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Use of Ultrasonication as a Rapid Pretreatment Method for MALDI-TOF MS of Mycobacterial Samples

Katsunao Niitsuma[1*], Shizuko Koshiba[2], Miwako Saitou[1], Tomoko Suzuki[1], Kinuyo Chikamatsu[3], Akiko Takagi[3] and Satoshi Mitarai[3,4]

[1]*Department of Infectious Diseases and Pulmonary Medicine, Fukushima Prefectural Medical University Aizu Medical Center, Fukushima, Japan*

[2]*Inspection Department, Fukushima Prefectural Minami Aizu hospital, Fukushima, Japan*

[3]*Bacteriology Division, Department of Mycobacterium Reference and Research, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan*

[4]*Department of Basic Mycobacteriosis, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*

[*]**Corresponding author:** Katsunao Niitsuma, Department of Infectious Diseases and Pulmonary Medicine, Fukushima Prefectural Medical University Aizu Medical Center, Fukushima, Japan, Tel: 81242752100; E-mail: kaniizuma1024@yahoo.co.jp

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Abstract

Background: In Japan, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is used as a simple and accurate method for mycobacterial identification, but existing pretreatment techniques are time-consuming and laborious.

Objective: We characterized a new pretreatment technique that extracts proteins using ultrasonic disruption.

Methods: We compared this new technique with the current pretreatment method and examined the usefulness of ultrasonication, including its use in mycobacterial inactivation. A total of 174 mycobacterial isolates were tested, including 50 *Mycobacterium tuberculosis* complex strains, 57 *Mycobacterium avium* strains, 55 *Mycobacterium* intracellulare strains, and 12 *Mycobacterium kansasii* strains. The ultrasonic pretreatment method was performed with or without heat-pretreatment at 95°C for 30 minutes, in parallel with the current conventional method. For all tested strains, the mycobacterial identification agreed when comparing the new and conventional methods.

Results: Samples prepared by ultrasonication without heat pretreatment exhibited multiple significant differences when compared with samples prepared by ultrasonication with heat pretreatment or by conventional methods. However, most scores were over 2.0, and the lowest score exceeded 1.7. Furthermore, the new techniques could be performed in only 10 minutes for diagnosis. In addition, we confirmed that 1 minute of ultrasonic pretreatment yielded complete inactivation of *M. tuberculosis* complex strains.

Conclusion: The more rapid technique of protein extraction/inactivation by ultrasonication without heat pretreatment is expected to be highly useful in clinical laboratory settings.

Keywords: Ultrasonication; Protein extraction method; Mycobacterial inactivation; MALDI-TOF MS; Mycobacterial identification

Abbreviations: MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry; MTC: *Mycobacterium tuberculosis* Complex; NTM: Non-tuberculous Mycobacteria; MAV: *M. avium*; MIN: *M. intracellurare*; RIT: Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association; AMC: Aizu Medical Center; MGIT: Mycobacteria Growth Indicator Tube; SVs: Score Values; PIP: Peak Incidence Power; DF: Duty Factor; CI: Confidence Interval; HCCA: α cyano-4-hydroxy acid

Introduction

The genus *Mycobacterium* is composed of 175 species (with 13 subspecies), including the *Mycobacterium tuberculosis* complex (MTC), non-tuberculous mycobacteria (NTM), and *Mycobacterium leprae* [1]. Approximately 30 of these species are considered pathogenic to humans, with roles in causing respiratory, skin and soft tissue, or disseminated infections.

In the diagnosis of mycobacterial infection, it is important to promptly confirm the presence of the bacterium and to identify which species is present. Rapid discrimination between MTC and NTM is especially important for managing of patients who are discharging bacteria and thereby compromising infection control at medical facilities. Rapid identification of the species of NTM also is necessary for judging patient infectiousness and to facilitate drug selection.

In Japan, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is used as a simple and accurate method for mycobacterial identification. However, this method requires time-consuming and laborious pretreatment of samples [2-8].

Adams et al. recently developed a new protein extraction method using ultrasonication [9] that saves time. In the current study, we compared the ultrasonic method and the current method, and moreover compared ultrasonication with and without heat pretreatment for mycobacterial inactivation. We found that the new

method allowed for rapid, safe, and specific protein extraction for MALDI-TOF MS.

Materials and Methods

We used clinical isolates obtained at either the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (RIT) or the Fukushima Prefectural Medical University Aizu Medical Center (AMC). The panel included 174 strains of four species of mycobacteria, including 50 MTC strains, 57 *M. avium* (MAV) strains, 55 *M. intracellurare* (MIN) strains, and *M. kansasii* strains, all of which had already been identified at the species level by conventional methods using polymerase chain reaction, DNA-DNA hybridization, 16s-ribosomal RNA sequencing. Overall, the 174-strain panel corresponded to 30, 27, 25 and 10 isolates of the respective species obtained as fresh strains that had been cultured in solid medium at RIT, and 20, 30, 30 and 2 isolates of the respective species obtained as stock strains that had been cultured in liquid medium at our hospital.

We compared three pretreatment methods: The current conventional method (Bruker Daltonics®, Bremen, Germany), ultrasonication with heat pretreatment (95˚C for 30 minutes, to inactivate mycobacteria), and ultrasonication without heat pretreatment. The resulting samples were analyzed by MALDI-TOF MS (MALDI Biotyper, Bruker Daltonics®, Bremen, Germany), and the differences in MS waveforms, peak intensities, and SVs were compared.

The sonicator was an Acousticsorbilizer (Adaptive Focused AcousticsTM M220 ultrasonicator, Covaris®, MA) with microtubes (microTUBETM, MA) containing silica glass beads. Ultrasonication was performed at 18˚C, using 1 minute of irradiation at 40 watts PIP and 50% DF [9].

Peptide extraction

For preparation by any of the three methods, the following procedure was performed. From colonies grown on solid medium, a 1 to 4 μL loopful of mycobacterial growth was collected, suspended in an eppendorf screw-cap tube with 300 μL of sterile water, and vortexed for 1-2 minutes. *Mycobacterium* Growth Indicator Tubes (MGIT 960 System; Becton Dickinson®, NJ) were used as the liquid medium. From the liquid medium with cultured mycobacterial growth, 500-1000 μL of fluid, including precipitate was collected by sterile transfer pipette, placed in a 1.5 mL eppendorf screw-cap tube, and centrifuged at 15,000 rpm for 2 minutes. After the supernatant was removed, the pellet was resuspended in 300 μL of sterile water by vortexing for 1-2 minutes.

For both ultrasonication with heat pretreatment and the conventional method, these 300 μL suspensions were first heated for 30 minutes in a 95˚C heat block and then returned to room temperature. For preparation by ultrasonication with heat pretreatment, the sample was centrifuged at 15,000 rpm for 2 minutes, and the supernatant was removed completely. The pellet was then resuspended by pipetting up and down in 50 μL of a 1:1 mixture of 100% acetonitrile and 70% formic acid. The suspension was transferred to a microtube with glass beads and irradiated for 1 minute using the AFATM M220 ultrasonicator. The sample was then centrifuged at 15,000 rpm for 2 minutes and 1 μL of the resulting supernatant was transferred to a MALDI Biotyper target plate.

For preparation by the conventional method, a heated sample was combined with 900 μL of 100% ethanol and vortexed for 1 minute. After centrifugation at 15,000 rpm for 2 minutes, the supernatant was completely removed, and the remaining pellet was air dried at room temperature. The pellet was then transferred to a tube containing 20-25 μL of zirconia silica beads and 20-25 μL of 100% acetonitrile and vortexed for 5 minutes, 20-25 μL of 70% formic acid was then added to the tube followed by vortexing for another 2 minutes. The sample was then centrifuged at 15,000 rpm for 2 minutes and 1 μL of the resulting supernatant was placed on the target plate.

For ultrasonication without heat pretreatment, the sample was resuspended in 300 μL of sterile water and centrifuged at 15,000 rpm for 2 minutes and the supernatant was removed completely. The pellet was then subjected to 1 minute of ultrasonication as described above. The sample was then centrifuged at 15,000 rpm for 2 minutes and 1 μL of the resulting supernatant was placed on the target plate.

The 1 μL sample supernatants placed on the target plate were allowed to air dry at room temperature. A 1 μL aliquot of α cyano-4-hydroxy acid (HCCA) matrix solution was placed on each dried spot, and the spot was again allowed to air dry at room temperature. Spots were arrayed on each target plate as 6 rows of 8 spots each, with each row consisting of 3, 3 and 2 spots of samples prepared by ultrasonication with heat pretreatment, ultrasonication without heat pretreatment, and the conventional method, respectively. The spots were analyzed by MALDI-TOF MS [4,5].

Mycobacterial identification by MALDI Biotyper

MS was obtained by Flex Control software (Bruker Daltonics®, Bremen, Germany). MALDI Biotyper 3 (Mycobacteria Library 2.0) was used as the database to perform mycobacterial identification.

Mycobacterial inactivation using ultrasonication equipment

Assessments of the effect of ultrasonication on mycobacterial inactivation were performed independently at the Aizu Medical Center and RIT. At the Aizu Medical Center, we tested 10 strains of non-heat-treated MTC with high infectious risk from among the MTC members of our mycobacterial panel. These 10 strains were randomly selected to include 5 strains each from solid medium and liquid medium. For each MTC strain, the sample consisted of a 1 μL loopful of growth from solid medium, which corresponds to a mean cell density of 2.8×10^8 CFU/mL, or from liquid medium, which corresponds to a mean cell density of 10^4-10^7 CFU/mL [10]. As described above, the optimized protocol consisted of ultrasonication at 18˚C for 1 minute at a setting of PIP 40 W/DF 50%. The entire content of each micro TUBETM was then transferred to a MGIT 960 automated culture detection system containing 0.8 mL of supplement (Becton Dickinson®, NJ) and incubated for 6 weeks or until a positive growth signal was obtained, whichever came first.

At RIT, the test of inactivation by ultrasonication used the *M. tuberculosis* type strain (H37Rv ATCC27294) and 2 MCT clinical isolates (J-18 and J-20). First, suspensions (at OD530 ~ 0.2) of each mycobacterial strain in Myco Broth (Kyokuto Pharmaceutical Industrial®, Tokyo, Japan) were prepared. To count CFU, each solution was diluted to 10-1-10-5 with physiological saline, and 100 μL of each dilution was spread on 7H10 solid medium; following incubation at 37˚C for 6 weeks, the number of colonies was counted and used to calculate the density of the original suspension. Second, 2 ml of each of the suspensions in Myco Broth was centrifuged at 11,000 rpm for 5

minutes; each resulting pellet was resuspended in 150 µL of an a 1:1 mixture of acetonitrile and 70% formic acid in water. These suspensions were ultrasonicated as described above. As much as possible of each ultrasonicated sample was collected and 50 µL of each sample, neat and diluted 10-fold, were separately spread on 7H10 solid medium and incubated for up to 6 weeks.

Results

We compared three pretreatment methods: the current conventional method (Bruker Daltonics®, Bremen, Germany), ultrasonication (Covaris®, MA) with heat pretreatment (95°C, 30 minutes) to inactivate mycobacteria, and ultrasonication without heat pretreatment [9].

Mycobacterium species consisted of 50 samples of MTC, 57 samples of MAV, 55 samples of MIN, and 12 samples of *M. kansasii*, from the Research Institute of Tuberculosis, RIT, and AMC as shown in Table 1. All species were isolated from clinical samples.

Mycobacterium species	No. of strains RIT	AMC	Total
M. tuberculosis complex	30	20	50
M. avium	27	30	57
M. intracellulare	25	30	55
M. kanasasii	10	2	12
All species			174

Table 1: *Mycobacterium* species tested in this study. *Mycobacterium* species that were tested in current study are shown. Samples were from the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (RIT) and Aizu Medical Center (AMC).

Three different methods for protein extraction were designed: ultrasonication with heat preparation (Figure 1A), ultrasonication with no-heat preparation (Figure 1B), and the current method (Figure 1C). In general, to extract proteins of species, the method using zirconia/silica beads had been common [11].

As shown in Figure 1, protein extraction times by these procedures ranged from 10 minutes to 40 minutes for setting up MALDI-TOF MS. It was clear that ultrasonication with no-heat preparation was most rapid method. Next, to assess the inactivation of mycobacterium, 5 MCT strains, each grown on both solid and liquid media, were used as samples. Samples were sonicated for 1 minute, and the resulting contents of each microtube were used to inoculate liquid medium. The resulting cultures exhibited no growth in MGIT 960 System after 6 weeks of incubation. Ultrasonication without heat pretreatment was intended to reduce the time needed for pretreatment of samples for MALDI-TOF MS without compromising safety.

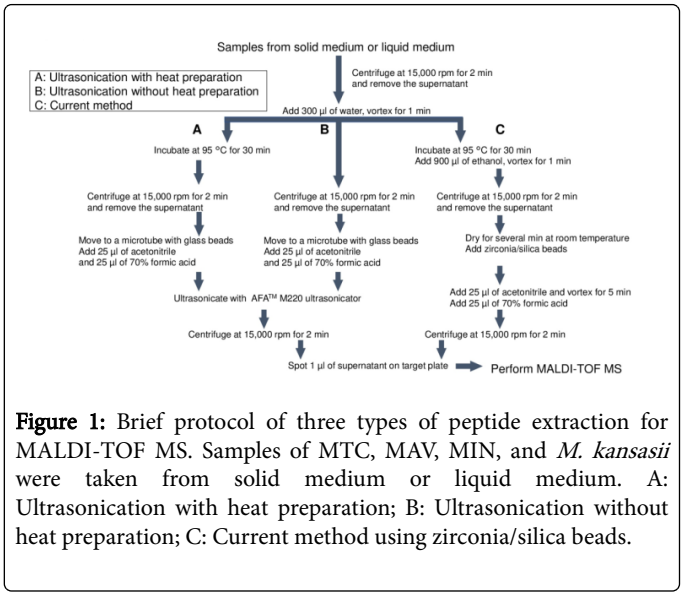

Figure 1: Brief protocol of three types of peptide extraction for MALDI-TOF MS. Samples of MTC, MAV, MIN, and *M. kansasii* were taken from solid medium or liquid medium. A: Ultrasonication with heat preparation; B: Ultrasonication without heat preparation; C: Current method using zirconia/silica beads.

The resulting score values (SVs) showed log confidence cut-offs of ≥ 1.7 for all tested strains. By setting ≥ 1.7 to >2.0 as the genus-level cut-off and ≥ 2 as the species-level cut-off, identifications agreed with those obtained via the conventional method. Pretreatment by the ultrasonication and conventional methods yielded MSs with the same waveforms and peak intensity patterns for the respective mycobacterial strains [12,13]. To compare SVs, we examined the mean values. Frequencies at the species level for each strain were compared in terms of culture medium.

With solid medium, frequencies of MTC strains with heat pretreatment, without heat pretreatment, and conventional pretreatment were 93.3%, 90.0%, and 96.7%, respectively (Figure 2). Those of MAV strains were 92.6%, 66.7%, and 88.9%, respectively (Figure 3), and those of MIN strains were 28.0%, 56.0%, and 84.0%, respectively (Figure 4). Although most frequencies exceeded 90%, the MIN strains showed low frequencies of 28% with heat pretreatment and 56% without heat pretreatment, and the SV means were 1.989 (1.774-2.321) and 2.019 (1.758-2.275), respectively. Means of other groups showed high SVs, with values exceeding 2.1 and even achieving 2.362 for MTC strains prepared by conventional pretreatment. Ten strains of *M. kansasii* showed frequencies of 80, 80, and 70% at the species level (Figure 5).

With liquid medium, the frequencies of MTC strains with heat pretreatment, without heat pretreatment, and conventional pretreatment were 95.0%, 80.0%, and 100%, respectively (Figure 2). Those of MAV strains were 66.7%, 66.7%, and 84.6%, respectively (Figure 3), and those of MIN strains were 87.0%, 87.0%, and 73.9%, respectively (Figure 4). MAV strains showed low frequencies of 66.7 and 66.7% with and without heat pretreatment, respectively; the corresponding SV means were 2.053 (1.779-2.313) and 2.060 (1.861-2.252), respectively. Means of other strains and groups showed high SV values of more than 2.12 (MTC strain conventional pretreatment showed 2.310), with the exception of MTC strains without heat pretreatment, which showed a frequency of 2.075. Two strains of *M. kansasii* (Figure 5) satisfied species-level frequencies.

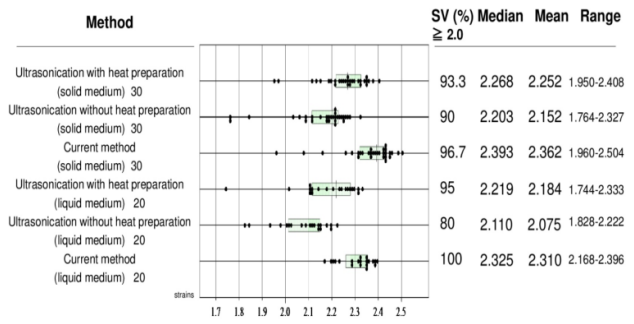

Figure 2: Distribution of SVs of the Mycobacterium tuberculosis complex. SVs of MTC in each type of method from solid or liquid medium are shown. Numbers indicate ≥ 2.0 score value (%) and log confidence scores (median, mean, range) for each of 20 or 30 strains.

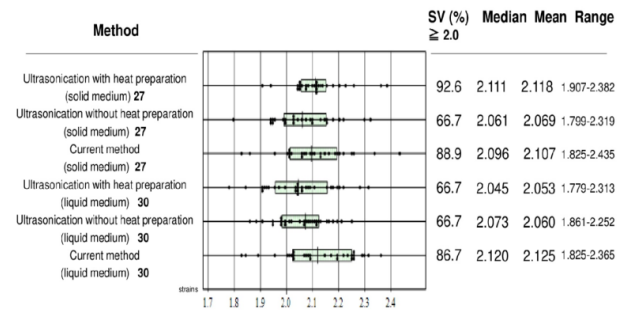

Figure 3: Distribution of SVs of *Mycobacterium avium*. SVs of MTC in each type of method from solid or liquid medium are shown. Numbers indicate ≥ 2.0 score value (%) and log confidence scores (median, mean, range) for each of 27 or 30 strains.

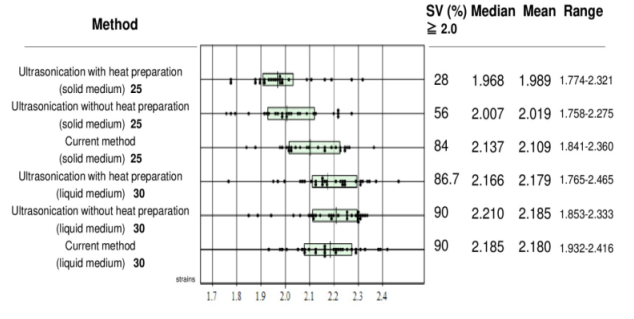

Figure 4: Distribution of SVs of *Mycobacterium intracellulare*. SVs of MTC in each type of method from solid or liquid medium are shown. Numbers indicate ≥ 2.0 score value (%) and log confidence scores (median, mean, range) for each of 25 or 30 strains.

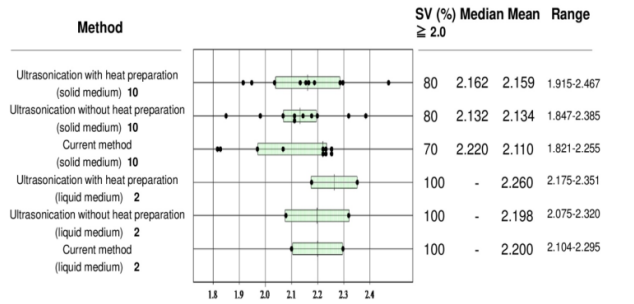

Figure 5: Distribution of SVs of *Mycobacterium kansasii*. SVs of MTC in each type of method from solid or liquid medium are shown. Numbers indicate ≥ 2.0 score value (%) and log confidence scores (median, mean, range) for each of 10 or 2 strains.

Ultrasonication without heat pretreatment was intended to reduce the time needed for pretreatment of samples for MALDI-TOF MS.

Additionally, according to a bactericidal test performed at RIT, the average cell density was 4.6×10^7 CFU/ml, ranging from 2.1×10^7 to 7.6×10^7 CFU/ml, corresponding to an actual average bacterial count of 9.2×10^7 CFU in 150 µl of specimen. Nonetheless, no bacterial growth was observed in either the stock solution or a 10-fold dilution over the course of 4 weeks of culturing after inoculation with the sonicated samples.

Discussion and Conclusion

The rapid diagnosis of mycobacterial infection provides clinicians with prompt feedback regarding mycobacterial presence/absence in patients, along with species identification. In the current study, we found that ultrasonication without heat pretreatment was a more rapid, safe, and accurate technique, and should be highly useful in a clinical laboratory setting.

For pretreatment, an early version of protein extraction by the conventional method (Bruker Daltonics ®, Bremen, Germany) required many steps to inactivate mycobacteria, had a high infection risk, and took about 40 minutes to prepare for MALDI-TOF MS (Figure 1). Adams et al. recently developed the use of Adaptive Focused Acoustics (AFATM) and ultrasonication, and optimized the ultrasonication protocol for clinical use in identification at the species level of mycobacteria grown in solid media, shortening the needed time compared to conventional extraction/inactivation methods [9]. They performed ultrasonication at 18 ℃ using 1-3 minutes of irradiation at 40 W peak incidence power (PIP) and 50% duty factor (DF), and it safely provided a higher average log confidence score [9]. In the present work, we used settings of 18 ℃, 40 W PIP, and 50% DF with a shorter exposure time of 1 minute. The ultrasoncation method greatly shortens the time compared to the conventional method.

Identification of mycobacteria using MALDI-TOF MS also inactivates the mycobacteria, since the extraction of protein requires destroying the thick mycobacterial cell wall. We were concerned that 1 minute of ultrasonic irradiation might not be sufficient to achieve cell wall destruction, protein extraction, and cellular inactivation, given that standard methods for mycobacterial inactivation incorporate heat treatment with 95 ℃ for 30 minute. We therefore evaluated the utility of

ultrasonication with and without heat pretreatment, specifically by applying heat prior to the 1 minute of ultrasonication. We compared techniques employing ultrasonication to the conventional heat-based method using MTC, MAV, and MIN strains. *M. kansasii* strains were excluded from our statistical evaluation since only 12 isolates of this species were included in our panel.

As a first step, we compared ultrasonication with or without heat pretreatment and from solid or liquid medium. For MTC strains, most comparisons between pretreatments for each medium and between media showed significant differences. Obtained values of mean and range (95% confidence interval (CI)) were as follows: 2.252 (1.950-2.408) for sonication with heat pretreatment from solid medium; 2.152 (1.764-2.327) for sonication without heat pretreatment from solid medium; 2.184 (1.744-2.333) for sonication with heat pretreatment from liquid medium; and 2.075 (1.828-2.222) for sonication without heat pretreatment from liquid medium. The obtained values exceeded the species-level thresholds, and the ranges between groups with significant differences exceeded the 1.7 log confidence cut-off value. For all pretreatment methods and media, SVs consistently exceeded the 1.7 log confidence cut-off value.

For MAV strains, most of the comparisons between pretreatments for each medium and between media did not show significant differences. Obtained values of mean and range (95% CI) were as follows: 2.118 (1.907-2.382) for sonication with heat pretreatment from solid medium; 2.069 (1.799-2.319) for sonication without heat pretreatment from solid medium; 2.053 (1.779-2.313) for sonication with heat pretreatment from liquid medium; and 2.060 (1.861-2.252) for sonication without heat pretreatment from liquid medium. The only significant difference was for the comparison between pretreatment methods from liquid medium, but the 95% CI for this comparison was greater than the 1.7 log confidence cut-off value. Therefore, our study suggests that similar results can be expected for MAV strains with any pretreatment method.

For MIN strains, sonication with and without heat pretreatment showed significant differences between media. Obtained mean values and ranges (95% CI) were as follows: 1.989 (1.774-2.321) for sonication with heat pretreatment from solid medium; 2.179 (1.765-2.465) for sonication without heat pretreatment from solid medium; 2.179 (1.765-2.465) for sonication with heat pretreatment from liquid medium; and 2.185 (1.853-2.333) for sonication without heat pretreatment from liquid medium. Means showed differences in protein extraction not by pretreatment methods but by media. However, as with MAV strains, 95% CIs for MIN strains were greater than the 1.7 log confidence cut-off value.

Comparing ultrasonication with and without heat pretreatment to the conventional method, some differences were found: For MTC strains, all comparisons except those between media using the conventional method, for MAV strains, comparisons between ultrasonication with and without heat pretreatment from liquid medium; for MIN strains, all comparisons except those between pretreatment methods with liquid medium as with MTC strains. Even when significant differences were found, the detection ranges of SV values and 95% CIs of each of the groups were greater than the 1.7 log confidence cut-off value, and the means of virtually all comparisons with each medium or between media showed relatively high values. Notably, for MTC strains mean scores with solid medium and liquid medium were 2.362 and 2.310, respectively. Although ultrasonication with heat pretreatment and the conventional method may have given slightly higher SVs, most SVs were greater than 1.7, the means were greater than the species level, and the 95% CIs were greater than the genus level. Therefore, the three methods do not have meaningful differences.

Regarding inactivation of mycobacteria, 1 minute ultrasonication in place of heat pretreatment at 95°C for 30 minutes provided inactivation of up to 10^8 CFU/mL of *M. tuberculosis* group bacteria, species that would otherwise represent a high infectious risk. Since NTM strains are considered to have no infectivity, heat pretreatment at 95°C for 30 minutes may not be important, and shortening the procedure by removing this step might be desirable. In fact, these cultures were negative. However, infectious strains such as members of MTC must be inactivated at clinical sites. We have already confirmed that 1 minute of ultrasonic pretreatment yielded complete inactivation of strains of the *M. tuberculosis* complex. Therefore, our results suggest that the use of ultrasonication permits subsequent procedures to be performed without risk of infection, meaning that the resulting sample can be safely used for MALDI-TOF MS.

In summary, we confirmed that the ultrasonication method without heat pretreatment shortened the time required by six-fold, is safe, and accurately facilitated protein extraction for MALDI-TOF MS. We expect that speed and ease of this preparation technique will provide clinical laboratories with savings in both time and cost.

Conflict of Interest Statement

None

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