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Identification of Anaerobes in Clinical Specimens Comparison between the RapID ANA II System and the Bruker MALDI-TOF MS System utilized in the Clinical Laboratory

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This thesis, written under the direction of the candidate's thesis advisor and approved by the program chair, has been presented to and accepted by the Clinical Laboratory Sciences in partial fulfillment of the requirements for the degree of Master of Science Clinical Laboratory Sciences.

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Identification of Anaerobes in Clinical Specimens

Comparison between the RapID ANA II System and the Bruker MALDI-TOF MS System utilized in the Clinical Laboratory

By

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A culminating project, submitted to the faculty of Dominican University of California in partial fulfilment of the requirements for the degree of Master of Science in Clinical Laboratory Sciences.

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Abstract

Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has been revealed as an invaluable platform for identifying anaerobic bacteria in the clinical laboratory over traditional methods such as the RapID ANA II System.

A qualitative comparison is made, through the analysis of methodologies and specifications, to determine whether the RapID ANA II system or Bruker MALDI-TOF MS is more suitable for identifying anaerobic organism in the clinical laboratory. Based on the data reviewed, the MALDI-TOF MS is a more intuitive platform within the clinical laboratory due to its increased specificity, cost-effectiveness, and shorten turnaround time for the identification of anaerobes.

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Introduction

Anaerobic bacteria grow in the absence of oxygen. The major types of anaerobes are obligate anaerobes, facultative anaerobes, and aerotolerant anaerobes. Obligate anaerobes only grow in the absence of oxygen. Facultative anaerobes do not require oxygen to grow but will use it if available. Aerotolerant anaerobes grow best without oxygen but can tolerate its presence. Anaerobes are part of the normal flora found on the human body. Under normal circumstances, anaerobic bacteria live on the body as beneficial commensals. The major sources of anaerobes are situated in the mouth, mucosal membrane surfaces, gastrointestinal and genital tracts.[1]

Prevotella and Peptostreptococcus species, which are part of our normal anaerobic gram-negative and gram-positive oral flora, have characteristics to protect the human body. [2] In a study conducted on children with a history of group A β-hemolytic streptococci (GABHS) pharyngotonsillitis, children with less Prevotella and Peptostreptococcus anaerobes cultured from their tonsils have an increased likelihood of having recurring GABHS infections than children with more of these anaerobes.[2] This phenomenon is known as bacterial interference and colonization resistance. [2,3] However, in humans with immunocompromised status, trauma or disease, GABHS infections may lead to serious anaerobic infections or death. [3] Therefore, it is imperative that the laboratory can identify anaerobes accurately and timely.

A specimen must be obtained before anaerobes can be identified. Due to the fastidious nature of anaerobes, it is often difficult to culture and grow them if collection techniques are not properly followed. [4] Some anaerobes are killed within seconds once they encounter molecular oxygen. [5] Therefore, using proper collection techniques is

important to enhance anaerobic growth with better outcomes. When anaerobes are stored in room temperature with the appropriate anaerobic transport tube, anaerobes will survive 24 to 72 hours. However, the survival of certain species of anaerobes is greatly diminished after 48 hours. [5] As a result, the ability to rapidly identify the correct type of anaerobic bacteria is extremely important when treating patients with anaerobic infections.

Methods

Given the need for the rapid identification of anaerobes, different tools have been developed to aid laboratorians in identifying anaerobes. The “golden standard” for identifying bacterial species is using whole-genome sequencing (WGS) and 16S ribosomal ribonucleic acid (rRNA) gene sequencing. [6,7] However, due to the cost and lengthy turnaround time of sequencing, these two methods are not the ideal options for identifying anaerobes in the clinical laboratory. At present, Remel RapID ANA II and the Bruker MALDI-TOF MS appear to be the most commonly used methods in anaerobic identification. A qualitative comparison is conducted to determine which of these two methods is more desirable for identifying anaerobic organism in the clinical laboratory via thorough analysis of their methodologies and specifications.

Remel RapID ANA II System

The Remel RapID ANA II system is a chromogenic, single sub-substrate qualitative method using enzyme technology tests to identify clinically significant anaerobes from human specimens. The assay is comprised of a plastic disposable RapID panel with ten reaction cavities, RapID inoculation fluid, RapID ANA II reagent, and RapID Spot Indole reagent. The test organism must first be dispensed into the RapID inoculation fluid prior to the inoculation of the cavity. After the inoculation of the panel, the organism must be incubated for 4-6 hours. Upon the completion of incubation, reagents must be added into underlined wells to produce a colorimetric change which indicates the presence of enzymes. The resulting pattern must then be

entered into an Electronic RapID Compendium (ERIC) database used to identify the anaerobic bacteria.[8]

Matrix-Assisted Laser Desorption and Ionization (MALDI) System

The MALDI is an ionization technique that uses a matrix to form ions from larger molecules for molecular identification. [11] First, the user must transfer a bacterial colony grown from a patient sample (which is first mixed with or without formic acid depending on the organism being identified) onto a MALDI "target" plate. The bacterial isolate is then embedded with the matrix for protein extraction. [12,13] Figure 1 below depicts the preparation of a sample plate for sample analysis (Clark et al., 2013). After the isolate embedded with the matrix is dried, the plate is inserted into the ionization assembly of the instrument. A nitrogen laser with a wavelength of 337 nm then heats the matrix embedded sample rapidly, causing the sample to vaporize into ions. The ions subsequently enter the mass spectrometer for identification. [14,15]

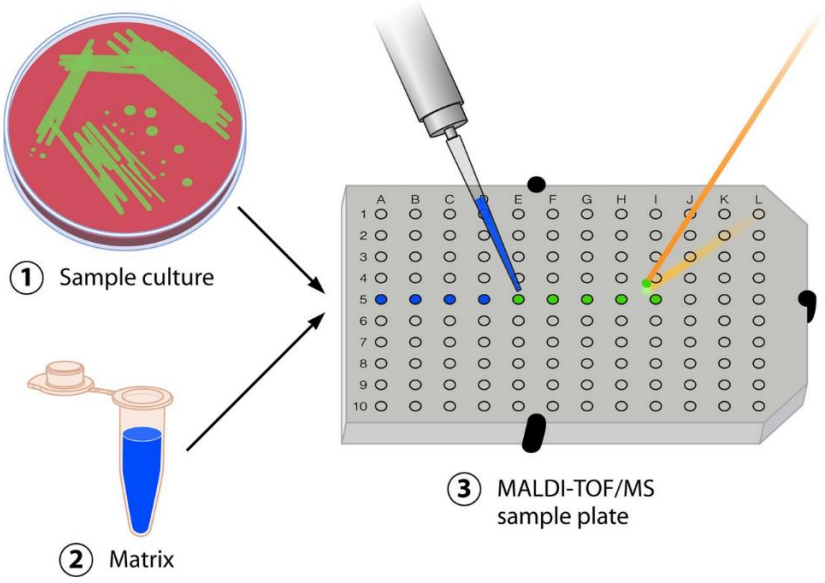

Figure 1 Schematic for the preparation of MALDI-TOF MS plate for sample identification. Growth from the sample culture is applied to target slide and overlaid with matrix. Once dried, it is ready for instrument anaylsis. (Clark et al., 2013)

The mass spectrometer used in this assay is Time-of-Flight Mass Spectrometry (TOF MS). In this stage, charged ions from the vaporized sample are divided based on their mass-to-charge ratio (m/z). Smaller ions with a greater charge will reach the detector quicker than larger ions with a smaller charge. [14] The detected biomolecules generate a spectrum that is matched with a database of known organisms. The matching provides a confidence level which aids in the identification of the sample. [15] The detailed steps of the MALDI-TOF MS is presented in Figure 2 below (Croxatto et al., 2012) . [16]

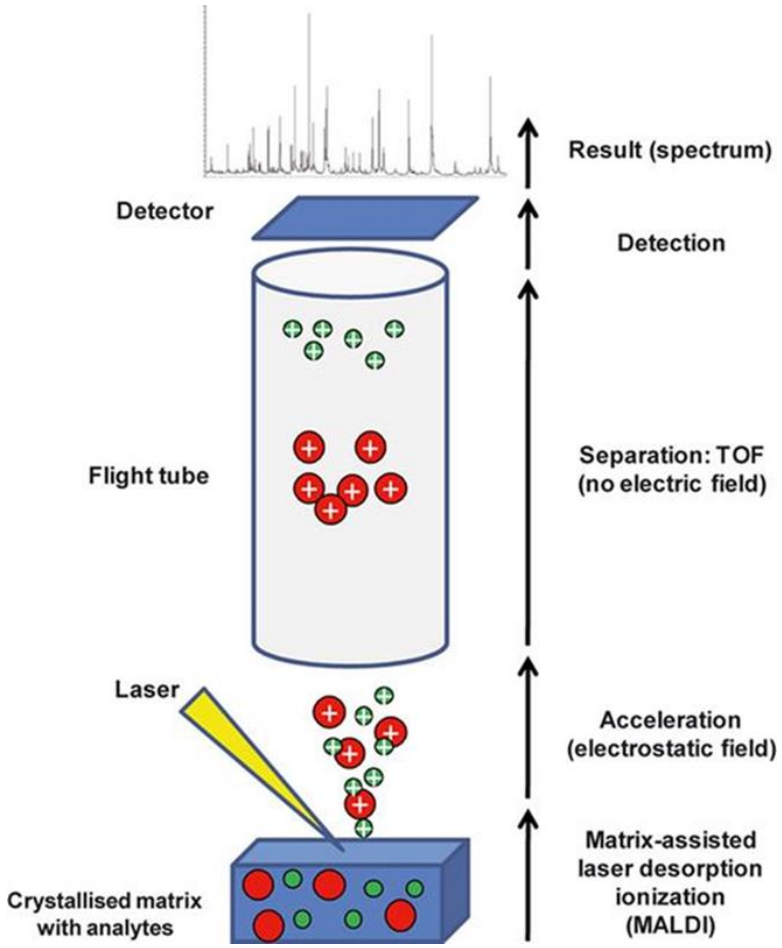

Figure 2 Identification process via the MALDI-TOF MS system. Matrix-embedded sample is introduced into a mass spectrometer where a laser strikes the sample resulting in vaporized ions. The ions enter a flight tube where they are sorted based on mass-to-charge charge ratio (m/z) and read by the detector. A spectrum is then generated that is matched with a database of known organisms. (Croxatto et al., 2012)

The Bruker Biotyper system generates a confidence score between 0.00-3.00, indicating the identification confidence of the organism being analyzed. A score of 2.00-3.00 (green) indicates a high degree of confidence, 1.70-1.99 (yellow) reflects a low degree of confidence, and below 1.70 (red) means no identification.

Results

RapID ANA II System

Research has shown that the accuracy of the RapID ANA II manifested discrepant results for anaerobic identification. In a performance study conducted by Marler et al., the RapID ANA II test was thoroughly evaluated for its ability to identity 566 anaerobes. The successful rates of identifying correct species under this method are summarized in Table 1 below:

Isolate	Correct identification (% of total)
Gram-negative bacilli	62% of 204
Nonsporeforming gram positive bacilli	70% of 69
Clostridium isolates	74% of 130
Anaerobic cocci	72% of 163

Table 1 Identification assessment of 566 anaerobes using the RapID ANA II test, adapted from "Evaluation of the new RapID-ANA II system for the identification of clinical anaerobic isolates," Marler et al., J. Clin. Microbiol. 29: 874-878.

The RapID ANA II kit performed well with spot indole negative Bacteroides fragilis group, correctly identifying 26 of 28 (93%) isolates. All strains of Parabacteroides distasonis as well as 19 of 27 (70%) of Bacteroides vulgatus were also accurately identified. On the contrary, the spot indole positive Bacteroides fragilis group proved problematic with the RapID ANA II panel. For Bacteroides ovatus and Bacteroides uniformis, 1 of 29 (3%) and 4 of 14 (29%) were correctly identified, respectively. Bacteroides ovatus was often confused with Bacteroides uniformis along with Bacteroides thetaiotaomicron. [9,10] Overall, the RapID ANA II system correctly identified approximately 68% of the 566 anaerobic isolates to the genus and species level. [9]

The limitations of the RapID ANA II system should be carefully assessed when using this method in the clinical laboratory. The technologist must ensure that

pure culture isolates with enough colonies be used to achieve a #3 McFarland standard, and the RapID ANA II tray be incubated between 4-6 hours at 35-37°C. Setting up the test outside of the manufacturer's instructed parameters or using a mixed culture may lead to misidentification. [8] Additionally, the technologist must have adequate knowledge of anaerobes to resolve discrepant results as well as sufficient access to additional tests that may help identify each isolate. Additional tests (including gram staining, aerotolerance, specimen source, and growth on selective agars) should be used in conjunction with the RapID ANA II system. [8]

Bruker MALDI-TOF MS System

Numerous studies indicated that Bruker MALDI-TOF MS is a robust platform which allows users to identify anaerobic organisms with a high level of confidence. In a study conducted by Schmitt et al. in 2012, this system was evaluated for its capability to correctly identifying 252 clinical isolates of anaerobic bacteria (see Table 2 below).

Total Isolates	Species identification	Genus identification	No identification
252	179 (70.8%)	232 (91.7%)	20 (7.9%)

Table 2 Evaluation of 252 clinical isolates of anaerobic bacteria using Bruker MALDI-TOF MS, adapted from "Identification of Anaerobic Bacteria by Bruker Biotyper Matrix-Assisted Laser Desorption and Ionization-Time of Flight Mass Spectrometry with On-Plate Formic Acid Preparation," Schmitt et al., *Journal of Clinical Microbiology,51*(3), 782-786.

The anaerobes were identified using Bruker's cut-off values. A total of 179 (70.8%) and 232 (91.7%) of anaerobic isolates were identified correctly to the genus and species level using the manufacturers cut-off scores. Twenty (7.9%) of the clinical isolates had no identification because they received a score below 1.70. [18] This study demonstrated that using the Bruker Biotyper was a great alternative for identifying anaerobic bacteria.

In a subsequent study involving a larger collection of anaerobes for the evaluation of the Bruker Microflex LT mass spectrometer equipped with the MALDI Biotyper 3.0 software, the results achieved were even more promising. Barreau et al., conducted a study with 1,325 anaerobes to determine the capability of the Bruker MS. The isolation of anaerobic specimens was from blood culture (362), abscesses and liquid collection (287), tissue samples (319), osteo-articular samples (144), sinus samples (115), lymph nodes (21), cerebral spinal fluid (20), pleural samples (32) and other samples (25). [19] The Bruker MS correctly identified 92.5% of isolates to the species level and 98.9% identified to genus level. 14 of the 1325 samples could not be identified to the genus level. [19] Table 3 below summarizes the findings from the study.

Barreau et al. stated that the improvements in identification were due to software upgrades. Barreau et al. further pointed out that this method should be considered the new gold standard for the routine identification of clinical anaerobes.

Total Isolates	Species identification (score ≥ 1.9)	Genus identification (score <1.9 ≥ 1.7)	No identification (Score < 1.7)
1,325	1,225 (92.5%)	86 (6.5% genus only)	14 (1.0%)

Table 3 Evaluation of the Bruker MALDI-TOF MS with Biotyper 3.0 software for the identification of 1,325 anaerobic isolates, adapted from "Improving the identification of anaerobes in the clinical microbiology laboratory through MALDI-TOF mass spectrometry," by Barreau et al., 2013, Anaerobe 22, 123-125. Copyright by 2013 Elsevier Ltd.

The MALDI-TOF MS must be validated to ensure it produces reliable and reproducible results before implementing it within the clinical laboratory. To properly validate this platform, the laboratory must follow the regulatory guidelines established by the College of American Pathologists (CAP) and the Clinical Laboratory Improvement Amendments (CLIA) for method validation.

According to CAP All Common Checklist, a minimum of 20 samples should be tested. [20,21] Likewise, CLIA suggests a minimum of 20 samples to be run for method validation. However, majority of other sources suggest running at least 40 specimens to detect discrepancies. Ideally speaking, method validation should be performed over a period of five days with a confidence score of at least 90%. However, if discrepancies are observed, the validation should be extended for five more days. [22]

Discussion

Cost effectiveness is a key consideration when incorporating a new platform in the clinical laboratory. The new method should yield a positive return of investment, allowing resources and personnel to be allocated elsewhere in the department for quality improvements. When comparing the two platforms discussed earlier (see Table 4 below), the cost per test and versatility of each method should be carefully analyzed.

	RapID ANA II	Bruker MALDI-TOF MS
Turnaround time	4-6 hours	~15 minutes
Accuracy	68% to genus and species	92.5% to genus and species
Cost	$9.19/test	$0.50/test

Table 4 Comparison of the RapID ANA II and Bruker MALDI-TOF MS based on turnaround time, accuracy, and cost-effectiveness.

The Department of Veteran Affairs lists a 20 pack RapID ANA II kit for the price of $152.16. [23] The kit includes the ANA II reagent but does not include the spot indole or RapID inoculation fluid that must be purchased separately. The rapid spot indole reagent is listed for $11.12, and the RapID inoculation fluid costs $20.43 for a pack of 20. [23] The price of the Bruker MALDI-TOF MS costs $150,000. [24] In addition, the Bruker requires additional consumables to run a sample. The consumables required to operate the MALDI TOF MS are pipette tips, target slides, matrix, and formic acid. When we factor in the RapID ANA II kit with the spot indole reagent, the cost is $9.19 per test. Using the MALDI-TOF MS; however, only costs $0.50 per test with a much higher level of accuracy in identification (92.5% with Bruker vs. 68% with RapID) [24,25]

This data shows that the clinical laboratory could run 18 anaerobe samples with MALDI-TOF MS for the cost of running one sample with RAPID ANA II. In addition, the test under RapID ANA II requires the kit to be incubated for 4 hours, applying reagents,

interpreting of each enzymatic test, and entering enzyme results into the ERIC database for identification. In contrast, the MALDI-TOF MS can identify a sample in approximately 15 minutes. [25]

The savings of incorporating MALDI-TOF MS are more profound than what have been discussed earlier. In a study conducted in a Taiwanese laboratory from July to December 2012, 52,500 isolates were analyzed (47,300 aerobes and 5,200 anaerobes). This study revealed a bi-annual savings of $84,000 compared to conventional biochemical methods. [26] If the 5,200 anaerobes were to be identified using RapID ANA, it would cost $42,284 compared to $2,600 using the MALDI-TOF Apparently, MALDI-TOF MS is much more versatile than RapID because the former could also identify yeast and aerobic organisms; thereby, significantly improving the financial health of the entire laboratory.

In a study conducted by the University of North Carolina Hospitals from April 2013 to March 2014, using MALDI-TOF MS could realize an annual savings of $73,646, which would compensate for the cost of the equipment within 3 years. [27] Nevertheless, cost savings also depend on the volume received at each laboratory, with larger institutions being able to offset the cost of the instrument much quicker.

Conclusion

The Bruker MALDI-TOF MS provides a more robust platform for identifying anaerobes within the clinical laboratory due to the following factors:

- Identification of anaerobes can be efficiently accomplished within 15 minutes since it does not require an aerotolerance test. The quicker turnaround time allows clinicians to treat patients more effectively and reduce the duration of hospital visits. In contrast, the RapID ANA II method recommends a 24-hour aerotolerance test and an additional 4-6 hours of incubation for identification.
- MALDI-TOF MS offers 18 times more savings than RapID ANA II, costing $0.50 compared to $9.19 per test and with a much higher successful rate in identification.
- Bruker's database of organism continues to expand through software upgrades, which allows for the timely detection of anaerobic organisms with increased accuracy.
- Numerous studies have shown that MALDI-TOF MS is more sensitive and specific than conventional methods for identifying anaerobes.

In conclusion, MALDI-TOF MS has emerged as a superior method of identification over the RapID ANA II system within the clinical laboratory. The advent of MALDI-TOF MS has demonstrated increased specificity, cost-effectiveness, and decreased turnaround time. Due to the infancy of MALDI-TOF MS, it may require an alternative method of identification for confirmation, such as 16S RNA and WGS sequencing. However, research has proven the MALDI-TOF MS is capable of continuous improvements through a growing database. The MALDI-TOF MS has flourished in the clinical laboratory for the routine identification of anaerobes.

Isolates	Species identification (score ≥ 1.9)	Genus identification (score < 1.9 ≥ 1.7)
Actinobacteria (417)	387 (92.3%)	30 (7.7%)
Propionibacterium (375)	350 (96.9%)	25 (7.1%)
Atopobium (7)	7 (100%)	
Bifidobacterium (8)	6 (75%)	2 (15%)
Eggerthella (10)	8 (80%)	2 (20%)
Slackia exigua (12)	12 (100%)	
Actinobaculum (4)	3 (75%)	1 (25%)
Brevibacterium frigotolerans (1)	1 (100%)	
Clostridiales (343)	314 (90.8%)	29 (9.2%)
Anaerotruncus colihominis (1)	1 (100%)	
Finegoldia (98)	86 (86.1%)	12 (13.9%)
Peptostreptococcus (9)	9 (90%)	1 (10%)
Anaerococcus (32)	24 (66.7%)	8 (33.3%)
Parvimonas (82)	77 (93.5%)	5 (6.5%)
Peptoniphilus (66)	65 (98.5%)	1 (1.5%)
Clostridium (43)	41 (95.1%)	2 (4.9%)
Tissierella praeacuta (1)	1 (100%)	
Bilophila wadsworthia (3)	3 (100%)	
Dialister microaerophilus (2)	2 (100%)	
Eubacterium (3)	3 (100%)	
Ruminococcus gnavus (2)	2	
Lactobacillus (12)	12 (100%)	
Fusobacterium (86)	76 (86.9%)	10 (13.1%)
Bacteriodales (442)	425 (96%)	17 (4%)
Porphyromonas (17)	14 (78.6%)	3 (21.4%)
Bacteroides (299)	292 (97.6%)	7 (2.4%)
Parabacteroides (11)	11 (100%)	
Prevotella (80)	74 (91.9%)	6 (8.1%)
Veillonella (29)	29 (100%)	
Alistipes finegoldii (1)	1 (100%)	
Butyricimonas (4)	3 (75%)	1 (25%)
Odoribacter splanchnicus (1)	1 (100%)	
Erysipelotrichales (9)	9 (100%)	
Solobacterium moorei (8)	8	
Turicibacter sanguinis (1)	1	
Proteobacteria (2)	2 (100%)	
Desulfovibrio (2)	2 (100%)	
Overall (1311)	**1225 (92.5%)**	**86 (6.5%)**

Table 5 Identification of 1,311anaerobe isolates at the genus and species level using the Bruker MALDI-TOF MS, adapted from "Improving the identification of anaerobes in the clinical microbiology laboratory through MALDI-TOF mass spectrometry," by Barreau et al., 2013, Anaerobe 22, 123-125. Copyright by 2013 Elsevier Ltd.

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