Detection and Identification of Vibrio Species Using Whole-Cell Protein Pattern Analysis

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Outbreaks of foodborne diseases associated with Vibrio species such as V. parahaemolyticus, V. vulnificus, and V. cholerae frequently occur in countries having a dietary habit of raw seafood consumption. For rapid identification of different Vibrio species involved in foodborne diseases, whole-cell protein pattern analysis for 13 type strains of 12 Vibrio species was performed using SDS-PAGE analysis. Pathogenic Vibrio species such as V. parahaemolyticus, V. vulnificus, V. cholerae, V. alginolyticus, V. fluvialis, and V. mimicus were included in the 12 Vibrio species used in this study. Each of the 12 Vibrio species showed clearly specific band patterns of its own. Two different strains of V. parahaemolyticus showed two different SDS-PAGE whole-cell protein patterns, giving the possibility of categorizing isolated strains in the same V. parahaemolyticus species into two subgroups. The 36 Vibrio isolates collected from sushi restaurants in Busan were all identified as V. parahaemolyticus by comparing their protein patterns with those of Vibrio type strains. The identified isolates were categorized into two different subgroups of V. parahaemolyticus. The whole-cell protein pattern analysis by SDS-PAGE can be used as a specific, rapid, and simple identification method for Vibrio spp. involved in foodborne diseases at the subspecies level.

Keywords: Vibrio, foodborne, SDS-PAGE, protein pattern

The genus Vibrio is a group of bacteria that can cause serious illnesses in humans. The bacteria are Gram-negative, rod-shaped, facultative anaerobes that grow in saline aquatic environments [44]. Vibrio infection is seasonal, with a peak in the late summer and early fall, coinciding with warm water temperatures higher than 20°C. Vibrio cholerae, V. parahaemolyticus, and V. vulnificus are considered as major human pathogens around the world [44]. There are also occasional reports of foodborne or waterborne infections caused by V. alginolyticus [13, 45], V. mimicus [35], and V. fluvialis [29]. Vibrio species are commonly found in tidal rivers and bays. The pathogens can infect people of all ages who consume contaminated fish or shellfish and have close contact with contaminated water or cooking utensils. Until now, 12 species of Vibrio have been known to be pathogenic to human beings [24]. According to the Korea Food & Drug Administration, there were 177 incidences of outbreaks associated with V. parahaemolyticus between 2002 and 2010, with as many as 3,736 patients having suffered from the disease in Korea [22]. The value of patient per incidence of outbreak was 21.1, ranking 4th among foodborne disease outbreaks in Korea in 2011 [22].

V. parahaemolyticus is one of the most common waterborne pathogens in the world, causing acute gastroenteritis associated with intake of contaminated raw or undercooked seafood, especially shellfish during summer [12, 27, 40]. V. parahaemolyticus is rarely detected in seawater unless the water temperature reaches 14°C [38]. Genes in V. parahaemolyticus, such as the thermostable direct hemolysin gene (tdh) and TDH-related hemolysin gene (trh) that are considered as the major virulence factors of V. parahaemolyticus [17], and the thermostable hemolysin gene (tlh), B subunit of DNA gyrase gene (gyrB), and toxR gene that are involved in the regulation of gene expression in V. parahaemolyticus [21, 42], were
used as markers for the detection of \textit{V. parahaemolyticus} [4]. In the Food and Drug Administration (FDA), the \textit{tdh}, \textit{trh}, and \textit{toxR} genes are selected to detect \textit{V. parahaemolyticus} by PCR and the \textit{toxR} gene for \textit{V. parahaemolyticus} by RT-PCR [23]. \textit{V. vulnificus} is the leading cause of seafood-borne mortality in the United States. Approximately 85% of \textit{V. vulnificus} infections occur between May and October when the water temperature is over 20°C [33]. \textit{V. vulnificus} causes foodborne diseases via eating of contaminated seafood or causes wound infections via swimming, fishing, or seafood handling [6, 15, 18, 31, 34]. Detection of \textit{V. vulnificus} through biochemical or immunological tests using a monoclonal antibody to a species-specific intracellular antigen is introduced in the FDA BAM (Bacteriological Analytical Manual) [40]. \textit{V. cholerae} has been the major causative strain of cholera disease in the world. \textit{V. cholerae} detection usually employs the \textit{toxR} gene sequence to design PCR primers [14]. \textit{V. alginolyticus} is a pathogen of several marine animals and humans. Studies on the pathogenicity of \textit{V. alginolyticus} suggested that some extracellular enzymes showing strong proteolytic activities could be important virulence factors [1, 2, 13]. \textit{V. mimicus} is closely related to \textit{V. cholerae}; therefore, it was first described as a biochemically atypical \textit{V. cholerae} [9]. Unlike \textit{V. cholerae}, most \textit{V. mimicus} do not produce cholera toxin (CT) [8]. Symptoms of the human infections are diarrhea, nausea, vomiting, abdominal cramps, and fever [7]. \textit{V. fluvialis} was first isolated by Furniss \textit{et al.} [11] and its clinical symptoms of gastroenteritis are very similar to those caused by \textit{V. cholerae} [28]. Information about proteins in the cell provides various functions of bacteria related to their environment such as internalization of nutrients, secretion of by-products, and bacterial cell adhesion to biological and abiotic surfaces. In pathogenic bacteria, proteins in the cell are associated with their virulence and survival during the infection process and play major roles in the interaction with human cells [3]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for estimating the apparent molecular weight (MW) of a protein. Since SDS-PAGE of whole-cell proteins reveals homogeneity of patterns among strains of the same species under standardized conditions, identification of bacteria based on SDS-PAGE patterns correlates closely with the genotyping results [5]. This suggests that it could be an effective method for rapid bacterial classification at the subspecies levels without performing any pre-identification. Different species of \textit{Listeria monocytogenes} [32], \textit{Leuconostoc} spp. [43], \textit{Streptococcus} spp. [41], and \textit{Lactococcus} spp. [3, 10] have been identified by using SDS-PAGE. In the case of \textit{Vibrio} spp., researches on SDS-PAGE analysis for \textit{V. parahaemolyticus} [19] and \textit{V. vulnificus} [25, 28] were performed but with limited numbers of strains.

In this study, whole-cell protein pattern analysis was performed with 13 \textit{Vibrio} spp. type strains and 36 isolates from sushi restaurants in Busan, Korea, expecting a simple, rapid, and accurate identification of pathogenic \textit{Vibrio} spp. that cause foodborne diseases and economic loss in the aquaculture industry.

### MATERIALS AND METHODS

#### Bacterial Strains and Growth Conditions

A total of 13 \textit{Vibrio} spp. type strains (Table 1) were obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea), or American Type Culture Collection (ATCC, Rockville, MD, USA). A total of 36 \textit{Vibrio} spp. isolates from sushi restaurants in Busan were provided by Busan Metropolitan City Research Institute of Public Health and Environment. All the \textit{Vibrio} strains were grown on Tryptic Soy Broth (TSB; Sparks, MD, USA) or TSB agar [1.5% agar (w/v)] containing 3% NaCl at 30°C for 12 h. Liquid cultures were incubated with shaking at 200 rpm (Shaking Incubator HK-S115C; Korea Machinery Plant, Hwasung, Korea).

#### SDS-PAGE of Whole-Cell Proteins and Identification of Isolated Strains

SDS-PAGE of whole-cell proteins was performed as described by Kim \textit{et al.} [20]. \textit{Vibrio} species type strains and isolates were cultured for 12 h at 30°C in 15 ml of TSB and centrifuged (Centrifuge 5415R; Eppendorf, Hamburg, Germany) at 13,000 × g for 15 min at 4°C. The pellet was washed with 50 mM Tris-HCl buffer (pH 8.0) and suspended in 50 μl of 50 mM Tris-HCl buffer (pH 8.0). Fifty mg of glass beads (425–600 micron diameter; Sigma, St. Louis, MO, USA) was added to a tube, and the cells were vortexed (G560; Scientific Industries, Inc., Bohemia, NY, USA) for 7 min. The pellet was resuspended in an equal volume of sample buffer (2× SDS sample buffer; 50 mM Tris-HCl (pH 6.8), glycerol 20%, SDS 4%, 2-mercaptoethanol 4%, bromophenol blue 0.3%). For protein denaturation,

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textit{Vibrio} species & Strains & Human pathogenicity* \\
\hline
\textit{V. parahaemolyticus} & ATCC 17802 & Y \\
\textit{V. parahaemolyticus} & ATCC 27969 & Y \\
\textit{V. vulnificus} & ATCC 33815 & Y \\
\textit{V. alginolyticus} & KCCM 40513 & Y \\
\textit{V. fluvialis} & KCCM 40287 & Y \\
\textit{V. algoinfesta} & KCCM 40861 & N \\
\textit{V. aextiriansus} & KCCM 40863 & N \\
\textit{V. harveyi} & KCCM 40866 & N \\
\textit{V. mediterranei} & KCCM 40867 & N \\
\textit{V. diazotrophicus} & KCCM 41666 & N \\
\textit{V. campbellii} & KCCM 41986 & N \\
\textit{V. mimicus} & KCCM 42257 & Y \\
\textit{V. cholerae} NAG & KDCD 13589 & Y \\
\hline
\end{tabular}
\caption{List of the \textit{Vibrio} species type strains used in this study.}
\label{table1}
\end{table}

*Refer to Seafood safety information system [24].
Cluster analysis of SDS-PAGE whole-cell protein profiles was performed with the numerical taxonomic system using a multivariate statistics program (NTSYSpc, version 2.02j; Exeter Software, Setauket, NY, USA) [36]. For each strain, a data record was constructed in which each band of particular MW was represented as either being present (value 1) or not present (value 0). Jaccard's similarity coefficient was estimated to convert the differences of protein band patterns into numerical data using NTSYSpc software. Jaccard's coefficients are defined as a/(a+b+c), where a is the number of positive matches (i.e., bands common to two samples), and b and c refer to the number of bands present only in each sample, respectively [37]. The unweighted pair group method using arithmetic averages (UPGMA) clustering was performed using the SHAN option of NTSYSpc, and a dendrogram representing the relationship between all strains tested was derived from the TREE option. The whole-cell protein patterns of the 36 isolates were compared with those of *Vibrio* species type strains to identify the isolated strains.

### RESULTS AND DISCUSSION

#### Whole-Cell Protein Profile of *Vibrio* Type Strains

The reproducibility of the SDS-PAGE analysis was investigated by electrophoretic runs of duplicate protein extracts of *Vibrio* cells at the log phase. The average value for the reproducibility was about 96 ± 1% (data not shown). The whole-cell protein patterns of the 13 *Vibrio* spp. type strains are shown in Fig. 1. Each *Vibrio* spp. had specific major protein band patterns ranging from 18 kDa to 116 kDa. Heterogeneity in SDS-PAGE protein patterns among different *Vibrio* species was clearly found (Fig. 1). *V. parahaemolyticus* had major protein bands of approximately 35 kDa, 38 kDa, and 48 kDa, which were not found in *V. vulnificus* or *V. cholerae*. *V. vulnificus* had major protein bands of approximately 37.5 kDa and 45 kDa, and *V. cholerae* had three major protein bands of approximately 35 kDa, 45 kDa, and 58 kDa. Although the 37 kDa or 37.5 kDa and 45 kDa bands in *V. vulnificus* and *V. cholerae* were hardly separated, *V. cholerae* has an extra major protein band of 58 kDa that does not exist in *V. vulnificus*. *V. alginolyticus* had three major bands of 40 kDa, 45 kDa, and 56 kDa. *V. fluvialis* had four major bands of 37 kDa, 47 kDa, 57 kDa, and 62 kDa and could be differentiated from others by the consecutive larger bands of 57 kDa and 62 kDa. *V. mimicus* had one extra major band of 65 kDa in addition to the major bands of 35 kDa, 38 kDa, 45 kDa, and 55 kDa that could differentiate this species from others. Marhual et al. [30] reported the same band patterns with about the same sizes for *V. parahaemolyticus* and *V. alginolyticus* as the results from this study. Tall et al. [39] reported 8 major protein bands on the SDS-PAGE protein profile for *V. fluvialis*. The protein band pattern was similar to the results from this study but the band intensities of smaller bands were stronger and those of larger bands were weaker compared with the results of this study. This difference is possibly due to the strain variance and the varied amounts of protein bands on the gels from different SDS-PAGE runs, a consequential difference in the selection of major bands among more than eight total protein bands shown on the gel. We tested 2 type strains of *V. parahaemolyticus*. There were two different protein patterns (lane 1 vs. lane 2 in Fig. 1) for the two *V. parahaemolyticus* strains. The difference can be noticed on the protein bands with sizes of 35 kDa, 38 kDa, and 40 kDa (Fig. 1). One group has all three protein bands (lane 1 in Fig. 1), but another group is missing the upper 40 kDa bands (lane 3 in Fig. 1). This result can be used to differentiate the protein patterns of the isolated strains into two different subgroups of *V. parahaemolyticus*.

These SDS-PAGE results gave homogeneity and heterogeneity of whole-cell protein patterns for different species of *Vibrio* type strains. Using these results, the species or subspecies level identification of large numbers of isolates from contaminated food samples would be possible without performing any pre-identification experiments.
Reports on simultaneous detection of *Vibrio* species using PCR [12], immunoassay [16], and protein analysis [19] are available, but those are focused only on a few well-known pathogenic *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The selective-differential media have also been widely used to isolate and differentiate several major *Vibrio* species. However, those media have a low detection limit, particularly when applied to isolates. For example, only 51% of bacterial isolates from environmental samples on TCBS (thiosulfate citrate bile salts sucrose) agar, one of the selective media for *Vibrio* species, were identified as members of the genus *Vibrio* [16]. Thus, the whole-cell protein pattern analysis is a useful tool to differentiate *Vibrio* from other bacteria even at the species level.

**UPGMA Clustering of 15 Vibrio Type Strains and Isolated Strains Based on the SDS-PAGE Whole Cell Protein Pattern**

A dendrogram (Fig. 2) was successfully generated based on the SDS-PAGE whole-cell protein patterns of 13 *Vibrio* type strains. Protein band patterns of 6 randomly selected isolates among 36 isolates were used together with the results of 13 type strains. The range of protein band size to generate the dendrogram was 20–100 kDa, which included all of the strain-specific protein bands. *V. parahaemolyticus* ATCC 27969 was in the same subgroup with 3 isolates (a, b, and c). *V. parahaemolyticus* ATCC 17802 that showed different protein band patterns from *V. parahaemolyticus* ATCC 27969 (lanes 1 and 2 in Fig. 1) was in another subgroup with 3 other isolates (d, e, and f). All 8 strains including two *V. parahaemolyticus* type strains and 6 isolates were in the same group, showing about 88% of similarity (Fig. 2). Other *Vibrio* species closely related to *V. parahaemolyticus* with approximate 60% similarity were *V. vulnificus* and *V. alginolyticus* (Fig. 2). They are classified as human pathogens in Korea [24] along with *V. parahaemolyticus*. *V. mimicus* had been considered as atypical *V. cholerae*, and these two species appeared in the same subgroup with approximate 65% similarity (Fig. 2). This suggests that they share the common hypothetical ancestors, according to the results in this study. Both *V. mimicus* and *V. cholerae* are human pathogens. Numerical analysis of the SDS-PAGE data for the 13 *Vibrio* type strains showed a relationship with similarity of at least 56%.

**Identification of Isolated Vibrio Species Using Whole-Cell Protein Profile**

The whole-cell protein pattern analysis using SDS-PAGE was applied to 36 isolates from sushi restaurants in Busan,
Korea. All 36 isolates were identified as *V. parahaemolyticus* and their whole-cell protein patterns were differentiated into two subgroups. Protein patterns of one subgroup (29 out of 36 isolates) were identical to those of *V. parahaemolyticus* ATCC 27969, and patterns for another subgroup (7 out of 36 isolates) were identical to those of *V. parahaemolyticus* ATCC 17802. The SDS-PAGE patterns of 6 selected isolates are shown with those of *V. parahaemolyticus* ATCC 27969, ATCC 17802, and patterns for another type strains by several differences in protein band patterns (Fig. 3). In addition, isolates a, b, and c showed three consecutive protein bands of 35 kDa, 38 kDa, and 40 kDa that are identical in pattern with *V. parahaemolyticus* ATCC 27969 (lanes 1–4 in Fig. 3), but isolates d, e, and f showed only two protein bands of 35 kDa and 38 kDa, with the upper 40 kDa band missing, that are identical in pattern with *V. parahaemolyticus* ATCC 17802 (lanes 5–8 in Fig. 3).

In conclusion, the results from SDS-PAGE protein patterns and dendograms would be helpful for the rapid identification and grouping of various *Vibrio* species from environmental samples, and also enable the differentiation of pathogenic and nonpathogenic *Vibrio* strains that would be very important for the epidemiological study of foodborne disease outbreaks.

**Fig. 3.** SDS-PAGE whole-cell protein profiles of *V. parahaemolyticus* ATCC 27969, *V. parahaemolyticus* ATCC 17802, and 6 selected *Vibrio* isolates from sushi restaurants in Busan, Korea. Lanes: M, Protein molecular weight markers (kDa); 1, *Vibrio parahaemolyticus* ATCC 27969; 2–4, isolates a–c; 5, *V. parahaemolyticus* ATCC 17802; 6–8, isolates d–f.

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**References**


