Examination of conditions for regular internal quality control in identification of microorganisms using MALDI-TOF MS

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Received 12 June, 2020/Accepted 26 October, 2020

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was approved for medical use in 2011, and is currently used as a rapid, accurate and low-cost technique for bacterial identification. Microbiological testing and internal accuracy control in Japan are mainly implemented in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI). However, few facilities perform internal accuracy control of bacterial identification by MALDI-TOF MS. Therefore, we examined the procedures for internal accuracy control of bacterial identification using MALDI-TOF MS in daily work at clinical laboratories in the seven hospitals.

Key words: Identification of microorganisms / Internal quality control / Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
Mass spectrometry (MS) is currently the most important method for proteome analysis. Anhalt and Fenselau (1975) first performed bacterial identification using MS based on peptide spectrum matching, and confirmed seven identified strains: Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Salmonella sp, Proteus morganii, and Providencia rettgeri. Bacterial identification with MS is an easy-to-use method with regard to sample preparation and measurement, and only requires about 5 minutes for identification of one strain (Freiwald and Sauer, 2009; Seng et al., 2009). The technique makes it easy to identify species and strains without cumbersome pretreatment of samples (Bizzini et al., 2010; De Bel et al., 2010; Sauer and Klem, 2010; Seng et al., 2009; Sogawa et al., 2011; Sogawa et al., 2012; van Veen et al., 2010).

Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) was approved for medical use in Japan in 2011 and is now used for bacterial identification. The Act Partially Amending the Medical Practitioners’ Act on quality and accuracy assurance of specimen examination was passed and promulgated in June 2017. The relevant amended ordinances of the Ministry of Health, Labour and Welfare (Ordinance for Enforcement of the Medical Care Act and Regulation for Enforcement of the Act on Clinical Laboratory Technicians) were promulgated in July 2018 and came into effect on December 1, 2018. The amendment defined legal criteria for accuracy control in clinical laboratory facilities, and many facilities have now obtained an International Standardization Organization (ISO) 15189 certificate as the international standard for clinical laboratory testing, including microbiology tests.

Valentine et al. (2005) performed bacterial identification using Yersinia enterocolitica, Bacillus subtilis, and Escherichia coli W3110 purchased from ATCC®. MALDI-TOF MS was used to identify colonies incubated on four different basic media, with no effect of the media found. TOF MS was used to identify colonies incubated on four second generations, respectively. Each time, the strain used for identification with MS is an easy-to-use method with regard to sample preparation and measurement, and only requires about 5 minutes for identification of one strain (Freiwald and Sauer, 2009; Seng et al., 2009). The technique makes it easy to identify species and strains without cumbersome pretreatment of samples (Bizzini et al., 2010; De Bel et al., 2010; Sauer and Klem, 2010; Seng et al., 2009; Sogawa et al., 2011; Sogawa et al., 2012; van Veen et al., 2010).

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Clinical microbiology testing measures the presence of microorganisms; therefore, the results are likely to change due to strains, commercial media and measurement method. With regard to internal accuracy control, many facilities have previously used Escherichia coli. However, E. coli is used for MALDI-TOF MS calibration, and this makes its use for internal quality control unsuitable. Commercial media do not affect bacterial identification (Valentine et al., 2005), but can influence identification reliability rates. There are three testing procedures used: direct measurement of colonies, measurement of colonies with formic acid, and measurement of proteins extracted from colonies (Yamashita, 2017; Hanaiwa et al., 2018).

Three university hospitals (Chiba, Kyushu and Asahikawa Medical) examined procedures for internal accuracy control of bacterial identification using MS (Sogawa et al., 2020). Based on the results, use of Enterobacter aerogenes (ATCC13048) and Enterococcus faecalis (ATCC19433), 18-24 hr culture on the same blood agar media after inoculation was proposed, with fine tuning at 6-month to one-year intervals for the MALDI Biotyper (recommended by Bruker Japan) and once per approx. 2,500 specimens for the VITEK MS (recommended by bioMérieux Japan Ltd.). We performed these procedures for internal accuracy control of bacterial identification using MS in seven facilities.

Enterobacter aerogenes (ATCC13048) and Enterococcus faecalis (ATCC19433) purchased from American Type Culture Collection (ATCC) were used in the study. Bacterial cultures were produced by adding a solution in a freeze-dried microbial pellet (E. aerogenes and E. faecalis) and were applied to Trypticase Soy Agar with 5% sheep blood with a cotton swab. After aerobic incubation for 24 hr at 37°C, colonies were suspended in Microbank™ and cryopreserved at −80°C. The freeze-dried microbial pellet, the strain preserved in Microbank™, and the strain used for internal accuracy control were defined as the zero, first and second generations, respectively. Each time, the strains were inoculated on Nissui Plate Sheep Blood Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) from Microbank™ and aerobically incubated at 35°C for 18 to 24 hr. Colonies growing on blood agar media were used for testing. Mass spectra were obtained using MALDI Biotyper (Bruker Daltonik GmbH, Bremen Germany) and VITEK MS (bioMérieux Japan Ltd., Tokyo, Japan) and identified by MALDI Biotyper ver.3.0 and Myla ver. 2.0 software. Fine tuning of MALDI-TOF MS was performed immediately before 8-week continuous measurement by consultants from Bruker Japan and bioMérieux Japan Ltd. HCCA portioned reagents (Bruker Daltonik GmbH) and 99% formic acid solution diluted in 70% (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan: LC-MS grade) were used for measurements with MALDI Biotyper; MS-CHCA
matrix reagent and MS-FA reagent (both bioMérieux Japan Ltd.) were used for measurements with VITEK MS.

Plates were prepared by the on-plate testing method without or with formic acid: in the former, small amounts of colonies on blood agar were scraped out using a toothpick and swabbed as a spot on a target plate thinly and evenly, and then fully dried after adding 1 µL of matrix reagent; in the latter, colonies were similarly swabbed as a spot on a target plate, dried after adding 1 µL of formic acid, and then fully dried after adding 1 µL of matrix reagent. With the MALDI Biotyper, Shinshu and Miyazaki university hospitals used on-plate testing without formic acid, whereas Chiba and Saga university hospitals used the method with formic acid. For the VITEK MS, plates were prepared by the on-plate testing method without formic acid at Kyushu, Mie and Ryukyu university hospitals. A strain was inoculated as 5 spots per blood culture medium and measured. Identification rates were defined based on strains found using MALDI-TOF MS. As reliability indexes that indicate accuracy at the bacterial species level, an identification probability per strain of 99.9% was used for the VITEK MS.

E. aerogenes and E. faecalis were measured once a week for 8 weeks at Chiba, Saga, Shinshu and Miyazaki using the MALDI Biotyper, and at Kyushu, Mie and Ryukyu using the VITEK MS. The three facilities using the VITEK MS identified E. aerogenes and E. faecalis incubated on blood agar media. The identification probabilities were 99.9% for 5 weeks at Kyushu, 6 weeks at Ryukyu, and 8 weeks at Mie (Table 1). Of the four facilities using the MALDI Biotyper, Chiba and Saga used on-plate testing with formic acid, and Shinshu and Miyazaki used similar testing without formic acid. E. aerogenes and E. faecalis incubated on blood agar media were identified with reliability indexes of 2.480 to 2.561 (2.519±0.023) and 2.403 to 2.442 (2.425±0.016) at Chiba, 2.505 to 2.542 (2.522±0.013) and 2.345 to 2.413 (2.386±0.021) at Saga, 2.512 to 2.613 (2.556±0.036) and 2.279 to 2.439 (2.377±0.051) at Shinshu, and 2.504 to 2.542 (2.527±0.011) and 2.228 to 2.438 (2.359±0.056) at Miyazaki, respectively (Table 2).

In clinical practice, definitive diagnosis and therapeutic strategy depend on the results of specimen examination. Therefore, accuracy assurance and control of testing systems is very important. Microbiological testing and internal accuracy control in Japan are mainly implemented in accordance with the Clinical and Laboratory Standards Institute (CLSI). However, few facilities perform accuracy control of bacterial identification using MALDI-TOF MS, despite having a definition of accuracy control. Therefore, we examined procedures for internal accuracy control of bacterial identification using MALDI-TOF MS in daily work at seven facilities.

Gram-negative bacillus E. aerogenes and Gram-positive cocci E. faecalis were used as standard strains for internal accuracy control for the following reasons. E. aerogenes structurally has thin peptidoglycan layers, which means that ribosome-derived proteins in this

| TABLE 1. VITEK MS score rates for E. aerogenes and E. faecalis |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 1st week | 2nd week | 3rd week | 4th week | 5th week | 6th week | 7th week | 8th week |
| Kyusyu University Hospital |         |          |          |          |          |          |          |          |
| E. aerogenes     | 99.9     | 99.9     | 99.9     | 99.9     | no data  | no data  | no data  | no data  |
| E. faecalis      | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     |
| Mie University Hospital |         |          |          |          |          |          |          |          |
| E. aerogenes     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     |
| E. faecalis      | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     |
| Ryukyu University Hospital |         |          |          |          |          |          |          |          |
| E. aerogenes     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | no data  | no data  | no data  |
| E. faecalis      | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | 99.9     | no data  | no data  |

As reliability indexes that indicates accuracy at the bacterial species level, an identification probability per strain of 99.9% was used for the VITEK MS.
| Hospital                     | 1st week  | 2nd week  | 3rd week  | 4th week  | 5th week  | 6th week  | 7th week  | 8th week  |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| **Chiba University Hospital** |           |           |           |           |           |           |           |           |
| *E. aerogenes*              | 2.520±0.062 | 2.533±0.022 | 2.539±0.019 | no data | 2.532±0.037 | 2.561±0.049 | 2.540±0.047 | 2.480±0.018 |
| *E. faecalis*               | 2.437±0.019 | 2.408±0.027 | 2.411±0.015 | no data | 2.441±0.025 | 2.433±0.036 | 2.403±0.038 | 2.442±0.034 |
| **Saga University Hospital** |           |           |           |           |           |           |           |           |
| *E. aerogenes*              | 2.542±0.026 | 2.528±0.014 | 2.519±0.026 | 2.505±0.038 | 2.519±0.023 | 2.539±0.014 | 2.505±0.042 | 2.517±0.038 |
| *E. faecalis*               | 2.399±0.056 | 2.345±0.035 | 2.368±0.021 | 2.392±0.037 | 2.413±0.044 | 2.394±0.024 | 2.367±0.040 | 2.398±0.021 |
| **Shinshu University Hospital** |       |           |           |           |           |           |           |           |
| *E. aerogenes*              | 2.594±0.045 | 2.522±0.043 | 2.512±0.063 | 2.524±0.015 | 2.613±0.052 | 2.554±0.046 | 2.589±0.029 | 2.538±0.048 |
| *E. faecalis*               | 2.337±0.166 | 2.366±0.101 | 2.394±0.059 | 2.421±0.060 | 2.349±0.067 | 2.428±0.014 | 2.279±0.102 | 2.439±0.038 |
| **Miyazaki University Hospital** |         |           |           |           |           |           |           |           |
| *E. aerogenes*              | 2.542±0.032 | 2.504±0.034 | 2.530±0.024 | 2.526±0.020 | 2.528±0.025 | 2.540±0.044 | 2.518±0.061 | 2.524±0.010 |
| *E. faecalis*               | 2.364±0.038 | 2.438±0.043 | 2.378±0.037 | 2.386±0.031 | 2.355±0.027 | 2.378±0.030 | 2.348±0.092 | 2.228±0.073 |

As reliability indexes that indicates accuracy at the bacterial species level, a score of 2.000-3.000 was used for the MALDI Biotyper.
strain are likely to be ionized (Yamashita, 2017). In contrast, *E. faecalis* has thick cell walls and a firm structure, which makes it unlikely that ribosome-derived proteins will be ionized (Yamashita, 2017). *E. aerogenes* and *E. faecalis* were grown on Nissui Plate Sheep Blood Agar, and in bacterial identification the reliability indexes with the MALDI Biotyper were ≥2.000 and the identification probability with the VITEK MS was 99.9% for all strains. Therefore, these strains are appropriate for internal accuracy control. *Escherichia coli* is used for calibration of the MALDI Biotyper and VITEK MS and is not appropriate for use as a standard for internal accuracy control.

If MALDI-TOF MS conditions (laser power, detector sensitivity) are inconsistent, even using the same strains, calibrants, matrix reagents and solvents, the spectra and reliability indexes are affected (Sogawa et al., 2020). In addition to use of the same strains and reagents, we fine-tuned the MALDI-TOF MS system immediately before continuous measurements for 8 weeks to compare MALDI-TOF MS results at each facility under the same conditions. In bacterial identification of strains in the seven facilities, the reliability indexes of the MALDI Biotyper were ≥2.200 and the identification probability of the VITEK MS was 99.9% for all strains. Facilities had different conditions and devices, but there was no problem with bacterial identification at the species level and identification after fine tuning using the procedures at the seven facilities.

The fine-tuning interval of the MALDI Biotyper was from 6 months to one year, as recommended by Bruker Japan, whereas that of the VITEK MS was once per approx. 2,500 specimens, as recommended by bioMérieux Japan Ltd. The MALDI Biotyper could not find a peak or had a reliability index of <2.000 for several species, although the device passed the standards for maintenance by the manufacturer after change-out of the detector and laser. Consequently, data were acquired using an on-plate testing method with formic acid at Chiba and Saga. The data acquisition period for the VITEK MS varied from 5 weeks at Kyushu to 6 weeks at Ryukyu and 8 weeks at Mie. Data acquisition was stopped at some facilities because the number of test specimens reached 2500 during the study period, which is the limit of fine tuning.

Based on examination of the procedures for internal accuracy control of bacterial identification using MALDI-TOF MS, our proposed method is as follows. (1) Internal quality control should be performed once a week. (2) The appropriate standard strains for internal quality control are second generation of *Enterobacter aerogenes* and *Enterococcus faecalis*. (3) Quality control strains should be inoculated on Nissui Plate Sheep Blood Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) from MicroBank™ and aerobically incubated at 35°C for 18 to 24 hr. (4) Colonies growing on blood agar media are used for testing, with measuring plates prepared for on-plate testing without or with formic acid. (5) Mass spectra are obtained using MALDI Biotyper and VITEK MS, and identified by MALDI Biotyper ver. 3.0 and Myla ver. 2.0 software. (6) The reliability indexes that indicate accuracy at the bacterial species level are a score of ≥2,200 for all 5 spots and other bacterial species not displayed in the top 5 scores for the MALDI Biotyper, and an identification probability per strain of 99.9% for the VITEK MS. (7) Fine tuning should be performed at 6-month to one-year intervals for the MALDI Biotyper, and once per approx. 2,500 specimens for the VITEK MS. We note that our recommended procedure is derived from one approach, and other valid procedures may be available. We also plan to use these procedures to examine external accuracy control at other facilities using MALDI-TOF MS.

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