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Identification of Waterborne Microbial Pathogens by Matrix-assisted Laser-desorption/ionization Time-of-flight Mass Spectrometry and the Biotyper 2.0 Databases

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On the basis of various guidelines for drinking-water quality, five species of bacteria (*Helicobacter pylori*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Legionella pneumophila*, and *Campylobacter jejuni*) were selected as representatives of waterborne microbial pathogens. Matrix-associated laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was conducted to identify the five bacteria, and the MALDI-TOF MS results were compared with results obtained through 16S rRNA identification. The MALDI-TOF MS analysis yielded score values of 1.60 ± 0.19, 2.41 ± 0.09, 2.37 ± 0.16, 2.19 ± 0.15, and 1.85 ± 0.08 for *H. pylori*, *S. typhimurium*, *Y. enterocolitica*, *L. pneumophila,* and *C. jejuni*, respectively. In addition, the MALDI-TOF identification results were well-correlated with those from the 16S rRNA identification. This study suggests that MALDI-TOF-based identification is an inexpensive, rapid, reproducible, and accurate alternative method for the identification of waterborne microbial pathogens in aquatic environments.

Key words: MALDI-TOF mass spectrometry, waterborne microbial pathogens, 16S rRNA identification.

Infections and diseases caused by waterborne and foodborne microbial pathogens are a worldwide threat to human health[1]. In developing countries, mortality due to bacterial diarrhea is a major international health issue[2, 3]. In the case of developed countries, management of waterborne pathogenic microorganisms that can cause bacterial diarrhea is strictly implemented. One aspect of such management is the listing of microorganisms that are considered waterborne pathogens. This can help to clarify the focus of public health agencies by specifying organisms known to be hazardous to human health. In the United States, the US-EPA specifies a large number of waterborne microbial pathogens in the contamination candidate list (CCL)[4]. The priority of microorganisms list (PML) is the list of waterborne and foodborne microbial pathogens designated as management targets by Korean government. World Health Organization (WHO) and Australian government also designated Guidelines for Drinking-water Quality and Australian Drinking Water Guidelines respectively in order to put emphasis on managing such waterborne microbial pathogens[5, 6].

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Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be used to analyze the protein composition in bacterial cells, and has been highlighted as an accurate and rapid protein identification technology[7-9]. This method yields reproducible and species-specific spectral patterns in a process requiring minimal time and expense[9, 10].

MATERIALS AND METHODS

Bacteria and culture

Five major waterborne microbial pathogens were selected for testing the applicability of MALDI-TOF profiling to the identification of pathogens of concern in drinking water safety. Three reference bacterial strains [*Campylobacter jejuni* (KCTC-5327), *Helicobacter pylori* (KCTC-12083), and *Legionella pneumophila* (ATCC-33152)] were purchased from the Korean Collection of Type Cultures (KCTC, Daejeon, Korea) and from the American Type Culture Collection (ATCC, Manassas, VA). *Salmonella typhimurium* and *Yersinia enterocolitica* were kindly provided from Prof. Kun-Ho Seo (Konkuk University, Korea). All bacteria were grown on Brain Heart Infusion Agar over 24 h under microaerobic conditions (GENbox microaer; BioMerieux, Marcy l'Etoile, France).

Sample preparation for MALDI-TOF MS analysis

A single colony of each cultured isolate was used for extraction of bacterial proteins for the MALDI-TOF MS analysis, which was carried out the same day. The extraction process was carried out according to the manufacturer's protocol. In brief, a single colony was suspended in 300 µL of distilled water, 900 µL of 100% ethanol was added, and the solution was mixed by vortexing. After centrifugation at 13,000 rpm for 2 minutes, supernatants were discarded and cell pellets were dried at room temperature. Next, 50 µL of 70% formic acid was added to the bacterial pellet and the components were mixed by vortexing. Then, 50 µL of 100% acetonitrile was added and the mixture was centrifuged at 13,000 rpm for 2 minutes. At this point, the supernatant contained the bacterial extract, and 1 µL of this supernatant was transferred to the MALDI target plate (Bruker Daltonik GmbH, Bremen, Germany) and dried. The samples were overlaid with 2 µL of MALDI matrix [a saturated solution of a-cyano-4-hydroxycinnamic acid (Bruker Daltonik GmbH) in 50% acetonitrile/2.5% trifluoroacetic acid/47.5% water] and crystallized by air-drying at room temperature.

MALDI-TOF MS identification

MALDI-TOF analysis was conducted using the Microflex MALDI-TOF MS instrument, which yielded automated measurements, and MALDI Biotyper software (Biotyper Library v 2.0, Bruker Daltonik GmbH) for protein identification. The analyzed mass range was 2~20 k m/z and each spectrum was obtained after 300 shots by 60Hz in an automatic acquisition mode. A mass to charge range of 2~20 kDa was used for the identification approach. The identification was conducted in quadruplicate and high-score cutoff values were applied to each measurement according to the manufacturer's instructions. According to this score system, a score of >2 is recommended for probable species identification and secure genus identification, and a score of greater than 2.3 is recommended for a highly probable species identification.

16S rRNA gene sequencing and sequence analysis

For sequencing of 16S rRNA genes, bacterial colonies were picked and transferred into 100 µL distilled water and heated at 100°C for 10 minutes. The tubes were allowed to cool to room temperature and then were centrifuged at 12,000 x g for 5 minutes. A sample of the resulting supernatant (1 µL) was used as the template for the 16S rRNA amplification PCR. The primers and PCR conditions used were previously published[11]. The amplified PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and were sequenced via automated DNA sequencing by Solgent (Dajeon, Korea).

RESULTS

Selection of microbial pathogens

To determine the applicability of MALDI-TOF MS analysis to detection and identification of waterborne pathogenic bacteria, five different bacteria were selected by referencing the three CCLs of the USEPA[4, 12, 13], the WHO Guidelines for Drinking-water Quality[6], the Australian drinking water guidelines[5], and the Korean PML, including

Table 1. Selection of five bacterial species on the basis of water safety guidelines from various governments and organizations

Bacteria	Korean PML	Korean PML candidates	US-EPA CCL	WHO GDQ	ADWG	MALDI -DB
Helicobacter pylori		○	○		○	○
Salmonella typhimurium	○			○	○	○
Yersinia enterocolitica				○	○	○
Legionella pneumophila	○		○	○	○	○
Campylobacter jejuni	○		○	○	○	○

GDQ, Guidelines for Drinking-water Quality; PML, priority of microorganisms list; CCL, contamination candidate list; ADWG, Australian drinking water guidelines

Table 2. Bacterial identification using MALDI-TOF MS and 16S rRNA sequencing in five selected pathogenic strains

Bacterial strains	Species determined by MALDI-TOF MS (score)	Species determined by 16S rRNA gene sequencing (GenBank Acc. Number)
Helicobacter pylori	*Helicobacter pylori* (1.60 ± 0.19)	*Helicobacter pylori* (AY593988.1)
Salmonella typhimurium	*Salmonella* sp. (2.41 ± 0.09)	*Salmonella typhimurium* (AF170176.1)
Yersinia enterocolitica	*Yersinia enterocolitica* (2.37 ± 0.16)	*Yersinia enterocolitica* (AF366378.1)
Legionella pneumophila	*Legionella pneumophila* (2.19 ± 0.15)	*Legionella pneumophila* ssp. (AE017354.1)
Campylobacter jejuni	*Campylobacter jejuni* (1.85 ± 0.08)	*Campylobacter jejuni* (AF550629.1)

PML candidates. CCLs 1 and 2 of the USEPA included *H. pylori* and *Mycobacterium avium-intracellularae* as targets for management. In addition, CCL3 was revised in 2009 also include *Campylobacter jejuni*, *Escherichia coli* (O157), *Legionella pneumophila*, *Salmonella enterica*, and *Shigella sonnei*[4]. The WHO guidelines for drinking water listed 12 bacteria, including *Shigella sonnei*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Legionella pneumophila*, and *Campylobacter jejuni*[6]. The Australian Drinking Water Guidelines 6 listed 13 different bacteria, including *Shigella sonnei*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Legionella pneumophila*, and *Campylobacter jejuni*[5]. The Republic of Korea designated six bacteria (*Shigella* sp., *Salmonella* sp., *Campylobacter jejuni*, *Legionella pneumophila*, *Mycobacterium avium* complex, and enterohemorrhagic *E. coli*), and an additional four bacteria (*Vibrio* sp., *Helicobacter pylori*, *Leptosporidium* sp., and *Clostridium* sp.) were listed as "PML candidates". Based on these guidelines, five species (*Helicobacter pylori*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Legionella pneumophila*, and *Campylobacter jejuni*) were selected (Table 1).

Identification of MALDI-TOF MS

The MALDI-TOF analysis yielded spectral and score values for each species (Fig. 1 and Table 2). The score values for *H. pylori*, *S. typhimurium*, *Y. enterocolitica*, *L. pneumophila*, and *C. jejuni* were 1.60 ± 0.19, 2.41 ± 0.09, 2.37 ± 0.16, 2.19 ± 0.15, and 1.85 ± 0.08, respectively. These scores (2.0 to 2.3 or greater) indicated that *S. typhimurium*, *Y. enterocolitica*, and *L. pneumophila* could be identified to the species level. Unfortunately, it was not possible to obtain a reliable identification result for *H. pylori*, and the *C. jejuni* values, 1.7 to 2.0,

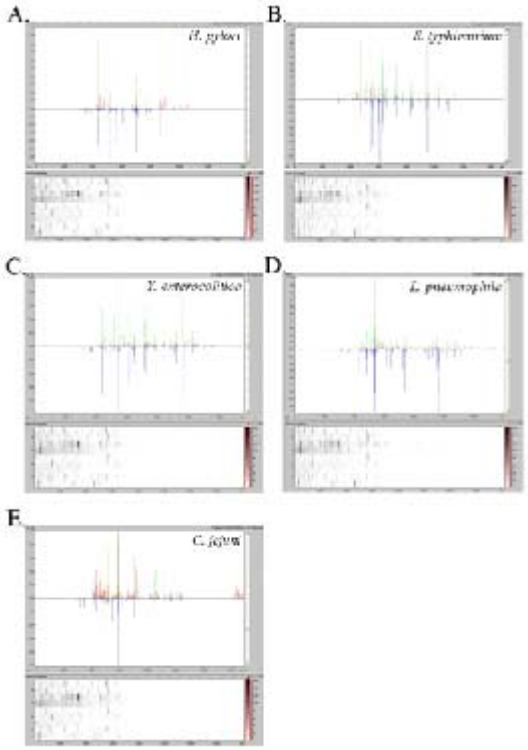

Fig. 1. Mass spectra of five bacterial strains from MALDI-TOF MS analysis

were sufficient for only probable identification. The identification results determined by MALDI-TOF MS were in agreement with those based on 16S rRNA sequencing (Table 2).

DISCUSSION

Traditionally, to isolate and identify these microorganisms, culture methods and phenotypic tests, such as Gram-staining, biochemical reactions, and fatty-acid patterns, have been used[14]. While effective, these methods have disadvantages, including misidentification due to limited biochemical activity and variable morphology, and their time-consuming, error-prone, and labor intensive nature[15-17]. Nucleic acid-based identification methods have overcome many of these limitations. Species-specific PCR, PCR-RFLP, and 16S rRNA gene sequencing are now used extensively, and identification using 16S rRNA gene sequencing has become the gold standard for bacterial identification[8, 18, 19]. Nucleic acid-based methods, however, require a careful quality control process and skilled technicians because they are very sensitive, and this can lead to cross-contamination between samples or false-positive results[9, 20]. Another disadvantage of PCR is that it can overestimate the number of bacteria, since it detects both dead and live bacteria without distinction between the two[21]. To address this, specific PCR methods have been developed that can detect live bacteria selectively through treatment with ethidium bromide monoazide and propidium monoazide before conducting the PCR[22-24], but the requirement for highly skilled technicians remains, due to the sensitivity of PCR. In recent years, to address the disadvantages, various identification technologies based on proteins, such as enzyme-linked immunosorbent assay and rapid diagnostic test, have been developed. The proof of principle of using MALDI-TOF MS for bacterial species identification was already demonstrated a decade ago[25]. Since then, the MALDI-TOF method has gained acceptance as a convenient, rapid, and precise method for the identification of microorganisms[17]. Government agencies, however, may be slow to accept new techniques without strong evidence of their efficacy for the specific task of that agency. In this study, five different waterborne pathogenic microorganisms were selected and identified using MALDI-TOF, and the results of the identification compared with those from 16S rRNA sequencing, which is considered a gold-standard method. In the case of *H. pylori*, it failed to show a high level of reliability, with an undesirable MALDI-TOF MS score value of 1.60 ± 0.19. In the case of *C. jejuni*, the method yielded only a probable identification result for genus, with a score value of 1.85 ± 0.08. It was reported that the identification of *Helicobacter* and *Campylobacter* using MALDI-TOF MS is not possible due to the fact that the modified cefoperazone deoxycholate agar used to culture these species interrupts the ionization of the biomolecules[26]. The extraction of a sufficient biomass of bacteria is also difficult because the bacteria are strongly bonded to the surface of the agar[26]. This exemplifies a prime drawback of this technique: the results of MALDI-TOF are greatly affected by the composition of culture media, growth types, culture conditions, and growth conditions[27]. These factors also affected the present results, leading to the low score values for

the MALDI-TOF analysis of *Helicobacter pylori* and *Campylobacter jejuni* in this study. Therefore, it can be concluded that the use of MALDI-TOF analysis for bacterial identification or development of bacterial protein profiling databases will first require the optimization of various analysis conditions, such as different growth and culture conditions[28]. The complete agreement with 16S rRNA sequencing results, however, indicates that the rapid MALDI-TOF MS method can provide accurate identification of the five different waterborne pathogenic microorganisms tested in this study.

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REFERENCES

1. Ashbolt, N.J. Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology*, 2004; **198**(1–3): 229–38.
2. Liu, L., Johnson, H.L., Cousens, S., Perin, J., Scott, S., Lawn, J.E., Rudan, I., Campbell, H., Cibulskis, R., Li, M., Mathers, C., Black, R.E., Child Health Epidemiology Reference Group of WHO and UNICEF. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet*, 2012; **379**(9832): 2151–61.
3. WHO. WHO methods and data sources for global causes of death 2000 2011. 2013; World Health Organization, Geneva. Available from: http://www.who.int/healthinfo/statistics/GlobalCOD_method.pdf
4. USEPA. Fact Sheet: Final Third Drinking Water Contaminant Candidate List (CCL 3). 2009; EPA 815-F-09-001. United States Environmental Protection Agency, Washington, DC.
5. NHMRC, NRMMC. Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy. 2011; National Health and Medical Research Council, National Resource Management Ministerial Council, Commonwealth of Australia, Canberra, Australia.
6. WHO. Guidelines for Drinking-water Quality. 2008; World Health Organization, Geneva. Available from: http://www.who.int/water_sanitation_health/dwq/fulltext.pdf
7. Alispahic, M., Christensen, H., Hess, C., Razzazi-Fazeli, E., Bisgaard, M., Hess, M. Identification of Gallibacterium species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry evaluated by multilocus sequence analysis. *Int. J. Med. Microbiol.*, 2011; **301**(6): 513–22.
8. Mellmann, A., Cloud, J.L., Andrees, S., Blackwood, K., Carroll, K.C., Kabani, A., Roth, A., Harmsen, D. Evaluation of RIDOM, MicroSeq, and Genbank services in the molecular identification of Nocardia species. *Int. J. Med. Microbiol.*, 2003; **293**(5): 359–70.
9. Stevenson, L.G., Drake, S.K., Shea, Y.R., Zelazny, A.M., Murray, P.R. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. *J. Clin. Microbiol.*, 2010; **48**(10): 3482–6.
10. Hsieh, S.Y., Tseng, C.L., Lee, Y.S., Kuo, A.J., Sun, C.F., Lin, Y.H., Chen, J.K. Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. *Mol. Cell. Proteomics*, 2008; **7**(2): 448–56.
11. Lee, G.C., Kim, Y.S., Kim, M.J., Oh, S.A., Choi, I., Park, J.G., Chong, C.K., Kim, Y.Y., Lee, K., Lee, C.H. Presence, molecular characteristics and geosmin producing ability of Actinomycetes isolated from South Korean terrestrial and aquatic environments. *Water Sci. Tech.*, 2011; **63**(11): 2745–51.
12. USEPA. Announcement of the Drinking Water Contaminant Candidate List. 1998; W–97–11; FRL–5972–5. United States Environmental Protection Agency, Washington, DC.
13. USEPA. Fact Sheet: The Drinking Water Contaminant Candidate List – The Source of Priority Contaminants for the Drinking Water Program. 2005; EPA 815-F-05-001. United States Environmental Protection Agency, Washington, DC.
14. O'hara, C.M. Manual and automated instrumentation for identification of Enterobacteriaceae and other aerobic gram-negative bacilli. *Clin. Microbiol. Rev.*, 2005; **18**(1): 147–62.
15. Kiska, D.L., Kerr, A., Jones, M.C., Caracciolo, J.A., Eskridge, B., Jordan, M., Miller, S., Hughes, D., King, N., Gilligan, P.H. Accuracy of four commercial systems for identification of Burkholderia cepacia and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J. Clin. Microbiol.*, 1996; **34**(4): 886–91.

16. McMenamin, J.D., Zaccone, T.M., Coenye, T., Vandamme, P., LiPuma, J.J. Misidentification of Burkholderia cepacia in US cystic fibrosis treatment centers: an analysis of 1,051 recent sputum isolates. *Chest*, 2000; **117**(6): 1661–5.
17. Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G., Wilson, D., Lasala, P., Kostrzewa, M., Harmsen, D. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J. Clin. Microbiol.*, 2008; **46**(6): 1946–54.
18. Becker, K., Harmsen, D., Mellmann, A., Meier, C., Schumann, P., Peters, G., von Eiff, C. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of Staphylococcus species. *J. Clin. Microbiol.*, 2004; **42**(11): 4988–95.
19. Zbinden, A., Böttger, E.C., Bosshard, P.P., Zbinden, R. Evaluation of the colorimetric VITEK 2 card for identification of gram-negative nonfermentative rods: comparison to 16S rRNA gene sequencing. *J. Clin. Microbiol.*, 2007; **45**(7): 2270–3.
20. Putignani, L., Paglia, M.G., Bordi, E., Nebuloso, E., Pucillo, L.P., Visca, P. Identification of clinically relevant yeast species by DNA sequence analysis of the D2 variable region of the 25-28S rRNA gene. *Mycoses*, 2008; **51**(3): 209–27.
21. Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., Morató, J. Viable real-time PCR in environmental samples: can all data be interpreted directly? *Microb. Ecol.*, 2011; **61**(1): 7–12.
22. Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F., Watanabe, H. Specific detection of viable Legionella cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. *Appl. Environ. Microbiol.*, 2009; **75**(1): 147–53.
23. Hein, I., Schneeweiss, W., Stanek, C., Wagner, M. Ethidium monoazide and propidium monoazide for elimination of unspecific DNA background in quantitative universal real-time PCR. *J. Microbiol. Methods*, 2007; **71**(3): 336–9.
24. Nam, S., Kwon, S., Kim, M.J., Chae, J.C., Maeng, P.J., Park, J.G., Lee, G.C. Selective detection of viable Helicobacter pylori using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction. *Microbiol. Immunol.*, 2011; **55**(12): 841–6.
25. Claydon, M.A., Davey, S.N., Edwards-Jones, V., Gordon, D.B. The rapid identification of intact microorganisms using mass spectrometry. *Nat. Biotechnol.*, 1996; **14**(11): 1584–6.
26. Alispahic, M., Hummel, K., Jandreski-Cvetkovic, D., Nöbauer, K., Razzazi-Fazeli, E., Hess, M., Hess, C. Species-specific identification and differentiation of Arcobacter, Helicobacter and Campylobacter by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis. *J. Med. Microbiol.*, 2010; **59**(Pt 3): 295–301.
27. Williams, T.L., Andrzejewski, D., Lay, J.O., Musser, S.M. Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. *J. Am. Soc. Mass Spectrom.*, 2003; **14**(4): 342–51.
28. Ilina, E.N., Borovskaya, A.D., Malakhova, M.M., Vereshchagin, V.A., Kubanova, A.A., Kruglov, A.N., Svistunova, T.S., Gazarian, A.O., Maier, T., Kostrzewa, M., Govorun, V.M. Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic Neisseria. *J. Mol. Diagn.*, 2009; **11**(1): 75–86.