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Identification of clinically relevant *Corynebacterium* strains by Api Coryne, MALDI-TOF-mass spectrometry and molecular approaches

Identification des souches cliniques de corynébactéries par Api Coryne, spectrométrie de masse de type MALDI-TOF et les techniques moléculaires

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ABSTRACT

We evaluated the Bruker Biotyper matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) for the identification of 97 *Corynebacterium* clinical in comparison to identification strains by Api Coryne and MALDI-TOF-MS using *16S rRNA* gene and hypervariable region of *rpoB* genes sequencing as a reference method. *C. striatum* was the predominant species isolated followed by *C. amycolatum*. There was an agreement between Api Coryne strips and MALDI-TOF-MS identification in 88.65% of cases. MALDI-TOF-MS was unable to differentiate *C. aurimucosum* from *C. minutissimum* and *C. minutissimum* from *C. singulare* but reliably identify 92 of 97 (94.84%) strains. Two strains remained incompletely identified to the species level by MALDI-TOF-MS and molecular approaches. They belonged to *Cellulomonas* and *Pseudoclavibacter* genus. In conclusion, MALDI-TOF-MS is a rapid and reliable method for the identification of *Corynebacterium* species. However, some limits have been noted and have to be resolved by the application of molecular methods.

Mots clés :
Identification
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Api Coryne
SM-MALDI-TOF
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Séquençage
16S rRNA

RÉSUMÉ

Objet de l'étude. – Le but de notre travail est d'évaluer l'intérêt de la spectrométrie de masse MALDI-TOF pour l'identification d'espèces de *Corynebacterium* d'importance médicale, en comparant ses performances à celles du système biochimique Api Coryne et les approches moléculaires (séquençage des gènes *rpoB* et *ARNr 16S*)

Méthode. – Quatre-vingt-dix-sept souches cliniques appartenant au genre *Corynebacterium* isolées au CHU F. Hached, Sousse, Tunisie de différents prélèvements cliniques ont été étudiées à la fois en spectrométrie de masse (Microflex, Bruker Daltonics) et par les galeries Api Coryne. Lorsqu'il existait une discordance entre les identifications MALDI-TOF et Api Coryne, l'identification génotypique (*16 rRNA* et *rpoB*) était prise en compte et considérée comme référence.

Résultats. – Parmi les 97 isolats analysés, 88,65 % ont été identifiés de façon concordante par les systèmes Api Coryne et Microflex. Quatre-vingt-douze isolats, 94,84 % ont été correctement identifiés à l'espèce par spectrométrie de masse, mais ce système demeure incapable de différencier *C. aurimucosum* du *C. minutissimum* et du *C. singulare*. Enfin, deux souches, ni l'identification Microflex ni celle de l'Api Coryne n'était fiable et l'identification génétique de ces souches reste insuffisante suggérant deux nouvelles espèces.

Conclusion. – Les galeries miniaturisées présentent certaines limites et sont insuffisantes pour l'identification fiable des corynébactéries. Les résultats obtenus montrent que les performances de la spectrométrie de masse sont comparables à celle des techniques moléculaires de référence.

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1. Introduction

The genus *Corynebacterium* includes Gram-positive bacteria, facultative anaerobe with worldwide distribution [1]. This genus contains more than 100 species isolated mostly from animals or humans (http://www.dsmz.de/support/bacterial-nomenclature-up-to-date-downloads.html accessed February 18, 2014). *Corynebacterium* species are ubiquitous microorganism of the human skin and mucous membranes and, when isolated from various clinical materials, are traditionally considered as contaminants [2]. However, it has increasingly been recognized as opportunistic pathogen [3], and isolated as the etiological factor of various infections such as pneumonia [4] vertebral osteomyelitis [5] septicemia [6] and endocarditis [7]. The identification of *Corynebacterium* species is challenging and it is becoming increasingly difficult to distinguish *Corynebacterium* species based on their biochemical profiles [8] because it always needs many and diverse biochemical tests that are not available in the Api Coryne system [9]. Also, the underlying database is only infrequently updated and it does not provide enough discrimination among newly described species [10]. Manual analyses are time-consuming, and require large amounts of biological material, which can be a major disadvantage. Molecular methods, such as *rpoB* and *16S rRNA* gene sequencing, have been demonstrated to have complementary value, but they are not practical for routine use due to their high cost [11]. Recently, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF-MS) have been increasingly applied in clinical microbial identification [11]. It is a fast, cheap, and accurate approach [12,13]. Published studies reporting identification of *Corynebacterium* strains using MALDI-TOF-MS presented several limitations. Most of them have focused on toxigenic species *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* or did not include all species [11,14–16]. Nevertheless, despite this promising data were reported elsewhere, MALDI-TOF-MS had not been officially launched for clinical use in Tunisia. In the present study, we report the findings of a large scale evaluation of the Brucker Biotyper MS system and commercial methods for the identification of 97 *Corynebacterium* clinical strains isolated from various specimens belonging to 12 different species. To the best of our knowledge, no similar studies have been performed in developing countries including Tunisia.

2. Materials and methods

2.1. Bacterial strains

The 97 strains evaluated in this study were recovered over a period of five years (2007 to 2012) from clinical specimens isolated from in and out-patients and routinely submitted for culture to the microbiology laboratory of the Hospital Farhat Hached, Sousse, Tunisia.

2.2. Biochemical identification

In routine, in addition to colonies' morphology, Gram coloration and catalase production, identification was performed by Api Coryne V.2 strips (bioMérieux, France) as recommended by the manufacturer. Test for susceptibility to the vibriostatic agent O/129 (150 μg biorad, France) was done in junction to Api Coryne V.2 to differentiate *C. striatum* (sensible) from *C. amycolatum* (resistant). All strains were stored in Brain Heart Infusion broth supplemented with 20% of glycerol at −80 °C.

2.3. MALDI-TOF mass spectrometry

The 97 strains were sub-cultured on 5% horse blood agar (biorad, France), incubated aerobically for 24–48 h at 37 °C and analyzed by MALDI-TOF-MS using the following protocol: a small amount of a colony was transferred to a metallic MALDI-TOF MSP 96 plate (Bruker Daltonic GmbH) and then covered with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid HCCA, 50% acetronitrile, 2.5% trifluoroacetone) and allowed to visibly dry at room temperature. Each sample was spotted at least in duplicate to achieve proper identification and to verify reproducibility. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonic, Wissembourg, France) via the Flex Control software (version 3.0). The spectrum was imported into the BioTyper software (version 2.0; Bruker, Karlsruhe, Germany). Identification score criteria used were those recommended by the manufacturer: a score ≥ 2.000 indicated species-level identification, a score of 1.700–1.999 indicated identification to the genus level, and a score of 1.700 was interpreted as no identification. We required minimum difference of 10% between the top and next closest score for a different genus or species [15]. When that first attempt at identification by MS did not show level of confidence results, a small amount of the organism from a single colony was smeared directly onto a spot on the MALDI-TOF-MS steel plate and overlaying with 1 μl of 70% formic acid [16].

3. Molecular identification

Partial sequencing of the hypervariable region of the *rpoB* gene and *16S rRNA* gene was performed for all the strains. Bacterial DNA was first extracted using MagNA Pure Lc (Roche, France) according to the manufacturer recommendations. Polymerase chain reaction (PCR) was carried out in a final volume of 50 μl as described previously [1,17] using the primers C2700F and C3130R and universal oligonucleotides for the amplification of partial *rpoB* gene 16S rRNA gene respectively. PCR-amplified fragments sequencing on both stands was performed as previously described [18,19] by a 3500 D X Genetic Analyzer Applied Biosystem sequencer. Obtained sequences were aligned and interpreted via the program BLAST of National Center BioInformatic (NCBI http://blast.ncbi.nlm.nih.gov/) and the program BioInformatic Bacteria Identification (BIBI http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi). *rpoB* based identification to the species level required ≥ 96.6% sequence identity with 2% separation between species. *16S rRNA* gene based identification to the species level required ≥ 99% identity and ≥ 0.8% separation between species [12,17,18]. For *C. diphtheriae* strain, the *toxA* gene was detected by classical PCR, as previously described [9].

4. Results

4.1. Biochemical identification

Among 97 strains identified by Api Coryne V2.0, 86 (88.65%) belonged to *C. striatum/amycolatum* species (Table 1). These species can be differentiated according to their colonies' morphology. *C. striatum* colonies on Columbia agar base with 5% horse blood were non-hemolytic, creamy white to yellowish with an entire edge. However, *C. amycolatum* characteristically produces flat, dry, whitish-gray and matte colonies. Complementary test based on O/129 (150 μg, Biorad, France) susceptibility showed that among them, 14/86 (16.27%) were resistant and so belonged to *C. amycolatum*. Other species identified by Api Coryne were as follows: *Corynebacterium* CDC Group G ($n = 3$), *C. coylae/afermentans* ($n = 1$), *C. diphtheriae* ($n = 1$), *C. macginleyi* ($n = 1$), *C. jeikeium* ($n = 1$) and *C. pseudodiphtheriticum* ($n = 1$). The 3 remained strains presented unusual colonies' morphology. For the first strain, the culture was sterile after 24 hours of incubation at 37 °C on Columbia agar with 5% sheep blood (bioMérieux, France), Chocolate agar, PolyViteX agar (bioMérieux) and MacConkey agar (bioMérieux) at 37 °C in 5% CO_2. Two days later, under the same conditions described above culture yielded small and gray colonies on Columbia agar with 5% sheep blood (bioMérieux, France). Api Coryne inoculated twice gave *C. auris* profile. For the two other strains, grown bacterial colonies were pale, yellow and smooth on Columbia agar with 5% sheep blood (bioMérieux, France). These strains were identified by Api Coryne V.2 as *Cellulomonas/Microbacterium*.

4.2. MALDI-TOF-MS identification

MALDI-TOF-MS identified 74 out of 97 (76.28%) *Corynebacterium* strains to the species level by direct colony testing. Among the 23 remained strains (scores < 2.000), 16 reached the confident level after the application of formic acid (scores ≥ 2.000) (Table 1).