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Rapid Identification of *Vibrio* Species Isolated from the Southern Coastal Regions of Korea by MALDI-TOF Mass Spectrometry and Comparison of MALDI Sample Preparation Methods

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Vibrio species are generally recognized as pathogens predominant in seafood along coastal areas. The food industry has sought to develop efficient microbial detection methods. Owing to the limits of conventional methods, this study aimed to establish a rapid identification method for *Vibrio* isolated from Korea, based on matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Four different preparation procedures were compared to determine the appropriate means to pretreat *Vibrio* species, using 17 isolates and five reference strains. Extended direct transfer and full formic acid extraction methods using bacterial colonies on agar plates revealed very low identification rates. Formic acid and trifluoroacetic acid (TFA) extractions using bacterial broth cultures were also performed. All *Vibrio* isolates and reference strains prepared by TFA extraction were successfully identified to the species level (17/22, 77.3%) and to the genus level (5/22, 22.7%). Thus, TFA extraction was considered the most appropriate method to pretreat *Vibrio* species for MALDI-TOF MS. The remaining 33 isolates and two reference strains were prepared by TFA extraction and analyzed by MALDI-TOF MS. Overall, 50 isolates were identified to the species level (40/50, 80%) and to the genus level (10/50, 20%). All isolates were identified as 43 *V. alginolyticus*, six *V. parahaemolyticus*, and one *V. vulnificus* species. *V. alginolyticus* and *V. parahaemolyticus* were isolated from fish offal (87.5% and 12.5%, respectively), seawater (91.3%, 8.7%), and shellfish (62.5%, 37.5%), whereas *V. alginolyticus* and *V. vulnificus* were isolated from sediment (90.9% and 9.1%, respectively). This study established a reliable system of MALDI-TOF MS preparation and analysis for *Vibrio* identification.

Keywords: *Vibrio*, MALDI-TOF MS, formic acid extraction, trifluoroacetic acid extraction, extended direct transfer extraction

Introduction

The *Vibrio* genus is a heterogeneous group within the class Gammaproteobacteria and is predominant in marine and coastal environments [1]. In some countries, *Vibrio* species have been one of the major causes of foodborne-disease outbreaks associated with seafood [2, 3]. Several *Vibrio* species are pathogenic in fish, marine invertebrates, and mammals [4]. *V. parahaemolyticus* and *V. vulnificus* cause human infection, including acute gastroenteritis, septicemia, and wound infection, upon exposure to contaminated water or contaminated undercooked seafood [5]. The World Health Organization (WHO) has reported that *V. parahaemolyticus* is a common cause of diarrheal disease worldwide, and choleragenic *V. cholerae* causes devastating disease and is an important pathogen in many developing countries [6]. The WHO has also reported the risk assessment of *V. vulnificus* in raw oysters in the United States [7]. *V. alginolyticus* can

cause skin infections in humans, often as a result of contact with sea water [8]. It has been responsible for gastroenteritis [9, 10] and peritonitis [11] in humans, and it has also been reported that infection with *V. alginolyticus* can cause mortality in immunocompromised patients [12]. Therefore, the rapid detection of *Vibrio* species is necessary to prevent food contamination and to protect human health.

Recently, various genomic-based methods have been applied to identify and type *Vibrio* species. These routine methods use pulsed-field gel electrophoresis [13–16], multilocus sequence typing [14, 17], real-time PCR [18,19], repetitive extragenic palindromic PCR [20], and multiplex PCR [21, 22]. However, many genomic-based identification and typing methods for *Vibrio* species are fastidious and time-consuming, requiring one or more days to distinguish genera and species [23].

Therefore, this study focused on establishing a method for *Vibrio* identification using matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which can provide fast and reliable bacterial discrimination. Recently, MALDI-TOF MS has been frequently used for bacterial identification in various fermented foods, including fermented cereal foods available in Côte d'Ivoire, long-term-aged kimchi (a traditional Korean fermented vegetable), traditional Vietnamese fermented meat products, and yoghurts [24–27]. Our laboratory has also produced successful results for MALDI-TOF-based identifications of *Pediococcus* and *Weissella* species from local salted and fermented foods [28, 29].

This study aimed to develop a rapid identification method for *Vibrio* species isolated from environmental or seafood samples. Four different preparation procedures were compared to generate high identification rates of samples using MALDI-TOF MS analysis.

Materials and Methods

Vibrio Strains

The *Vibrio* species in this study included seven reference strains obtained from culture centers and 50 isolates from coastal seawater, fish offal, sediment, and shellfish in Korea. The culture centers were the Korean Culture Center of Microorganisms (KCCM, Korea), the Korean Collection for Type Cultures (KCTC, Korea), and the American Type Culture Collection (ATCC, USA). Our previous study aimed to develop a *Vibrio* multiplex PCR assay using isolates from local areas of Korea [22]. Primers and proteins of target genes used for multiplex PCR assay are shown in Table 1. These isolates were used for establishing a rapid identification method by MALDI-TOF MS analysis in the present study.

Sample Preparation for MALDI-TOF MS

Seventeen randomly selected isolates and five reference strains were used to compare four different preparation procedures for MALDI-TOF MS analysis. The extended direct transfer (EDT) procedure and full formic acid (EX) extraction were performed according to the manufacturer's standard procedures (Bruker Daltonics, Germany). Trifluoroacetic acid (TFA) and formic acid (FA) extraction procedures were followed according to the methods described by Hazen *et al*. [30] and Kuda *et al*. [31], respectively.

Table 1. Primers and proteins of target genes for *Vibrio* multiplex PCR used in this study.

Primer name	Source of gene[a]	Target genus or species	Primer sequences (5′-3′)[b]	Protein of target gene
VP 1155272 F	NC_004605.1 (c115272-1154856)	*Vibrio parahaemolyticus*	5′ AGCTT ATTGG CGGTT TCTGT CGG	Hypothetical protein VPA1095
VP 1155272 R			5′ CKCAA GACCA AGAAA AGCCG TC	
VC C634002 F	NC_002506.1 (c634002-633547)	*Vibrio cholerae*	5′ CAAGC TCCGC ATGTC CAGAA GC	Hypothetical protein VCA0694
VC C634002 R			5′ GGGGC GTGAC GCGAA TGATT	
VV 2055918 F79	NC_005139.1 (2055918-2056664)	*Vibrio vulnificus*	5′ CAGCC GGACG TCGTC CATTT TG	Hypothetical protein VV2055
VV 2055918 R			5′ ATGAG TAAGC GTCCG ACGCG T	
VA 1198230 F	NC_CH902589.1 (1198230-1198616)	*Vibrio alginolyticus*	5′ ACGGC ATTGG AAATT GCGAC TG	Whole genome shotgun sequence
VA 1198230 R			5′ TACCC GTCTC ACGAG CCCAA G	
VM C727581F	NZ_ADAF01000001.1 (c727581-726859)	*Vibrio mimicus*	5′ ATAAA GCGGG CTTGC GTGCA	Contig43, whole genome shotgun sequence
VM C727581R			5′ GATTT GGRAA AATCC KTCGT GC	
VG C2694352 F46	NC_004603.1 (c2694352-2693309)	*Vibrio* genus	5′ GTC ARA TTG AAA ARC ART TYG GTA AAG G	Recombinase A
VG C2694352 R734			5′ ACY TTR ATR CGN GTT TCR TTR CC	

[a]Reference sequence numbers of chromosomes in GenBank at the NCBI, and the position of the gene.

[b]Mixed base: K = G + T; R = A + G; Y = C + T; N = A + C + G + T.

EDT Extraction

Single bacterial colonies of samples and references were picked up and placed onto a 96-spot polished steel target plate (MSP 96; Bruker Daltonics). Then, 1 µl of 70% formic acid and 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in acetonitrile: water: trifluoroacetic acid (50.0: 47.5: 2.5 (v/v)) were overlaid onto the dried samples. Finally, each bacterial mixture was crystallized at room temperature.

EX Extraction

A loopful of each bacterial sample being tested was suspended in 300 µl of sterile deionized H_2O and added to 900 µl of absolute ethanol. The bacterial suspension was then vortexed and centrifuged at 16,200 ×*g* for 5 min. The pellet was resuspended in 25 µl of 70% formic acid and added to 25 µl of pure acetonitrile. Another centrifugation was performed using the same conditions, and 1 µl of the resulting supernatant was placed on the target plate (Bruker Daltonics). After air-drying, HCCA matrix solution was overlaid and crystallized as described for the EDT extraction.

TFA Extraction

The protocol was conducted as previously described, with slight modification [30]. Briefly, 1 ml of each bacterial culture was centrifuged at 16,200 ×*g* for 5 min, and the supernatant was discarded. The remaining pellet was then washed in 1 ml of 50% ethanol with centrifugation for 5 min at 16,200 ×*g*. The pellet was resuspended in 20 µl of 1% TFA, and 1.2 µl of the resulting supernatant was placed on the target plate (Bruker Daltonics) followed by drying at room temperature. The HCCA matrix solution was then overlaid and crystallized as described for the EDT extraction.

FA Extraction

The procedure was slightly modified from the method of a previous study [31]. In brief, 1 ml of each bacterial culture was centrifuged at 16,200 ×*g* for 5 min, and the supernatant was decanted. The pellet was washed in 1 ml of 70% ethanol with centrifugation for 5 min at 16,200 ×*g*. Then, the washed pellet was resuspended in 100 µl of 25% formic acid with centrifugation for 5 min at 16,200 ×*g*, and 1 µl of the supernatant was placed on the target plate (Bruker Daltonics). After drying, HCCA matrix solution (Bruker Daltonics) was overlaid and crystallized as described for the EDT extraction.

MALDI-TOF MS Analysis

After crystallization, the measurements were carried out on a Microflex LT bench-top mass spectrometer (Bruker Daltonics) using Flexcontrol software equipped with 240 laser shot steps.

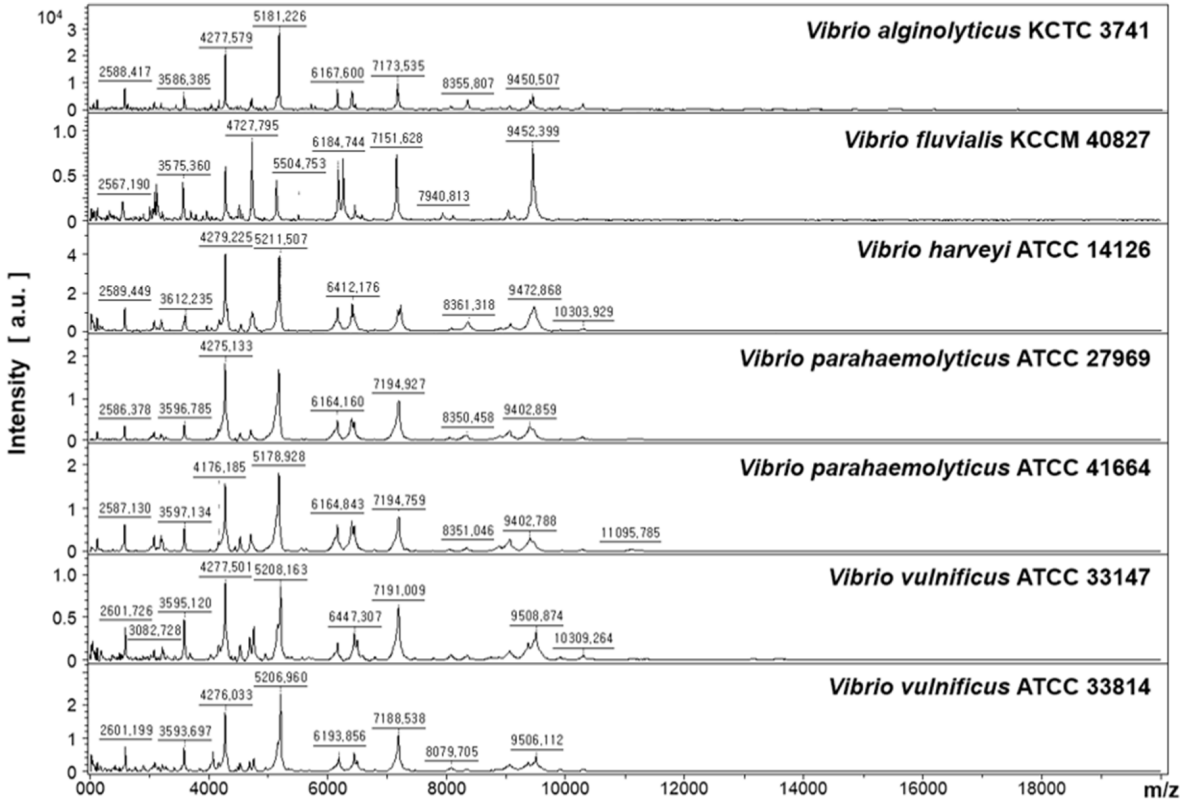

Fig. 1. Mass spectra of seven *Vibrio* reference strains in the range from 2,000 to 12,000 Da.

The reference stains are *V. alginolyticus* KCTC 3741, *V. fluvialis* KCCM 40827, *V. harveyi* ATCC 14126, *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 41664, *V. vulnificus* ATCC 33147, and *V. vulnificus* ATCC 33814. a.u., arbitrary units.

Table 2. Comparison of the identification performance of four MALDI-TOF preparation methods.

Number/ reference	Area	Source/ strain No.	Multiplex PCR result	MALDI-TOF results							
				Detected species (EDT[b])	Score[a]	Detected species (EX[c])	Score	Detected species (TFA[d])	Score	Detected species (FA[e])	Score
Isolates											
KHU0021	Pusan	Fish offal	*Vibrio* species	*V. alginolyticus*	1.496	*V. alginolyticus*	1.797	*V. alginolyticus*	1.936	*V. mytili*	1.710
KHU0023	Pusan	Fish offal	*V. alginolyticus*	*V. alginolyticus*	1.412	No peaks found	<0	*V. alginolyticus*	2.027	*V. alginolyticus*	1.861
KHU0025	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	1.755	No peaks found	<0	*V. alginolyticus*	2.122	*V. alginolyticus*	2.067
KHU0026	Pusan	Seawater	*V. alginolyticus*	No peaks found	<0	No peaks found	<0	*V. alginolyticus*	2.045	*V. alginolyticus*	1.266
KHU0029	Pusan	Seawater	*Vibrio* species	No peaks found	<0	No peaks found	<0	*V. alginolyticus*	2.056	*V. alginolyticus*	1.874
KHU0030	Pusan	Seawater	*V. parahaemolyticus*	*V. parahaemolyticus*	1.664	No peaks found	<0	*V. parahaemolyticus*	2.103	*V. parahaemolyticus*	1.551
KHU0031	Pusan	Seawater	*Vibrio* species	No peaks found	<0	No peaks found	<0	*V. alginolyticus*	2.062	*V. parahaemolyticus*	1.519
KHU0032	Pusan	Fish offal	*V. parahaemolyticus*	*V. parahaemolyticus*	1.723	*V. parahaemolyticus*	1.901	*V. parahaemolyticus*	1.912	*V. parahaemolyticus*	1.677
KHU0043	Pusan	Shellfish	*V. parahaemolyticus*	No peaks found	<0	*V. parahaemolyticus*	1.975	*V. parahaemolyticus*	2.018	No peaks found	<0
KHU0044	Pusan	Shellfish	*V. parahaemolyticus*	*V. parahaemolyticus*	1.534	*V. parahaemolyticus*	1.951	*V. parahaemolyticus*	2.120	*V. parahaemolyticus*	1.407
KHU0046	Pusan	Shellfish	*V. alginolyticus*	No peaks found	<0	No peaks found	<0	*V. alginolyticus*	2.012	*V. alginolyticus*	2.209
KHU0005	Tongyoung	Sediment	*Vibrio* species	*V. alginolyticus*	1.759	No peaks found	<0	*V. alginolyticus*	1.995	*V. alginolyticus*	1.838
KHU0007	Tongyoung	Sediment	*Vibrio* species	No peaks found	<0	*V. alginolyticus*	1.844	*V. alginolyticus*	2.027	*V. furnisii*	1.270
KHU0008	Tongyoung	Sediment	*Vibrio* species	No peaks found	<0	No peaks found	<0	*V. alginolyticus*	1.934	*V. alginolyticus*	1.856
KHU0013	Pusan	Seawater	*Vibrio* species	*V. mytili*	1.516	*V. alginolyticus*	1.524	*V. alginolyticus*	2.220	*V. alginolyticus*	1.802
KHU0015	Pusan	Seawater	*Vibrio* species	No peaks found	<0	*V. vulnificus*	1.317	*V. alginolyticus*	1.920	*V. alginolyticus*	1.549
KHU0017	Pusan	Sediment	*V. vulnificus*	*V. vulnificus*	1.673	No peaks found	<0	*V. vulnificus*	2.298	*V. vulnificus*	1.934
Reference strains											
V. parahaemolyticus		ATCC[f] 27969	-	No peaks found	< 0	No peaks found	< 0	*V. parahaemolyticus*	2.104	*V. parahaemolyticus*	1.763
V. parahaemolyticus		ATCC 41664	-	*V. parahaemolyticus*	1.514	No peaks found	< 0	*V. parahaemolyticus*	2.131	*V. parahaemolyticus*	1.850
V. vulnificus		ATCC 33147	-	No peaks found	< 0	*V. vulnificus*	2.060	*V. vulnificus*	2.271	No peaks found	< 0
V. vulnificus		ATCC 33814	-	No peaks found	< 0	*V. vulnificus*	2.144	*V. vulnificus*	2.150	*V. vulnificus*	0.854
V. alginolyticus		KCTC[g] 3741	-	No peaks found	< 0	*V. alginolyticus*	2.300	*V. alginolyticus*	2.000	*V. alginolyticus*	1.879

[a]The meanings of log score values obtained were as follows: ≥2.000: species-level identification; 1.700–1.999: genus-level identification; ≤1.699: unreliable identification.

[b]EDT: Extended direct transfer method.

[c]EX: Full formic acid extraction.

[d]TFA: Trifluoroacetic acid extraction (Hazen *et al*. [30]).

[e]FA: Formic acid extraction (Kuda *et al*. [31]).

[f]ATCC: American Type Culture Collection.

[g]KCTC: Korean Collection for Type Culture.

Generated raw spectra data were collected within a mass range from 2,000 to 20,000 Da following calibration with a bacterial test standard (BTS; Bruker Daltonics). The parameter conditions were as follows: ion source 1, 20.0 kV; ion source 2, 18.2 kV; lens, 6.0 kV; initial laser power, 25%; and maximal laser power, 35%. The resulting spectra for each of the reference strains were analyzed using Flexanalysis software 3.4 (Bruker Daltonics), and selected spectra were uploaded into the Biotyper 3.0 software to generate a single mean spectrum for each reference strain using the master spectra library creation method of the Biotyper software. The recorded spectra for each sample were matched to a reference library database in the Biotyper software that includes the mass spectrometry profiles of 5,627 species. Integrated pattern-matching algorithms were recorded as logarithmic scores with maximum values of 3.0. The database includes 82 *Vibrio* profiles with 52 different species: *V. alginolyticus*, four profiles; *V. parahaemolyticus*, eight profiles; and *V. vulnificus*, nine profiles (Bruker Daltonics, MALDI-TOF systems overview).

Results and Discussion

Multiple Preparation Methods Were Used to Compare the Performance of *Vibrio* Identification

Four different procedures for the preparation step of MADLI-TOF MS analysis were compared using 17 sample strains and five reference strains. Species-specific MALDI-TOF MS profiles for seven reference strains are shown in Fig. 1. The reference strains were used as positive controls to confirm our procedures. All reference strains were identified to the species level with log scores ≥2.0 (Tables 2 and 3). To compare and confirm the MALDI-TOF results, previous identification data generated by our research group using multiplex PCR were used (Tables 2 and 3) [22]. The EDT and EX extractions were first conducted and showed low identification rates compared with the other extraction methods. For the EDT extraction, only three isolates (13.6%) were correctly identified at the genus level (log scores between 2 and 1.7), and seven isolates were inconclusively identified with log scores ≤1.7. Twelve isolates (54.5%) were not identified, having no peaks. For the EX extraction, only three isolates (13.6%) were identified at the species level (log scores ≥2.0) and five (22.7%) at the genus level. In these two procedures (EDT and EX extractions), no peaks were found for many isolates and reference strains of *Vibrio*. This could have been due to the high viscosity of *Vibrio* species. The high viscosity of *Vibrio* species from agar plates seemed to interfere with the bacterial membrane lysis step. Incomplete membrane lysis led to a lack of protein for MALDI-TOF analysis, which meant that two procedures (EDT and EX) were not able to release enough proteins from the bacterial cells. The FA and TFA extractions use bacterial broth cultures, whereas the EDT and EX extractions use bacterial colonies on agar.

Therefore, FA and TFA extractions were conducted. The FA extraction method yielded higher identification rates (9.1% and 45.5%) at the species and genus levels than the EDT and EX extraction methods, respectively, although it was not high enough to be considered acceptable. However, the slightly modified TFA extraction method described by Hazen *et al*. [30] showed the highest identification rate to the species level (17/22, 77.3%) and to the genus level (5/22, 22.7%). This indicates that TFA is a better chemical reagent for *Vibrio* species than the formic acid used in the EDT, EX, and FA extractions. A previous study has reported that TFA can be recommended for protein identification by MALDI-TOF MS analysis because it produces specific internal cleavage products and generates optimal lengths of peptides [32]. Table 4 depicts the identification rates of each *Vibrio* species. Ratios were calculated as the number of identified samples or references using MALDI-TOF MS divided by that using multiplex PCR. There was no major trend found for the identification rates among *Vibrio* species. Similarly, no correlation was shown between the *Vibrio* species and the four procedures. The bacterial identification is likely to be dependent on their structures when it comes to choosing extraction methods. Based on our comparison of these four preparation procedures, the TFA extraction procedure was chosen for all further MALDI-TOF MS preparations used in this study.

Vibrio Isolates Prepared by TFA Extraction Were Identified by MALDI-TOF MS

Thirty-three additional isolates and two reference strains were identified by MALDI-TOF MS, in comparison with the results of multiplex PCR (Table 3). Twenty-eight of the 33 isolates were unequivocally identified to the species level with high log scores (≥2) and five isolates were identified to the genus level when compared with the MALDI Biotyper database (Table 3). All of these identified species were identical to the 16S rRNA sequencing results (data not shown).

Overall, the 50 isolates were correctly identified to the species level (40/50, 80%) or to the genus level (10/50, 20%) (Table 5). All scores of the isolates identified to the genus level were higher than 1.9, with an exception of only one isolate showing a score of 1.876. These identified species were *V. alginolyticus* (36/50, 72.0%), *V. parahaemolyticus* (3/50, 6.0%), and *V. vulnificus* (1/50, 2.0%) to the species level, whereas *V. alginolyticus* (7/50, 14.0%) and *V. parahaemolyticus*

Table 3. Identification data for 33 isolates[a].

Number[a]/reference	Area	Source/strain no.	Multiplex PCR result	MALDI-TOF MS result[b]	
				Detected species	Score
Isolates					
KHU0001	Tongyoung	Seawater	*Vibrio* species	*V. alginolyticus*	2.177
KHU0002	Tongyoung	Seawater	*Vibrio* species	*V. alginolyticus*	2.027
KHU0003	Tongyoung	Seawater	*Vibrio* species	*V. alginolyticus*	2.017
KHU0004	Tongyoung	Seawater	*Vibrio* species	*V. alginolyticus*	2.157
KHU0006	Tongyoung	Sediment	*Vibrio* species	*V. alginolyticus*	2.115
KHU0009	Tongyoung	Sediment	*Vibrio* species	*V. alginolyticus*	1.989
KHU0010	Tongyoung	Sediment	*Vibrio* species	*V. alginolyticus*	2.031
KHU0011	Tongyoung	Sediment	*Vibrio* species	*V. alginolyticus*	2.014
KHU0012	Pusan	Seawater	*Vibrio* species	*V. alginolyticus*	2.064
KHU0014	Pusan	Seawater	*Vibrio* species	*V. parahaemolyticus*	1.876
KHU0016	Pusan	Sediment	*Vibrio* species	*V. alginolyticus*	2.165
KHU0018	Pusan	Shellfish	*Vibrio* species	*V. alginolyticus*	2.158
KHU0019	Pusan	Shellfish	*Vibrio* species	*V. alginolyticus*	2.024
KHU0020	Pusan	Fish offal	*Vibrio* species	*V. alginolyticus*	1.911
KHU0022	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.027
KHU0024	Pusan	Fish offal	*Vibrio* species	*V. alginolyticus*	2.053
KHU0027	Pusan	Seawater	*Vibrio* species	*V. alginolyticus*	2.081
KHU0028	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.037
KHU0033	Pusan	Sediment	*V. alginolyticus*	*V. alginolyticus*	2.018
KHU0034	Pusan	Sediment	*V. alginolyticus*	*V. alginolyticus*	2.090
KHU0035	Pusan	Fish offal	*V. alginolyticus*	*V. alginolyticus*	2.019
KHU0036	Pusan	Fish offal	*V. alginolyticus*	*V. alginolyticus*	2.156
KHU0037	Pusan	Fish offal	*V. alginolyticus*	*V. alginolyticus*	2.081
KHU0038	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.059
KHU0039	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.232
KHU0040	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.066
KHU0041	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.024
KHU0042	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.084
KHU0045	Pusan	Shellfish	*V. parahaemolyticus*	*V. parahaemolyticus*	1.929
KHU0047	Pusan	Shellfish	*V. alginolyticus*	*V. alginolyticus*	1.981
KHU0048	Pusan	Shellfish	*V. alginolyticus*	*V. alginolyticus*	2.111
KHU0049	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.041
KHU0050	Pusan	Seawater	*V. alginolyticus*	*V. alginolyticus*	2.129
Reference strains					
		KCCM[c] 40827		*V. fluvialis*	2.095
		ATCC[d] 14126		*V. harveyi*	2.051

[a]Among 50 isolates, 17 are shown in Table 2.

[b]Isolates were prepared by the trifluoroacetic acid extraction procedure.

[c]KCCM: Korean Culture Center of Microorganisms.

[d]ATCC: American Type Culture Collection.

(3/50, 6.0%) were identified to the genus level. Based on the multiplex PCR detection, 26 of 50 isolates were correctly identified to the species level. The remaining isolates (24/50, 48%) were identified only to the genus level,

Table 4. Comparison of the identification rates of four different preparation procedures for MADLI-TOF MS analysis.

Bacteria	Identification rate according to extraction method				Number of strains in database[a]
	EDT	EX	TFA	FA	
Isolated strains					
Vibrio species (n[b] = 8)	0[c], 1[d] (0%, 12.5%)[e]	0, 2 (0%, 25.0%)	4, 4 (50.0%, 50.0%)	0, 5 (0% , 62.5%)	-
V. alginolyticus (n = 4)	0, 1 (0%, 25.0%)	0, 0 (0%, 0%)	4, 0 (100%, 0%)	2, 1 (50.0%, 25.0%)	4
V. parahaemolyticus (n = 4)	0, 1 (0%, 25.0%)	0, 3 (0%, 75.0%)	3, 1 (75.0%, 25.0%)	0, 0 (0%, 0%)	8
V. vulnificus (n = 1)	0, 0 (0%, 0%)	0, 0 (0%, 0%)	1, 0 (100%, 0%)	0, 1 (0%, 100%)	9
Subtotal (n = 17)	0, 3 (0%, 17.6%)	0, 5 (0%, 29.4%)	12, 5 (70.6%, 29.4%)	2, 7 (11.8%, 41.2%)	
Reference strains					
V. alginolyticus KCTC 3741	0, 0 (0%, 0%)	1, 0 (100%, 0%)	1, 0 (100%, 0%)	0, 1 (0%, 100%)	4
V. parahaemolyticus ATCC 27969	0, 0 (0%, 0%)	0, 0 (0%, 0%)	1, 0 (100%, 0%)	0, 1 (0%, 100%)	8
V. parahaemolyticus ATCC 41664	0, 0 (0%, 0%)	0, 0 (0%, 0%)	1, 0 (100%, 0%)	0, 1 (0%, 100%)	8
V. vulnificus ATCC 33147	0, 0 (0%, 0%)	1, 0 (100%, 0%)	1, 0 (100%, 0%)	0, 0 (0%, 0%)	9
V. vulnificus ATCC 33814	0, 0 (0%, 0%)	1, 0 (100%, 0%)	1/0 (100%, 0%)	0, 0 (0%, 0%)	9
Subtotal (n = 5)	0, 0 (0%, 0%)	3, 0 (60%, 0%)	5, 0 (100%, 0%)	0, 3 (0%, 60%)	
Total (n = 22)	0, 3 (0%, 13.6%)	3, 5 (13.6%, 22.7%)	17, 5 (77.3%, 22.7%)	2, 10 (9.1%, 45.5%)	
	3/5 (60.0%)	3/5 (60.0%)	3/5 (60.0%)	3/5 (60.0%)	
	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	
	3/5 (60.0%)	3/5 (60.0%)	3/5 (60.0%)	3/5 (60.0%)	

[a]Number of *Vibrio* strains in the Biotyper taxonomy database.

[b]Number of bacterial isolates.

[c]Number of correctly identified isolates (log score ≥2.0) to the species level by MALDI-TOF MS.

[d]Number of correctly identified isolates (log score between 2.0 and 1.7) to the genus level by MALDI-TOF MS.

[e]Rates of correctly identified isolates to the species and genus levels, respectively.

See Table 2 for the extraction descriptions (EDT, EX, TFA, and FA).

Table 5. Comparison of the identification results from MALDI-TOF MS and PCR for 50 isolated strains[a].

Multiplex PCR result	MALDI-TOF MS result	
	Species level	Genus level
Vibrio species (n = 24)	17 (*V. alginolyticus*)	6 (*V. alginolyticus*)
		1 (*V. parahaemolyticus*)
V. alginolyticus (n = 20)	19	1
V. vulnificus (n = 1)	1	0
V. parahaemolyticus (n = 5)	3	2
Total (n = 50)	36 (*V. alginolyticus*)	7 (*V. alginolyticus*)
	3 (*V. parahaemolyticus*)	3 (*V. parahaemolyticus*)
	1 (*V. vulnificus*)	

[a]Isolates were prepared by the trifluoroacetic acid extraction procedure.

and no species-level information was provided. In contrast, MALDI-TOF MS assigned *Vibrio* species identifications for all isolates. For this reason, we concluded that MALDI-TOF MS with a TFA preparation procedure was the most reliable method to identify members of the genus *Vibrio*. In terms of isolation origins, *V. alginolyticus* and *V. parahaemolyticus* were isolated from fish offal (7/8 and 1/8, respectively), seawater (21/23, 2/23), shellfish (5/8, 3/8), respectively, whereas *V. alginolyticus* and *V. vulnificus* were from sediment (10/11 and 1/11, respectively). *V. alginolyticus*

was predominant in all isolation origins.

In summary, we successfully developed a system of MALDI-TOF MS preparation and analysis useful for the specific identification of all *Vibrio* species isolated from Korea. The performance of four preparation procedures was investigated using *Vibrio* species. Owing to the high viscosity of the genus *Vibrio*, only the TFA extraction procedure presented reliable and consistent identification rates. Although further investigation is needed, the present study suggests that this MALDI-TOF MS-based identification of *Vibrio* can be applied to the food industry for diagnostic purposes.

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