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SHORT COMMUNICATION

Antibiotic Resistance and Integron of *Vibrio cholerae* Detection from School Street Foods in Jakarta

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Street foods represent foods and beverages prepared by vendors in streets or other public places, i.e. schools. Food safety issues perceive street foods as a potential major public risk. Street foods contaminated with toxigenic *Vibrio cholerae* may lead to serious poisoning to school-age children. In this study, 17 isolates of *V. cholerae* were obtained from nine (45%) of total 20 street foods samples collected in Jakarta. Five (29%) were confirmed to be *V. cholerae* O1, serotype Ogawa using biochemical tests and serological identification. Of the 17 *V. cholerae* isolates 47% proved to be resistant to ampicillin, 35% to trimethoprim, 17.6% to tetracycline, and 17.6% to streptomycin. A class 1 integrons bearing streptomycin/spectinomycin resistant gene cassette of *aad*A1c were discovered on isolate Vc25n. This may leads to horizontal transfer of the antibiotic resistant genes to other bacteria.

Key words: foods, *Vibrio cholerae*, antibiotic resistance, integron

Street foods defined as foods and beverages prepared and sold by vendors in streets and other public places for immediate consumption without further processing or preparation (WHO 1996). Street foods or its equivalent "street-vended foods" which are largely an urban phenomenon in developing countries. Food hygiene of street vended food and personnel hygiene of the vendors have been a great concern in developing country especially in Asia and Pacific (FAO 2004). In Jakarta, they can be found in clusters around places of work, hospitals, railway stations, bus stations and schools. In schools, the subject of children as consumers of street foods deserves special attention related to their potential for serious food poisoning outbreaks, particularly due to the microbiological contamination.

Cholera is a persistent life-threatening diarrheal disease in Indonesia, caused by *Vibrio cholerae* and transmitted through water and contaminated foods. *Vibrio cholerae* is classified in serogroups according to its somatic antigen O. Serotypes Ogawa, Inaba, and rarely found Hikojima are part of the O1 group. Particularly in Indonesia, seven years of surveillance efforts throughout Indonesian archipelago (1993-1999) showed that *V. cholerae* O1, Ogawa serotype was predominant etiology in all 17 investigated diarrheal outbreaks (Simanjuntak *et al*. 2001). In recent community-based surveillance study in North Jakarta has found an overall incidence of cholera of 0.5 per 1000 diarrheal cases per year, with the highest incidence (4.0 per 1000 diarrheal cases per year) occurred in young children (Agtini *et al.* 2005).

Vibrio cholerae begins the abrupt onset of watery diarrhea within incubation period of 6 to 48 hours. Vomit and initial stool may exceed one liter, leading to hypovolemic shock. Muscle cramps may accompany as water and electrolytes are lost from body tissues. Weak pulse, loss of skin turgor and scaphoid abdomen are characteristics of cholera. The disease runs its course in two to seven days; the outcome depends upon the extent of water and electrolyte loss, and the adequacy of water and electrolyte repletion therapy (Sack *et al.* 2004). Watery diarrhea may rapidly lead to metabolic acidosis, potassium depletion, and ultimately vascular collapse and death if treatment is not promptly given.

Treatments for cholera patients consist of rehydration and antibiotic therapy. The rehydration therapy can be conducted through oral rehydration salt (ORS) solution or intravenous (Ringer's lactate). Antibiotic therapy is essential in treating cholera patients (Sack *et al.* 1978). Antibiotic agents reduce the duration of illness, the volume of stool, and duration of shedding of *Vibrios* in the feces (Sack *et al.* 2004). â-lactam antibiotics such as ampicillin and aminoglycosides group such as streptomycin are commonly used as antibiotic agents in the treatment of infection by both gram-negative (e.g. *Vibrio*) and gram-positive organisms. Other antibiotic agents for treating cholera patients are tetracycline, trimethoprim-sulfametho-xazole, erythromycin, ciprofloxacin, and azithromycin (WHO 1999; Sack *et al.* 2004).

Due to increasing of resistancy level and the emergence of multi-antibiotic resistance microorganism, i.e. *V. cholerae* (Sack *et al*. 2001; Shi *et al*. 2006), the determination of *V. cholerae* susceptibility becomes crucial for the optimal antibiotic therapy (Fluit *et al.* 2001). Emergence of resistance to multiple antibiotics is a serious clinical problem to the treatment and containment of the cholera disease, specifically since antibiotic resistant *V. cholerae* were found during 1995-2001 from diarrheal patients in provinces of Indonesia (Tjaniadi *et al.* 2003). Selecting antibiotic for treatment of cholera

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patients is more essential nowadays since changes in the drug sensitivity pattern were observed recently in *V. cholerae* O1 (Iwanaga *et al.* 2004) and discoveries of Superintegrons (SIs) in the *V. cholerae* genome (Biskri *et al.* 2005).

Integrons are genetic element capable to incorporate resistance genes (cassette-associated genes) by site-specific recombination then convert them to functional genes. Integron have been characterized into four different classes according to sequences of their integrase (*int*) genes, and those most frequently detected in clinical isolates belong to class I (Iwanaga *et al*. 2004). Some information and studies are available for the distribution and importance of class 1 integrons in encoding antibiotic resistance in *V. cholerae* (Falbo *et al.* 1999; Dalsgaard *et al.* 2000a; Dalsgaard *et al.* 2000b; Dalsgaard *et al.* 2001).

Integrons consist of an integrase gene (*intI*), a recombination site (*attI*), and a resident promoter (Pc). The integrase mediates site-specific recombination between the *attI* site and a target recombination sequence termed 59-base element (or *attC* site). The 59-base element is usually found with a single open reading frame (ORF) associated in a covalently closed circular structure, called a gene cassette (Stokes & Hall 1989; Hall *et al.* 1991; Collis & Hall 1992). Nucleid acid-based detection systems often offer rapid and sensitive methods to detect presence of cassette genes. Currently, more than 70 different antibiotic resistant cassette genes have been characterized in integrons (Fluit & Schmitz 2004).

Two kinds of beverages per school were collected (July through August, 2005) from street food vendors in five primary school of North Jakarta and five of South Jakarta. Collected street foods samples were immediately placed in cooler box in order to maintain the microbial number in samples during transportation to the laboratory.

In order to isolate the bacteria, beverages samples were filtered using sterile filter paper, using microbiology aseptic procedures and transferred to micro-filter vacuum pump containing 0.2 ìm filter membrane (Millipore). The filter membrane containing expected bacteria was then placed in tryptone soya rich enrichment broth and incubated at 37 ^{o}C. Inoculated enrichment broth was then subcultured to thiosulfate citrate bile-salt sucrose (TCBS) agar after 24 hr of incubation for 18-24 hours incubation period at 37 ^{o}C.

Yellow shiny colonies resembling those of *V. cholerae* were picked from TCBS agar and identified by biochemical and serologic methods (WHO 1999). *Vibrio cholerae* isolates were confirmed on the basis of following criteria: (i) oxidase positive, (ii) lysine decarboxilase (LD) positive, (iii) producing acid slant over an acid butt on Triple Sugar Iron Agar (TSIA), and (iv) producing alkaline slant over an acid butt on Kligler's Iron Agar (KIA). Oxidase test conducted with fresh growth suspected *V. cholerae* isolates from Heart Infusion Agar (Oxoid, Hampshire, England) smeared in filter papers containing oxidase reagent N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (Merck). TSIA and KIA slants are inoculated with suspected *V. cholerae* by stabbing the butt and streaking the surface of the medium, then the result examined after 18-24 hours of incubation period at 37 ^{o}C. Lysine decarboxilase Broth inoculated with suspected bacteria then examined after incubation for 18-24 hours at 37 ^{o}C.

Isolates agglutinating to *V. cholerae* polyvalent O1 antiserum (Biofarma, Bandung, Indonesia) were further characterized by serology with Ogawa- and Inaba- specific monovalent antisera (Biofarma, Bandung, Indonesia) using slide agglutination procedures (WHO 1999). This serological test was conducted using control positive of *V. cholerae* serotype Inaba from clinical isolate provided by *Balai Pengembangan Laboratorium Kesehatan* (BPLK) Ministry of Health. Fresh growths from nonselective agar (HIA) were used after less than 18 hours incubation at 37 ^{o}C to conduct serology tests.

Antibiotic resistance testing of all 17 *V. cholerae* isolates was performed by using disc diffusion test (Kirby-Bauer methods). Isolates were assessed as being resistant, intermediate or susceptible to ampicillin (10 µg), streptomycin (10 µg), trimethoprim (5 µg) and tetracycline (30 µg) (Oxoid, Hampshire, England) according to standard cut-off zone sizes according to the National Committee for Clinical Laboratory Standards (NCCLS) document M100-S9 (NCCLS 1999).

Whole-cell DNA of all 17 *V. cholerae* isolates, including five *V. cholerae* O1 serotype Ogawa isolates was screened for the presence of class 1 integrons by PCR (94 ^{o}C 2'; 55 ^{o}C 2'; 72 ^{o}C 3') using Platinum *Taq* polymerase (Invitrogen, Groningen, The Netherlands) and primers corresponding to the 5' Conserved Sequence (CS) (5' GGCATCCAAGC AGCAAG 3') and 3'CS (5' AAGCA GACTTGACCTGA 3') regions of class 1 integrons (Levesque *et al.* 1995). PCR Detection of class 1 integron was using positive control of *Escherichia coli* Vy2a and Vi5a (*Int*$^{+}$) (Waturangi *et al.* 2003).

The PCR amplified were sequenced by using a Big Dye® Terminator v3.1 Cycle Sequencing Kit Reagent (Applied Biosystems, Foster City, Calif). Products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). The identities of the DNA sequences determined were analyzed by comparison with the gene sequences in the databases using ORF Finder and BLAST nucleotide tools from NCBI (http://www.ncbi .nlm.gov).

Total of 17 suspected *V. cholerae* isolates was found from nine (45%) variety of street foods (beverages) of total 20 beverages collected, which were sold by vendor outside ten different primary schools in Jakarta (Table 1). Five and twelve *V. cholerae* isolates were obtained from primary school in North and South Jakarta, respectively. Five (29%) of 17 *V. cholerae* isolates showed agglutinations with polyvalent O1 antiserum, while the other 12 isolates are considered as *V. cholerae* non-O1. All 5 isolates of *V. cholerae* showed positive reactions with Ogawa antiserum and negative with Inaba antiserum (Table 2).

Detection of integron using specific primer showed one of the 5 *V. cholerae* serotype Ogawa isolates yielded ~1.1 kb amplicon with primers corresponding to the 5' Conserved Sequence (5'CS) and 3'CS regions of class 1 integrons. Sequence analysis of the amplicon showed a *NTP_transf_2* cassette gene for streptomycin-spectinomycin (Sp-Sm) resistance was carried by class 1 integron in *V. cholerae* serotype Ogawa isolated from street foods in Jakarta. The

predicted product of the 567 bp *NTP_transf_2* cassette gene (Figure 1) consists of 188 amino acids.

Findings of *V. cholerae* isolates were the first indication of poor sanitation of street foods sold in primary schools in Jakarta. Then this indication made street foods need further attention if consumed by school-age children in Jakarta.

Antibiotic resistance test performed *V. cholera* isolates from street foods in Jakarta showed resistance to commonly used first line antibiotics in developing countries in cholera treatment (Saha *et al*. 2005). As many as 47% of the isolates resistance to Ampicillin (25 ìg), 35% resistance to Trimethoprim (5 ìg), 17.6% resistance to Tetracycline (30 ìg), and 17.6% resistance to Streptomycin (10 ìg). Findings from this research will benefit further developments of cholera antibiotics therapy.

The predominance occurrence of *V. cholerae* of Ogawa serotype to Inaba serotype in street foods collected in Jakarta might correlated with previously cholera cases study, where *V. cholerae* Ogawa was notable as the ubiquitous predominant cholera causing agent (98%) in sporadic diarrheal disease in Indonesia (Simanjuntak *et al*. 2001). Antibiotic resistance determination showed *V. cholera* isolates obtained from street foods in Jakarta showed resistance to commonly used first line antibiotics.

The predicted product of 567 bp *NTP_transf_2* cassette gene 98% identical *aad*A1 genes that encoded streptomycin-spectinomycin adenyltransferase protein of *V. cholerae* (GenBank accession no. BAE66662). The initiation codon, ATG, of the streptomycin and spectinomycin resistance gene cassette is located at bp 76, and the gene continues for the next 536 bp, or 178 amino acids. The gene was designated *aad*A1c because it has the same features as the known and characterized *aad*A1 genes but with a different sequence. An *aadA* gene encodes adenylylation enzyme that modifies streptomycin and spectinomycin, and *aadA* genes are the only characterized genes that encode both streptomycin and spectinomycin resistance. The presence of *aadA* genes related with class 1 integrons in *V. cholerae* O1 strains also have been reported in various regions such as Vietnam (Dalsgaard *et al.* 1999) and Thailand (Dalsgaard *et al.* 2000b). A ribotyping study should be conducted to ascertain whether the O1 strains showed a ribotype R1 identical to O1 strains that established cholera emergence from both previous studies. Thus, it can determine it is likely or not O1 strains of *V. cholerae* in Indonesia were transferred between Vietnam or Thailand and potential of causing an emergence.

Moreover, detection of integron and characterization of antibiotic resistance gene cassette within *V. cholerae* isolated in Jakarta are important in understanding the epidemiology of *V. cholerae*, particularly in Indonesia. This finding is also expected to be a contribution for cholera treatment in Indonesia.

Further study need to be conducted about *aad*A1, other *aad*A genes, and also other antibiotic resistance genes encoded in class 1 integrons and their association including their possibility of transfer to other bacteria. Findings of

Table 1. Sample collection from primary schools in Jakarta (July-August 2005)

Observation dates	School	District	Street food samples
27/07/2005	SDN A	North Jakarta	a Shreded iced with coconut slices (Doger) drinks b Orange-flavored drinks
03/08/2005	SDN B	North Jakarta	a Pineapple-flavored drinks b Mixed-flavor drinks
03/08/2005	SDN C	North Jakarta	a Sour sop-flavored drinks b Iced Nata-de-coco drinks
08/08/2005	SDN D	North Jakarta	a Green-artificial lemonades b Mixed-fruit drinks
08/08/2005	SD E	North Jakarta	a Orange-flavoured drinks b Iced Tea
27/07/2005	SDN P	South Jakarta	a Blue-artificial lemonades b Iced Sirsak drinks
22/08/2005	SDN Q	South Jakarta	a Cocoa-flavored drinks b Grape-flavored drinks
22/08/2005	SD R	South Jakarta	a Cocoa-flavored drinks b Iced Tea
29/08/2005	SDN S	South Jakarta	a Yellow-artificial lemonades b Iced Tea
29/08/2005	SDN T	South Jakarta	a Cocoa-flavored drinks b Iced Tea

Table 2. *Vibrio cholerae* serological and antibiotic resistance tests

V. cholerae biochemically positive	School	Polyvalent O1 antiserum	Monovalent Ogawa/Inaba antiserum	Antibiotic resistance testing (Kirby-Bauer Methods)							
				Amp 10		S 10		W 5		Te 30	
2	South Jakarta	Positive	Ogawa	7	R	20	S	13	I	12	R
23	North Jakarta	Negative	Negative	16	I	18	S	0	R	25	S
25	North Jakarta	Positive	Ogawa	12	R	13	I	15	I	20	S
28	North Jakarta	Negative	Negative	16	I	19	S	0	R	28	S
30	North Jakarta	Positive	Ogawa	18	S	19	S	15	I	26	S
40	South Jakarta	Negative	Negative	18	S	18	S	0	R	25	S
54	South Jakarta	Negative	Negative	18	S	18	S	27	S	22	S
73	North Jakarta	Positive	Ogawa	0	R	18	S	12	I	17	I
81	South Jakarta	Negative	Negative	25	S	23	S	14	I	29	S
82	South Jakarta	Negative	Negative	0	R	8	R	26	S	17	I
83	South Jakarta	Negative	Negative	18	S	0	R	0	R	29	S
87	South Jakarta	Negative	Negative	16	I	12	I	14	I	28	S
93	South Jakarta	Negative	Negative	0	R	10	R	11	I	21	S
94	South Jakarta	Positive	Ogawa	0	R	14	I	0	R	0	R
96	South Jakarta	Negative	Negative	0	R	23	S	0	R	22	S
97	South Jakarta	Negative	Negative	16	I	16	S	15	I	25	S
111	South Jakarta	Negative	Negative	0	R	16	S	15	I	0	R

Amp: Ampicilin, S: Streptomycin, W: Trimethoprim, Te: Tetracycline, R: resistance, I: intermediate, S: sensitive

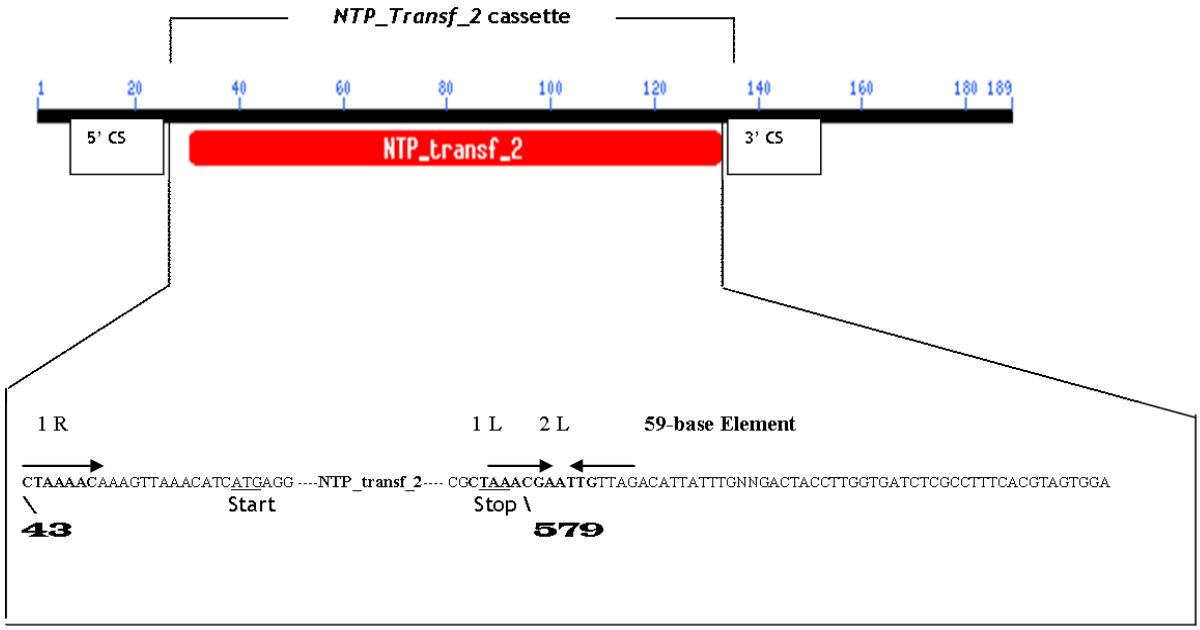

Figure 1. Schematic representation of a *NTP_transf_2* gene cassette detected in this study. The *aadA1* reading frame are shown as arrow, the 5' and 3' Conserved Segment (5'CS, 3'CS) of class 1 integron are shown as boxes. The translational start (ATG) and stop (TAA) codons are underlined. The putative *IntI* 1 integrase binding domains 1L, 2L, and 1R are indicated by arrows. Numbers indicating important positions of bases in the 59-base element.

17 *V. cholerae* isolates in street foods (beverages) from vendors located in primary schools in Jakarta deserved raise concerns to their potential of cholera outbreaks, especially since street foods played an important role in school-age children's diet. Resistance of *V. cholerae* isolates to commonly used antibiotics in cholera treatment is more worrying as genetic element of antibiotic resistant gene captures, a class 1 integron also discovered. The class 1 integron, bearing streptomycin/spectinomycin-resistant gene finding may leads to horizontal transfer of antibiotic resistant genes to other bacteria.

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