

Use of Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) for Detection of *Escherichia coli* in Clinical Urine Samples

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Urinary tract infections (UTIs) are very common and are becoming a concern in the medical community due to the growing prevalence of antimicrobial resistance in causative pathogens. The poor analytic performance or long time requirements of current diagnostic tests contribute to this problem, as antibiotic treatment often needs to be administered before the cause of the infection is confirmed, which frequently leads to prescription of antibiotics based solely on often inadequate preliminary clinical observations or test results. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) has been successfully used to identify cultured microorganisms, but protocols that employ MALDI to directly identify microorganisms in clinical samples are still under development. This study aims to develop a simple, fast, and cost-effective MALDI method that could potentially diagnose UTIs by accurately detecting *Escherichia coli* in clinical urine samples. The protocol was developed using cultured *E. coli*, which was smeared directly onto the MALDI plate then overlaid with α -Cyano-4-hydroxycinnamic acid (HCCA) matrix. The samples were identified using Bruker MALDI Biotyper software. Using this protocol, MALDI could reliably identify *E. coli* samples with high Bruker Biotyper quality values. Clinical urine samples were spotted directly onto the MALDI plate either without preparation or after a simple centrifugation, then overlaid and analyzed in the same way. MALDI was able to identify *E. coli* in some samples, but in many cases identification failed. Further work is required to determine the cause of the failures, and what can be done to improve the analysis quality to increase the likelihood of successful detection.

Urinary tract infections (UTIs) are one of the most common infections, accounting for 130 to 175 million hospital visits worldwide each year (1). Additionally, approximately 40% of hospital-acquired infections are UTIs (1,2). While the incidence of UTIs remains unchanged in recent years, these infections are becoming more of a healthcare concern due to the widespread increase in antimicrobial resistance in the pathogens that commonly cause them (1).

The current methods used to diagnose UTIs are part of the problem. Urine culture is the gold standard for reliable confirmation of UTIs (3), however the culture and isolation of bacterial pathogens from urine samples requires 18-24 hours, and the reliable biochemical identification of the isolates demands a further 18-24 hours, bringing the turnaround time for traditional biochemical identification to 36-48 hours total (4, 5). This is sometimes increased to up to 72 hours in the case of microorganisms that are difficult to isolate or identify (1, 6). The length of time required for urine culture often limits its clinical applicability, as patients often need to be treated before the results are available, even though 60-80% of urine samples collected for UTI diagnosis turn out to be negative for pathogen growth (7, 8, 9). In the meantime, patients are usually given empirical treatment based solely on clinical symptoms or a few rapid assays, such as urine dipstick tests for nitrite and leukocyte counts (1, 2). Screening tests, such as flow cytometry or automated microscopic urine sediment analysis, are also used to quickly predict negative samples, but they have very high sensitivity to avoid false negatives, and do not give information on the identity of the infection (3). Although fairly efficient time-wise, the overall analytical specificity

of these tests is low, and the antibiotics prescribed based on their results or empirical observations may be unnecessary or not optimal for treatment of the infection (1, 4). Molecular methods for UTI diagnosis have also been introduced, but while they are reliable, they are also often expensive and complex, and therefore not suitable for routine clinical use (6, 10, 11). Because of the lack of immediate or reliable clinical applicability of the currently available diagnostic methods, antibiotic treatment given based on symptoms and the results of rapid tests is often unnecessary, unsuitable, or inadequate (5). In addition to being less effective, inappropriate use of antibiotics also contributes to the growing problem of antimicrobial resistance in pathogens, so the ability to quickly confirm the identity of the causative microorganism could help guide earlier appropriate antibiotic use (1, 12).

However, if a diagnostic method could be developed that is cost-effective, simple, and could provide accurate results in a short enough time that they could be used to decide patient treatment, many of the concerns currently surrounding the treatment of UTIs could be addressed. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) has been used to provide rapid and reliable identification of microorganisms in other areas of microbiology, and it is an analytical technique that shows promising applications to clinical microbiology and medical diagnostics. MALDI is capable of detecting macromolecules in complex mixtures, such as identifying proteins in biological samples, as it is a soft ionization technique and can ionize molecules for separation via mass spectrometry without fragmentation

(13, 14). MALDI is able to identify microorganisms by collecting mass spectra “fingerprints” of their characteristic cellular components, and comparing these to reference libraries collected in a database (4, 6, 13). These databases are constantly growing and being contributed to, but many common microorganisms are already well represented.

The use of MALDI in medical microbiology laboratories is relatively new and it is still being explored. It has been used fairly extensively to identify cultured microorganisms, and it has been shown to be able to classify them very accurately at the genus and species level, and even down to differentiating between stains of the same microorganism in some cases (6, 10, 15), including the identification of markers of antibiotic-resistant strains (16). However, these assays still require the time-consuming growth of microbial cultures, which delays the analysis for 18-24 hours, therefore limiting the clinical applicability of MALDI analysis (1, 17). The direct use of MALDI on clinical samples has not been thoroughly explored, but several studies have demonstrated that MALDI may be able to accurately identify microorganisms directly from clinical samples such as blood and urine (1, 2, 3, 5, 17). Apart from the initial cost of purchasing the instrument (which most western laboratories and hospitals now possess), the cost of consumables per sample is low, an estimated \$0.20/sample (2, 18). MALDI also boasts high efficiency of sample analysis and high throughput; for isolated microorganisms, MALDI is capable of identifying one isolate in approximately 6 minutes at approximately 22-32% the cost of conventional phenotypic methods (4), and it is likely that at least some of this efficiency and affordability could be carried over to direct identification. Some studies into direct identification using urine samples have demonstrated that they can produce results for multiple samples in under one hour (5). Developing protocols that could enable patient samples to be run directly using MALDI could provide a valuable tool for diagnosing UTIs that has the potential to surpass current tests in affordability, efficiency, and reliability (6, 19).

Compared to other patient samples, urine samples are a more suitable specimen for direct analysis using MALDI due to the high bacterial concentration during infection compared to other patient samples, such as blood or stool samples (1). The threshold for UTI diagnosis is usually 10^5 CFU or higher (1, 5, 6). MALDI is sensitive enough to detect many microorganisms at these concentrations, as a signal with a sufficient signal-to-noise ratio can be generated from a sample containing $<10^4$ CFU/mL of microorganisms, although for direct identification up to 10^6 CFU/mL may be necessary and the threshold varies among different species (4, 5, 17). MALDI also has difficulty resolving multiple kinds of microorganisms in one sample, but most UTIs are monomicrobial (over 90%) so this issue should be minimized (1, 5, 9). Additionally, urine samples tend to contain fewer proteins and other microorganisms than blood or stool samples, so the signal of the target microorganism is generally more distinct (20). In this study, we chose to analyse *Escherichia coli* as an experimental microorganism because it is the causative agent of 70-95%

of UTIs (1, 2, 8, 9), is strongly represented in commercially available MALDI databases, and MALDI has been shown to produce the best results when detecting gram-negative bacteria (9).

The current study is directed at further developing the protocol for potential diagnosis of urinary tract infections via direct sample analysis using MALDI that was initially developed during the summer of 2015. The end goal of this research is to establish and optimize a protocol for MALDI operation and sample preparation in order to identify *E. coli* in urine samples quickly and reliably. Once finalized, this protocol could provide an efficient, cost-effective, and clinically applicable method for diagnosing UTIs caused by *E. coli*, which will be suitable for use in hospital laboratories.

MATERIALS AND METHODS

α -Cyano-4-hydroxycinnamic acid (HCCA) was the matrix used in this study, and it was purchased from Sigma Aldrich, Oakville, ON, Canada. The solid matrix was stored at -18°C prior to solubilisation. The matrix was prepared by dissolving solid HCCA in the MALDI organic solvent (detailed below) at a concentration of 10 mg/mL, according to instructions from the manufacturer of the MALDI (Bruker, Milton, ON, Canada). Once prepared, it was stored at room temperature for up to a week.

Bacterial Test Standard (BTS) was also purchased from Bruker. Solubilized BTS was prepared by solubilizing in the MALDI organic solvent, according to manufacturer instructions and stored in individual microfuge tubes at -20°C . Before use, one microfuge tube of BTS was thawed at room temperature for 20-30 minutes or at body temperature for 5 minutes before being spotted onto the MALDI plate. Frozen solubilized BTS could be stored at -18°C or below for up to 5 months.

The organic solvent used for solubilisation of BTS and HCCA was a solution of 50% acetonitrile, 47.5% HPLC-grade water, and 2.5% trifluoroacetic acid (TFA). It was prepared using chemicals purchased from Sigma Aldrich.

Pure formic acid was also purchased from Sigma Aldrich, and was diluted to 70% with HPLC-grade water. This was overlaid over the sample spots in some assays, as it has been shown to be a useful step to improve cell lysis and protein extraction (6).

A 96-spot ground steel MALDI target plate and Microflex LTC MALDI TOF MS, both from Bruker, were used for analysis of all samples.

All clinical urine samples were collected from patients by Royal Inland Hospital (RIH), and were transported to Thompson Rivers University in a chilled container, in accordance with Transport of Dangerous Goods guidelines.

Protocol development. MALDI protocol for identifying cultured *E. coli* was developed by using solid *E. coli* cultured in the lab. *E. coli* was grown on nutrient agar overnight at 37°C . The *E. coli* plates were freshly cultured and not refrigerated before use. The most successful method for identifying solid *E. coli* was determined to be the direct smear method, in which a colony of *E. coli* is smeared onto the MALDI plate using a toothpick, then overlaid with 1 μL of HCCA matrix.

BTS was used as a calibrant, healthy urine was used as a negative control, and healthy urine spiked with *E. coli* was used as a positive control. Prepared BTS was spotted and overlaid with 1 μL of HCCA matrix. Healthy urine was collected immediately prior to testing, and spiked urine was prepared by suspending one colony of cultured *E. coli* in 1 mL of healthy urine. Healthy and spiked urine were spotted and analyzed in the same way as the clinical urine samples.

Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	not reliable identification	(-)	red

FIG. 1 Meaning of Bruker Biotyper score values.

Treatment of clinical urine samples. All clinical urine samples from RIH were transported to Thompson Rivers University in a chilled container, in accordance with TDG guidelines, and immediately frozen at -20°C (frozen samples) or refrigerated at 4°C (unfrozen samples).

The unfrozen urine samples were analyzed the same day that they were acquired from RIH. Samples had to be acquired by RIH and cultured before they were given to us, which means that all urine samples were collected at least one day prior to analysis, if not more.

MALDI plate spotting protocol. Prior to spotting, any frozen samples were thawed completely, and the appearance of all samples was noted. The samples were swirled to suspend any solid material, then 1 µL of each was applied to one spot of the MALDI plate using a micropipet. Each sample was spotted in duplicate, and allowed to dry completely before the next layer was applied. After the sample spots has dried completely, 1 µL of prepared HCCA matrix was spotted over them and allowed to dry. At this point, the samples were ready to be assayed and the plate was transported to the MALDI.

Formic acid extraction protocol. All samples were spotted in two sets, both containing duplicate spots of each sample. The second set of samples was overlaid with 1 µL 70% formic acid and allowed to dry completely, prior to being overlaid with 1 µL HCCA matrix as described above and analyzed in the same way. The goal of this extraction was to improve the identification by lysing the bacterial cells to release more molecules that could be ionized by the MALDI and detected by the mass spectrometer.

Centrifugation protocol. 1 mL aliquots of each sample were pipetted into individual 1.5 mL microfuge tubes and centrifuged at approximately 800 x g for 10 minutes to pellet the cells at the bottom of the tubes. The majority of the supernatant was removed, and the pellet and small amount of remaining supernatant was mixed with a pipet tip then spotted in 1 µL aliquots onto the MALDI plate in duplicate. The centrifuged sample spots were allowed to dry before being overlaid with matrix as described above, with a second set of spots being overlaid with formic acid as described above. The purpose of the centrifugation procedure was to attempt to concentrate the bacterial cells in the sample.

Sample culture. 10 and 100 µL aliquots of several urine sample were plated on nutrient agar plates and incubated at 37 °C overnight. The bacteria cultured from the samples were then plated in the same way as the solid *E. coli* and analyzed with the MALDI to confirm that the samples contained *E. coli*. The samples were streaked using a 4 way-streak to prioritize isolation of colonies for MALDI analysis over quantification of cells in urine samples. The plated samples were then incubated overnight at 37 °C to see if any microbial colonies would grow. One colony from each plate (10 and 100 µL of each sample) was analyzed if present, and sample sets both with and without the 70% formic acid overlay were run. The cultured clinical urine samples were plated the same way and analyzed using the same MALDI parameters as the solid cultured *E. coli* used in the protocol development.

MALDI parameters and protocol. Once prepared and spotted onto the MALDI plate, samples were placed in the MALDI and

analyzed using Bruker Biotyper 3 and Biotyper RTC (Real Time Classification) software. Spectra were collected either using the “MBT_FC.par” Flex Control method in Linear and Positive mode, or directly in Biotyper RTC using the default settings for identification of microorganisms. The Biotyper software compares the spectra acquired from the samples to those from the Bruker database of microorganisms, and classifies them based on the similarities. The software then assigns a quality value ranging from 0-3.000 along with the classification, with 0.000-1.699 meaning no reliable identification, 1.700-1.999 meaning a probable genus identification, 2.000-2.299 meaning a secure genus identification and a probable species identification, and 2.300-3.000 meaning a highly probable species identification (Fig.1).

RESULTS

Three sets of clinical urine samples were analysed and different techniques/treatments were applied to each in an effort to improve the identification (Fig. 2). The first set consisted of 20 urine samples, which were frozen at -20°C for several months prior to analysis. *E. coli* was not successfully identified in any runs of this sample set, but the data allowed us to investigate the effect of storage time and temperature prior to MALDI analysis of clinical urine samples.

The second set consisted of 9 clinical urine samples that were obtained from RIH and analyzed on the same day, and were refrigerated at 4°C prior to analysis. Not only did the second sample set allow us to investigate the effect of shorter and longer storage at different temperatures, but many different experiments were run. The samples were centrifuged to increase the concentration of microorganisms spotted onto the MALDI plate, and they were also cultured and the solid cultured bacteria were analyzed, to confirm that they did contain *E. coli*. *E. coli* was successfully identified in both replicates of one sample in this set, when it was centrifuged and overlaid with 70% formic acid.

The third and final set consisted of 11 clinical samples, which, like sample set 2, were acquired and analyzed on the same day. However, for this sample set *E. coli* was only identified in one replicate of one sample, and with a low quality score.

Sample Set 1: Frozen urine samples. Our first sample set examined the ability of MALDI to analyze urine samples that had been stored frozen and thawed prior to analysis. To investigate this, we analyzed 20 clinical urine samples that had been stored for several months at -20°C. BTS was used as the analytical standard, and solid cultured *E. coli* and spiked healthy urine (that had not been frozen) were used as positive controls. Healthy urine was used as a

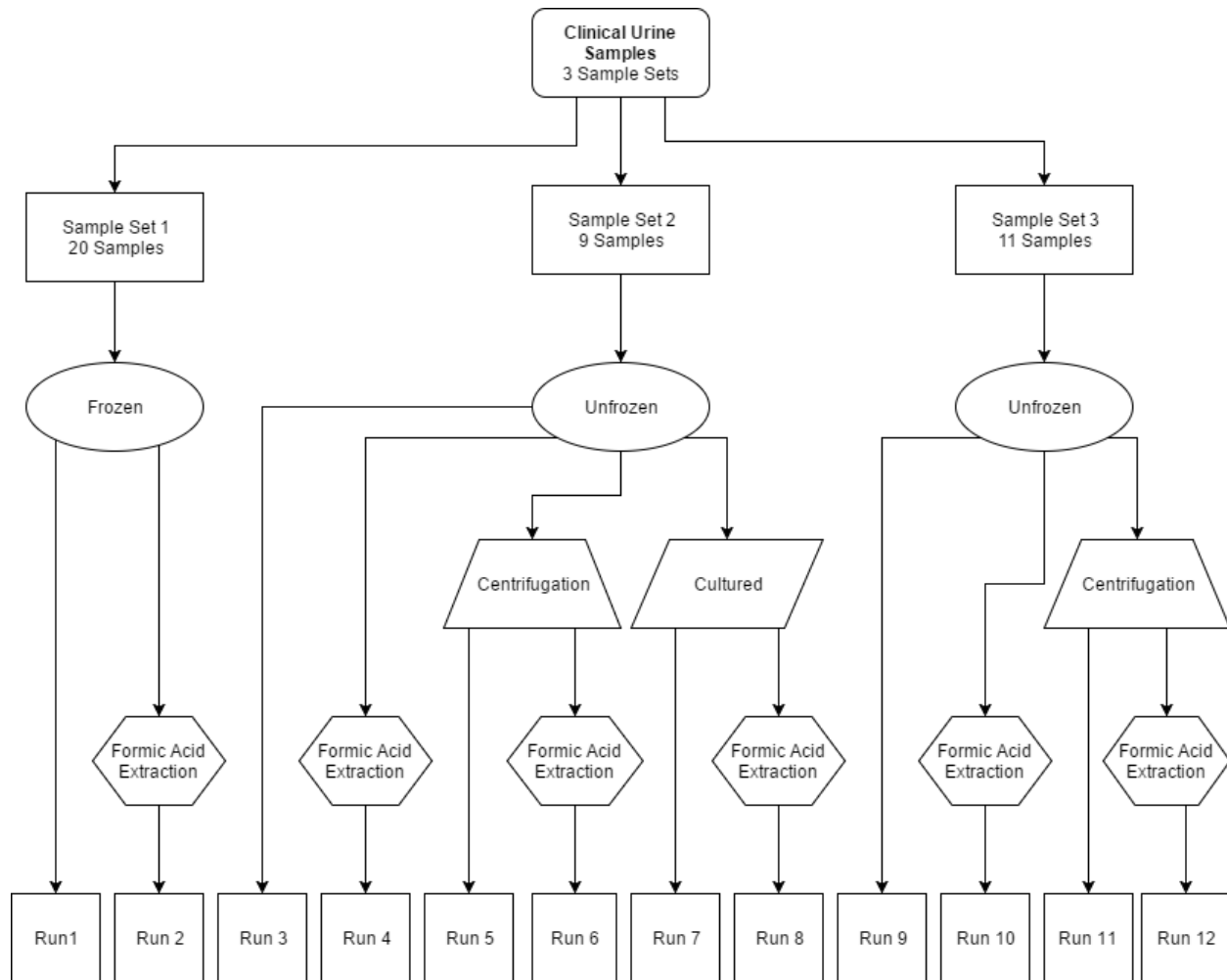


FIG. 2 Flowchart of sample treatments.

negative control. We also used the 70% formic acid extraction to evaluate what effect it had on the results.

Run 1: MALDI analysis was able to successfully identify *E. coli* in all standard and positive control samples with quality scores in at least the probable (+) range, and did not detect any *E. coli* in the negative control. However, identification of *E. coli* was not obtained for any of the clinical urine samples in this run (Fig.S1).

Run 2: The results of the MALDI analysis with the 70% formic acid extraction were similar to Run 1. The quality values for the successfully identified control samples may show some slight improvement, as all standards and positive controls were identified with quality scores in the secure (++) range. However, *E. coli* was still not successfully identified in any of the clinical urine samples (Fig. S2).

Sample Set 2: Unfrozen urine samples. For our second sample set we used 9 urine samples that were freshly acquired from RIH to investigate if storage temperature had any effect on the analysis of urine samples. These samples were never frozen, and were stored at 4°C at all times prior to analysis. The samples were analyzed the same day that they were acquired from RIH. It is unknown how long

beforehand the samples were collected by RIH, but it is likely 1-2 days prior to testing. The experimental standard, positive controls, and negative controls employed were the same as for sample set 1.

Run 3: The Biotyper RTC software failed to identify *E. coli* in any of the urine samples in this set. Solid cultured *E. coli* and BTS were successfully identified in all cases with quality scores in the probable (+) range or higher, but healthy urine samples spiked with cultured *E. coli* were not identified at any concentration, even though they had been successfully identified with high quality values in previous assays (Fig. S3).

Run 4: Once again, samples overlaid with 70% formic acid showed similar results to those with no formic acid, with possibly slightly higher quality values (with all successful identifications in the range of secure (++) or higher). However, like Run 3, none of the clinical samples or spiked urine samples were successfully identified (Fig. S4).

Sample Set 2.2: Centrifuged unfrozen urine samples. For the next two runs, a simple centrifugation process was used on the urine samples in an effort to increase the

concentration of bacteria in the plated samples so that MALDI could better detect *E. coli*. 1mL of each sample was centrifuged at approximately 800g for 10 minutes to cause the bacteria to collect in a pellet. The supernatant was then drained off, and the semi-solid pellet was smeared on the MALDI plate for analysis. When centrifuged, all urine samples and the 1 mL spiked urine sample produced visible pellets of a fair size (approximately 5-10 μ L in volume), except for Sample 4, which produced no visible pellet. Due to the absence of a pellet, Sample 4 was not included in this assay. The standard (BTS) was not included in this assay because the quantities available were too small to be centrifuged, and the negative control (healthy urine) did not produce a pellet when centrifuged, because it contained little to no bacteria.

Run 5: *E. coli* was not successfully identified in any samples in this run, including the spiked urine positive control (Fig. S5).

Run 6: The only successful identification in this sample set was obtained when the 70% formic acid extraction was applied to the centrifuged samples. Both duplicate spots of Sample 5 were successfully identified as containing *E. coli*, with a genus identification and a probable species identification (++) (Fig. S6).

Samples Set 2.3: Cultured unfrozen urine samples. For the 3rd set of analyses using Sample Set 2, all samples were cultured on nutrient agar to investigate what microorganisms they contained, and at what concentration. Plates were prepared using 10 μ L and 100 μ L of each urine sample, and all plates were incubated overnight at 37°C. All urine samples produced colonies after one day, and all cultures appeared to be pure, with all colonies exhibiting the same colour and colony morphology, and all colonies appeared to be *E. coli* based on macroscopic morphology. Some samples, such as Sample 4, produced fewer colonies, but this observation was only qualitative since the colonies were not distinct in most cases and could not be counted. However, the 10 μ L plates of every sample produced at least 100 colonies, so the concentration of *E. coli* in the samples should be approximately 10⁴ CFU/mL at the very least, which should be enough to be detectable by MALDI. The healthy urine negative control was also plated to see if it contained any microorganisms that could possibly interfere with the identification. No colonies grew on the 10 μ L plate, but a single colony grew on the 100 μ L plate.

Run 7: All cultures were successfully identified as *E. coli* with quality scores in the secure (++) range for at least one replicate of each sample. The single colony obtained from the 100 μ L negative control plate was successfully identified by MALDI as *E. coli*, which suggested that the healthy urine negative control may contain *E. coli*, but at a concentration of approximately 10 CFU/mL, which is much below both the microorganism load for UTI quantification and the detection limit of the MALDI (Fig. S7).

Run 8: In this case, the quality scores obtained when the 70% formic acid extraction was used were slightly worse. In Sample 6, *E. coli* was only successfully detected in one replicate, and the quality score was only in the probable (+) range (Fig. S8).

Sample Set 3: Unfrozen urine samples. The third and final samples set consisted of 11 clinical urine samples. Like the samples in set 2, they were acquired from RIH and analyzed on the same day, and were stored at 4°C at all times in between collection and analysis. This sample set was done to increase the number of trials and acquire more data.

Run 9: The Biotyper RTC software failed to identify *E. coli* in any of the 11 urine samples in Sample Set 3. Solid cultured *E. coli* was successfully identified in both replicates, but BTS was only successfully identified in one replicate, even though it had been consistently identified in prior analyses (Fig. S9).

Run 10: The use of 70% formic extraction did not seem to improve the identification quality. No experimental samples were successfully identified in this run, and BTS was not successfully identified in either replicate (Fig. S10).

Sample Set 3.1: Centrifuged unfrozen urine samples. The samples in Sample Set 3 were then concentrated via centrifugation, following the same method as Sample Set 2. All samples produced visible pellets of varying sizes.

Run 11: No samples were successfully identified in this run (Fig. S11).

Run 12: One centrifuged sample that had been overlaid with 70% formic acid was identified as *E. coli* (Sample 6, Sup. Fig. 12), but with a quality score only in the probably genus identification (+) range, and only for one of the two replicate spots.

DISCUSSION

Sample set 1 (frozen). The direct MALDI analysis both with and without the formic acid extraction procedure failed to positively identify *E. coli* in any of the clinical urine samples in this sample set. This could be due to a multitude of reasons.

The urine samples acquired from RIH in Sample Set 1 were blind samples, so it is unknown what tests have been performed on them prior to acquisition, and what the results of those tests may have been. Some of the urine samples tested may not contain *E. coli* in the first place, and some of them could contain multiple microbial species which would hinder the successful identification of *E. coli* if it were present.

Additionally, the urine samples were frozen in order to preserve them, and it is possible that freezing could damage the samples. This analysis was completed in April 2016, and the urine samples had been frozen since November 2015, so it is also possible that they underwent some deterioration during that period of time. Likely a significant factor in the failed identification is that none of the samples were freshly thawed prior to this analysis. Samples 1-10 had been thawed approximately 5 months prior, and then kept at 4 °C. The extended storage time of these samples could potentially hinder their analysis, but they were run nonetheless to evaluate whether old samples would produce any useful results. Samples 11-20, while thawed much more recently, were still not thawed immediately prior to analysis. Samples

11-15 were thawed 3 days prior to the analysis, while samples 16-20 were thawed 1 day prior. No frozen samples remained at the time of the analysis.

However, the analyses conducted on these samples when they were fresh were not useful, because the TRU MALDI instrument was not properly calibrated prior to maintenance completed by a Bruker technician on April 21st of 2016. While many analyses were attempted before this date, the MALDI failed to identify any samples, including BTS and solid *E. coli*, likely because it was not properly calibrated with the standard method.

While the current results indicate that the method is not working according to its purpose, there are many factors that could be causing this, and its potential should still be investigated. *E. coli* has previously been identified with good quality values using the same protocol earlier in this project, prior to the technical difficulties with the TRU MALDI instrument, so this indicates that this identification is likely possible. If the factors mentioned above can be eliminated, a clearer picture can be obtained of the potential MALDI has for direct analysis of urine samples for diagnostic purposes. Acquisition and analysis of unfrozen clinical urine samples that have tested positive for *E. coli* infection could be very helpful in the next steps of this research.

Samples set 2 (unfrozen). The unfrozen urine samples were not successfully identified by spotting them directly onto the plate without any sample preparation, although this had worked in the past (July 2015). Interestingly, the spiked healthy urine spots could also not be identified in this analysis, even though they were effectively identified in previous runs. A possible reason for this could be that the cultured *E. coli* used for the spikes had not been freshly cultured and had been stored at 4 °C for several days. Fresh *E. coli* was not used for this run because the urine samples were obtained on less than a day's notice, and *E. coli* had not been plated the day before.

The simple centrifugation protocol was employed in an attempt to increase the number of bacterial cells spotted onto the MALDI plate. The procedure was simple and did not require any unusual equipment, and added approximately 15 minutes to the total analysis time for 20 samples. If a laboratory possessed a microfuge capable of holding many samples at a time, then the increase to the turn-around time would likely be small even when running many samples. While it would be more optimal to run samples without any preparation, centrifugation to concentrate the samples prior to MALDI analysis does not significantly increase the complexity or time consumption of the diagnostic procedure.

Out of the centrifuged samples, one sample was able to be identified, which shows that the identification was likely improved by the centrifugation, but the reason why only the one sample could be identified and others could

not is unknown. Sample 5 did not seem to have a significantly larger concentration of cells than other samples, and observation based on the size of the pellet obtained and the density of colonies when cultured. However, Sample 5 did have a different appearance than the other samples: it was completely clear and colourless, and looked more like water than urine. The other samples varied in colour and cloudiness, and the appearance of each sample has been listed in Appendix 1. Some samples had unusual colours that seemed to be due to a dye or indicator, and not naturally occurring.

Additionally, Sample 5 was only identified when 70% formic acid extraction was used, even though it has not seemed to have significant effects on previous analyses. A possible reason for this could be that the intent of the formic acid extraction is to lyse the cells to release molecules for the MALDI to detect, and since the urine samples had not been frozen, the cells were still intact. However, cultured samples have been successfully identified without the formic acid extraction, and the cultured cells should also still be intact. We are unsure why the formic acid extraction seems to have allowed the successful identification of Sample 5.

Culture and analysis of the clinical urine samples indicated that all of them contain *E. coli*, and that if other microorganisms are present it is at a very low concentration. Therefore, the likelihood of multiple microorganisms hindering the MALDI identification is low, especially since the majority of UTIs are monomicrobial.

However, the possibility that other compounds could interfere with the identification is still possible. The urine samples other than Sample 5 were of varying colours, and some had suspended material, were heavily cloudy, or appeared to contain indicators. Additionally, some urine samples collected at RIH have boric acid added as a preservative. It is possible that additives such as indicators or preservatives could interfere with the MALDI analysis and hinder successful identification.

Samples set 3 (unfrozen). Similar to the results for Sample Set 2, none of the unfrozen urine samples were identified when spotted directly onto the MALDI plate, regardless of whether or not the formic acid extraction technique was employed.

The simple centrifugation procedure was done on the samples in Set 3, but with fewer positive results than Set 2, as no samples were identified with good (++) scores, and only one replicate of one sample was even identified at all.

It is still possible that other compounds, such as additives or preservatives added by the hospital labs, could interfere with the analysis. Additionally, the fairly significant time delay between sample collection by the hospital and sample analysis could result in deterioration of the samples, therefore hindering the classification. Because the samples need to be cultured by the hospital

before they can be passed over for analysis, the samples are at least a day old by the time they can be analyzed, if not older. If samples could be analyzed within several hours of collection instead of several days, it is possible that it could improve the quality of the analysis.

FUTURE DIRECTIONS

As a fairly small number of unfrozen samples from RIH have been run since the calibration issues with the MALDI were solved, it could be beneficial to run more unfrozen urine samples. While samples that had already tested positive for *E. coli* were specifically requested in this instance, it could be beneficial to run samples without any additives from previous tests, and without any preservatives if possible, to eliminate the possibility that additives could be interfering with the analysis. Additionally, the delay between sample collection by the hospital and sample analysis could be causing some deterioration that could hinder analysis. It has been observed by some studies that the protein profile of urine samples begins to degrade after only 2 days of storage at 4°C (20), so it would likely be beneficial to run some samples much sooner after they are collected, possibly even within hours of collection instead of days.

In addition to using fresher samples, it could be beneficial to study how storage and temperature affects analysis quality, by testing replicate spiked urine samples that have been frozen or refrigerated at regular intervals over a period of several days and weeks, to examine how the signal changes over time with possible sample decay.

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