Detection of genes encoding *ompW* and *ctxA* of *Vibrio cholerae* isolated from shrimp and shellfish at Kedonganan fish market, Bali-Indonesia

Rian Ka Praja[1*], I Dewa Made Sukrama[2], Ni Nengah Dwi Fatmawati[2]

[1]Alumnus of Master Program in Biomedicine, Faculty of Medicine, Udayana University
[2]Department of Clinical Microbiology, Faculty of Medicine, Udayana University
Jl. PB. Sudirman, Denpasar, Bali
*Corresponding author: riankapraja@gmail.com

ABSTRACT

Contamination of pathogenic bacteria in food can lead to the emergence of foodborne disease. One of foodborne disease which often occurs in some developing countries such as Africa, Southeast Asia, and Latin America is cholera which is caused by *Vibrio cholerae*. The disease is transmitted through beverages and food, especially contaminated seafood. *V. cholerae* has several virulence factors including the outer membrane protein W *(ompW)* and cholerae toxin *(ctx).*The *ompW* acts as a protective barrier and can also be used as a marker specific species of *V. cholerae* and cholerae toxin is an enterotoxin responsible for the incidence of diarrhea in a cholera outbreak produced by pathogenic *V. cholerae*. This study was an observational study to determine the level of contamination of *V. cholerae* by detecting the outer membrane protein W *(ompW)* and cholerae toxin subunit A *(ctxA)* gene of *V. cholerae* in shrimp and shellfish sold at Kedonganan fish market. Samples were taken using total sampling technique and obtained 24 samples consisting of 14 shrimp samples and 10 shellfish samples. Samples were examined using culture methods and biochemical tests, and then further tested using Duplex Polymerase Chain Reaction (dPCR) to detect *ompW* and *ctxA* gene. The dPCR assay results showed 8 out of 14 (57.1%) samples from shrimp and 1 out of 10 (10%) samples from the shellfish positive carried *ompW* gene, and found no positive samples carrying the *ctxA* gene in samples derived from shrimp and shellfish. Chi square test analysis results indicated contamination of *V. cholerae* in shrimp was higher than shellfish based on *ompW* gene ($p<0.05$). It can be concluded that the shrimp and shellfish at Kedonganan fish market are contaminated by *V. cholerae*. Further research is needed to detect the virulence factors besides *ompW* and *ctxA* of *V. cholerae* in seafood.

Keywords: Foodborne disease, *Vibrio cholerae*, *ompW* gene, *ctxA* gene, and Duplex Polymerase Chain Reaction (dPCR).

INTRODUCTION

One of the most common foodborne diseases in some developing countries such as Africa, Southeast Asia, and Latin America is cholera caused by *Vibrio cholerae* transmitted through beverages and food, especially contaminated seafood (Lesmana, 2004; Madigan et al., 2015). In severe form, the disease is characterized by severe diarrhea with stools resembling rice water which can rapidly lead to dehydration and even death if left untreated (Faruque et al., 1998; Ryan and Ray, 2004).

Vibrio cholerae is a facultative anaerobic, Gram negative, non-spore forming, curved rod, about 1.04-1.06 µm long (Maheshwari et al., 2011a). *V. cholerae* is a very heterogeneous species with 206 serotypes of O antigen (Olaniran et al., 2011). *V. cholerae* can be divided into 2 types based on its pathogenicity, *V. cholerae* serogroup O1/O139 and *V. cholerae* serogroup non-O1/non-O139. *V. cholerae* O1 consists of the classical biotype and El Tor, the two biotypes consist of Inaba, Ogawa and Hikojima serotypes (Borroto, 1997; Kharirie, 2013). *V. cholerae* serogroup O1 and O139 are pathogenic *Vibrio* groups (Dziejman et al., 2002; Ryan and Ray, 2004; Olaniran et al., 2011).

The expression of the virulence genes is a factor that contributes to the pathogenicity of *V. cholerae*. Some virulence factors present in *V. cholera* are *ToxR regulator*, cholerae toxin (consisting of *ctxA* and *ctxB*), toxin-coregulated pilus subunit *(TcpA),* outer membrane protein U *(ompU)*, outer membrane protein W *(ompW)*, accessory cholerae enterotoxin *(Ace)*, and zonula occludens toxin *(Zot)* (Waturangi et al., 2013; Ramazanzadeh et al., 2015). Cholerae toxin is responsible for the occurrence of diarrhea in cholera outbreaks (Sikora, 2013, Pal, 2014). The *ctx* gene is usually owned by *V. cholerae* pathogens of serogroups O1 and O139 (Maheshwari et al., 2011a; Dalusi et al., 2015) but non-O1/O139 are known to carry *ctx* genes such as the outbreak that occurred in the United States in 2011 caused by *V. cholerae* serogroup O75 that was transmitted through raw oyster consumption (Onifade et al., 2011). Besides producing toxins, to cause a disease *V. cholerae* must be able to adapt to certain environmental conditions in the body. This adaptability depends on the outer membrane protein W encoded by the *ompW* gene (Alizadeh et al., 2013). Outer membrane consists of phospholipids,

lipopolysaccharides, and proteins that act as a physical protector between bacteria and their environment (Lin et al., 2002).

Some cholera pandemics have occurred, the first to sixth pandemic caused by the classic *V. cholerae* O1 biotype. The most important pandemic is the seventh pandemic caused by *V. cholerae* O1 El Tor biotype that occurred in Sulawesi in 1961 because it first occurred in Indonesia, then spread to Java, Sarawak and Kalimantan, then to the Philippines, Sabah, Taiwan and almost all Southeast Asia in 1962. During 1963 to 1969 the pandemic spread to Asia mainland. The eighth pandemic occurred in late 1992 in Madras and elsewhere in India and Bangladesh. Although the symptoms appear were cholera but the cause was *V. cholerae* non-O1 which then became *V. cholerae* serogroup O139 (Faruque et al., 1998).

Several studies on the existence of *V. cholerae* related to fishery products have been conducted by several researchers, including research on fishery products such as shrimp, crab and fish by Adeleye et al. (2010) in Lagos, Nigeria found 6.8% contaminated with *V. cholerae*, Jones et al. (2014) found a prevalence of 8.8% in *Crassostrea virginica* and 3.3% in *Mercenaria mercenaria*. Not only marine fishery products, Sousa et al. (2004) conducted a study on the shellfish *(Crassostrea rhizophorae)* raised in the Cocó River in Brazil, found that *V. cholerae* was the most common detected species (33.3%). In Bali, there were several previous studies on *V. cholerae*, Wijaya et al. (2013) and Ananta et al. (2013) found contamination of *V. cholerae* on fish preservative ice in Badung and Denpasar, and Widyastana et al. (2015) found contamination of *V. cholerae* of 7.4% in some fishery products such as shrimp and shellfish sold in several traditional markets in Denpasar City. Some research on *V. cholerae* conducted in Bali is still using phenotypic method. Research on the presence of toxigenic *V. cholerae* is very important to prevent outbreak in the future (Waturangi et al., 2013).

Kedonganan fish market is the largest and famous fish market in Bali. Various types of fish can be found in the market. Kedonganan fish market gets a supply of fish not only from Kedonganan local fishermen, but also gets supplies from other districts, even to other provinces (Hariyanto et al., 2015). This market is always crowded by tourists both local and foreign. Kedonganan fish market is located in Jimbaran area not far from Ngurah Rai Airport, South Kuta District.

Detection of *V. cholerae* from the clinical specimens and the environment can be done by conventional method (culture) and molecular diagnostic method using Polymerase Chain Reaction (PCR) (Kharirie, 2013). Cultural methods are performed on specific media for the growth of *V. cholerae* i.e. TCBS medium then followed by biochemical test (CDC, 2010). The diagnosis using this culture method is only able to diagnose the presence of *V. cholerae* but cannot be used to detect the virulence factor carried by the bacteria. To determine the virulence factor possessed by *V. cholerae* is usually used PCR method with specific target genes that act as a virulence factor (Huq et al., 2012; Waturangi et al., 2013; Ramazanzadeh et al., 2015). In addition, the PCR method is a more sensitive and specific molecular laboratory test, fast and accurate when compared to conventional methods (Angeliya and Kurdiwa, 2013; Kharirie, 2013).

Based on the description above and considering the absence of data about the presence of *V. cholerae* in Bali conducted using molecular technique to determine the virulence factors, so that this research is interesting to do.

MATERIALS AND METHODS

Sample

Samples of shrimp and shellfish were taken using total sampling technique from all of the fish sellers at Kedonganan fish market.

Isolation of *Vibrio cholerae*

Bacterial isolation began with enrichment process. In this process each sample was cut into small parts and crushed first. After that, 1 gram of sample was placed in 9 mL of Alkaline Peptone Water (APW) medium then homogenized and then incubated in incubator at 37°C for 6 hours. After the sample was incubated, the colonies were moved to TCBS medium, incubated at 37°C for 24 hours. Suspected colonies are yellow with size 2-3 mm and then the colonies were moved to TSA for further identification (Widyastana et al., 2015).

Identification of *Vibrio cholera*

Identification of *V. cholerae* was performed by oxidase test. Colonies of bacteria that grown on TSA medium were taken up one loop then transferred to

oxidase paper. Positive results are indicated by the change of paper color to purple in 5-10 seconds (CDC, 2010).

DNA Genomic Extraction

DNA genomic extraction was performed by boil cell extraction method. Bacterial colonies were placed into 200 µL TE then heated for 10 minutes at 100ºC. Further cooled using ice for 1-3 minutes and then centrifuged 8,000 rpm for 1 minute. The supernatant was transferred into a new eppendorf tube and stored at -80°C until use (Praja et al., 2016).

Amplification of *ompW* and *ctxA* genes with dPCR

The dPCR reaction to amplify the *ompW* and *ctxA* gene consisted of 2 µL template DNA, ompW primers (ompW-F, -R) 0.5 µM, ctxA primers (ctxA-F, -R) 0.2 µM, PCR Mix Promega 12,5 µL and nuclease free water 7 µL with total volume 25 µL. The PCR machine was programmed with pre-denaturation 95ºC for 2 minutes (1 cycle), denaturation 95ºC for 1 minute, annealing 53ºC for 1 minute, extension 72ºC for 1 minute (35 cycles), and post-extension 72ºC for 5 minutes (1 cycle). The arrangement of primers used is shown in the table below.

Table 1 Sequence of *ompW* and *ctxA* primers

Target	Primer	Sequence (5'-3')	Size	Reference
ompW	ompW-F	CACCAAGAAGGTGACTTTATTGTG	588 bp	Nandi et al., 2000
	ompW-R	GAACTTATAACCACCCGCG		
ctxA	ctxA-F	CTCAGACGGGATTTGTTAGGCACG	302 bp	
	ctxA-R	TCTATCTCTGTAGCCCCTATTACG		

Electrophoresis of dPCR results

Electrophoresis was performed using 1.5% agarose gel added with 1 µL Biotium Gel Red™ Nucleic Acid. Electrophoresis used TBE 0.5 X at 50 volts for 60 minutes. The gel was documented using the gel documentation tool. In the result of electrophoresis we can see the pattern of separation of DNA bands whose size is known by comparison with the size of DNA Ladder bands (100 bp).

Data Analysis

Comparative analysis was done by using Chi square test to compare the presence of *ompW* and *ctxA* genes of *V. cholerae* between shrimp and shellfish sold at Kedonganan fish market.

RESULTS

Total samples in this study were 24 samples consisting of 14 shrimp samples and 10 shellfish samples. Based on the dPCR test, 8 of 11 shrimp isolates and 1 of 9 shellfish isolates carried the *ompW* gene while there were no isolates carrying the *ctxA* gene in samples from shrimp and shellfish. The dPCR result is shown in Figure 1 and isolation, identification, and gene detection results are listed in the Table 2 and Table 3 below.

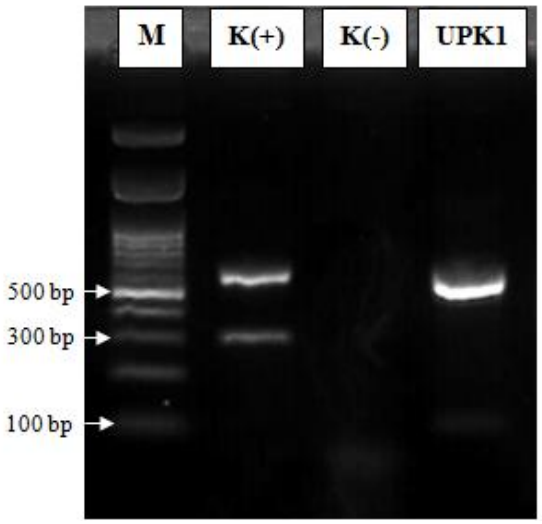

Figure 1 Electrophoregram of the dPCR result

Legend:
M = Marker 100bp DNA *ladder,*
K(+) = Positive control of *ompW* (588 bp) and *ctxA* (302 bp) *V. cholerae* O1 Ogawa serotype,
K(-) = Negative control,
UPK1 = Shrimp from Kedonganan fish market 1.

Table 2 Isolation, identification, and gene detection results from shrimp samples

No	Sample	APW	TCBS	Oxidase	dPCR	
					ompW	*ctxA*
1	UPK1	√	(+)	(+)	(+)	(-)
2	UPK2	√	(-)	-	-	-
3	UPK3	√	(+)	(+)	(+)	(-)
4	UPK4	√	(+)	(+)	(-)	(-)
5	UPK5	√	(-)	_	-	-
6	UPK6	√	(+)	(+)	(+)	(-)
7	UPK7	√	(+)	(+)	(+)	(-)
8	UPK8	√	(+)	(+)	(+)	(-)
9	UPK9	√	(+)	(+)	(-)	(-)
10	UPK10	√	(-)	_	-	-
11	UPK11	√	(+)	(+)	(+)	(-)
12	UPK12	√	(+)	(+)	(+)	(-)
13	UPK13	√	(+)	(+)	(-)	(-)
14	UPK14	√	(+)	(+)	(+)	(-)
Total		**14**	**11**	**11**	**8**	**0**
Percentage		**100**	**78,5%**	**78,5%**	**57,1%**	**0%**

Legend: UPK = Shrimp from Kedonganan fish market, √ = cultured, (+) = positive, (-) = negative, - = not tested

Table 3 Isolation, identification, and gene detection results from shellfish samples

No	Sample	APW	TCBS	Oxidase	dPCR	
					ompW	*ctxA*
1	KPK1	√	(+)	(+)	(-)	(-)
2	KPK2	√	(+)	(+)	(-)	(-)
3	KPK3	√	(+)	(+)	(-)	(-)
4	KPK4	√	(-)	_	-	-
5	KPK5	√	(+)	(+)	(-)	(-)
6	KPK6	√	(+)	(+)	(-)	(-)
7	KPK7	√	(+)	(+)	(-)	(-)
8	KPK8	√	(+)	(+)	(+)	(-)
9	KPK9	√	(+)	(+)	(-)	(-)
10	KPK10	√	(+)	(+)	(-)	(-)
Total		**10**	**9**	**9**	**1**	**0**
Percentage		**100%**	**90%**	**90%**	**10%**	**0%**

Legend: KPK = Shellfish from Kedonganan fish market, √ = cultured, (+) = positive, (-) = negative, - = not tested

It can be explained descriptively about the presence of *ompW* gene isolated from shrimp (57.1%) was higher than shellfish (10%) while *ctxA* gene was not found in isolates of shrimp and shellfish. The comparison data on the presence of gene encoding *ompW* in shrimp and shellfish at Kedonganan fish market were analyzed using Chi square test presented in Table 4.

Table 4 Comparative analysis of o*mpW* gene in shrimp and shellfish

Sample	Total Sample	*ompW* Gene		X^2
		Positive	Negative	
Shrimp	14	8	6	0,019^s
Shellfish	10	1	9	

Description: s = significant

Based on the result of Chi square test above, there were significant differences between the presence of *ompW* gene of *V. cholerae* isolated from shrimp and shellfish ($p < 0.05$), obtained 8 positive isolates of *ompW* gene from 14 shrimp samples and 1 positive isolate of *ompW* gene from 10 shellfish samples. Chi square test was not performed for *ctxA* gene because no shrimps and shellfish were positive to carry the *ctxA* gene.

DISCUSSION

The *ompW* gene is a gene encoding virulence factor associated with the adaptability of *V. cholerae* in certain environments and is often used as a marker of specific species of *V. cholerae* (Nandi et al., 2000; Tamrakar et al., 2007; de Menezes et al., 2014). Detection of the presence of *ompW* gene is a confirmation of previous biochemical tests. The 9 isolates (8 shrimp samples and 1 shellfish sample) which positively carried the *ompW* gene it can be ascertained that the isolate was *V. cholerae*. Based on *ompW* gene detection results, it can be ascertained that shrimp and shellfish at Kedonganan fish market contaminated with *V. cholerae.* This study strengthens previous research conducted by Ananta et al. (2013), Wijaya et al. (2013) and Widyastana et al. (2015) related to the contamination of *V. cholerae* in fish preservative ice and contamination in shrimp and shellfish in Bali.

The results of this study are in accordance with research conducted by Vengadesh et al. (2012) found shrimp, shellfish, fish, and squid sold in wet market and supermarket in Malaysia contaminated with *V. cholerae*. In this study, the

presence of contamination of *V. cholerae* was relatively high based on *ompW* genes as much as 57.1% in shrimp and 10% in shellfish. The results of contamination findings in seafood based on *ompW* target gene were also found by Sathiyamurti et al. (2013) of 35.9% in fish and 47.4% in shellfish in the fish market in Tamil Nadu, India, then Maheshwari et al. (2011b) found 45.71% fish, 57.14% crabs and 17.1% shrimp contaminated with *V. cholerae*. The smaller contamination of *V. cholerae* was reported by Jones et al. (2014) in oysters *(Crassostrea virginica)* 8.8% and shellfish *(Mercenaria mercenaria)* 3.3% in Long Island, USA.

Based on the results of this study, 8 of 14 shrimp samples (57.1%) and 1 of 10 shellfish samples (10%) were positively contaminated by *V. cholerae* and based on Chi square test analysis found contamination in shrimp was higher than shellfish ($p < 0.05$). The basic interaction between *V. cholerae* and shrimp is the production of chitinase that can degrade chitin which is part of the exoskeleton of the shrimp. *V. cholerae* can use chitin as a source of energy, carbon, and nitrogen (Markov et al., 2015). In the aquatic environment, chitin will be colonized by the chitinolytic bacteria (Gooday et al., 1991). Xibing et al. (2007) reported the chemotactic properties of *V. cholerae* to colonize the chitin that belongs to shrimp. This symbiotic relationship enhances the survival of *V. cholerae* in aquatic environments (Kirn et al., 2005). Based on the anatomical structure, the shrimp have very much chitin on the body's exoskeleton so it will be a factor that attracts *V. cholerae* to colonize on the part whereas the shellfish get contamination from ingestion of organisms such as plankton and algae. This is what causes contamination of shrimp higher than shellfish ($p < 0.05$).

Chi square analysis was not conducted on the comparison of *ctxA* gene presence between samples from shrimp and shellfish because the results showed no positive isolates carrying *ctxA* gene from 14 shrimp samples and 10 shellfish samples. The presence of *ctxA* in *V. cholerae* derived from environmental samples is highly erratic or random and the incidence rate is very small. In contrast to samples derived from clinical samples are very often found the existence of these genes, especially those from outbreak cases (Nishibori et al., 2011; Khariri, 2012).

The results of this study are supported by previous research results, Koralage et al. (2012) and Traoré et al. (2014) found no isolates of *V. cholerae* carrying *the ctxA* gene from seafood samples. Rajpara et al. (2013) isolated *V. cholerae* derived

from water samples, zooplankton, and phytoplankton but based on PCR test was known that the isolates did not carry the gene encoding virulence factors of *ctxA, ctxB, tcpA* and *Zot.* Narwati (2011) also reported that all isolates of *V. cholerae* isolated from Kalimas River, Surabaya negative *ctxA* gene. However, it is important to monitor the presence of toxigenic *V. cholerae* from environmental samples because the outbreak usually results from contamination of the environment. Amizar (2011) reported that 20% of the total samples of shrimp, shellfish and crab were positively contaminated by *V. cholerae* carrying *ctxA* gene in Padang City, West Sumatera and Muara Angke, North Jakarta. Blake et al. (1980), Hill et al. (2011), and Khariri (2012) found a match between *V. cholerae* derived from the environment such as seafood and drinking water sources with *V. cholerae* outbreaks.

Although all isolates of *V. cholerae* isolated from shrimp and shellfish did not carry the *ctxA* gene, it does not mean that the isolates cannot cause disease in humans. In addition *V. cholerae* has *Ace* gene encoding accessory cholerae enterotoxin and *Zot* gene encoding zonula occludens toxin which can cause diarrhea but is usually mild and sporadic in contrast to *ctxA* capable of causing death and into a worldwide pandemic (Faruque et al., 1998; Waturangi et al., 2013).

CONCLUSION

Shrimp and shellfish at Kedonganan fish market were contaminated with *V. cholerae* 57.1% and 10% based on *ompW* gene, and no *V. cholerae* isolates from shrimp and shellfish were positive carrying *ctxA* gene.

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