Medical Microbiology

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

Mohammed Suliman AlMogbel*

Molecular Diagnostic and Personalized Therapeutics Unit, College of Applied Medical Sciences, University of Ha’il, Hail, Saudi Arabia

ARTICLE INFO

Article history:
Received 25 May 2015
Accepted 20 August 2015
Available online 2 March 2016
Associate Editor: Afonso Luís Barth

Keywords:
Clostridium species
MALDI–TOF-MS
VITEK 2
16S rRNA

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.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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
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 510bp of 16S rRNA gene was performed according to the previously described method, using the universal primers 27F (5’-AGAGTTTGTATCMTGGCTCAG-3’) and 519R (5’-GWATTACGGCGGCKGCTG-3’).\(^7\) The sequencing of the 510bp 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 level.

| 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. sordelli | 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 were misidentified. Among C. sordelli, 19% (4/21) of the isolates were misidentified. (iii) 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, 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. 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. 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. 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. 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.

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

**Funding**

This work was supported by the University of Ha’il.

**Conflict of interest**

None to declare.

**References**

1. Stevens DL, Aldape MJ, Bryant AE. Life-threatening clostridial infections. *Anaerobe*. 2012;18(2):254–259.
2. Keto-Timonen R, Heikinheimo A, Eerola E, Korkeala H. Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. *J Clin Microbiol*. 2006;44(11):4057–4065.
3. Ramlachan N, Anderson RC, Andrews K, Laban G, Nisbet DG. Characterization of an antibiotic resistant *Clostridium hathewayi* strain from a continuous-flow exclusion chemostat culture derived from the cecal contents of a feral pig. *Anaerobe*. 2007;13(3–4):153–160.
4. Alam SI, Dixit A, Tomar A, Singh L. Comparative genomic analysis of a neurotoxicogenic *Clostridium* species using partial genome sequence: phylogenetic analysis of a few conserved proteins involved in cellular processes and metabolism. *Anaerobe*. 2009;16(2):147–154.
5. La Scola B, Fournier PE, Raoult D. Burden of emerging anaerobes in the MALDI–TOF and 16S rRNA gene sequencing era. *Anaerobe*. 2011;17(3):106–112.
6. Chean R, Kotsanas D, Francis MJ, et al. Comparing the identification of Clostridium spp. by two matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry platforms to 16S rRNA PCR sequencing as a reference standard: a detailed analysis of age of culture and sample preparation. *Anaerobe*. 2014;30(12):85–89.
7. Gerhard H, Ulrich S, Giuseppe V, et al. Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. *Microbiology*. 2003;149(pt 1):67–75.
8. Mailafia S, Michael O, Kwaja E. Evaluation of microbial contaminants and. antibiogram of Nigerian Paper Currency Notes (Naira) circulation in Gwagwalada, Abuja, Nigeria. *Nigerian Vet J*. 2013;34(1):726–735.
9. Alqumber MA. *Clostridium difficile* in retail baskets, trolleys, conveyor belts, and plastic bags in Saudi Arabia. *Saudi Med J*. 2014;35(10):1274–1277.
10. Grosse-Herrenthey A, Maier T, Gessler F, et al. Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization–time of flight mass spectrometry (MALDI–TOF MS). *Anaerobe*. 2008;14(4):242–249.
11. Mory F, Alauzet C, Matuszewski C, Riegel F, Lozniewski A. Evaluation of the New Vitek 2 ANC card for identification of medically relevant anaerobic bacteria. *J Clin Microbiol*. 2009;47(6):1923–1926.
12. Blairon L, Mazza ML, Wybo I, Piéard D, Dediste A, Vandenberg O. Vitek 2 ANC card versus BI, crystal anaerobe and RapID ANA II for identification of clinical anaerobic bacteria. *Anaerobe*. 2010;16(4):355–361.
13. Nagy E, Maier T, Urban E, Terhes G, Kostrzewa M. Species identification of clinical isolates of Bacteroides by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *Clin Microbiol Infect*. 2012;15(8):796–802.
14. Schmitt BH, Cunningham SA, Dailey AL, Gustafson DR, Patel R. Identification of anaerobic bacteria by Bruker Biotyper matrix-assisted laser desorption ionization–time of flight mass spectrometry with on-plate formic acid preparation. *J Clin Microbiol*. 2013;51(3):782–786.
15. Garner O, Mochon A, Branda A, et al. Multi-centre evaluation of mass spectrometric identification of anaerobic bacteria using the VITEK MS system. *Clin Microbiol Infect*. 2014;20(4):335–339.