Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry as a tool for rapid diagnosis of potentially toxigenic *Corynebacterium* species in the laboratory management of diphtheria-associated bacteria

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The rapid identification of the potentially toxigenic *Corynebacterium* species, *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* is essential for diagnosis and treatment of diphtheria and diphtheria-like diseases. We used matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) in comparison with classical microbiological and molecular methods on 116 *Corynebacterium* strains. All 90 potentially toxigenic *Corynebacterium* strains collected by the German National Consiliary Laboratory on Diphtheria in a period of more than ten years were correctly identified by MALDI-TOF MS. We propose an algorithm for fast and reliable diagnosis of diphtheria incorporating MALDI-TOF MS, real-time *tox* PCR and Elek testing.

Introduction

Diphtheria is a potentially fatal disease caused by toxigenic strains of the three *Corynebacterium* species, *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* harbouring lysogenic beta-corynephages bearing the *tox* gene. In parallel with the vaccination-related decrease in diphtheria incidence worldwide in the second half of the 20th century, laboratory skills in the diagnosis of diphtheria have declined in many parts of the world. This holds true not only for routine microbiological laboratories, but even for diphtheria reference centres as revealed by an international external quality assessment organised by the European Diphtheria Surveillance Network (DIPNET) [1] with support of the European Commission [2]. Diphtheria incidence has fallen in most European countries after the 1990s diphtheria epidemic in the Newly Independent States of the Former Soviet Union. However, according to data from the World Health Organization (WHO), a country located in the European Union, Latvia, had the highest diphtheria incidence globally in 2008; in 2009, Latvia had the third highest diphtheria incidence worldwide with 0.27 per 100,000 population, which was the highest incidence in the WHO European region [3,4].

The genus *Corynebacterium* contains clinically relevant species including those causing diphtheria as well as opportunistic commensals. Identification of suspected isolates usually relies on phenotypic methods such as biochemical reactions, and molecular techniques including PCR and sequencing. The *tox* genes are detected by classical or real-time PCR [5,6] and diphtheria toxin production is usually detected by Elek test in specialised laboratories [7]. Species identification by molecular methods, for example *rpoB* gene sequencing [8] is time-consuming and requires trained staff. However, for supporting clinical decisions a fast and accurate species differentiation is needed to distinguish potentially toxigenic *Corynebacterium* spp. from harmless species or opportunistic pathogens.

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a new technology for species identification based on the protein composition of microbial cells. Although first descriptions of this method were published more than ten years ago [9,10], a wider use in routine laboratories became possible only recently when successful species identification for different genera was shown [11-14]. The most prominent advantages are its speed and its low running costs provided that a quality-controlled

database of reference spectra including all relevant microorganisms is available.

Here we describe the evaluation of MALDI-TOF MS for the identification of potentially toxigenic *Corynebacterium* spp. Based on these results, we propose an algorithm incorporating MALDI-TOF MS, real-time *tox* PCR and Elek testing into the rapid and resource-effective laboratory diagnosis of diphtheria.

Materials and methods

We recovered 116 *Corynebacterium* isolates from routine examination of human and veterinary clinical specimen submitted to the German Consiliary Laboratory for Diphtheria between 1997 and 2010. The isolates were cultured after aerobic or microaerophilic incubation at 35 °C on sheep blood agar (Oxoid) and Hoyle´s Tellurite agar (BD Diagnostics). After Gram staining and determination of catalase activity, the isolates were identified by API Coryne (bioMerieux).

For MALDI-TOF MS, single colonies of a subculture were taken and subjected to a short protein extraction protocol with ethanol and formic acid according to the Bruker Daltonics protocol. 1 µl of the supernatant was transferred onto the target plate in duplicate for drying at room temperature. The samples were overlaid with 1 µl of saturated α-cyano-4-hydroxycinnamic acid (HCCA) solution in 50% acetonitrile/2.5% trifluor acetic acid (v/v) as MALDI matrix (Bruker Daltonics). Measurements were performed on a Microflex LT mass spectrometer (Bruker Daltonics) with a standard pattern matching algorithm (BioTyper 2.0 Software). Resulting log(score) values above 2.0 are required for reliable identification on species level and values between 1.7 and ›2.0 for genus level. Log(score) values below 1.7 cannot be rated as valid according to the manufacturer's instructions.

RpoB gene sequencing was done as described before, with more than 95% similarity considered as cut-off for reliable species identification [15]. Toxigenicity was tested both by real-time PCR [6] and Elek test.

Results

MALDI-TOF MS was performed on 116 *Corynebacterium* spp. strains (Table). The reference database provided by Bruker (n=3,287) contained 138 single reference spectra of 71 *Corynebacterium* species including all relevant human pathogenic and most opportunistic species with one or more spectra. For each of the three potentially toxigenic species *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans*, four reference spectra of different strains were included, among them reference strains DSM44123T, DSM44287T, DSM46628 and DSM46325T from the German Collection of Microorganisms and Cell Cultures (DSMZ).

Ninety tested isolates were *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, and all of them were correctly identified to the species level by MALDI-TOF MS and the API Coryne system. Of those, toxigenic (n=8) and non-toxigenic (n=82) strains yielded comparable log(scores). MALDI technology and *rpoB* gene sequencing [8] showed identical results for 115 (99,1%) of all 116 tested *Corynebacterium* strains (Table). The log(score) ranged between 2.0 and 2.5 indicating a reliable species identification. Only one isolate determined as *C. tuberculostearicum* by *rpoB* sequencing yielded a lower log(score) of 1.8 – albeit also for *C. tuberculostearicum* - by MALDI-TOF. Therefore, MALDI-TOF could only identify this isolate to the genus level; the same was the case with API Coryne.

In 104 of 116 strains (88.8%), biochemical identification by API Coryne yielded identical results as *rpoB* gene sequencing, including in all potentially toxigenic *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* strains. Twelve strains showed unreliable or ambiguous API results and were therefore only identified to genus level (*Corynebacterium* spp.) (Table). These isolates were concordantly identified by *rpoB* sequencing and MALDI-TOF MS except for the single isolate of *C. tuberculostearicum*.

In conclusion, 99.1% of the tested Corynebacteria were correctly identified by MALDI-TOF MS when compared to *rpoB* gene sequencing. Moreover, both the positive and negative predictive values for identification of potentially toxigenic *Corynebacterium* species were 100% with MALDI-TOF.

Discussion and conclusions

For laboratory-confirmed diagnosis of diphtheria a fast and reliable identification of putative pathogenic Corynebacteria isolates is crucial. So far, this has been done biochemically, e.g. with API Coryne, which takes at least 16 hours after isolation of suspicious colonies from screening plates (typical growth, positive catalase reaction, Gram-positive coryneform rods), and may often yield unclear results. This might be mainly due to the fact that the current apiweb reference database (version V3.0) provides defined API codes for most clinically relevant human-pathogenic species (including 18 *Corynebacterium* species, three *Corynebacterium* groups (group F-1, group G and renale group) and 25 additional species of Gram-positive rods, whereas species rarely isolated from clinical specimens such as *C. ammoniagenes*, *C. camporealensis*, *C. casei*, *C. confusum* and *C. xerosis* are not yet included. Therefore, *rpoB* gene sequencing seems to be more reliable and comprehensive, but it can take up to several days until a result is available. However, API Coryne correctly identified all 90 isolates of the three potentially toxigenic *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*. The same was true for *rpoB* gene sequencing and MALDI-TOF. In contrast to the two other methods, MALDI-TOF MS analysis is much faster, allowing species identification of one isolate within 15 minutes.

The four *C. diphtheriae* biovars are usually differentiated by classical microbiology. A differentiation of

Table

Identification results of *Corynebacterium* strains, Germany, 1997–2010 (n=116)

rpoB gene sequencing (500 bp fragment)	Number of isolates	API Coryne [a]	Number of isolates	MALDI-TOF MS with log(score) in brackets	Number of isolates
Brevibacterium stationis[b]	1	*Corynebacterium* sp. (1000324)	1	*Brevibacterium stationis* (2.3)	1
C. accolens	2	*C. accolens*	2	*C. accolens* (2.1 each)	2
C. ammoniagenes	1	*Corynebacterium sp.* (1001304)	1	*C. ammoniagenes* (2.5)	1
C. amycolatum	3	*Corynebacterium* sp (300324, 2100324, 1000324)	3	*C. amycolatum* (2.2 each)	3
C. camporealensis	1	*Corynebacterium* sp. (3000104)	1	*C. camporealensis* (2.0)	1
C. casei	1	*Corynebacterium* sp. (3000325)	1	*C. casei* (2.0)	1
C. confusum	1	*Corynebacterium* sp. (3100304)	1	*C. confusum* (2.1)	1
C. coyleae	1	*Corynebacterium* sp (2000304)	1	*C. coyleae* (2.1)	1
C. diphtheriae[c]	78	*C. diphtheriae*	78	*C. diphtheriae* (2.4 ± 0.1)	78
C. macginleyi	1	*C. macginleyi*	1	*C. macginleyi* (2.2 ± 0.1)	1
C. propinquum	2	*C. propinquum*	2	*C. propinquum* (2.2 each)	2
C. pseudodiphtheriticum	5	*C. pseudodiphtheriticum*	5	*C. pseudodiphtheriticum* (2.3 ± 0.1)	5
C. pseudotuberculosis	4	*C. pseudotuberculosis*	4	*C. pseudotuberculosis* (2.3± 0.1)	4
C. striatum	3	*C. striatum* 2 *Corynebacterium* sp.(2110325) 1	3	*C. striatum* (2.4 ± 0.2)	3
C. tuberculostearicum	1	*Corynebacterium* sp. (0100105)	1	*Corynebacterium* sp. (1.8)	1
C. ulcerans	8	*C. ulcerans*	8	*C. ulcerans* (2.4 ± 0.1)	8
C. urealyticum	2	*C. urealyticum*	2	*C. urealyticum* (2.4 ± 0.1)	2
C. xerosis	1	*Corynebacterium* sp. (3110325)	1	*C. xerosis* (2.5)	1

[a] API codes for ambiguous or unreliable species identification are put in brackets.

[b] *B. stationis* was recently proposed to be assigned to the genus *Corynebacterium* [16].

[c] Including reference strains *C. diphtheriae* biovar *belfanti* (NCTC 10356), *C. diphtheriae* biovar *gravis* (NCTC 3984, NCTC 10648), *C. diphtheriae* biovar *intermedius* (ATCC 51280, ATCC 51279); *C. pseudotuberculosis* (DSM 20689, DSM 7180), *C. pseudodiphtheriticum* ATCC 10700 and clinical isolates of *C. diphtheriae* (2 isolates), *C. diphtheriae* biovar *belfanti* (14 isolates), *C. diphtheriae* biovar *gravis* (26 isolates), *C. diphtheriae* biovar *intermedius* (2 isolates), *C. diphtheriae* biovar *mitis* (34 isolates).

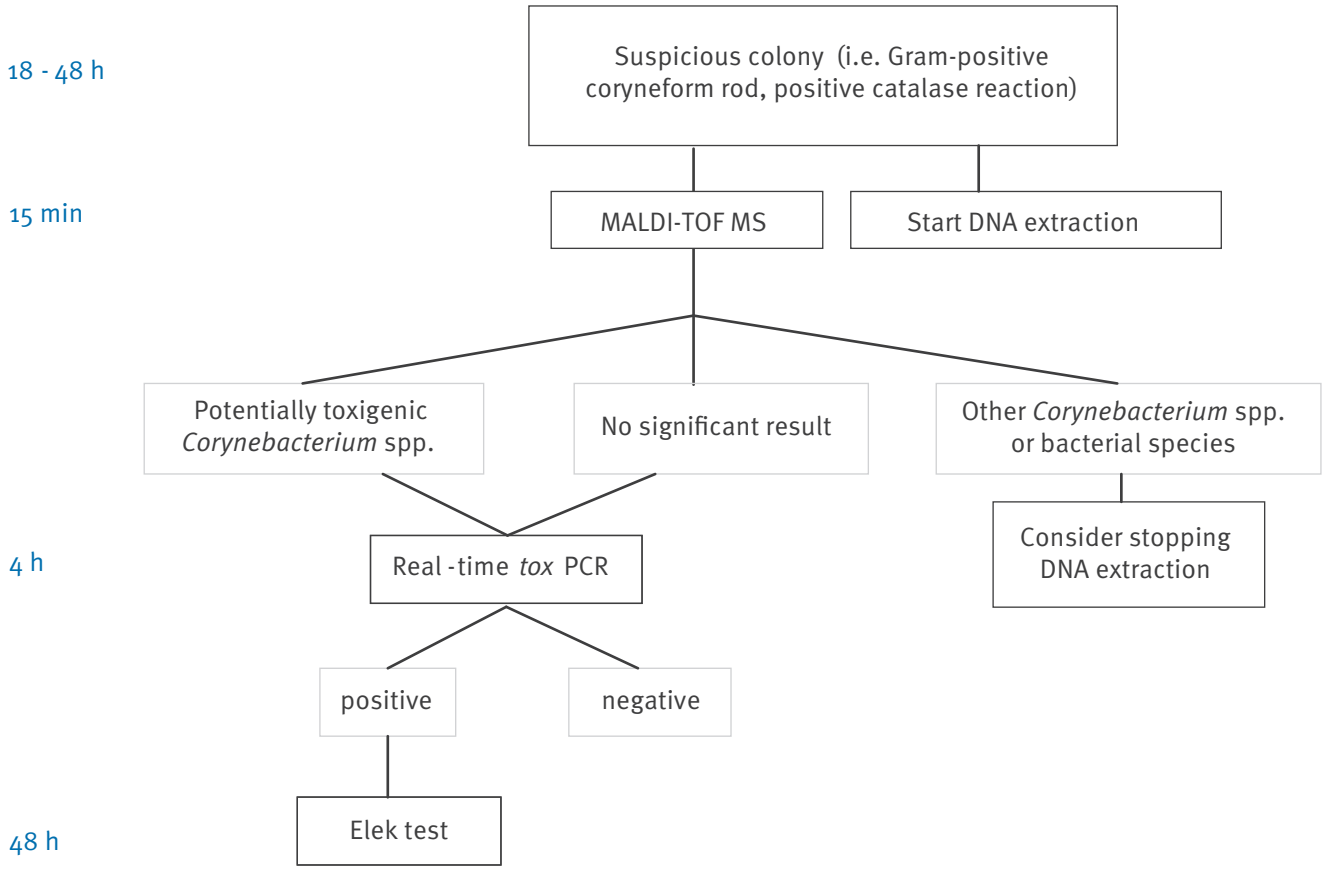
biovars by MALDI-TOF MS seems currently not to be reliable, since the available database of reference spectra and the routine analysis tools contain so far only one single strain for each biovar. Therefore, the generation of a database comprising several reference spectra for each of the four biovars is needed. Cluster analysis of these spectra and possibly modified algorithms will show whether it might be possible to distinguish those closely related biovars within the *C. diphtheriae* species. More important than rapid determination of the biovar is testing isolates of the three potentially toxigenic Corynbacterium spp. for toxigenicity. However, the diphtheria toxin is much larger than the ribosomal proteins analysed for species identification by the Microflex LT mass spectrometer and therefore cannot be directly measured in the current system. As MALDI-TOF MS is now being applied for the differentiation of bacterial strains [17] studies trying to differentiate toxigenic from non-toxigenic by MALDI-TOF MS may be warranted.

In conclusion, species identification of potentially toxigenic *Corynebacterium* spp. colonies can be accomplished by MALDI-TOF MS within 15 minutes. In this scenario, MALDI-TOF MS technology could be used as a rapid screening method helping to decide whether suspicious colonies should be analysed for the presence of the *tox* gene by real-time PCR [6]. Since diphtheria antitoxin for diagnostic purposes is increasingly difficult to obtain in many countries worldwide [18], it might be both reasonable and feasible to test only *tox* PCR-positive strains for diphtheria toxin production by Elek test. In any case, due to the existence of *tox*-positive, non-toxigenic strains it is important to perform the Elek test on all *tox*-positive strains [19]. A proposal for the rapid and cost-effective identification of toxigenic *Corynebacterium* isolates incorporating MALDI-TOF MS and real-time *tox* PCR is depicted in the Figure.

To our knowledge, our study is the first one in the rapidly evolving field of MALDI-TOF-based bacterial identification [14] which evaluates the use of this new technology on potentially toxigenic corynebacteria. Nevertheless it is of pivotal importance to further develop and maintain the bacterial database with a focus on other diphtheroid bacteria. Since accurate and fast diphtheria laboratory diagnosis is not only a matter of acute patient management, but also an important issue in public health due to international notification and management requirements, there is an urgent need for a reliable, robust and fast laboratory method for diagnosing toxigenic diphtheria-causing corynebacteria, especially in the light of the continuing loss of laboratory expertise even in national reference laboratories for diphtheria [2].

Figure

Proposed algorithm for rapid identification of toxigenic *Corynebacterium* spp.



*Erratum: The affiliation of S Schubert was corrected on 3 November 2010.

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