of this study was to identify the *Clostridium* spp. obtained from currency notes in Saudi Arabia using MALDI–TOF-MS.

Materials and methods

Study design and bacterial isolation

In this study, 144 *Clostridium* spp. were isolated in sterile tubes from 320 currency notes (1-Riyal) collected separately from the Ha'il region in September 2014. The notes were collected in sterile tubes to avoid cross contamination and then transferred into new sterile tubes containing sterile brain heart infusion (BHI) broth. The tubes were vortexed for 30 s followed by incubation in a shaker incubator for 4 h at 37 °C. The tubes were vortexed again for 30 s and incubated at 37 °C overnight. The samples were sub cultured on blood agar (BA) plates containing 50 μg of metronidazole and 10 μg gentamicin discs (Oxoid, UK). The plates were incubated for 48–72 h at 37 °C using anaerobic jars (Oxoid, UK). All colonies that were susceptible to metronidazole and resistant to gentamicin were selected and sub cultured on two separate blood agar plates and incubated aerobically and anaerobically. All isolates that grew anaerobically and not aerobically were designated anaerobic bacteria and were selected for further identification.

Identification of bacterial isolates by MALDI–TOF-MS

Isolates were identified by MALDI–TOF-MS (Bruker Daltonics, Bremen, Germany) using a formic acid-based direct, on-plate preparation method.[6] In this method, one microliter of 70% formic acid per well was deposited onto the MALDI–TOF MS steel anchor plate (BigAnchor 96-well plate; Bruker Daltonics). The colonies were spread into the formic acid and allowed to dry. The dried mixture was overlain with 2 μl of matrix solution (α-cyano-4-hydroxycinnamic acid (HCCA); Bruker Daltonics), dissolved in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid and allowed to dry prior to analysis using a MALDI Biotyper. A MicroFlex LT mass spectrometer (Bruker Daltonics) was used for the analysis. The spectra were analyzed using Bruker Biotyper 3.0 software. The manufacturer-recommended cutoff scores were used for identification, with scores of ≥2.000 indicating identification to the species level, scores between 1.700 and 1.999 indicating identification to the genus level, and scores of <1.700 indicating no identification. The isolates producing scores of <1.700 were retested once, and the highest score was used for the final analysis.

Identification of bacterial isolates by VITEK 2

Additionally, the bacterial isolates were identified using a VITEK 2 (bioMérieux, France) according to the manufacturer's guidelines for anaerobic identification.

Identification of bacterial isolates by the 16S rRNA gene sequence

The identification of the isolates was performed with the 16S rRNA gene sequence. The DNA of the bacterial isolates was extracted, and amplification of the 510 bp of 16S rRNA gene was performed according to the previously described method, using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCG-CGGCKGCTG-3′).[7] The sequencing of the 510 bp PCR product was performed using the above forward and reverse primers on an Applied Biosystems 3500 Genetic Analyzer according to the manufacturer's instructions. The sequence results were analyzed using GenBank (http://www.ncbi.nlm.nih.gov).

Results

The identification of different *Clostridium* spp. from our study using MALDI–TOF-MS is presented in Table 1. The results showed that of 144 *Clostridium* spp., MALDI–TOF-MS could correctly identify 88.8% (128/144) to the species level and 92.3% (133/144) to the genus level. The *Clostridium* spp. identified at the species level and genus level were as follows: (i) *C. perfringens*, 94.8% (92/97) of the isolates were identified to the species

Table 1 – Identification of 144 *Clostridium* species using MALDI–TOF-MS at a log score ≥2.000 (species level) and ≥1.700 (genus level).

Species	MALDI–TOF MS at log (score)			
	≥2.000	≥1.700	Not reliable	Misidentification
C. perfringens	92	95	2	0
C. subterminale	17	17	0	4
C. sordelli	7	8	1	4
C. sporogenes	12	13	0	0
Total	128	133	3	8

Table 2 – Comparison of the identification of *Clostridium* species by MALDI–TOF-MS, VITEK 2 and 16S rRNA.

Species	MALDI–TOF MS	VITEK 2	16S rRNA
C. perfringens	95	78	97
C. subterminale	17	20	21
C. sordellii	8	5	13
C. sporogenes	13	9	13
Total	133	112	144

level with a score ≥2.0, and 98% (95/97) of the isolates were identified to the genus level with a score of 1.7–2.0. Among *C. perfringens*, 2% of the isolates (2/97) yielded no reliable identification with a score <1.7. (ii) Among *C. subterminale*, 81% (17/21) of the isolates were identified to the species level with a score ≥2.0 and 81% (17/21) of the isolates to the genus level with a score 1.7–2.0. Among *C. subterminale*, 19% (4/21) of the isolates were misidentified. (iii) Among *C. sordelli*, 53.8% (7/13) of the isolates were identified to the species level with a score ≥2.0, and 61% (8/13) of the isolates to the genus level with a score of 1.7–2.0. Among *C. sordelli*, 7.6% (1/13) of the isolates yielded no reliable identification with a score <1.7, and 30% (4/13) of the isolates were misidentified. (iv) Among *C. sporogenes*, 92.3% (12/13) of the isolates were identified to the species level with a score ≥2.0, and 100% (13/13) of the isolates were identified to the genus level with a score 1.7–2.0.

The identification of *Clostridium* spp. from our study using VITEK 2 is presented in Table 2. The results showed that of 144 *Clostridium* spp., VITEK 2 could identify 77.7% (112/144) correctly. The following *Clostridium* spp. were identified: *C. perfringens*, 80% (78/97), *C. subterminale*, 95% (20/21), *C. sordelli*, 38% (5/13) and *C. sporogenes*, 69% (9/13). Additionally, 12.5% (18/144) of the isolates were not identified, and 9.7% (14/144) were misidentified by VITEK 2.

By using the sequence analysis of 16S rRNA, all the isolates (144/144) were successfully identified, and 76% (110/144) of the *Clostridium* isolates were identified by 16S rRNA, MALDI–TOF-MS and VITEK 2 (Fig. 1). Individually, 81% (79/97) of *C. perfringens* were identified by all 3 methods, and 16.5% (16/97) were identified by MALDI–TOF-MS and 16S rRNA only. Among *C. sporogenes*, 69% (9/13) were identified by all of the methods,

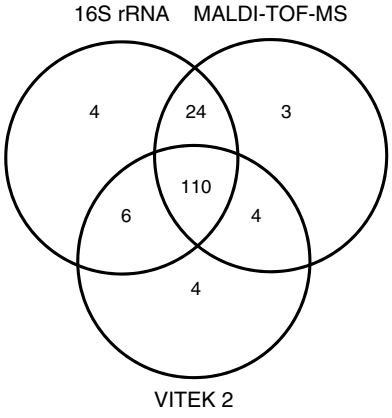

Fig. 1 – Summary of *Clostridium* spp. identified by Bruker MALDI–TOF-MS compared to VITEK 2 identification. The total number of isolates, *n* = 144.

and 30% (4/13) were identified by MALDI–TOF-MS and 16S rRNA only. A total of 30% (4/13) of the isolates of *C. sordelli* were identified by all 3 methods, 30% (4/13) were identified by MALDI–TOF-MS and 16S rRNA only, and 7.7% (1/13) were identified by 16S rRNA and VITEK 2 only. Of the strains, 81% (17/21) of the isolates of *C. subterminale* were identified by all 3 methods, and 29% (6/21) were identified by 16S rRNA and VITEK 2.

Discussion

The members of the genus *Clostridium* are found in soil, aquatic sediment, decaying plants, vertebrates and insects. Additionally, they comprise an essential part of the anaerobic flora of humans and are present in gastrointestinal and vaginal flora. The genus has been reported from other sources, such as paper currency and retail surfaces.[8,9] This research, which is aimed at the rapid and accurate identification of *Clostridium* species, is crucial because of the capability of these organisms to produce toxins. The toxins produced by *Clostridium* spp. comprise the major causes of the infections associated with the species, which may range from localized wound infections to systemic life threatening diseases. The infections caused by *Clostridium* spp. include gas gangrene, antibiotic associated colitis, neutropenic enterocolitis and neurological syndromes, such as tetanus and botulism.[1] From a treatment perspective, it is essential to identify particular bacteria in a routine microbiology laboratory. Bacterial identification is normally based on phenotypic tests, including morphology, Gram staining and a biochemical pattern. Although some of these tests are performed rapidly, complete identification is routinely achieved within hours to several days based on the nature of the micro-organism. Thus, these conventional, time-consuming procedures delay appropriate treatment of patients.

Anaerobic bacteria such as *Clostridium* spp. are complex in nature, and their isolation, culture and identification in a routine diagnostic microbiology laboratory are tedious and time consuming. The most reliable and accurate identification of anaerobes has been attributed to sequencing of the 16S rRNA gene. This technique has been shown to be highly proficient and is considered the “gold standard”. Regardless of the wide acceptability of 16S rRNA sequence identification of anaerobic bacteria, this technique remains inapplicable for most routine microbiology laboratories because it is too expensive and time-consuming for routine use. Several techniques have been employed for the purpose of correctly identifying or classifying anaerobic bacteria.[10] VITEK 2 and MALDI–TOF-MS are two of the automation methods now widely used in routine microbiology. Compared with the results of the traditional method, several studies have shown promising results in identifying anaerobic bacteria by VITEK 2 However, for *Clostridium* spp. identification, this system remains unsatisfactory. In our study, 78% of the *Clostridium* isolates were identified by VITEK 2. These results were slightly different from those previously published (44.4% and 64.3%) by Mory et al. and Blairon et al.[11,12] Identification of microbes by MALDI–TOF-MS has been in use for more than a decade and has been shown to be a rapid, less expensive and accurate method for identifying microbes,

including anaerobic bacteria.[12,13] In our study, MALDI–TOF-MS could correctly identify 89% (128/144) of the microbes to the species level and 92% (133/144) to the genus level. These results are consistent with those published by Schmitt et al.[14] In his study, comprising 179 anaerobic bacteria, 70.8% and 91.7% were identified by MALDI–TOF-MS to the species and genus level, respectively. His results further demonstrated that among the 179 anaerobic bacteria tested, 28 isolates of *Clostridium* species yielded results of 89% and 93% for identification to the species and genus level, respectively. Garner et al.,[15] reported a 96% identification rate by MALDI–TOF-MS at both the genus and the species levels of 108 *Clostridium* isolates. Several studies have compared MALDI–TOF-MS and VITEK 2 for the identification of anaerobic bacteria to demonstrate which technique is more suitable, and most of these studies clearly concluded that the MALDI–TOF-MS technique is rapid, reliable and more accurate.

Our results suggest that MALDI–TOF-MS performs better and is superior to VITEK 2 for the identification of *Clostridium* spp. and that it could be successfully incorporated into the routine identification of the clinical strains of *Clostridium* spp.

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Conflict of interest

None to declare.

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