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Taxonomy/systematics

Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry (MALDI–TOF MS)

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ABSTRACT

Diverse techniques were applied to effect the identification and classification of isolated clostridial strains. Nevertheless, the correct identification of clostridial strains remains a laborious, time-consuming task which entails a not inconsiderable degree of expertise. In addition to this, traditional methods based on the metabolic properties of the bacteria require rigorously standardized media and growth conditions to assure the attainment of reproducible results. Although DNA-based methods, like the PCR of a species specific gene, are known to yield precise and reproducible results, their degree of effectivity is circumscribed by the fact that even the incidence of a toxin encoding gene is not necessarily linked to nor consequently indicative of the presence of an infectious disease. Moreover, most of these methods postulate an initial assumption concerning the expected bacterial species involved before the choice of PCR primer for use can be made. Consequently, the scope of these methods is restricted to that of targeted analyses. The 16S rDNA sequencing which is assumed to be the gold standard for bacterial classification having the unequivocal advantage of being capable of determining even uncultivable bacteria is nonetheless a time-consuming and costly technique. In the present study we describe the utilization of matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry (MALDI-TOF MS) for whole cell fingerprinting in combination with a dedicated bioinformatic software tool to distinguish between various clostridial species. Total 64 clostridial strains of 31 different species each displayed a mass spectrum unique to the strain involved, to the effect that it was also possible not only to differentiate between the strains examined, but also to establish to which species the individual strains belonged to. Starting with a single colony it was possible to correctly identify a *Clostridium* species within minutes. It was even possible to identify species which are normally difficult to differentiate by traditional methods, such as *C. chauvoei* and *C. septicum*. With the results obtained we were able to assemble a dendrogram of the *Clostridium* species which showed considerable similarities to dendrograms based upon 16S rDNA sequencing data. To conclude, our findings indicate that, inasmuch as the MALDI–TOF MS technology employed is based on a high-quality reference database, it may serve as an effective tool for the swift and reliable identification and classification of *Clostridia*.

1. Introduction

The genus *Clostridium* was described for the first time by Prazmowski in 1880. In the present day it is taxonomically classified under the phylum Firmicutes, class Clostridia, order Clostridiales, the family Clostridiaceae. Due to the fact that only four criteria (Gram-positive stain, spore-forming nature, anaerobic growth, and the absence of the dissimilatoric reduction of sulfate) are used to classify a bacterium as a member of the genus

Abbreviations: BoNT, botulinum neurotoxin; MALDI–TOF MS, matrix-assisted laser desorption and ionization–time-of-flight mass spectrometry; TFA, trifluoroacetic acid; AN, acetonitrile; CHCA, α-cyano-hydroxy-cinnaminic acid; *m/z*, mass-to-charge ratio

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Clostridium, this genus currently includes well over 150 species. One of these, the neurotoxin-producing *C. botulinum*, has become the object of widespread interest not only for being the source of one of the most toxic of bacterial toxins, but also for reason of its clinical benefits. There are also several other well-known species of the genus *Clostridium*, which are pathogenic to animals and humans and responsible for severe, and often fatal illnesses [1], but the genus *Clostridium* also includes a number of organisms with enormous potential for use in biotechnological processes and medical treatments [2]. Although *Clostridia* form a very heterologous group, the task of differentiating between a number of very closely related species is sometimes difficult. Therefore, the identification of an isolated strain is a time-consuming process, which sometimes requires expensive micro-molecular techniques or even inoculation tests in animals to determine the pathogenicity of the organism or the toxicity of the toxins involved.

In the past, several techniques were employed for the purpose of correctly identifying or classifying isolated clostridial strains. Holdeman et al. [3] wrote a helpful manual for the identification of clostridial strains based on their biochemical and gas-chromatographical properties. Nevertheless, the correct identification of clostridial strains on the basis of these properties is still a time-consuming process which is not easily manageable for the inexperienced user. An additional disadvantage of the task of analysing the metabolic products of *Clostridia* with the aid of gas chromatography is the necessity of rigorously standardized media and growth conditions to obtain reproducible results. In addition to this, a number of clostridial species are represented only by a single strain. For this reason, identification based on biochemical methods may be rendered inaccurate by the natural intraspecific variation occurring in metabolic processes within a range of various strains within one and the same species. On the other hand, *Clostridia* of medical significance, such as, *C. baratii* or *C. absonum*, can be phenotpically distinguished alone on the basis of a few metabolic features or even only on the basis of the toxins produced. Polymerase chain reaction (PCR) is a widely used technique for the identification of *Clostridia* in clinical samples. For PCR-based methods even the isolation or cultivation of strains is no longer necessary due to the fact that genes encoding pathogenic factors can be identified in nearly every clinical specimen. A number of PCR procedures have been developed which have subsequently found widespread use in diagnostics, such as, for instance, the ones employed in the detection of the botulinum neurotoxin (BoNT)-forming gene of toxigenic *Clostridia* like *C. botulinum*. Other PCR procedures are used to determine the toxin type of *C. perfringens*. There is, however, a disadvantage of these techniques. The incidence of a gene is not inevitably indicative of the presence of a disease. Moreover, many types of genes simply are not expressed. In yet many other cases, though the presence of *C. perfringens* can be established in faeces, it will not have caused a disease in the organism from which the faeces originated. A further disadvantage of techniques operating exclusively on principles of molecular biology is the fact that to effect a proper selection of the primer to be employed, one must have prior knowledge of the causative disease. This entails prior testing of the microbes in question using macromorphological and micromorphological (Gram's method) techniques to determine which pathogen is actually present and to subsequently determine which biochemistry-based method is best suited. The aforementioned procedure is rendered unnecessary by the MALDI–TOF MS technique.

Nowadays, the comparison of 16S rDNA sequence homology is the most widely accepted technique for the classification of bacteria. It provides information about relationships between species and strains on a genetic basis. It is generally assumed that a divergence of more than 3% in sequence homology results in the formation of a new species [4]. In contrast, Stackebrandt and Hippe [5] found up to a 99.3% similarity between *C. haemolyticum* and *C. botulinum*-producing neurotoxin types C and D. They also reported a 98.5% sequence similarity within a group of clostridial species including *C. septicum* and *C. chauvoei*, which are of major medical and veterinary importance. It is especially in the field of veterinary medicine that the latter two species have to be carefully differentiated from one another because *C. chauvoei* is the causative agent of malignant edema or black quarter disease, a disease listed by the Office International des Epizooties (OIE).

The objective of the present study is to establish whether MALDI-TOF MS fingerprinting, when combined with a dedicated bioinformatic tool, can be used to distinguish between various clostridial species and serve as an effective tool for the swift and reliable identification of clostridial species.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Every strain (see Table 1) was cultured anaerobically for 36 h at a temperature of 37 °C on a substrate of Columbia Blood Agar (bioMerieux) which was subjected to a 16 h-period of prior incubation under anaerobic conditions. For this purpose we used unsupplemented agar because all the clostridial strains used displayed a satisfactory rate of growth when cultured on such agar plates. Microbes of the same strain were cultivated repeatedly and examined in order to attain a wider range of measurements. Various types of agar (peptone yeast extract glucose agar with 5% horseblood [3], reinforced clostridial medium [Sifin, Germany] supplemented with 1.5% Agar and 5% horseblood) were used to establish whether the type of agar involved had an effect on the mass spectra displayed by the individual strains when tested later with the MALDI–TOF MS technique.

To trigger varying degrees of sporulation, cultures were started (following the procedure described above) on Columbia blood agar and harvested after periods of anaerobic incubation of 64 and 96 h, respectively. The degree of sporulation attained was then examined with the help of a light-optical microscope and rated with a score (+, ++, +++).

2.2. General sample preparation

To effect the inactivation of vegetative cells and spores, about 10 mg of cell material of the cultured strain involved was collected and put into 50 μl of an 80% solution of trifluoroacetic acid (TFA). The cells were carefully suspended and left for 5 min in this 80% TFA solution to ensure complete inactivation. Inactivation efficiency was tested by means of culturing the TFA supernatants over a period of several days in the course of a preliminary test involving spore-forming species of *Clostridia*. After suspending and inactivation, 150 μl of water was added to the suspension, which was then mixed. A further 200 μl of acetonitrile (AN) was subsequently added and mixed in similar manner, and the suspension centrifuged for 2 min at 6500*g*. An amount of 1 μl clear supernatant was transferred to the MALDI target and allowed to dry. Five MALDI target positions were then prepared in parallel. This procedure was repeated twice to minimize the effect of variations arising in the process of sample preparation.

Table 1
Clostridial reference strains used in this study

	Lab. no.	Strain	Origin	Designation
1	1001	*C. absonum*	NCTC[a]	10 984
2	1011	*C. butyricum*	DSM[b]	552
3	1018	*C. paraperfringens*	NCTC	10 986
4	1020	*C. difficile*	NCTC	11 206
5	1021	*C. clostridioforme*	NCTC	11 224
6	1023	*C. chauvoei*	NCTC	8070
7	1024	*C. chauvoei*	NCTC	8596
8	1025	*C. novyi* A	NCTC	538
9	1026	*C.septicum*	NCTC	547
10	1027	*C. bifermentans*	NCTC	1341
11	1028	*C. botulinum* A	NCTC	7272
12	1029	*C. botulinum* B	NCTC	7273
13	1030	*C. botulinum* C	NCTC	8264
14	1031	*C. botulinum* D	NCTC	8265
15	1032	*C. botulinum* E	NCTC	8266
16	1033	*C. botulinum* F	NCTC	10 281
17	1035	*C. coccoides*	NCTC	11 035
18	1036	*C. histolyticum*	NCTC	503
19	1037	*C. perfringens* A	NCTC	8237
20	1038	*C. perfringens* B	NCTC	4964
21	1041	*C. perfringens* C	NCTC	10 720
22	1046	*C. sphenoides*	NCTC	507
23	1047	*C. spiroforme*	NCTC	11 211
24	1048	*C. tertium*	NCTC	541
25	1049	*C. tetani* type 1	NCTC	279
26	1050	*C. tetanomorphum*	NCTC	2909
27	1051	*C. villosum*	NCTC	11 220
28	1069	*C. haemolyticum*	ATCC[c]	9650
29	1070	*C. sordellii*	ATCC	9714
30	1072	*C. beijerinkii*	ATCC	25 752
31	1073	*C. botulinum* G	ATCC	27 322
32	1074	*C. cadaveris*	ATCC	25 783
33	1076	*C. chauvoei*	ATCC	10 092
34	1077	*C. cochlearium*	ATCC	17 787
35	1078	*C. hastiforme*	ATCC	33 268
36	1079	*C. inocuum*	ATCC	14 501
37	1080	*C. lentoputrescens*	ATCC	17 794
38	1082	*C. novyi*	ATCC	17 861
39	1083	*C. paraputrificum*	ATCC	17 796
40	1084	*C. baratii*	ATCC	25 782
41	1087	*C. sporogenes*	ATCC	3584
42	1089	*C. tetani*	ATCC	10 779
43	1739	*C. botulinum* D	ATCC	9633
44	1740	*C. botulinum* C	Germany[d]	CMM 27/2
45	1968	*C. perfringens* B	NCTC	3110
46	1971	*C. perfringens* B	ATCC	3626
47	2142	*C. botulinum* D	France[e]	Pasteur 1873-D
48	2145	*C. botulinum* C	Erfurt[f]	REB1455
49	2150	*C. perfringens* D	NCTC	8346
50	2256	*C. botulinum* C2	Netherlands[g]	AO 28
51	2257	*C. botulinum* C1	Netherlands	468C
52	2267	*C. botulinum* A	Spain[h]	551
53	2269	*C. botulinum* B	Spain	4610
54	2270	*C. botulinum* CoG	Spain	4615
55	2271	*C. botulinum* E	Spain	4611
56	2272	*C. botulinum* F	Spain	4612
57	2273	*C. bifermentans*	Sweden[i]	35 297
58	2274	*C. bifermentans*	Sweden	35 556 A
59	2276	*C. botulinum* A	Sweden	7735
60	2292	*C. botulinum* A	New Zealand[j]	4997
61	2293	*C. botulinum* B	New Zealand	75-3074
62	2294	*C. botulinum* C	New Zealand	5295
63	2295	*C. botulinum* D	New Zealand	35ka29
64	2297	*C. botulinum* F	New Zealand	83-4304

Listed here according to origin and designation are the clostridial reference strains which were used in this study.
[a] National Collection of Type Cultures.
[b] Deutsche Sammlung für Microorganismen.
[c] American Type Culture Collection.
[d] Germany, unknown origin.
[e] Institut Pasteur, Paris.
[f] Nationales Referenzzentrum für Clostridien, Erfurt.
[g] ID-DLO, Lelystad.
[h] Colleción española de cultivos tipo, Valencia.
[i] Culture Collection, University of Göteborg.
[j] New Zealand Reference Culture Collection, Porirua.

2.3. Matrix and standard preparation

A CHCA matrix (α-cyano-hydroxy-cinnaminic acid) (Bruker Daltonik GmbH; #V201344) was used as a saturated solution in a solvent mixture. The standard organic solvent mixture was comprised of 2.5% TFA and 50% AN in water. All chemicals used were of the highest quality (Merck, designated to be eminently suitable for HPLC or MALDI-based techniques). Before each MALDI run *E. coli* DH5α was measured to serve as the positive control and calibration standard. Known ribosomal proteins of *E. coli* were employed for the task of calibration (RL36, RS22, RL34, RL33meth, RL32, RL29, and RS19).

2.4. Instrumentation and data acquisition

The MALDI–TOF MS measurement was performed using a Bruker microflex LT mass spectrometer (Bruker Daltonik GmbH) equipped with a nitrogen laser. Spectra were recorded in the linear positive mode within a mass range of 2000–20 000 Da. The initial "matrix blast" considerably increased the quality of the spectra acquired. The acquisition of spectra was performed both manually and automatically just above the "desorption level" of laser energy to maximize the resolution of the peak pattern. A minimum resolution of 600 had to be reached within the range of 4000–12 000 Da.

The acquisition of spectra was performed both manually and automatically using the Bruker flexControl in the autoexecute mode. The spectra were collected as a sum of 10 times 50 laser shots ranged across a target spot. Twenty independent spectra were collected for each *Clostridia* species.

2.5. Data processing using Bruker BioTyper™

The spectra were analysed using Bruker BioTyper™ 1.1 software, whereby raw spectra were directly handled by the program. The spectra were subsequently smoothed and baseline corrected. In addition to the afore-mentioned processes, the peaks were selected. Using peak lists of approx. 20 spectra as a basis, a reference peak list (the main spectrum) of each species was calculated. The threshold for peak acceptance was set at S/N (signal to noise) of two. The spectra were aligned with each other before the reference spectra were calculated. After alignment, peaks with an m/z (mass-to-charge ratio) difference of less than 250 ppm were considered to be identical. The main spectra were stored in libraries. A database of all *Clostridia* species measured in course of the study was employed as the basis for the identification procedures as well as for taxonomical observations and comparisons.

2.6. Identification of clinical isolates

The clostridial database generated by the reference strains listed in Table 1 was used to determine whether the clinical clostridial isolates (field strains) were identified correctly by MALDI-TOF MS on the basis of their biochemical properties, their toxin type, or by sequencing.

The field strains (see Table 2) were cultured anaerobically as explained above for 36 h at 37 °C on a growth substrate of Columbia Blood Agar. For details concerning sample preparation, the acquisition of spectra, and data processing, kindly refer to the section dealing with the preparation of reference strains. The generated library of reference strains was used to identify the clinical isolates.

Table 2
Clostridial field strains used in this study

Lab. no.	ID	Origin	Field strain[a]
1	S96	Soil	*C. botulinum* A
2	05131/2-3	Corn-based silage	*C. butyricum*
3	05117/3 4	Biogas slurry	*C. butyricum*
4	07065/11	Rabbit gut	*C. inocuum*
5	0509n2	Cattle faeces	*C. perfringens*
6	0509n8	Cattle faeces	C. perfringens
7	0509n12	Cattle faeces	*C. perfringens*
8	05145/5	Biogas slurry	*C. perfringens*
9	05148/1	Biogas slurry	*C. perfringens*
10	05055/11	Rabbit gut	*C. perfringens*
11	05055/91	Rabbit gut	*C. perfringens*
12	05203/4	Cattle slurry	*C. perfringens*
13	269	Chicken faeces	*C. perfringens*
14	I WS	Chicken faeces	*C. perfringens*
15	06124/3 1	Chicken faeces	*C. perfringens*
16	P10	Chicken faeces	*C. perfringens*
17	P51	Chicken faeces	*C. perfringens*
18	07065/1	Rabbit gut	*C. perfringens*
19	07131-Le	Piglet liver	*C. perfringens*
20	07131-Co	Piglet colon	*C. perfringens*
21	07144-nie	Piglet kidney	*C. perfringens*
22	07144-Jej1	Piglet jejunum	*C. perfringens*
23	PS1	Human faeces	*C. perfringens*
24	07124	Malignant edema horse	*C. septicum*
25	05196/1e8	Soil	*C. sporogenes*

Listed here according to origin and designation are the clostridial field strains which were used in this study.
[a] For identification procedure of field strains see Table 4.

3. Results and discussion

3.1. Comparison of different culture conditions

Differences occurring owing to the particular culture medium involved displayed only minimal effects on the mass spectra fingerprints. In the case of variations observed to occur only in the lower mass range between 2000 and 4000 Da, only few peaks were affected. Nevertheless, an attempt was made to standardize the whole procedure of culture and harvesting in order to ensure that all samples were prepared the same way. An effect on the mass spectra could also be observed in the course of measuring sporulated cultures of *Clostridia*. Initially, we had no interest in testing spores, but we realized that spores may have an unfavourable effect on the exact identification. For this reason we used cultures with a higher degree of sporulation.

Spores display slight deviations in fingerprint patterns because they contain high amounts of proteins which are necessary to ensure survival even in suboptimal conditions. The more advanced the state of sporulation the cell subjected to testing was in, the greater the difference occurring in the spectra acquired within one and the same strain when comparing sporulated to non-sporulated cells. To preclude the influence of spores upon the mass spectra presented in this study, all strains were prepared from fresh cultures to rule out the incidence of sporulation. In any case spore spectra could also be used to differentiate *Clostridia*.

3.2. Acquisition of MALDI– TOF MS spectra and annotation of characteristic proteins

The process of sample preparation leads to cell death, the rupturing of the cell wall and the ejection of proteins out of the cytoplasm. Through centrifugation larger particles of cell material like cell wall fragments are eliminated and only soluble components remain in the supernatant which is subsequently subjected to mass spectrometry. The molecular mass of proteins measured fell within a range of 2000–20 000 Da. The spectra of most of the examined species proved to differ considerably from each other. Generally, less than 5% of the peaks were identical between two species leading to identification scores significantly below the MALDI BioTyper species threshold of 2.0. To determine which of the cell proteins were contributing to the peak pattern, a comparison of the molecular masses of the proteins subjected to measurement and the molecular masses of a number of well-described proteins was performed. The data were acquired from the expasy database [6]. As one of the best-described *Clostridium* species, *C. perfringens* was used to attribute a number of ribosomal proteins to the measured peaks of the spectra *C. perfringens* displayed. In addition, there were a number of different strains present in our own culture collection. Fig. 1 presents a typical mass spectrum of this species showing the molecular masses of the measured proteins in Dalton, and the intensity of the peaks. In the MALDI process the intensity of the peaks is not absolutely related to the quantity of protein present in the cell. Instead, a correlation between the ionization efficiency coupled with the protein quantity exists in relation to the ability to produce an intense peak in the protein pattern.

In Table 3, the acquired data from the expasy database [6] concerning certain ribosomal proteins of *C. perfringens* are compared to the data acquired by MALDI. Eleven of the most prominent peaks derived from the peak pattern of *C. perfringens* are listed. Certain proteins of the 50s and the 30s ribosomal subunit of the bacterium are listed by name (left column) and by molecular mass (second column), while the third column lists the most probable explanation to the molecular mass determined by MALDI. Seven of these proteins measured by MALDI find perfect resemblances among the ribosomal proteins previously described. Four of the measured peaks manifest ribosomal proteins after methionine loss, a regular post-translational modification. These findings are in accordance with those of previously mentioned publications [7].

For other species equivalent attempts were made. Nonetheless, there are still only a few molecular masses of clostridial proteins of various clostridial species known.

3.3. Distinct mass spectrometric pattern for Clostridia species

With the aid of the MALDI technique we were able to establish distinct patterns for every strain of the 64 *Clostridia* tested in the course of the study. With the spectra we acquired, it was possible to build up a special database for the 31 clostridial species involved in this study. After being subjected to repeated cultivation, every species was identified correctly within the clostridial database with the help of blinded measurement performed on the strains included in the study. In addition to this, several environmental isolates were identified correctly with use of the clostridial database.

With the software used, a score-oriented dendrogram was generated on the basis of all the clostridial reference strains tested (see Fig. 2). The dendrogram demonstrated considerable similarities to dendrograms generated after analysing the 16S rRNA gene sequencing [8] and the 16S rDNA [9]. In a similar manner as to that established by the findings of Collins and East [8] who analysed the phylogenetic position of *C. botulinum* within the clostridial cluster I, we discovered that the four metabolic groups of *C. botulinum* split into four distinct MALDI patterns. The proteolytic *C. botulinum* types A, B, and F of the metabolic group one form a cluster together with *C. sporogenes*. The 16S rRNA gene

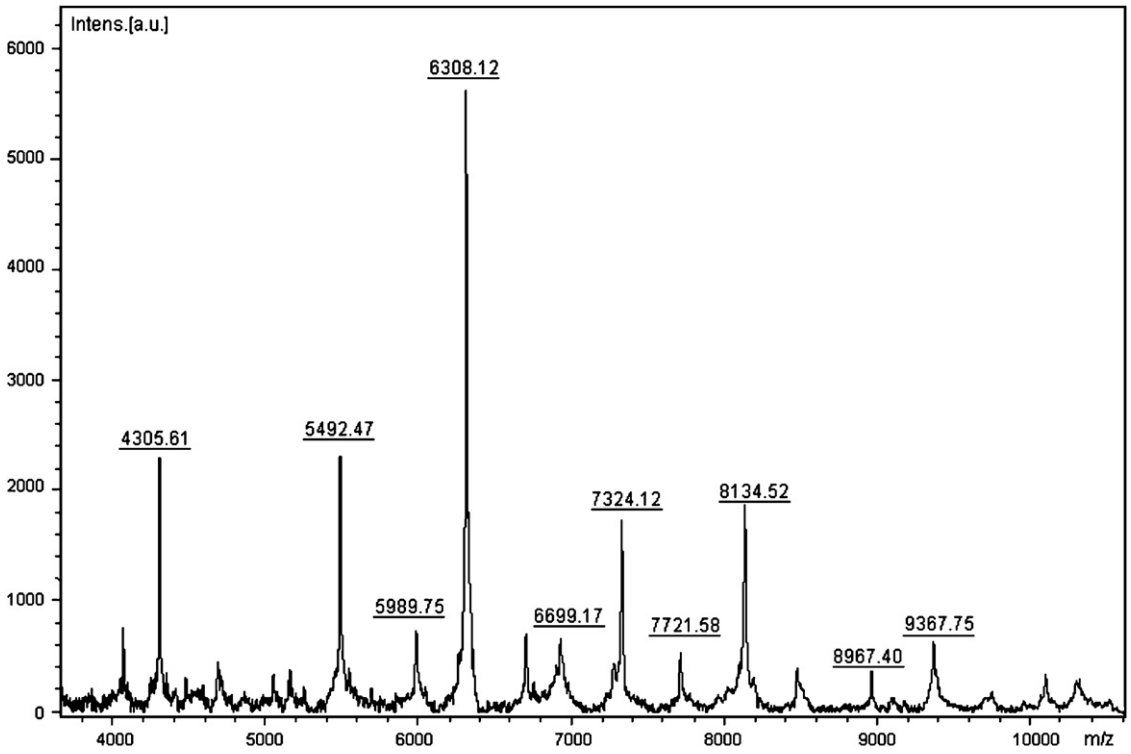

Fig. 1. Mass spectrum of *C. perfringens*. A typical mass spectrum of this species. The *x*-axis marks molecular masses in Dalton, while *y*-axis shows the intensity of the peaks. Selected peaks are marked with their specific molecular masses. With reference to Table 3 the molecular masses are linked to the corresponding ribosomal protein.

Table 3
Mass peaks observed by *C. perfringens*

Ribosomal protein *C. perfringens*	Annotated molecular weight (Da)	Observed molecular weight by MALDI–TOF (Da)	Comment
50s RP L36	4305	4305.61	
50s RP L34	5492	5492.47	
50s RP L33	5992	5989.75	
50s RP L30	6308	6308.12	
50s RP L32	6833	6699.17	Δ 133 (methionine loss)
50s RP L35	7455	7324.12	Δ 131 (methionine loss)
50s RP L31	7721	7721.58	
50s RP L29	8134	8134.52	
RP S4 (fragment)	8967	8967.52	
30s RP S16	9098	8967.12	Δ 131 (methionine loss)
30s RP S20	9499	9367.75	Δ 131 (methionine loss)

The molecular masses of some ribosomal proteins of *C. perfringens* are compared with the mass peaks determined by MALDI.

sequencing analyses demonstrated that these species are related at species level [8]. The saccharolytic *C. botulinum* type E as member of the metabolic group II and *C. argentinense* (*C. botulinum* type G) as member of the metabolic group IV split into two different clusters, which themselves were clearly distinguishable from each other. The metabolic group III, comprised of *C. botulinum* types C and D, proved to be different to all the other *C. botulinum* types whenever either the 16S rDNA analyses [9] or the MALDI system were employed. The fact that *C. haemolyticum* is very closely related to *C. botulinum* types C and D was evident whenever two techniques of analysis, MALDI and 16S rDNA were employed.

The genetic diversity of 174 *C. botulinum* strains had been analysed only recently [10]. The authors demonstrated the clustering of *C. botulinum* in four different groups, and subsequently identified four (types A and B), respectively, five (type E) distinct lineages within these groups. Only four of the *C. botulinum* strains used in this study were also characterized by the MALDI technique; one strain for each of the types A, B, C, and D. In accordance with the work of Hill et al. [10] the types A and B clustered in group one of the proteolytic strains, and types C and D clustered in the third group where toxin types C and D are located. With the aid of the MALDI technique we were able to differentiate between *C. novyi* types A and *C. botulinum* types C and D. These species are not capable of being differentiated on the basis of biochemical or phenotypical properties. They are considered to be very closely related [11,12]. The group III *C. botulinum* types C and D and *C. haemolyticum* formed one subcluster while two other *C. botulinum* type C strains formed a second subcluster within this group. This phenomenon had been previously described by Nakamura et al. [13].

With respect to all *C. botulinum* species it should be taken into consideration that with MALDI–TOF MS, it is not possible to determine whether the isolate subjected to testing is a neurotoxin-producing strain or not.

The strain numbers 2276, 1031, and 1739 *C. botulinum* types A and D were found to be non-toxic [14], but nonetheless fit into the dendrogram at the correct place. Surprisingly, the strain designated with the number 1740, a *C. botulinum* type C strain with an unknown origin, and strain number 2295, a *C. botulinum* type D strain originating from New Zealand were identified as a member of the *C. botulinum* metabolic group I. As stated previously [14], these strains produce type B or type A *C. botulinum* neurotoxin, respectively, and therefore were correctly identified as being members of the corresponding strains.

Other closely related species, e.g. *C. septicum* and *C. chauvoei*, were differentiated correctly by MALDI, while the 16S rDNA analysis showed only a 1.5% difference [5].

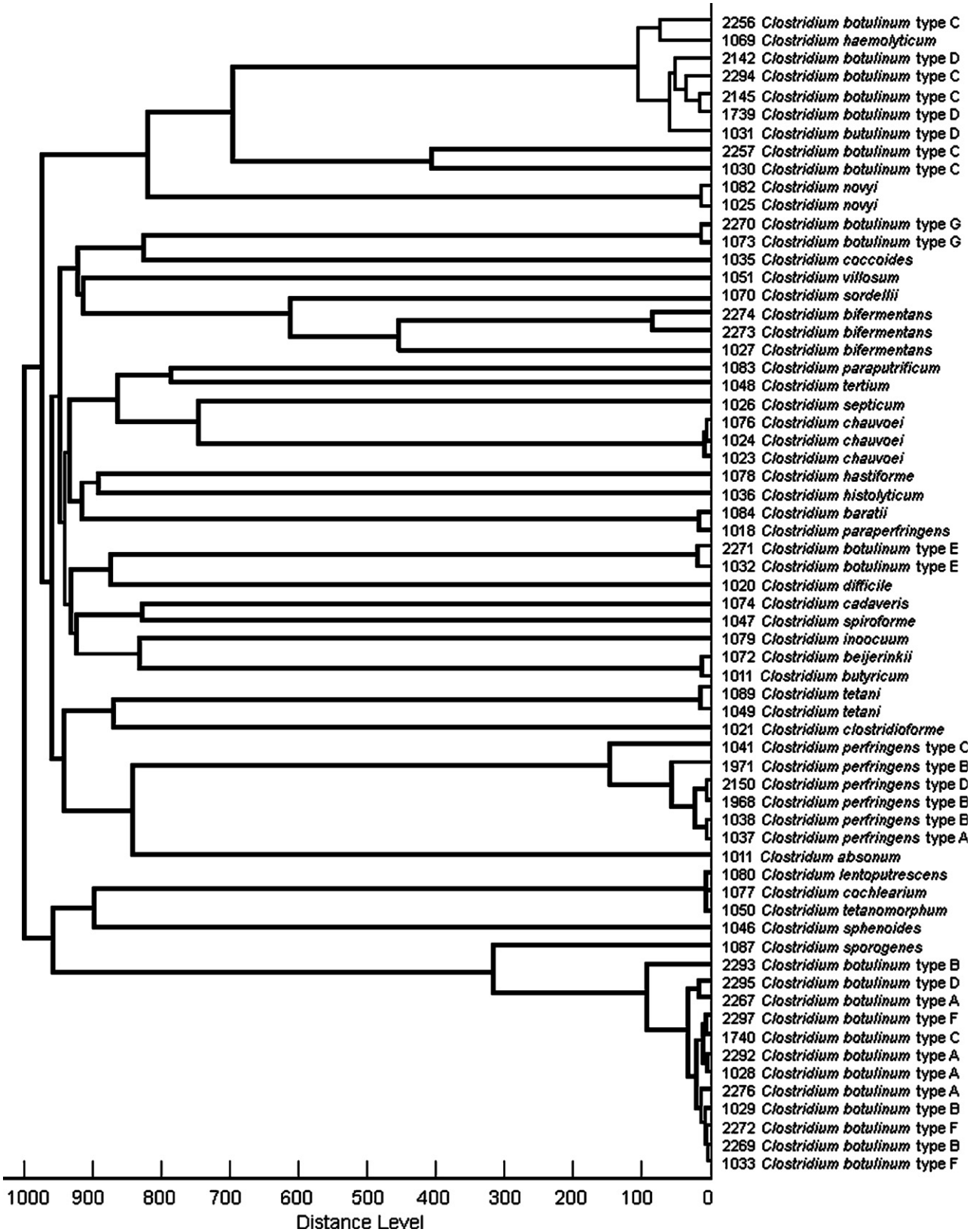

Fig. 2. Score-oriented dendrogram of *Clostridia* species tested. Dendrogram of 64 tested *Clostridia* of 31 different species. Strains of the same species cluster into clearly distinguishable groups, such as, the four metabolic groups of *C. botulinum* which are scattered among the other clostridial species forming distinct groups within the particular species.

With the MALDI technique it was possible to confirm that *C. baratii* and *C. paraperfringens* are truly one species. A uniform designation has already been proposed by Cato et al. [15]. It would be practical to proceed similarly with respect to the heterotypic synonyms *C. lentoputrescens* and *C. cochlearium* [13]. In the same manner, the strain designated as 1050 *C. tetanomorphum* (NCTC

Table 4
Characterization of the field strains used in this study

lab.-no.	ID	Identified as	By means of	MALDI result
1	S96	*C. botulinum* A	BoNT-PCR[a]	*C. botulinum* group ABF
2	05131/2-3	*C. butyricum*	Biochemical tests[b]	*C. butyricum*
3	05117/3 4	*C. butyricum*	Biochemical tests	*C. butyricum*
4	07065/11	*C. inocuum*	Sequencing[c]	*C. inocuum*
5	0509n2	*C. perfringens*	Biochemical tests	*C. perfringens*
6	0509n8	*C. perfringens*	Biochemical tests	*C. perfringens*
7	0509n12	*C. perfringens*	Biochemical tests	*C. perfringens*
8	05145/5	*C. perfringens*	Biochemical tests	*C. perfringens*
9	05148/1	*C. perfringens*	Biochemical tests	*C. perfringens*
10	05055/11	*C. perfringens*	Biochemical tests	*C. perfringens*
11	05055/91	*C. perfringens*	Biochemical tests	*C. perfringens*
12	05203/4	*C. perfringens*	Biochemical tests	*C. perfringens*
13	269	*C. perfringens*	Biochemical tests	*C. perfringens*
14	I WS	*C. perfringens*	Biochemical tests	*C. perfringens*
15	06124/3 1	*C. perfringens*	Biochemical tests	*C. perfringens*
16	P10	*C. perfringens*	Biochemical tests	*C. perfringens*
17	P51	*C. perfringens*	Biochemical tests	*C. perfringens*
18	07065/1	*C. perfringens*	Biochemical tests	*C. perfringens*
19	07131-Le	*C. perfringens*	Biochemical tests	*C. perfringens*
20	07131-Co	*C. perfringens*	Biochemical tests	*C. perfringens*
21	07144-nie	*C. perfringens*	Biochemical tests	*C. perfringens*
22	07144-Jej1	*C. perfringens*	Biochemical tests	*C. perfringens*
23	PS1	*C. perfringens*	Biochemical tests	*C. perfringens*
24	07124	*C. septicum*	Sequencing	*C. septicum*
25	05196/1e8	*C. sporogenes*	Biochemical tests	*C. sporogenes*

Shown here are the identification results of the field strains used in this study. The strains were identified in the course of the routine diagnostical procedure, which was accomplished by means of biochemical testing, sequencing or PCR and compared with the MALDI results.
[a] BoNT PCR [17].
[b] Biochemical test [3].
[c] Sequencing with primers 609V and 699R [18].

2909), which surprisingly proved to be indistinguishable from *C. cochlearium*, has been established to be a *C. cochlearium* by other authors [16].

3.4. Identification of clinical isolates

Table 4 displays the characterization of the field strains used in this study. The clostridial strains were roughly identified during the routine diagnostical procedure of our institutes. Whenever the biochemical testing of the field strains proved to be unsatisfactory, additional characterization procedures (see Table 4) were employed. All strains subject to testing were identified by MALDI–TOF MS according to the results obtained through the traditional diagnostic techniques [3,17,18]. The entire procedure of identification for the characterization of the field strains by MALDI–TOF MS after cultivation, was accomplished in a total of 10 min (8 min for sample preparation and 2 min for measurement).

4. Conclusions

All 64 clostridial strains of the 31 different species studied displayed a characteristic mass spectrometric pattern which can be utilized to effect an unequivocal identification of the species involved. With the MALDI–TOF technique it was possible to identify a single colony of an unknown *Clostridium* species within minutes. Processing hundreds of samples per day is possible. In consequence, under ideal conditions, species identification conducted upon an unknown clinical sample in question can be completed within a period of 36–48 h from the time it is taken into receipt by the diagnostic laboratory. The speed of the process is limited only by the growth rate of the bacterium. We conclude that for the characterization of clostridial strains, the MALDI–TOF MS technique is not only swift, reliable, and accurate, but also eminently facile in its application and use. Nonetheless, the afore-mentioned technique for the quick and easy differentiation of microorganisms should not be construed to be a replacement for established techniques of identification (e.g. 16S DNA sequencing). The objective of the present study is, after all, only to present the potential of the MALDI technique. In any case, the said method affords smaller-scale laboratory operations the possibility of performing a swift identification of microbes with only a minimal financial outlay. Test systems operating on the basis of biochemical principles which are indicated for certain bacterial groups could be dispensed with. In consequence, this would render unnecessary and often inordinately drawn-out process of identification characteristic to such systems. In addition to this, the accompanying cost-intensive provisioning with consumables required by such techniques is also no longer necessary.

In the course of the present study it was possible to demonstrate that the use of the MALDI–TOF MS technique as previously described by Mellmann et al. [19] is also eminently suitable for use in the identification of *Clostridia*.

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References

[1] Hatheway CL. Toxigenic clostridia. Clin Microbiol Rev 1990;3:66–98.
[2] Dürre P. From Pandora's box to cornucopia: *Clostridia*—a historical perspective. In: Bahl H, Dürre P, editors. *Clostridia*. Biotechnology and medical applications. Weinheim, Germany: Wiley-VCH; 2001.
[3] Holdemann LV, Cato EP, Moore WEC. Anaerobe laboratory manual. 4th ed. Blacksburg: VPI Anaerobe Laboratory; 1977.
[4] Stackebrandt E, Goebel BM. Taxonomic note: a place for DNA–DNA reassociation and 16 rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994;44:846.
[5] Stackebrandt E, Hippe H. Taxonomy and systematics. In: Bahl H, Dürre P, editors. *Clostridia*. Biotechnology and medical applications. Weinheim, Germany: Wiley-VCH; 2001.
[6] 〈http://au.expasy.org/srs5/〉.
[7] Ryzhov V, Fenselau C. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Anal Chem 2001;73(4):746–50.
[8] Collins MD, East AK. Phylogeny and taxonomy of the foodborne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol 1998; 84:5–17.
[9] Stackebrandt E, Kramer I, Swiderski J, Hippe H. Phylogenetic basis for a taxonomic dissection of the genus *Clostridium*. FEMS Immunol Med Microbiol 1999;24:253–8.
[10] Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, et al. Genetic diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 2007;189(3):818–32 [Epub November 17, 2006].
[11] Keto-Timonen R, Nevas M, Korkeala H. Efficient DNA fingerprinting of *Clostridium botulinum* types A, B, E, and F by amplified fragment length polymorphism analysis. Appl Environ Microbiol 2005;71(3):1148–54.
[12] Hutson RA, Thompson DE, Collins MD. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F and related *Clostridia* as revealed by small-subunit rRNA gene sequences. FEMS Microbiol Lett 1993;108(1):103–10.
[13] Nakamura S, Kimura I, Yamakawa K, Nishida S. Taxonomic relationships among *Clostridium novyi* types A and B, *Clostridium haemolyticum* and *Clostridium botulinum* type C. J Gen Microbiol 1983;129:1473–9.
[14] Loch PV. Kulturell-biochemische und molekularbiologische Untersuchungen sowie Pathogenitätsprüfung von *Clostridium botulinum*-Stämmen-Ein Beitrag zur Taxonomie. Inaugural Dissertation, Tierärztliche Hochschule, Hannover; 2000.
[15] Cato EP, Holdeman LV, Moore WEC. *Clostridium perenne* and *Clostridium paraperfringens*: later subjective synonyms of *Clostridium barati*. Int J Syst Bacteriol 1982;32:77–81.

[16] Wilde E, Hippe H, Tosunoglu N, Schallehn G, Herwig K, Gottschalk G. *Clostridium-tetanomorphum* sp.-nov., nom.-rev. Int J Syst Bacteriol 1989; 39:127–34.
[17] Takeshi K, Fujinaga Y, Inoue K, Nakajima H, Oguma K, Ueno T, et al. Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction. Curr Top Microbiol Immunol 1996;40(1): 5–11.
[18] Ackermann G, Tang YJ, Kueper R, Heisig P, Rodloff AC, Silva Jr J, et al. Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in gyrA. Antimicrob Agents Chemother 2001;45: 2348–53.
[19] Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, et al. Evaluation of matrix-assisted laser desorption ionization–time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 2008;46(6): 1946–54 [Epub 2008 April 9, 2008].