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Thesis for the Degree of Doctor of Engineering

Development of PCR system targeting *groEL* gene as a useful phylogenetic marker for detection of human pathogenic *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*

by

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August 2013

Development of PCR system targeting *groEL* gene as a useful phylogenetic marker for detection of human pathogenic *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*

인체 병원성 *Vibrio cholerae*, *V. parahaemolyticus* 와 *V. vulnificus* 검출을 위한 *groEL* 유전자 표적 PCR 시스템의 개발

Advisor: **Professor In-Soo Kong**

by

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A thesis submitted in partial fulfillment of the requirements
for the degree of Doctor of Engineering
in Department of Biotechnology, The Graduate School,
Pukyong National University

August 2013

DECLARATION

I, the undersigned, hereby certify that the work contained in this thesis is my own original work and has not previously been submitted by me in its entirety or in part to any other university for a degree.

Name: **Muhammad Tofazzal Hossain**

Signature: .....

Date: 2013.07.01

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DEDICATION

This work is dedicated to my parents Muhammad Nabi Hossain and Mrs. Tahmina Hossain, aunt Bilkis Parvin, wife Sadia Islam Tama and only beloved son Muhammad Sadik Hussain

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BRIEF CONTENTS

Development of PCR system targeting *groEL* gene as a useful phylogenetic marker for detection of human pathogenic *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*

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SUMMARY

Vibrio species are naturally diverse bacteria that inhabit aquatic environments and marine animals as symbionts and commensals. Among the identified *Vibrio* species, *Vibrio cholerae* (Vc), *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) are of major concern as they are pathogenic to animals including humans. *Vibrio* infections remain a serious threat to public health. In the last decade, *Vibrio* disease outbreaks have created a painful awareness of the personal, economic, societal, and public health costs associated with the impact of contaminated water and seafood in the aquatic environment. Vc is the etiological agent of cholera, an acute dehydrating diarrhoea that occurs in epidemic form throughout the world, particularly in developing countries. Vp is a leading pathogen that causes seafood-borne gastroenteritis worldwide. Another pathogen, Vv, causes gastroenteritis, septicaemia and severe wound infection with a high mortality in susceptible persons. Moreover, both Vp and Vv strains can

cause diseases in aquatic organisms, including economically important fish and shrimp. Filter feeders act as carrier in transferring these pathogens. Accurate detection is necessary before drinking water, consumption of raw seafood or during outbreak of any disease produced by these species. In this study, the efficiency of *groEL* gene, which has proven as a good marker in detection of pathogens, was checked for the detection of above mentioned *Vibrio* species accurately by PCR assays. Genomic DNA was purified from pure cultures of 22 *Vibrio* and 10 non-*Vibrio* enteric species. All available sequences of *groEL* gene among *Vibrio* and non-*Vibrio* enteric species were accepted from GenBank using the BLASTN search program. Potential oligonucleotide primer sets were designed to detect Vc, Vp and Vv by simplex, duplex or multiplex PCR. After optimizing PCR conditions, the specificity and sensitivity test of PCR assays were performed. Shellfish homogenates, flounder and sterilized seawater were artificially inoculated with the target *Vibrio* species to check the efficiency of developed PCR methods.

First target was to detect Vp specifically and for that PCR conditions were standardized and tested to evaluate the specificity of primers. A 510 bp band was appeared only from Vp by PCR. Notably, the detection was shown to be functional at high annealing temp above 68°C. The *groEL* primers detected 100 pg and 1 ng of DNA purified from Vp culture and artificially infected oyster tissue,

respectively. Second target was to develop a multiplex PCR assay for detection and differentiation of Vp strains using primer sets; a species-specific marker, *groEL,* and two virulence markers, *tdh* and *trh*. After standardization of multiplex PCR, the sensitivity and efficacy of this method was validated using artificially inoculated shellfish and seawater. The expected sizes of amplicons were 510 bp, 382 bp, and 171 bp for *groEL*, *tdh,* and *trh*, respectively. PCR products were sufficiently different in size, and the detection limits of the multiplex PCR for *groEL*, *tdh* and *trh* were each 200-pg DNA. Specific detection and differentiation of virulent from non-virulent strains in shellfish homogenates and seawater was also possible after artificial inoculation with various Vp strains.

Third target was to develop a duplex PCR assay using two sets of primers targeting the *groEL* gene for the accurate simultaneous detection of Vc and Vv. The primer sets were found to be specific for these two species and could detect both target bacteria without any ambiguity, even among closely related species. For both species, the detection limit was 100 pg from purified genomic DNA. The duplex PCR showed high specificity and sensitivity for each species and was sufficient for the detection of Vc and Vv from artificially infected shellfish tissue, flounder, and even inoculated seawater. Final target was to develop an effective multiplex PCR assay for the simultaneous detection of three important *Vibrio* species, Vc, Vp and Vv using the

groEL gene. Three species-specific primer sets were designed to target Vc, Vp and Vv. A total of 131 *Vibrio* and non-*Vibrio* strains were used to determine the specificity and sensitivity of primers. The primers produced specific PCR fragments from all target species strains and did not cross react with other *Vibrio* and non-*Vibrio* species. This PCR method showed good efficiency in detecting co-existing target species in the same sample with a detection limit of 100 pg of Vc, Vp and Vv from mixed purified DNA. Detection of three target species was also possible from artificially inoculated shellfish, flounder and seawater.

We found that this *groEL* gene is a good marker for the specific detection of *Vibrio* species. All the results suggest that *groEL* gene-targeted PCR assay may be suitable and reliable method for the species-specific detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* from clinical and environmental samples.

CHAPTER 1

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CHAPTER 1

GENERAL INTRODUCTION

The genus *Vibrio* belongs to the family *Vibrionaceae*, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium* (Atlas 1997). Vibrios are clearly very important inhabitants of the riverine, estuarine, and marine aquatic environments (Colwell 2006). They are commonly associated with marine living species and include many important pathogens for farmed animals and humans who consume contaminated seafood or polluted drinking water. *Vibrio* species have been isolated from seawater, sea mud, or sea foods in Asia, North America, Australia, New Zealand, Africa, and Europe. It has been reported that vibrios are the predominant bacteria in the digestive tracts of oysters, clams and mussels (Sugita *et al.* 1981; Kueh and Chan 1985), prawns (Yasuda and Kitao 1980), and Artemia (Puente *et al.* 1992). In general, *Vibrio* species are frequently detectable in summer, but during winter they are less common, possibly because of the occurance of a viable but nonculturable stage. However, in tropical and subtropical waters, the variation in vibrio populations is low (Nishibuchi 2006).

All vibrios are ubiquitous in estuarine waters and all species except *Vibrio cholerae* and *V. mimicus*, require sodium chloride supplementation in media for their growth. There are more than 30 species in the genus *Vibrio* and 12 of them are pathogenic to humans (Table 1.1). All of the pathogenic vibrios have been reported to cause foodborne disease, and among them *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are considered the most significant agents. Members of the *Vibrio* genus are Gram-negative, straight or curved, nonspore-forming rods (McLaughlin 1995). They are motile by a

Table 1.1: *Vibrio* species which are associated with human infections

	Occurrence in human clinical specimens*	
	Intestinal	Non-intestinal
V. alginolyticus	-	++
V. carchariae	-	+
V. cholerae O1 and O139	++++	+
V. cholerae non-O1/non-O139	++	++
V. cincinnaitiensis	-	+
V. damsela	-	+
V. fluvialis	++	-
V. furnissii	++	-
V. hollisae	++	-
V. metschnikovii	+	+
V. mimiscus	++	+
V. parahaemolyticus	++++	+
V. vulnificus	+	+++

*The symbol (+) refers to the relative frequency of each organism in clinical specimens and (-) indicated that the organism was not found (Dalsgaard 1998).

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single polar flagellum and are aerobic or facultatively anaerobic. Most species produce oxidase and catalase, and ferment glucose without producing gas (McLaughlin 1995). *Vibrio* species can frequently be isolated in high numbers from bivalves, crustaceans, finfish, sediment, and plankton (Kelly 1982: Oliver *et al*. 1982; Tamplin *et al*. 1982; O'Neil *et al*. 1992; DePaola *et al*. 1994). In general, higher densities of the organisms are found in oyster digestive tissue (Tamplin and Capers 1992; DePaola *et al*. 1997) as compared to muscle tissue.

Cholera has been categorized as one of the emerging and re-emerging enteric diarrheal disease in developing countries (Satcher 1995) and is classified as Category B bioterrorism by Centre for Disease Control and Prevention (WHO 2008). This disease is caused by *V. cholerae* which continues to be a worldwide health concern. Two distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemic that progressively affect many countries in multiple continents over the course of many years (Kaper *et al*. 1995). *V. cholerae* is classified into two serotypes: O1 and non O1 (Chatterjee and Maiti 1984). Cholera is one of the three diseases requiring notification to WHO under the International Health Regulation.

Cholera is usually transmitted by ingestion of contaminated water (Tauxe and Blake 1992). Sewage contamination of ground water is also responsible for the epidemic of *V. cholerae* (Pathak *et al*. 1993). The toxigenic *V. cholerae* is a native flora of the aquatic environment which is transmitted

through drinking water and still remains the leading cause of morbidity and mortality in many developing countries (Chomvarin *et al*. 2007). Vegetables may be contaminated during washing with polluted water. This can also occur when contaminated water is injected into fruits, such as watermelons, to preserve their weight and taste (Feachem 1981). Spices, including raw onions and garlic, can support the survival of *V. cholerae* for 2-3 days at ambient temperature (Felsenfeld 1967). Fishes are likely to be contaminated by *V. cholerae* when the surrounding water is contaminated by the sewage or other environmental sources of *V cholerae* O1. It has been shown that *V. cholerae* can survive in seawater in association with zooplankton. Seafoods, including mollusks, crustaceans, crabs, and oysters, feed on plankton and can become infected with *V. cholerae* (DePaola 1981). Garate-Lizarranga *et al.* (2006) reported that *V. cholerae* adhere strongly to shellfishs digestive tract and cannot be removed efficiently by rinsing the shellfish or depuration. Consumption of raw oysters correlated strongly with gastrointestinal infections in which *V. cholerae* has been implicated as causative agent (Rippey 1994). Contamination of meat of animal origin occurs exogenously during processing, cooking, storage or consumption, which may act as source of this organism (Maheshwari *et al*. 2011).

V. parahaemolyticus is frequently found in seawater, sediments, plankton, finfish and shellfishes (Fishbein *et al*. 1974; Pavia *et al*. 1989), which causes acute gastroenteritis characterized by diarrhea, vomiting and abdominal cramps through consumption of contaminated raw fish or shellfish (Rippey 1994). It also causes traveller's diarrhea, wound infection, ear infection and secondary septicemia in humans (Pavia *et al*. 1989). In humans, traveler's

diarrhea is caused after consumption of contaminated raw or partially cooked fish or shell fish, particularly oysters or exposure to a marine environment (Khan *et al.* 2002). Etiological studies on acute diarrheal diseases in gangetic plain areas have shown that gastroenteritis caused by *V. parahaemolyticus* ranks second to cholera in terms of incidence (Sakazaki *et al*. 1971). Although most often it induces a self-limiting watery diarrhea, it occasionally causes bloody diarrhea and rarely sudden cardiac arrhythmia (Honda *et al.* 1976). Clinical manifestations of *V. parahaemolyticus* include diarrhea, abdominal cramps, nausea, vomiting, headache, fever and chills (Takeda 1983). Primary septicemia is reported in the individuals with chronic illness (Cook *et al*. 2002) and becomes life-threatening to people having underlying medical conditions such as liver disease or immune disorders. This organism is known to be completely destroyed in cooked foods, especially in South Asian countries cooked with plenty of spices. Illness due to *V. parahaemolyticus* was reported in many countries like China, Indonesia, Vietnam, Australia, Great Britain, France, Germany, Italy, Canada and United States (CDC 1998 and 1999), but it is most prevalent foodborne pathogen in many Asian countries, where seafood is often consumed (Pan *et al*. 1997).

V. vulnificus is an opportunistic human pathogen that is highly lethal and is responsible for the overwhelming majority of reported seafood-related deaths in the United States (Feldhusen 2000; Oliver and Kaper 2007). This bacterium is a part of the natural flora of coastal environments worldwide

and has been isolated from water, sediments, and a variety of seafood, including shrimp, fish, oysters, and clams (Tamplin *et al*. 1982; Myatt and Davis 1989; O'Neil *et al*. 1992; DePaola *et al*. 1994; Hoi *et al*. 1998; Bisharat *et al*. 1999; do Nascimento *et al*. 2001; Baffone *et al*. 2006; Oliver 2006b; Mahmud *et al*. 2008). Consumption of seafood (primarily raw oysters) containing *V. vulnificus* can result in a severe, fulminant systemic infection. Characteristics of this disease include fever, chills, nausea, hypotensive septic shock, and the formation of secondary lesions on the extremities of patients (Bowdre *et al*. 1983; Klontz *et al*. 1988; Chuang *et al*. 1992; Hlady and Klontz 1996; Strom and Paranjpya 2000; Oliver 2006a). This primary septicemia is the most lethal infection caused by *V. vulnificus*, with an average mortality rate exceeding 50% (Hlady and Klontz 1996; Feldhusen 2000). In addition to septicemia, *V. vulnificus* can produce serious wound infections that typically result from exposure of open wounds to water harboring the bacterium (Oliver 2005). Wound infections are frequently contracted as a result of recreational swimming, fishing injuries, or seafood handling (Howard *et al*. 1986; Bisharat *et al*. 1999).

V. cholerae, *V. parahaemolyticus* and *V. vulnificus* as food and/or water-borne pathogens pose a significant threat to public health, leading to a substantial economic burden in many countries (Chen *et al.* 2012). For this reason, the availability of rapid, sensitive and specific diagnostic methods for the detection of these pathogens from contaminated seafood or polluted water is important. Nishibuchi (2006) mentioned that it has become

impossible to establish a comprehensive scheme to differentiate *Vibrio* species using only biochemical characteristics. Classical methods based on biochemical and microbiological tests are time-consuming (3–5 days), labour-intensive, unreliable, and involve evaluation of large number of samples (Peeler *et al*. 1992). To overcome from these problems, researchers have sought molecular genetic identification methods that are quicker and more definitive than biochemical tests (Gonzalez *et al*. 2004; Gray *et al*. 2005). Polymerase chain reaction (PCR) assays are becoming increasingly popular for microbial testing due to their relative ease of use, efficiency, and low cost, and are highly specific for the detection of small number of pathogens (Aznar and Alarcon 2002; Fan *et al*. 2008; Tunung *et al*. 2010). Various PCR-based methods have been reported for *Vibrio* species identification, such as real-time PCR, microarrays and conventional PCR. However, the first two detection methods are costly due to the requirement for expensive instruments, whereas the PCR method that detects single or multiple species targets is effective. Multiplex PCR has been proven to provide rapid and highly sensitive methods for the specific detection of microorganisms (Fan *et al.* 2008) and can be easily performed in diagnostic laboratories. Several PCR-based detection methods for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* have been developed. Many virulence genes, such as *omp*, *ctx*, *zot*, *ace*, *tcp*, *rtx*, *sto* and *hly* in *V. cholerae*; *tdh*, *trh* and *toxR* in *V. parahaemolyticus*; and *vvh*, *viuB* and *toxR* in *V. vulnificus* have been targeted for species-specific detection by uniplex

or multiplex PCR (Lalitha *et al.* 2008; Neogi *et al.* 2010; Teh *et al.* 2010). The use of virulence genes as identification markers may be of significance because their existence may be linked to pathogenesis. However, when applied to environmental samples, there is a potential risk of misidentification because such genes might transfer among bacteria. The mobilization of phages leads to genome recombination and the emergence of new toxigenic strains in *V. cholerae* (Waldor and Mekalanos 1996; Karaolis *et al*. 1998; Zhang *et al*. 2003). CTXϕ could also be horizontally transferred between *V. cholera*e and *V. mimicus* (Boyd *et al*. 2000). Spreading of the gene *tdh* among *Vibrio* species is supposed to be mediated by an insertion sequence (Terai *et al*. 1991). Although most *Vibrio* species are nonpathogenic, they are considered to provide a large reservoir of the known virulence genes. Some, including *ctxA* and *tdh*, may be horizontally transferred, leading to new pathogenic strains (Nishibuchi *et al*. 1996; Boyd *et al*. 2000). The mobility of the virulence genes and a successful transfer may cause the transformation of a nonpathogenic strain to pathogenic strain (Nishibuchi *et al*. 1996; Boyd *et al*. 2000; Hentschel *et al*. 2000; Faruque and Nair 2002). Virulence genes homologous to *V. parahaemolyticus* and *V. cholerae* virulence determinants are widely distributed among some *Vibrio* species, such as, *V. parahaemolyticus*, *V. cholerae* non-O1, *V. mimicus*, *V. hollisae*, *V. fluvialis* and *V. alginolyticus* (Nishibuchi *et al*. 1996; Sechi *et al*. 2000). Neogi *et al.* (2010) also reported that among closely related *Vibrio* species, horizontal transfer of toxigenic genes can equip the

nontoxigenic strains with epidemic potential. Therefore, it is important to conduct surveillance on the total population (both toxigenic and nontoxigenic) of these three target species. A suitable phylogenetic marker is necessary for the detection of all strains of a particular species. Many PCR methods have been developed targeting housekeeping genes like 16S rRNA, 23S rRNA, *pho*, *amiB*, *dnaJ*, *gyrB*, *rpoA* and *rpoB* (Thompson *et al.* 2005; Nhung *et al.* 2007). However, the sensitivity and specificity of these assays remain an issue, as false-positive and -negative results of *V. parahaemolyticus* PCR assays occurred (Croci *et al.* 2007).

The *groEL* gene encodes a 60 kDa subunit (also known as HSP 60 and 60 kDa chaperonin) of heat shock proteins (HSPs). HSPs are an evolutionary conserved family of proteins whose expression increases in response to a variety of different metabolic insults. Structurally, GroEL is a dual-ringed tetradecamer, with both the *cis* and *trans* rings consisting of seven subunits each (Fig. 1.1). The conformational changes that occur within the central cavity of GroEL cause for the inside of GroEL to become hydrophillic, rather than hydrophobic, and is likely facilitates protein folding. Most notable function of HSPs is their role as molecular chaperones, facilitating the synthesis and folding of proteins throughout the cell. In addition, heat shock proteins have been shown to participate in protein assembly, secretion, trafficking, protein degradation, and the regulation of transcription factors (Robert 2003; Mogk *et al.* 2011). Under stress situations, including environmental (heat shock, exposure to heavy metals or

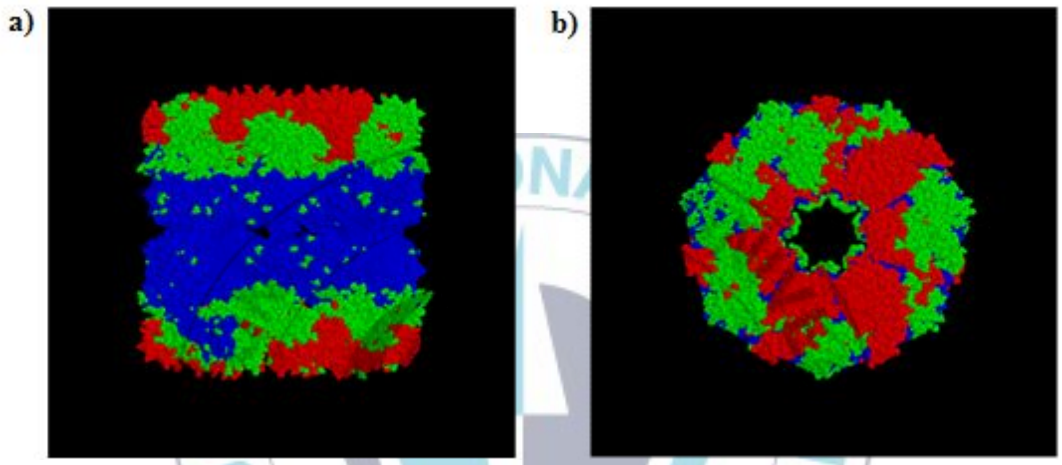
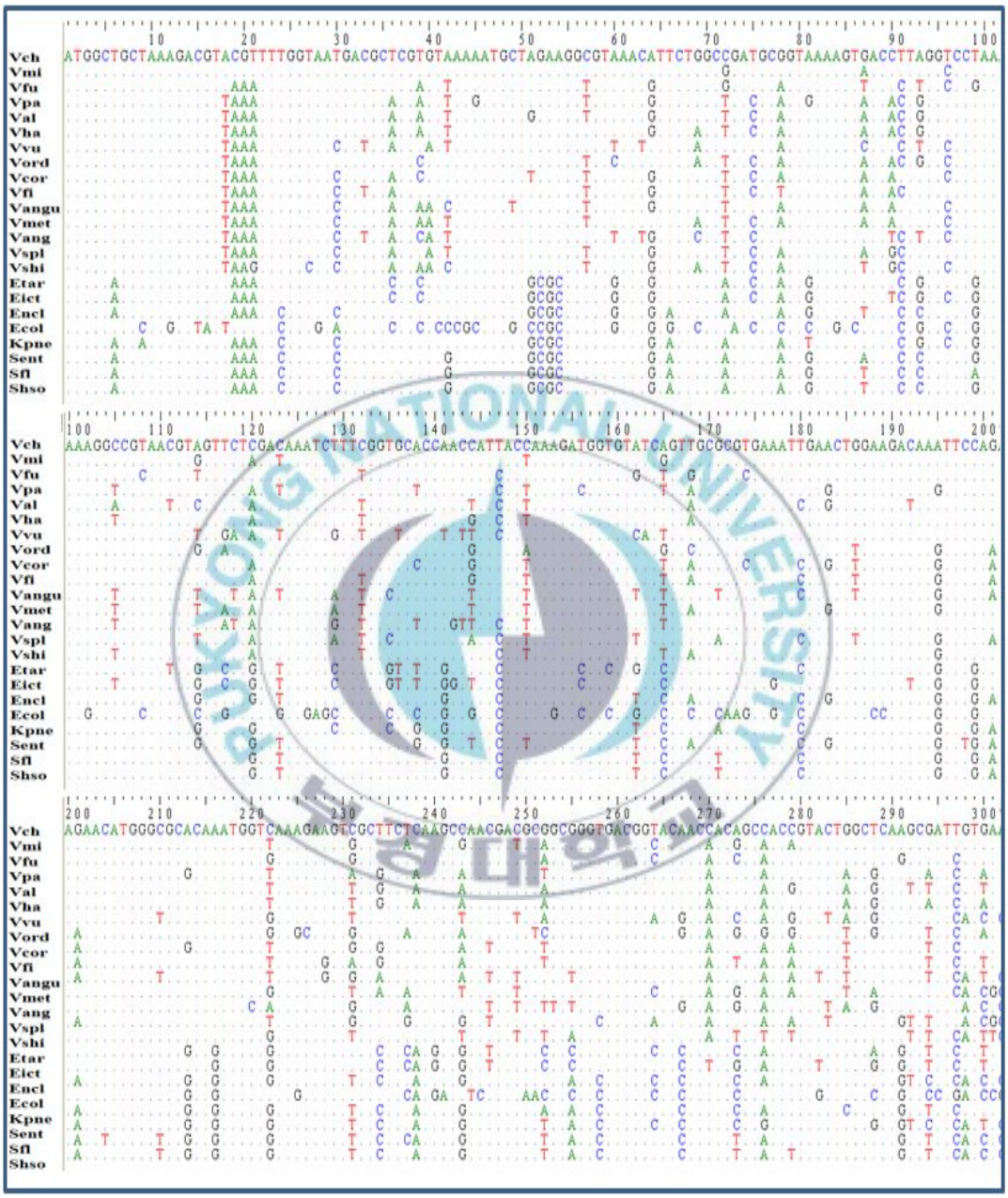


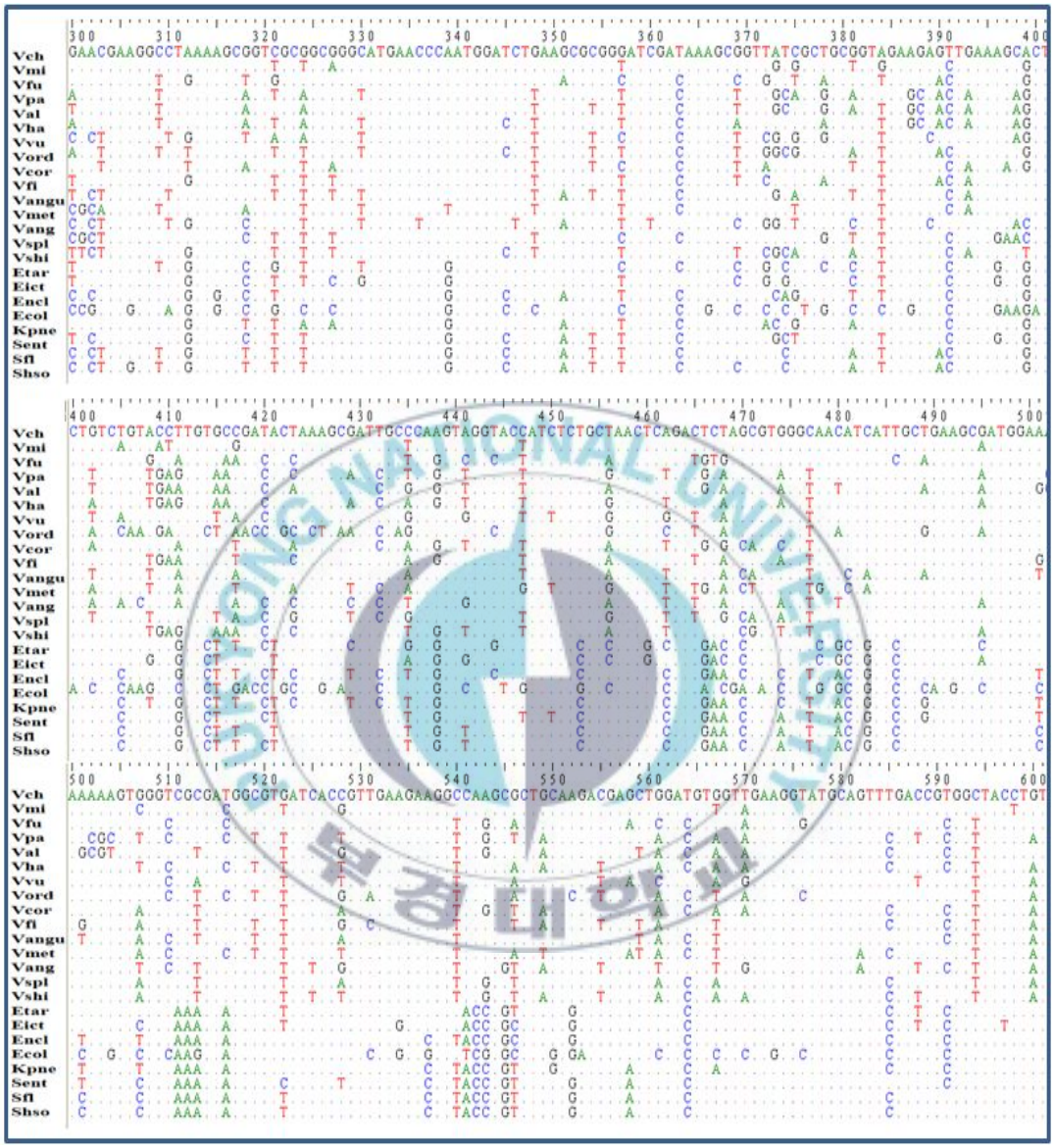
Fig. 1.1 A side-view of the protein GroEL (a) and a top-view of the protein GroEL (b). Collected from https://en.wikipedia.org/wiki/GroEL.

UV radiation), pathological (infections or fever, malignancies, inflammation or autoimmunity) or physiological stress (growth factor deficiency, cell differentiation, hormonal stimulation or tissue development) (Tomanek 2002; Segal *et al*. 2006), HSP synthesis is markedly increased to protect cells from damage. HSPs range in size from 27 to 110 kDa and are divided into five groups based on molecular weight and function (Moseley 1997). Major families of HSPs are low molecular weight (LMW) HSPs, HSP60, HSP70 and HSP90 that have an approximated molecular mass of 60, 70, and 90 kDa respectively, and high molecular weight (HMW) HSPs. HSP60 proteins have been vigorously studied for their chaperone function in protein folding and for their cooperation with other chaperones (particularly HSP70) in cellular trafficking (Horwich *et al*. 2007). More than 150 homologues of HSP60 sequences are currently available with pair wise similarity extending from 40 to 100% at the amino acid level (Karlin and Brocchieri 2000). The *groEL* gene also has the potential to serve as a general phylogenetic marker because of its ubiquity and conservation in nature. The heat shock protein is of great importance for maintaining cellular normal physiological function. It has been well documented that the *groEL* gene is one of the most conserved systems in nature (Kwok *et al*. 1999; Rebecca *et al*. 2002; Karuna *et al*. 2003; Lee *et al*. 2003; Chang *et al*. 2003; Eric *et al*. 2004; Sensu *et al*. 2004; Giuseppe *et al*. 2008). Despite of conserved nature of the *groEL* gene, the level of interspecies variation of *groEL* sequence is greater. Therefore, the *groEL* gene may be a good target gene for species

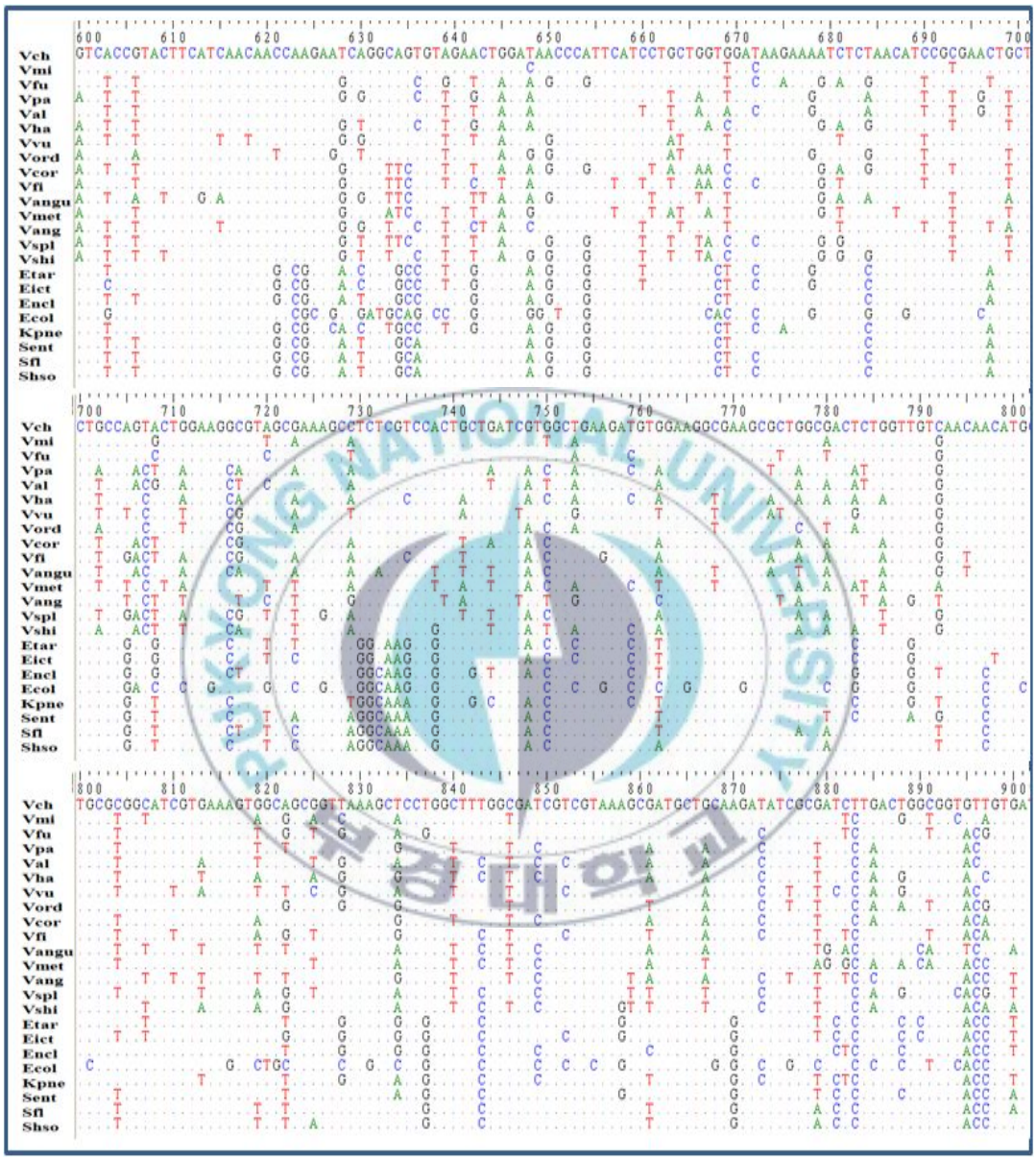
classification (Yushan *et al*. 2010). Yushan *et al*. (2010) reported that according to the phylogenetic analysis of *groEL* gene, it can be devided into 5 clusters in bacteria. They also mentioned that the highest homology exists in the same genus of bacteria with closest interrelationship and the homology of bacteria belonging to the same family is higher than those of bacteria of different families. The nucleotide sequences of the *groEL* genes of 10 *Vibrio* species was compared and the identity was found between 80 and 90% (Kim *et al*. 2010). Figure 1.2 represents the comparison of nucleotide sequences of *groEL* genes of 12 *Vibrio* and 8 non-*Vibrio* enteric species with that of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* using ClastalW program. The nucleotide sequence identity in *Vibrio* species varies between 80 and 92% (Table 1.2) and in non-*Vibrio* species varies between 66 and 80% (Table 1.3). The superiority of the *groEL* gene compared to 16S rRNA and 23S rRNA has already been reported in the detection of *Vibrio* species (Nishibuchi 2006; Yushan *et al*. 2010). This gene has been shown to be a suitable marker for the successful typing and detection of *V. anguillarum*, *Campylobacter jejuni*, *Bifidobacterium*, *Salmonella*, and *Staphylococcus* species on account of its complete database (Goh 1996; Satheesh *et al*. 2002; Karenlampi *et al*. 2004; Kim *et al*. 2010; Yushan *et al*. 2010; Junick and Blaut 2012).



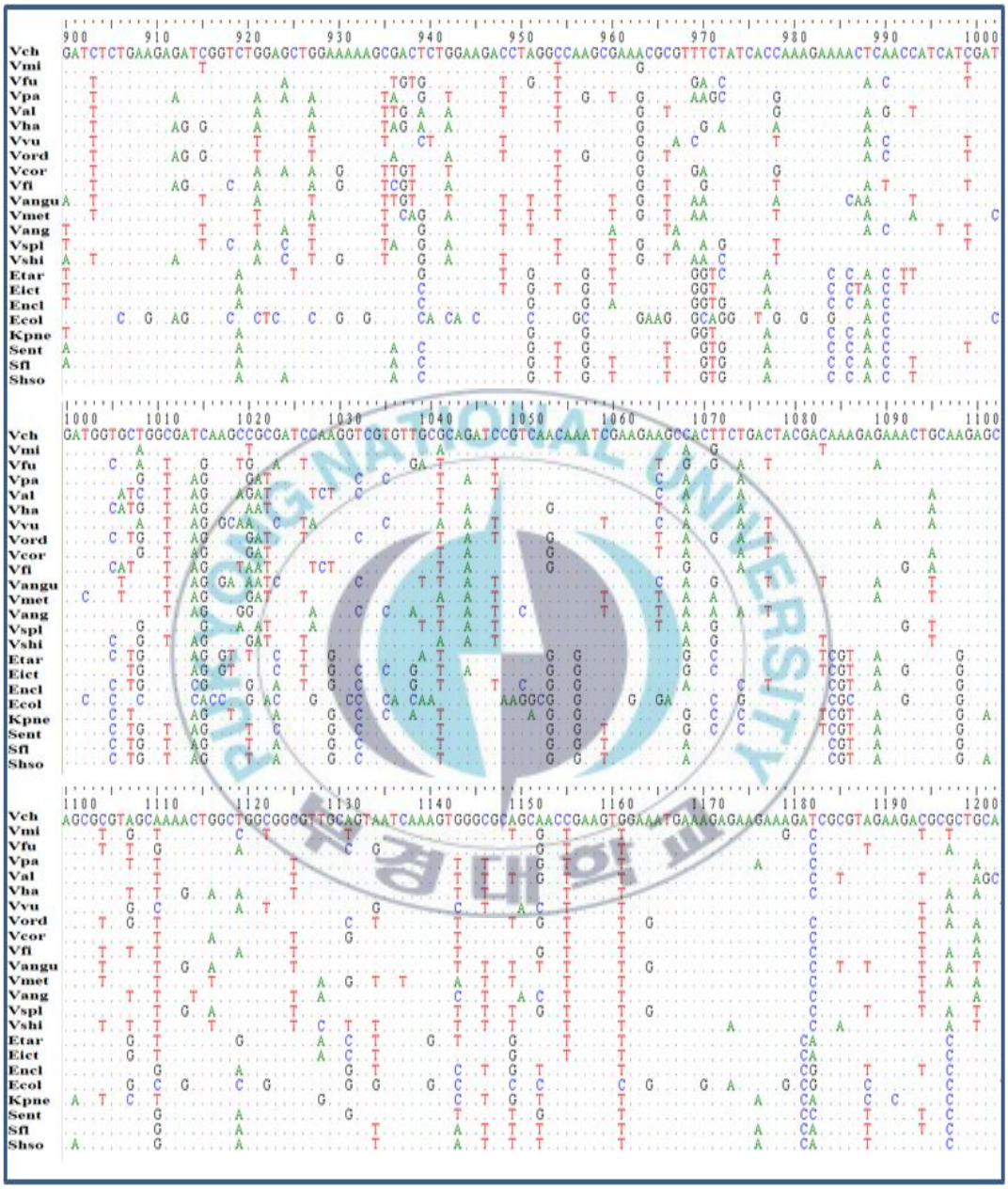
Nucleotide sequences of *groEL* gene in *Vibrio* and non-*Vibrio* enteric species



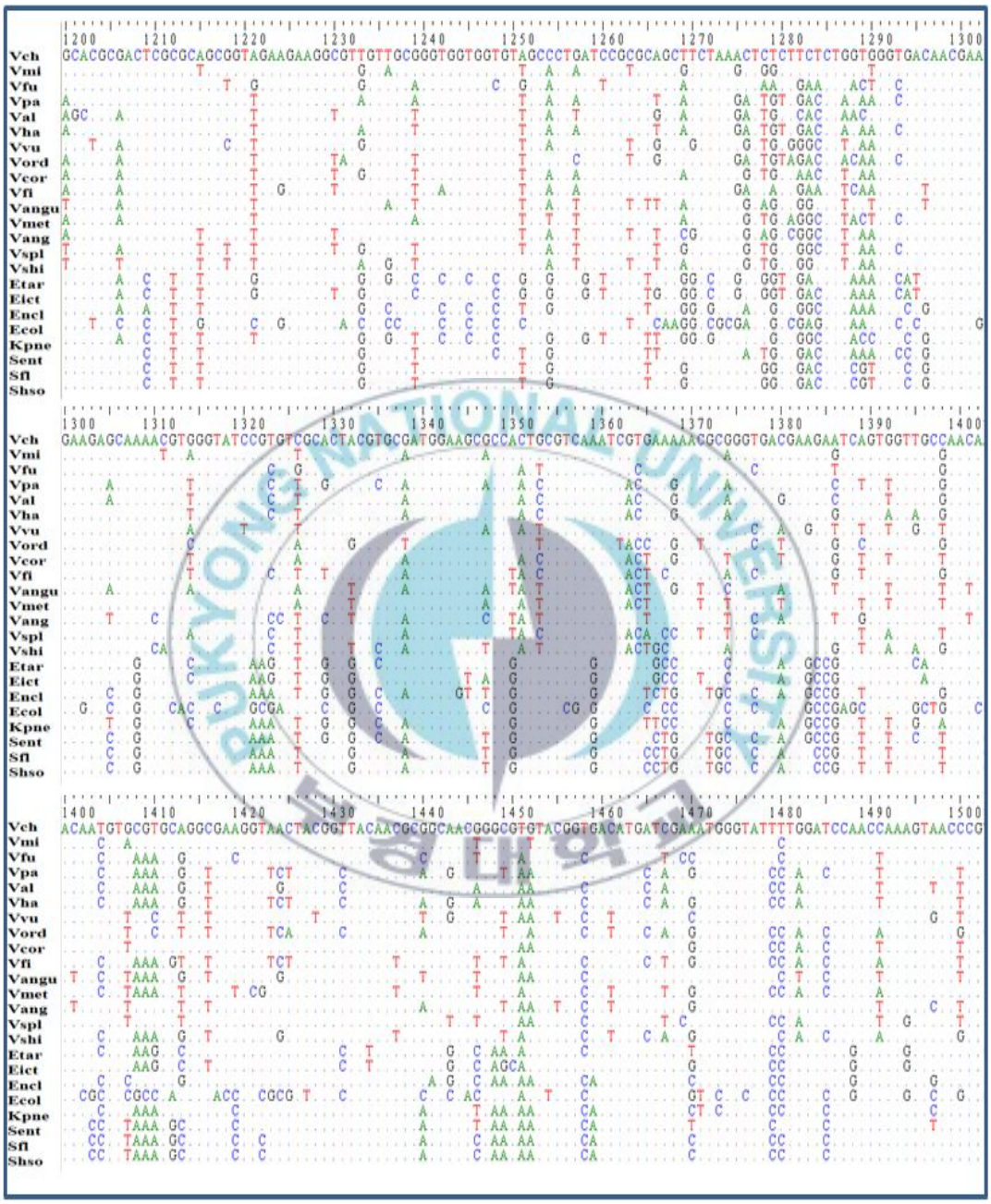
Nucleotide sequences of *groEL* gene in *Vibrio* and non-*Vibrio* enteric species



Nucleotide sequences of *groEL* gene in *Vibrio* and non-*Vibrio* enteric species



Nucleotide sequences of *groEL* gene in *Vibrio* and non-*Vibrio* enteric species



Nucleotide sequences of *groEL* gene in *Vibrio* and non-*Vibrio* enteric species

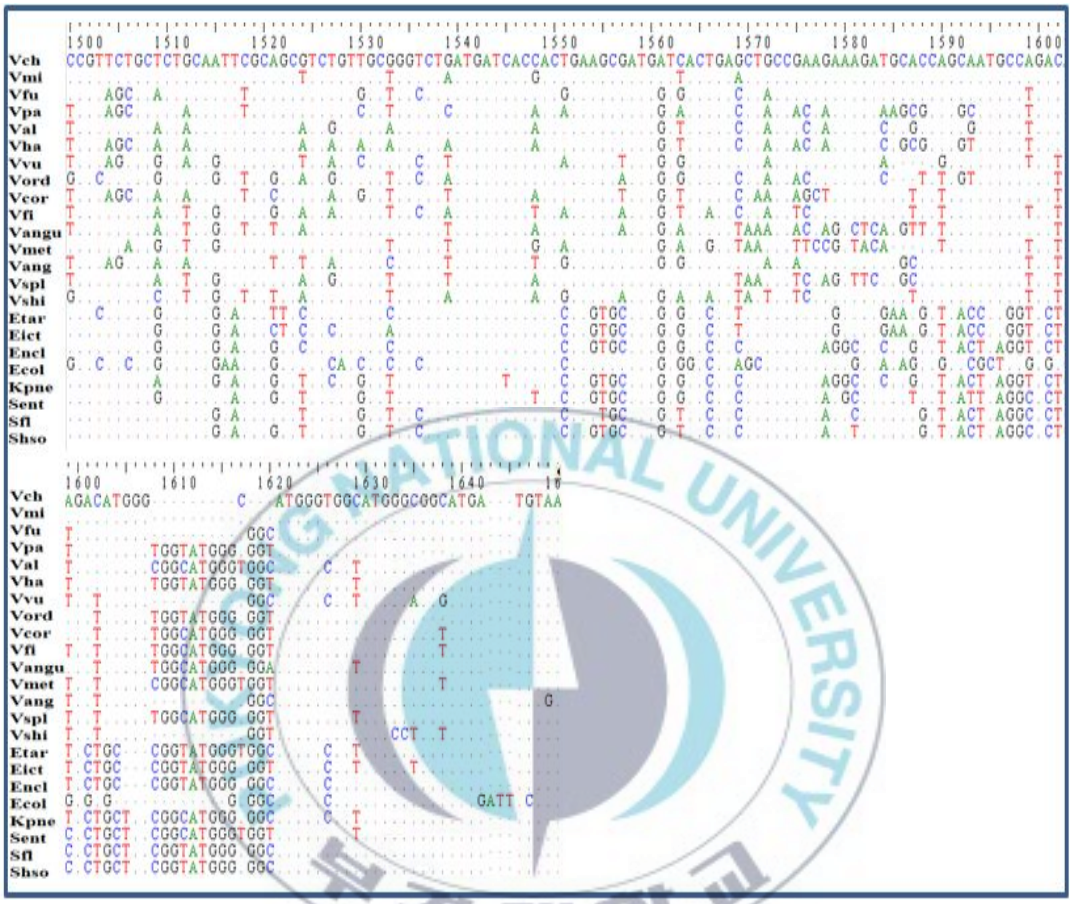


Figure 1.2 Comparison of nucleotide sequences of *groEL* gene among *Vibrio* and non-*Vibrio* enteric species. Vch: *V. cholarae*; Vmi: *V. mimicus*; Vfu: *V. furnessii*; Vpa: *V. parahaemolyticus*; Val: *V. alginolyticus*; Vha: *V. harveyi*; Vvu: *V. vulnificus*; Vord: *V. ordalli*; Vcor: *V. coralliilyticus*; Vfi: *V. fischeri*; Vangu: *V. angustum*; Vmet: *V. metschnikovii*; Vang: *V. anguillarum*; Vspl: *V. splendidus*; Vshi: *V. shiloi*; Etar: *Edwardsiella tarda*; Eict: *E. ictaluri*; Encl: *Enterobacter cloacae*; Ecol: *Escherichia coli*; Kpne: *Klebsiella pneumoniae*; Sent: *Salmonella enteritidis*; Shfl: *Shigella flexneri* and Shso: *S. sonnei*. Identical nucleotide sequences are indicated by dots.

Table 1.2 The *groEL* gene sequence identity for each pair of *Vibrio* species determined by multiple alignment using ClustalW program

	Vcho	Vmim	Vfur	Vpar	Valg	Vhar	Vvul	Vord	Vcor	Vfis	Vangu	Vmet	Vang	Vspl	Vshi
Vcho		**92.4**	**86.6**	**81.2**	**82.1**	**82.2**	**81.1**	**83.8**	**82.0**	**80.2**	**81.0**	**81.9**	**82.9**	**81.9**	**81.0**
Vmim	**99.4**		**86.0**	**81.6**	**82.5**	**82.7**	**82.2**	**83.6**	**82.8**	**81.6**	**81.4**	**81.8**	**82.4**	**82.8**	**82.6**
Vfur	**94.5**	**94.1**		**82.9**	**83.1**	**83.6**	**81.6**	**83.7**	**82.7**	**80.2**	**81.6**	**81.8**	**83.5**	**81.5**	**82.3**
Vpar	**91.7**	**91.7**	**92.5**		**90.2**	**92.4**	**84.9**	**87.9**	**85.5**	**83.1**	**83.3**	**82.2**	**81.4**	**83.7**	**86.0**
Valg	**91.2**	**91.4**	**92.8**	**96.7**		**90.3**	**83.6**	**87.6**	**87.1**	**84.2**	**83.4**	**81.2**	**81.6**	**85.1**	**84.5**
Vhar	**91.6**	**91.4**	**92.7**	**97.0**	**95.6**		**85.7**	**88.2**	**88.2**	**83.9**	**84.3**	**81.9**	**82.4**	**85.6**	**85.9**
Vvul	**91.4**	**91.4**	**90.5**	**93.7**	**92.3**	**93.7**		**84.3**	**84.6**	**81.1**	**82.5**	**80.7**	**82.3**	**81.6**	**82.4**
Vord	**93.0**	**92.8**	**94.7**	**94.8**	**94.5**	**95.0**	**92.1**		**89.0**	**86.8**	**86.1**	**82.6**	**82.0**	**88.2**	**85.7**
Vcor	**92.1**	**91.9**	**94.1**	**94.7**	**95.6**	**96.3**	**92.8**	**95.8**		**84.7**	**84.2**	**81.1**	**80.8**	**86.0**	**85.7**
Vfis	**89.5**	**89.7**	**92.1**	**92.1**	**92.5**	**92.5**	**89.9**	**94.1**	**93.0**		**86.5**	**81.4**	**81.3**	**86.7**	**84.5**
Vangu	**91.0**	**90.8**	**93.0**	**92.3**	**92.5**	**93.2**	**90.6**	**95.4**	**93.6**	**94.8**		**81.2**	**81.8**	**85.4**	**86.4**
Vmet	**92.3**	**92.4**	**92.3**	**90.6**	**91.0**	**90.5**	**88.3**	**91.7**	**90.5**	**90.8**	**90.8**		**86.6**	**82.3**	**82.6**
Vang	**92.1**	**92.3**	**92.4**	**91.4**	**91.2**	**90.6**	**89.2**	**91.9**	**90.3**	**91.4**	**91.0**	**95.7**		**82.0**	**81.2**
Vspl	**91.0**	**90.8**	**92.3**	**92.1**	**91.9**	**92.5**	**89.7**	**95.4**	**93.2**	**93.7**	**93.9**	**91.7**	**92.1**		**86.1**
Vshi	**91.5**	**91.7**	**92.8**	**93.2**	**93.0**	**93.2**	**90.5**	**95.0**	**94.3**	**92.7**	**93.0**	**91.7**	**91.3**	**94.7**	

Identity (%) of the *groEL* nucleotide sequences is shown above the diagonal, and the deduced amino acid sequence similarity with GroEL proteins is shown below the diagonal. Vcho *V. cholarae*; Vmim *V. mimicus*; Vfur *V. furnessii*; Vpar *V. parahaemolyticus*; Valg *V. alginolyticus*; Vhar *V. harveyi*; Vvul *V. vulnificus*; Vord: *V. ordalli*; Vcor: *V. coralliilyticus*; Vfis *V. fischeri*; Vangu: *V. angustum*; Vmet: *V. metschnikovii*; Vang: *V. anguillarum*; Vspl: *V. splendidus*; Vshi: *V. shiloi*.

Table 1.3 The *groEL* gene sequence identity for each pair of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, and non-*Vibrio* species determined by multiple alignment using ClustalW program

	Vpar	Vcho	Vvul	Etar	Eict	Enclo	Ecol	Kpne	Sent	Shfle	Shson
Vpar		**81.3**	**84.9**	**76.2**	**76.3**	**76.3**	**65.9**	**76.3**	**77.6**	**77.8**	**77.8**
Vcho	**92.3**		**81.2**	**79.0**	**78.7**	**79.4**	**69.1**	**78.8**	**79.2**	**80.2**	**80.0**
Vvul	**93.7**	**91.9**		**75.7**	**76.0**	**76.0**	**67.4**	**75.0**	**77.1**	**77.1**	**77.0**
Etar	**86.1**	**85.3**	**85.0**		**94.8**	**88.2**	**73.7**	**87.7**	**86.9**	**86.5**	**86.5**
Eict	**85.9**	**85.3**	**85.0**	**98.9**		**86.4**	**72.7**	**86.3**	**85.6**	**85.3**	**85.3**
Enclo	**84.6**	**85.5**	**84.6**	**93.6**	**93.4**		**72.9**	**91.6**	**91.6**	**91.4**	**91.2**
Ecol	**75.1**	**76.5**	**76.0**	**76.2**	**75.6**	**77.3**		**72.3**	**71.2**	**70.7**	**71.0**
Kpne	**84.6**	**84.8**	**84.4**	**94.1**	**93.6**	**96.1**	**77.0**		**90.1**	**89.8**	**90.0**
Sent	**85.7**	**85.2**	**85.4**	**93.7**	**93.2**	**97.6**	**77.3**	**96.1**		**93.8**	**93.6**
Shfle	**85.7**	**85.4**	**85.2**	**94.3**	**93.6**	**96.8**	**77.1**	**96.3**	**98.9**		**98.9**
Shson	**85.7**	**85.4**	**85.2**	**94.3**	**93.6**	**96.8**	**77.1**	**96.3**	**98.9**	**100**	

Identity (%) of the *groEL* nucleotide sequences is shown above the diagonal, and the deduced amino acid sequence similarity with GroEL proteins is shown below the diagonal. Vpar *V. parahaemolyticus*; Vcho: *V. cholerae*; Vvul: *V. vulnificus*; Etar: *Edwardsiella tarda*; Eict: *E. ictaluri*; Enclo: *Enterobacter cloacae*; Ecol: *Escherichia coli*; Kpne: *Klebsiella pneumoniae*; Sent: *Salmonella enteritidis*; Shfle: *Shigella flexneri*; Shson: *S. sonnei*.

Considering the above points, the *groEL* gene has been selected as a target gene to investigate its significance in the identification of important three *Vibrio* species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. This gene was targeted to develop simplex, duplex or multiplex PCR assays for the specific detection of these species accurately throughout the study. For this reason, the objectives were focused on 4 targets:

Target 1: Application of *groEL* gene for the species-specific detection of *V. parahaemolyticus* by PCR,

Target 2: Multiplex PCR for the detection and differentiation of *V. parahaemolyticus* strains using *groEL*, *tdh* and *trh* genes,

Target 3: Detection of *V. cholerae* and *V. vulnificus* by duplex PCR specific to *groEL* gene, and

Target 4: Development of a *groEL* gene-based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

References

Atlas RM (1997) Bacterial diversity. In: Fishback JE (ed) *Principles of Microbiology*. Brown Publishers, Boston.

Aznar R and Alarcon B (2002) On the specificity of PCR detection of *Listeria monocytogenes* in food: a comparison of published primers. *Systematic and Applied Microbiology* **25**: 109–19.

Baffone W, Tarsi R, Pane L, Campana R, Repetto B, Mariottini GL and Pruzzo C (2006) Detection of free-living and plankton-bound vibrios in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity associated properties. *Environmental Microbiology* **8**: 1299–1305.

Bisharat N, Agmon V, Finkelstein R, Raz R, Ben-Dror G, Lemer L, Soboh S, Colodner R, Cameron DN, Wykstra DL, Swerdlow DL and Farmer JJ (1999) Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. *Lancet* **354**: 1421–4.

Bowdre JH, Hull JH and Cocchetto DM (1983) Antibiotic efficacy against *Vibrio vulnificus* in the mouse: superiority of tetracycline. *Journal of Pharmacology and Experimental Therapeutics* **22**: 595–8.

Boyd EF, Moyer KE, Shi L and Waldor MK (2000) Infectious CTXphi and the *Vibrio* pathogenicity island prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. *Infection and Immunity* **68**: 1507–13.

Centers for Disease Control and Prevention (CDC) (1998) Outbreak of *Vibrio parahaemolyticus* infections association with eating raw oysters -Pacific Northwest, 1997. *Morbidity and Mortality Weekly Report* **47**: 457−62.

Centers for Disease Control and Prevention (CDC) (1999) Outbreak of *Vibrio parahaemolyticus* infection association with eating raw oysters and clams harvested from Long Island Sound -Connecticut, New Jersey and New York, 1998. *Morbidity and Mortality Weekly Report* **48**: 48−51.

Chang YH, Shangkuan YH, Lin HC and Liu HW (2003) PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. *Applied and Environmental Microbiology* **69**: 4502−10.

Chatterjee SN and Maiti M (1984) Vibriophages and Vibriocins: physical, chemical and biological properties. *Advances in Virus Research* **29**: 263−312.

Chen J, Tang J, Liu J, Cai Z and Bai X (2012) Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *Journal of Applied Microbiology* **112:** 823−30.

Chomvarin C, Namwat W, Wongwajana S, Alam M, Thaew-Nonngiew K, Sinchaturus A and Engchanil C (2007) Application of duplex- PCR in rapid and reliable detection of toxigenic Vibrio cholerae in water samples in Thailand. *Journal of General and Applied Microbiology* **53**: 229−37.

Chuang YC, Yuan CY, Liu CY, Lan CK and Huang AH (1992) *Vibrio*

vulnificus infection in Taiwan: report of 28 cases and review of clinical manifestations and treatment. *Clinical Infectious Diseases* **15**: 271−6.

Colwell RR (2006) A global and historical perspective of the Genus *Vibrio*. In: Thompson FL, Austin B and Swings J (ed) *The Biology of Vibrios*. ASM press, Washington, p. 3−11.

Cook DW, O'Leary P, Hunsucker JC, Sloan EM, Bowers JC, Blodgett RJ and Depaola A (2002) *Vibrio vulnificus* and *Vibrio parahaemolyticus* in US retail shell oysters: A National Survey from June 1998 to July 1999. *Journal of Food Protection* **65**: 79−87.

Croci L, Suffredini E, Cozzi L, Toti L, Ottaviani D, Pruzzo C, Serratore P, Fischetti R, Goffredo E, Loffredo G and Mioni R (2007) Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology* **102**: 229−37.

Dalsgaard A (1998) The occurance of human pathogenic *Vibrio* spp. and *Salmonella* in aquaculture. *International Journal of Food Science and Technology* **33**: 127−38.

Depaola A (1981) *Vibrio cholerae* in marine foods and environmental waters: Literature review. *Journal of Food Science* **46**: 66−70.

DePaola A, Capers GM and Alexander D (1994) Densities of *Vibrio vulnificus* in the intestines of fish from the US Gulf Coast. *Applied and Environmental Microbiology* **60**: 984−8.

DePaola A, McLeroy S and McManus G (1997) Distribution of *Vibrio vulnificus* phage in oyster tissues and other estuarine habitats. *Applied*

and Environmental Microbiology **63**: 2464−7.

do Nascimento SM, dos Fernandes Vieira RH, Theophilo GN, dos Prazeres Rodrigues D and Vieira GH (2001) *Vibrio vulnificus* as a health hazard for shrimp consumers. *Revista do Instituto de Medicina Tropical de São Paulo* **43**: 263−6.

Eric G, Christophe H, Chi ZL, Carol AG (2004) A chaperone network controls the heat shock response in *E. coli*. *Genes and Development* **18**: 2812−21.

Fan H, Wu Q and Kou X (2008) Co-detection of five species of water-borne bacteria by multiplex PCR. *Life Science Journal* **4**: 47−54.

Faruque SM and Nair GB (2002) Molecular ecology of toxigenic *Vibrio cholerae*. *Microbiology and Immunology* **46**: 59−66.

Feachem RG (1981) Environmental aspects of cholera epidemiology. I. A review of selected reports of endemic and epidemic situations during 1961−1980. *Tropical Diseases Bulletin* **78**: 675−98.

Feldhusen F (2000) The role of seafood in bacterial foodbourne diseases. *Microbes and Infection* **2**: 1651−60.

Felsenfeld O (1967) The cholera problem. Warren H. Green, St. Louis, Missouri, p. 165.

Fishbein M, Webz B, Landry WL and MacFachern B (1974) *Vibrio parahaemolyticus* isolated in the U.S.: 1969-1972. In: Fujino T, Sakaguchi G, Sakazaki R and Takeda Y (eds), *International Symposium on Vibrio parahaemolyticus*. Saikon Publication, Tokyo, p. 53.

Garate-Lizarranga I, Bustillos-Guzman JJ, Lopez-Cortes DJ, Hernandez-Sandoval F, Erler K and Luckas B (2006) Paralytic shellfish toxin profiles in netphyto plankton samples from bahia. concepcion, gulf of California, Mexico. *Marine Pollution Bulletin* **52**: 800−6.

Giuseppe B, Vincenzina F, Danilo E, Maria A, Olimpia P and Francesco V (2008) *Lactobacillus* strain diversity based on partial *hsp*60 gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. *Applied and Environmental Microbiology* **74**: 208−15.

Goh SH (1996) *groEL* gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *Journal of Clinical Microbiology* **34**: 818−23.

Gonzalez SF, Krug MJ, Nielsen ME, Santos Y and Call DR (2004) Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. *Journal of Clinical Microbiology* **42**: 1414 –9.

Gray J, Carolyn E, Michele M, Cheryl A, Jaonne D and David A (2005) Nucleic acid amplification strategies for DNA microarray-based pathogen detection. *Proceedings of the National Academy of Sciences* **102**: 10109–14.

Hentschel U, Stelnert M and Hacker J (2000) Common molecular mechanisms of symbiosis and pathogenesis. *Trends in Microbiology* **8**: 226−31.

Hlady WG and Klontz KC (1996) The epidemiology of *Vibrio* infections in Florida, 1981−1993. *The Journal of Infectious Diseases* **173**: 1176−83.

Høi L, Larsen JL, Dalsgaard I and Dalsgaard A (1998) Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Applied and Environmental Microbiology* **64**: 7−13.

Honda T, Takeda Y, Miwatani T, Kato K and Nimura Y (1976) Clinical features of patients suffering from food poisoning due to *Vibrio parahaemolyticus*, especially on changes in electrocardiograms. *The Japanese Association for Infectious Diseases* **50**: 216−23.

Horwich AL, Fento WA, Chapman E and Farr GW (2007) Two families of chaperon: Physiology and mechanism. *Annual Review of Cell and Development Biology* **23**: 115−45.

Howard R, Brennaman B and Lieb S (1986) Soft tissue infections in Florida due to marine *Vibrio* bacteria. *The Journal of the Florida Medical Association* **73**: 29−34.

Junick J and Blaut M (2012) Quantification of human fecal *Bifidobacterium* species by use of quantitative real-time PCR analysis targeting the *groEL* gene. *Applied and Environmental Microbiology* **78**: 2613−22.

Kaper JB, Morris JG and Levine MM (1995) Cholera. *Clinical Microbiology Reviews* **8**: 48−86.

Karaolis DK, Johnson JA, Bailey CC, Boedeker EC, Kaper JB and Reeves PR (1998) A Vibrio cholerae pathogenicity island associated with epidemic and pandemic strains. *Proceedings of the National Academy of*

Sciences USA **95**: 3134−39.

Karenlampi RI, Tolvanen TP and Hanninen ML (2004) Phylogenetic Analysis and PCR-Restriction Fragment Length Polymorphism Identification of *Campylobacter* Species Based on Partial *groEL* Gene Sequence. *Journal of Clinical Microbiology* **42**: 5731−38.

Karlin S and Brocchieri L (2000) Heat shock protein 60 sequence comparisons: duplication, lateral transfer and mitochondrial evolution. *Proceedings of the National Academy of Sciences* **97**: 11348−53.

Karuna P, Yasuyuki N, Timothy DR, Artem C, Jeffrey K, Caixia S, Colleen CN and Robert CB (2003) Molecular analysis of the multiple GroEL proteins of *Chlamydiae*. *Journal of Bacteriology* **185**: 1958−66.

Kelly MT (1982) Effect of temperature and salinity on *Vibrio* (*beneckea*) *vulnificus* occurrence in a Gulf coast environment. *Applied and Environmental Microbiology* **44**: 820−4.

Khan AA, McCarthy S, Wang RF and Cerniglia CE (2002) Characterization of United States outbreak isolates of *Vibrio parahaemolyticus* using enterobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3:K6 isolates. *FEMS Microbiology Letters* **206**: 209−14.

Kim DG, Kim YR, Kim EY, Cho HM, Ahn SH and Kong IS (2010) Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. *Fisheries Science* **76**: 803−10.

Klontz KC, Lieb S, Schreiber M, Janowski HT, Baldy LM and Gunn RA

(1988) Syndromes of *Vibrio vulnificus* infections. Clinical and epidemiological features in Florida cases, 1981−1987. *Annals of Internal Medicine* **109**: 318−23.

Kueh CS and Chan KY (1985) Bacteria in bivalve shellfish with special reference to the oyster. *Journal of Applied Bacteriology* **59**: 41−7.

Kwok AY, Su SC, Reynolds RP, Wilson JT, Coulthart M, Mutharia L and Chow AW (1999) Species identification and phylogenetic relationships based on partial *groEL* gene sequences within the genus *Staphylococcus*. *International Journal of Systematic Bacteriology* **49**: 1181−92.

Lalitha P, Siti Suraiya MN, Lim KL, Lee SY, Nur Haslindawaty AR, Chan YY, Ismail A, Zainuddin ZF and Ravichandran M (2008) Analysis of *lolB* gene sequence and its use in the development of a PCR assay for the detection of *Vibrio cholerae*. *Journal of Microbiological Methods* **75**: 142−4.

Lee JH, Park HS, Jang WJ, Koh SE, Kim JM, Shim SK, Park MY, Kim YW, Kim BJ, Kook YH, Park KH and Lee SH (2003) Differentiation of *Rickettsiae* by *groEL* gene analysis. *Journal of Clinical Microbiology* **41**: 2952−60.

Maheshwari M, Nelapati K and Kiranmayi B (2011) *Vibrio cholerae* − A review. *Veterinary World* **4**: 423−8.

Mahmud ZH, Neogi SB, Kassu A, Mai Huong BT, Jahid IK, Islam MS and Ota F (2008) Occurrence, seasonality and genetic diversity of *Vibrio vulnificus* in coastal seaweeds and water along the Kii Chanel, Japan.

FEMS Microbiology Ecology **64**: 209−18.

McLaughlin JC (1995) *Vibrio*. In: JoBaron E, Pfaller MA, Tenover FC, Yolken RH and Murray PR (eds) *Manual of clinical microbiology*. ASM Press, Washington, p. 465−76.

Mogk A, Huber D and Bukau B (2011) Integrating protein Homeostasis strategies in prokaryotes. *Cold Spring Harbor Perspectives in Biology* **3**: 10−19.

Moseley PL (1997) Heat shock proteins and heat adaption of the whole organism. *Journal of Applied Physiology* **83**: 1413−17.

Myatt DC and Davis GH (1989) Isolation of medically significant *Vibrio* species from riverine sources in South East Queensland. *Microbios* **60**: 111−23.

Neogi SB, Chowdhury N, Asakura M, Hinenoya A, Haldar S, Saidi SM, Kogure K, Lara RJ and Yamasaki S (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology* **51**: 293−300.

Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, Iihara H, Goto K, Masaki T, Miyasaka J and Ezaki T (2007) The *dnaJ* gene as a novel phylogenetic marker for identification of *Vibrio* species. *Systematic and Applied Microbiology* **30**: 309−15.

Nishibuchi M (2006) Molecular Identification. In: Thompson FL, Austin B and Swings J (eds) *The Biology of Vibrios*. American Public Health

Association, Washington, p. 44−64.

Nishibuchi M, Janda JM and Ezaki T (1996) The thermostable direct hemolysin gene (*tdh*) of *Vibrio hollisae* is dissimilar in prevalence to and phylogenetically distant from the *tdh* genes of other vibrios: implications in the horizontal transfer of the *tdh* gene. *Microbiology and Immunology* **40**: 59−65.

O'Neil KR, Jones SH and Grimes DJ (1992) Seasonal incidence of *Vibrio vulnificus* in the Great Bay estuary of New Hampshire and Maine. *Applied and Environmental Microbiology* **58**: 3257−62.

Oliver JD (2005) Wound infections caused by *Vibrio vulnificus* and other marine bacteria. *Epidemiology and Infection* **133**: 383−91.

Oliver JD (2006a) *Vibrio vulnificus*. In: Thompson FL, Austin B and Swing J (eds) *The biology of vibrios*. ASM Press, Washington, p. 349−66.

Oliver JD (2006b). *Vibrio vulnificus*. In: Belkin S and Colwell RR (eds) *Oceans and health: pathogens in the marine environment*. Springer Science, New York, p. 253−276.

Oliver JD and Kaper JB (2007) *Vibrio* species. In: Doyle MP and Beuchat LR (eds) *Food microbiology: fundamentals and frontiers*. ASM Press, Washington, p. 343−79.

Oliver JD, Warner RA and Cleland DR (1982) Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. *Applied and Environmental Microbiology* **44**: 1404−14.

Pan TM, Wang TK, Lee CL, Chien SW and Horng CB (1997) Food-borne disease outbreaks due to bacteria in Taiwan, 1986-1995. *Journal of Clinical Microbiology* **5**: 1260−2.

Park HS, Lee JH, Jeong EJ, Koh SE, Park TK, Jang WJ, Park KH, Kim BJ, Kook YH and Lee SH (2004) Evaluation of *groEL* gene analysis for identification of *Borrelia burgdorferi*. *Journal of Clinical Microbiology* **42**: 1270−3.

Pathak SP, Gautam AR, Ramteke PW and Bhattacherjee JW (1993) Incidence of pathogenic Vibrio cholerae non-O1 in sewage contaminated ground water. *Indian Journal of Microbiology* **33**: 235−9.

Pavia AT, Bryan JA, Maher KL, Hester Jr. TR and Farmer JJ (1989) *Vibrio carchariae* infection after ashark bite. *Annual International Medicine* **3**: 85−6.

Peeler JT, Houghtby GA and Rainosek AP (1992) The most probable number technique. In: Downes FP and Ito K (eds) *Compendium of methods for the microbiological examination of food*. American Public Health Association, Washington, p. 105−20.

Puente ME, Vega-Villasante F, Holguin G and Bashan Y (1992) Susceptibility of the brine shrimp Artemia and its pathogen *Vibrio parahaemolyticus* to chlorine dioxide in contaminated sea-water. *Journal of Applied Bacteriology* **73**: 465−71.

Rebecca S, Wong Y and Chow AW (2002) Identification of enteric pathogenes by heat shock protein 60 kDa (GroEL) gene sequences.

FEMS Microbiology Letters **206**: 107−11.

Rippey SR (1994) Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews* **7**: 419−25.

Robert J (2003) Evaluation of heat shock protein and immunity. *Development and Comparative Immunology* **27**: 449−64.

Sakazaki R, Tamura K, Prescott LM, Bencic Z, Sanyal SC and Sinha R (1971) Bacteriological examination of diarrhoeal stools in Culcutta. *Indian Journal Medical Research* **59**: 1025−34.

Satcher D (1995) Emerging infections: getting ahead of the cure. *Emerging Infectious Diseases Journal* **1**: 1−6.

Satheesh N, Thong KL, Tikki P and Martin A (2002) Characterization of *Salmonella s*erovars by PCR single-strand conformation polymorphism analysis. *Journal of Clinical Microbiology* **40**: 2346−51.

Sechi LA, Dupre I, Deriu A, Fadda G and Zanetti S (2000) Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. *Journal of Applied Microbiology* **88**: 475−81.

Segal BH, Wang X, Dennis CG, Youn R, Repsky EA, Manjili MH and Subjeck JR (2006) Heat shock proteins as adjuvants in infections and cancer. *Drug Discovery Today* **11**: 534−40.

Strom MS and Paranjpye RN (2000) Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and Infection* **2**: 177−88.

Sugita H, Tanaami H, Kobashi T and Deguchi Y (1981) Bacterial flora of coastal bivalves. *Bulletin of Japanese Society for Scientific Fisheries* **47**:

655−61.

Takeda Y (1983) Thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Pharmacology and Therapeutics* **19**: 123−46.

Tamplin M, Rodrick GE, Blake NJ and Cuba T (1982) Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. *Applied and Environmental Microbiology* **44**: 1466−70.

Tamplin ML and Capers GM (1992) Persistence of *Vibrio vulnificus* in tissues of gulf coast oysters, *Crassostrea virginica*, exposed to seawater disinfected with UV light. *Applied and Environmental Microbiology* **58**: 1506−10.

Tauxe RV and Blake PA (1992) Epidemic cholera in Latin America. *The Journal of the American Medical Association* **267**: 1388−90.

Teh CSJ, Chua KH and Thong KL (2010) Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using multiplex PCR based on *gyrB* and *pntA* genes. *Journal of Applied Microbiology* **108**: 1940−5.

Terai A, Baba K, Shirai H, Yoshida O, Takeda Y and Nishibuchi M (1991) Evidence for insertion sequence-mediated spread of the thermostable direct hemolysin gene among *Vibrio* species. *Journal of Bacteriology* **173**: 5036−46.

Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB and Swings J (2005) Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Applied and*

Environmental Microbiology **71**: 5107−15.

Tomanek L (2002) The Heat-Shock Response: Its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). *Integrative and Comparative Biology* **42**: 797−807.

Tunung R, Margaret SP, Jeyaletchumi P, Chai LC, Zainazor TC, Ghazali FM, Nakaghuchi Y, Nishibuchi M and Son R (2010) Prevalence and quantification of *Vibrio* in raw salad vegetables at retail level. *Journal of Microbiology and Biotechnology* **20**: 391–6.

Waldor MK and Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910−4.

WHO (2008). Cholera. Fact sheets no. 107, Geneva.

Yasuda K and Kitao T (1980) Bacterial flora in the digestive tract of prawn, *Penaeus japonicus* Bate. *Aquaculture* **19**: 229−34.

Yushan H, Lei L, Weijia L and Xiaoguang C (2010) Sequence analysis of the *groEL* gene and its potential application in identification of pathogenic bacteria. *African Journal of Microbiology Research* **4**: 1733−41.

Zhang DL, Rajanna C, Sun W and Karaolis DK (2003) Analysis of the *Vibrio* pathogenicity island-encoded Mop protein suggests a pleiotropic role in the virulence of epidemic *Vibrio cholerae*. *FEMS Microbiology Letters* **225**: 311−8.

CHAPTER 2

Application of *groEL* gene for the species-specific detection of *Vibrio parahaemolyticus* by PCR

(Published in Letters in Applied Microbiology)

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CHAPTER 2

Application of *groEL* gene for the species-specific detection of *Vibrio parahaemolyticus* by PCR

Abstract

Vibrio parahaemolyticus is a significant cause of human gastrointestinal disorders and is transmitted through ingestion of raw or undercooked contaminated seafood. We used the *groEL* gene for the species-specific detection of *V. parahaemolyticus* from artificially inoculated shellfish, fish, and seawater. The nucleotide sequences of 24 *Vibrio* and 7 non-*Vibrio* species were compared, and less conserved regions were selected for the designing of primer sets. To detect *V. parahaemolyticus* specifically, PCR conditions were standardized and tested to evaluate the specificity of primers. A 510 bp band was appeared only from *V. parahaemolyticus* by PCR. Notably, the detection was shown to be functional at high annealing temp above 68°C. The *groEL* primers detected 100 pg and 1 ng of DNA purified from *V. parahaemolyticus* culture and artificially infected oyster tissue, respectively. The *groEL* gene is a potential marker for the species-specific detection of *V. parahaemolyticus* and could be used to detect this bacterium in contaminated food by PCR. PCR using primers designed from *groEL* gene provide an efficient method for the accurate identification of *V. parahaemolyticus* from contaminated samples.

Keywords: *groEL* gene, PCR, shellfish, species-specific, *V. parahaemolyticus*

2.1 Introduction

V. parahaemolyticus, which is widely distributed in the marine environments, is a halophilic organism that has received extensive attention due it being a major seafood-borne pathogen throughout the world. The vast majority of environmental *V. parahaemolyticus* isolates are nonvirulent, but this bacterium is a leading cause of gastroenteritis related to raw or undercooked seafood consumption in the United States and Asia (Iwamoto *et al*. 2010; Noorlis *et al*. 2011).

Accurate identification of *V. parahaemolyticus* in samples from outbreak areas and the environment is very important in the context of public health. Different molecular technologies are replacing culture-based approaches. To enhance sensitivity, the use of unique oligonucleotide primers based on target DNA sequences also results in absolute specificity (Venkateswaran *et al*. 1998). Specific or universal genes, including toxin genes and 16s rRNA genes, have been used in PCR assays as target markers for the detection of different strains of *V. parahaemolyticus*. Unfortunately, there is often very high nucleotide similarity among these genes in bacterial species, especially those within the same genus, and the absence of toxin genes in nonvirulent strains has prevented these toxin genes from being useful targets for species-specific identification of bacterial pathogens (Chizhikov *et al*. 2001). Therefore, accurate identification of *V. parahaemolyticus* requires new and more specific targets to reduce the risk of both false-positive and false-negative results in PCR assays.

The *groEL* gene, which encodes a 60 kDa subunit (known as HSP60, 60 kDa chaperonin and heat shock protein), is one of the most conserved genes in nature (Giuseppe *et al*. 2008). Nishibuchi (2006) suggested that the *hsp60* gene sequence might be able to use for the identification of *Vibrio* species. Yushan *et al*. (2010) reported that the *groEL* gene is more heterogeneous than 16S rRNA and 23S rRNA genes, and might be applicable in interspecies differentiation. It has already been used as a target gene in the typing and identification of *Salmonella* species, *Staphylococcus* species, *Campylobacter jejuni*, and *Vibrio anguillarum* on account of its complete database (Kim *et al*. 2010; Yushan *et al*. 2010). In this study, we selected the *groEL* gene as a target gene to design suitable primers for PCR and investigated its significance in the specific and accurate identification of *V. parahaemolyticus* irrespective of phenotype, serotype, and virulence status. We also used these primers for the detection of *V. parahaemolyticus* from artificially inoculated shellfish, flounder and seawater.

2.2 Materials and Methods

2.2.1 Bacterial strains and media

A total of 70 strains of *V. parahaemolyticus*, 40 strains of other *Vibrio* species and ten strains of non-*Vibrio* enteric species used in this study are listed in Table 2.1. All the *Vibrio* and non-*Vibrio* species were cultured and maintained on Brain Heart Infusion agar (BD, Franklin Lakes, NJ).

2.2.2 Oligonucleotide primers

Potential oligonucleotide primers gro-vp1: 5′-AGGTCAGGCTAAGCGCGTAA GC-3′ and gro-vp2: 5′-GTCACCGTATTCACCCGTCGCT-3′ were designed for the specific amplification of a 510 bp amplified segment of the *groEL* gene of *V. parahaemolyticus* (Fig. 2.1) and analyzed for specificity by comparison with known gene sequences of other *Vibrio* and non-*Vibrio* species available in GenBank using BLASTN search program provided by the National Center for Biotechnology Information. The primer sets were commercially synthesized by Cosmo Genetech, Seoul, Korea.

2.2.3 PCR assays

PCR assays were performed with 16S rRNA primers (27F and 1492R) using a thermal cycler (2720 Thermal cycler; Applied Biosystems, California, USA) according to Kim *et al*. (2008). Two sets of reaction parameters were applied during PCR using *groEL* primers. The first set comprised initial denaturation at 94°C for 5 min; 30 cycles of amplification (denaturation at 94°C for 30 s, primer annealing at 69°C for 30 s, primer extension at 72°C

for 30 s) and final extension of the incompletely synthesized DNA at 72°C for 7 min. The second set comprised initial denaturation at 94°C for 5 min; 30 cycles of amplification, each consisting of 30 s at 94°C and 30 s at 72°C (for primer annealing); and final extension of the incompletely synthesized DNA at 72°C for 7 min. Amplified products (3 µl) were analyzed by 1.2% agarose gel electrophoresis.

2.2.4 Specificity of detection

The species specificity of the *groEL* primers were evaluated by amplifying purified genomic DNA from all of the strains listed in Table 2.1. Genomic DNA was purified from pure cultures of all *Vibrio* and non-*Vibrio* strains by phenol-chloroform extraction and ethanol precipitation method described by Ausubel *et al*. (1998). Briefly, 1 ml overnight culture of each species was taken in eppendorf tube and centrifuged at 12000 rpm for 2.5 min. Supernatant was discarded and pellet was resuspended with 600 µl distilled water (DW). 17.5 µl of 20% SDS and 4 µl of protease K (20 mg/ml) was added and then the mixture was incubated at 37°C for 1 h. After incubation, 100 µl of 5M sodium chloride solution was added and mixed by inversion. Then, 350 µl phenol and 350 µl chloroform was added, mixed by vortexing and centrifuged at 12000 rpm for 10 min. The supernatant was collected, equal amount of phenol and chloroform was added and centrifuged. Similar procedure was repeated at least three times and then finally supernatant was collected. The supernatant was washed two times using 100% ethanol and centrifuged at 12000 rpm for 10 min maintaining

4°C. The supernatant was discarded, 70% ethanol was added and centrifuged at 12000 rpm for 10 min maintaining 4°C. Finally, supernatant was discarded, vacuum dried for 15 min and stored at –20°C until use.

2.2.5 Detection of V. parahaemolyticus in artificially infected shellfish and flounder

Four species of shellfish (*Crassostrea gigas*, *Saxidomus purpuratus*, *Mytilus coruscus* and *Scapharca subcrenta*) purchased from the local markets were homogenized separately with sterilized artificial seawater and aliquoted to 15 ml. The homogenates of each species were divided into three groups. Homogenates of the first group were inoculated with *V. parahaemolyticus* KCCM 41664 (1.5×10^5 CFU/ml) in 100 µl 1 × PBS, and those of the second group were inoculated with *V. parahaemolyticus* KCCM 41664 (1.5×10^5 CFU/ml) in addition with *V.cholerae* and *V. vulnificus*. Homogenates of the third group were kept as an uninoculated negative control. After incubation at 37 for 5 h and 18 h, total DNA was extracted from all tissues using a commercial DNA extraction kit (Qiagen, Hilden, Germany), and PCR was then performed.

Nine apparently healthy flounders (*Paralichthys olivaceus*) were reared in aerated plastic containers (15 l). Two groups were intraperitoneally injected with same amount of *Vibrio* species used to infect shellfish tissues, and a third group was used as an uninoculated control. The internal organs (gill, liver, intestine and kidney) from inoculated and uninoculated fishes were collected 48 h postinfection. Total DNA was extracted from the fish tissues

using a DNA extraction kit (Qiagen), and PCR amplification was performed maintaining optimized concentrations of reagents and temperature cycling parameters.

2.2.6 Detection of V. parahaemolyticus in seawater

To examine whether *V. parahaemolyticus* in seawater can be detected by PCR with *groEL* primers, seawater sample artificially inoculated with *V. parahaemolyticus* and seawater sample from containers of infected flounders were prepared, respectively. About 300 ml of natural sterilized seawater was inoculated with *V. parahaemolyticus* KCCM 41664 (1.5×10^5 CFU/ml) and incubated at 37°C for 24 h. Another seawater sample was collected from the containers rearing two groups of infected flounder after 48 h. DNA was extracted from seawater using the same procedure described by Cilliers *et al*. (2000). Briefly, inoculated seawater was collected and filtered using 0.2 μm membrane filter (Advantec, Japan). The filter paper was placed in a plastic conical tube and 5 ml DW was added. Contents of filter paper was removed in DW by vortexing and then centrifuged at 12000 rpm for 5 min. Supernatent was discarded and pellet was resuspended with 100 μl of DW and boiling at 100°C for 15 min. The suspension was cooled in ice immediate after boiling and centrifuged at 13000 rpm for 5 min. The supernatant was collected and stored at –20°C until use. Detection of *V. parahaemolyticus* in seawater was performed by PCR using the extracted DNA and *groEL* primers.

2.2.7 Sensitivity of detection

To check the sensitivity of detection, 1 μg of chromosomal DNA purified from *V. parahaemolyticus* and 1 μg of extracted total DNA from artificially infected oyster tissue were tenfold serially diluted in distilled water (dilution range 1 μg–0.1 pg) and subjected of each dilution to PCR amplification.

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Table 2.1 Strains used in this study

	Microorganisms	Source or reference	PCR reaction[a]
1	*V. aestuarianus*	KCCM 40863	-
2	*V. alginolyticus*	KCTC 2472, E	-
3	*V. anguillarum*	KCTC 2711, J-O-2, J-O-3, YT, NB10, E	-
4	*V. campbellii*	KCCM 41986	-
5	*V. cholerae*	KCCM 41626	-
6	*V. cincinnatiensis*	KCTC 2733	-
7	*V. damsella*	E	-
8	*V. diazotrophicus*	KCCM 41606	-
9	*V. fluvialis*	ATCC 33809	-
10	*V. furnissii*	KCTC 2731, E	-
11	*V. harveyi*	KCCM 40866	-
12	*V. hollisae*	KCCM 41680	-
13	*V. logei*	KCTC 2721	-
14	*V. mediterranei*	KCCM 40867	-
15	*V. metschnikovii*	KCTC 2736	-
16	*V. mimicus*	ATCC 33653	-
17	*V. natriegens*	KCCM 40868	-
18	*V. navarrensis*	KCCM 41682	-
19	*V. nereis*	KCCM 41667	-
20	*V. ordalii*	KCCM 41669	-
21	*V. parahaemolyticus*	KCCM 41664, KCCM 11965, KCTC 2471, 37 E strains, 30 C strains	+ +
22	*V proteolyticus*	KCTC 2730	-
23	*V. tubiashii*	KCTC 2728	-
24	*V. vulnificus*	KCCM 41665, KCTC 2962, KCTC 2980, KCTC 2981, KCTC 2982, KCTC 2983, KCTC 2985, KCTC 2986, KCTC 2987, 2 E	- - -
25	*Aeromonas hydrophila*	KCTC 2358	-
26	*Escherichia coli* BL21	L	-
27	*E. coli* 0157	E	-
28	*Edwardsiella tarda*	E	-
29	*Enterobacter cloacae*	E	-
30	*Klebsiella oxyta*	E	-
31	*K. pneumoniae*	E	-
32	*Salmonella typhi*	E	-
33	*Shigella flexneri*	E	-
34	*Shigella sonnei*	E	-

L, laboratory collection; E, environmental source; C, Clinical source; ATCC: American Type Culture Collection, USA; KCCM: Korean Culture Center of Microorganisms, Korea; KCTC: Korean Collection for Type Cultures, Korea; a +, only one amplification product of 510 bp; -, no amplification products.

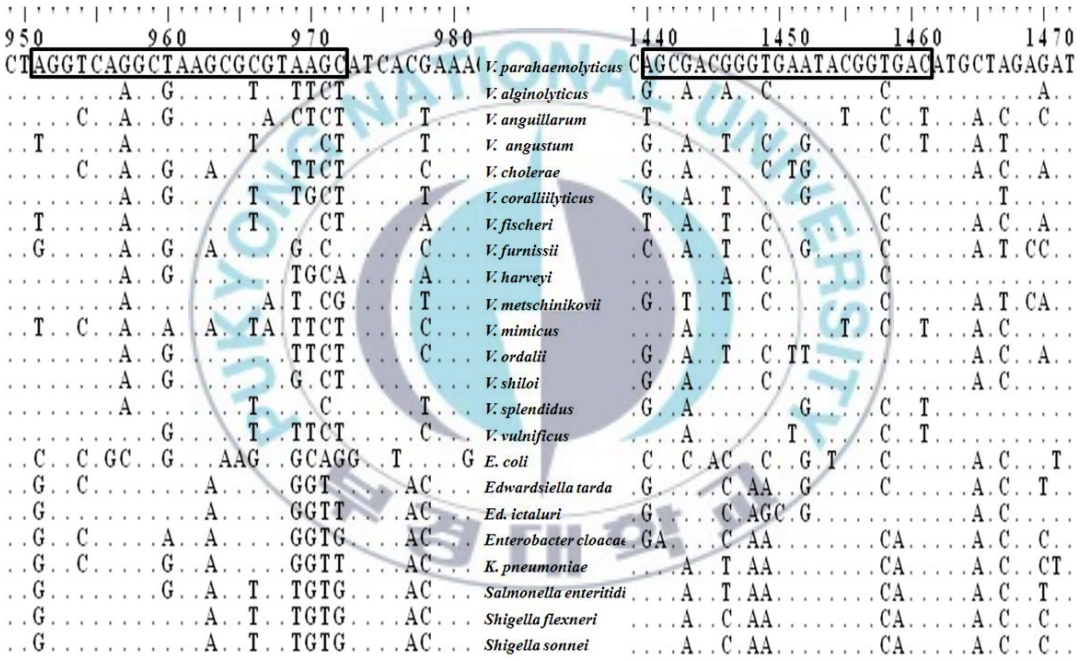

Figure 2.1 Location of primer sequences in *groEL* gene. Primer regions are indicated by boxes: left box for forward and right box for reverse primer. Nucleotides sequence identical to those of the *V. parahaemolyticus* are indicated by dots.

2.3 Results

2.3.1 Specificity of the groEL primers

A band of 1466 bp was appeared from all 24 *Vibrio* and seven non-*Vibrio* species after PCR using 16S rRNA primers (Fig. 2.2). Amplification of *V. parahaemolyticus* DNA by PCR with *groEL* primers yielded a 510 bp fragment at both 69°C (Fig. 2.3a) and 72°C (Fig. 2.3b), whereas no products were obtained from the 50 non-*V. parahaemolyticus* bacterial strains (Table 2.1). Similar patterns were observed with DNAs extracted from all 70 strains of *V. parahaemolyticus* preserved in our laboratory (data not shown).

2.3.2 Detection of V. parahaemolyticus in artificially infected shellfish, flounder and seawater

We were able to accurately and specifically detect *V. parahaemolyticus* in infected tissues from all shellfish (both infected groups). In all cases, a 510 bp amplification product was produced at both annealing temperatures 69°C (Fig. 2.4) and 72°C (results not presented). Tissue homogenates of shellfish not inoculated with *V. parahaemolyticus* showed no amplification of the target gene segments (Fig. 2.4). The *groEL* primers were also able to detect *V. parahaemolyticus* from all samples obtained from the injected flounders (Table 2.2) and from all of the seawater samples in PCR reactions with annealing temperatures of 69 and 72°C (Fig. 2.5, Table 2.2).

2.3.3 Sensitivity of the groEL primers

The sensitivity of the PCR assay for detecting *V. parahaemolyticus* is shown in Figure 2.6. The limit for detecting the *groEL* target in purified DNA was 100 pg. With this amount of template, 510 bp bands were produced with annealing temperatures of 69 and 72°C. The *groEL* primers were able to detect as little as 1 ng total DNA purified from infected oyster tissue.

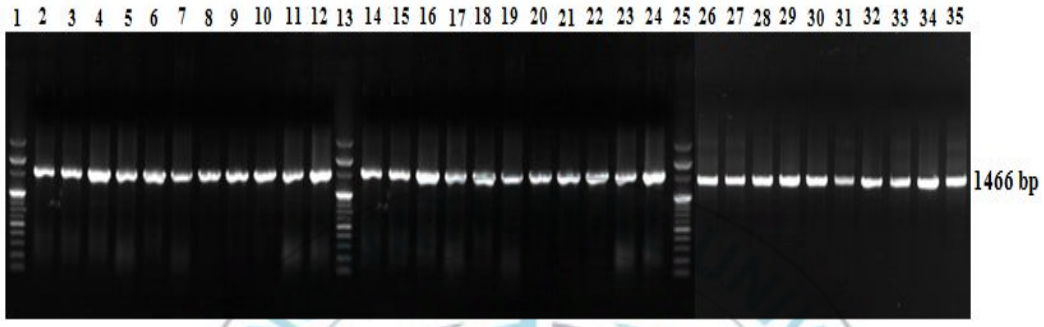

Figure 2.2 Agarose (1.2%) gel electrophoresis of DNA products amplified from *Vibrio* and non-*Vibrio* species by PCR using the universal 16S rRNA primer set. Lanes 1, 13 and 25 100 bp DNA ladder; lanes 2-12, 14-24 and 26-35: *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. cincinnatiensis*, *V. damsella*, *V. diazotrophicus*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. logei*, *V. mediterranei*, *V. metschinikovii*, *V. mimicus*, *V. natriegens*, *V. navarrensis*, *V. nereis*, *V. ordalii*, *V. parahaemolyticus*, *V. proteolyticus*, *V. vulnificus*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *E. ictaluri, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca*, *K. pneumoniae, Salmonella typhi*, *Shigella flexneri,* and *S. sonnei*, respectively.

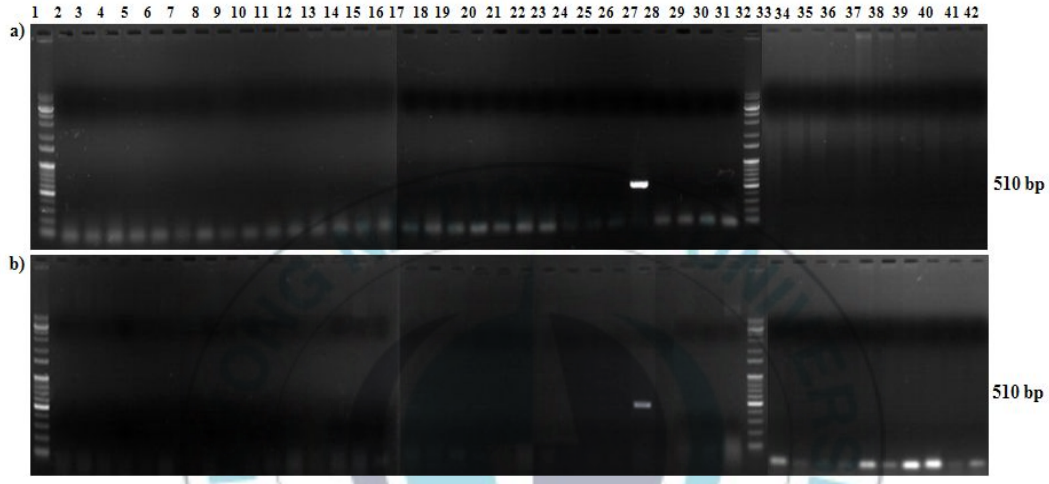

Figure 2.3 Agarose (1.2%) gel electrophoresis of DNA products amplified from *Vibrio* and non-*Vibrio* species by PCR using (a) *groEL* primers and annealing temp 69°C, (c) *groEL* primers and annealing temp 72°C. Lanes 1 and 32, 1 kb DNA ladder; lanes 2–31, *Vibrio* species and lanes 33–42, non-*Vibrio* species; lane 27, *V. parahaemolyticus*.

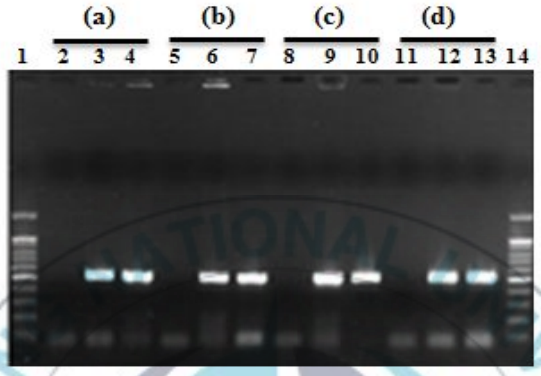

Figure 2.4 Detection of *V. parahaemolyticus* in artificially infected shellfish homogenates. (a) Oyster (*Crassostrea gigas*), (b) Purple washington clam (*Saxidomus purpuratus*), (c) Hard shelled mussel (*Mytilus coruscus*), (d) Half crenate ark (*Scapharca subcrenata*). Lanes 1 and 14, 100 bp DNA ladder; lanes 2, 5, 8 and 11, uninoculated shellfishes; lanes 3, 6, 9 and 12, incubated for 5 h after inoculation; lanes 4, 7, 10 and 13, incubated for 18 h after inoculation.

Figure 2.5 Detection of *V. parahaemolyticus* directly from inoculated artificial seawater and seawater containing infected fish. Annealing temp 69°C (left side of marker) and 72°C (right side of marker); lane 5, 100 bp DNA ladder; lanes 1 and 5, Uninoculated seawater; lanes 2 and 6, seawater inoculated with *V. parahaemolyticus*; lanes 3 and 8 seawater containing infected fish injected with *V. parahaemolyticus*; lanes 4 and 9 seawater containing infected fish injected with *V. cholera*, *V. parahaemolyticus* and *V. vulnificus*.

Table 2.2 Artificially inoculated samples: PCR results obtained with *groEL* target primers for the detection of *V. parahaemolyticus*

Source of sample	PCR analysis
Shellfish homogenates	
Crassostrea gigas	++
Saxidomus purpuratus	++
Mytilus coruscus	++
Scapharca subcrenata	++
Water	
Sterilized seawater after inoculation with *V. parahaemolyticus*	++
Seawater containing infected fish	++
Fish (Flounder)	
Gill	+
Liver	+
Intestine	+
Kidney	+

++, Strong band; +, Light band

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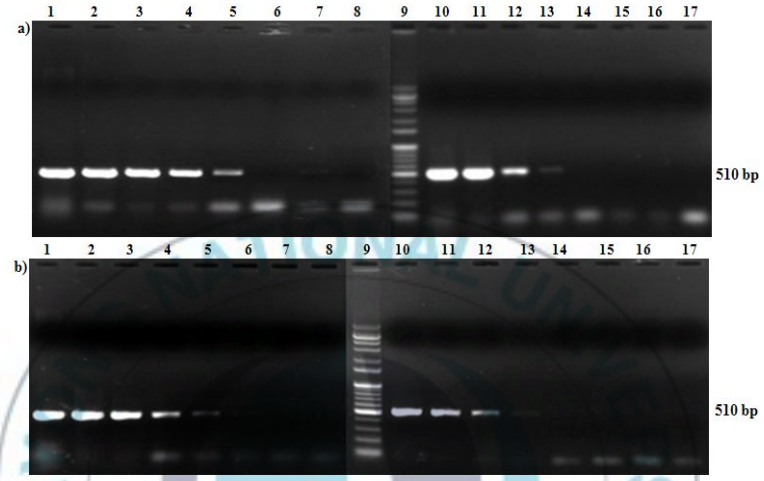

Figure 2.6 Agarose (1.2%) gel electrophoresis showing sensitivity of detection of the PCR-amplified DNA from various concentrations of DNA of *V. parahaemolyticus* purified from cultured cells and infected oyster tissues: (a) annealing temp 69°C; (b) annealing temp 72°C; lane 9, 1 kb DNA ladder, lanes 1-8, chromosomal DNA: 1, 1 µg ; 2, 100 ng; 3, 10 ng; 4, 1 ng; 5, 100 pg ; 6, 10 pg; 7, 1 pg; 8, 0.1 pg; lane 10–17, infected oyster DNA: 1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg.

2.4 Discussion

Analyses of 16S rRNA sequences have already proven inadequate for the differentiation of *V. parahaemolyticus* from closely related species (Ruimy *et al*. 1994). There are still false-positive and false-negative results in PCR assays targeting *tlh*, *toxR* and pR72H fragments for the identification of *V. parahaemolyticus* (Croci *et al*. 2007). Recently, Shuijing *et al*. (2010) used the *irgB* gene as a species-specific marker for the identification of *V. parahaemolyticus*. However, the authors only used ten *Vibrio* species to compare the nucleotide sequences and to check the specificity of this gene.

Recently, we compared the nucleotide sequences of the *groEL* genes of ten *Vibrio* species and found the identity to be between 80 and 90% (Kim *et al*. 2010). We also analyzed the *groEL* sequences of those species in addition with 14 more *Vibrio* and seven non-*Vibrio* species, and found the similarity to be 81–92% and 65–78% in *Vibrio* and non-*Vibrio* spp., respectively (data not shown). We found that the nucleotide number 951–972 and 1438–1460 in *groEL* gene of *V. parahaemolyticus* showed the highest variation compared with other *Vibrio* and non-*Vibrio* species. During optimization of PCR annealing temperature, *V. parahaemolyticus* yielded a clear and specific band at temperatures of 69°C and also at 72°C. Hocker and Roux (1996) reported that both specificity and yield in PCR increase at high annealing temperatures. In our study, the result that high annealing temperature was required for the production of specific band may be due to the similarity in *groEL* nucleotide sequences among *Vibrio* species. In our

previous study, the high annealing temperature (67°C) was also used in the PCR detection of *V. anguillarum* using the *groEL* gene (Kim *et al*. 2010). The use of high annealing temperature reduced PCR time by at least 1 h resulting in the rapid detection of *V. parahaemolyticus*.

The *groEL* primers successfully detected *V. parahaemolyticus* in all of the artificially inoculated shellfish samples, indicating that the *groEL* primers are highly specific for this bacterium. We also detected *V. parahaemolyticus* in different flounder tissues after artificial infection, but the band was weaker. This may be due to any inhibitory materials in the fish tissues. The detection limits for the primers were found to be 100 pg and 1 ng for DNA purified from bacteria and infected oyster tissue, respectively. Kim *et al*. (2010) also tested the sensitivity of *groEL* primers to detect *V. anguillarum* and found that they were able to detect as little as 1 and 10 ng purified DNA from bacteria and infected oyster tissue, respectively. However, in the present study, we found that the *groEL* gene primers were more sensitive in the detection of *V. parahaemolyticus*.

In conclusion, the PCR primers targeted to the *groEL* gene are highly specific for *V. parahaemolyticus* and were able to accurately detect all tested strains of this species. Further studies using different food samples, such as retail shellfish, aquaculture fish and water, are necessary to verify the efficacy of the PCR assay we have developed.

2.5 References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1998) *Current Protocols in Molecular Biology*. Wiley and Sons, New York.

Chizhikov V, Rasooly A, Chumakov K and Levy DD (2001) Microarray analysis microbial virulence factors. *Applied and Environmental Microbiology* **67**: 3258–63.

Cilliers M, Du Preez M, Venter SN, Brozel VS and Theron J. 2000. Detection of small numbers of toxigenic *Vibrio cholerae* from environmental water samples by a novel polymerase chain reaction procedure. *The WISA 2000 Biennial Conference*, Sun City, South Africa p. 1-7.

Croci L, Suffredini E, Cozzi L, Toti L, Ottaviani D, Pruzzo C, Serratore P, Fischetti R, Goffredo E, Loffredo G and Miono R (2007) Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology* **102**: 229–37.

Giuseppe B, Vincenzina F, Danilo E, Maria A, Olimpia P and Francesco V (2008) *Lactobacillus* strain diversity based on partial *hsp60* gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. *Applied and Environmental Microbiology* **74**: 208–15.

Hecker KH and Roux KH (1996) High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR.

Biotechniques **20**: 468–75.

Iwamoto M, Ayers T, Mahon BE and Swerdlow DL (2010) Epidemiology of seafood associated infections in the United States. *Clinical Microbiology Reviews* **23**: 399–411.

Kim DG, Ahn SH, Kim LH, Park KJ, Hong YK and Kong IS (2008) Application of the *rpoS* gene for species-specific detection of *Vibrio vulnificus* by real-time PCR. *Journal of Microbiology and Biotechnology* **18**: 1841–7.

Kim DG, Kim YR, Kim EY, Cho HM, Ahn SH and Kong IS (2010) Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. *Fisheries Science* **76**: 803–10.

Nishibuchi M (2006) The Biology of Vibrios. In: hompson FL, Austin B and Swings J (eds) *Molecular Identification*. American Society for Microbiology, Washington, p. 44–64.

Noorlis A, Ghazali FM, Cheah YK, Tuan Zainazor TC, Ponniah J, Tunung R, Tang JYH, Nishibuchi M, Nakaguchi Y and Son R (2011) Prevalence and quantification of *Vibrio* spp. and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *International Food Research Journal* **18**: 673–9.

Ruimy R, Breittmayer V, Elbaze P, Lafay B, Boussemart O, Gauthier M and Christen R (1994) Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from smallsubunit rRNA sequences. *International Journal of Systematic*

Bacteriology **44**: 416–26.

Shuijing Y, Wanyi C, Dapeng W, Xiaohua H, Xinna Z and Xianming S (2010) Species-specific PCR detection of the food-borne pathogen *Vibrio parahaemolyticus* using the *irgB* gene identified by comparative genomic analysis. *FEMS Microbiology Letters* **307**: 65–71.

Venkateswaran K, Dohmoto N and Harayama S (1998) Cloning and nucleotide sequence of the gyrB gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. *Applied and Environmental Microbiology* **64**: 681–7.

Yushan H, Lei L, Weijia L and Xiaoguang C (2010) Sequence analysis of *groEL* gene and its potential application in identification of pathogenic bacteria. *African Journal of Microbiology Research* **4**: 1733–41.

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CHAPTER 3

Multiplex PCR for the detection and differentiation of *Vibrio parahaemolyticus* strains using the *groEL*, *tdh* and *trh* genes

(Published in Molecular and Cellular Probes)

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CHAPTER 3

Multiplex PCR for the detection and differentiation of V*ibrio parahaemolyticus* strains using *groEL*, *tdh* and *trh* genes

Abstract

Vibrio parahaemolyticus is a significant cause of human gastrointestinal disorders worldwide, transmitted primarily by ingestion of raw or undercooked contaminated seafood. In this study, a multiplex PCR assay for detection and differentiation of *V. parahaemolyticus* strains was developed using primer sets for a species-specific marker, *groEL,* and two virulence markers, *tdh* and *trh*. Multiplex PCR conditions were standardised, and extracted genomic DNA of 70 *V. parahaemolyticus* strains were used for identification. The sensitivity and efficacy of this method was validated using artificially inoculated shellfish and seawater. The expected sizes of amplicons were 510 bp, 382 bp, and 171 bp for *groEL*, *tdh,* and *trh*, respectively. PCR products were sufficiently different in size, and the detection limits of the multiplex PCR for *groEL*, *tdh* and *trh* were each 200-pg DNA. Specific detection and differentiation of virulent from non-virulent strains in shellfish homogenates and seawater was also possible after artificial inoculation with various *V. parahaemolyticus* strains. This newly developed multiplex PCR is a rapid assay for detection and differentiation of pathogenic *V. parahaemolyticus* strains, and could be used to prevent disease outbreaks and protect public health by helping the seafood industry maintain a safe shellfish supply.

Keywords: Detection and differentiation, *groEL,* multiplex PCR, shellfish, *tdh*, *trh*, *Vibrio parahaemolyticus*

3.1. Introduction

Among the medically important *Vibrio* species, *Vibrio parahaemolyticus* is recognised as the leading cause of human gastroenteritis associated with seafood consumption (Oliver 2006; Nhung *et al*. 2007), and is an important seafood-borne pathogen globally (Kaysner and DePaola 2001). It is a Gram-negative, facultative halophilic bacterium common in aquatic environments worldwide, including estuaries and seawater, and is frequently isolated from zooplankton, coastal fish, and shellfish (Robert-Pillot *et al*. 2004).

Vibrio infections are more frequently encountered in coastal countries due to the greater consumption of raw or undercooked seafood; shellfish and other bivalves are of particular concern, as bacteria are known to accumulate in these species during filter feeding. Bacterial loads in seafood display seasonal variation, typically peaking during the summer months. These elevated bacterial loads increase the likelihood of outbreaks of food-borne illness, and are a significant cause for concern within the seafood industry (Panicker *et al*. 2004). Although the gastroenteritis caused by *V. parahaemolyticus* is self-limiting, the infection is capable of causing life-threatening septicaemia in people with underlying conditions, such as liver disease or immune disorders (Su and Liu 2007). Risk assessment for *V. parahaemolyticus* in seafood is an increasingly important issue in countries with high-level seafood consumption, including Korea, Japan, Taiwan, and China. Therefore specific, sensitive, and rapid detection of this bacterium is important for public health (No *et al*. 2011).

Detection of virulent strains in clinical and food samples using traditional culture methods remains difficult. Virulent strains exhibit no obvious growth phenotypes to differentiate them from non-virulent strains, and their populations are generally very small relative to other bacteria (Takahashi *et al*. 2005). At the same time, classical methods based on biochemical and microbiological tests are time-consuming, labour-intensive, unreliable, and involve evaluation of large numbers of samples (Peeler *et al*. 1992). Alternatively, polymerase chain reaction (PCR) assays are becoming increasingly popular for microbial testing due to their relative ease of use, efficiency, and low cost, and are highly specific for the detection of small numbers of pathogens (Hossain *et al*. 2012). Initially, PCR using primers targeting the *tdh* or *trh* genes, which encode the thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), respectively, were developed for detection of virulent *V. parahaemolyticus* strains (Honda and Iida 1993; Kim *et al*. 1999; Alam *et al*. 2002). However, this assay cannot be used to detect *V. parahaemolyticus* strains that lack either of the two genes. Sometimes non-pathogenic strains might be an important reservoir of virulence genes. The *tdh* gene of *V. parahaemolyticus* may be horizontally transferred, leading to the development of new pathogenic strains (Nishibuchi *et al*. 1996; Xie *et al*. 2005). For this reason, screening of total environmental population of this species is also important. Specific marker genes such as 16S rRNA, *tlh*, *gyrB*, *toxR*, pR72H fragment and *irgB* have been used to positively identify *V. parahaemolyticus* by PCR (Lee *et al*. 1995; Venkateswaran *et al*. 1998; Bej *et al*. 1999; Kim *et al*. 1999; Yu *et*

al. 2010), but provide no information regarding pathogenic potential. Thus many researchers have developed multiplex PCR assays for the specific detection of virulent and non-virulent *V. parahaemolyticus* strains using both species-specific gene and toxin genes (Rizvi and Bej 2010; Yu *et al*. 2010; No *et al*. 2011). However, the sensitivity and specificity of these assays remain an issue, as false-positive and -negative results of *V. parahaemolyticus* PCR assays occurred (Croci *et al*. 2007).

We have selected the *groEL* gene which is well established as a powerful phylogenetic marker (Junick and Blaut 2012) for the detection of many enteric bacteria, including *Vibrio* species (Kim *et al*. 2010; Yushan *et al*. 2010; Hossain *et al*. 2013a & b). This gene product is known to be a heat shock protein in bacteria and its expression increases during stress conditions (environmental or inside host body) (Chowdhury *et al*. 1996; Mukhopadhyay *et al*. 2006). The superiority of *groEL* compared to 16S rRNA and 23S rRNA for detection of *Vibrio* species has also been reported (Nishibuchi 2006; Hossain *et al*. 2013b). The purpose of the present study was not only to detect pathogenic strains but also to detect the total population (both pathogenic and non-pathogenic strains) of *V. parahaemolyticus*. Considering this, a multiplex PCR assay was developed targeting the species-specific marker *groEL*, and the toxin genes *tdh* and *trh*. This assay is capable of simultaneous detection of both virulent and non-virulent *V. parahaemolyticus* strains, which may reduce the incidence of associated illness in humans.

3.2. Materials and Methods

3.2.1 Bacterial strains and microbiological media

A total of 70 *V. parahaemolyticus* strains were analysed in this study, including the laboratory strains KCCM 41664 (*tdh*$^-$ and *trh*$^-$), KCCM 11965 (*trh*$^+$), and KCTC 2471 (*tdh*$^+$), 30 clinical strains, and 37 environmental strains. Other *Vibrio* strains used are listed in Table 3.1. All strains were cultured in brain–heart infusion (BHI; BD, Franklin Lakes, NJ, USA) broth with 0.5–3% NaCl. BHI agar plates containing 3% NaCl were used to count *V. parahaemolyticus* for infection experiment.

3.2.2 DNA extraction

Genomic DNA of all *Vibrio* strains was extracted by phenol–chloroform precipitation, and identified by 16S rRNA sequencing (Hossain *et al*. 2012). Template DNA was also extracted from all *V. parahaemolyticus* strains using a simple boiling method, as described by Hossain *et al*. (2013b).

3.2.3 Oligonucleotide primers

Nucleotide sequences, locations, melting temperatures (Tm) of oligonucleotide primers specific for *groEL*, *tdh* and *trh*, and size of amplicons following PCR amplification are described in Table 3.2. A set of *groEL* primers validated previously for detection of *V. parahaemolyticus* were also used (Hossain *et al*. 2012). Primers for the detection of *tdh* were designed using regions conserved in both *tdh*1 (Accession no. BA000032) and *tdh*2 (Accession no. JQ029159), allowing for amplification of either gene.

Similarly, fragments located in well-conserved regions of *trh*1 (Accession no. DQ359749) and *trh*2 (Accession no. AB112354) were selected to design primers for *trh* genes. All oligonucleotide primers were synthesised commercially by Cosmo Genetech, Seoul, Korea.

3.2.4 Optimisation of the multiplex PCR assay

Uniplex PCR was performed using primers for *groEL*, *tdh* and *trh* in a total reaction volume of 50 µl, under conditions similar to our previous study (Hossain *et al*. 2012). The PCR reaction comprised 1 µg of purified DNA, 4 µl of dNTP mixture (200 µmol/l of each dNTP), 0.25 µl of ExTaq polymerase (Takara Bio, Japan), 0.5 µmol/l of specific primers, and sterile distilled water to a final volume of 50 µl. PCR conditions were as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 69°C, extension for 30 s at 72°C, and a final extension step for 5 min at 72°C in a PCR thermal cycler (2720 Thermal cycler; Applied Biosystems, Carlsbad, CA, USA). PCR products were resolved by electrophoresis in a 1% agarose gel. The multiplex PCR assay was standardised using the PCR conditions and reagents described above, with the exception of primer concentrations. For this assay, different concentrations of the *groEL*, *tdh* and *trh* primers were used in a single tube reaction to produce amplicons with good intensities.

3.2.5 Detection limits and multiplex PCR assay

The sensitivity of the three primer sets used in the multiplex PCR assay was determined following the methods of Hossain *et al*. (2013b). Briefly, purified chromosomal DNA (2 μg each) from *V. parahaemolyticus* strains KCCM 11965 and KCTC 2471 were combined, diluted serially in sterile distilled water in 10-fold steps, and tested using the newly developed multiplex PCR assay by addition of primers for *groEL*, *tdh* and *trh* in a single reaction (Table 3.2). Chromosomal DNA was also extracted from one clinical strain (P26) isolated from human, which contained both the *tdh* and *trh* genes, by boiling. The DNA from this strain was then used for sensitivity testing. Multiplex PCR detection of virulent and non-virulent *V. parahaemolyticus* strains was carried out by addition of the three primer sets to a single reaction. The PCR amplification conditions were as described above.

3.2.6 Detection of V. parahaemolyticus strains in artificially inoculated shellfish and seawater

The applicability of our multiplex PCR assay to accurate detection of target species with differentiation of virulent from non-virulent strains was confirmed using artificially inoculated shellfish homogenates and seawater. Oyster (*Crassostrea gigas*) tissue homogenates were inoculated with *V. parahaemolyticus* strains KCCM 41664 (1.4×10^5 CFU/ml), KCCM 11965 (9×10^5 CFU/ml), or KCTC 2471 (4×10^5 CFU/ml); moreover the latter two strains were used incombination. At the same time, tissue homogenates

from oysters, blood clams (*Tegillarca granosa*), thick shell mussels (*Mytilus coruscus*), and Manila clams (*Tapes philippinarum*) were inoculated with *V. parahaemolyticus* strain P26. Inoculated tissues were incubated at 37°C for 5 h; chromosomal DNA was extracted from each sample using a DNA extraction kit ((NucleoGen Biotech, Siheung, Korea)). Similarly, sterilised seawater (~300 ml) was incubated at 37°C for 24 h following inoculation with the same strains. Bacterial chromosomal DNA was extracted according to the methods described by Hossain *et al.* (2013b), and used as the template for multiplex PCR assay.

2.7 Sensitivity of PCR assays using spiked oyster and flounder meat

Oyster tissues and flounder meat were processed and spiked following the methods of No *et al*. (2011). Briefly, *V. parahaemolyticus* strain P26 (4×10^6 CFU/ml) was cultured in BHI broth and diluted serially in 10-fold steps. Aliquots (100µl) of each dilution were inoculated into 900 µl of oyster and flounder meat homogenates. Spiked samples were mixed well and centrifuged at 1,000 × *g* for 2 min. Supernatants were then transferred to a fresh tube and centrifuged at 10,000 × *g* for 5 min to pellet bacterial cells. Pellets were resuspended in 100 µl of distilled water and crude DNA was extracted by heating at 100°C for 20 min, followed by immediate cooling on ice. Aliquots of 2 µl of DNA lysate were used as template DNA for PCR assays.

Table 3.1 Strains used in this study

	Microorganisms	Source or reference
1	*Vibrio aestuarianus*	KCCM 40863
2	*V. alginolyticus*	KCTC 2472
3	*V. anguillarum*	KCTC 2711
4	*V. campbellii*	KCCM 41986
5	*V. cholerae*	KCTC 2715
6	*V. cincinnatiensis*	KCTC 2733
7	*V. damsella*	E
8	*V. diazotrophicus*	KCCM 41606
9	*V. fluvialis*	ATCC 33809
10	*V. furnissii*	KCTC 2731
11	*V. harveyi*	KCCM 40866
12	*V. hollisae*	KCCM 41680
13	*V. logei*	KCTC 2721
14	*V. mediterranei*	KCCM 40867
15	*V. metschnikovii*	KCTC 2736
16	*V. mimicus*	ATCC 33653
17	*V. natriegens*	KCCM 40868
18	*V. navarrensis*	KCCM 41682
19	*V. nereis*	KCCM 41667
20	*V. ordalii*	KCCM 41669
21	*V. parahaemolyticus*	KCCM 41664, KCCM 11965, KCTC 2471, E (37 strains), C (30 strains)
22	*V. proteolyticus*	KCTC 2730
23	*V. tubiashii*	KCTC 2728
24	*V. vulnificus*	KCCM 41665

C, clinical strain; E, environmental strain; ATCC: American Type Culture Collection, USA; KCCM: Korean Culture Center of Microorganisms, Korea; KCTC: Korean Collection for Type Cultures, Korea.

Table 3.2 Oligonucleotide primers, amplicon size, Tm value, and sources of gene sequences used for multiplex PCR

Target gene	Primer	Sequence	Position within gene (bp[a])	Length (nt[b])	Primer Tm (°C) (calculated)[c]	Amplicon size (bp)	Source
groEL	GRO-1	5′-AGGTCAGGCTAAGCGCGTAAGC-3′	951-972	22	70	510	Hossain *et al*. (2012)
	GRO-2	5′-GTCACCGTATTCACCCGTCGCT-3′	1440-1460	21	68		
tdh	TDH-1	5′-TATCCATGTTGGCTGCATTCAAAAC-3′	125-154	25	70	382	This study
	TDH-2	5′-TCTTCACCAACAAAGTTAGCTACA -3′	482-506	25	70		
trh	TRH-1	5′-TTCAACGGTCTTCACAAAATCAGA-3′	346-369	24	66	171	This study
	TRH-2	5′-AAACATATGTCCATTTCCGCTCTC-3′	493-516	24	68		

[a]Base pair of DNA

[b]Nucleotide

[c]Tm (°C) = 2 (A+T) + 4 (G+C)

3.3. Results

3.1 Uniplex and duplex PCR amplification

The primers for *groEL* amplified the expected 510-bp DNA segment from *V. parahaemolyticus* KCCM 41664. Similarly, the primers for *tdh* amplified a 382-bp segment from KCTC 2471, and those for *trh* amplified a 171-bp segment from KCCM 11965 (Fig. 3.1). Distinguishable amplicons were produced when duplex PCR was performed using mixed DNA of *V. parahaemolyticus* KCTC 2471 and KCCM 91665 (Fig. 3.1).

3.2 Optimisation of multiplex PCR

Various concentrations of the primer sets for *groEL*, *tdh* and *trh* were used to produce bands of good intensities. After adjusting the individual primer concentrations [0.5 μmol/l (gro1/gro2), 1.0 μmol/l (tdh1/tdh2) and 1.5 μmol/l (trh1/trh2)] for each multiplex PCR amplification, three fragments of the expected molecular weights were produced; the intensities of the bands produced upon resolution by electrophoresis were comparable (Fig. 3.1). Nonspecific amplification products were not evident in any of the reactions (Fig. 3.1). Amplicons of *groEL* (510 bp), *tdh* (382 bp) and *trh* (171 bp) were simultaneously generated in a multiplex reaction system from genomic DNA of *V. parahaemolyticus*. No bands were detected from non-target *Vibrio* species used in this study (data not presented).

The newly developed multiplex PCR was applied to 70 strains of *V. parahaemolyticus*. All 70 strains showed PCR amplification of *groEL*, 36 showed amplification of *tdh* and four showed amplification of *trh*; one strain

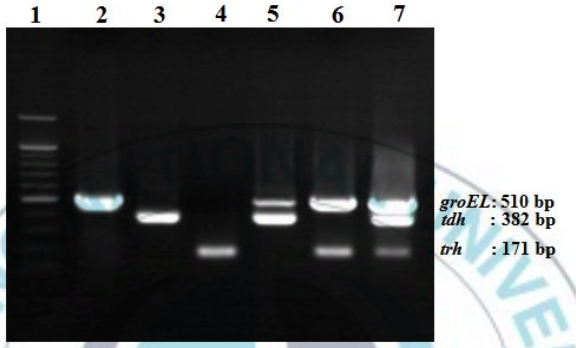

Fig. 3.1 Agarose (1%) gel electrophoresis of PCR products amplified from *V. parahaemolyticus* strains during standardisation of the multiplex PCR assay. Lanes: (1) 100-bp DNA ladder, (2) non-virulent strain KCCM 41664, (3, 5) virulent strain KCTC 2471, (4, 6) virulent strain KCCM 11965, (7) both virulent strains, (2–4) uniplex PCR, (5–6) duplex PCR and (7) multiplex PCR.

showed simultaneous amplification of both *tdh* and *trh* (Fig. 3.2). Based on this analysis, 30 strains were identified as non-virulent and 40 as virulent using this multiplex PCR assay.

3.3 Detection limits

The multiplex PCR assay functioned efficiently using DNA templates purified from *V. parahaemolyticus* KCTC 2471 and KCCM 11965 individually as well as in combination, and a qualitative decrease in amplicon intensity occurred with decreasing DNA concentration. The detection limit of mixed genomic DNA in multiplex PCR was 200 pg (Fig. 3.3a). When cell lysates from *V. parahaemolyticus* strain P26 were used to determine the detection limit, the *groEL*, *tdh* and *trh* primers were capable of detecting as few as 400 cells per tube (Fig. 3.3b).

The spike test was also carried out using oyster and flounder meat homogenates inoculated with 10-fold serial dilutions of *V. parahaemolyticus* strain P26. *V. parahaemolyticus* strain P26 was detected at levels above 4×10^4 CFU/g in spiked oyster samples and above 4×10^5 CFU/g in spiked meat samples by multiplex PCR (data not presented).

3.4 Detection of virulent and non-virulent V. parahaemolyticus strains in artificially inoculated shellfish and seawater

Virulent and non-virulent strains were detected by multiplex PCR in all artificially inoculated shellfish homogenates. Oyster homogenates were inoculated with three strains and bands were visualised by gel electrophoresis

after multiplex PCR using extracted DNA as the template (Fig. 3.4). All four types of shellfish inoculated with *V. parahaemolyticus* strain P26 showed a positive reaction (Fig. 3.4). Furthermore, this newly developed multiplex PCR also detected and differentiated virulent from non-virulent strains of *V. parahaemolyticus* from artificially inoculated seawater samples (Fig. 3.4).

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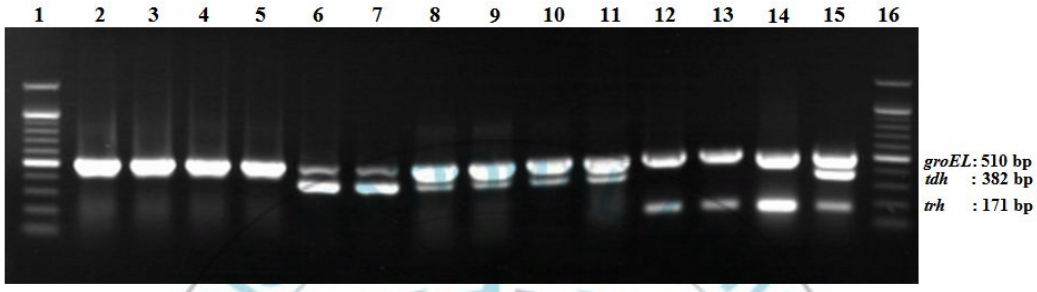

Fig. 3.2 Multiplex PCR assay for detection of *V. parahaemolyticus* targeting *groEL*, *tdh* and *trh*, respectively. Lanes: (1and 16) 100-bp DNA ladder, (2–5) non-virulent field strains, (6–11) virulent strains containing *tdh*, (12–14) virulent strains containing *trh*, and (15) virulent strain containing both genes.

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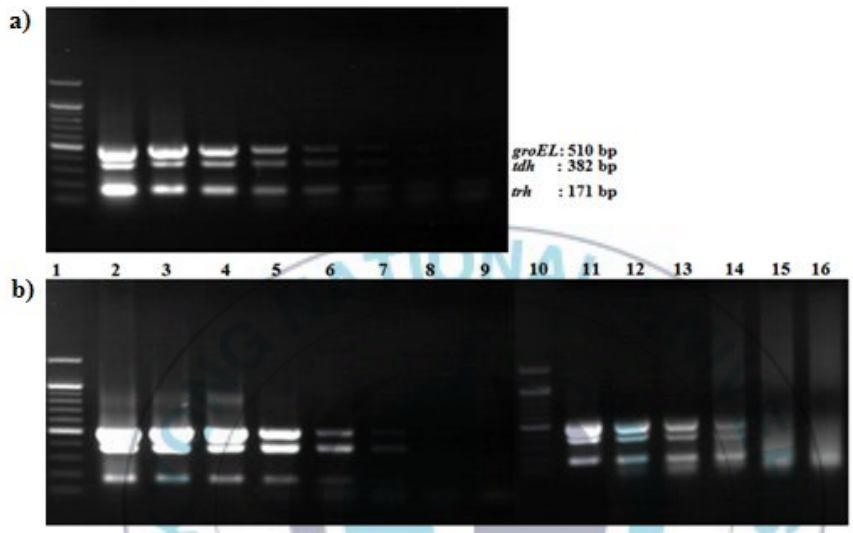

Fig. 3.3 Detection limit of multiplex PCR targeting *groEL*, *tdh* and *trh* of *V. parahaemolyticus*. (a) 10-fold serial dilutions (2 μg to 200 pg) of mixed purified DNA of KCTC 2471 and KCCM 11965; (b) 10-fold serial dilutions of lysate DNA of strain P26 (2–9) and oyster tissue spiked with 10-fold serial dilutions of cultured strain P26 (11-16).

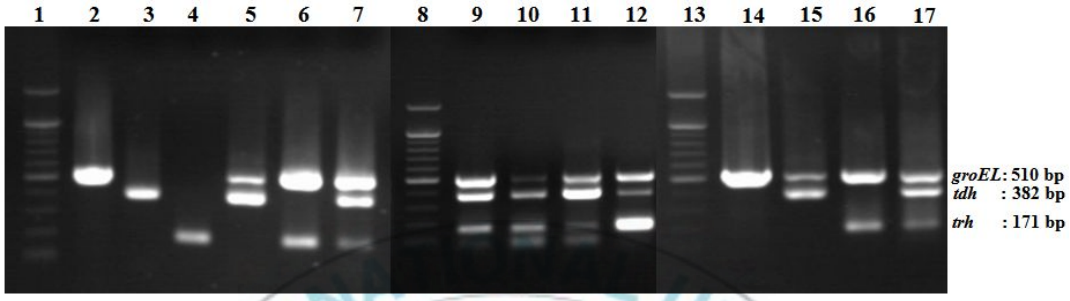

Fig. 3.4 Detection and differentiation of virulent and non-virulent strains of *V. parahaemolyticus* from artificially inoculated shellfish homogenates and seawater. Lanes: (1, 8, and 13) 100-bp DNA ladder, (2–7) oyster tissue homogenates inoculated with KCCM 41664, KCTC 2471 and KCCM 11965 strains, (9–12) oyster, Manila clam, thick shell mussel and blood clam, respectively inoculated with strain P26, (14–17) seawater inoculated with KCCM 41664, KCTC 2471, and KCCM 11965 strains.

3.4. Discussion

Pathogenesis of *V. parahaemolyticus* is based on the presence of virulence factors: the thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) or both, encoded by the *tdh* and *trh* genes, respectively (Honda and Iida 1993; Alam *et al*. 2002). The detection of *tdh* and *trh* in *V. parahaemolyticus* is necessary to determine the risk posed to human health by the presence of this species. Therefore, many molecular assays targeting the genes responsible for the production of TDH and TRH have been developed (Bej *et al*. 1999; Nordstrom *et al*. 2007; Nemoto *et al*. 2009), including PCR-based methods targeting the major virulence genes *tdh* and *trh* as diagnostic markers (Bej *et al*. 1999). However, this detection method can reveal only the presence or absence of both genes. The specificity of target sequences is crucial for the accurate identification of a particular bacterial species. In the environment, closely related *Vibrio* species can exchange genetic elements, such as virulence genes, or undergo deletion of a particular gene (Wong *et al*. 2012), resulting in the possibility of false-positive or false-negative results if only virulence genes are targeted. Furthermore, there is significant nucleotide sequence similarity among the *tdh* or *trh*-like genes of some *V. alginolyticus*, *V. cholerae*, *V. mimicus* and *V. hollisae* strains, which may prevent these toxin genes from being useful targets for identification of *V. parahaemolyticus* (Nishibuchi *et al*. 1996; Neogi *et al*, 2010). To overcome this problem, multiplex PCR assays targeting the species-specific gene and toxin genes have been developed (Bej

et al. 1999; Haldar *et al*. 2010; Rizvi and Bej 2010; Yu *et al*. 2010; No *et al*. 2011; Wong *et al*. 2012). It is difficult to differentiate *V. parahaemolyticus* from *V. alginolyticus*, *V. harveyi* and *V. campbellii* using 16S rRNA and 23S rRNA genes as the species-specific target due to their high sequence similarity (Croci *et al*. 2007; Haldar *et al*. 2010); these genes also cannot be used to distinguish *Aeromonas* from *Vibrio* species (Teh *et al*. 2010). In case of multiplex PCR using pR72H fragment, *tlh* or *toxR* genes for the identification of *V. parahaemolyticus*, there were still false-positive and -negative results in PCR analysis (Junick and Blaut 2012). Recently, Yu *et al*. (2010) and Liu *et al*. (2012) used the *irgB* gene and IAC, respectively as a species-specific marker for the identification of *V. parahaemolyticus*. However, the authors only used 10 *Vibrio* species to compare the nucleotide sequences and to check the specificity. Teh *et al.* (2010) also developed a multiplex PCR assay using *gyrB* and *pntA* genes to detect pathogenic and nonpathogenic *Vibrio* species, but they did not confirm its efficacy in a mixed population.

In contrast, we previously found the *groEL* gene to be highly specific for detection of *V. anguillarum*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Kim *et al*. 2010; Hossain *et al*. 2013 a & b) from 24 *Vibrio* species. We also developed a multiplex PCR for simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* using *groEL* as the target, which allows accurate detection of these species even in mixed bacterial cultures (Hossain *et al*. 2013b). This gene has also been used for

typing and identification of *Campylobacter jejuni*, *Bifidobacterium*, *Salmonella* and *Staphylococcus* species (Yushan *et al*. 2010; Junick and Blaut 2012). The primers for *groEL* used in this study had already been validated for species-specific detection of *V. parahaemolyticus*, even in artificially inoculated shellfish homogenates, flounder tissues, and seawater (Hossain *et al*. 2012). A multiplex PCR method was developed in the present study using species-specific *groEL*, in addition to the virulence markers *tdh* and *trh*, for detection and differentiation of *V. parahaemolyticus* strains. The primer sets produced amplicons of various sizes that were readily distinguishable by electrophoresis. Recently, No *et al*. (2011) developed a multiplex PCR method using *hns*, *tdh* and *trh* for the specific detection and differentiation of *V. parahaemolyticus* strains. However, the sensitivity of only *hns* for detection of a particular strain was examined in their study. The detection limit of mixed primers for detection of *V. parahaemolyticus* strains was 200 pg DNA, consistent with the findings of our previous study (Hossain *et al*. 2012 and 2013). Serial dilution of chromosomal DNA from one field isolate positive for both pathogenic genes yielded similar levels of sensitivity (Fig. 3b), indicating that our newly developed multiplex PCR method is capable of identifying any *V. parahaemolyticus* strain present at levels above the detection limit.

In conclusion, our data indicate successful PCR amplification of specific markers for accurate detection and differentiation of *V. parahaemolyticus* strains. The multiplex PCR assay targeting *groEL*, *tdh* and

trh genes successfully worked to detect total and pathogenic strains of *V. parahaemolyticus*, which has a potential to be applied in food industry, diagnostics and taxonomic studies. Further evaluation of this newly developed method using clinical and environmental samples is necessary to verify its detection efficacy.

3.5 References

Alam MJ, Tomochika K, Miyoshi S and Shinoda S (2002) Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Island Sea, Japan. *FEMS Microbiology Letters* **208**: 83–7.

Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD and Kaysner CA (1999) Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *Journal of Microbiological Methods* **36**: 215–25.

Chowdhury R, Sahu GK and Das J (1996) Stress response in pathogenic bacteria. *Journal of Bioscience* **21**: 149–160.

Croci L, Suffredini E, Cozzi L, Toti L, Ottaviani D, Pruzzo C, Serratore P, Fischetti R, Goffredo E, Loffredo G and Miono R (2007) Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology* **102**: 229–37.

Haldar S, Neogi SB, Kogure K, Chatterjee S, Chowdhury N, Hinenoya A, Asakura M and Yamasaki S (2010) Development of a haemolysin gene-based multiplex PCR for simultaneous detection of *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus*. *Letters in Applied Microbiology* **50**: 146–52.

Honda T and Iida T (1993) The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct hemolysin and related hemolysin. *Reviews in Medical Microbiology* **4**: 106–13.

Hossain MT, Kim EY, Kim YR, Kim DG and Kong IS (2012) Application of *groEL* gene for the species-specific detection of *Vibrio*

parahaemolyticus by PCR. *Letters in Applied Microbiology* **54**: 67–72.

Hossain MT, Kim YR, Kim EY, Lee JM and Kong IS (2013a) Detection of *Vibrio cholerae* and *Vibrio vulnificus* by duplex PCR specific to the *groEL* gene. *Fisheries Science* **79**: 335–40.

Hossain MT, Kim EY, Kim YR, Kim DG and Kong IS (2013b) Development of a *groEL* gene-based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *V. vulnificus*. Journal of Applied Microbiology **114**: 448–56.

Izumiya H, Matsumoto K, Yahiro S, Lee J, Morita M, Yamamoto S, Arakawa E and Yamamoto S (2011) Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Molecular and Cellular Probes* **25**: 174–6.

Junick J and Blaut M (2012) Quantification of human fecal *Bifidobacterium* species by use of quantitative real-time PCR analysis targeting the *groEL* gene. *Applied and Environmental Microbiology* **78**: 2613–22.

Kaysner CA and DePaola A (2001) Vibrios. In: Downes FP and Ito K (eds) *Compendium of methods for the microbiological examination of food*. American Public Health Association, Washington, p. 405–20.

Kim DG, Kim YR, Kim EY, Cho HM, Ahn SH and Kong IS (2010) Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. *Fisheries Science* **76**: 803–10.

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S and

Nishibuchi M (1999) Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. *Journal of Clinical Microbiology* **37**: 1173–7.

Lee CY, Pan SF and Chen CH (1995) Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. *Applied and Environmental Microbiology* **61**: 1311–17.

Liu B, He X, Chen W, Yu S, Shi C, Zhou X, ChenJ, Wang D and Shi X (2012) Development of a real time PCR assay for rapid detection of *Vibrio parahaemolyticus* from seafood. *Protein and Cell* **3**: 204–12.

Mukhopadhyay S, Miller RD, Sullivan ED, Theodoropoulos C, Mathews SA, Timms P and Summersgill JT (2006) Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. *Infection and Immununity* **74**: 3853–63.

Nemoto J, Sugawara C, Akahane K, Hashimoto K, Kojima T, Ikedo M, Konuma H and Hara-Kudo Y (2009) Rapid and specific detection of the thermostable direct hemolysin gene in *Vibrio parahaemolyticus* by loop-mediated isothermal amplification. *Journal of Food Protection* **72**: 748–54.

Neogi SB, Chowdhury N, Asakura M, Hinenoya A, Haldar S, Saidi SM, Kogure K, Lara RJ and Yamasaki S (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology* **51**: 293–300.

Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, Iihara H, Goto K, Masaki T, Miyasaka J and Ezaki T (2007) The *dnaJ* gene as a novel phylogenetic marker for identification of *Vibrio* species. *Systematic and Applied Microbiology* **30**: 309–15.

Nishibuchi M, Janda JM and Ezaki T (1996) The thermostable direct hemolysin gene (*tdh*) of *Vibrio hollisae* is dissimilar in prevalence to and phylogenetically distant from the *tdh* genes of other vibrios: implications in the horizontal transfer of the *tdh* gene. *Microbiology and Immunology* **40**: 59–65.

Nishibuchi M (2006) The Biology of Vibrios. In: Thompson FL, Austin B and Swings J (eds) *Molecular Identification*. American Public Health Association, Washington, p. 44–64.

No AR, Okada K, Kogure K and Park KS (2011) Rapid detection of *Vibrio parahaemolyticus* by PCR targeted to the histone-like nucleoid structure (H-NS) gene and its genetic characterization. *Letters in Applied Microbiology* **53**: 127–33.

Nordstrom JL, Vickery MC, Blackstone GM, Murray SL and DePaola A (2007) Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* in oysters. *Applied and Environmental Microbiology* **73**: 5840–7.

Oliver JD (2006) *Vibrio vulnificus* In: Belkin S and Colwell RR (eds) *Oceans and Health: Pathogens in the Marine Environment*. Springer Science, New York, p. 253–76.

Panicker G, Cell DR, Krug MJ and Bej AK (2004) Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Applied and Environmental Microbiology* **70**: 7436–44.

Peeler JT, Houghtby GA and Rainosek AP (1992) The most probable number technique. In: Downes FP and Ito K (eds) *Compendium of methods for the microbiological examination of food*. American Public Health Association, Washington, p. 105–20.

Rizvi AV and Bej AK (2010) Multiplex real-time PCR of *tlh*, *tdh* and *trh* genes in *Vibrio parahaemolyticus* and its rapid detection in shellfish and Gulf of Mexico water. *Antonie van Leeuwenhoek* **98**: 279–90.

Robert-Pillot A, Guenole A, Lesne J, Delesmont R, Fournier JM and Quilici ML (2004) Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. *International Journal of Food Microbiology* **91**: 319–25.

Su YC and Liu C (2007) *Vibrio parahaemolyticus*: A concern of seafood safety. *Food Microbiology* **24**: 549–58.

Takahashi H, Hara-Kudo Y, Miyasaka J, Kumagai S and Konuma H (2005) Development of a quantitative real-time polymerase chain reaction targeted to the toxR for detection of *Vibrio vulnificus*. *Journal of Microbiological Methods* **61**: 77–85.

Teh CSJ, Chua KH and Thong KL (2010) Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using a multiplex PCR based on *gyrB* and *pntA* genes. *Journal of Applied*

Microbiology **108**: 1940–5.

Venkateswaran K, Dohmoto N and Harayama S (1998) Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. *Applied and Environmental Microbiology* **64**: 681–7.

Wong HC, You WY and Chen SY (2012) Detection of toxigenic *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* in oyster by multiplex-PCR with internal amplification control. *Journal of Food and Drug Analysis* **20**: 48–58.

Xie ZY, Hu CQ, Chen C, Zhang LP and Ren CH (2005) Investigation of seven *Vibrio* virulence genes among *V. alginolyticus* and *V. parahaemolyticus* strains from the coastal mariculture systems in Guangdong, Chaina. *Letters in Applied Microbiology* **41**: 202–7.

Yu S, Chen W, Wang D, He X, Zhu X and Shi X (2010) Species-specific PCR detection of the food-borne pathogen *Vibrio parahaemolyticus* using the *irgB* gene identified by comparative genomic analysis. *FEMS Microbiology Letters* **307**: 65–71.

Yushan H, Lei L, Weijia L and Xiaoguang C (2010) Sequence analysis of the *groEL* gene and its potential application in identification of pathogenic bacteria. *African Journal of Microbiology Research* **4**: 1733–41.

CHAPTER 4

Detection of *Vibrio cholerae* and *Vibrio vulnificus* by duplex PCR specific to *groEL* gene

(Published in Fisheries Science)

PUKYONG NATIONAL UNIVERSITY 부경대학교

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Abstract

Vibrio cholerae and *V. vulnificus* are of major concern due to their effect on public health throughout the world. It is therefore imperative to identify a gene and method that are suitable for the accurate species-specific detection of these two species. A duplex polymerase chain reaction (PCR) assay was developed using two sets of primers targeting the *groEL* gene for the accurate simultaneous detection of *V. cholerae* and *V. vulnificus*. The nucleotide sequence of the *groEL* gene was compared with the sequences of other *Vibrio* and non-*Vibrio* species. The specificity of two primer sets for duplex PCR was checked using 24 *Vibrio* and 8 non-*Vibrio* species. The primer sets were found to be specific for these two species and could detect both of the target bacterial species without any ambiguity, even when comparing closely related species. For both species, the detection limit was 100 pg of purified genomic DNA. The duplex PCR showed high specificity and sensitivity for each species and was sufficient for the detection of *V. cholerae* and *V. vulnificus* from artificially infected shellfish tissue, flounder, and even inoculated seawater. This method is simple and cost-effective, and can be utilized for the simultaneous detection of both species, thus representing an effective tool for both epidemiologist and ecologist.

Keywords: Duplex PCR, flounder, *groEL* gene, shellfish, *V. cholerae*, *V. vulnificus*

4.1 Introduction

Vibrio species are found in aquatic environments, particularly estuaries, marine coastal waters, and sediments, as well as in aquaculture settings in temperate and tropical climates (Hsieh *et al*. 2008). Among these *Vibrio* species, the *Vibrio cholerae* O1 and O139 serogroups and some strains of other serogroups (non-O1/non-O139) are mainly responsible for cholera epidemics (Sack *et al*. 2004). In addition, the opportunistic pathogen *V. vulnificus* can cause gastroenteritis, septicemia, and wound infections with high fatality rates in immunocompromised individuals and those with chronic liver disease (Oliver 2005; Jones and Oliver 2009). A major source of *V. cholerae* and *V. vulnificus* infection is the consumption of raw or slightly cooked seafood, particularly shellfish (Oliver and Kaper 2001; Ottaviani *et al*. 2009). Therefore, the specific and rapid detection of these two *Vibrio* species is important from ecological and epidemiological perspectives.

The traditional methods that are used to detect *Vibrio* species are time-consuming: 3–5 days are usually required to obtain clear results. It has already been proven that molecular techniques, especially polymerase chain reaction (PCR) are able to detect the targeted microorganism with high specificity and sensitivity when discrimination among closely related species is required within one day. Simplex or multiplex PCR assays have been developed to detect the virulence genes of *V. cholerae* and *V. vulnificus* (Lalitha *et al*. 2008; Han and Ge 2010; Neogi *et al*. 2010; Teh *et al*. 2010). It is also important that the environmental non-O1/non-O139 type strains of *V. cholerae*, which cause cholera-like diarrhea, do not possess either the *ctx* operon or other virulence genes (Lalitha *et al*. 2008). When

these genes are applied to investigate environmental samples, there is a potential risk of misidentification, because horizontal transmission of toxigenic genes to nontoxigenic strains of closely related *Vibrio* species may occur (Faruque *et al*. 1998). The absence of toxin genes in nonvirulent strains has prevented these toxin genes from being useful targets for species-specific identification of bacterial pathogens (Chizhikov *et al*. 2001). Therefore, a PCR method based on a phylogenetic marker would be valuable (Izumiya *et al*. 2011).

PCR assays based on 16S rRNA sequences have also been developed, but these assays do not appear to be suitable for species discrimination within the *Vibrio* genus (Lalitha *et al*. 2008). In the last few years, new PCR methods that target housekeeping genes, such as *pho*, *amiB*, *dnaJ*, *gyrB*, *rpoA*, and *rpoB*, have been developed (Jeyasekaran *et al*. 2011). Although the *groEL* gene—which encodes a 60 kDa subunit known as HSP60, 60 kDa chaperonin, and heat shock protein—is known to be one of the most strongly conserved genes, the *groEL* gene in bacteria is more heterogeneous than the 16S rRNA and 23S rRNA genes (Nishibuchi 2006; Yushan *et al*. 2010). The *groEL* gene is reportedly applicable to interspecies differentiation as a target gene. We recently developed PCR for *V. parahaemolyticus* (Hossain *et al*. 2012) and DNA array detection methods (Kim *et al*. 2012) using the *groEL* gene. Because *V. cholerae* and *V. vulnificus* are the important *Vibrio* species in the context of public health, the aim of this study was to develop a duplex PCR for the simultaneous identification of these two species with high sensitivity using the *groEL* gene, a potential species-specific marker.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth media

A total of 50 *Vibrio* strains including the target species (9 *V. cholerae* and 11 *V. vulnificus*) as well as reference strains representing 22 other *Vibrio* species and 8 other non-*Vibrio* enteric bacterial species were examined in this study (Table 4.1). *Vibrio* and non-*Vibrio* species, with the exception of *E. coli* were grown aerobically in brain heart infusion (BHI) broth (BD, Franklin Lakes, NJ, USA) supplemented with 0.5–3% (wt/vol) sodium chloride. *E. coli* was grown in Luria-Bertani (LB) broth (USB, Cleveland, OH, USA). BHI agar was also used for the bacterial count during the determination of the infection dose in shellfish and flounder.

4.2.2 Primer design

All available sequences of the *groEL* gene among *Vibrio* and non-*Vibrio* enteric species were retrieved from GenBank using the BLASTN search program provided by the National Center for Biotechnology Information. The nucleotide sequences were compared using ClustalW to design species-specific oligonucleotide primer sets groVc1–groVc2 for *V. cholerae* (Accession No. NC002505) and groVv1–groVv2 for *V. vulnificus* (Accession No. NC004459) (Fig. 4.1). Potential oligonucleotide primers were designed to detect *V. cholerae* and *V. vulnificus* for specific amplicons of 418 and 192 bp, respectively. The primer sets were commercially synthesized by Cosmo Genetech, Seoul, Korea.

Table 4.1 Strains used in this study

	Microorganisms	Source or reference
1	*Vibrio aestuarianus*	KCCM 40863
2	*V. alginolyticus*	KCTC 2472, E
3	*V. anguillarum*	KCTC 2711, J-O-2, J-O-3, YT, NB10
4	*V. campbellii*	KCCM 41986
5	*V. cholerae*	KCCM 41626, KCTC 2715, 2 C, 5 E
6	*V. cincinnatiensis*	KCTC 2733
7	*V. damsella*	E
8	*V. diazotrophicus*	KCCM 41606
9	*V. fluvialis*	ATCC 33809
10	*V. furnissii*	KCTC 2731, E
11	*V. harveyi*	KCCM 40866
12	*V. hollisae*	KCCM 41680
13	*V. logei*	KCTC 2721
14	*V. mediterranei*	KCCM 40867
15	*V. metschnikovii*	KCTC 2736
16	*V. mimicus*	ATCC 33653
17	*V. natriegens*	KCCM 40868
18	*V. navarrensis*	KCCM 41682
19	*V. nereis*	KCCM 41667
20	*V. ordalii*	KCCM 41669
21	*V. parahaemolyticus*	KCCM 41664, KCCM 11965, KCTC 2471
22	*V. proteolyticus*	KCTC 2730
23	*V. tubiashii*	KCTC 2728
24	*V. vulnificus*	KCCM 41665, KCTC 2962, KCTC 2980, KCTC 2981, KCTC 2982, KCTC 2983, KCTC 2985, KCTC 2986, KCTC 2987, 2 E
25	*Aeromonas hydrophila*	KCTC 2358
26	*Edwardsiella tarda*	KCTC 12267, E
27	*Enterobacter cloacae*	E
28	*Escherichia coli*	L, E
29	*Klebsiella oxytoca*	E
30	*K. pneumoniae*	E
31	*Salmonella* Typhi	E
32	*Shigella flexneri*	E

L, laboratory collection; C, clinical strain; E, environmental strain; ATCC: American Type Culture Collection, USA; KCCM: Korean Culture Center of Microorganisms, Korea; KCTC: Korean Collection for Type Cultures, Korea.

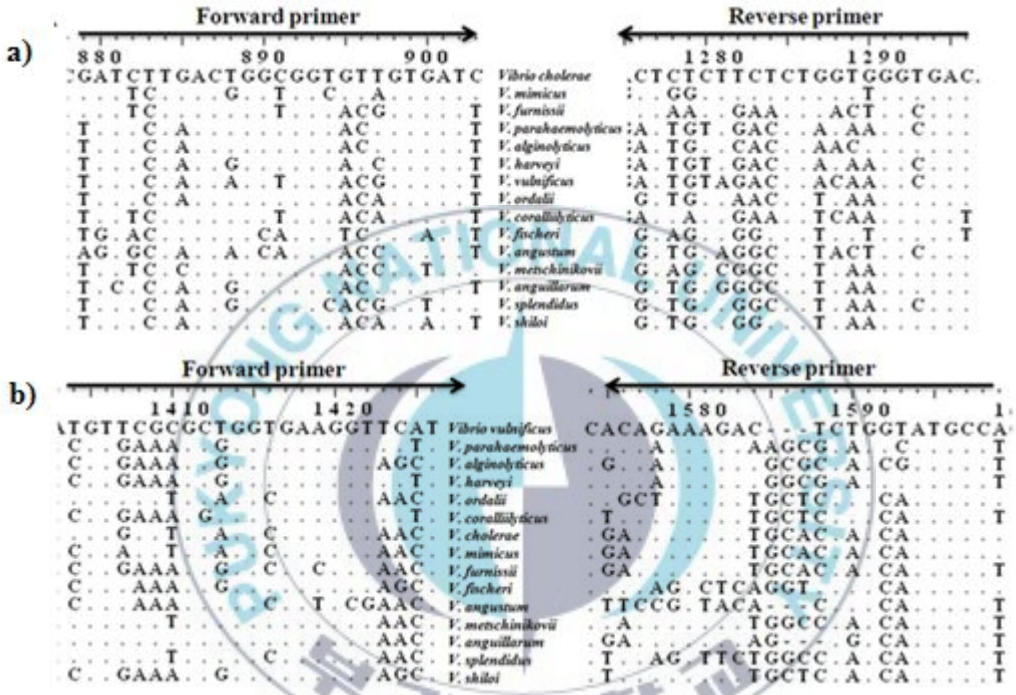

Figure 4.1 Comparison of primer sequences of *Vibrio cholerae* (a) and *V. vulnificus* (b) to other *Vibrio* species. Identical nucleotide sequences are indicated by dots.

4.2.3 Duplex PCR assay

Total genomic DNA was purified from the pure cultures of all *Vibrio* and non-*Vibrio* strains using phenol-chloroform extraction and ethanol precipitation methods as described by Ausubel *et al*. (1998), and was identified by a 16S rRNA primer set following the method of Hossain *et al*. (2012). The conditions and reaction mixtures of the simplex PCR assay were optimized to obtain the proper intensity for amplicons of *V. cholerae* and *V. vulnificus* using *groEL* gene primers following Hossain *et al*. (2012). In the duplex PCR assay, the PCR solution comprised 1 µl purified DNA; 5 µl of 10× PCR buffer with $MgCl_2$ (2 mmol/l); 4 µl of dNTP mixture (200 µmol/l of each dNTPs); 0.25 µl of Ex*Taq* polymerase (Takara Bio, Japan); 50 pmol of each primer for groVc1, groVc2, groVv1, and groVv2; and up to 50 µl of sterile distilled water. PCR conditions were optimized as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 69°C, extension for 30 s at 72°C, and a final extension step for 5 min at 72°C. The PCR products were subjected to analysis by 1% agarose gel electrophoresis. The specificity and sensitivity of the duplex PCR assay were determined by the method of Hossain *et al*. (2012). DNA was also extracted from overnight broth culture (1 ml) of *V. cholerae* and *V. vulnificus* following the methods of Kim *et al*. (2008), and then the lysate was serially diluted tenfold for a sensitivity test.

4.2.4 Detection of V. cholerae and V. vulnificus from artificially inoculated shellfish, flounder, and seawater

Oysters (*Crassostrea gigas*) and blood clams (*Tegillarca granosa*) purchased from the local markets were homogenized separately and infected with *V. cholerae* and *V. vulnificus* according to the methods described by Hossain *et*

al. (2012). Briefly, each shellfish homogenate was divided into four groups. Homogenates of two groups were separately infected with 3.5×10^6 CFU/ml of *V. cholerae* and 9×10^6 CFU/ml of *V. vulnificus*. One group was infected with a mixture of these two species and another group was kept as an uninoculated control. DNA was extracted from the infected tissue homogenates after 5 h of enrichment at 37°C. Sixteen apparently healthy flounders (*Paralichthys olivaceus*) were reared in aerated plastic containers (15 l) and divided into four groups. Fish of three groups were intraperitoneally injected with *V. cholerae*, *V. vulnificus*, and mixed species at the same infection dose used to infect shellfish tissues; fish of the other group were used as uninoculated controls. The internal organs (gill, liver, spleen, intestine and kidney) from inoculated and uninoculated fish were collected at 48 h post-infection. Total DNA was extracted from the fish tissues and shellfish homogenates using a DNA extraction kit (NucleoGen Biotech, Siheung, Korea). To examine whether *V. cholerae* and *V. vulnificus* can be detected in seawater by duplex PCR with *groEL* primers, sterilized seawater was artificially inoculated with *V. cholerae*, *V. vulnificus*, or a mixture of these two species. The DNA from the inoculated seawater was extracted following the methods of Hossain *et al*. (2012). The detection of *V. cholerae* and *V. vulnificus* from artificially inoculated shellfish homogenates, infected organs of flounder, and inoculated seawater, was performed by duplex PCR using the extracted DNA as a template and *groEL* primers while maintaining optimized concentrations of reagents and temperature cycling parameters.

4.3 Results

4.3.1 Specificity of V. cholerae and V. vulnificus primers

There was a band of 1466 bp appeared from all tested *Vibrio* and non-*Vibrio* species after PCR using 16S rRNA primers (data not shown). The groVc1–groVc2 primer sets amplified a 418 bp band for *V. cholerae* and groVv1–groVv2 primer sets amplified a 192 bp band for *V. vulnificus* reference strains by both simplex and duplex PCR (Fig. 4.2a). A specific amplicon was also produced for all of the test strains of these two species (data not presented). No amplicon of a specific size was observed for nontarget *Vibrio* or other non-*Vibrio* enteric species when the primer sets were tested for specificity in the detection of *V. cholerae* and *V. vulnificus* by duplex PCR. Non-specific amplicons of different sizes did not appear in any case (Fig. 4.2b).

4.3.2 Sensitivity for PCR detection

The sensitivity of both simplex and duplex PCR assays for the detection of *V. cholerae* and *V. vulnificus* are shown in Figure 4.3. The *groEL* primers were able to detect 100 pg for *V. cholerae* and 1 ng for *V. vulnificus* when purified DNA of each species was diluted, but detection down to 100 pg was possible upon the dilution of mixed purified DNA of both species (Fig. 4.3a). For cell lysate dilution, the detection limit, were 140 and 50 CFU for *V. cholerae* and *V. vulnificus*, respectively (Fig. 4.3b).

4.3.3 Detection of V. cholerae and V. vulnificus from artificially inoculated shellfish, flounder, and seawater

The *groEL* primer set was able to specifically and accurately detect *V. cholerae* from infected tissue homogenates of oysters and blood clams. In all cases, a 418 bp amplification product was produced (Fig. 4.4b) from the extracted DNA following duplex PCR. Detection of *V. cholerae* was also possible from all organs obtained from the injected flounder (Fig. 4.4b) and from all of the seawater samples by duplex PCR (Fig. 4.4a). *V. vulnificus* was specifically and accurately detected from inoculated tissues of both shellfish, organs of flounder, and seawater. In all cases, a 192 bp amplification product was produced (Fig. 4.4a–c). No amplification of the target gene segments of *V. cholerae* or *V. vulnificus* was observed in uninoculated tissues or seawater (data not presented). For samples inoculated with mixed species, both species were accurately detected by duplex PCR (Fig. 4.4a–c).

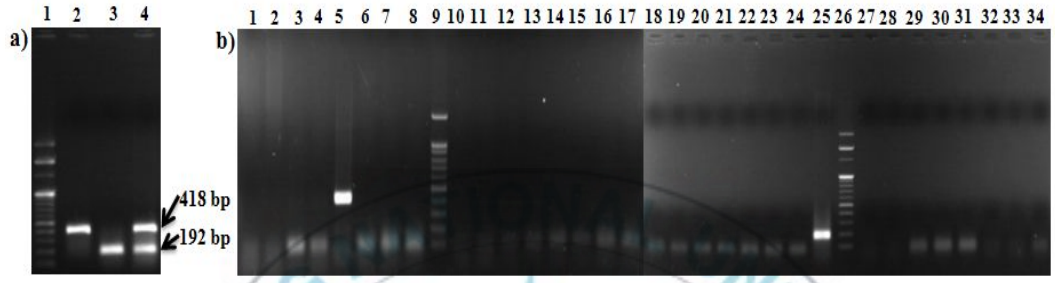

Figure 4.2 Agarose (1%) gel electrophoresis of DNA products amplified from *V. cholerae* and *V. vulnificus* (a) and specificity of duplex PCR using *Vibrio* and non-*Vibrio* species (b) using *groEL* primers. (a) Lane 1: 100 bp DNA ladder; lane 2: *V. cholerae*; lane 3: *V. vulnificus*; and lane 4: Both species. (b) Lane 9 and 26, 100 bp DNA ladder; lanes 1–8, 10–25 and 27–34: *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. cincinnatiensis*, *V. damsella*, *V. diazotrophicus*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. hollisae*, *V. logei*, *V. mediterranei*, *V. metschnikovii*, *V. mimicus*, *V. natriegens*, *V. navarrensis*, *V. nereis*, *V. ordalii*, *V. parahaemolyticus*, *V. proteolyticus*, *V. tubiashii*, *V. vulnificus*, *Aeromonas hydrophila*, *Edwardsiell tarda*, *Enterobacter cloacae*, *Escherichia coli, Klebsiella oxytoca*, *K. pneumoniae*, *Salmonella* Typhi, and *Shigella flexneri*, respectively.

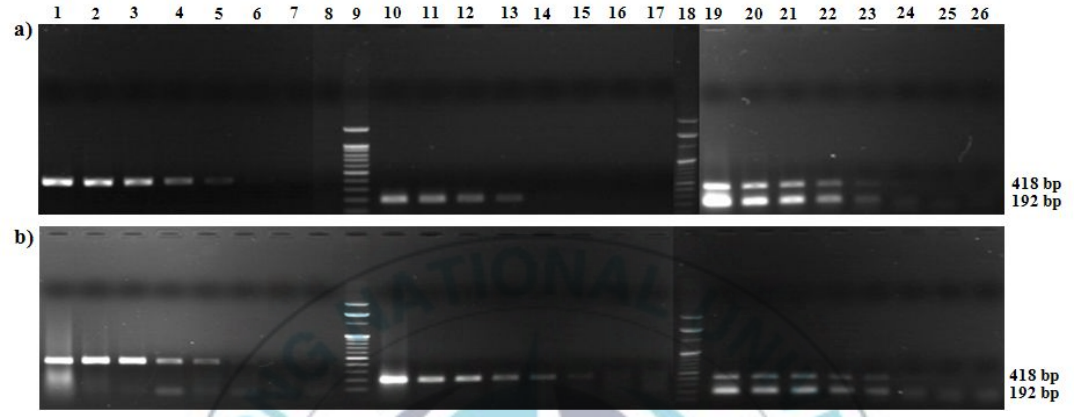

Figure 4.3 Sensitivity of duplex PCR for the detection of *V. cholerae* and *V. vulnificus*. Lanes 9 and 18: 100 bp DNA ladder; (a) Ten–fold serial dilution of purified chromosomal DNA (1 µg to 0.1 pg) of cultured cells; lanes 1–8: *V. cholerae*; lanes 10–17: *V. vulnificus*; and lanes 19–26: mixed species. (b) Ten–fold serial dilution of cell lysate of overnight culture of *V. cholerae* (lanes 1–8), *V. vulnificus* (lanes 10–17), and mixed species (19–26).

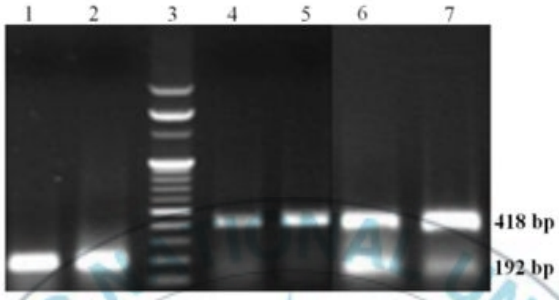

Figure 4.4 Detection of *V. cholerae* and *V. vulnificus* by duplex PCR directly from inoculated seawater. Lane 3, 100 bp DNA marker; seawater inoculated with *V. vulnificus* (lanes 1 and 2), *V. cholerae* (lanes 4 and 5), and a mixer of these two species (lanes 6 and 7). (b) DNA extracted from oyster (*Crassostrea gigas*) homogenates artificially infected with *V. vulnificus* (lane 1), *V. cholerae* (lane 4), and both species (lane 6); DNA extracted from blood clam (*Tegillarca granosa*) homogenates artificially infected with *V. vulnificus* (lane 2), *V. cholerae* (lane 5), and both species (lane 7).

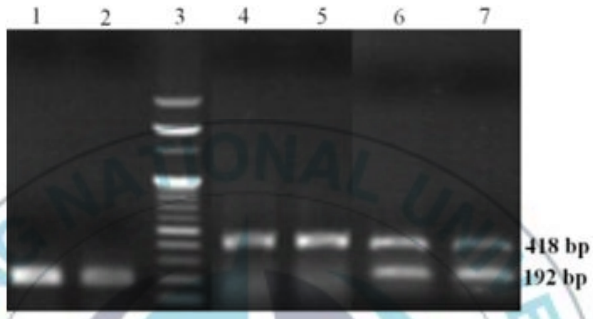

Figure 4.5 Detection of *V. cholerae* and *V. vulnificus* by duplex PCR directly from artificially inoculated shellfish homogenates. DNA extracted from oyster (*Crassostrea gigas*) homogenates artificially infected with *V. vulnificus* (lane 1), *V. cholerae* (lane 4), and both species (lane 6); DNA extracted from blood clam (*Tegillaria granosa*) homogenates artificially infected with *V. vulnificus* (lane 2), *V. cholerae* (lane 5), and both species (lane 7).

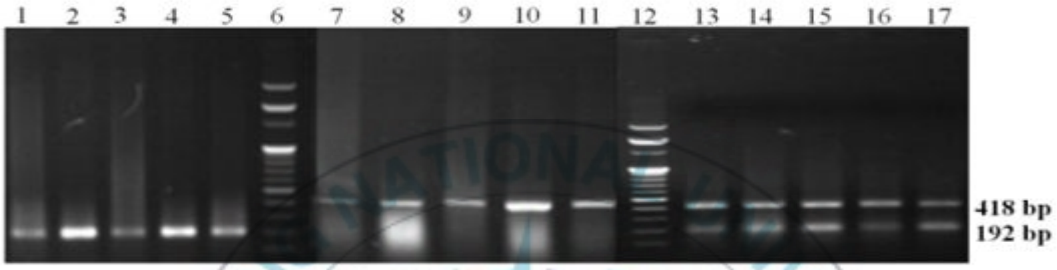

Figure 4.6 Detection of *V. cholerae* and *V. vulnificus* by duplex PCR directly from infected flounder organs. Lanes 6 and 12, 100 bp DNA marker; detection from organs (gill, liver, spleen, intestine, and kidney, respectively) of flounder artificially infected with *V. vulnificus* (lane 1–5), *V. cholerae* (lane 7–11), and mixed species (13–17).

4.4 Discussion

The simplicity and rapidity of PCR is its most important advantage over other DNA-based detection methods or immunoassays. Toxigenic genes are not suitable for the species-specific detection of *V. cholerae* and *V. vulnificus*. Several toxigenic genes, such as *omp*, *ctx*, *zot*, *ace*, *tcp*, *rtx*, *sto*, and *hly* in *V. cholerae* and *vvh*, *viuB*, and *toxR* in *V. vulnificus* have been previously targeted for PCR-based identification of these two species (Lalitha *et al*. 2010; Teh *et al*. 2010), but the methods excluded the vast majority of strains that do not possess the targeted toxigenic genes. Nhung *et al*. (2007) and Tarr *et al*. (2007) did not verify the multiplex PCR method using target genes to detect *V. cholerae* and *V. vulnificus* with a mixed population of targeted species in the same sample. A smaller difference in size was also problematic in the PCR method used by Nhung *et al*. (2007). A recent study using RNA colony hybridization has been shown to be useful for monitoring *V. cholerae* in environmental samples, but could not differentiate *V. cholerae* from *V. mimicus* (Neogi *et al*. 2010). In Tarr *et al*. (2007), the 16S rRNA target failed to distinguish *Aeromonas* from *Vibrio* species. The *rpoB* gene, which is potentially able to differentiate different *Vibrio* strains up to the species level, required further DNA sequencing (Mollet *et al*. 1997).

On the other hand, the primers designed from the *groEL* gene of *V. cholerae* and *V. vulnificus* detected these two species without any ambiguity due to false-positive results from nontarget species, even closely related

species, by duplex PCR. Both primer sets were found to be highly specific and accurate for *V. cholerae* and *V. vulnificus*. Our PCR assay showed good efficacy with a mixed population of targeted species in the same sample, and the species-specific primer sets produced amplicons of various sizes that are easily distinguishable by electrophoresis.

In this study, the detection levels of purified genomic DNA were 100 pg and 1 ng for *V. cholerae* and *V. vulnificus*, respectively, when simplex PCR was used for amplification. A similar detection limit was also observed in our previous study when *groEL* gene primers were used to detect *V. parahaemolyticus* and *V. anguillarum* (Kim *et al.* 2010; Hossain *et al.* 2012). We obtained an almost identical sensitivity pattern in both simplex and duplex PCR. The *groEL* primer sets of both *V. cholerae* and *V. vulnificus* successfully detected the specific species accurately from all artificially inoculated samples by duplex PCR (Fig. 4a–c).

In conclusion, the duplex PCR assay developed in this study can be considered a highly sensitive and specific assay for the simultaneous detection of *V. cholerae* and *V. vulnificus*. This simple and rapid method will aid epidemiologists, physicians, and ecologists in predicting the possible risk associated with these two species in biological, environmental, and food samples. An evaluation of this newly developed duplex PCR method for the simultaneous detection of *V. cholerae* and *V. vulnificus* in environmental and clinical samples is required to verify its efficacy.

4.5 References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1998) *Current Protocols in Molecular Biology*. Wiley and Sons, New York.

Chizhikov V, Rasooly A, Chumakov K and Levy DD (2001) Microarray analysis of microbial virulence factors. *Applied and Environmental Microbiology* **67**: 3258–63.

Faruque SM, Asadulghani, Saha MN, Alim AR, Albert MJ, Islam KM and Mekalanos JJ (1998) Analysis of clinical and environmental strains of nontoxigenic *Vibrio cholerae* for susceptibility to CTXΦ: molecular basis for origination of new strains with epidemic potential. *Infection and Immunity* **66**: 5819–25.

Han F and Ge B (2010) Multiplex PCR assay for simultaneous detection and characterization of *Vibrio vulnificus* strains. *Letters in Applied Microbiology* **51**: 234–40.

Hossain MT, Kim EY, Kim YR, Kim DG and Kong IS (2012) Application of *groEL* gene for the species-specific detection of *Vibrio parahaemolyticus* by PCR. *Letters in Applied Microbiology* **54**: 67–72.

Hsieh JL, Fries JS and Noble RT (2008) Dynamics and predictive modeling of *Vibrio* spp. in the Neuse River Estuary, North Carolina, USA. *Environmental Microbiology* **10**: 57–64.

Izumiya H, Matsumoto K, Yahiro S, Lee J, Morita M, Yamamoto S, Arakawa E and Ohnishi M (2011) Multiplex PCR assay for

identification of three major pathogenic *Vibrio* spp., *Vibrio cholera*, *Vinrio parahaemolyticus*, and *Vibrio vulnificus*. *Molecular and Cellular Probes* **25**: 174–6.

Jeyasekaran G, Raj KT, Shakila RJ, Thangarani AJ and Sukumar D (2011) Multiplex polymerase chain reaction-based assay for the specific detection of toxin-producing *Vibrio cholerae* in fish and fishery products. *Applied Microbiology and Biotechnology* **90**: 1111–8.

Jones MK and Oliver JD (2009) *Vibrio vulnificus*: disease and pathogenesis. *Infection and Immunity* **77**: 1723–33.

Kim DG, Kim YR, Kim EY, Cho HM, Ahn SH and Kong IS (2010) Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. *Fisheries Science* **76**: 803–10.

Kim JS, Lee GG, Kim J, Kwon JY and Kwon ST (2008) The development of rapid real-time PCR detection system for *Vibrio parahaemolyticus* in raw oyster. *Letters in Applied Microbiology* **46**: 649–54.

Kim YR, Kim EY, Kim DG, Kim YO, Hossain MT and Kong IS (2012) DNA array with the *groESL* intergenic sequence to detect *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Analytical Biochemistry* **424**: 32–4.

Lalitha P, Siti Suraiya, MN, Lim KL, Lee SY, Nur Haslindawaty AR, Chan YY, Ismail A, Zainuddin ZF and Ravichandran M (2008) Analysis of *lolB* gene sequence and its use in the development of a PCR assay for the detection of *Vibrio cholerae*. *Journal of Microbiological Methods* **75**:

142–144.

Mollet C, Drancourt M and Raoult D (1997) *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology* **26**: 1005–11.

Neogi SB, Chowdhury N, Asakura M, Hinenoya A, Haldar S, Saidi SM, Kogure K, Lara RJ and Yamasaki S (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology* **51**: 293–300.

Nhung PH, Ohkusu K, Miyasaka J, Sun XS and Ezaki T (2007) Rapid and specific identification of 5 human pathogenic *Vibrio* species by multiplex polymerase chain reaction targeted to *dnaJ* gene. *Diagnostic Microbiology and Infectious Disease* **59**: 271–5.

Nishibuchi M (2006) The Biology of Vibrios. In: Thompson FL, Austin B and Swings J (eds) *Molecular Identification*. American Public Health Association, Washington, p. 44–64.

Oliver JD (2005) Wound infections caused by *Vibrio vulnificus* and other marine bacteria. *Epidemiology and Infection* **133**: 383–91.

Oliver JD and Kaper JB (2001) *Vibrio* species. In: Doyle MP, Beuchat LR and Montville TJ (eds) *Food Microbiology: Fundamentals and Frontiers*. ASM Press, Washington.

Ottaviani D, Leoni F, Rocchegiani E, Santarelli S, Masini L, Trani VD, Canonico C, Pianetti A, Tega L and Carraturo A (2009) Prevalence and

virulence properties of non-O1 and non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. *International Journal of Food Microbiology* **132**: 47–53.

Sack DA, Sack RB, Nair GB and Siddique AK (2004) Cholera. *Lancet* **363**: 223–33.

Tarr CL, Patel JS, Phur ND, Sowers EG, Bopp CA and Strockbine NA (2007) Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *Journal of Clinical Microbiology* **45**: 134–40.

Teh CSJ, Chua KH and Thong KL (2010) Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using multiplex PCR based on *gyrB* and *pntA* genes. *Journal of Applied Microbiology* **108**: 1940–5.

Yushan H, Lei L, Weijia L and Xiaoguang C (2010) Sequence analysis of the *groEL* gene and its potential application in identification of pathogenic bacteria. *African Journal of Microbiology Research* **4**: 1733–41.

CHAPTER 5

Development of a *groEL* gene–based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*

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Development of a *groEL* gene–based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*

Abstract

Among more than 70 identified *Vibrio* species, *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* are of major concerns as they are pathogenic to animals, including humans. All three species are commonly associated with seawater, sediment, shellfish, and the intestinal contents of fish. Multiplex polymerase chain reaction (PCR) has been proven to provide rapid and highly sensitive methods for the specific detection of microorganisms and can be easily performed in diagnostic laboratories. The objective of this study was to develop an effective multiplex PCR for the simultaneous detection of three important *Vibrio* species, *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* using the *groEL* gene, a potential phylogenetic marker. The nucleotide sequence of *groEL* gene was compared with the sequences of other *Vibrio* and non-*Vibrio* species. Three species-specific primer sets were designed to target *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. A total of 131 *Vibrio* and non-*Vibrio* strains were used to determine the specificity and sensitivity of primers. The efficacy of the multiplex PCR method was checked using the samples collected from artificially inoculated seawater, shellfish homogenates and fish.

The primers produced specific PCR fragments from all target species strains and did not cross react with other *Vibrio* and non-*Vibrio* species. This PCR method showed good efficiency in detecting co-existing target species in the same sample with a detection limit of 100 pg of all target species from mixed purified DNA. Detection of three target species was also possible from artificially inoculated shellfish, flounder and seawater. The *groEL* gene is a potential marker for accurate simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, and could be used to detect these species in environmental and clinical samples. This newly developed multiplex PCR is a useful and cost-effective method that is applicable in a disease-outbreak prediction system and may provide an effective tool for both the epidemiologist and ecologist.

Keywords: *groEL* gene, multiplex PCR, seawater, shellfish, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*

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5.1 Introduction

Vibrio species are naturally diverse bacteria that inhabit aquatic environments and marine animals as symbionts and commensals (Izumiya *et al.* 2011). Among more than 70 identified *Vibrio* species, *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* are of major concern as they are pathogenic to animals, including humans (Oliver 1989; Thompson *et al.* 2004; Tracz *et al.* 2007). All three species are commonly associated with seawater, sediment, shellfish and the intestinal contents of fish (Wong *et al.* 2012). Under optimum conditions, these species are incorporated in high quantities by aquatic organisms, especially by filter feeders such as mussels, clams and oysters, which concentrate these bacteria in their muscle due to their filter-feeding habit. Infection by *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* occurs through ingestion of contaminated seafood or exposure to aquatic environments (Oliver 2006; Ottaviani *et al.* 2009). *V. cholerae* is the etiological agent of cholera, an acute dehydrating diarrhoea that occurs in epidemic form throughout the world, particularly in developing countries (Faruque and Nair 2002), whereas the non-O1 and non-O139 strains are involved in sporadic infection. *V. parahaemolyticus* is a leading pathogen that causes seafood-borne gastroenteritis worldwide, including developed countries such as the United States and Japan (Nair *et al.* 2007). Another pathogen, *V. vulnificus*, causes gastroenteritis, septicemia and severe wound infection with a high mortality in susceptible persons (Jones and Oliver 2009). Moreover, both *V. parahaemolyticus* and *V. vulnificus* strains can

cause diseases in aquatic organisms, including economically important fish and shrimp (Thompson *et al.* 2004).

Various polymerase chain reaction (PCR)–based methods have been reported for *Vibrio* species identification. These methods include real-time PCR, micro-arrays and multiplex PCR. However, the first two detection methods are costly due to the requirement for expensive instruments, whereas the multiplex PCR method that detects single or multiple species targets is effective. Multiplex PCR has been proven to provide rapid and highly sensitive methods for the specific detection of microorganisms (Fan *et al.* 2008) and can be easily performed in diagnostic laboratories. Many virulence genes, such as *omp*, *ctx*, *zot*, *ace*, *tcp*, *rtx*, *sto* and *hly* in *V. cholerae*; *tdh*, *trh* and *toxR* in *V. parahaemolyticus*; and *vvh*, *viuB* and *toxR* in *V. vulnificus* have been targeted for species-specific detection by uniplex or multiplex PCR (Lalitha *et al.* 2008; Neogi *et al.* 2010; Teh *et al.* 2010). According to Neogi *et al.* (2010), to accurately detect particular individual species, it is critical to address unresolved complications such as the precise differentiation of *V. parahaemolyticus* from closely related species, the simultaneous detection of all target species in a sample and the coexistence of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in costal environments, diseased animals, seafood or aquaculture (Gopal *et al*. 2005; Mahmud *et al*. 2008).

In the environment, *Vibrio* species can exchange genetic elements such as virulence genes or undergo deletion of a particular gene (Izumiya *et al.*

2011), resulting in the possibility of obtaining false-positive or false-negative results when targeting virulence genes. To overcome this problem, new PCR methods have been developed targeting housekeeping genes like *pho*, *amiB*, *dnaJ*, *gyrB*, *rpoA* and *rpoB* (Thompson *et al.* 2005; Nhung *et al.* 2007). The *groEL* gene encodes the chaperonin GroEL (synonyms are Cpn 60, GroL, Hsp 60, and Mop A), which plays an essential role in the control of cellular stress and is also a powerful phylogenetic marker (Junick and Blaut 2012). The superiority of the *groEL* gene compared to 16S rRNA and 23S rRNA has already been reported in the detection of *Vibrio* species (Nishibuchi 2006; Yushan *et al.* 2010). This gene has been shown to be a suitable marker for the successful detection of many bacteria including *Vibrio* species (Kim *et al*. 2010; Yushan *et al.* 2010; Hossain *et al*. 2012; Kim *et al*. 2012). Therefore, the aim of this study was to develop a suitable multiplex PCR method using the *groEL* gene that can be used for accurate simultaneous species-specific detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

5.2 Materials and Methods

5.2.1 Bacterial culture and DNA extraction

A total of 131 bacterial strains were used in this study (Table 5.1), including 9 *V. cholerae* strains, 70 *V. parahaemolyticus* strains, and 11 *V. vulnificus* strains. All *Vibrio* species were cultured in brain heart infusion (BHI; BD, Franklin Lakes, NJ) broth with 2.5% sodium chloride, while the other bacterial strains were cultured in Luria–Bertani (LB, USB, Cleveland, OH, USA) or BHI broth. Genomic DNA of all *Vibrio* and non-*Vibrio* strains purified and identified by 16S rRNA in our previous study (Hossain *et al.* 2012) were used as template DNA. Template DNA was also extracted from the target *Vibrio* species by the simple boiling method as described by Kim *et al.* (2008).

5.2.2 Oligonucleotide primers

All available sequences of the *groEL* gene among *Vibrio* and non-*Vibrio* species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov /Genbank/) and sequences were aligned using the ClustalW program. Species-specific conserved regions of the *groEL* gene for each of the three target species were identified and specific primers were designed (Fig. 5.1). Based on mismatches between *groEL* gene sequences of each target species and those of other *Vibrio* species, primers gro Vc1: 5′–GATCTTGACTGG CGGTGTTGTG–3′ and groVc2: 5′–GTCACCCACCAGAGAAGAGAGT–3′ for *V. cholerae*, groVp1: 5′–GTCAGGCTAAGCGCGTAAGCA–3′ and groVp2: 5′–GCATGCCTGCGCTTTCTTTTTG–3′ for *V. parahaemolyticus*, and groVv1: 5′–GTTCGCGCTGGTGAAGGTTCA–3′ and groVv2: 5′–TGG

CATACCAGAGTCTTTCTGTG–3′ for *V. vulnificus* were designed for the specific amplification of 418, 644, and 192 bp fragments, respectively.

5.2.3 Multiplex PCR assay and its efficiency test

PCR conditions were optimised using a 50 µl reaction mixture for each tube containing 1 µl of DNA template, 10 × PCR buffer containing $MgCl_2$, 0.2 $mmolL^{-1}$ of dNTP, 0.6 U of Taq polymerase (Takara Bio, Otsu, Shiga, Japan) and variable concentrations of each primer set. The final concentration of each primer set was standardised to obtain proper intensity for each amplicon. PCR conditions were optimized as follows: initial denaturation of 5 min at 94°C followed by 30 cycles each having denaturation for 30 s at 94°C, annealing for 30 s at 69°C and extension for 30 s at 72°C, and final extension step for 5 min at 72°C in a PCR thermal cycler (2720 Thermal cycler; Applied Biosystems, Carlsbad, CA, USA). The PCR products were subjected to 1% agarose gel electrophoresis. The PCR protocol was verified with all strains belonging to the target as well as non-target species (Table 5.1). The specificity and sensitivity test of the multiplex PCR was performed according to Hossain *et al*. (2012). The efficiency of multiplex PCR was also checked using variable DNA concentrations in mixed conditions. DNA representing 10^6 CFU of *V. cholerae* was mixed with a DNA mixture of *V. parahaemolyticus* and *V. vulnificus* representing 10^5, 10^4 and 10^3 CFU of each bacterium. Similarly, DNA representing 10^6 CFU of *V. parahaemolyticus* or *V. vulnificus* was mixed with DNA representing a similarly lower number of two other species. The multiplex PCR was carried out under optimal conditions.

Table 5.1 Strains used in this study

	Micro-organisms	Source or reference	Uniplex PCR[a]			Multiplex PCR[a]
			Vc	Vp	Vv	
1	*Vibrio aestuarianus*	KCCM 40863	-	-	-	-
2	*V. alginolyticus*	KCTC 2472, 3 E	-	-	-	-
3	*V. anguillarum*	KCTC 2711, J-O-2, J-O-3, YT, NB10	-	-	-	-
4	*V. campbellii*	KCCM 41986	-	-	-	-
5	*V. cholerae*	KCCM 41626, KCTC 2715, 2 C, 5 E	+	-	-	+
6	*V. cincinnatiensis*	KCTC 2733	-	-	-	-
7	*V. damsella*	E	-	-	-	-
8	*V. diazotrophicus*	KCCM 41606	-	-	-	-
9	*V. fluvialis*	ATCC 33809	-	-	-	-
10	*V. furnissii*	KCTC 2731, E	-	-	-	-
11	*V. harveyi*	KCCM 40866	-	-	-	-
12	*V. hollisae*	KCCM 41680	-	-	-	-
13	*V. logei*	KCTC 2721	-	-	-	-
14	*V. mediterranei*	KCCM 40867	-	-	-	-
15	*V. metschnikovii*	KCTC 2736	-	-	-	-
16	*V. mimicus*	ATCC 33653	-	-	-	-
17	*V. natriegens*	KCCM 40868	-	-	-	-
18	*V. navarrensis*	KCCM 41682	-	-	-	-
19	*V. nereis*	KCCM 41667	-	-	-	-
20	*V. ordalii*	KCCM 41669	-	-	-	-
21	*V. parahaemolyticus*	KCCM 41664, KCCM 11965, KCTC	-	+	-	+
		2471, 37 E, 30 C	-	+	-	+
22	*V. proteolyticus*	KCTC 2730	-	-	-	-
23	*V. tubiashii*	KCTC 2728	-	-	-	-
24	*V. vulnificus*	KCCM 41665, KCTC 2962, KCTC 2980,	-	-	+	+
		KCTC 2981, KCTC 2982, KCTC 2983,	-	-	+	+
		KCTC 2985, KCTC 2986, KCTC 2987,	-	-	+	+
		2 E	-	-	+	+
25	*Aeromonas hydrophila*	KCTC 2358	-	-	-	-
26	*Escherichia coli*	L, E	-	-	-	-
27	*Edwardsiella tarda*	KCTC 12267, E	-	-	-	-
28	*E. ictaluri*	ATCC 33202	-	-	-	-
29	*Enterobacter cloacae*	E	-	-	-	-
30	*Klebsiella oxytoca*	E	-	-	-	-
31	*K. pneumoniae*	E	-	-	-	-
32	*Salmonella typhi*	E	-	-	-	-
33	*Shigella flexneri*	E	-	-	-	-
34	*S. sonei*	E	-	-	-	-

L, laboratory collection; C, clinical strain; E, environmental strain; ATCC: American Type Culture Collection, USA; KCCM: Korean Culture Center of Microorganisms, Korea; KCTC: Korean Collection for Type Cultures, Korea; [a]+, only amplification product of 418 bp for *Vibrio cholerae*, 644 bp for *V. parahaemolyticus* and 192 bp for *Vibrio vulnificus*; -, no amplification products. Vc, Vp and Vv represents *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively.

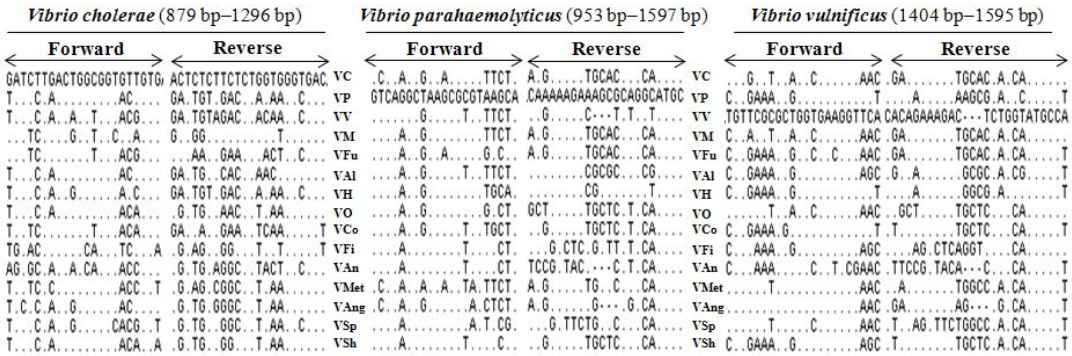

Figure 5.1 Comparison of primer sequences used for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* with those for other *Vibrio* species. VC: *Vibrio cholerae*, VP: *V. parahaemolyticus*, VV: *V. vulnificus*, VM: *V. mimicus*, VFu: *V. furnissii*, VAl: *V. alginolyticus*, VH: *V. harveyi*, VO: *V. ordalii*, VCo: *V. coralliilyticus*, VFi: *V. fischeri*, Van: *V. angustum*, VMet: *V. metschinikovii*, VAng: *V. anguillarum*, VSp: *V. splendidus* and VSh: *V. shiloi*. Identical nucleotide sequences are indicated by dots.

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5.2.4 Evaluation of multiplex PCR in shellfish homogenates and flounder

To test the applicability of this multiplex PCR method for accurate identification of three targeted species from shellfish, tissue homogenates of oyster (*Crassostrea gigas*), blood clam (*Tegillarca granosa*), thick shell mussel (*Mytilus coruscus*) and Manila clam (*Tapes philippinarum*) were used in a spiking test as described by Kumar *et al.* (2006). Briefly, 100 µl of one, two or three target species (*V. cholerae*: 6×10^7 CFU/ml, *V. parahaemolyticus*: 9×10^6 CFU/ml and *V. vulnificus*: 1×10^7 CFU/ml) was added to shellfish homogenates (15 ml) and mixed by vortexing. One milliliter of the spiked shellfish homogenates were then transferred to tryptone broth (5% tryptone + 2% NaCl) for enrichment at 37°C for 5 h. Total DNA was extracted from tissues using a DNA extraction kit (NucleoGen Biotech, Siheung, Korea), and multiplex PCR amplification was performed maintaining optimised concentrations of reagents and temperature cycling parameters.

Twenty healthy flounders (*Paralichthys olivaceus*) were reared in aerated plastic containers (15 l) and divided into five groups. Fish from four groups were intraperitoneally injected with *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* separately and in combination with the same infectious dose used to infect shellfish tissues, and the last group of fish was kept as a non-inoculated control. The internal organs (gill, liver, spleen, intestine and kidney) of inoculated and non-inoculated fish were collected at 48 h post-infection. Total DNA was extracted from tissues using a DNA

extraction kit and multiplex PCR amplification was performed.

5.2.5 Detection in artificially inoculated seawater

Sterilized seawater (~300 ml) was incubated at 37°C for 24 h after artificial inoculation with *V. cholerae* (6×10^4 CFU/ml), *V. parahaemolyticus* (9×10^3 CFU/ml) and *V. vulnificus* (1×10^4 CFU/ml) separately and in combination. The inoculated seawater and seawater containing infected flounder was collected and bacterial chromosomal DNA was extracted following the method of Hossain *et al.* (2012).

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5.3 Results

5.3.1 Uniplex and duplex PCR amplification

Three sets of primers designed to target three *Vibrio* species were used in uniplex and duplex PCRs to amplify 418 bp for *V. cholerae*, 644 bp for *V. parahaemolyticus* and 192 bp for *V. vulnificus* (Fig. 5.2a). Distinguishable amplicons were produced when duplex PCR was performed using DNA template mixtures of any two of the three target species (Fig. 5.2a).

5.3.2 Multiplex PCR

PCR amplification of mixed genomic DNA from three species with each set of primers produced a single DNA fragment of the expected molecular weight (Fig. 5.2a). This suggests that the primers specific for the individual pathogens used in this study and would not generate false-positives in the PCR reaction. Multiplex PCR enabled simultaneous amplification of all three targets with comparable band intensities using the PCR cycling parameters.

5.3.3 Specificity of V. cholerae, V. parahaemolyticus and V. vulnificus primers

The newly developed multiplex PCR produced amplicons of the expected sizes; that is, 418 bp for *V. cholerae*, 644 bp for *V. parahaemolyticus* and 192 bp for *V. vulnificus* (Fig. 5.2b). Furthermore, the PCR products were sufficiently different in size to be distinguishable by agarose gel electrophoresis. Specific amplicons were also produced from all strains of the three species (Table 5.1). No amplified products were obtained with

other *Vibrio* and non-*Vibrio* enteric species used in this study (Fig. 5.2b). The results demonstrated that the primers groVc1–groVc2, groVp1–groVp2 and groVv1–groVv2 are sufficiently specific for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively.

5.3.4 Evaluation of the detection limit and efficiency of multiplex PCR

The multiplex PCR worked efficiently with DNA templates from each species individually as well as in combination with three species (Fig. 5.3). There was a qualitative decrease in amplicon intensity with decreasing DNA concentration. The detection limit for genomic DNA in the uniplex PCR was 100 pg for all three species (Fig. 5.3a). The detection limit of mixed genomic DNA in multiplex PCR was also 100 pg for all three *Vibrio* species (Fig. 5.3a). When cell lysate was used to determine the detection limit, the sensitivities of *groEL* primers for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were 140, 130 and 50 CFU, respectively (data not presented). When variable DNA concentrations were used, specific amplicons with conspicuous band intensities were produced even with 100-fold differences in cell numbers among different species; *e.g.* 10^5 CFU *V. cholerae* along with 10^3 CFU of each of *V. parahaemolyticus* and *V. vulnificus* per tube (Fig. 5.3b). However, a 1000-fold difference in cell density resulted in generation of bands representing only the species with the greater cell density.

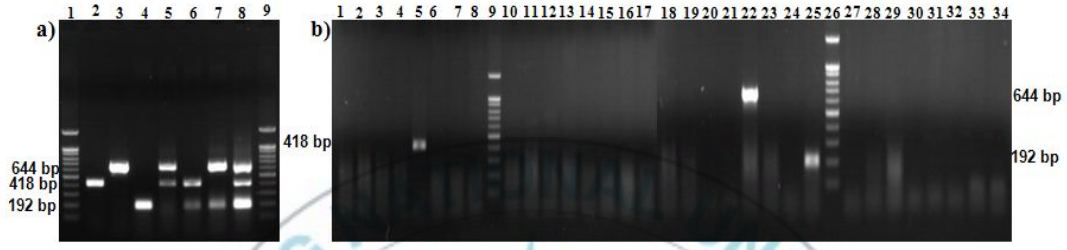

Figure 5.2 Agarose (1%) gel electrophoresis of PCR products of *V. cholerae* (Vc), *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) amplified during standardisation of multiplex PCR (a) and specificity testing using *Vibrio* and non-*Vibrio* species (b). (a) Lanes 1 and 9, 100 bp DNA ladder; lane 2, Vc; lane 3, Vp; lane 4, Vv; lane 5, Vc and Vp; lane 6, Vc and Vv; lane 7, Vp and Vv; lane 8, Vc, Vp and Vv. (b) Lanes 9 and 26: 100 bp DNA ladder; lanes 1-8, 10-25 and 27-34: *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. cincinnatiensis*, *V. damsella*, *V. diazotrophicus*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. hollisae*, *V. logei*, *V. mediterranei*, *V. metschinikovii*, *V. mimicus*, *V. natriegens*, *V. navarrensis*, *V. nereis*, *V. ordalii*, *V. parahaemolyticus*, *V. proteolyticus*, *V. tubiashii*, *V. vulnificus*, *Aeromonas hydrophila*, *Edwardsiell tarda*, *Enterobacter cloacae*, *Escherichia coli, Klebsiella oxytoca*, *K. pneumoniae*, *Salmonella typhi* and *Shigella flexneri*, respectively.

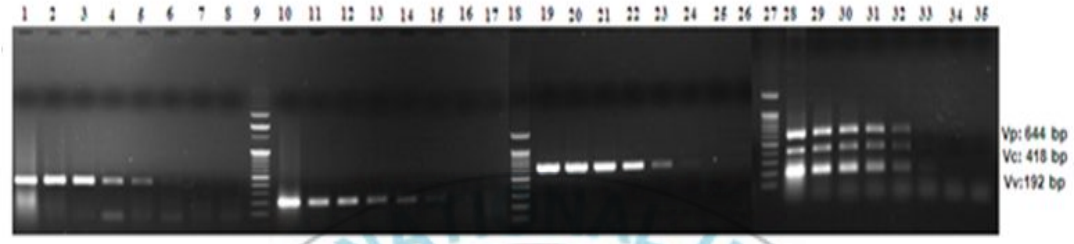

Figure 5.3 Sensitivity for detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (A). 10-fold serial dilution (1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg and 0.1 pg) of purified chromosomal DNA of cultured cells; Lanes 9, 18 and 27: 100 bp DNA ladder; lanes 1–8, *V. cholerae*; lanes 10–17, *V. vulnificus*; lanes 19–26, *V. parahaemolyticus* and lanes 28–35, mixed chromosomal DNA of the three species.

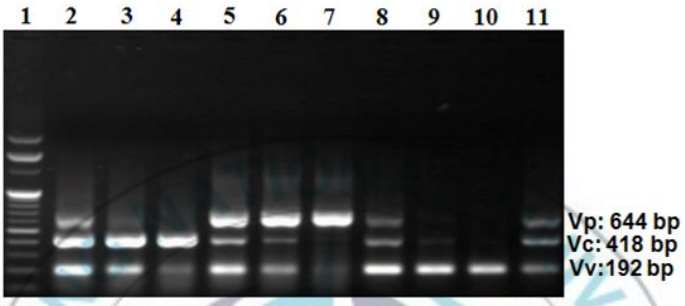

Figure 5.4 Efficacy of multiplex PCR for detection of targets using variable DNA template concentrations. Vc, Vp and Vv represents *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively. Lane 1, 100 bp DNA ladder; Lanes 2, 3 and 4, 10^5 CFU of *V. cholerae* mixed with 10, 100 and 1000-fold fewer CFUs of the other two species, respectively. A similar strategy was followed for lanes 5–7 and lanes 8–10 with 10^5 CFU of *V. parahaemolyticus* and *V. vulnificus*, respectively; lane 11, positive control.

5.3.5 Detection of V. cholerae, V. parahaemolyticus and V. vulnificus from artificially inoculated shellfish homogenates, flounder, and seawater

Target *Vibrio* species were detected by multiplex PCR from all artificially inoculated shellfish homogenates. Manila clam homogenates were inoculated with one, two or three target species, and bands were visualized by gel electrophoresis after multiplex PCR using extracted DNA as the template (Fig. 5.4a). Similar results were observed for all shellfish species investigated (data not presented). DNA extracted from all flounder organs tested positive for the three target *Vibrio* species by multiplex PCR (Fig. 5.4b). This newly developed multiplex PCR detected *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* from all inoculated seawater samples and from seawater containing infected fish (Fig. 5.4b).

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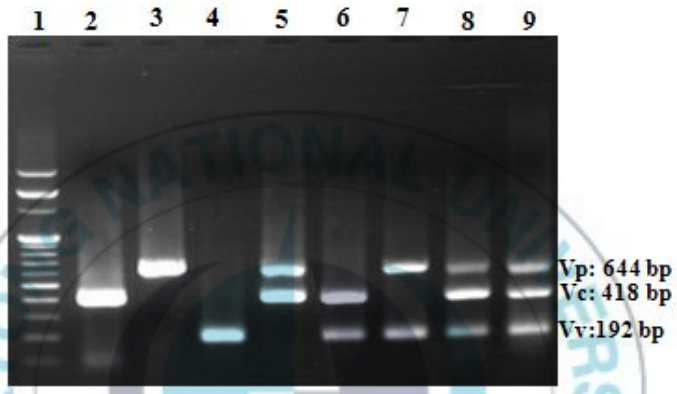

Figure 5.5 Detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in inoculated samples by multiplex PCR. Vc, Vp and Vv represent *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively. DNA extracted from Manila clam homogenates (*Tapes philippinarum*) infected with Vc (lane 2), Vp (lane 3), Vv (lane 4), Vc + Vp (lane 5), Vc + Vv (lane 6), Vp + Vv (lane 7) and Vc + Vp + Vv (lane 8) after 5 h enrichment; lane 1, 100 bp DNA ladder; lane 9, positive control.

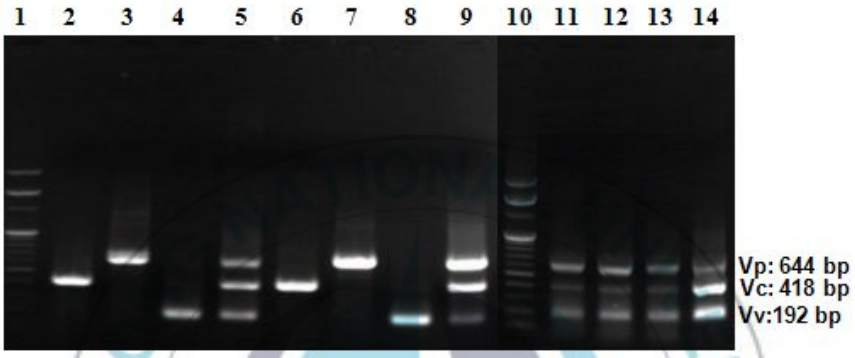

Figure 5.6 Detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in inoculated samples by multiplex PCR. Vc, Vp and Vv represent *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively. Seawater inoculated with Vc (lane 2), Vp (lane 3), Vv (lane 4) and all three species (lane 5); seawater containing infected fish inoculated with: lane 6, Vc; lane 7, Vp; lane 8, Vv and lane 9, all three species; lanes 1 and 10, 100 bp DNA marker; Detection in organs of flounders inoculated with Vc, Vp and Vv together (lanes 11, 12, 13 and 14; gill, liver, kidney and intestine, respectively).

5.4 Discussion

Food-borne pathogens pose a significant threat to public health, leading to a substantial economic burden in many countries (Chen *et al.* 2012). For this reason, the availability of rapid, sensitive and specific diagnostic methods for the detection of disease-causing pathogens is important. Molecular diagnosis protocols have provided effective methods for the diagnosis of bacterial agents because they permit specific and sensitive detection (Gonzalez *et al.* 2004). Several PCR-based detection methods for detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* have been developed. The use of virulence genes as identification markers may be of significance because their existence may be linked to pathogenesis. However, when applied to environmental samples, there is a potential risk of misidentification because such genes might transfer among bacteria. Neogi *et al.* (2010) reported that among closely related *Vibrio* species, horizontal transfer of toxigenic genes can equip the nontoxigenic strains with epidemic potential. Therefore, it is important to conduct surveillance on the total population (both toxigenic and nontoxigenic) of these three target species. A suitable phylogenetic marker is necessary for the detection of all strains of a particular species. The *groEL* gene, which has been established as a good marker for species-specific detection of various bacteria including *V. anguillarum* and *V. parahaemolyticus*, was used for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in this study. It is also important to detect pathogenic strain of particular species. Many virulence genes have

been used as target marker for the specific detection of pathogenic strains. But, during disease outbreak or screening of samples, the first choice is to detect particular pathogen at species level instead of strain level. So, our developed multiplex PCR method is suitable in this context and if necessary, the pathogenic strains can be confirmed by using virulence marker.

Pinto *et al.* (2005) developed a collagenase-targeted multiplex PCR with high specificity to detect *Vibrio* species, but they did not include *V. vulnificus*, an organism of public health concern in their assay. The PCR detection assay developed by Nhung *et al*. (2007) was specific only for pathogenic *Vibrios*, and failed to identify non-pathogenic *Vibrios*. Teh *et al*. (2010) also developed a multiplex PCR assay using *gyrB* and *pntA* genes to detect pathogenic and non-pathogenic *Vibrio* species, but they did not confirm its efficacy in a mixed population. Nhung *et al*. (2007) proposed a *dnaJ* gene based multiplex PCR but did not verify their method with a mixed population; differences among amplicon sizes were also problematic. Tarr *et al*. (2007) targeted *sodB*, *flaE* and *hsp* genes to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, but they did not verify with mixed populations. According to Neogi *et al*. (2010), the method developed by Bauer and Rorvik (2007) failed to differentiate *V. parahaemolyticus* from *V. alginolyticus* and the method developed by Grim *et al*. (2009) could not differentiate *V. cholerae* from *V. mimicus*. Neogi *et al*. (2010) used *toxR* and *vvhA* genes for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by multiplex PCR and successfully detected these three species.

They performed both specificity and sensitivity tests using a mixed population but used only pond water to confirm the efficiency of the developed method, instead of seawater or shellfish.

The multiplex PCR developed in this study successfully detected *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* without false-positive results from non-target species. The species-specific primer sets also produced amplicons of various sizes that were easily distinguishable by electrophoresis. The detection levels of both uniplex and multiplex PCR assays from mixed, purified genomic DNA of the three target species were similar. Efficiency was also good in a mixed population similar to that described by Neogi *et al.* (2010). We used seawater, shellfish and flounder as samples for artificial infection to confirm the efficiency and accuracy of three primer sets. Wong *et al.* (2012) mentioned that detection of bacteria in food by PCR is often hindered by the presence of inhibitors. Enrichment procedures can minimise the interference of the PCR inhibitors and increase the concentration of the target microorganisms. Enrichment for 5–12 h was applied during detection of *Vibrio* species from fish, fishery products, shellfish and water (Hossain *et al.* 2012; Jeyasekaran *et al.* 2011; Malayil *et al.* 2011; Wong *et al.* 2012). In this study, we extracted DNA from inoculated shellfish homogenates and seawater before and after enrichment for 5 h. Detection was possible in seawater samples before and after enrichment, but weak amplicons were observed in shellfish samples before enrichment. Enrichment resulted in detection with a strong signal, indicating that the multiplex PCR assay was

able to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* from the artificially inoculated samples.

In conclusion, the multiplex PCR assay developed in this study is highly sensitive and specific for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Further evaluation of this newly developed method using environmental and clinical samples is necessary to verify its detection efficacy, which will ultimately assist the epidemiologists, physicians and ecologists to investigate these three important *Vibrio* species.

5.5 References

Bauer A and Rørvik LM (2007) A novel multiplex PCR for the identification of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*. *Letters in Applied Microbiology* **45**: 371–5.

Chen J, Tang J, Liu J, Cai Z and Bai X (2012) Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *Journal of Applied Microbiology* **112**: 823–30.

Fan H, Wu Q and Kou X (2008) Co-detection of five species of water-borne bacteria by multiplex PCR. *Life Science Journal* **4**: 47–54.

Faruque SM and Nair GB (2002) Molecular ecology of toxigenic *Vibrio cholerae*. *Microbiology and Immunology* **46**: 59–66.

Gonzalez SF, Krug MJ, Nielsen ME, Santos Y and Call DR (2004) Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. *Journal of Clinical Microbiology* **42**: 1414–9.

Gopal S, Otta SK, Kumar S, Karunasagar I, Nishibuchi M and Karunasagar I (2005) The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *International Journal of Food Microbiology* **102**: 151–9.

Grim CJ, Zo YG, Hasan NA, Ali A, Chowdhury WB, Islam A, Rashid MH, Alam M, Morris JG, Huq A and Colwell RR (2009) RNA colony blot hybridization method for enumeration of culturable *Vibrio cholerae* and *Vibrio mimicus* bacteria. *Applied and Environmental Microbiology* **75**: 5439–44.

Hossain MT, Kim EY, Kim YR, Kim DG and Kong IS (2012) Application of *groEL* gene for the species-specific detection of *Vibrio parahaemolyticus* by PCR. *Letters in Applied Microbiology* **54**: 67–72.

Izumiya H, Matsumoto K, Yahiro S, Lee J, Morita M, Yamamoto S, Arakawa E and Ohnishi M (2011) Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholera*, *Vinrio parahaemolyticus*, and *Vibrio vulnificus*. *Molecular and Cellular Probes* **25**: 174–6.

Jeyasekaran G, Raj KT, Shakila RJ, Thangarani AJ and Sukumar D (2011) Multiplex polymerase chain reaction-based assay for the specific detection of toxin-producing *Vibrio cholerae* in fish and fishery products. *Applied Microbiology and Biotechnology* **90**: 1111–8.

Jones MK and Oliver JD (2009) *Vibrio vulnificus*: disease and pathogenesis. *Infection and Immunity* **77**: 1723–33.

Junick J and Blaut M (2012) Quantification of human fecal Bifidobacterium species by use of quantitative real-time PCR analysis targeting *groEL* gene. *Applied and Environmental Microbiology* **78**: 2613–22.

Kim DG, Kim YR, Kim EY, Cho HM, Ahn SH and Kong IS (2010) Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. *Fisheries Science* **76**: 803–10.

Kim JS, Lee GG, Kim J, Kwon JY and Kwon ST (2008) The development of rapid real-time PCR detection system for *Vibrio parahaemolyticus* in raw oyster. *Letters in Applied Microbiology* **46**: 649–54.

Kim YR, Kim EY, Kim DG, Kim YO, Hossain MT and Kong IS (2012) DNA array with the *groESL* intergenic sequence to detect *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Analytical Biochemistry* **424**: 32–4.

Kumar HS, Parvati A, Karunasagar I and Karunasagar I (2006) A *gyrB*-based PCR for the detection of *Vibrio vulnificus* and its application for direct detection of this pathogen in oyster enrichment broth. *International Journal of Food Microbiology* **111**: 216–20.

Lalitha P, Siti Suraiya MN, Lim KL, Lee SY, Nur Haslindawaty AR, Chan YY, Ismail A, Zainuddin ZF and Ravichandran M (2008) Analysis of *lolB* gene sequence and its use in the development of a PCR assay for the detection of *Vibrio cholerae*. *Journal of Microbiological Methods* **75**: 142–4.

Mahmud ZH, Neogi SB, Kassu A, Mai Huong BT, Jahid IK, Islam MS and Ota F (2008) Occurrence, seasonality and genetic diversity of *Vibrio vulnificus* in coastal seaweeds and water along the Kii Channel, Japan. *FEMS Microbiology Ecology* **64**: 209–18.

Malayil L, Turner JW, Mote BL, Howe K and Lipp EK (2011) Evaluation of enrichment media for improved PCR-based detection of *V. cholerae* and *V. vulnificus* from estuarine water and plankton. *Journal of Applied Microbiology* **110**: 1470–5.

Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y and Sack DA (2007) Global dissemination of *Vibrio parahaemolyticus* serotype

O3:K6 and its serovariants. *Clinical Microbiology Reviews* **20**: 39–48.

Neogi SB, Chowdhury N, Asakura M, Hinenoya A, Haldar S, Saidi SM, Kogure K, Lara RJ and Yamasaki S (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology* **51**: 293–300.

Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, Iihara H, Goto K, Masaki T, Miyasaka J and Ezaki T (2007) The *dnaJ* gene as a novel phylogenetic marker for identification of *Vibrio* species. *Systematic and Applied Microbiology* **30**: 309–15.

Nishibuchi M (2006) The Biology of Vibrios. In: hompson FL, Austin B and Swings J (eds) *Molecular Identification*. American Society for Microbiology, Washington, p. 44–64.

Oliver JD (1989) *Vinrio vulnificus* In: Doyle MP (ed), *Foodborne Bacterial Pathogens*. Marcel-Dekker Inc., New York, p. 569–600.

Oliver JD (2006) *Vibrio vulnificus* In: Belkin S and Colwell RR (eds) *Oceans and Health: Pathogens in the Marine Environment*. Springer Science, NewYork, p. 253–276.

Ottaviani D, Leoni F, Rocchegiani E, Santarelli S, Masini L, Trani VD, Canonico C, Pianetti A, Tega L and Carraturo A (2009) Prevalence and virulence properties of non-O1 and non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. *International Journal of Food Microbiology* **132**: 47–53.

Pinto AD, Ciccarese G, Tantillo G, Catalano D and Forte VT (2005) A collagenase-targeted multiplex PCR assay for identification of *Vibrio alginolyticus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*. *Journal of Food Protection* **68**: 150–3.

Tarr CL, Patel JS, Phur ND, Sowers EG, Bopp CA and Strockbine NA (2007) Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *Journal of Clinical Microbiology* **45**: 134–40.

Teh CSJ, Chua KH and Thong KL (2010) Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using multiplex PCR based on *gyrB* and *pntA* genes. *Journal of Applied Microbiology* **108**: 1940–5.

Tracz DM, Backhouse PG, Olson AB, McCrea JK, Walsh JA, Ng LK and Gilmour MW (2007) Rapid detection of *Vibrio* species using liquid microsphere arrays and real-time PCR targeting the ftsZ locus. *Journal of Medical Microbiology* **56**: 56–65.

Thompson FL, Iida T and Swings J (2004) Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews* **68**: 403–31.

Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB and Swings J (2005) Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Applied and Environmental Microbiology* **71**: 5107–15.

Wong HC, You WY and Chen SY (2012) Detection of toxigenic *Vibrio*

cholerae, *V. parahaemolyticus* and *V. vulnificus* in oyster by multiplex-PCR with internal amplification control. *Journal of Food and Drug Analysis* **20**: 48–58.

Yushan H, Lei L, Weijia L and Xiaoguang C (2010) Sequence analysis of the *groEL* gene and its potential application in identification of pathogenic bacteria. *African Journal of Microbiology Research* **4**: 1733–41.

CONCLUSION

The studies were conducted to develop suitable PCR methods for the detection of three important pathogenic *Vibrio* species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The *groEL* gene is a suitable phylogenetic marker and highly conserved among bacterial species. A simplex PCR was developed for the species-specific detection of *V. parahaemolyticus*, a multiplex PCR assay to differentiate strains of this species, a duplex PCR assay to detect *V. cholerae* and *V. vulnificus*, and another multiplex PCR assay was developed for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* using the primers designed from *groEL* gene. All available nucleotide sequences of the *groEL* gene of *Vibrio* and non-*Vibrio* enteric species were downloaded from GenBank, and sequences were aligned using the ClustalW program. The similarity in nucleotide sequences of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* varies between 80–90% and 66–80% when compared with other *Vibrio* and non-*Vibrio* species, respectively. The primer sets designed from the *groEL* gene produced highly specific band for particular target species without producing nonspecific band for other species. The sensitivity pattern of the primer sets were almost similar, i.e. detection limit was between 1 ng to 100 pg. Each of the primer set was able to detect target species from artificially inoculated shellfish homogenates, flounder organs and seawater, and even from mixed bacterial population. These findings indicate that the *groEL* gene is a species-specific marker for the detection of target *Vibrio*

species and can be used by the epidemiologists, physicians and ecologists to predict the possible risk associated with *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in biological, environmental and food samples.

인체 병원성 *Vibrio cholerae*, *V. parahaemolyticus* 와 *V. vulnificus* 검출을 위한 *groEL* 유전자 표적 PCR 시스템의 개발

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요약

수산생물 및 해양 환경에 존재하는 병원성 *Vibrio* 균은 인체 및 양식 수산물에 심각한 질병을 유발하며 심지어 사망에까지 이르게 하는 치명적인 균이다. 특히, *Vibrio cholerae* (Vc), *V. parahaemolyticus* (Vp) 및 *V. vulnificus* (Vv)는 대표적인 병원성 *Vibrio* 균주로 잘 알려져 있으며, 이들은 막대한 개인적, 경제적, 사회적 손실을 야기한다. Vc는 콜레라를 유발하는 병원균으로서 감염시 심각한 탈수 증세를 동반한 설사를 일으킨다. 콜레라의 경우 전염성이 매우 뛰어나므로 개발도상국과 같은 나라에서 특히 위험한 균주로 여겨지고 있다. Vp는 일반적으로 Vp가 오염된 어패류의 섭취를 통해 감염되며 장염을 일으키는 대표적인 장염 비브리오 균이다. 또한 Vv는 비브리오 패혈증 균으로서 상처 감염증 (wound infection) 또는 원발성 패혈증(primary septicemia)를 유발시키며 설사, 통증과 함께 다양한 피부 염증이 발생하는 감염성 질환을 일으킨다. 국내에서는 매년 20-40명의 환자가 발생하며 치사율이 50% 이상에 이르게 하는 병원성 균이다. 특히 접근성이 높은 어류 및 새우 등을 포함한 해양생물의 경우 물 속 미생물을 걸러서 먹이를 섭취하는 여과 섭식자(Filter feeders)로서, 이러한 기능이 사람에게 병원성 비브리오를 옮기는 중요한 수단으로 작용한다. 또한 최근 해산물이 미래의 식량자원으로서 각광받음에 따라 이들의 소비가 증가하고 있고, 특히 한국, 일본 및 중국의 경우 해산물을 날것으로 섭

취하는 경우가 많으므로 *Vibrio* 감염에 대한 주의가 더욱 요구되는 실정이다. 한편, 미생물의 유전자 수준에서의 판별을 위하여 16S rRNA 및 다양한 병원성 유전자 (*ctx, vvh, toxR, hly, omp, trh, tdh*) 등을 이용한 primer set가 고안되어 있지만, 16S rRNA는 종간에서도 매우 높은 상동성을 나타내므로 종간의 판별이 매우 어렵다는 단점이 있으며 병원성 유전자는 비병원성 미생물은 검출되지 않으며 목적 유전자 이외의 또다른 병원성 인자를 가지는 병원성 미생물은 검출되지 않을 수 있다는 단점이 있다. 따라서 이를 보완하기 위한 마커 유전자로서 house keeping gene이 사용되고 있으며, 최근 다양한 미생물의 검출을 위하여 *groEL* 유전자가 적절한 마커 유전자로 인식되고 있는 실정이다. 따라서 본 연구에서는, *groEL* 유전자를 이용하여 위험균으로 인식되고 있는 Vc, Vp 및 Vv를 PCR, duplex PCR 및 multiplex PCR을 이용하여 쉽고 빠르게 검출하는 방법을 개발하고자 하였으며, 본 연구를 통하여 실제 수산물에 감염된 인체 유해 병원성 *Vibrio* 균의 존재를 쉽고 정확하게 진단하여 안전한 수산물의 유통 및 양식장 어패류의 Vibrio성 대량 폐사를 예방하고자 하였다.

첫째, PCR method를 이용하여 Vp를 검출하기 위하여 Vp의 *groEL* 유전자에 특이적인 primer를 제작하였으며, 환경으로부터 스크리닝 한 Vp를 포함한 70개의 Vp sample로부터 510bp의 뚜렷한 PCR 증폭산물을 확인 할 수 있었다. 특히, 본 연구에서 제작된 primer의 annealing 온도는 68°C 이상으로서 좀더 빠르고 특이적인 검출을 하는데 용이하였다. Vp *groEL* primer는 chromosomal DNA 와 감염 조직으로부터 분리한 DNA를 각각 100pg, 1ng까지 검출할 수 있었다. 둘째, Vp를 strain 수준에서 구분하고 검출하기 위하여 특이적인 마커로서 *groEL*(510bp) 및 두 병원성 마커인 *tdh*(382bp)와 *trh*(171bp)를 사용하여 multiplex PCR assay를 구축하였다. 최적화된 multiplex PCR 조건의 민감도 및 효율성을 확인하기 위하여 Vp가 감염된 조개 및 해수를 이용하였으며, 모든 sample에서 효과적으로 검출 및 strain 구분이 가능하였다. 세가지 primer set를 이용한 multiplex 방법의 검출 한계는 200pg이었다. 셋째, PCR을 이용하여 Vc와 Vv를 동시에 검출하는 duplex PCR 방법을 구축하고자 각각의 *Vibrio*에 특이적인 *groEL* primer를 제작하였으며, 제작된 primer를 이용하여

primer의 특이성 및 민감도를 확인하였다. Vc(418bp)와 Vv(192bp)는 유연관계가 가까운 *Vibrio* 종 사이에서도 특이적으로 검출 가능하였으며, 100pg의 chromosomal DNA까지 검출 가능하였다. 또한 실제 sample에 적용 가능 여부를 확인하기 위하여 조개, 넙치 및 해수를 이용하여 실험한 결과 효율적으로 Vc와 Vv를 동시에 검출할 수 있었다. 넷째, 인체 병원성 *Vibrio*인 Vc(418bp), Vp(644bp) 및 Vv(192bp)를 동시에 검출하기 위한 multiplex PCR assay를 구축하기 위하여 각각의 *groEL* 유전자에 species-specific한 primer set를 제작하였다. Primer의 특이성과 민감도를 확인하기 위하여 총 131 종의 *Vibrio*와 non-*Vibrio* strain을 사용하였으며, 각각의 목적하는 strain을 특이적으로 검출할 수 있었다. Multiplex PCR의 검출 한계는 100pg의 DNA였으며, 세 *Vibrio* 종이 감염된 조개, 넙치 및 해수 sample에서도 특이적이고 정확한 검출이 가능하였다.

본 연구에서는 병원성 *Vibrio* 종을 simplex 특이적으로 검출하기 위한 마커로서 *groEL* 유전자를 이용하였으며, Vc, Vp 그리고 Vv를 PCR, duplex PCR 및 multiplex PCR을 이용하여 검출하기 위한 확실하고 적합한 마커임을 확인하였다.

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APPENDIX

List of articles published during doctoral study

Sl no	Authors	Title	Year	Journal	Volume & page	IF
1	**Hossain MT**, Kim EY, Kim YR, Kim DG and Kong IS	Application of *groEL* gene for the species-specific detection of *Vibrio parahaemolyticus* by PCR	2012	*Lett Appl Microbiol*	54: 67-72	1.647
2	**Hossain MT**, Kim YR, Kim EY, Lee JM and Kong IS	Detection of *Vibrio cholerae* and *Vibrio vulnificus* by duplex PCR using *groEL* gene	2013	*Fish Sci*	79: 335–340	0.96
3	**Hossain MT**, Kim EY, Kim YR, Kim DG and Kong IS	Development of a *groEL* gene-based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*	2013	*J Appl Microbiol*	114: 448-456	2.337
4	**Hossain MT**, Kim YO and Kong IS	Multiplex PCR for the detection and differentiation of V*ibrio parahaemolyticus* strains using *groEL*, *tdh* and *trh* genes	2013	*Mol Cell Probes*	DOI: http://dx.doi.org/10.1016/j.mcp.2013.04.001	2.078
5	Kim YR, Kim EY, Kim DG, Kim YO, **Hossain MT** and Kong IS	DNA array with the *groESL* intergenic sequence to detect *Vibrio parahaemolyticus* and *Vibrio vulnificus*	2012	*Anal Biochem*	424: 32-34	3.2
6	Heo WS, Kim EY, Kim YR, **Hossain MT** and Kong IS	Salt effect of nisin Z isolated from a marine fish on the growth inhibition of *Streptococcus iniae*, a pathogen of streptococcosis	2012	*Biotechnol Lett*	34: 315-320	1.768
7	Kim YR, Kim EY, **Hossain MT**, Oh R, Heo WS, Lee JM, Cho YC and Kong IS	Effect of a probiotic strain, *Enterococcus faecium*, for the immune responses of olive flounder (*Paralichthys olivaceus*)	2012	*J Microbiol Biotechnol*	22: 526-529	1.224