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Identification of clinically relevant *Corynebacterium* spp., *Arcanobacterium haemolyticum* and *Rhodococcus equi* by MALDI-TOF MS.

Running title: Gram-positive bacilli identified by MALDI-TOF MS.

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22 **ABSTRACT**

23 The identification of 83 *Corynebacterium* spp., 13 *Arcanobacterium haemolyticum* and 10
24 *Rhodococcus equi* by conventional methods (API Coryne complemented with 16SrRNA
25 sequence analysis) was compared with MALDI-TOF. The correlation between API and
26 MALDI-TOF was 89%. MALDI-TOF is a rapid and accurate system to identify the above
27 mentioned microorganisms.

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Corynebacteria are widespread in nature. Pathogenic *Corynebacterium* include *Corynebacterium diphtheriae* and non-diphtheroid *Corynebacterium*. The non-diphtheroid *Corynebacterium* are found in the mucosa and normal skin flora of humans and animals. Some species as *Corynebacterium amycolatum, Corynebacterium jeikeium*, *Corynebacterium striatum*, *Corynebacterium urealyiticum* and *Corynebacterim xerosis*, are relevant human pathogens, mainly in immuno-compromised patients (4). The API Coryne™V2.0 system (Biomerieux, Marcy-L'Etoile, France), complemented with conventional phenotypic test, is most commonly used in routine laboratories for identification of these microorganisms. However, this method is time-consuming and not always giving reliable identification at the species level (2). Identification by means of 16S rRNA sequencing is more specific but also slow, and it is expensive.

Arcanobacterium haemolyticum is an obligate parasite of the pharynx of humans; sporadically, it causes pharyngeal or skin lesions (11). *Rhodococcus equi* is the most important pathogenic species of the genus *Rhodococcus*, causing several infections such as necrotizing pneumonia and enteritis, mainly in immunocompromised patients such HIV-positive patients (14). *A. haemolyticum* and *R. equi* are also routinely identified with the API Coryne™V2.0 system. In the case of *R. equi*, reliable identification frequently requires confirmation by molecular methods including PCR and DNA sequencing (7).

During the last years, Matrix-assisted laser desorption/ionisation time-of- flight mass spectrometry (MALDI-TOF MS) has been increasingly applied in clinical microbial diagnostics for species identification of bacterial and fungal pathogens (13). MALDI-TOF MS identified successfully at species level a group of 119 *Corynebacterium* spp. clinical isolates including 78 *C. diphtheriae* and 31 non-diphtheroid corynebacteria (6). In a recent report (3) MALDI-TOF discriminated *Corynebacterium aurimucosum* from *Corynebacterium minutissimum*, two closely-related *Corynebacterium* species previously considered as difficult

to differentiate (5).

The aim of this study was to assert if MALDI-TOF MS can be used as a routine method for a fast and reliable identification of *Corynebacterium* spp. clinical isolates at the species level in our laboratories. Seventy-three clinical strains isolated at the Clinical Microbiology Laboratory, Hospital Universitario Marqués de Valdecilla, Santander, Spain, were initially identified by API Coryne^{TM}V2.0 and other conventional phenotypic methods (4) as *C. jeikeium* (18), *Corynebacterium pseudodiphthericum* (16), *C. striatum* (15), *C. amycolatum* (12), *C. urealyticum* (9), *Corynebacterium glucuronolyticum* (3), *Rhodococcus equi* (11) and *Arcanobacterium haemolyticum* (13). The strains *C. striatum* ATCC 6940 and *C. urealyticum* ATCC 43042 were also included in the study. Toxin producing species as Corynebacterium diphtheriae were not added in this study because of lack of samples in this concrete collection. The 75 strains were analyzed by MALDI-TOF MS through the following procedure: a small amount of a colony growth on blood agar was transferred to a metallic MALDI-TOF MSP 96 plate (BrukerDaltonikGmbH, Bremen, Germany) and thereafter covered with 1 µL of matrix (saturated alpha-cian-4-hydroxycinamic acid in 50 % of acetonitril and 2.5 % of trifluoroacetic acid The plate was then left to dry at room temperature for 5 minutes). When first attempt of identification by MS did not show results with a high level of confidence, samples were identified using formic acid/acetonitrile extraction. The reference strain *Escherichia coli* K12 (genotype GM48) was used as a standard for calibration and as reference for quality control. Measurements were performed by a Microflex II mass spectrometer (Bruker Daltonik) equipped with a 60 Hz laser. The proteic spectra obtained with the spectrometer were processed with Bruker MALDI Biotyper v2.0 software (BrukerDaltonik GmbH) and compared with the reference spectra present in the database, showing the 10 most similar patterns for each isolate. The analogy is demonstrated as a score (≥ 2: identification at species level, 1.7-1.999: identification at genus level and < 1.7: no

reliable identification). Discrepancies between API Coryne™V2.0 and MALDI-TOF MS identification were resolved by sequencing partially the 16S rRNA gene.

All the isolates identified as *C. amycolatum, C. glucuronolyticum, C. striatum, C. urealyticum* and *A. haemolyticum* by API Coryne™V2.0 were also identified by MALDI-TOF MS as such, scoring ≥ 2 (Table 1). One out of 19 strains identified as *C. jeikeium* by API Coryne™V2.0 was identified as *C. amycolatum* by MALDI-TOF MS. 16S rRNA sequencing assigned this isolate to *C. amycolatum. C. jeikeium* is recognized as one of the *Corynebacterium* species most frequently associated with human infectious diseases (8), causing sepsis, endocarditis, pneumonia and other infections, while *C. amycolatum* is considered an emergent pathogen (9). From 7 strains identified as *C. minutissimum* by API Coryne™V2.0, 3 were also identified by MALDI-TOF MS, 3 were assigned to *C. aurimucosum* and one was assigned to *C. amycolatum,* (Table 1). 16S rRNA sequence confirmed that indeed theses isolates belonged to the species identified by MALDI-TOF. However, when the *rpoB* gene was sequenced the results obtained by MALDI-TOF were confirmed with the exception of two *C. minutissimum* strains which were identified as *C. aurimucosum*, therefore further investigation is needed to evaluate the use of MALDI-TOF MS to identify this species. It has been previously reported that API Coryne™V2.0 did not discriminate among these three species because their biochemical profiles are very similar, needing complementary tests for a reliable identification (12). Discrepancies between API Coryne™V2.0 and MALDI-TOF MS were also found in 5 of the 16 strains identified as *C. pseudodiphthericum* by API Coryne™V2.0, which were given to *C. propinquum* by MALDI-TOF MS. 16S rRNA sequencing confirmed these 5 isolates as true *C. propinquum*. Indeed there was also a total correlation between the identification by MALDI-TOF MS and sequencing of the *rpoB* gene. *C. pseudodiphthericum* and *C. propinquum* are part of the oropharyngeal microbiota. They have been reported as a cause of pneumonia and

endocarditis. Both species are phenotypically analogous but most of the *C. pseudodiphthericum* produce urease whereas the *C. propinquum* are urease-negative.

From 11 isolates identified presumptively as *R. equi* by API Coryne^{TM}V2.0, MALDI-TOF MS identified all except one, which was identied as *Dietzia maris*. 16S rDNA sequencing identified this isolate as *D. maris*. We have recently reported that 8 of 15 clinical isolates presumptively identified by API Coryne as *R.equi* at Hospital Marqués de Valdecilla, Santander, belonged in fact to the genus *Dietzia* (10).

Since most of the non-diphtheroid *Corynebacterium*, *A. haemolyticum,* and *R. equi,* are actually well-recognized human pathogens, their reliable identification at the species level is necessary. API Coryne^{TM}V2.0 is, at the present time, the most common method to identify these bacteria in routine microbiological laboratories, but its trustworthiness at the species level is limited. Moreover, it takes at least 16 hours after isolation of suspicious colonies from screening plates. On the contrary, MALDI-TOF MS analysis is much faster, rendering species identification of one isolate in less than 10 minutes. This study demonstrates that MALDI-TOF MS is a rapid and consistent system to routinely identify at the species level clinical isolates belonging to the abovementioned microorganisms.

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172 **Table. Comparative results between the identification methods used in this study.**

Microorganism	Number of strains	API Coryne V2.0	MALDI-TOF	16S rRNA
Corynebacterium amycolatum	12	12	12	--
Corynebacterium glucuronolyticum	3	3	3	--
Corynebacterium jeikeium	19	19	18 *C. jeikeium* 1 *C. amycolatum*	-- 1 *C. amycolatum*
Corynebacterium minutissimum	7	7	3 *C. minutissimum** 3 *C. aurimucosum* 1 *C. amycolatum*	-- 3 *C. aurimucosum* 1 *C. amycolatum*
Corynebacterium pseudodiphtheriticum	16	16	11 *C. pseudodiphtheriticum*** 5 *C. propinquum**	-- 5 *C. propinquum*
Corynebacterium striatum	16	16	16	--
Corynebacterium urealyticum	10	10	10	--
Rhodococcus equi	11	11	10 *R. equi* 1 *Dietzia maris*	-- 1 *D. maris*
Arcanobacterium haemolyticum	13	13	13	--

173 * Two out of these three strains were identified as *C. aurimucosum* by sequencing the *rpoB*
174 gene.
175 ** All *C. pseudodiphtheriticum* and *C. propinquum* identified by MALDI-TOF MS were
176 confirmed by sequencing the *rpoB* gene.