Prevalence and quantity of pathogenic *Escherichia coli* in ice-based beverages in Bogor, Indonesia

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ABSTRACT

Aims: Pathogenic *Escherichia coli* is one of global concern because of their effect on public health. The aims of this study were to determine the prevalence of *E. coli*, i.e. enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) in ice-based beverages sold in street food vendors in Bogor and to determine virulence factor and quantity of labile toxin (LT), stable toxin (ST), intimin (EAE), shiga-like toxin1 (STX1), shiga-like toxin 2 (STX2), and plasmid invasion (INV).

Methodology and results: Identification and quantification of pathogenic *E. coli* in ice-based beverages (n=85) were performed using real-time PCR (RT-PCR) assay adding propidium monoazide (PMA) prior to samples extraction. Extracted samples were analyzed using electrophoresis gel to define positive genome. The analyzed result showed 55.3% samples were positive genome and those samples were analyzed using RT-PCR to define virulence factor and determined concentration of bacteria. The result showed virulence factor of pathogenic on ice-based beverages (pudding with coconut milk ice, flavored ice, mix fruits ice, coconut ice, and orange ice) were only labile toxin (LT). The prevalence value was 8.2% with varied cycle threshold (Ct) numbers between 28.2 ± 0.3 to 32.8 ± 3.0 and the melting temperature (Tm) was 78.5 °C. The enterotoxigenic *E. coli* (ETEC) concentration in samples were 1.8×10^1 - 2.0×10^2 cells/mL.

Conclusion, significance and impact study: Iced-based beverage samples in Bogor contained 8.2% of ETEC. Based on the consumption data and the concentration of ETEC in iced-based beverages, the number of ETEC exposure in ice-based beverages consumed by the consumer in Bogor (2.9×10^3 - 2.4×10^4 cells) was lower than infection dose set by FDA. However, this result could be considered as an early warning of health problems.

Keywords: Concentration, ETEC, ice-based beverages, prevalence

INTRODUCTION

Diarrheal disease is a global problem with high morbidity in many countries, especially in developing countries with a high prevalence of diarrheal disease cases. The food outbreaks data in 2017 showed 5,293 cases. It caused 2,041 illnesses, 3 deaths, with attack rate (AR) of 38.56% and case fatality rate (CFR) of 0.15%. While food snack outbreaks occurred at 24.35%. The source of food poisoning mostly was caused by snack and culinary foods (NADFC, 2018). Ice-based beverages used ice cubes or crushed ice (block ice) to cool the drinks. Ice cubes were made by ice tank machine that was filled with ammonia gas as refrigerant. Water was flowed inside column tank and froze for at least 12-24 h every production batch. Hot

refrigerant was flown to release the ice column, then they were cut into a cube shape. In general, block ice was made from water that poured into big square can, which was submerged into brine pond which was -12 °C and the process took about 24-48 h. Poor hygiene and sanitation in ice-based beverages production can cause microbial contamination. Coliform and *E. coli* could be used as an indicator of the organism for fecal contamination in hygienic practices. The previous studies also already reported the presence of pathogenic bacteria such as *E. coli*, *Salmonella* spp., and *Vibrio cholerae* in ice (Waturangi *et al.*, 2012a, Waturangi *et al.*, 2012b, Nababan *et al.*, 2017).

Study of Food and Environmental Hygiene Department (2005) showed that from 89 samples,

8 samples were contaminated by coliform (9%). In Greece, 22% of 100 ice samples were detected *E. coli* and 31% of samples were detected Coliform (Gerokomou *et al.*, 2011). In addition, Canada noted that the proportion of gastrointestinal disease due to contamination of pathogenic bacteria in surface water was 0.11%. Pathogenic *E. coli* bacteria were the most common bacteria found in surface water contamination (Chandran and Mazumbar, 2015).

In Indonesia, ice cube and ice crush were used in various drinks and sold in many places, especially street vendors. The practice of using *E. coli* as a hygiene indicator had been studied in some areas in Indonesia (Lubis, 2012; Wahyuni *et al.*, 2013; Ritonga *et al.*, 2014). Septiani *et al.* (2018) reported that critical points, that caused contamination in ice drink, existed in every part of the supply chain. Rahayu *et al.* (2017) also reported that many ice-based beverages street vendors in Bogor did not comply with good hygiene practices (GHP). Rahayu *et al.* (2017) categorized ice-based beverages street vendors into two groups, the compliance and non-compliance to GHP. According to the report, there were ten critical factors affecting the hygiene of food observed, such as: 1) Accessibility of flowing water to wash hands and tools; 2) Cleanliness of clothes; 3) Cleanliness of nails; 4) Cleanliness of hands; 5) Cleanliness of tools; 6) Ice crushing process; 7) Washing hands before handling ice-based beverages; 8) Availability of trash bin; 9) Pets around street vendors spot; 10) Wounds on hands. The non-compliance with GHP can cause ice-based beverages to be contaminated by pathogenic organism, especially pathogenic *E. coli*.

Pathogenic *E. coli* is classified by the virulence characteristics, each group causes disease through different mechanisms. The attachment properties of the intestinal epithelium cells are encoded by the gene in the plasmid as an intermediate toxin. Six groups of diarrhea-causing *E. coli* bacteria are known as diarrheagenic *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Kaper *et al.*, 2004). Four of the six types of *E. coli*, ETEC, EPEC, EHEC, and EIEC are known to be foodborne illness (FDA, 2018) bacteria. Symptoms caused by infection of these four types of *E. coli* are diverse, including: abdominal pain, diarrhea, mild dysentery, bloody diarrhea, and acute hemolytic uremic syndrome (HUS), and thrombocytopenic purpura (TTP). In fact, such infections can cause death (FDA, 2017). Infection doses of ETEC, EPEC, EHEC and EIEC in healthy adults were estimated to be at least 10^8 ; 10^6 ; 10^{10} ; and 10^6 cells, respectively (FDA, 2018).

The current biochemical reaction method was only able to detect the presence of *E. coli* in general, but not its virulence. Method of detecting the presence of pathogenic bacteria in food based on virulence factor could be run by using RT-PCR to obtain relatively fast and accurate results. However, the use of RT-PCR method also had challenges in detecting pathogenic *E.*

coli bacteria in contaminated food. RT-PCR detects all bacteria DNA from viable cells (living cells) and non-viable cells (dead cells), which could lead to overestimation. Overestimation of bacterial detection could happen during RT-PCR analysis, because of living and dead cell DNA would be fully amplified by RT-PCR (Wang and Levin, 2006). The number of pathogenic *E. coli* that was small in sample must be known accurately using RT-PCR analysis. Living and dead cells could be differentiated by using propidium monoazide (PMA) which entered dead bacterial cell but could not enter living bacteria cells prior to the DNA extraction (Cawthorn and Witthuhn, 2008). PMA had the ability to enter into membranes and DNA of dead bacteria cells with light exposure. The result of crosslinking between PMA and DNA bands could inhibit DNA target amplification during analysis using RT-PCR.

MATERIALS AND METHODS

Determination and sampling of ice-based beverages samples

The preparation stage was held by collecting ice-based beverages samples based on good handling practices (GHP) to non-compliance category vendors (Rahayu *et al.*, 2017). Ice-based beverages samples were taken from 85 ice-based beverages vendors in seven areas in Bogor City, contained of six types of ice-based beverages, i.e. jelly grass ice (eight samples), coconut ice (21 samples), pudding with coconut milk ice (six samples), orange ice (12 samples), mix fruits ice (16 samples) and ice tea (five samples). Samples were taken with two replicates, then put into sterile plastic bags with label and stored in a cool box, then brought to laboratory (Rahayu *et al.*, 2016). The time limit between sampling in vendors and sample preparation was not over 2 h. As much as 10 g of samples was put into 90 mL of Brain Heart Infusion Broth (BHIB), then homogenized with stomacher for 60 sec. The mixture of sample and BHIB solution was incubated at 37 °C for 3 h for resuscitation of bacteria cells.

DNA extraction from Ice-based beverages samples and genome analysis

Samples were extracted by Chelex 100 (Sigma Aldrich, USA) and microwave combination methods of Komalasari (2017) with modification. The modification was conducted by adding propidium monoazide (PMA) (Biotium Inc, USA) to differentiate living or dead bacteria DNA (Li and Chen, 2012). PMA was dissolved with aquabidest as a test stock in 10 mM solution and stored at -20 °C in dark condition (Xiao, 2015). About 10 mL of the resuscitation sample was put in 15 mL centrifuge tube, then centrifuged for 25 min at 3,500 rpm. Pellet of the bacteria cells was added by 0.5 mL of Buffer Pepton Water (BPW), then removed to 2.0 mL micro-centrifuge tube and added 2.0 µL of 10 mM PMA solution. Samples with PMA were incubated at 25-27 °C in dark conditions for 5 min, then shaken three or four times to homogenize the sample

mixture. The sample was put under the LED blue light with wavelength 450-500 nm at a distance of 20 cm for 2 min, in order to get photo-induce-cross-linking (Li and Chen, 2012).

Samples with PMA treatment, then centrifuged again for 5 min at 8,000× g; 4 °C. The pellets were re-suspended with 1,000 µL TE buffer (10 mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0) and homogenized using vortex. The mixture was centrifuged for 5 min at 8,000× g; 4 °C. Pellets were re-suspended with 100 µL TES buffer test (10 mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0; 0.5% SDS), then heated using microwave for 3 min at 100 °C. About 150 µg proteinase K and 20 µg RNase (Thermo Fisher, USA) were added into the mixture solution. The mixture solution was re-heated using microwave for 3 min at 100 °C. After the destruction process, the mixture solution was incubated for 2 min at 25-27 °C, then added with 150 µL of TE buffer and 25 mg Chelex 100. The mixture solution was put back into the microwave in the same conditions (3 min; 100 °C, then centrifuged at 10,000× g for 5 min at 4 °C. Supernatant containing DNA was taken then precipitated with sodium acetate 3 M and 2.5× ethanol 95%, the mixture solution then incubated at -20 °C for 20 h. The mixture solution was incubated then washed twice with 1 mL of 70% ethanol, dried completely at room temperature then dissolved in 40 µL aquabidest (DNA extract). DNA extract was analyzed using gel electrophoresis in 75 V for 45 min (Powerpack BioRad, USA) using 2% of agarose (2 g of agarose in 100 mL TAE). The resulted gel was soaked in ethidium bromide solution for 30 min, then rinsed by aquadest and analyzed using gel documentation under UV light. A positive genome is a band of genomic DNA detected on electrophoresis that shows sufficient DNA concentrations of the genome to be analyzed. DNA extract with positive genome was analyzed to measure its purity and concentration using NanoDrop 2000 spectrophotometer (Thermo Fisher, USA).

Detection of pathogenic *E. coli* bacteria with real time PCR (RT-PCR)

The analysis of *E. coli* bacterial detection was performed on four types of *E. coli* which are known to be associated with food contamination (FDA, 2018). The detection process using simplex RT-PCR method was based on six virulence factors that characterized by each type of *E. coli*. ETEC bacteria (enterotoxigenic *E. coli*) was characterized by virulence of labile toxin (LT) and stable toxin (ST), EPEC (enteropathogenic *E. coli*) was characterized by virulence of intimin (EAE), EHEC (enterohaemorrhagic *E. coli*) was characterized by virulence of shiga toxin 1 (STX1) and 2 (STX2), and EIEC (enteroinvasive *E. coli*) was characterized by invasive plasmid virulence (Kaper *et al.*, 2004).

The virulence factors and the primary sequences used in the study were presented in Table 1. The reaction volume was carried out at 20 µL, contained 1× Dynamo flash SYBR Green master mix (Thermo Fisher, USA), 1 µL template DNA, and primer gene concentration 0.125-0.25 µM (Nurjanah *et al.*, 2017). The cycle conditions for RT-PCR were carried out at 95 °C for 3 min (pre-denaturation), followed by 30 cycles of 95 °C denaturation for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. The dissociation stage (melting) was run by raising the temperature from 60 to 90 °C at a speed of 0.5 °C for 10 sec.

Standard curve of virulence factor

A serial dilution of DNA extract range between 0.01-100 ng was used as DNA template in standard curve from a virulence factor as positive control. This serial dilution reaction for RT-PCR was prepared as the same composition as RT-PCR reaction samples. The RT-PCR reaction sample mixture was analyzed using RT-PCR in the same condition as the analyzed samples.

Table 1: The virulence factors and primary sequences were used in this study.

Types	Gene targets	Primer sequences (5'-3')	Reference	Concentration (µM)	Tm products ¹ (°C)	Tm products ² (°C)
ETEC	LT	F- AGCGGCGCAACATTTTCAG R- TTGGTCTCGGTCAGATATGTGATTC	Hardegen <i>et al.</i> , 2010	0.25	77	78.5
	ST	F- TTAATAGCACCCGGTACAAGCAGG R- CCTGACTCTTCAAAAGAGAAAATTAC	Toma <i>et al.</i> , 2003	0.125	NA	75.0
EPEC	EAE	F- TGATAAGCTGCAGTCCGAATCC R- CTGAACCAGATCGTAACGGC	Hegde <i>et al.</i> , 2012	0.125	81	81.6
EHEC	STX1	F- CAGTTAATGTGGTGGCGAAGG R- CACCAGACAATGTAACCGCTG	Sjoling <i>et al.</i> , 2014	0.125	79	79.8
	STX2	F- ATCCTATTCCCGGGAGTTTACG R- GCGTCATCGTATACACAGGAGC	Sjoling <i>et al.</i> , 2014	0.125	82	82.7
EIEC	INV	F- TTTCCCTCTTGCTGCATATGCGC R- CTCACCATACCATCCAGAAAGAAG	Kim <i>et al.</i> , 2010	0.125	78	77.1

¹Theoretical Tm product values (amplicons). Based on Science Launcher's Biochemistry and Molecular/Cell Biology (2017).

²Actual Tm product values (amplicons). Based on Nurjanah *et al.* (2017).

NA, Not available.

Standard curve was made by plotting Ct number versus log pathogenic *E. coli* DNA concentration, in order to get linear equation of $Y = aX + b$.

Quantification of ETEC in samples

The results of RT-PCR were Tm and Ct number; Tm could be used to identify the positive virulence factor, then Ct number could be used to determine cell number in samples based on bacteria DNA number by plotting linear regression of standard curve. The quantity of DNA obtained from standard curve was used to calculate predicted cell number which was the result of the conversion from the calculation of copy number using the following formula (Staroscik, 2004):

$$Cn = \frac{X \times A}{L \times W \times C} \dots \text{Equation 1}$$

Cn = Copy number

X = Amount of DNA (ng)

A = Avogadro number = 6.022×10^{23} number/mol

L = Length of template ETEC NCCP 15470 = 4.79×10^6 bp (Kwon *et al.*, 2017)

W = Average weight of 1 bp dsDNA = 650 g/mol bp

C = Conversion g form to ng = 1×10^9 ng/g

The total Cn was obtained by multiplying Cn with ice-based beverages daily consumption patterns (assuming 1 g = 1 mL; density = 1), then total Cn was converted into cells unit. The Cn value than were converted to cell number using the following approach: The copy number (Cn) value was the number of copies of genes from one bacterial cell. According to Thermo Fisher (2018), 1 cell of *E. coli* contained DNA as much as 1.7×10^{-2} pg and 1 Cn of a gene can produce 5.2×10^{-3} pg DNA. So, in one of *E. coli* cell there have 3.3 Cn $\approx$ 3 Cn.

$$\text{Cell number of } E. coli = \frac{Cn}{3} \dots \text{Equation 2}$$

Cn = copy number

RESULTS AND DISCUSSION

The presence of pathogenic *E. coli* genome in ice-based beverages

The results of DNA extraction using a combination of Chelex 100 and microwave methods were analyzed by electrophoresis gel. The results of electrophoresis gel analysis of positive genome from each sample can be seen in Table 2. The number of positive genomes was based on the proportion of samples with positive genomes compared to all samples analyzed on every type of the samples. Samples with positive genomes, known also as positive DNA bands, were shown by the availability of DNA mark on the gel electrophoresis result (Figure S1). The DNA mark on the gel means that there was enough bacteria DNA to be detected and analyzed in the next analysis. Positive DNA bands were further analyzed for the purity of DNA. The results of extracted

DNA purity using a combination of chelex and microwave methods showed various purity values, between 1.5-2.0 (Table 2). According to Nolan *et al.* (2006) DNA was expressed purely if the ratio between absorbance of 260 and 280 were in the range of 1.8-2.0. Purity values less than 1.8 or more than 2.0 indicated that there was still impurities matrix involved in DNA extraction. Impurities in DNA extract were protein, RNA, lipids, or material of DNA extraction (Thermo Fisher, 2018). Some DNA extracts from ice-based beverages samples had purity values from the expected range while others were containing matrix of fat or lipid and protein that caused low DNA purity.

Table 2: The result of electrophoresis gel of positive genomes from each sample (n=47).

Name of samples	Positive genomes (%)	Concentration (ng/uL)	Purities (A260/A280)
Jelly grass ice	2.4	951.1	2.0
Coconut ice	18.8	248.3	1.8
Pudding with coconut milk ice	9.4	284.5	1.5
Orange ice	8.2	63.8	1.9
Mix fruits ice	9.4	385.7	2.0
Flavoured ice	5.9	234.9	1.9
Ice tea	1.2	-	-
Total positive genome	55.3	-	-

The prevalence of pathogenic *E. coli*

DNA extraction on positive DNA bands in electrophoresis gel assay were resulting DNA genome which then analyzed using RT-PCR. The analysis found that from 85 samples that were tested, seven samples (8.2%) were positive LT, which was one of the ETEC virulence factor. LT was the only virulence factor detected in samples. Samples with positive LT were defined by comparing Tm result of RT-PCR analysis to Tm of LT virulence factor reference and the Ct number under 40th cycle with single peak of Tm curve ($78.5 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$) (Table 1) (Nurjanah *et al.*, 2017). The 1 $^\circ\text{C}$ temperature difference was tolerated according to the previous study by Mangold *et al.* (2005) that showed the difference of tolerance Tm in control samples which was $\pm 1 \text{ }^\circ\text{C}$. The positive LT virulence factor can be seen in Table 3 while Ct and Tm value can be seen in Table 4 (melting curve and amplification plot can be seen in Figure S2).

According to Kaper *et al.* (2004), ETEC has colonization factor attached on small intestine. ETEC produced exotoxin which was labile toxin (LT), and molecular weight was 80,000 g/mol controlled by plasmid genetic. Exotoxin has two units, i.e. A unit and B unit. A unit was facilitated by B unit to enter small intestine epithelium cell to activate adenylate cyclase. Adenylate

cyclase made cyclic adenosine mono phosphate (cAMP) raised significantly, caused water (H₂O) and chloride (Cl⁻) hypersecretion and inhibit sodium (Na⁺) reabsorption. Because intestine lumen was bigger due to water, hypermotility was happened and caused diarrhea in several days.

Table 3: The result of RT-PCR positive virulence from samples (n=85).

Name of samples	Positive virulence					
	LT	ST	EAE	STX 1	STX 2	INV
Jelly grass ice	0/8	0/8	0/8	0/8	0/8	0/8
Coconut ice	2/21	0/21	0/21	0/21	0/21	0/21
Pudding with coconut milk ice	1/8	0/8	0/8	0/8	0/8	0/8
Orange ice	1/12	0/12	0/12	0/12	0/12	0/12
Mix fruits ice	1/16	0/16	0/16	0/16	0/16	0/16
Flavoured ice	2/15	0/15	0/15	0/15	0/15	0/15
Ice tea	0/5	0/5	0/5	0/5	0/5	0/5
Total positive samples	7/85	0/85	0/85	0/85	0/85	0/85
Percentage (%)	8.2	0.0	0.0	0.0	0.0	0.0

Table 4: The result of Ct and Tm value of LT positive.

Name of samples	Positive LT (ETEC)		Tm value reference ¹ (°C)
	Ct average	Actual Tm value (°C)	
Pudding with coconut milk ice	29.9 ± 0.1	78.8 ± 0.4	78.5
Flavoured ice	28.4 ± 0.7	79.2 ± 0.4	
	29.8 ± 1.4	79.0 ± 0.0	
Mix fruits ice	28.2 ± 0.3	79.0 ± 0.0	
Coconut ice	30.9 ± 1.1	79.3 ± 0.4	
	32.8 ± 0.0	79.0 ± 0.0	
Orange ice	31.8 ± 3.6	79.3 ± 0.0	

¹Nurjanah *et al.* (2017).

Labile toxin of ETEC was the most common virulence factor found in food samples such as water and ice. The results of this study are supported by research conducted by Nababan *et al.* (2017) who found that 0.7% of ice-based beverages samples from three provinces in Indonesia were contaminated by ETEC. Kamaruzaman *et al.* (2017) found out that the source of contamination on ice-based beverages comes from ice cubes that added to ice-based beverages.

Feng (2015) states that ETEC infection was more common to cause outbreaks by the consumption of contaminated water. The infections caused by ETEC

often cause travellers to have diarrhea in the developing country they are visiting (traveller's diarrhea). In addition, children under 2 years old are also the most at-risk populations of ETEC bacteria (Qodri *et al.*, 2007).

Standard curve of LT gene target in ETEC

Standard curve was a curve between Ct number and serial of log DNA concentration. The result of the analysis showed that Ct number increased equally with the decreasing of log DNA concentration. The linear regression result was $Y = -4.38X + 17.24$, $R^2 = 0.97$ (Figure 1). The equation result by standard curve was used in quantification calculating DNA concentration from the samples with positive LT, which Y was Ct and X was DNA concentration (ng) in positive samples.

Figure 1: Standard curve plot of real time-PCR assay.

The quantity of ETEC in positive samples

Quantification of ETEC cell number in positive LT samples was carried out by plotting Ct number (Y) to linear equation of the standard curve. According to the linear equation generated from the standard curve in Figure 1, it showed that DNA concentration on positive LT was 2.8×10^{-4} – 1.3×10^{-3} ng (Table 5). Bacteria cell number in LT positive samples was counted using Cn approachment in Equation 1. The Cn values for each LT positive samples were 25; 26.4–54.2; 59.6; 5.4–15.2; 9.2 copy number/g for pudding with coconut milk, flavoured ice, mix fruits ice, coconut ice and orange ice respectively.

According to Rahayu *et al.* (2019) ice-based beverages daily consumption patterns for coconut milk, flavoured ice, mix fruits ice, coconut ice and orange ice were 181.5; 132.9; 99.6; 180.3; 89.7 mL/day respectively. Based on Equations 1 and 2, bacteria cells number were obtained for each LT positive samples. The cells number consumed by the customer per day in Bogor from pudding with coconut milk was 1.5×10^4 cells, from flavoured ice were 1.2 and 2.4×10^4 cells, from mix fruits ice was 2.0×10^4 cells, from coconut ice were 3.3 and 8.8×10^3 cells and from orange ice was 2.9×10^3 cells (Table

Table 5: Copy number of positive LT.

Name of samples	Daily consumption (mL/day) ¹	DNA concentration (ng) ²	Cn of samples (Cn/g) ³	Amount of ETEC in samples (cell/mL) ⁴	Amount of ETEC consumed per day (cell) ⁵
Pudding with Coconut milk ice	181.5	1.3×10^{-3}	2.6×10^2	8.5×10^1	1.5×10^4
Flavoured ice	132.9	2.8×10^{-3}	5.5×10^2	1.8×10^2	2.4×10^4
Mix fruits ice	132.9	1.4×10^{-3}	2.6×10^2	8.7×10^1	1.2×10^4
Coconut ice	99.6	3.2×10^{-4}	6.1×10^2	2.0×10^2	2.0×10^4
Orange ice	180.3	2.8×10^{-4}	1.5×10^2	4.9×10^1	8.8×10^3
	180.3	1.4×10^{-3}	5.4×10^1	1.8×10^1	3.3×10^3
	89.7	4.7×10^{-4}	9.7×10^1	3.2×10^1	2.9×10^3

¹Based on Rahayu *et al.* (2019).

²Obtained from linear equations on standard curve as shown in Figure 1.

³Obtained from equation 1.

⁴Obtained from equation 2. Assuming 1 cell equal with 3 Cn (copy number).

⁵Obtained from daily consumption (mL/day) x amount of ETEC in sample (cell/mL).

5). These exposures of ETEC in iced-based beverages were still lower than the infective dose set by FDA for ETEC (10^8 cells) (FDA, 2018).

CONCLUSION

The conclusions obtained from this study were the prevalence pathogenic *E. coli* in ice-based beverages in Bogor, which LT used as the virulence factor for ETEC, was 8.2% (n=85). The quantity of ETEC in ice-based beverages was 1.8×10^1 - 2.0×10^2 cells/ mL. The number of ETEC cells found in ice-based beverages consumed by the consumer in Bogor was lower than FDA reference. Although this number is below the infection dose (1×10^8), this result could be considered as an early warning of health problems.

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SUPPLEMENTARY INFORMATION

Figure S1: Electrophoresis result from positive genome of ice sample from pudding with coconut milk ice (lane 4 and 6), jelly grass ice (lane 5), and orange ice (lane 7).

Figure S2: Melting curve of RT-PCR for positive LT in ice sample. Melting temperature of LT was 79.0 °C.