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Species identification of clinical isolates of *Bacteroides* by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry

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Abstract

Bacteroides fragilis and related species are important human pathogens involved in mixed infections of different origins. The *B. fragilis* group isolates are phenotypically very similar, grow more slowly than aerobic bacteria and, accordingly, are frequently misidentifed with classical or automated phenotypical identification methods. Recent taxonomic changes and new species accepted as members of the *Bacteroides* genus are not included in the different databases of commercially available identification kits. The use of matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was therefore evaluated for the species identification of 277 clinical isolates of the *Bacteroides* genus. Species identification was carried out with MALDI Bruker Daltonik Biotyper software (Bruker Daltonik GmbH, Bremen, Germany) by comparing the mass spectrum of each strain with the mass spectra of the 3260 reference strains currently available. The results of conventional phenotypical identification of the isolates were used as a reference. 16S rRNA gene sequencing was performed for a selection of the strains that gave discrepant results and for all those inconclusively identified by MALDI-TOF MS; 270 isolates (97.5%) were unequivocally identified [log(score) ≥2.0] by comparison with the reference strains present in the MALDI Biotyper database. Of the 23 isolates for which the MALDI-TOF MS species identification differed from the conventional phenotypical identification, 11 were sequenced. The sequencing data confirmed the MALDI-TOF MS result in ten cases and, for the remaining isolate, the sequencing data did not lead to the determination of the species, but only to that of the genus (*Bacteroides* sp.). The discriminating power and identification accuracy of MALDI-TOF MS proved to be superior to that of biochemical testing for *Bacteroides thetaiotaomicron*, *Bacteroides ovatus* and *Bacteroides uniformis*.

Keywords: Bacteroides, clinical isolates, identification, MALDI-TOF MS, sequencing
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Introduction

Bacteroides fragilis and related species are important components of the commensal flora in the lower intestinal tract of mammals, but are also important and frequently isolated as opportunistic anaerobic pathogens causing severe infections, including intra-abdominal, pelvic, lung and brain abscesses, peritonitis and sepsis [1]. Bacteraemia due to *B. fragilis* group isolates contributes appreciably to morbidity and mortality [1–3]. Infections caused by *Bacteroides* strains, alone or in mixed cultures, are especially significant because they exhibit resistance mechanisms to almost all groups of antibiotics and the level of resistance to some antibiotics may be extremely high [4]. Resistance to the most potent antibiotics, such as carbapenems, *beta*-lactam and *beta*-lactamase inhibitor combinations and fourth-generation fluoroquinolones, has slowly increased in Europe over the past 15 years [5,6]. Correct identification at the species level is necessary because the resistance to different anti-anaerobic drugs may differ according to the species [7]. Presumptive identification may be performed with antibiotic discs and spot tests (Wadsworth method) [8]; for example, at the genus level (*Bacteroides*

ides fragilis group). Definitive species-level identification requires a battery of biochemical tests, low molecular weight fatty acid profiling and, occasionally, molecular genetic methods, such as 16S rRNA gene sequencing. Routine laboratories can identify *Bacteroides* isolates using different substrates in a conventional manner or via commercially available identification kits that require a shorter incubation period in an aerobic environment (i.e. detection of preformed enzymes) or a longer incubation in an anaerobic environment (i.e. detection of inducible enzymes). However, these phenotypic methods do not always clearly distinguish closely related species and definitive identification requires a lengthy incubation after isolation of these bacteria from clinical specimens.

The taxonomy of *Bacteroides* has undergone significant changes in recent years [9]. The presumed genus *Bacteroides* was found to contain species from several genera. Most of the species previously included among the *Bacteroides* were placed into two new genera, *Porphyromonas* and *Prevotella* [9], but other genera have subsequently been described for further species that do not belong in these three major groups (e.g. *Anaerorhabdus*, *Dichelobacter*, *Dialister*, *Fibrobacter*, *Megamonas*, *Mitsuokella*, *Rikenella*, *Sebaldella*, *Tannerella*, *Tissierella* and *Alistipes*) [8]. The new *Bacteroides* spp. *Bacteroides nordii* and *Bacteroides salyersae*, found in clinical specimens [10], can be misidentified as *Bacteroides stercoris* and *Bacteroides uniformis* with routine biochemical tests. The recently described *Bacteroides goldsteinii* was phylogenetically close to *Bacteroides distasonis* and *Bacteroides merdae* as classified by 16S rRNA gene sequencing [11]. However, these latter three species were recently reclassified as *Parabacteroides*, *Parabacteroides distasonis*, *Parabacteroides merdae* and *Parabacteroides goldsteinii*; they are phenotypically similar to members of the *Bacteroides* genus (*senso stricto*) but are distinct phylogenetically [12].

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to constitute a useful and simple method for the rapid identification of microorganisms associated with infectious diseases and also for discriminating between different subtypes of pathogens [13–15]. The present study aimed to set up a database for the most frequently isolated anaerobic *Bacteroides* spp. and to test the applicability of MALDI-TOF MS profiling for the identification of *Bacteroides* spp.

Materials and Methods

Isolates

The 277 clinical isolates analysed in the present study were collected during a Europe-wide antibiotic resistance surveillance of *Bacteroides* spp, the results of which will be published soon.

Their identification was performed in the collecting laboratories with different phenotypical methods, such as classical biochemical tests, rapid ID 32A (ATB) and API20 ANA (bioMérieux SA, Marcy-l'Etoile, France). In some cases, only genus-level identification was available. Because identification at the species level may be important for the evaluation of differences in antibiotic resistance of *Bacteroides* strains, the central laboratory (Szeged) re-identified all strains received for the study. Part of the present work involved parallel classical biochemical tests [8] and analysis with MALDI Biotyper software. All strains were cultured on Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) cattle blood, haemin (1 mg/L) and vitamin K_1 (5 mg/L) for 24–48 h at 37°C in an anaerobic chamber (Bactron from Sheldon Manufacturing Inc., Cornelius, OR, USA). Cryobank vials (Mast Diagnostics, Reinfeld, Germany) were used for long-term strain storage. ATCC 25285 *B. fragilis*, DSM2151 *B. fragilis* and ATCC 29742 *Bacteroides thetaiotaomicron* were used as control strains.

Sample preparation for MALDI-TOF MS measurement

One colony of each bacterial strain that had been subcultured for 24 h was transferred into an Eppendorf vial and carefully suspended in 300 μL of bidistilled water. Ethanol (900 μL) was added to the suspension and mixed well. At this stage, the stabilized samples were sent from the Szeged reference laboratory to the Bruker laboratory in Leipzig. There, the samples were centrifuged (13 000 ***g*** for 2 min), the supernatants were removed and the pellets were dried at room temperature. Each bacterial pellet was re-suspended in 50 μL of 70% aqueous formic acid and 50 μL of acetonitrile. After centrifugation (13 000 ***g*** for 2 min), 1 μL of the supernatant was spotted onto a sample position on a ground-steel MALDI target plate and dried at room temperature. Subsequently, a further 2-μL aliquot of MALDI matrix (a saturated solution of HCCA (α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot, which was again dried. The MALDI target plate was subsequently introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation.

Instrumentation and data acquisition

Samples were analysed with a microflex LT or ultraflex TOF/TOF MALDI-TOF instrument (Bruker Daltonik GmbH, Bremen, Germany). Spectra were acquired in the linear, positive ion mode with a laser frequency of 20 Hz (microflex) or 200 Hz (ultraflex). Parameter settings for microflex