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# Rapid Detection of Virulence Genes in *Vibrio cholerae* from Edible Ice in Jakarta

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*Vibrio cholerae* is a bacteria that lives naturally in an aquatic environment. It causes a waterborne disease which is called cholera. Infection of waterborne disease occurs via the fecal-oral route, mostly through drinking water. As we know, ice is made from city water sources and it is commonly used in beverages. Most of publications about *V.cholerae* come from clinical samples, while little is known about the presence of these bacteria in potable water, especially in ice. In this study, we isolated *V. cholerae* from ice in Jakarta and continued with detection of the virulence genes. We recovered *V. cholerae* from ice samples and then continued with detection of virulence genes including *toxR, ctxA, ompU, tcpA, ace, zot* using multiplex PCR. The results indicated that all of the samples were non-toxigenic strains, but were classified as pathogenic strains because they have at least one of the virulence genes present. The presence of pathogenic *V. cholerae* in edible ice needs to be emphasized since they have some of the virulence factors and also the class 1 integron.

*Keywords: Vibrio cholerae, ice, multiplex PCR, virulence genes,*

*Vibrio cholerae* is primarily an inhabitant of the aquatic environment and water plays an important role in the transmission and epidemiology of cholera. Infection of waterborne disease happens via fecal-oral route, mostly through drinking water.

The majority of beverages in Jakarta are provided with ice cubes, made from local water sources. Since Indonesia lacks a public-health inspection program for monitoring ice quality, we suspect that ice may be a major source of water borne diseases, especially bacterial enteropathogens including *V. cholerae*. Most publications on *V. cholerae* come from clinical samples while little is known about the presence of these bacteria in local water, especially in the form of ice.

The pathogenicity of *V. cholerae* depends on combination properties of its virulence genes. There are at least six virulence genes in *V. cholerae*, the regulator factor *toxR, cholera toxin enzymatic subunit* A (*ctxA*), *toxin-coregulated pilus* (*tcpA*), *outer membrane protein* (*ompU*), *accessory cholera toxin* (*ace*) and *zonula occludens toxin* (*zot*). ToxR regulated genes are divided into four general classes, namely cholera toxin (CTX) genes, toxin co-regulated pilus (TCP) genes, accessory colonization factor (ACF) genes and ToxR-activated genes (TAG) of unknown function (Peterson and Mekalanos 1988; Matson *et al.* 2007). Toxin co-regulated pilus (TCP) has the function of being an essential colonization factor (Taylor *et al.* 1987).

The A and B subunits of cholera toxin are encoded by the ctx operon. It is now known that the ctx genes lie within the genome of a lysogenic filamentous phage (CTXö). OmpU protein has the potential role in the adhesion of *V. cholerae* to mammalian cells. The accessory cholera enterotoxin (*ace*) has recently been identified as a third toxin of *V. cholerae* which alters ion transport and causes fluid accumulation. Zonula occludens toxin (*zot*) has potential function to increase the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction (Baudry *et al.* 1992).

Here we report the isolation of *V. cholerae* from edible ice in Jakarta and the detection the presence of virulence genes using multiplex PCR.

## MATERIALS AND METHODS

**Sample Collection.** Ice cubes were collected from five different regions of Jakarta. Each region of Jakarta was represented by three ice suppliers. We also collected ice cubes from street vendors and restaurants around Jakarta. Ice cubes were collected and melted at room temperature. Aliquots of samples were combined with Alkaline Peptone Water (APW) (Oxoid) with a ratio 1:1. Incubations were undertaken for 24 hours at 37ºC.

**Isolation of *V. cholerae*.** Enriched samples in APW were diluted by adding 0.85% of NaCl (w/v). Diluted samples were spread onto thiosulfate-citrate-bile-sucrose (TCBS) (Oxoid) agar plates and incubated at 37ºC for 18 to 24 hours. Yellow single colonies which grown on TCBS plates were randomly selected for PCR detection. Each sample was chosen from 10-15 single colonies. The selected colonies were grown overnight at 37ºC.

**Preparation of PCR Template.** A 5 mL aliquot of Luria Broth (LB) was inoculated with the selected colony to obtain template DNA for multiplex PCR. Incubation was done for 24 hours at 37ºC with shaking at 100 rpm. To prepare template DNA, 1.5 mL of the culture was centrifuged, resuspended in sterile distilled water, and boiled for 10 min.

**Multiplex PCR Assay.** A master mix was prepared for all of the samples into one reaction tube. The master mix for each sample contains 25 µL of Go-Taq Master Mix Kit (Promega), 10.5 µL of $ddH_2O$ and 12 µL of primers. We used six pairs of primer for detection of six virulence genes of *V.*

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*cholerae*, consisting of *toxR, ctxA, ompU, tcpA, ace, zot* 25 pmol.uL$^{-1}$ for each primer (Eurogentec). The sequences of the primers are listed in Table 1. Isolates from a previous study were used as a positive control. The multiplex PCR reaction was carried out as described by Singh *et al.* (2002). PCR products were separated by 1.8% w/v agarose gel (Promega) in 0.5x TBE buffer. The gel was visualized using Gel-Doc (Biorad).

**Serological Assay.** The *V. cholera* strains which were found to possess one or another of the virulence genes sought were examined for agglutination by the somatic-O-antigen sero-grouping by using polyvalent-O and monovalent-Ogawa and Inaba serum (Biofarma, Bandung, Indonesia). Some of the isolates were confirmed as virulent through biochemical assays by using Microbact™ GNB 12A + 12B (Oxoid).

**Antimicrobial Resistance Assays**. Positive colonies were inoculated on brain-heart-infusion-agar (BHIA) (Oxoid) plates using cotton swabs and seven antimicrobial disc (Oxoid) was put on the surface of an agar plate (streptomycin 10 µg, tetracycline 30 µg, erythromycin 15 µg, ciprofloxacin 5 µg, kanamycin 30 µg, sulphamethaxole-trimethoprim 25 µg, ampycilin 10 µg). Each disc must be pressed down to ensure complete contact with the agar surface. Plates were incubated overnight at 37ºC. The zone of inhibition was measured by reduced diameter of the clear zone with in the diameter of the disc. The results were analyzed according to the 2003 NCCLS standard.

## RESULTS

From 15 ice samples, we selected 330 isolates to be detected for the presence of virulence genes and the class 1 integron. Each sample was alternated by 15 until 20 isolates. All of the presumptive colonies of *V. cholerae* were analyzed by multiplex PCR assay (Fig 1). The results show that there are 92.06% isolates which were positive for *toxR*, 15.87% which were positive for *ompU*, 7.94% which were positive for *zot* and *tcp*, 87.3% which were positive for the class 1 integron. Five isolates showed negative for *tox*R detection, but these isolates were positive for *tcp*A gene detection (Table 2). None of the isolates were positive for *ctx* or *ace*, indicating that all of the isolates were non-toxigenic strains because they having at least one virulence gene. We used 7 antibiotics (streptomycin 10 µg, tetracycline 30 µg, erythromycin 15 µg, ciprofloxacin 5 µg, kanamycin 30 µg, sulphamethaxole-trimethoprim 25 µg, ampycilin 10 µg) to showed the occurrence of antibiotic resistance gene. All of the isolates have at least one antibiotic resistant gene (Table 3). There are 53.97% that resistant to ampycilin, 44.44% that resistant to sulphamethaxole-trimethoprim, 58.73% that resistant to erythromycin, 66.67% that resistant to streptomycin, 50.79% that resistant to tetracycline, 25.40% that resistant to ciprofloxacin, and 66.67% that resistant to kanamycin. Result of serological assay showed only eight isolates were belonging to O1 serogroup, 1 isolate is Inaba-O1 and 7 isolates are Ogawa-O1. Therefore most of the isolates belong to the non-O1 serogroup (Table 2).

## DISCUSSION

**Multiplex PCR.** The presence of ToxR protein is important for the regulation of other virulence genes in pathogenic *V. cholerae* (Bina *et al.* 2003; Stonehouse *et al.* 2008). Usually the presence of *tox*R gene is important for the function of other virulence genes, since it play as a

Table 1 Primer sequence used in these study

| Primer | Sequences | Amplicon size (bp) | Tm | References |
|---|---|---|---|---|
| *ctxA*-F | CGGGCAGATTCTAGACCTCCTG | 564 | 53.4 | Fields *et al.* (1992) |
| *ctxA*-R | CGATGATCTTGGAGCATTCCCAC | | 51.9 | |
| *zot*-F | TCGCTTAACGATGGCGCGTTTT | 947 | 49.7 | Rivera *et al.* (2001) |
| *zot*-R | AACCCCGTTTCACTTCTACCCA | | 49.7 | |
| *ace*-F | TAAGGATGTGCTTATGATGGACACCC | 316 | 52.9 | Shi *et al.* (1998) |
| *ace*-B | CGTGATGAATAAAGATACTCATAGG | | 47.7 | |
| *ompU*-F | ACGCTGACGGAATCAACCAAAG | 869 | 49.7 | Rivera *et al.* (2001) |
| *ompU*-B | GCGGAAGTTTGGCTTGAAGTAG | | 49.7 | |
| *tcpA*-F | CACGATAAGAAAACCGGTCAAGAG | 620 | 50.6 | Rivera *et al.* (2001) |
| *tcpA*-B | TTACCAAATGCAACGCCGAATG | | 47.9 | |
| *toxR*-F | CCTTCGATCCCCTAAGCAATAC | 779 | 49.7 | Rivera *et al.* (2001) |
| *toxR*-B | AGGGTTAGCAACGATGCGTAAG | | 49.7 | |

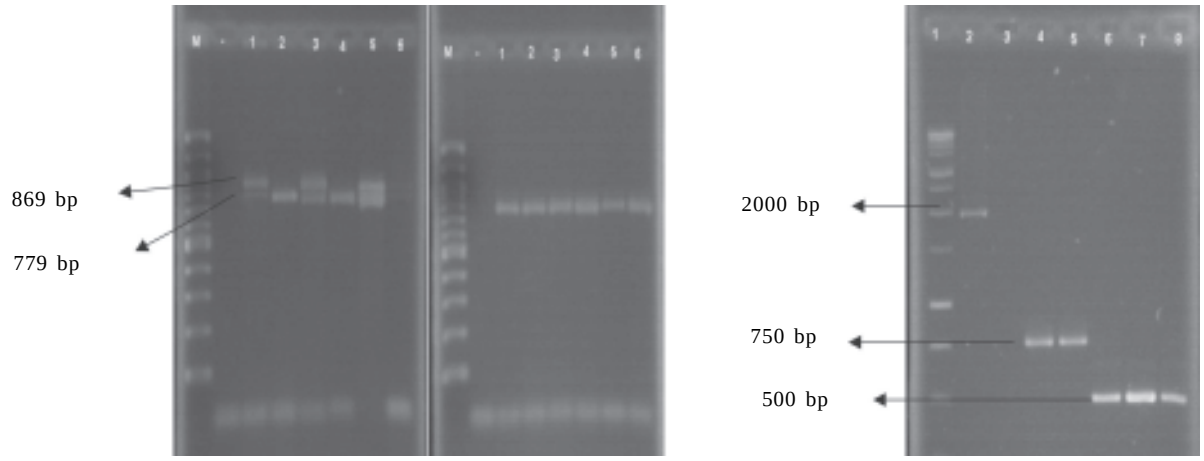


Fig 1 Results of multiplex PCR and PCR of class 1 integron detection (Left) M, marker 100 bp (New England BioLabs); - negative control; 1, 3, 5, 6, isolates JA, MH, WL, JG *toxR* and *ompU* positive; 2 and 4, isolates JC, LE *toxR* positive. (Centre) M, marker 100bp (New England BioLabs); -, negative control; 1, 2, 3, 4, 5, 6, isolates JM, JH, BB, CA, PC, MC *toxR* positive. (Right) 1, marker 1 kb ladder (Fermentas); 2, positive control (Waturangi *et al.* 2003); 3, negative control; 4 and 5, class 1 integron with the amplicon size 750 bp; lane 6, 7, 8, class 1 integron with the amplicon size 500 bp.

regulator for other genes (Matson *et al.* 2007). One of the possibilities is horizontal gene transfer from pathogenic strains to non-pathogenic strains, and the transfer is not included the *tox*R gene (Covacci *et al.* 1997). Our five isolates were also negative in *ctx*A detection. This is also contrary to the current assumption that most cholera toxin (CT)-positive isolates are also positive for TCP. None of the detected isolates showed a positive result for *ctxA* and *ace* which indicates that all of the isolates do not have the genetic potential to produce CT.

OmpU has a potential role in the adhesion of *V. cholerae* to mammalian cells. Five isolates showed positive result in *zot* gene detection. The presence of these protein could increase the permeability of the small intestinal mucosa by affecting the structure of the intracellular tight junction (Baudry *et al.* 1992). Although there are a lot of isolates which do not have any virulence genes detected in this study except for the *tox*R gene, it does not indicate that these bacteria could not evoke diarrhea. Because there are some cases of mild diarrhea that are caused by non-pathogenic *V. cholerae* by an unknown mechanism (Ramamurthy *et al.* 1993), and because there are some virulence genes that have not been detected in the present study. These non-pathogenic strains may be pathogenic strains, referenced by the current hypothesis that some pathogenic bacteria have evolved from non-pathogenic strains of the same species via horizontal transfer of virulence genes (Covacci *et al.* 1997).

**Serological and Antimicrobial Resistance Assay.** Our results cannot indicate that the non-O1 serogroup bacteria are non-pathogenic strains. Because the non-O1 strains have been reported to be involved in the emergence of a newer variant of *V.cholerae*, O139 strains could occur in epidemic and pandemic forms (Bik *et al.* 1995). Also, a lot of isolates in the present study contain many virulence genes. There are several isolates showed resistance to almost all of the antibiotic resistance genes. This finding need to aware since the virulence properties of the isolates and their relation with diarrheal disease.

## ACKNOWLEDGEMENT

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Table 2 Results of multiplex PCR and serological assay

| Region | Sample | toxR | ctxA | ompU | ace | zot | tcpA | Serogroup |
|---|---|---|---|---|---|---|---|---|
| West Jakarta | JC | v | - | - | - | - | - | Non-O1 |
| | JA | v | - | v | - | - | - | Non-O1 |
| | JE | v | - | - | - | - | - | Non-O1 |
| | JT | v | - | - | - | - | - | Non-O1 |
| | JF | v | - | v | - | - | - | Inaba-O1 |
| | LA | v | - | - | - | - | - | Non-O1 |
| | LB | v | - | - | - | - | - | Non-O1 |
| | LC | v | - | - | - | - | - | Non-O1 |
| | JD | v | - | - | - | v | - | Non-O1 |
| | JG | v | - | v | - | - | - | Ogawa-O1 |
| | GH | v | - | - | - | - | - | Non-O1 |
| | GI | v | - | - | - | - | - | Non-O1 |
| | LE | v | - | - | - | - | - | Non-O1 |
| | LK | v | - | - | - | - | - | Non-O1 |
| | JB | v | - | - | - | - | - | Non-O1 |
| | LH | v | - | - | - | - | - | Non-O1 |
| | JM | v | - | - | - | - | - | Non-O1 |
| | JH | v | - | - | - | - | - | Non-O1 |
| | LM | v | - | - | - | - | - | Non-O1 |
| Central Jakarta | MG | v | - | - | - | - | - | Non-O1 |
| | OG | - | - | - | - | - | v | Non-O1 |
| | OJ | - | - | - | - | - | v | Non-O1 |
| | OB | - | - | - | - | - | v | Ogawa-O1 |
| | OD | - | - | - | - | - | v | Non-O1 |
| | OE | - | - | - | - | - | v | Non-O1 |
| | NB | v | - | - | - | - | - | Non-O1 |
| | NJ | v | - | - | - | - | - | Non-O1 |
| | OK | v | - | - | - | - | - | Non-O1 |
| | QF | v | - | - | - | - | - | Non-O1 |
| | MH | v | - | v | - | - | - | Ogawa-O1 |
| | PC | v | - | - | - | - | - | Non-O1 |
| | MC | v | - | - | - | - | - | Non-O1 |
| | QH | v | - | - | - | - | - | Non-O1 |
| | XF | v | - | - | - | - | - | Non-O1 |
| | XG | v | - | - | - | - | - | Non-O1 |
| | XA | v | - | - | - | - | - | Non-O1 |
| | VB | v | - | - | - | - | - | Non-O1 |
| | VL | v | - | - | - | - | - | Non-O1 |
| | WK | v | - | v | - | - | - | Ogawa-O1 |
| | WD | v | - | v | - | - | - | Ogawa-O1 |
| | WC | v | - | v | - | - | - | Ogawa-O1 |
| | WE | v | - | v | - | - | - | Non-O1 |
| | WF | v | - | v | - | - | - | Ogawa-O1 |
| | WL | v | - | v | - | - | - | Non-O1 |
| | VI | v | - | - | - | - | - | Non-O1 |
| North Jakarta | DF | v | - | - | - | v | - | Non-O1 |
| | DK | v | - | - | - | - | - | Non-O1 |
| | DB | v | - | - | - | v | - | Non-O1 |
| | DA | v | - | - | - | v | - | Non-O1 |
| | DC | v | - | - | - | - | - | Non-O1 |
| | DE | v | - | - | - | v | - | Non-O1 |
| South Jakarta | BB | v | - | - | - | - | - | Non-O1 |
| | BE | v | - | - | - | - | - | Non-O1 |
| | BA | v | - | - | - | - | - | Non-O1 |
| | EG | v | - | - | - | - | - | Non-O1 |
| | EK | v | - | - | - | - | - | Non-O1 |
| | EG | v | - | - | - | - | - | Non-O1 |
| | EH | v | - | - | - | - | - | Non-O1 |
| | EC | v | - | - | - | - | - | Non-O1 |
| East Jakarta | CA | v | - | - | - | - | - | Non-O1 |
| | CL | v | - | - | - | - | - | Non-O1 |
| | CB | v | - | - | - | - | - | Non-O1 |
| | CE | v | - | - | - | - | - | Non-O1 |

Table 3 Results of class 1 integron detection and antibiotic resistance assay

| Region | Sample | Amp | SXT | E | S | TE | CIP | K |
|---|---|---|---|---|---|---|---|---|
| West Jakarta | JC | S | S | I | R | R | R | I |
| | JA | S | R | R | R | S | I | R |
| | JE | S | S | S | I | S | S | S |
| | JT | R | R | R | R | R | I | R |
| | JF | S | S | R | R | R | R | I |
| | LA | S | S | I | R | S | S | R |
| | LB | S | I | I | R | S | S | R |
| | LC | S | S | I | R | S | S | R |
| | JD | S | S | R | R | R | R | I |
| | JG | S | S | R | S | S | R | R |
| | GH | R | R | I | S | R | R | R |
| | GI | S | S | R | S | S | S | S |
| | LE | R | R | R | R | R | I | I |
| | LK | S | S | R | I | R | S | S |
| | JB | S | S | R | R | R | I | R |
| | LH | R | R | R | I | R | R | S |
| | JM | R | S | I | R | S | S | S |
| | JH | R | R | R | I | S | S | R |
| | LM | S | S | S | I | R | S | R |
| Central Jakarta | MG | R | R | R | R | I | S | S |
| | OG | R | R | R | R | R | I | I |
| | OJ | R | R | R | R | I | S | R |
| | OB | R | S | S | S | I | I | R |
| | OD | R | I | R | R | R | I | R |
| | OE | R | R | R | R | R | I | R |
| | NB | R | R | R | R | I | I | S |
| | NJ | R | R | R | R | R | S | R |
| | OK | R | R | R | R | R | I | I |
| | QF | S | I | I | I | R | S | R |
| | MH | R | R | R | R | S | S | S |
| | PC | S | I | S | R | S | S | R |
| | MC | R | R | R | I | I | I | R |
| | QH | R | I | S | R | R | I | R |
| | XF | S | S | S | I | I | R | R |
| | XG | S | S | I | I | I | R | S |
| | XA | S | S | S | R | R | R | R |
| | VB | R | R | R | R | R | I | I |
| | VL | R | R | S | S | S | S | R |
| | WK | R | R | R | R | R | I | R |
| | WD | R | R | R | R | I | R | R |
| | WC | R | R | R | R | R | I | R |
| | WE | R | R | R | R | R | I | R |
| | WF | R | R | R | R | R | I | I |
| | WL | R | R | R | R | R | R | I |
| | VI | S | S | S | R | I | S | R |
| North Jakarta | DF | R | R | R | R | I | S | R |
| | DK | S | I | I | R | S | S | R |
| | DB | S | S | I | R | S | S | R |
| | DA | S | S | I | R | S | S | R |
| | DC | S | I | S | R | S | S | R |
| | DE | S | I | I | R | S | S | R |
| South Jakarta | BB | R | I | R | R | R | I | S |
| | BE | R | R | R | R | R | I | R |
| | BA | R | I | I | S | S | S | R |
| | EG | S | I | R | S | R | I | R |
| | EK | R | I | I | S | S | R | I |
| | EG | R | R | S | S | S | I | R |
| | EH | R | R | R | S | S | S | I |
| | EC | S | I | R | R | R | I | R |
| East Jakarta | CA | S | S | I | I | R | R | R |
| | CL | R | R | R | I | R | R | R |
| | CB | S | I | R | R | R | R | R |
| | CE | S | S | R | R | R | R | R |

Amp, Ampycilin; SXT, sulphamethaxole-trimethoprim; E, erythromycin; S, streptomycin; TE, tetracycline; CIP, ciprofloxacin; K, kanamycin, R = resistant; I, intermediate; S, sensitive; v, present.

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