Evaluation of Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry for Clinical Microbiology Applications

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Abstract

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MALDI-TOF-MS has the ability to streamline identification of microorganisms in the microbiology lab. In this project, we focused on two main aims. First, the implementation of this technology for direct blood culture identification. Within this aim we determined the limit of detection for the protein purification method we planned on using in our laboratory, determined handling conditions for the samples, and evaluated the MALDI-TOF-MS for concordance with our current workflow and reports. Second, customization of the commercial library for *Cryptococcus gattii* was performed. Within this aim, we improved representation of the species in the MALDI-TOF-MS library, and also explored ways to determine the subspecies based on gathered spectra.

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Chapter One – Introduction

Accurate and rapid identification of microorganisms has long been an important goal of clinical microbiology laboratories. To this end, technology is continually being adapted to aid in the standardization of lab processes and the confidence of the identification of bacteria. Polymerase Chain Reaction, Florescent *In Situ* Hybridization probes for identities, and automated microtiter dilution for sensitivities, are just some of the technologies that have been developed to help differentiate pathogens from normal flora and provide clinicians with antibiotic resistance information. Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) is one of the newer technologies present in the microbiology laboratory.

MALDI-TOF-MS TECHNOLOGY

Time-of-flight mass spectrometry is based on having multiple ions excited at the same moment. These ions then enter a field-free drift pathway where they are able to separate based on their mass and charge. At the end of the pathway, a detector records the time of flight[1]. Because multiple ions are excited simultaneously and there is no selection for particular ions in the drift pathway, large ranges of masses can be analyzed in one run. Also ions tend to stay in their molecular form and not fragment into daughter ions, as seen in other mass spectrometry methods[2].

MALDI technology relies on matrix and laser compatibility to create the excited ions for the mass spectrometer. The matrix should be a substance that can maximally absorb energy at the wavelength of its paired laser. For UV lasers that are used in the Bruker and bioMerieux systems, the matrix should be an aromate, in this case, α-cyano-4-hydroxycinnaminic acid[1]. The addition of matrix has expanded laser desorption/ionization application from small aromatic residues of 1000 Daltons or less to large and less volatile substances[1]. MALDI-TOF mass spectrometers also have low noise level in the high mass region. Matrix ions are easily distinguished from molecular ions of interest, allowing analysis of spectra over large m/z ratios[2].

Modern MALDI-TOF-MS has the ability to analyze a variety of substances including large biomolecules. It was originally recognized for its ability to look at a potentially unlimited mass range (upwards of 350 kDa) with analytes at femtomolar concentrations[3]. As the technology has progressed, it has been applied to increasingly diverse scientific problems. Two major companies that have come out with instruments suited for clinical microbiology laboratory use are Bruker Daltonics and bioMerieux. bioMerieux recently acquired third party software and the accompanying database from Shimadzu and AnagnosTec GmbH. The Bruker microflex LT and the bioMerieux Vitek MS both rely on sample placed on metal targets that are then subjected to MALDI-TOF-MS. Spectra are recorded, analyzed with proprietary algorithms, and then compared to commercial libraries assembled for each platform.

One of the primary differences in these two platforms is that the Vitek MS is a reflectron MALDI-TOF-MS. A reflectron redirects the sample ions back down the drift pathway, essentially doubling the length that the ions have to separate by mass to charge ratio. The microflex on the other hand, is a linear MALDI-TOF-MS, which has lower resolution because of the shorter drift pathway.

Another difference between the platforms is in how the scores are calculated and interpreted. For the Bruker microflex, the raw sample spectra are compared to a library of reference main spectra. Main spectra are an average of multiple raw spectra of a single strain to reach a representative spectrum. To calculate the score, peak position, intensity and frequency are taken into consideration and a log score from 0.000 to 3.000 is generated. These scores are then assigned to categories based on how reliable the identification is: unreliable identification, secure genus level identification, or secure species level identification. For the Vitek MS, raw sample spectra are converted to a list of peaks and their associated intensities. This peak list is compared to a reference SuperSpectra, which is an average of at least 15 individual strains. Individual reference spectra can also be used in analysis, if appropriate SuperSpectra have not provided an adequate match. Scores are then generated based on probability of a match and given a confidence value[4]. This confidence value is considered reliable identification above 90%, probable identification between 85-89.9% and unreliable identification below 85%[5].

MICROBIOLOGICAL APPLICATIONS

Initial applications of MALDI-TOF technology in microbiology focused on lyophilized cells in which proteins were extracted for analysis[6,7]. These early data showed peaks at unique mass to charge ratios by species indicating that MALDI-TOF-MS could be adapted for bacterial identification. It was noted that the greatest variation occurred in between 1kDa and 20kDa m/z range[6]. Whole microorganisms were subjected to testing using many of the settings determined in the cellular extract experiments. The use of α-cyano-4-hydroxycinnaminic acid for the matrix and UV wavelength lasers for desorption of microbiological samples has become standard.

MALDI-TOF-MS proved its usefulness in whole colony identification of bacteria starting with rapid identification of intact food borne pathogens. Bacteria were distinguished from each other. Furthermore, they were matched to known reference spectra[8]. Expanding upon this initial application, MALDI-TOF-MS has become a mainstay for bacterial identification. The method is highly accurate and efficient overall[9–11]. Some bacterial species do pose problems for identification by MALDI-TOF-MS. These organisms are frequently closely related or are already hard to distinguish using biochemical methods. Some examples include differentiating *Streptococcus mitis* group and *Streptococcus pneumoniae*, *Shigella* species and *E. coli*, and *Listeria* species from each other[4].

One effort to eliminate this problem of poor identification used the *Streptococcus mitis* group as a model. In order to improve identification among *Streptococcus mitis* and *S. pneumoniae*, seven unique peaks were identified as discriminatory between the species. These peaks are represented as six MALDI Profiles that define the *Streptococcus mitis* group species[12]. MALDI Profile 1 represented *Streptococcus pneumoniae,* while MALDI Profiles 5 and 6 represented non-pneumococcal species in the group such as *S. oralis* and *S. mitis*. This discovery showed that with additional analysis, it is possible to find subtle differences between closely related organisms and improve MALDI-TOF-MS reliability.

Over the decade, development of commercial reference libraries and the exploration of novel applications have continued to expand the use of this technology. One such clinical application is the identification of bacteria from specimens or samples instead of pure growth on solid media.

One such sample is blood culture. Blood culture has the advantage of enriching for bacteria. Currently, positive blood cultures are Gram stained and subcultured to solid agar based media for definitive identification. MALDI-TOF-MS has increasingly been applied to blood cultures, allowing for definitive organism identification within hours of culture positivity[13–15].

Sepsis is an infection in which prompt initiation of appropriate antibiotics has a direct effect on patient outcomes. Patient mortality increases for every hour that antibiotics are delayed after hypotension associated with sepsis[16,17]. While initial treatment may be empiric for the most probable organism, narrowing treatment quickly is important to decrease the development of resistance and also to decrease the cost of hospital treatment[18,19]. Rapid identification of organisms in blood cultures can help ensure that antibiotic coverage is appropriate and good antibiotic stewardship is practiced[20]. A study by Vlek, *et al.* tracked time to bacterial identification and appropriateness of antibiotic coverage over consecutive blood cultures. For the first 164 samples, standard culture was used for identification. The next 89 samples were processed using an in-house preparation method and identified with MALDI-TOF-MS. The difference in median time to identification was 28.8 hours. The cultures were then sent for antibiotic susceptibilities, regardless of identification method. The time to first antibiotic switch was measured after identification at 24 hours in the standard group and 17.5 hours in the MALDI-TOF-MS group. This switch resulted in patients receiving appropriate antibiotics in 64.0% of cases compared with 75.3% of cases, respectively[20]. In this study, rapid identification of causative agents allowed providers to prescribe appropriate antibiotics in 11% more patients and decrease inappropriate therapy by just under 10% of patients.

The introduction of MALDI-TOF-MS to the clinical lab has reduced turnaround time while keeping costs low when compared with traditional manual workup[21,22]. The cost of identification on the MALDI-TOF platform is estimated at $0.50 per sample, compared with $1.98 for traditional biochemical identification. Adding the Sepsityper kit for processing blood cultures raises the cost to $5.15 per sample[22]. This cost may recouped by other aspects of patient care, such as decreased stay or more judicious use of antibiotics.

PROJECT GOALS

At the beginning of this project, MALDI-TOF-MS was just becoming available to the clinical microbiology laboratory. Various laboratories were validating identification of colonies of bacteria and working on expanding reference libraries. Some of the first attempts to identify bacteria from blood culture began in 2009[13]. The work by La Scola and Raoult tested two preparation methods for extracting proteins from bacteria in blood cultures. It was discovered that formic acid and acetonitrile extraction improved Gram positive identification to 67% over the 37% achieved with acetonitrile with triflouroacetic acid extraction. With either extraction, Gram negative organisms were already identified correctly almost 90% of the time[13]. This work launched efforts to optimize protein extraction and improve identification rates for blood cultures[14,15,23]. Differential centrifugation to separate red blood cells and ammonium chloride lysis of cells were used to clean up varying amounts of blood culture in order to analyze sample using MALDI-TOF-MS.

This project looked at various aspects of MALDI-TOF-MS technology to evaluate the expansion of use to direct identification of bacteria from blood culture at the University of Washington. Sample processing conditions, limit of detection and workflow were analyzed. A prospective direct identification study was performed on 243 unique aerobic blood cultures. Additionally, an underrepresented organism, *Cryptococcus gattii*, was selected and added to the reference library to show that the technology can grow with the institution and its unique patient population. *Cryptococcus gattii* is an organism endemic to the region that the University of Washington Medical Center serves.

Chapter Two – Evaluating Preanalytical Variables in MALDI-TOF-MS Workflow

Sepsis has been identified as a critical state in which identification information early on can change patient outcomes by influencing antibiotic choices[16,20]. The effect of inappropriate antibiotic treatment has been identified by Kumar *et al.* to have a 7.6% decrease in survival rates in patients with hypotension due to sepsis[16]. Reliable identification of organisms is therefore an important quality of a rapid identification system. Recognizing interferences and preanalytical variables that impact reliability is an important first step in implementing the new technology.

Human and other non-bacterial proteins present in blood culture media can be a source of interference for MALDI-TOF-MS identification, and must be removed prior to analysis. A variety of processing methods have been published, including differential centrifugation to eliminate blood cells, use of clot separator tubes to separate blood cells followed by centrifugation to eliminate any remaining debris, and in-house developed lysis buffers to eliminate red blood cells[14,24–26]. The commercial availability of a dedicated processing kit (Sepsityper, Bruker Daltonics) affords the opportunity for greater standardization between laboratories[27–29]. The Sepsityper kit provides lysis and washing buffers along with mass spectroscopy grade microcentrifuge tubes. Total processing time for a sample is around twenty minutes, and many samples can be batched together. This system was chosen based on work done by Loonen *et al.*, who shown that use of the Sepsityper kit resulted in higher rates of species level identification[25].

Positive blood cultures would ideally be processed for MALDI-TOF-MS immediately after culture positivity occurs. However, because of the technical components and hands-on time involved, batching of samples is more feasible. In many previous studies, processing for MALDI-TOF-MS has occurred within 8 hours of culture positivity. Such a time frame may not be practical for all laboratories. Positive blood cultures are not necessarily removed from incubation on a 24-hour basis in all laboratories, nor is MALDI-TOF-MS necessarily performed on all shifts[25,30]. Also the additional processing time of 20 minutes for the Sepsityper kit necessitates some level of batched work. Therefore, the impact of post-positivity incubation on the performance of MALDI-TOF-MS for organism identification directly from positive blood cultures was investigated.

RESULTS

Limit of detection for MALDI-TOF-MS had previously been determined by inoculating resuspended colonies into sterile blood culture media at known concentrations[31]. Christner *et al.* determined that a concentration of 10^6 cfu/mL was equivalent to sterile media and that concentrations at or above 10^7 cfu/mL resulted in species level identification. However, the study was performed on blood culture media without blood and utilized a stepwise centrifugation method for processing a 3 mL sample. For our study, we wanted to determine the limit of detection in a specimen that more closely mimicked a patient sample. To achieve this, we inoculated bacteria into blood culture media with whole blood and processed 1 mL of sample.

The VersaTREK blood culture system (Trek Diagnostics) uses 0.5uL to 10mL whole blood drawn into 80 mL culture bottles. To determine the limit of detection using the

Sepsityper kit, dilutions of a 3.0 McFarland density suspension of ATCC organisms representing three classes of organisms (Gram negative bacteria, Gram positive bacteria and yeast) were inoculated in to blood culture media containing 5mL of whole blood. Samples were then immediately processed using the Sepsityper kit. It was noted that the resulting cell pellet was much smaller than seen in samples that were allowed to grow to similar densities over time (data not shown). As shown in Table 1, acceptable scores were achieved for *E. coli* and *S. aureus* at a concentration of 1 x 10^8 cfu/mL and 4.5 x 10^7 cfu/mL, respectively. An acceptable identification was not reached for the *C. albicans*. This is primarily due to the fact that yeast cells are larger and therefore an identical density does not result in an equivalent cfu/mL as shown in Table 1.

The implication from these data is that, in general, blood cultures will have to reach a minimum cfu/mL of 4.5 x 10^7 to 10^8 organisms to provide a positive identification. This information was taken into consideration in all future experiments as a potential explanation of low scores.

TABLE 1 - Limit of organism detection for Sepsityper processing. Mean cfu/mL and identification scores were determined for each organism. Samples were run in triplicate. Target concentrations were based on McFarland densities of *E. coli*. Samples were diluted from 3.0 McFarland saline suspension (~10^9 cfu/mL for *E. coli*). Target concentrations were 10^6 cfu/mL, 10^7 cfu/mL, 10^8 cfu/mL. Biomass at the end of Sepsityper processing was much less when compared to a culture allowed to grow up to a similar cfu/mL from a low concentration in a blood culture bottle. Scores above 1.8 show a species identification (green), 1.6-1.8 a genus identification (yellow), and less than 1.6 an unacceptable identification (pink).

Species	10^{-3} of 3.0 McFarland		10^{-2} of 3.0 McFarland		10^{-1} of 3.0 McFarland	
	Average cfu/mL	Average score	Average cfu/mL	Average score	Average cfu/mL	Average score
E. coli	1.1 10^6	0.51±0.87	1.3 10^7	1.48±1.28	1.0 10^8	2.31±0.03
S. aureus	5.3 10^5	0.00±0.00	9.5 10^6	0.76±1.31	4.5 10^7	2.20±0.43
C. albicans	1.3 10^4	0.68±0.96	1.1 10^5	1.32±1.14	1.4 10^6	1.44±1.25

Average scores are based on scores acquired from three replicates for each concentration of microorganism

To evaluate specimen handling and its effects on MALDI-TOF-MS identification, a larger selection of organisms were analyzed at various incubation conditions common to the clinical microbiology laboratory. Ten organisms representing Gram-positive, Gram-negative and anaerobic bacteria as well as yeast were selected. The strains were: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 35657), *Pseudomonas aeruginosa* (ATCC 27853), *Stenotrophomonas maltophilia* (patient isolate), *Bacteroides fragilis* (patient isolate), *Staphylococcus aureus* (ATCC 43300), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecium* (ATCC 51559), *Candida albicans* (ATCC 60193), and *Candida glabrata* (patient isolate).

Blood culture bottles with 5mL of whole blood added were inoculated with approximately 10^2 cfu/mL of the organism to be tested. All bottles were then incubated in the VersaTREK blood culture system (Thermo Scientific, TREK Diagnostic Systems, Cleveland, OH) until growth was detected by the instrument. At this time an aliquot was processed and then subsequent aliquots were held at 4°C, room temperature (RT) or 35°C for 24 hours to simulate samples being removed from the instrument and placed either in a refrigerator, on a bench or left in the instrument. (See Appendices for detailed growth conditions and methods.)

As shown in Table 2, many samples achieved a reliable species identification score (greater than 1.8, indicated in green). However, we observed poor scores for *S. maltophilia* and *B. fragilis*, despite obtaining species level identification scores when colonies of these strains were tested from solid media. *S. maltophilia* achieved only

TABLE 2A – Impact of post-positivity incubation conditions on the identification of microorganisms from blood culture. Mean identification scores of organisms were calculated from values obtained after Sepsityper processing for each post-positivity condition listed below. Each organism was run in triplicate. Species level identification scores are shown in green (≥1.8), genus level identification is indicated in yellow (1.6-1.8), and unacceptable identification in pink (≤1.6).

Organism	Initial processing	4°C, 24h	RT, 24h	35°C, 24h	Solid media
E. coli	2.38 ±0.04	2.24 ±0.14	2.32 ±0.06	2.14 ±0.01	2.18 ±0.12
K. pneumoniae	2.23 ±0.06	1.99 ±0.23	2.34 ±0.05	2.26 ±0.08	2.26 ±0.09
P. aeruginosa	1.96 ±0.62	2.19 ±0.13	2.38 ±0.06	2.31 ±0.08	2.32 ±0.03
S. maltophilia	1.78 ±0.57*	1.16 ±0.06*	1.76 ±0.27*	1.75 **	2.15 ±0.03
B. fragilis	1.47 **	1.66 ±0.81*	2.07 ±0.34*	1.91 ±0.61*	2.17 ±0.20
S. aureus	2.29 ±0.09	2.23 ±0.07	2.32 ±0.01	2.35 ±0.02	2.05 ±0.11
S. epidermidis	2.18 ±0.02	2.10 ±0.19	2.20 ±0.12	2.11 ±0.03	1.95 ±0.14
E. faecium	2.35 ±0.10	2.33 ±0.07	2.40 ±0.03	2.36 ±0.03	2.22 ±0.30
C. albicans	1.65 ±0.16	1.75 ±0.21	1.87 ±0.21	2.00 ±0.20	2.22 ±0.05
C. glabrata	1.44 ±0.01	1.41 ±0.26*	1.84 ±0.04	1.85 ±0.40	2.32 ±0.05

* One replicate generated no spectra, average of remaining two replicates.

** Two replicates generated no spectra, value for one replicate.

\+ P-value 0.005 between initial processing and hold condition, using Student's two-tailed paired T-test on the unaveraged data.

TABLE 2B - Colony counts for post-positivity incubation of microorganisms in blood culture. Range of colony counts from replicates for organisms. Blood cultures were held at each post-positivity condition listed below.

Organism	Initial processing	4°C, 24h	RT, 24h	35°C, 24h
E. coli	$7.3\ 10^{8}$ - $2.0\ 10^{9}$	$8.7\ 10^{8}$ - $1.5\ 10^{9}$	$8.0\ 10^{8}$ - $4.0\ 10^{9}$	$8.0\ 10^{8}$ - $1.4\ 10^{9}$
K. pneumoniae	$4.7\ 10^{8}$ - $5.7\ 10^{8}$	$4.7\ 10^{8}$ - $2.5\ 10^{9}$	$6.0\ 10^{8}$ - $3.7\ 10^{9}$	$5.0\ 10^{6}$ - $2.3\ 10^{9}$
P. aeruginosa	$1.2\ 10^{8}$ - $8.0\ 10^{8}$	$1.0\ 10^{8}$ - $6.3\ 10^{8}$	$5.0\ 10^{8}$ - $2.5\ 10^{9}$	$7.0\ 10^{8}$ - $1.4\ 10^{9}$
S. maltophilia	$3.3\ 10^{7}$ - $7.3\ 10^{7}$	$3.0\ 10^{7}$ - $1.4\ 10^{8}$	$5.7\ 10^{8}$ - $1.5\ 10^{9}$	$1.6\ 10^{8}$ - $2.3\ 10^{8}$
B. fragilis	$1.6\ 10^{9}$ - $5.0\ 10^{9}$	$1.2\ 10^{9}$ - $5.3\ 10^{9}$	$2.9\ 10^{9}$ - $6.0\ 10^{9}$	$4.0\ 10^{9}$ - $9.3\ 10^{9}$
S. aureus	$2.3\ 10^{6}$ - $4.0\ 10^{7}$	$4.0\ 10^{7}$ - $1.1\ 10^{8}$	$8.7\ 10^{7}$ - $4.3\ 10^{8}$	$4.7\ 10^{7}$ - $1.7\ 10^{8}$
S. epidermidis	$5.7\ 10^{6}$ - $2.3\ 10^{8}$	$2.3\ 10^{7}$ - $9.7\ 10^{7}$	$1.4\ 10^{8}$ - $1.0\ 10^{9}$	$1.5\ 10^{8}$ - $4.7\ 10^{8}$
E. faecium	$1.1\ 10^{8}$ - $1.2\ 10^{9}$	$1.3\ 10^{8}$ - $4.7\ 10^{8}$	$4.7\ 10^{8}$ - $2.0\ 10^{9}$	$5.3\ 10^{8}$ - $7.3\ 10^{8}$
C. albicans	$1.7\ 10^{6}$ - $2.7\ 10^{6}$	$2.1\ 10^{6}$ - $5.0\ 10^{6}$	$8.7\ 10^{6}$ - $2.3\ 10^{7}$ *	$9.0\ 10^{6}$ - $1.6\ 10^{7}$ *
C. glabrata	$4.3\ 10^{6}$ - $3.0\ 10^{7}$	$2.3\ 10^{5}$ - $1.3\ 10^{7}$	$4.0\ 10^{6}$ - $9.3\ 10^{7}$	$6.3\ 10^{6}$ - $7.7\ 10^{7}$

* P value 0.05 between initial processing and room temperature. P-value = 0.02 for 35°C, using Student's two-tailed paired T-test on individual data points.

unreliable and genus level identification from blood culture, with the scores at 4°C being significantly different from solid media (p-value <0.01). Despite having low scores, *B. fragilis* did not show statistically lower scores at 4°C, room temperature or 35°C when compared to solid media. Statistics were unable to be performed with respect to the initial processing, because only one run generated spectra. Interestingly, both organisms were noted to have smaller pellets after Sepsityper processing than the other organisms tested. Both *Candida* species tested produced lower scores at both initial processing and when held at 4°C for 24 hours. However, identification scores improved when aliquots were held at room temperature and 35°C (Table 2A). For *C. albicans*, the improvement in scores between initial processing and room temperature was not statistically significant (p-value = 0.39). For *C. glabrata*, the improvement between initial processing and room temperature was statistically significant (p-value = 0.005). When the scores are taken into consideration with the cfu/mL for each condition, it was noted that the improvement in scores for *C. albicans* may be attributable to the increase in organisms between the two conditions.

To further explore the phenomenon observed with the *Candida* species, we expanded the study to include additional patient isolates. Three patient isolates were selected for each of the four most commonly isolated *Candida* species. Isolates were inoculated into blood culture bottles and evaluated under the conditions tested above. Identification improvement was defined as the transition from an “unreliable” to a “genus level” or a “genus level” to a “species level” identification score. Replicates that did not generate spectra were not considered in the final evaluation of the data. *Candida albicans* and *C.*

tropicalis showed species level identification for all isolates at initial processing and at post-positivity incubation in 2 of 3 isolates (Table 3A). For one isolate of *C. albicans,* the 4°C incubation had a decreased score, but it was not statistically significant (p-value = 0.17). The decrease in score for *C. tropicalis* isolate 11 is significant across all conditions when compared to the initial processing score (p-value = 0.009 for 4°C, 0.03 for room temperature, and 0.05 for 35°C). One of three *C. parapsilosis* isolates showed some improvement in identification scores between initial processing and post-positivity incubation. *C. glabrata* showed some improvement in scores between initial processing and post-positivity incubation at room temperature or 35°C in two isolates. For *C. glabrata* isolate 4, the difference in scores between initial processing and room temperature incubation is statistically significant even though there is no score improvement as defined above (p-value 0.05). None of the *C. glabrata* isolates showed score improvement at 4°C incubation. Colony forming units per milliliter were also counted. *Candida glabrata* had slightly higher cfu/mL at initial processing than *C. albicans and C. parapsilosis* (p-value 0.02 and 0.03 respectively), but not *C. tropicalis*. No difference was seen between the other species at initial processing. *C. glabrata* isolate 4 showed a significant difference between cfu/mL at initial processing and 35°C post-positivity incubation (p-value 0.01). No other isolates showed a significant difference between cfu/mL at initial processing and any of the post-positivity incubation conditions.

TABLE 3A - Impact of post positivity incubation conditions on the identification of *Candida* species from blood culture. Mean identification scores of *Candida* species isolated from clinical were calculated from values obtained after Sepsityper processing for each post-positivity condition listed below. Each isolate was run in triplicate. Species level identification scores are shown in green (≥1.8), genus level identification is indicated in yellow (1.6-1.8), and unacceptable identification in pink (≤1.6).

Isolate Number	*Candida* species	Initial processing	4°C, 24hr	RT, 24hr	35°C, 24hr
1	*C. albicans*	2.01 ± 0.14	1.77 ± 0.24	1.89 ± 0.16	2.16 ± 0.05
2	*C. albicans*	1.83 ± 0.10	1.91 ± 0.12	1.98 ± 0.17	2.21 ± 0.03[+]
3	*C. albicans*	1.96 ± 0.09*	1.92 ± 0.09	2.05 ± 0.04	2.16 ± 0.06
4	*C. glabrata*	1.86 ± 0.10*	1.81 ± 0.26*	2.22 ± 0.05[+]	2.21 ± 0.18
5	*C. glabrata*	1.73 ± 0.26	1.64 ± 0.12	2.02 ± 0.20*	2.07 ± 0.22*
6	*C. glabrata*	1.75 ± 0.23	1.63 ± 0.07	1.91 ± 0.16	2.16 ± 0.11
7	*C. parapsilosis*	1.74 ± 0.46	1.90 ± 0.09	2.00 ± 0.10	2.08 ± 0.10
8	*C. parapsilosis*	1.96 ± 0.16*	2.08 **	2.08 ± 0.03	2.13 ± 0.08
9	*C. parapsilosis*	1.99 ± 0.06	1.93 ± 0.27	2.01 ± 0.25	2.22 ± 0.08
10	*C. tropicalis*	2.20 ± 0.07	1.94 ± 0.12	1.81 ± 0.20[+]	2.10 ± 0.01
11	*C. tropicalis*	2.12 ± 0.05	1.82 ± 0.01[+]	1.70 ± 0.15[+]	1.95 ± 0.06[+]
12	*C. tropicalis*	2.16 ± 0.07*	1.86 ± 0.17	1.94 ± 0.06	2.21 ± 0.05

* One replicate generated no spectra, average of remaining two replicates

** Two replicates generated no spectra, value for one replicate

\+ P value ≤0.05 between initial processing and hold condition, using Student's two-tailed paired T-test on unaveraged data.

TABLE 3B - Colony counts for post-positivity incubation of *Candida* species from blood culture. Range of colony counts (cfu/mL) from replicates of *Candida* species. Cultures were incubated post-positivity under the conditions listed below.

Isolate Number	*Candida* species	Initial processing	4°C, 24hr	RT, 24hr	35°C, 24hr
1	*C. albicans*	3.7 10^4 – 3.0 10^6	8.3 10^4 – 5.0 10^6	9.3 10^4 – 7.0 10^6	3.0 10^4 – 3.0 10^7
2	*C. albicans*	3.0 10^5 – 4.0 10^6	2.3 10^5 – 1.8 10^6	1.7 10^6 – 6.7 10^6	1.7 10^6 – 3.7 10^6
3	*C. albicans*	3.0 10^5 – 1.7 10^6	3.3 10^5 – 1.7 10^6	1.6 10^6 – 9.0 10^6	1.6 10^7 – 5.0 10^7
4	*C. glabrata*	1.7 10^6 – 7.0 10^6	3.0 10^6 – 1.7 10^7	7.3 10^6 – 3.3 10^7	5.0 10^7 – 6.7 10^7
5	*C. glabrata*	1.3 10^6 – 1.0 10^7	3.3 10^6 – 1.1 10^7	1.3 10^7 – 2.3 10^7	6.0 10^7 – 2.3 10^8
6	*C. glabrata*	1.4 10^6 – 1.8 10^7	1.7 10^6 – 2.3 10^7	5.7 10^6 – 4.7 10^7	2.3 10^7 – 1.3 10^8
7	*C. parapsilosis*	1.0 10^6 – 4.3 10^6	2.7 10^6 – 3.3 10^6	3.0 10^6 – 7.0 10^6	2.1 10^7 – 7.3 10^7
8	*C. parapsilosis*	7.3 10^5 – 2.0 10^6	1.1 10^6 – 1.7 10^6	1.7 10^6 – 4.0 10^6	2.3 10^7 – 5.3 10^7
9	*C. parapsilosis*	1.7 10^6 – 2.0 10^6	3.0 10^5 – 2.3 10^6	2.7 10^6 – 7.3 10^6	3.0 10^7 – 5.0 10^7
10	*C. tropicalis*	3.0 10^6 – 2.0 10^7	1.7 10^6 – 1.0 10^7	2.7 10^6 – 2.3 10^7	2.2 10^7 – 6.0 10^7
11	*C. tropicalis*	1.5 10^6 – 3.3 10^6	1.6 10^6 – 3.3 10^6	9.0 10^6 – 1.0 10^8	2.4 10^7 – 1.0 10^8
12	*C. tropicalis*	1.3 10^6 – 4.3 10^6	2.0 10^6 – 3.3 10^6	3.3 10^6 – 7.3 10^7	1.7 10^7 – 1.0 10^8

Because *C. glabrata* showed improvement in scores in two isolates from the yeast experiment and the isolate from the original post-positivity experiment, a time course was set up to explore how quickly the improvement was seen (Figure 1). Bottles were held at 35°C after positivity and sampled every 8 hours. At initial positivity the original isolate "O" and isolates 4 and 5 from the yeast experiment gave unreliable scores on average, while isolate 6 gave a genus level score. All four isolates scored high enough to be identified to species level after 8 hours of incubation at 35°C. This improvement in scores is unlikely to be due to an increase in colony counts. No significant difference was seen in cfu/mL between the time points Time 0 to 8 hours ($p = 0.10$), Time 8 to 16 hours ($p = 0.19$), Time 16 to 24 hours ($p = 0.16$) (Table 4).

It could be hypothesized that the improvement in scores would be associated with a higher match with the library strains. In Figure 2, the sample spectrum is displayed on the top with the best reference library match in blue underneath. Each peak is represented in red, yellow or green depending how well it matched the top reference spectrum peaks. The second line is the raw spectra used for analysis. We can see that the spectra differ in the later samples when compared with the initial processing. This is especially apparent in the 1000 to 5000 Da m/z range. The baseline flattens over time and there is less overall signal-to-noise interference on observation of the spectra.

FIGURE 1 – Identification scores for *Candida glabrata* over time. Patient isolates were grown up in blood culture and incubated at 35°C post-positivity. Samples were taken every eight hours and processed using the Sepsityper kit. The original patient isolate used for the post-positivity incubation is designated (O). Isolates from the yeast experiments are marked with the same designation as before (4, 5, 6). Genus level score is marked with an orange dashed line. Unreliable scores fall at or below the pink dashed line.

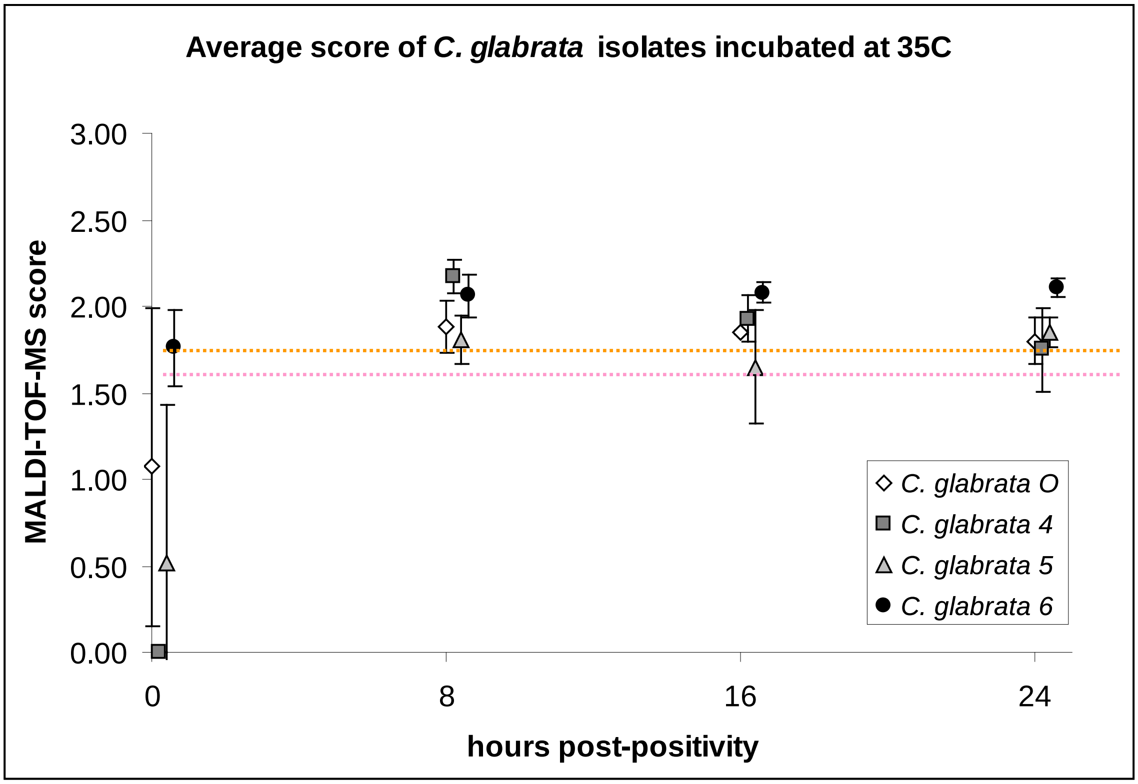
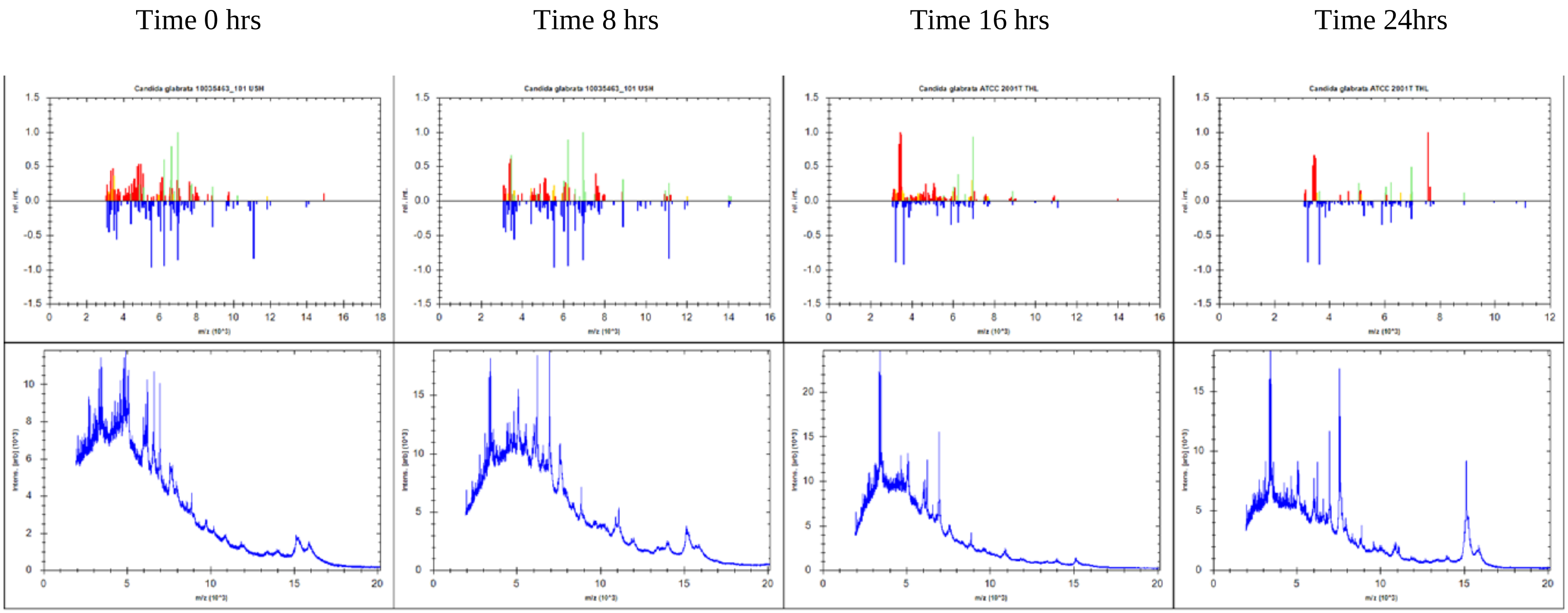


TABLE 4 Range of colony counts for time course of *Candida glabrata* identification scores. *Candida glabrata* isolates were held at 35°C post-positivity and sampled every 8 hours.

No significant difference is seen in average cfu/mL between 8 hour time points using Student's two-tailed paired T-test. Time 0 to 8 hrs (p = 0.10), Time 8 to 16 hrs (p = 0.19), Time 16 to 24 hrs (p = 0.16).

Isolate	Initial processing	35°C, 8 hr	35°C, 16 hr	35°C, 24 hr
O	3.67 10^{6} – 1.03 10^{7}	4.67 10^{7} – 7.00 10^{7}	4.33 10^{7} – 5.67 10^{7}	2.67 10^{7} – 5.67 10^{7}
4	2.67 10^{6} – 4.67 10^{6}	1.00 10^{7} – 2.33 10^{7}	1.67 10^{7} – 3.67 10^{7}	1.00 10^{7} - 1.33 10^{7}
5	6.00 10^{5} – 3.33 10^{6}	1.37 10^{7} – 3.00 10^{7}	4.00 10^{7} – 6.33 10^{7}	8.00 10^{6} – 4.33 10^{7}
6	6.00 10^{6} – 7.67 10^{6}	1.00 10^{7} – 3.00 10^{7}	3.00 10^{7} – 5.67 10^{7}	3.33 10^{7} – 7.33 10^{7}

FIGURE 2 Library comparisons of *Candida glabrata* spectra over time. Identification matches and raw spectra for one representative run on the original isolate of *Candida glabrata* shown below. The spectrum from the unknown sample is seen in the top portion of the identification match, with the closest library match in blue extending below the axis. Green peaks indicate major peaks that match exactly, yellow indicates key peaks that are not matched in intensity, unmatched peaks are indicated in red. The raw spectra, shown in the second row of figures, shows a change in spectrum appearance, especially between 1 and 5 kDa, over time.


Time 0 hrs

Time 8 hrs

Time 16 hrs

Time 24hrs

DISCUSSION

The limit of detection for organism identification by MALDI-TOF-MS directly from positive blood cultures was previously determined by Christner *et al*. to be 10^7 CFU/ml, with concentrations of 10^6 CFU/ml shown to be equivalent to sterile media[31]. This was determined by inoculating Bactec Plus Aerobic/F bottles without blood (BD, Frankin Lakes, NJ) with desired concentrations of laboratory strains (*E. coli* DH5α and *S. aureus* Newman), followed by processing a 6 mL sample using stepwise centrifugation.

In this study, we determined the limit of detection by simulating a patient sample as much as possible. Specimens were inoculated into blood culture media compatible with the VersaTREK blood culture system containing 5 mLs of whole blood, according to the manufacturer's recommendations. 1 mL of this mixture was then processed according to the Sepsityper protocol. The limit of detection for the Sepsityper kit in the presence of human blood is $5x10^7$ to 10^8 cfu/mL in this study. This sample size resulted in a cell pellet of approximately 0.5 μL volume. This is greatly reduced from the pellets of approximately 20 μL that we observe in bottles that are allowed to incubate and turn positive in a blood culture incubation system (data not shown). It is possible that this reduction in biomass could be due to the fact that the only bacteria present in the limit of detection study were the viable cells that achieved the desired density. In the post-positivity incubation, microorganisms were allowed to go through multiple generations and so some of the biomass observed could have been non-viable. Human blood cells have also been described as becoming part of the protein extraction if not fully lysed beforehand. Despite the limit of detection study, we observed valid species level identifications for both bacteria and yeast that grew 2×10^6- 5×10^6 cfu/mL in a number of instances (Table 2B). The inclusion of additional centrifugation and washing steps prior to Sepsityper processing was previously shown to yield a limit of detection of 5.9 $x10^5$ cfu/mL for *Candida* species[32]. The practical limit of detection for Sepsityper processing may therefore be lower than for differential centrifugation methods or spiked sample methods.

As shown in Table 2A, valid identification scores for *S. maltophilia* and *B. fragilis* were infrequently obtained, despite both organisms being identified to the species level when tested directly from colonies grown on solid media. This was evident by how many replicates failed to generate spectra, and by the average score for different incubation conditions. Even though the concentration of organisms present at the time of processing was comparable to the other bacteria tested (Table 2B), dramatically decreased pellet sizes were observed after incubation in Sepsityper lysis buffer (data not shown). These bacterial strains may interact with the lysis buffer in an unforeseen way and no longer be intact after treatment. Further work is needed to fully elucidate the issues with processing both *S. maltophilia* and *B. fragilis*, including analysis of other strains for both organisms.

The identification of *S. maltophilia* and *B. fragilis* by MALDI-TOF-MS directly from positive blood cultures has been variable in previous studies[13,23,29,33]. Collectively a variety of processing methods, including both the Sepsityper kit and in-house developed extraction methods, were employed and a relatively small number of samples were examined. Interestingly, the presence of elevated leukocyte numbers has previously been shown to correlate with decreased pellet sizes upon completion of Septityper processing[34]. The thought is that the free DNA from leukocytes prevents the bacteria from forming a pellet during centrifugation, and they are removed with the supernatant during processing. This is unlikely to account for our observations, as all blood in this study was obtained from healthy volunteers. Total colony forming units in the original sample is also an unlikely cause, as both *B. fragilis* and *S. maltophilia* both were present at higher levels than the limit determined in the spiking study. The optimal processing method for identification of *S. maltophilia* and *B. fragilis* directly from positive blood cultures by MALDI-TOF-MS is therefore unknown and requires further investigation with more strains and different processing techniques.

For all remaining bacteria in the study, scores of ≥2.0 were generally obtained subsequent to all post-positivity incubation conditions, including 4°C. Interestingly, this latter condition is generally considered to be deleterious to the identification of microorganisms by MALDI-TOF-MS (Dr. GongYi Shi, Bruker Daltonics, personal communication). Bacterial identification scores were generally improved following post-positivity incubation at either room temperature or 35°C, although this difference was not statistically significant ($p=0.13$ and $p=0.16$, respectively).

The identification of yeast directly from positive blood cultures by MALDI-TOF-MS is known to be problematic[32,35–37]. However, we observed improvements to the identification scores of yeast following post-positivity incubation at both room temperature and 35°C in our original experiment. In the case of *C. albicans*, this improvement most likely resulted from an increased concentration of organisms present after post-positivity incubation (RT: p=0.05, 35°C: p=0.007). In contrast, improved identification scores for *C. glabrata* were not accompanied by concomitant increases in organism concentrations (RT: p=0.32, 35°C: p=0.22).

To follow up on this, *Candida* species isolated from patients were processed as for the original ten organisms. Overall the trend of improved scores after room temperature or 35°C incubation held. After initial processing 9 of 12 *Candida* isolates had species level scores. With 24 hour incubation at 4°C, 9 strains had species level scores and at room temperature or 35°C, 11 and 12 isolates achieved species level scores, respectively. It was observed that the *C. glabrata* isolates continued to have lower scores at 4°C. These data suggest that the score improvement after additional post-positivity incubation may be limited to certain species of *Candida* and does not appear to be a feature of the genus as a whole. To further explore the degree of additional incubation time needed to achieve a score for species identification, a time course using the four *C. glabrata* isolates was set up (Figure 1). When bottles were held at 35°C, score improvements were seen in as little as 8 hours. This did not appear to be due to an increase in cellular concentration as there was no statistical difference between cfu/mL at one time point compared to the next subsequent time point. P values were determined by two tailed paired Student's T-test and showed the following: Time 0 to 8 hours (p = 0.10), Time 8 to 16 hours (p = 0.19), Time 16 to 24 hours (p = 0.16). In Figure 2, one representative run of the original *Candida glabrata* isolate is shown. A change in spectral appearance is observed by the increase in distinct peaks and smoothing of the baseline over the incubation times. For isolates 4 and 5, the increased incubation appeared to have a detrimental effect (Figure 1, squares and triangles respectively). It is believed that this is due loss of biomass during processing, since the same bottle was sampled over the course of 24 hours. It could also be that as these strains grow, they do not produce the exact pattern of proteins in the concentrations found in the reference strains making up the library. This change in protein expression would result in a lower identification score, since the algorithm used to match spectra looks at both m/z ratios and intensity of key spectral peaks.

Chapter Three – Evaluation of direct identification of bacteria from blood culture by MALDI-TOF MS

Having extensively analyzed the preanalytical variables that could affect the performance of MALDI-TOF-MS for blood culture identification, we evaluated the methodology prospectively. The goal is to implement this technology in the clinical microbiology laboratory. This would further decrease time to identification for microorganisms isolated from septic events. Providers could use this information to better direct patient care.

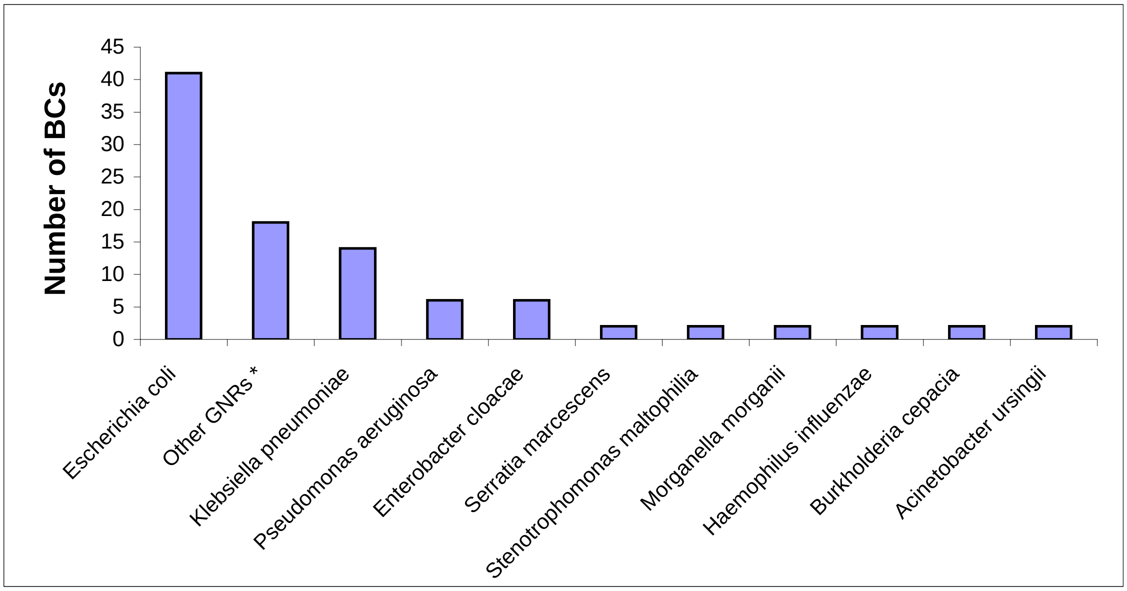
243 positive non duplicate blood cultures were accrued over a period of six months. Experimental aliquots were taken from excess sample material that was collected as part of routine clinical care. 105 were positive for Gram negative bacilli, 123 were positive for Gram positive cocci, 3 positive for Gram positive bacilli, and 7 were positive for yeast. Five samples were excluded due to processing errors. MALDI-TOF-MS identification was compared to conventional identification. Conventional identification consisted of a combination of MALDI-TOF identification of a subcultured colony by direct smear, biochemical reactions, PCR, and molecular identification.

RESULTS

Ninety seven of the one hundred and five blood cultures positive for Gram negative bacilli were monomicrobial. The majority of these were identified as *Escherichia coli* by conventional methods, followed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Figure 3). Eighty-four of these monomicrobial cultures were identified by mass spectrometry to the species level. Eighty-one of the eighty-four were concordant with the conventional identification (96.4%). The three that were discordant were an *E. coli* that was misidentified as *Raoultella terrigena* with a score of 2.20 on the microflex platform and two *Enterobacter cloacae* that were mis-identified at the species level, as an *E. koseri* and *E. asburiae*, with scores of 2.15 for both isolates.

An additional three cultures were identified to genus confidence and were concordant with the conventional identification. Ten samples fell below the reliable score cutoff of 1.6, including those samples that generated no spectra. Three of those that fell below the cutoff were considered to be correct. Two isolates matched the final reported species by conventional methods, while one of the samples was identified only as “fastidious Gram negative rods” and was called *Cardiobacterium hominis* by MALDI-TOF-MS. Five samples generated no spectra, and were designated with scores of 0.00.

FIGURE 3 Distribution of Gram negative rods identified by conventional workup. GNRs identified by conventional methods in this study. 27 total species were identified in 97 samples.

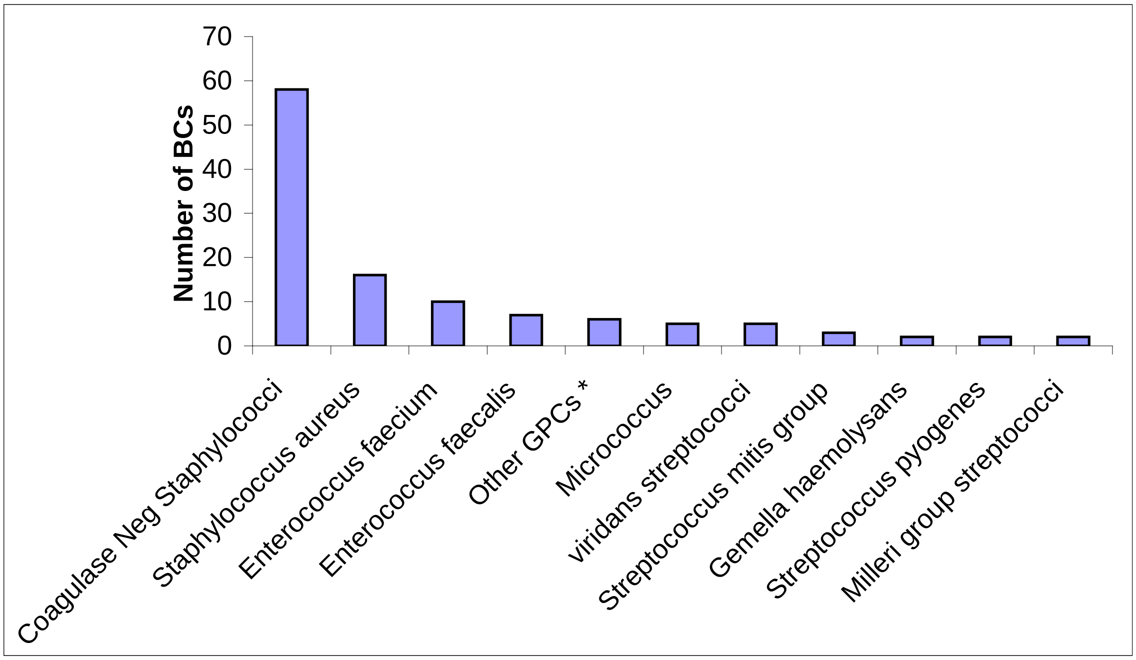


* Other GNRs included *Acinetobacter baumannii, Bacteroides fragilis, Campylobacter jejuni, Chryseobacterium indologenes, Citrobacter koseri, Enterobacter asburiae, Haemophilus parainfluenzae, Klebsiella oxytoca, Leptotrichia sp., Pantoea sp., Proteus mirabilis, Providencia stutzeri, Pseudomonas mendocina,* and *Serratia liquefaciens.*

One hundred sixteen of the one hundred twenty-three cultures positive for Gram positive cocci were monomicrobial. *Staphylococcus* species and *Enterococcus* species were most commonly identified in this study by conventional methods. Ninety-eight of these monomicrobial cultures were identified to the species level confidence by mass spectrometry with scores of ≥1.8. Ninety-three of the ninety-eight were concordant with the conventional identification (94.9%). The five that were discordant included a *S. aureus* that was mis-identified as *S. epidermidis* with a score of 2.21 by MALDI-TOF-MS. The remaining four were called *S. pneumoniae* and identified as *Streptococcus mitis* group or viridans streptococci by traditional methods.

An additional nine cultures were identified to genus level confidence and eight were concordant with the conventional identification. Eight samples fell below the reliable score cutoff of 1.6. Interestingly four of these were correct when compared with the conventional identification. A total of four isolates generated no spectra and were designated with scores of 0.00.

FIGURE 4 Distribution of Gram positive cocci identified by conventional workup. GPCs identified by conventional methods in this study. 19 total species were identified in 116 cultures.



* Other GPCs include *Enterococcus gallinarum, Granulicatella adiacens, Rothia mucilaginosa, Streptococcus agalactiae, Group G streptococci,* and *Streptococcus pneumoniae*.

The remaining monomicrobial cultures represented 3 gram positive bacilli and 7 yeasts. All gram positive bacilli scored above the genus cutoff score of 1.6. This was concordant with conventional workup, which stopped at genus identification. Of the yeasts, 4 scored above the reliable cutoff of 1.6 and were in agreement to the species with the conventional identification, and 3 fell below the cutoff score.

The samples that were determined to be polymicrobial were analyzed in two ways. First, it was determined if the bacteria identified by mass spectrometry matched with any one of the organisms identified by conventional workup. In 11 of 12 cases (91.7%) a species identification of greater than 1.8 was made for one of the organisms reported. The discordant culture called *S. pneumoniae* and was identified as mixed viridans streptococci and coagulase-negative streptococci by traditional methods. An additional culture had a correct identity to the genus level. The remaining three cultures fell below the cutoff score of 1.6. The second method of analyzing these data was to determine how many identifications were made. The final reports for these cultures identified a total of 34 organisms in the sixteen cultures. As previously stated, 12 organisms were identified concordantly using mass spectrometry. In this case only 35.5% of the total number of organisms was identified by mass spectrometry.

In an effort to determine whether or not the cutoff score could be lowered, concordance to the species level was analyzed. Lowering the cutoff score for species level identification could increase the cultures that can be reported without additional testing or culturing to confirm identification. Using a 1.6 cutoff score, 185 of 194 (95.4%)

monomicrobial cultures agreed to the species with conventional workup. For the 222 total monomicrobial cultures, 200 were correctly identified to the species level (90.1%). The 22 discordant results include those samples that generated no spectra (7) and those that were addressed above (9). In addition, six of the discrepant cultures fell below the 1.6 score cutoff. When discounting those that generated no spectra, agreement increases to 93.2%. Because no data were generated with these samples, it can be assumed that these seven cultures would either be re-extracted or identified by alternative means.

DISCUSSION

Overall, the Bruker microflex platform accurately identified organisms directly from blood culture using the lowered 1.6 score cutoff for species level confidence when compared to conventional methods used in our laboratory. This study demonstrated that the identification of Gram positive cocci was excellent using MALDI-TOF mass spectrometry. These organisms were correctly identified the species level (>1.8) 94.9% of the time. In contrast, previous studies have achieved species identification in only 53-76% of cultures[28,33,38]. This improvement could be due to the extra wash step recommended by Bruker (Dr. GongYi Shi, Bruker Daltonics, personal communication). Extra washes have been shown to improve the identification of yeast when using MALDI-TOF-MS[32,39]. Gram negative rods were also identified to species level at a higher frequency than previously reported. This study identified organisms correctly to the species level 96.4%, compared with 77-91%[28,33,38].

It could be argued that the *Enterobacter* species that were miscalled are part of the *E. cloacae* complex.

Limitations of this method for identification involve mixed cultures. Polymicrobial cultures are a recognized limitation because of the blended spectrum that results from the different organisms. This is especially problematic with organisms that Gram stain similarly, since the technologist is given no indication to look for mixed results on the mass spectrometer. We found that the predominant organism can be identified easily in these polymicrobial cultures. The other organisms are not reported, nor is there a hint that there might be a mixture, because the peaks in the generated spectra are not identified specifically. However, previous studies have demonstrated improvements in the performance of this technology. Chen *et al.* recently showed that by examining the top 10 matches, it is possible to determine the composition of the mixed culture about 20% of the time[30]. This limitation is not unique to MALDI-TOF-MS. Other new methods for rapid identification, such as Nanosphere, a microarray based platform, also have difficulties with polymicrobial cultures[40]. For the microarray methodology, multiple organisms can prevent target DNA from being detected because of low concentrations. In the meantime, subcultures of blood culture bottles should be continued in the laboratory to ensure a culture is not mixed. In this way, a preliminary identification could be put out rapidly and followed up with colony identification, should the sample be polymicrobial.

Another limitation is the similar protein profiles of some organisms. In this study we especially found *Streptococcus mitis* group frequently being misidentified as

Streptococcus pneumoniae. This misidentification is a known error and is listed in the Bruker Biotyper manual and on the results page of the software. In this case, direct bile solubility could be performed to differentiate these samples. The other major platform, Vitek MS (Biomeriuex, Durham, NC) has made strides in this area. In a study by Kärpänoja *et al*., *Streptococcus* species were correctly identified from blood culture 75% of the time on the Bruker MALDI Biotyper platform and 97% of the time using the bioMerieux Vitek MS[41]. There are other organisms that come with programmed alerts, such as *E. coli* and *Shigella* species and *Legionella* species. These alerts indicate that additional testing may be necessary due to the similarity of spectra that can be generated. Because the analysis and classification of samples is based on the majority proteins that are present that have distinct mass/charge ratios, this will continue to be a limitation until a unique peak is determined for each species.

Chapter Four – Improving identification of *Cryptococcus gattii* by developing a custom library

One of the biggest limitations of MALDI-TOF-MS technology is the number of organisms in the reference library. In the Bruker Biotyper software, this depth of coverage can be easily seen by how many reference spectra are available to test against. Common organisms such as *E. coli* are represented by multiple reference strains curated by many independent laboratories. Other organisms, such as *Leptotrichia* and *Salmonella*, are represented by one or two main spectra. These main spectra are made up of an average of individual spectra taken from the same sample extract multiple times. In some cases, there are only three or four individual spectra that are averaged, compared to the recommended 20 spectra (Dr. GongYi Shi, Bruker Daltonics, personal communication). By averaging more individual spectra, the noise should be averaged out and the important peaks should become more evident because of their presence in each replicate spectrum. *Cryptococcus gattii* was selected as an organism in which the depth of coverage should be improved because of the University of Washington's location in the Pacific Northwest, an area were *C. gattii* is endemic, and because of its status as a reportable organism.

C. gattii is an emerging pathogen that needs to be distinguished from other *Cryptococcus* and yeast infections. Immunocompetent patients show cryptococcomas in the lung or brain. These cryptococcomas have poor drug penetration that result in long treatment courses[42]. Diversifications of subspecies, especially in *C. gattii* have led to less conventional clinical presentations due to hyper-virulence of strains from the United States. Before the outbreak on Vancouver Island, British Columbia, the difference between *C. gattii* and the next closest species, *C. neoformans,* was thought to be an acute focal presentation needing prolonged treatment or surgical intervention. With the emergence of the VGIIa-c subspecies in 1999, azole resistance and mortality have also become important considerations to patient care. Both an increase in *in vitro* minimum inhibitory concentration of azoles and refractory response to clinical treatment have been observed. Otherwise healthy individuals may have cryptococcosis caused by *C. gattii* VGI and VGII strains[43].

The current gold standard for differentiating *Cryptococcus* subspecies for both *C. neoformans* and *C. gattii* is Multi Locus Sequence Typing (MLST)[44]. This method uses standardized primers that have been established for the following genes: CAP59, GPD1, LAC1, PLB1, SOD1, and URA5. These genes are present in all eight molecular types of these *Cryptococcus* species and are variable enough to classify isolates into their respective subspecies. MLST is currently performed in regional reference laboratories. The aims of this project were to increase representation of *C. gattii* in a custom library to improve MALDI-TOF-MS identification and to test the ability of this technology to differentiate the subspecies VGI-VGIII of *Cryptococcus gattii*.

One of the main reasons for subtyping *Cryptococcus gattii* is to track the spread of subtypes for epidemiology. The Vancouver, BC outbreak has been specifically tied to the VGIIa and VGIIb molecular types, while VGIIc has been established in Oregon as causing disease[45]. Also in the early 1990s, VGIII was found in samples collected in California[46]. Worldwide, Africa sees more cases of cryptococcosis cause by VGI and

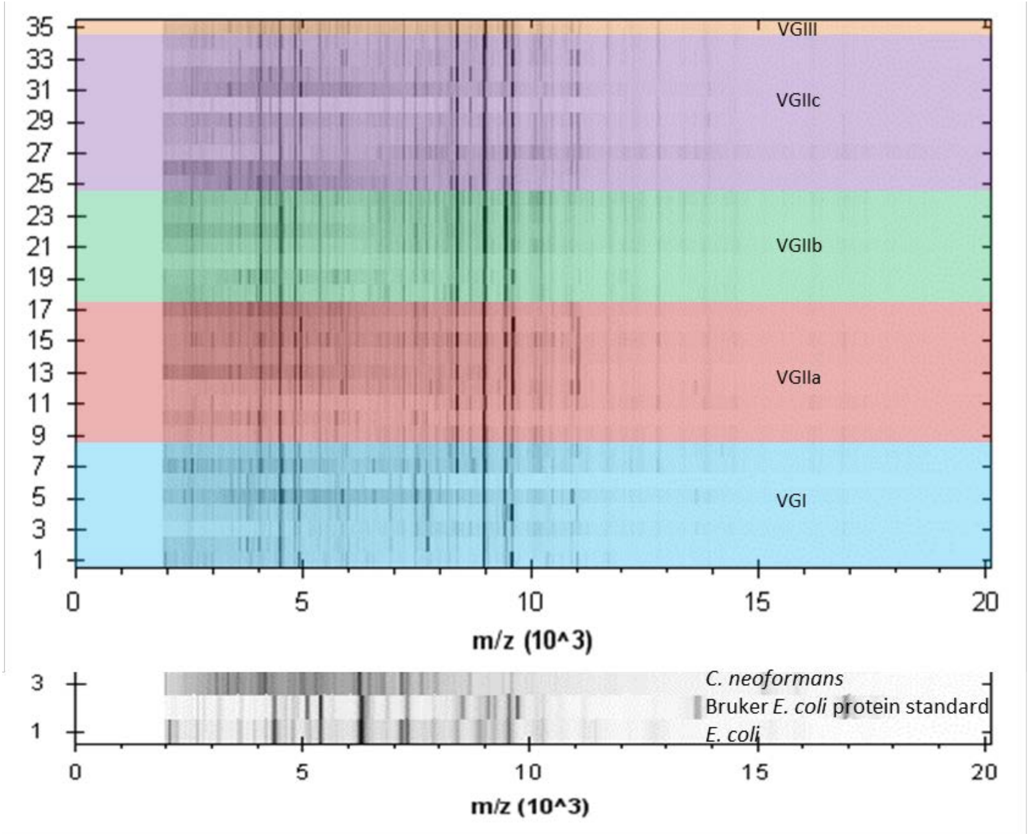
VGIV while Australia has cases linked to VGI and VGII, not a, b or c[47]. Clinical presentation of these molecular subtypes can vary from respiratory illness to meningitis, with differing morbidities associated with each strain and presentation[45,47]. These multiple molecular types also have varying azole resistance patterns. When taking geographic source and molecular type into consideration, a better treatment plan may be achieved[47].

RESULTS

The Bruker Biotyper library had two reference spectra for *Cryptococcus gattii* included in the commercial library. A supplemental reference library for *Cryptococcus gattii* was created using 48 hour growth of colonies grown on Inhibitory Mould Agar to increase depth of coverage for this organism. Colony extracts were run on the MALDI-TOF-MS and individual spectra gathered. A Main SPectra (MSP) representing the average spectra for one strain was collected into a supplemental reference library. 35 strains of *Cryptococcus gattii* with confirmed molecular types made up this library. Strains were provided by the British Columbia Center for Disease Control, Oregon State Public Health and the University of Washington. The molecular types represented were 8 VGI, 9 VGIIa, 7 VGIIb, 10 VGIIc, and 1 VGIII.

The molecular types were visually evaluated on a virtual gel to look for shifts and peaks that would differentiate the molecular types from each other (Figure 5). No obvious m/z shifts were observed in the gel.

FIGURE 5 Virtual gel of *Cryptococcus gattii* MSPs 35 strains representing 5 molecular types were used for the library. Each peak was given a virtual density and represented as a band on the gel. Darker bands indicate more intense peak. For comparison, *C. neoformans* and two *E. coli* sources are shown below.



To evaluate the effectiveness of the supplemental library, new extractions of the 35 strains making up the library were run in duplicate against the Bruker commercial library, followed by the commercial library with the supplemental reference library (Table 5). When identified with the Bruker library, all strains had *Cryptococcus gattii* as their top library match in both runs. Five strains were identified to the species level in each run, using the commercial library. When the supplemental library was added to the Bruker library and the strains identified with the combined database, 33 and 26 strains were identified to the species level. To prevent bias, the spectrum representing the strain being identified was excluded from the combined database. This improvement in identification was reflected in an increased average score for each run. The improvement in scores is statistically significant, p value < 0.01, and reflects the effectiveness of improving representation of a particular organism in the reference library.

TABLE 5 – Identification of *Cryptococcus gattii* strains identified with Bruker Biotyper commercial library versus Bruker Biotyper and supplemental libraries
Spectra were collected from fresh extractions. Saved spectra were reviewed against electronic libraries to eliminate variation between inquiries. The improvement in scores between the two library challenges is statistically significant (p-value <0.01)

	Bruker Biotyper library alone		Bruker Biotyper and supplemental libraries	
	Run 1	Run 2	Run 1	Run 2
Species level identification	5	5	33	26
Genus level identification	24	18	0	0
Unreliable identification	4	3	0	0
Total Strains	33	26	33	26
Average Score*	1.834	1.860	2.493	2.467

*Specimens that failed to generate spectra were excluded.

The strains making up the library were then run against the Bruker commercial library with the supplemental library to determine if molecular types would identify appropriately. All VGI strains that produced spectra identified correctly to the subspecies level (7/7, 1 no spectra). Only one strain of both VGIIa and VGIIb were identified correctly to the subspecies level. Six of seven VGIIc samples were correctly identified to the subspecies level, with one being misidentified as VGIIa and another generating no spectra. The VGIII strain was also correctly subtyped.

To determine why the VGII subspecies were less able to be subspeciated, a dendogram was created with Bruker Biotyper 3.0 software (Figure 6). The dendogram branches are created based on the commercial algorithm that evaluates spectral relatedness. The VGI molecular type formed a distinct clade on the dendogram. The VGIIa-c subtypes along with VGIII did not sort as discretely.

In an effort to increase distinction between subtypes, the library entries were analyzed with Flex Analysis (Bruker) and merged into a composite spectrum for each strain for peak analysis (Figure 7). The top ten peaks were determined for each composite spectrum by relative intensity. Peaks were then analyzed for presence between VG molecular types. Most major peaks were shared between subtypes.

FIGURE 6 Dendogram of *Cryptococcus gattii* subtypes Strains were used to create a reference library. The VGI subtype creates its own distinct clade, while the VGIIa-c and VGIII are not as distinct. It is apparent that there is some clustering by subtype

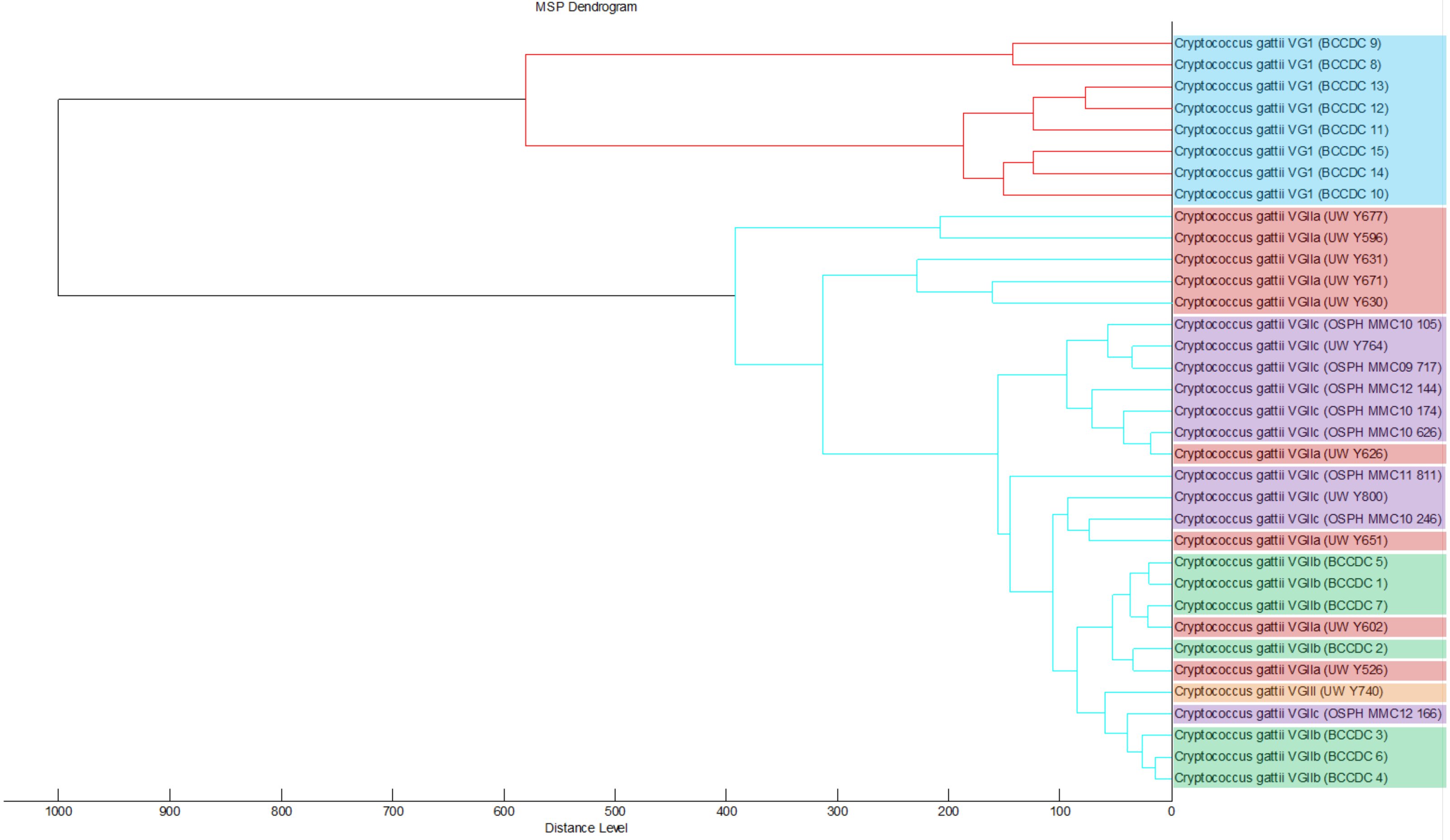
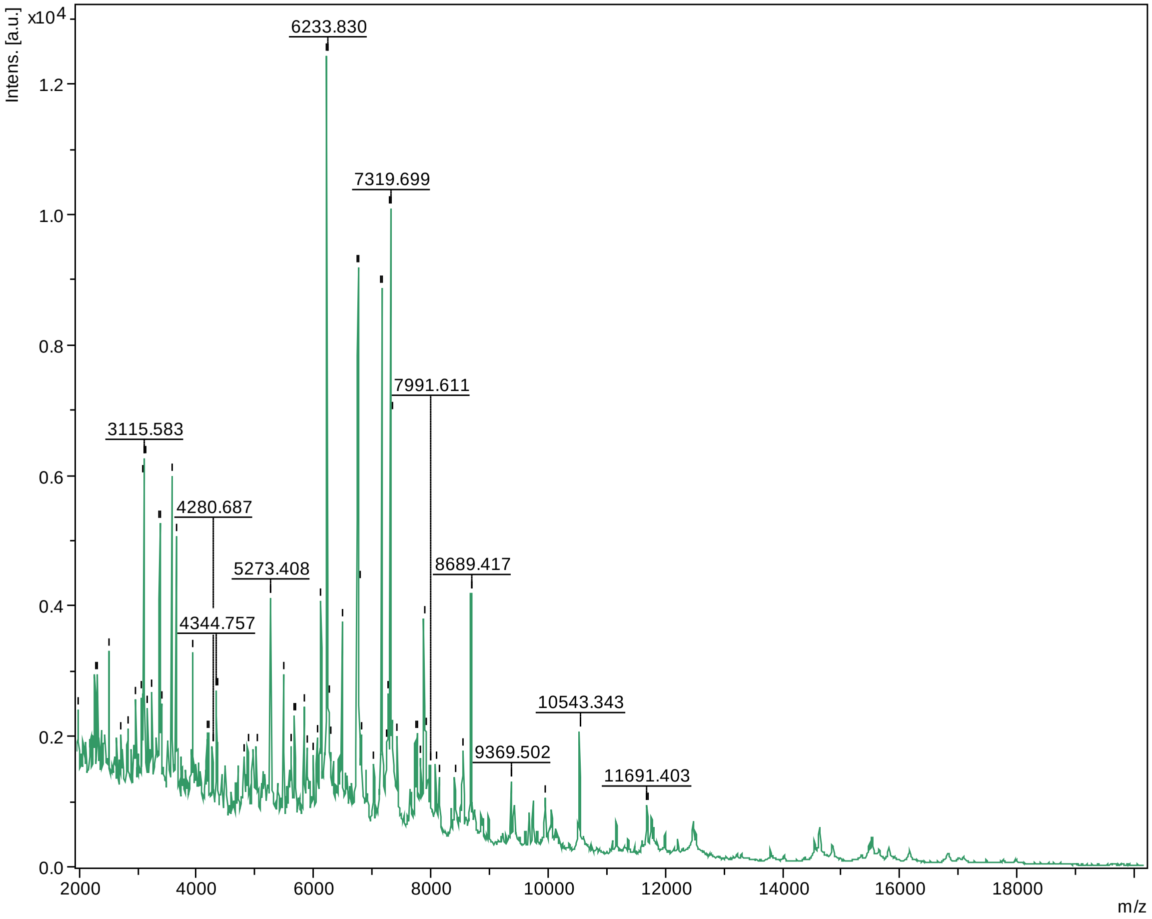


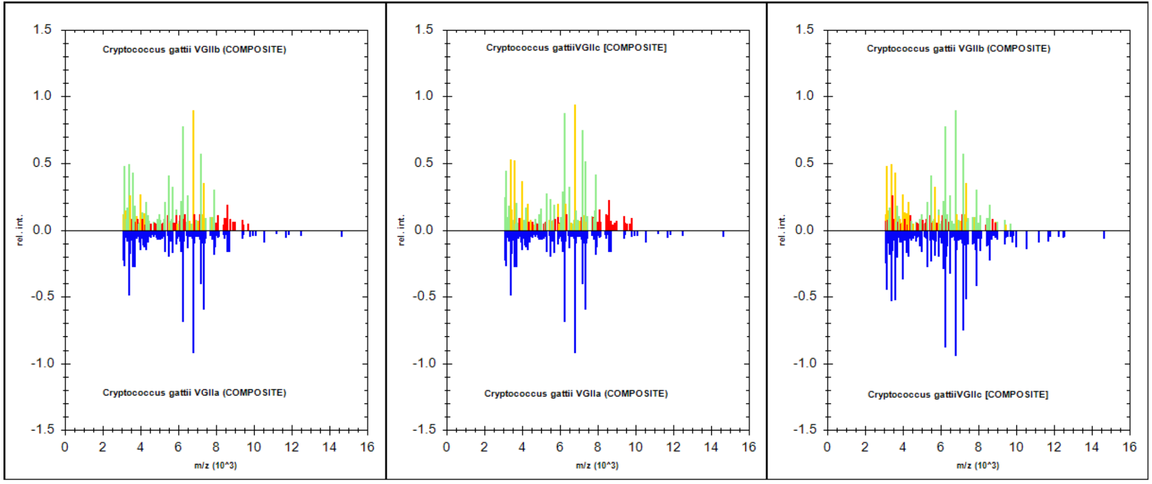
FIGURE 7 ***Cryptococcus gattii*** **Main SPectra** MSP was generated by finding peaks for 19-24 raw spectra of one sample in Flex Analysis and creating a composite spectrum. *Cryptococcus gattii* VGIIa UW Y602 shown. The base peak seen here is at 6233 m/z, with a relative intensity of 1.00 (100%).



Assuming that there were more subtle differences that were not picked up in the top ten peak lists, a challenge of *Cryptococcus gattii* specimens with blinded molecular types was run on the MALDI-TOF mass spectrometer to determine if the overall spectral pattern was enough to identify strains not part of the library. Again, all samples were identified as *Cryptococcus gattii*. The 10 blinded samples from the BC CDC were either VGI or VGIIb. Fifty percent of the time one of these two molecular types was identified. Confirmation of these results with the BC CDC will be determined. The remaining unknowns were correctly subtyped in eight of sixteen samples. Up to this point, *Cryptococcus gattii* has only been typed to major molecular groups: VGI, VGII, VGIII, VGIV[48].

The similarity between the VGII subtypes is emphasized when we look at composite spectra of each molecular type. For these spectra, the best raw spectra from all strains available in the library were merged into a new MSP and designated as composites. They were then electronically identified against each other using Flex Analysis software. From this analysis, it became evident that most major peaks used for library comparison are shared between the subtypes.

FIGURE 8 – Composite spectra from individual *C. gattii* VGII subtype spectra
Composite spectra were created from individual strain spectra from one molecular type. Spectra were run against each other using virtual library identification. Green peaks indicate major peaks that match exactly, yellow indicates key peaks that are not matched in intensity, mismatched peaks are indicated in red. As seen below, most major peaks are green or yellow, indicating the similar protein profiles of these strains.



DISCUSSION

As previously noted in the literature, entering more MSP into a supplemental library improves the identification of a particular organism[48,49]. All samples were correctly identified as *Cryptococcus gattii* to the genus level with the two commercial reference strains. Identification was improved to species level confidence after adding 35 strains to supplement the strains already included in the commercial Bruker library.

In analyzing the virtual gel, there are no obvious peak differences, indicated by density differences in bands, across the subtypes or molecular groups (Figure 5). Other organisms, such as *Propionibacterium acnes,* have shown distinct shifts of approximately 25 Da in banding patterns when viewed in the virtual gel. In those instances, MALDI-TOF mass spectrometry correctly identified specimens to the subspecies level[50]. For *Cryptococcus gattii*, the similar banding patterns point to similar protein production by the subspecies under the condition tested. MALDI-TOF spectra reflect the most abundant proteins available, usually ribosomal or cell wall proteins. The subspecies (VGIIa-c) may not have a significant change in protein production or modification, and are thus not able to be distinguished easily by mass spectrometry.

Other groups have used MALDI-TOF-MS technology to show that *Cryptococcus neoformans* and *C. gattii* can be consistently and discretely identified to the species and subspecies level[48,49,51,52]. In this study, all library strains were correctly typed to their major molecular group (i.e. VGIIa was identified as a VGII). Firacative *et al.* showed that separating out the major molecular groups with good resolution is possible with larger supplemental libraries, approximately 80 strains. They found that the different subtypes will create very distinct branches on a dendogram[48]. This was seen again in this study, as VGI created a separate branch from VGII and VGIII strains. While the resolution between subspecies was not as clear as desired, the dendogram does show clustering of the VGIIa and VGIIc subtypes (Figure 6). This indicates that perhaps additional manipulation of the spectra could enhance the differentiation of these subspecies. This could be achieved by weighting peaks, so that a peak at a certain mass to charge ratio or certain intensity is flagged as indicative for a particular subspecies.

To help resolve the discrepancies in subtyping the *C. gattii* molecular types, especially VGIIa, VGIIb, and VGIIc, we looked at the ten most prominent peaks in each strain, rounded to the nearest 5 Da. This cutoff was chosen because the Bruker Biotyper Real Time Classification software uses a minimum of 10 peaks over 1000 arbitrary units to identify an organism. Firacative *et al.* found that there were three peaks: 3250, 4000, and 6650 that were specific to their *C. gattii* strains. Furthermore they identified an additional three peaks: 7050, 7600, 7900 Da that were common to the *Cryptococcus* genus[48]. In the analysis of the strains from the Pacific Northwest used for this study, only 3250 and 7900 Da peaks could be found consistently. A window of +/- 50 Daltons was used to compensate for different culturing conditions from previous studies. When looking at the ten most prominent peaks in each sample, some differences do emerge. VGI spectra do not seem to have a significant peak at 6770 Da, unlike all the VGII and VGIII subtype samples. This void in the spectral pattern could be the reason that the VGI strains cluster together when visualized on a dendogram and are readily typed by mass spectrometry. In

75% of the VGII molecular type a peak of 7320 Da was observed in the top ten, which could be a unique peak to this subtype. The VGIII sample also had a potentially unique molecular weight peak at 3945 Da. This would have to be confirmed with more specimens from this molecular group. Further analysis of the peaks and relative intensity is required to confirm these observations. In order to determine distinct peaks, we need to know the resolution of the spectra. Analysis of the algorithm used to assign weights to peaks could assist in determining unique peaks at particular m/z ratios.

Chapter 5 – Conclusions and final remarks

MALDI-TOF-MS has the ability to streamline identification of microorganisms in the microbiology lab. In this project, we focused on two main aims. First, the implementation of this technology for direct blood culture identification. Within this aim we determined the limit of detection for the protein purification method we planned on using in our laboratory, determined handling conditions for the samples, and evaluated the MALDI-TOF-MS for concordance with our current workflow and reports. Second, customization of the commercial library for *Cryptococcus gattii* was performed. Within this aim, we improved representation of the species in the library, and also explored ways to determine the subspecies based on gathered spectra.

To initiate implementation of MALDI-TOF-MS for routine blood culture identification, we first determined the limit of detection for the Sepsityper, our protein purification kit for the study. We found that our limit of detection study and those performed by Christner, *et al.*, and Kroumova, *et al.*, did not reflect the minimum cfu/mL needed to obtain an adequate score when a sample was allowed to grow up in a blood culture system, as reflected in the post-positivity incubation. All three replicates to determine the limit of detection required at least 10^7 cfu/mL to get an acceptable species identification, whereas the samples collected that achieved a species level score had organism burdens of as low as $2x10^6$ cfu/mL[31,53].

MALDI-TOF-MS has the potential to greatly decrease turn-around times for microbial samples. This turn-around time cannot be fully achieved unless the technology is properly deployed in the laboratory. This study looked at the impact of post-positivity incubation to address the issue that not all blood cultures may be processed for mass spectrometry at time of positivity. Samples will likely be batched throughout the day and not all shifts may have staff that is trained to fully process the samples.

Only one previous study has attempted to assess the impact of post-positivity incubation on the performance of MALDI-TOF-MS for direct blood culture identification[54]. Blood cultures that did not turn positive during day shift were left overnight for unknown amounts of time. These cultures were noted and analyzed separately from bottles that were processed immediately. Additionally, Martiny *et al.*, did not compare the effects of post-positivity incubation directly using the same samples, nor were yeast included. By directly comparing the performance of MALDI-TOF-MS to initial processing for the same sample, we observed a generally improved performance following additional post-positivity incubation at room temperature and 35°C. This was most pronounced in the case of the two *Candida* species examined. Even though a 24-hour post-positivity incubation was employed in this study, we found that a similar trend was observed after lesser periods of incubation in the case of *C. glabrata*. Post-positivity incubation should be therefore be added into the list of variables (e.g., bottle type, processing method, etc.) known to impact the performance of MALDI-TOF-MS for blood culture identification[55–57].

In our prospective study, we found excellent correlation with the conventional workup identification. A concordance rate of 95.4% was achieved using an acceptable score of

1.6 or greater. When concordance was analyzed regardless of score a decrease in the agreement between MALDI-TOF-MS identification and conventional identification was seen, mostly due to samples that scored lower than 1.6 or generated no spectra. There were some high scoring samples that were in disagreement. One of the most common errors seen was *S. mitis* group being identified by mass spectrometry as *S. pneumoniae*. This limitation has been identified by the manufacturer of the technology as an inherent error based on the relatedness of these groups of organisms. Further work is needed to determine what caused misidentification in the non-*Streptococcus* samples.

Clinical implementation of this technology would allow for rapid identification of organisms in blood cultures. Sample processing takes approximately 40 minutes; 20 minutes for the Sepsityper kit to prepare the sample and 20 minutes for the MALDI-TOF-MS to reach an identification[22,33]. This could decrease turn-around time to the final report by at least 24 hours[22,30]. Data in the literature indicate the decrease in identification time can improve patient care and reduce costs by allowing for more accurate antimicrobial therapy[18,20]. There is an increased laboratory cost because of the increased hands on time to prepare the sample. For colony identification by mass spectrometry, the cost per sample has been estimated at around $0.50 each[11]. Direct identification of blood culture involves the use of the Sepsityper kit and increased technologist time resulting in a cost of $5.15 versus the $1.98 for conventional workup[22]. This cost to the patient may be offset by antibiotic therapy changes and a decreased stay in the hospital.

A final area of exploration was the improvement of organism representation in the reference library. Importantly, organisms that are recognized with a low identification score may actually not have a reference spectrum to compare to. This was seen in an isolate of *Leptotrichia* identified from our prospective study. It was identified by conventional methods only. The ability to add additional organisms of interest to the reference database makes this technology even more promising to clinical microbiology laboratories. Including organisms that may not be considered pathogens in a larger context, but a prevalent in a particular patient population is a great asset for a clinical laboratory.

One such organism was *Cryptococcus gattii*. In the commercially provided Bruker Biotyper library there were two strains included. These were supplemented with an additional 35 strains for which the molecular type (VG) was known. The library strains along with additional known isolates were run against the custom library along with the Bruker library. Identification at species level confidence (score >2.0) increased significantly.

Cryptococcus gattii was chosen as an important organism to increase coverage on the Bruker microflex platform because of its importance as a pathogen in immunocompetent patient populations and its presence in the Pacific Northwest as an endemic organism. Outbreaks have occurred in unconventional populations in the Pacific Northwest with the rise of the VGIIa, b and c subspecies as well. Identification of this species can be quickly accomplished with MALDI-TOF MS. The identification of both species and subspecies with this technology is attainable. Other groups have seen improvement by drastically increasing the number of strains in their supplemental library[48,52]. By entering more examples of subtypes, better identification of the subtypes is possible for the University of Washington. Eventually, even if the limit of the data is reached and we cannot improve identification past major molecular types, we can eliminate the need to send non-VGII samples off to be confirmed with MLST.

Overall, the addition of MALDI-TOF mass spectrometry to the microbiology laboratory represents an opportunity to decrease reporting time while maintaining confidence in results. This technology is flexible in that it can be used for colony identification off of many solid media and can be adapted for direct blood culture identification as well as typing applications. Adding reference spectra, for organisms of interest, to supplemental libraries would allow for customization of the platform for particular patient populations.

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Appendix I Organisms and culture conditions

For pre-analytical variables in MALDI-TOF workflow

For limit of detection studies, *Escherichia coli* (ATCC 25922, clinical source), *Staphylococcus aureus* (ATCC 43300, clinical source), and *Candida albicans* (ATCC 60193, clinical source).were used. Organisms were grown on Sheep Blood agar overnight at 35°C with 5% CO_2. Colonies were resuspended in saline to a McFarland of 3.0 before being serially diluted 1:10 in saline and added to 2mL aliquots of blood culture media and whole blood in a 17:1 ratio (80mL blood culture media, 5mL whole blood). One hundred microliters of suspensions were serially diluted for quantitative plating and 1 mL was processed with the Sepsityper kit before being identified on the Bruker microflex.

For post-positivity studies, ten organisms representing Gram-positive, Gram-negative and anaerobic bacteria and yeast were selected from the University of Washington stocks. The strains were: *Escherichia coli* (ATCC 25922, clinical source), *Klebsiella pneumoniae* (ATCC 35657, laboratory source), *Pseudomonas aeruginosa* (ATCC 27853, clinical source), *Stenotrophomonas maltophilia* (patient isolate), *Bacteroides fragilis* (patient isolate), *Staphylococcus aureus* (ATCC 43300, clinical source), *Staphylococcus epidermidis* (ATCC 12228, laboratory source), *Enterococcus faecium* (ATCC 51559, clinical source), *Candida albicans* (ATCC 60193, clinical source), *Candida glabrata* (patient isolate). Isolates were stored at -80°C and then cultured on Sheep Blood agar (Trypticase soy agar with 5% sheep blood; Remel, Lenexa, KS) and incubated overnight at 35°C with 5% CO_2. *B. fragilis* was culture on Brucella Agar with blood, hemin and vitamin K (Remel, Lenexa, KS) and incubated anaerobically at 35°C. All organisms were subbed once before inoculation into blood culture bottles. Colonies were then suspended in saline to a McFarland of 0.5. The culture was diluted to approximately 100-500 colony forming units (CFU) and inoculated into VersaTREK® REDOX 80 bottles (Thermo Scientific, TREK Diagnostic Systems, Cleveland, OH) along with 5ml of whole blood obtained from healthy volunteers. Bottles were incubated in the VersaTREK 528 instrument (TREK Diagnostic Systems, Cleveland, OH) for up to 5 days per the manufacturer's directions. Bottles were flagged for growth between 9 and 38 hours and all experiments were performed in triplicate.

Follow up testing was performed on *Candida* species. Three additional patient isolates for each of the following were selected for testing: *C. albicans, C. glabrata, C. parapsilosis,* and *C. tropicalis.* They were cultured and handled as described above.

For prospective comparison study

One milliliter aliquots were obtained from 243 patient blood cultures over a period of six months. The blood cultures were collected and worked up as part of routine patient care and therefore did not require an IRB as there was no additional sampling of patients. Aliquots were made by removing blood with a 3 mL sterile syringe and 23 gauge needle and storing blood in a 1.5 mL Eppendorf tube. Sepsityper processing was performed directly on these aliquots.

For library construction

Cryptococcus gattii VGI (7 strains) and VGIIb (8 strains), plus 10 undesignated subtypes were provided by the British Columbia Center for Disease Control. *Cryptococcus gattii* VGIIa (14), VGIIc (2), and VGIII (1) were provided by University of Washington Medical Center. *Cryptococcus gattii* VGIIc (19) were provided by the Oregon State Department of Public Health. Strains were stored at -80°C and were cultured on Inhibitory Mold Agar (Remel, Lenexa, KS) at 30°C for 48 hours. Strains were subbed once before being processed for mass spectrometry.

Appendix II – Determining colony forming units and limit of detection

Colony forming units per milliliter (CFU/mL) were determined by serial dilution. First original samples were diluted serially 1:10 in saline for 8 dilutions. Then three 10 microliter drops of each dilution were plated onto appropriate media, either blood agar, brucella agar or inhibitory mold agar. After incubation at 35°C, colonies were counted in an appropriate dilution. This dilution was determined to have between two and twenty colonies per 10 μL spot. In this method, one colony in each of the first three spots would be equivalent to 100 CFU/mL. The dilution range was from 10^2 to 10^9 cfu/mL.

Limit of detection was determined by resuspending colonies from solid media. First, colonies were grown on sheep blood agar at 35°C with 5%CO_2 for 24 hours. Colonies were then picked up with a swab and resuspended into 3mL sterile saline until a McFarland of 3.0 was achieved. Next, this original solution was serially diluted 1:10 twice in saline. 200uL of these final solutions were added to two milliliter aliquots of blood culture media and whole blood in a 17:1 ratio (80 mL media with 5 mL whole blood). One hundred microliters of the culture was used for determining colony forming units as stated above. One milliliter of the culture went to Sepsityper processing for identification score by mass spectrometry. Limit of detection was determined to be the concentration of cells for which a “species level” score (1.80 to 3.00) was achieved.

Appendix III Protein extraction with and without the Sepsityper kit

Protein extraction from colonies

Samples were extracted using the Bruker Ethanol Formic Acid extraction. Briefly, 2-3 colonies were suspended in 75% ethanol and centrifuged 2 minutes at 13,000 rpm. The supernatant was removed and the sample was allowed to air dry (15 minutes). Samples were resuspended in acetonitrile, based on volume (25-50uL), with an equal volume of 70% formic acid added and gently mixed. Samples were centrifuged again and the supernatant was used for MALDI-TOF identification.

Sepsityper kit processing

Within 3 hours of culture positivity, aliquots were removed and subjected to the following: 1) Immediate processing; 2) 4°C refrigeration, 24 hours; 3) Room temperature incubation, 24 hours; and 4) 35°C incubation, 24 hours. One hundred microliters of each sample was plated on sheep blood agar, brucella agar or inhibitory mold agar for quantitation. One milliliter of the same samples was processed with the Sepsityper Kit according to manufacturer's directions (Bruker Daltonics, Billerica, MA). Briefly, blood cells were lysed and the aliquot was centrifuged 13000 rpm for 1 minute and the supernatant removed. The sample was then resuspended in Wash Buffer and centrifuged again at 13000 rpm for 1 minute. A second wash was performed on the recommendation of the Bruker representative (Dr. GongYi Shi, Bruker Daltonics, personal communication). This pellet was then resuspended in 75% ethanol. The sample was centrifuged again and the supernatant removed. The remaining pellet was allowed to air dry before being resuspended in acetonitrile and formic acid in an equal volume. The volume of final suspension varied from 4 µL to 50 µL, dependent on pellet size. The sample was centrifuged a final time and the supernatant was used for MALDI-TOF identification.

For the prospective study, one milliliter aliquots from blood culture were batched once per shift (every eight hours) as performed in previous studies. The samples were processed by microbiology technologists using the Bruker Sepsityper kit, with the extra wash step described above. The samples were then extracted with ethanol and formic acid to create the final supernatant to run on the Bruker microflex MALDI-TOF MS. No colony counts were performed on these samples.

Appendix IV MALDI-TOF mass spectrometry identification

Bacterial identification from solid media was performed by taking a single colony off of solid media and smearing it on the target plate before overlaying the colony with matrix. The yeast isolates were extracted using the protein extraction from colonies method (Appendix II). One microliter aliquots of extraction supernatants were spotted onto a 96 place polished stainless steel target plate and allowed to dry at room temperature before proceeding. Samples were overlaid with 1µl of α-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics). After drying, samples were subjected to analysis using the Bruker Microflex LT system.

For colony identification, samples were run using the Bruker Biotyper 3.0 software and reference spectra library. Mass peaks with *m/z* ratios 2,000 to 20,000 were analyzed. Scores of 1.7 -2.0 and 2.0-3.0 were considered valid to the genus and species level, respectively. Scores of <1.7 were considered invalid.

The resulting spectra were analyzed as described previously with the Bruker Biotyper 3.0 software and reference spectra library using blood-culture specific parameters that excluded mass peaks with *m/z* ratios of <4,000[33]. For blood cultures, identification scores of 1.6-1.8 and 1.8-3.0 were considered valid to the genus and species level, respectively. Scores of <1.6 were considered invalid.

Appendix V Library Construction

Extract supernatants were spotted 8 times and each spot was read 3 times, for a total of 24 spectra acquired for each sample. Spectra were analyzed using Bruker Biotyper 3.0 software. Individual spectra were visually evaluated for noise before being averaged. Noisy or missing spectra were deleted and the remainder were used to form a Main Spectrum (MSP) for a library entry. Entries contain 19-24 averaged spectra. Thirty-five strains of *Cryptococcus gattii* with confirmed molecular types made up the supplemental library.