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Simultaneous detection of *ompW* and *ctxA* gene of *Vibrio cholera* isolated from ice used for marine product preservative by Duplex Polymerase Chain Reaction assay (dPCR)

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ABSTRACT

Background: Cholera is an infectious disease with symptom rice water stool diarrhea caused by *Vibrio cholerae*. Cholera transmitted by water or contaminated seafood. Ice usually used as a preservative marine products which vulnerable to contamination.

Objective: This study aimed to determine the bacterial contamination of *V. cholerae* carrying the *ompW* and *ctxA* genes on ice preservatives marine product in Kedonganan Fish Market, Badung-Bali.

Method: This study used 3 samples of ice that has not been used to preserve marine products and 20 samples of ice that has been used to preserve marine products. Samples were examined using bacterial culture methods and followed by dPCR assay to detect *ompW* and *ctxA* genes.

Result: The results showed all samples of ice that has not been used preservative marine products are not contaminated by *V. cholerae*, while 16 of 20 (80%) samples of ice that had used contaminated by *V. cholerae* based on *ompW* gene and found no samples that carry *ctxA* gene.

Conclusion: Ice agent (EA) was not contaminated by *V. cholerae*, while use ice sample marine product preservative contaminated with *V. cholerae* with 80% positive and none of isolates carry the gene *ctxA*.

Keywords: *Vibrio cholerae, ompW, ctxA*, duplex polymerase chain reaction

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INTRODUCTION

Infectious disease remains a major health problem that occurs in many developing countries, including Indonesia. Infectious disease is usually caused by infectious agents such as viruses, bacteria, fungi, parasites and prions. One of infectious disease that was being the pandemic in all over the word was cholera disease which caused by *Vibrio cholerae*.[1,2] Manifestation of cholera disease is severe diarrhea with feces resemble rice water, which can quickly lead to dehydration until death.[3,4] Transmission of cholera and its source originated from contaminated water, food, vegetable, ice, fish or fishery.[5,6]

The pathogenic *V. cholerae* produce cholerae toxins encoded by the genes *ctx*.[1] Additionally, *V. cholerae outer membrane protein W* encoded by gene *ompW* help it to survive and adapt in specific body conditions.[7,8] *Outer membrane proteins W* (*ompW*) is commonly used and recommended by many researchers as a specific genetic marker species of *V. cholerae*.[9,10]

Kedonganan Fish Market is the largest fish market in Bali. Ice usually used to maintain freshness and its quality at Pasar Ikan Kedonganan. The ice could probably contain bacteria *V. cholerae*.[11] It is certainly dangerous when ice for marine product preservatives is contaminated by *V. cholerae*. Therefore, the marine product can transmit *V. cholerae* to the consumer.

Duplex PCR is the development of PCR that can be used to detect two types of genes in one running. This method is more practical, time-saving and cost-effective than a single PCR or PCR Uniplex. Some studies used dPCR technique to detect pathogen agent. Yasmon *et al.* (2010) using dPCR for simultaneous detection of *Legionella species* and *Legionella pneumophila* in water samples in Jakarta.[12] Thanananta and Thanananta (2008) detect *E. coli* from water using dPCR with *lacZ* and *uidA* as the target gene.[13]

METHODS

The research was done by these following steps: 1) Sampling 2) Isolation of *V. cholerae* bacteria; 3) Identification of *V. cholerae*; 4) Genomic DNA Extraction of *V. cholerae*; 5) Gene Amplification *ompW* and *ctxA* with dPCR; 6) Electrophoresis dPCR results.

Samples were taken from the ice agent (EA), ice fish preservatives (EPI), ice squid preservative (EPC), ice shrimp preservatives (EPU), ice shells preservatives (EPK) and ice lobster preservatives (EPL). Isolation of *V. cholerae* was started by

culturing in specific media enrichment for Vibrio, Alkaline Peptone Water (APW). One milliliter of each thawed ice sample were taken and were added with 9 ml of APW then were incubated for 6 hours at 37°C. The growth of Vibrio was characterized by the presence of turbidity in the media APW, Furthermore the bacteria were cultured on media Thiosulfate Citrate Bile Salts Sucrose (TCBS), incubated at 37°C for 18-24 hours.

Presumptive colonies of *V. cholerae,* yellow in color, and measuring 2-3 µm, then taken to be were culture on Tryticase Soy Agar (TSA) for further identification. The identification of *V. cholerae* was done by using oxidase strip test. The positive result was indicated by the change of paper color to purple in 5-10 seconds.

Genomic DNA isolation was performed using *Boil Cell Extraction* (BCE) method. DNA amplification was performed using *duplex polymerase chain reaction* (dPCR) method. dPCR reaction to amplify genes *ompW* and *ctxA*, consists of 0,8µl DNA *template*, 0.5 mL of primary ompW 0,5µM concentration ompW-F (5'-CACCAAGAAGGTGACTTTATTGTG-3') ompW-R (5'-GAACTTATAACCACCCGCG-'3),[9] 0.5 mL primer ctxA 0.5M concentration ctxA-F (5'-CTCAGACGGGATTTGTTAGGCACG-'3) ctxA-R (5'-TCTATCTCTGTAGCCCCTATTAC G-'3),[9] 5 mL Master Mix Promega and 3.2 aquabidest. The dPCR protocol was as follows: 95°C for 120 s (1 cycle), 95°C for 60 s, 50°C for 60 s, 72°C for 60 s (35 cycles), and 72°C for 5 minute. The PCR products were examined by electrophoresis in 1.5% (w/v) TAE agarose gel. *V. cholerae* DNA band sizes for *ompW* gene was 588 bp and for *ctxA* gene is 301 bp.

RESULTS

Samples in this study came from Fish on Market Kedonganan Bali, Indonesia. The samples were 23 ice samples consisting of 3 samples of ice those have not used for preserving marine product or ice agent (EA) and 20 samples of ice that have been used to preserve marine products. The data from the isolation and detection of gene identification *ompW* and *ctxA* from ice preservative marine origin are shown in Table 1 and Table 2.

Table 1 **Results of Isolation, Identification and Detection of Gene *ompW* and *ctxA V. cholerae* Origin Sea Ice Preservatives results are yet used in Kedonganan Fish Market**

No	Code	APW	TCBS	Oxidase	dPCR	
					ompW	*ctxA*
1	EA1	√	(-)	-	-	-
2	EA2	√	(-)	-	-	-
3	EA3	√	(-)	-	-	-
Total		3	0	0	0	0
%		100	0%	0%	0%	0%

Description: √ = planted, (+) = positive, (-) = negative, - = not tested, EA= EsAgen (Not to be used as a preservative fish).

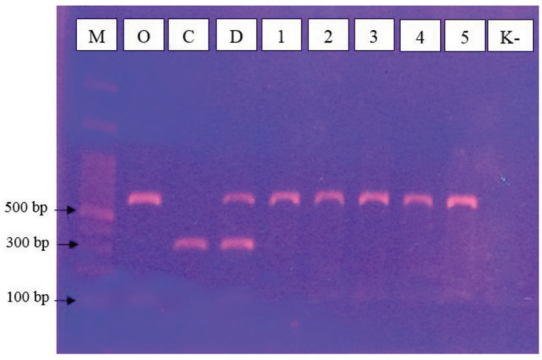

Figure 1 Electropherogram dPCR

Description:
M = Marker 100bp DNA ladder
O = Positive controls *ompW* gene *V. cholerae*
O1 = serotype Inaba
C = positive control *ctxA* gene *V. cholerae*
D = Duplex PCR gene *ompW* and *ctxA*
1 = Es Fish Preservatives 4 (EPI4)
2 = Ice Squid Preservatives 1 (EPC1)
3 = Ice Shrimp Preservatives 1 (EPU1)
4 = Es Crab Preservatives 1 (EPK1)
5 = Es Lobster Preservatives 1 (EPL1)
K = negative control

DISCUSSION

Based on the results obtained, the sample of EA was not contaminated by *V. cholerae*. On the other hands, were dPCR *ompW* genes can showed that use ice sample marine product preservative contaminated with *V. cholerae* with 80% positive and none of the samples carry the gene *ctxA*. Contamination was most likely derived from the marine products. Some studies showed that seafood contaminated by *V. cholerae.*[14,15]

OmpW gene is conserved genes of *V. cholerae* that is used as markers of *V. cholerae* specific species.[9] *OmpW* gene mechanism expression depends on environmental condition.[16] The use of gene *ompW* as a target *V. cholerae* has been widely used to detect a variety of samples, such as environmental and clinical samples.[10,17] In this study, the contamination of ice shrimp preservatives (EPU) and ice lobster preservatives (EPL) was higher than ice fish preservatives (EPI), squid (EPC) and crab (EPK). It was because of the exoskeletons of shrimp and lobster composed of chitin. Even tough chitin has chemotactic against *V. cholerae,* it is known as a source of energy, carbon, and nitrogen for *V. cholerae.*[18]

Table 2 **Results of Isolation, Identification and Detection of Gene *ompW* and *ctxA V. cholerae* Origin Sea Ice Preservatives results are already used in Kedonganan Fish Market**

No	Code	APW	TCBS	Oxidase	dPCR	
					ompW	*ctxA*
1	EPI1	√	(+)	(+)	(+)	(-)
2	EPI2	√	(+)	(+)	(+)	(-)
3	EPI3	√	(+)	(+)	(-)	(-)
4	EPI4	√	(+)	(+)	(+)	(-)
5	EPC1	√	(+)	(+)	(+)	(-)
6	EPC2	√	(+)	(+)	(-)	(-)
7	EPC3	√	(+)	(+)	(-)	(-)
8	EPC4	√	(+)	(+)	(+)	(-)
9	EPU1	√	(+)	(+)	(+)	(-)
10	EPU2	√	(+)	(+)	(+)	(-)
11	EPU3	√	(+)	(+)	(+)	(-)
12	EPU4	√	(+)	(+)	(+)	(-)
13	EPK1	√	(+)	(+)	(+)	(-)
14	EPK2	√	(+)	(+)	(+)	(-)
15	EPK3	√	(+)	(+)	(+)	(-)
16	EPK4	√	(+)	(+)	(-)	(-)
17	EPL1	√	(+)	(+)	(+)	(-)
18	EPL2	√	(+)	(+)	(+)	(-)
19	EPL3	√	(+)	(+)	(+)	(-)
20	EPL4	√	(+)	(+)	(+)	(-)
N		20	20	20	16	0
%		100	100%	100%	80%	0%

Description: √= planted, (+) = positive, (-) = negative, - = not tested, EPI = Ice Fish Preservatives, EPC = Ice Squid Preservatives, EPU = Ice Shrimp Preservatives, EPK = Ice Crab Preservatives, EPL = Ice Lobster Preservatives.

ctxA gene of *V. cholerae* is a gene that plays a role in the incidence of diarrhea in a cholera outbreak. The existence of *ctx* genes in *V. cholerae* bacteria derived from environmental samples are extremely rare, unlike the case with *V. cholerae* derived from clinical cases.[17,19,20] Although *V. cholerae* which were identified did not carry the gene *ctxA*, it does not mean that the bacteria cannot cause disease. *V. cholerae* has several virulence factor genes that can cause diarrhea besides *ctx* genes, such as *Zot* and *Ace* gene.[1]

CONCLUSION

As the conclusion in this study we found that ice agent (EA) was not contaminated by *V. cholerae*, while use ice sample marine product preservative contaminated with *V. cholerae* with 80% positive and non of the isolates samples carry the gene *ctxA*.

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