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Development of a rapid and simple immunochromatographic assay to identify *Vibrio parahaemolyticus*

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ABSTRACT

To rapidly and simply determine whether or not bacterial colonies growing on agar were *Vibrio parahaemolyticus*, we developed an immunochromatographic assay (VP-ICA) using two different monoclonal antibodies (designated mAb-VP34 and mAb-VP109) against the delta subunit of *V. parahaemolyticus*-F_0F_1 ATP synthase. The epitopes recognized by mAb-VP34 and mAb-VP109 were mapped to sequences of eight (^{47}LLTSSFSA^{54}) and six amino acid residues (^{16}FDFAVD^{21}), respectively. An amino acid sequence similarity search of the NCBI database using BLASTP showed that both epitopic amino acid sequences were present together only in *V. parahaemolyticus*. When 124 *V. parahaemolyticus* strains and 94 strains of 27 other *Vibrio* species or 35 non-*Vibrio* species were tested using the VP-ICA, the VP-ICA identified *V. parahaemolyticus* with 100% accuracy. The VP-ICA rapidly and simply identified the pathogen directly from a single agar colony within 30 min, indicating that VP-ICA will greatly reduce labor and time required to identify *V. parahaemolyticus* compared with conventional biochemical tests.

1. Introduction

Vibrio parahaemolyticus is a halophilic bacterium and a major food-borne pathogen that causes diarrheal illness in humans through consumption of seafood (Blake et al., 1980; Su and Liu, 2007; Yeung and Boor, 2004). Reducing the risk of *V. parahaemolyticus*-associated food-borne illness therefore requires closely monitoring its presence in foods. The common method for detecting *V. parahaemolyticus* in foods involves enrichment cultures that are first plated on selective agar such as TCBS agar or CHROMagar Vibrio agar (Gomez-Gil and Roque, 2006; Hara-Kudo et al., 2001; Kaysner and DePaola, 2004). However, when grown on selective agar, certain *Vibrio* species such as *Vibrio vulnificus*, *Vibrio mimicus*, *Vibrio harveyi*, or *Vibrio campbellii* produce colonies similar to those of *V. parahaemolyticus* (Shima et al., 2011; Su et al., 2005), requiring the utilization of time-consuming (>2 days) and labor-intensive biochemical tests for reliable identification (Kaysner and DePaola, 2004; Ministry of Health, Labor and Welfare, 2004). A rapid and simple method that can be used to discriminately detect *V. parahaemolyticus* colonies growing on selective agar is therefore required.

Assays using monoclonal antibodies (mAbs) are powerful tools for the rapid and simple identification of bacterial species, in comparison with culture methods (Marot-Leblond et al., 2006; Pongsunk et al., 1999; Qian et al., 2008). However, a mAb that reacts specifically with *V. parahaemolyticus* is not available.

We generated a specific mAb (designated mAb-VP34) against the delta subunit of the *V. parahaemolyticus* F_0F_1 ATP synthase (designated δ-subunit in the text that follows) that we used to develop a dot-blot method (VP-Dot assay) for identifying *V. parahaemolyticus* (Sakata et al., 2012). The δ-subunit consists of 177 amino acids, couples ATP synthesis to proton transport, and maintains homeostasis (Sakai et al., 1990; Thompson et al., 2007). Because mAb-VP34 reacted in ELISAs with all 140 *V. parahaemolyticus* strains tested and cross-reacted only with *Vibrio natriegens* among 97 strains belonging to 57 unrelated species (Sakata et al., 2012), we judged this mAb suitable for use in the VP-Dot assay. However, aside from its cross-reactivity with *V. natriegens*, the VP-Dot assay is too complex for routine use (Sakata et al., 2012).

The purpose of this study was to develop a more rapid, simple, and specific method for identifying *V. parahaemolyticus* compared with the VP-Dot assay. Because immunochromatography assays are rapid, simple, and do not require specialized equipment and technical skills (Bautista et al., 2002; Kawatsu et al., 2006; Park et al., 2003), we chose to develop such an assay. To this end, we generated a mAb (designated mAb-VP109) that recognizes an epitope different from that recognized by mAb-VP34 and successfully developed a sandwich-type immunochromatographic assay (VP-ICA) for rapid and specific identification of *V. parahaemolyticus* colonies growing on selective agar. Further, to support the specificity of the VP-ICA, we also characterized each epitope recognized by mAb-VP34 or mAb-VP109 and compared its sequence with that of the δ-subunits among *V. parahaemolyticus* strains and other bacterial species.

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2. Materials and methods

2.1. Bacterial strains

The 138 *V. parahaemolyticus* strains, 54 strains of 27 other *Vibrio* species, and 40 strains of 35 unrelated species analyzed here are listed in Table 1. The *V. parahaemolyticus* strains, including 65 different O- and K-antigen serotypes, were isolated between 1983 and 2012 and were confirmed using the most accurate species-specific PCR assay available that detects *toxR* (*toxR*-PCR) (Croci et al., 2007; Kim et al., 1999). Ninety-three strains isolated from clinical samples were provided by Kansai Airport Quarantine Station (Osaka, Japan). The identity of a *V. natriegens* strain isolated from seafood was verified using nucleotide sequence analysis of *atpA* (Thompson et al., 2007).

2.2. Nucleotide sequence analysis of the δ-subunit

The genes encoding the δ-subunit from *V. parahaemolyticus* and *V. natriegens* were PCR-amplified using primers described by Sakata et al. (2012). The amplicons were sequenced using the primers described in Table 2 using a BigDye Terminator, version 3.1, Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Sequences were determined using an Applied Biosystems 3130 Genetic Analyzer and analyzed with GENETYX software (GENETYX, Tokyo, Japan).

2.3. Epitope mapping

To map the epitopes recognized by mAb-VP34 and mAb-VP109, we constructed a library of fragments representing segments of the δ-subunit as well as the full-length sequence (Fig. 1A, B) using PCR-amplification of *V. parahaemolyticus* (V2409, serotype O3:K6) genomic DNA and then ligated the products to a pET-SUMO vector using a TA cloning kit (Champion pET-SUMO Expression System; Life Technologies). The primer pairs used are listed in Table 2. *Escherichia coli* BL21(DE3) was transformed with each construct, and the transformants were treated with B-PER II Bacterial Protein Extraction Reagent (Life Technologies). The reactivity of each extract with its respective mAb was tested using a dot-blot assay (Sakata et al., 2012) with each of the peroxidase-labeled mAbs. Uninduced BL21(DE3) cells transformed by each of the pET-SUMO vectors served as negative controls.

To identify the amino acid sequence recognized by each mAb, overlapping peptides (Fig. 1A, B) were synthesized, spotted on a nitrocellulose membrane (Pep-SPOT; JPT Peptide Technologies GmbH, Berlin, Germany), and tested for reactivity using a chemiluminescent dot-blot assay with each mAb according to the manufacturer's instructions.

Epitope sequences were used as queries for BLASTP analyses of the National Center for Biotechnology Information (NCBI) nonredundant protein database (nr).

2.4. Production of mAbs against different regions of the epitope recognized by mAb-VP34

A recombinant δ-subunit was expressed using the Champion pET-SUMO Expression System, as described previously (Sakata et al., 2012). The His-tagged SUMO-recombinant δ-subunit that was purified from whole cell lysates under nondenaturing conditions using a HisTrap HP Kit (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK) was used as an immunogen for the production of mAbs. Three female BALB/c mice (8 weeks old) were immunized intraperitoneally with 70 μg of the immunogen emulsified in Freund's complete adjuvant (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After 2, 4, 6, and 8 weeks, the mice were boosted intraperitoneally with 70 μg of the immunogen emulsified in Freund's incomplete adjuvant (Wako Pure Chemical Industries Ltd.). At 14 weeks, antibody titers were determined using an

Table 1
Bacterial strains used in this study.

Species	Number of strains	Source or strain no.[a]
Vibrio parahaemolyticus[b]	93	Clinical
	45	Seafood
Other *Vibrio* species		
Vibrio aestuarianus	1	NBRC15629^T
Vibrio alginolyticus	1	NBRC15630^T
	1	Unknown
Vibrio azureus	1	NBRC104587^T
Vibrio campbellii	1	NBRC15631^T
	1	Seafood
Vibrio cholerae	3	Clinical
Vibrio comitans	1	NBRC102076^T
Vibrio diazotrophicus	1	NBRC103148^T
Vibrio ezurae	1	NBRC102218^T
Vibrio fischeri	1	NBRC101058
Vibrio fluvialis	1	Clinical
	1	Seafood
Vibrio gazogenes	1	NBRC103151^T
Vibrio halioticoli	1	NBRC102217^T
Vibrio harveyi	1	RIMD2224001^T
	6	Seafood
Vibrio ichthyoenteri	1	NBRC15847^T
Vibrio inusitatus	1	NBRC102082^T
Vibrio mediterranei	1	NBRC15635^T
Vibrio metschinikovii	1	Clinical
	1	Seafood
Vibrio mimicus	2	Clinical
	2	Seafood
Vibrio natriegens	1	NBRC15636^T
	1	Seafood
Vibrio nereis	1	NBRC15637^T
Vibrio orientalis	1	NBRC15638^T
Vibrio penaeicida	1	NBRC15640^T
Vibrio proteolyticus	1	NBRC13287^T
Vibrio rarus	1	NBRC102084^T
Vibrio splendidus	1	NBRC101061
Vibrio tubiashii	1	NBRC15644^T
Vibrio vulnificus	1	NBRC15645^T
	1	Clinical
	10	Seafood
Other species		
Acinetobacter calcoaceticus	1	IAM12087^T
Aeromonas caviae	1	Clinical
Aeromonas hydrophila	1	IAM1646
Aeromonas sobria	1	IAM12333
Alcaligenes faecalis	1	IAM12369^T
Bacillus cereus	1	Food
Bacillus subtilis	1	ATCC6633
Campylobacter coli	1	ATCC43478
Campylobacter jejuni	1	ATCC33560^T
Citrobacter freundii	1	NBRC12681^T
Citrobacter koseri	2	Clinical
Cronobacter sakazakii	1	NBRC102416^T
Edwardsiella tarda	1	Clinical
Enterobacter aerogenes	1	JCM1235^T
	1	IAM12348^T
Enterobacter cloacae	1	IAM12349^T
Enterobacter intermedius	1	IAM14238^T
Escherichia coli	2	Clinical
	1	Food
Grimontia hollisae	1	Clinical
Klebsiella oxytoca	1	JCM1665^T
Klebsiella pneumoniae subsp. *ozaenae*	1	JCM1663^T
Klebsiella pneumoniae subsp. *pneumoniae*	1	NBRC14940^T
Listeria monocytogenes	1	Food
Listonella anguillarum	1	NBRC13266^T
Listonella pelagia	1	NBRC15639^T
Morganella morganii	1	JCM1672^T
Photobacterium damselae subsp. *damselae*	1	NBRC15633^T
Plesiomonas shigelloides	1	Clinical
Proteus vulgaris	1	IAM12542^T
Providencia alcalifaciens	1	Clinical
Pseudomonas aeruginosa	1	IAM1514^T
Raoultella ornithinolytica	1	ATCC31898^T
Raoultella planticola	1	ATCC43176

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