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Original article

Genetic, phenotypic and matrix-assisted laser desorption ionization time-of-flight mass spectrometry-based identification of anaerobic bacteria and determination of their antimicrobial susceptibility at a University Hospital in Japan

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ABSTRACT

The accuracies of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and the phenotypic method using VITEK 2 were compared to the accuracy of 16S rRNA sequence analysis for the identification of 170 clinically isolated anaerobes. The antimicrobial susceptibility of the isolates was also evaluated. Genetic analysis identified 21 Gram-positive species in 14 genera and 29 Gram-negative species in 11 genera. The most frequently isolated genera were *Prevotella* spp. (n = 46), *Bacteroides* spp. (n = 25) and *Clostridium* spp. (n = 25). MALDI-TOF MS correctly identified more isolates compared with VITEK 2 at the species (80 vs. 58%, respectively; $p < 0.01$) and genus (85 vs. 71%, respectively; $p < 0.01$) levels. More than 90% of the isolates of the three major genera identified (*Prevotella*, *Bacteroides*, and *Clostridium* species other than *Clostridium difficile*) were susceptible to beta-lactam/beta-lactamase inhibitor combinations, carbapenems, metronidazole and chloramphenicol. MALDI-TOF MS provided better identification results than VITEK2. Commonly used anti-anaerobic agents indicated that the isolates of the three most frequently identified anaerobic genera exhibited good antimicrobial susceptibility.

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1. Introduction

Anaerobes are the causative agents of severe infections and bacteremia. However, their routine identification remains limited in clinical microbiology laboratories because conventional identification methods for anaerobes are time-consuming and expensive, and they require an experienced microbiologist. Automated systems that utilize phenotypic methods are not always reliable for the identification of uncommon species; therefore, genetic analysis is needed for accurate identification [1]. Recently, an increasing body of literature has illustrated the utility of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for anaerobe identification [2–4]. Antimicrobial susceptibility surveillance data for anaerobic bacteria are limited in Japan; there have been only two studies written in English, and they only reported on the susceptibility of *Bacteroides* species [5,6]. Even in Japanese papers, few data for the other anaerobic species are available, although their antimicrobial activity may vary by region [7].

This study aimed to evaluate the accuracy of the identification of clinically isolated anaerobes by MALDI-TOF MS and the phenotypic method and to identify the antimicrobial susceptibility patterns of the clinical isolates determined by the genetic identification of their species.

2. Materials and methods

From June 2013 to May 2014, 170 anaerobic bacteria were isolated from 137 clinical samples collected at Kyoto University

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Hospital. The sources of the isolates included skin and subcutaneous tissue or pus (n = 42), female genital tracts (n = 27), surgical wounds (n = 21), intra-abdominal fluid (including aspirated ascites and bile) (n = 18), blood cultures (n = 16), stools (n = 16), intra-oral fluid or tissue (n = 12), muscles and bones (n = 10), urinary tracts (n = 3), intra-pleural fluid or tissue (n = 3), and central nervous systems (n = 2).

Species identification was performed using the VITEK 2 Anaerobic and *Corynebacterium* cards (bioMérieux, Marcy l'Etoile, France) and MALDI-TOF MS, and the results were compared with the 16S ribosomal ribonucleic acid (rRNA) gene sequences as references.

MALDI-TOF MS was performed using a Microflex LT mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Ethanol-formic acid extractions were performed on the samples according to the manufacturer's instructions [8]. The spectra were analyzed using MALDI Biotyper software (version 3.1, library version 3.3.1.0; Bruker Daltonics GmbH, Bremen, Germany). The data obtained were classified into the following categories: (I) correct identification to the species level, verified by the 16S rRNA gene sequence data with a log score $\geq$ 2.0; (II) correct identification to the genus level, verified by the 16S rRNA gene sequence data with a log score of 1.7–1.999 or a multiple choice within the same genus with the log score $\geq$ 1.7; (III) no identification, any results with a log score < 1.7; and (IV) misidentification, including an identification in which the genus was incorrect compared with the 16S rRNA gene sequence data [3].

The 16S rRNA gene was amplified by PCR using the 27f and 1492r primer pair under previously described conditions [9]. The amplified products were purified and directly sequenced. The obtained sequence data were compared with the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blast/cgi). A homology level >98.7% was considered to be sufficient for species identification [10].

The minimum inhibitory concentrations (MICs) of the following antimicrobial agents were tested: ampicillin, piperacillin, ampicillin/sulbactam, amoxicillin/clavulanate, piperacillin/tazobactam, cefoxitin, cefmetazole, flomoxef, imipenem, meropenem, moxifloxacin, chloramphenicol, clindamycin, linezolid and metronidazole. Vancomycin and teicoplanin were only tested on Gram-positive isolates. The testing was performed with a broth microdilution method according to the standard protocol established in 1993 by the Japanese Society of Chemotherapy using dry plates (Eiken, Tokyo, Japan) according to the manufacturer's instructions with the exception of metronidazole, which was tested with a gradient strip method (Etest strips, bioMérieux, Marcy l'Etoile, France). *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Eggerthella lenta* ATCC 43055 were used in each run for quality control of the susceptibility tests. The isolates were categorized by CLSI breakpoints [11], with the exception of vancomycin, which was categorized by the EUCAST breakpoint [12].

Statistical analysis consisted of Chi-square tests that were carried out to evaluate the accuracies of the identifications determined by MALDI-TOF MS and VITEK 2; a P value < 0.05 was considered to be statistically significant.

3. Results

One hundred seventy clinical anaerobic isolates comprised 81 Gram-positive strains and 89 Gram-negative strains. Genetic analysis identified 21 Gram-positive species in 14 genera and 29 Gram-negative species in 11 genera. The most frequently isolated genera were *Prevotella* spp. (n = 46, 27%), *Bacteroides* spp. (n = 25, 15%) and *Clostridium* spp. (n = 25, 15%).

Table 1 indicates that MALDI-TOF MS correctly identified more isolates compared with VITEK 2 at the species (80 vs. 58%,

Table 1
Performance of MALDI-TOF MS versus VITEK 2 in the identification of anaerobes.

Species determined by 16S rRNA gene sequencing analysis	Identification method							
	MALDI-TOF MS				VITEK 2 ANC card			
	Correct identification to		No identification	Mis-identification	Correct identification to		No identification	Mis-identification
	Species level	Genus level			Species level	Genus level		
Gram-negative isolates (n = 89)	70 (79%)	76 (85%)	2 (2%)	11 (12%)	47 (53%)	65 (73%)	10 (11%)	14 (16%)
Prevotella spp.[a] (n = 46)	40	44	0	2	20	36	5	5
Bacteroides spp.[b] (n = 25)	24	24	0	1	19	20	1	4
Fusobacterium spp.[c] (n = 8)	4	5	0	3	5	5	1	2
Other Gram-negative strains[d] (n = 10)	2	3	2	5	3	4	3	3
Gram-positive isolates (n = 81)	66 (81%)	69 (85%)	1 (1%)	11 (14%)	51 (63%)	55 (68%)	14 (17%)	12 (15%)
Clostridium spp.[e] (n = 25)	22	22	0	3	22	23	2	0
Peptostreptococcus spp.[f] (n = 13)	12	12	1	0	8	8	1	4
Parvimonas micra (n = 10)	7	7	0	3	4	4	3	3
Propionibacterium acnes (n = 7)	7	7	0	0	7	7	0	0
Finegoldia magna (n = 6)	6	6	0	0	6	6	0	0
Other Gram-positive strains[g] (n = 20)	12	15	0	5	4	7	8	5
All isolates (n = 170)	136 (80%)	145 (85%)	3 (2%)	22 (13%)	98 (58%)	120 (71%)	24 (14%)	26 (15%)

When an identification yielded an old name, it was considered to be a newly classified species (e.g., *Micromonas micros* was considered to be *Parvimonas micra*).
If less than five strains in a genus were isolated, they were categorized as other Gram-positive/negative strains.
[a] *P. bivia* (n = 20), *P. nigrescens* (n = 7), *P. buccae* (n = 6), *P. disiens* (n = 2), *P. timonensis* (n = 2), *P. denticola* (n = 2), *P. oris* (n = 2), *P. amnii* (n = 1), *P. buccalis* (n = 1), *P. corporis* (n = 1), *P. intermedia* (n = 1) and *P. oralis* (n = 1).
[b] *B. fragilis* (n = 18), *B. uniformis* (n = 2), *B. caccae* (n = 1), *B. heparinolyticus* (n = 1), *B. ovatus* (n = 1), *B. pyogenes* (n = 1) and *B. theaiotaomicrion* (n = 1).
[c] *F. nucleatum* (n = 7) and *F. mortiferum* (n = 1).
[d] *Campylobacter ureolyticus* (n = 2), *Parabacteroides distasonis* (n = 2), *Alloprevotella rava* (n = 1), *Brachyspira pilosicoli* (n = 1), *Cetobacterium somerae* (n = 1), *Dialister pneumosintes* (n = 1), *Dysgonomonas oryzarvi* (n = 1) and *Veillonella dispar* (n = 1).
[e] *C. perfringens* (n = 12), *C. difficile* (n = 11) and *C. ramosum* (n = 2).
[f] *P. anaerobius* (n = 12) and *P. stomatis* (n = 1).
[g] *Gardnerella vaginalis* (n = 3), *Slackia exigua* (n = 3), *Bifidobacterium dentium* (n = 2), *Eggerthella lenta* (n = 2), *Peptoniphilus asaccharolyticus* (n = 2), *Actinomyces odontolyticus* (n = 1), *Anaerococcus hydrogenalis* (n = 1), *Anaerococcus obesiensis* (n = 1), *Atopobium parvulum* (n = 1), *Bifidobacterium longum* (n = 1), *Bifidobacterium pseudocatenulatum* (n = 1), *Peptoniphilus harei* (n = 1) and *Shuttleworthia satelles* (n = 1).

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