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Original Article

Critical points and the presence of pathogenic bacteria in iced beverage processing lines

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Abstract

Introduction: Ice can be contaminated by pathogenic bacteria. This study aimed to identify critical points in iced beverage production and distribution lines to examine the presence of pathogenic bacteria in a beverage and its processing environment, as well as when water and ice used as main ingredients.

Methodology: The critical points were determined using the principles of Hazard Analytical Critical Control Point (HACCP) to analyze each processing and distribution step from the survey. Samples collected from the points of concern based on the critical points that were found were tested for pathogens by conventional method and molecular method using primers and polymerase chain reaction (PCR).

Results: *Escherichia coli* was found in 6.34% of samples, and 0.7% of them were confirmed as enterotoxigenic *Escherichia coli* (ETEC) by PCR. *Vibrio cholerae* was found in in 0.7% of water samples used to make iced beverages and in ice production, as well as in 2.12% of distribution and production tools. *Salmonella* Typhimurium was found in 1.4% of water samples used to make ice and ice products. *Staphylococcus aureus* was found in 2.02% of the surfaces of ice distribution and production tools and in 5.05% of production and distribution workers' hands. *S. aureus* counts ranged from 2.4×10^2 - 3.5×10^2 colonies/100 cm² surface area and 1.9×10^1 - 3.7×10^2 colonies/workers' hands.

Conclusion: Control on many critical points in iced beverage processing and distribution is required so that the beverages are safe for consumption.

Key words: critical points; iced beverages; pathogenic bacteria.

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Introduction

Iced beverages are a consumer favorite easily found in various places in Indonesia. However, the quality of the beverages, especially for those sold in primary schools, does not meet safety requirements. The total plate count and most probable number (MPN) coliform were previously found to be 48.6% and 46.7%, respectively, in 2012 and 36.0% and 40.5%, respectively, in 2013 [1]. Several previous studies also revealed the presence of pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., and *Vibrio cholerae* in ice and iced beverages [2-4]. These conditions indicate the potency of microbiological hazards in iced beverage production and distribution lines, which may lead to consumer health risks. The microbiological hazards may come from the ingredients, processing, or environment of the iced beverages. To determine the source of contamination, it is necessary to investigate along the iced beverage processing line, from upstream (producer) to downstream (vendor). This study aimed to determine the critical points along the iced beverage processing routes and to test for the presence of pathogenic bacteria, particularly pathogenic *E. coli*, *Staphylococcus aureus*, *Salmonella* spp., and *V. cholerae* along the examined routes.

Methodology

Survey of production and handling conditions for iced beverages

Determination of critical points began with a survey of the production and handling practices of iced beverages from three groups of respondents: ice

producers/manufacturers, distributors, and vendors of iced beverages. The survey aimed to collect information on the production and distribution process of iced beverages and ice cubes as a raw material in iced beverages. The survey was conducted using three questionnaires (one for each group of respondents) that were previously validated. The survey was conducted in a face-to-face interview with 136 iced-beverage vendors from 52 public schools, 19 ice distributors, and 36 ice manufacturers located in three big cities (City A, City B, and City C). The survey was conducted in May–September 2014, initiated by a survey of iced beverage vendors at targeted primary schools and continued in a survey to distributors and manufacturers from whom the previously surveyed vendors obtained the ice, if necessary. The vendor respondents were chosen to represent the vendor who used ice cubes in the beverage, the vendor who used shaved ice in the beverage, and the vendor who sold stick ice made by freezing homogenous beverages (*e.g.*, stick ice from tea or syrup or shaved ice with syrup). The schools were selected by considering non-compliant findings with respect to microbiological testing of iced beverages taken from those schools during routine inspection by the Indonesian National Agency of Drug and Food Control (NADFC) in 2011, 2012, and/or 2013.

Hazard analysis and determination of critical points

Flowcharts of ice and iced beverages from production to supply to consumer were constructed based on data and information obtained from the survey. The hazard analysis and determination of critical points at every step of the process in the flow chart were conducted according to the principles of Hazard Analysis Critical Control Point [5]. This study focused on *V. cholerae*, *Salmonella* Typhimurium, enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), enterohemorrhagic *Escherichia coli* (EHEC), and enteroinvasive *Escherichia coli* (EIEC).

Sampling at processing lines of iced beverages

Sampling locations were determined based on the hazard analysis in the previous step, which predicted expected sources of microbial hazards associated with the critical points. Three lines of iced beverage supply chains were selected as sampling points in each city based on their representativeness of all lines that were surveyed, included their critical points and the uniqueness of ice types and processes. Sampling was conducted in November–December 2014 in the three cities. Water samples, ice cubes, iced beverages, and frozen iced beverage samples were aseptically taken (at least 250 mL or g) and stored in a cool box to keep them frozen or chilled (for iced beverages) until arrival at the laboratory. Equipment surface samples were taken by swabbing or rinsing (if difficult to swab) using a sterile 0.85% NaCl solution. Swabbing was performed randomly on five unique areas of the equipment's surface. Likewise, samples from workers' hands were taken using a swab method on the workers' hands. Samples were analyzed immediately upon arrival at the laboratory.

Microbiological analysis

Microbiological analyses were performed to confirm the presence of pathogenic bacteria. The analytical parameters were quantitative for *E. coli* and *S. aureus* and qualitative for *V. cholerae* and *Salmonella* spp. These assays were performed by both conventional and molecular methods.

The conventional method of *E. coli* assay was based on the analytical method of National Quality Control Laboratory of Drug and Food (NQCLDF) No. 72 /MIK/06 (Most Probable Number Assay of *Escherichia coli* in food and beverages) [6] as well as ISO 7251:2005 (Microbiology of food and feed – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – MPN Technique) [7]. The basic principle of this method is to detect and enumerate *E. coli* in liquid culture using MPN techniques through a test phase presumptive 36 ± 1°C of incubation and confirmation test phase at 45 ± 1°C.

The conventional assay of *Salmonella* spp. and *S.* Typhimurium assay was conducted using analytical method NQCLDF No. 74/MIK/06 (*Salmonella* testing in food and beverages) [8] as well as ISO 6579:2002 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.) [9]. The principle of this method is to detect *Salmonella* spp. on liquid pre-enrichment non-selective medium buffered peptone water (Oxoid, Basingstoke, UK) at 37 ± 1°C for 18 ± 2 hours, selective enrichment Rappaport-Vassiliadis *Salmonella* (Oxoid, Basingstoke, UK) at 41.5 ± 1°C for 24 ± 3 hours, and Muller-Kauffmann tetrathionate-novobiocin broth (Oxoid, Basingstoke, UK) at 37 ± 1°C for 24 ± 3 hours on selective medium xylose lysine deoxycholate agar (Oxoid, Basingstoke, UK) and 1 other selective medium bismuth sulfite agar (Oxoid, Basingstoke, UK) at 37 ± 1°C for 24 ± 3 hours. The confirmation test was carried out via biochemical and serological assays.

Analysis of *V. cholerae* was performed using the analytical method of NQCLDF No. 81/MIK/06 (*V.*

cholerae testing in food and beverage) [10]. The principle of this method is to detect the growth of *V. cholerae* bacteria on specific media incubated at 37 ± 1°C for 18–24 hours and followed by biochemical tests.

Conventional methods for *S. aureus* followed the analytical method of NQCLDF No. 66/MIK/06 (*S. aureus* number testing in food and beverages) [11] and SNI ISO 6888-1:2012 (Microbiology of food and feed – Horizontal method for the enumeration of coagulase-positive staphylococci) technique using Baird-Parker agar medium (Oxoid, Basingstoke, UK) [12]. The principle of this method is to detect the growth of *S. aureus* on the appropriate plate medium by reduction of potassium tellurite, hydrolysis of egg yolk after incubation at 36 ± 1°C for 24–48 hours, and coagulation of rabbit plasma.

PCR testing was performed by selecting five specific colonies of *E. coli* bacteria, *S.* Typhimurium, and *V. cholerae* in their selective media and positive results of the MPN test or identification test for further analysis. Each colony was then streaked onto the brain-heart infusion medium (Oxoid, Basingstoke, UK) and incubated at 37°C for 24 hours. Afterwards, two loopfuls of bacterial colonies were taken for DNA extraction using a boiling method, and multiplex PCR was conducted. Go Taq Green Master Mix (Promega, Madison, USA), PCR-grade water (IDT, Coralville, USA), and primers (IDT, Coralville, USA) were used. The primers used in this study are shown in Table 1. *V. cholerae* Inaba (PROM-V-001), *S.* Typhimurium (ATCC 14028), and EPEC NR2 – ETEC NR2 *E. coli* were used as positive control bacteria. *P. aeruginosa* (ATCC 10145), *E. coli* (ATCC 25922), and *E. aerogenes* (ATCC 13048) were used as negative control bacteria. PCR products were identified using electrophoresis and GelDoc (BioRad, Hercules, USA). Agarose (1st Base, Singapore), tris borate EDTA (TBE) or tris acetate EDTA (TAE 0.5× (Vivantis, Oceanside, USA), Florosafe DNA stain (1st Base, Singapore), loading dye (Geneaid, New Taipei City, Taiwan), and DNA Ladder 100 bp (100 bp–3,000 bp) (Geneaid, New Taipei City, Taiwan) were used.

Results

Critical point of microbiology in iced beverages

The survey results from three cities showed varying steps used in each iced beverage processing line and showed that the critical points among them differed. Processing steps and critical points for each line in the three cities are shown in Table 2. The water used as raw material for ice making, ice cubes, the boiling of water, water filling into the mold, ice washing, ice downsizing,

Table 1. Primer used in this study.

Bacteria	Primers
ETEC (O167; O148)	LTL/elt_fwd: TCTCTATGTGCATACGGAGC LTR/elt_rvs: CCATACTGATTGCCGCAAT
EPEC (O152; O164)	SK1/eae_fwd: CCCGAATTCGGCACAAGCATAAGC SK2/eae_rvs: CCCGGATCCGTCTCGCCAGTATTCG
S. Typhimurium (antigen O4)	Rfbj-s: CCAGCACCAGTTCCAACTTGATAC Rfbj-as: GGCTTCCGGCTTTATTGGTAAGCA
S. Typhimurium (antigen H1-i)	Flic-s: ATAGCCATCTTTACCAGTTCCCCC Flic-as: GCTGCAACTGTTACAGGATATGCC
S. Typhimurium (antigen H2-1,2)	Fljb-s: ACGAATGGTACGGCTTCTGTAACC Fljb-as: TACCGTCGATAGTAACGACTTCGG
V. cholerae (regulator toxin)	toxR-B: AGGGTTAGCAACGATGCGTAAG toxR-F: CCTTCGATCCCCTAAGCAATAC
V. cholerae (outer membrane protein) *ompU*	ompU-F: ACGCTGACGGAATCAACCAAAG ompU-R: GCGGAAGTTTGGCTTGAAGTAG
V. cholerae (accessory *cholerae* enterotoxin) *ace*	Ace-F: TAAGGATGTGCTTATGATGGACACCC Ace-B: CGTGATGAATAAAGATACTCATAGG
V. cholerae (*Cholerae* toxin enzymatic sub unit A) *ctxA*	ctxA-F: CGGGCAGATTCTAGACCTCCTG ctxA-B: CGATGATCTTGGAGCATTCCCAC
V. cholerae (*Zonula occludens* toxin) *zot*	Zot-F: TCGCTTAACGATGGCGCGTTTT Zot-B: AACCCCGTTTCACTTCTACCCA
V.cholerae (Hemolysin A) hlyA	hlyA-F: GGCAAACAGCGAAACAAATACC hlyA-B: CTCAGCGGGCTAATACGGTTTA
V.cholerae (Toxin co-regulated pilus) *tcpA*	tcpA-F: CACGATAAGAAAACCGGTCAAGAG tcpA-B_Cl: TTACCAAATGCAACGCCGAATG tcpA-B_El: CGAAAGCACCTTCTTTCACACGTTG

ice storage, mixing before serving of the beverage, and the serving of iced beverages are processes that became critical points in many processing lines in this study.

Presence of pathogenic bacteria in iced beverage processing lines

The critical points were used as references to determine the sampling sites for microbiological analysis. The selected sampling sites were considered the entry point of microbial pathogens at the critical points identified.

The presence of pathogens in the three lines of iced beverage listed in Table 3 represent beverages with crushed ice in a plastic bag (line 1), beverage with crushed ice block (line 2), and beverage with ice crystals (line 3).

City A

Conventional testing results indicated the presence of *E. coli* on workers' hands in the distribution line and in vendors of iced beverages, as well as on the surface of the transport equipment in line 3, as high as 5.5×10^0 – 2.2×10^1 MPN/mL and 1.8×10^1 MPN/mL, respectively. Moreover, *E. coli* was also identified in the rinsing solution of packaging plastic for ice taken from the manufacturer in line 1, as high as 2.1×10^1 MPN/mL. However, further detection using PCR for the presence of pathogenic *E. coli* (EPEC, ETEC) gave negative results. *S. aureus* was also found in samples of workers' hands at the production and distribution lines of ice as high as 1.9×10^1 – 3.7×10^2 colonies/hand. The bacteria showed negative results in the testing of swabbed samples of hands of iced beverage vendors. At the distribution steps, pathogenic bacteria were also found on the surface of transport equipment (line 3), as high as 2.4×10^2 colonies/100 cm². *Salmonella* spp. and *V. cholerae* assays showed negative results.

Table 2. Processing steps and critical point in DKI Jakarta, East Java, and South Sulawesi.

Steps/Material	Line														
	DKI Jakarta									East Java				South Sulawesi	
	A	B	C	D	E	F	G	H	I	A*	B*	C*	D*	A*	B*
Water as raw material for ice	!	!	!	√	!	√	√	√	!						
Boiling of water				!		!	!	!							
Water filtration			!												
UV sterilization															
Chlorination															
Mixing with other material															
Water cooling and other material			√	√		√	√	√							
Water filling into plastic mold	!	!	√	!	!	!	!	!	!						
Lifting into cooling tub			√												
Freezing	√	√	√	√	√	√	√	√	√						
Ice cubes in plastic packaging/blocks	√	√	√	√	√	√	√	√		!	!	!	!	!	!
Soaking of ice in a bath			√												
Release of ice from the mold			√												
Ice sorting			!												
Packaging															
Storage 1	√	√	!	√	√		√	√	√						
Physical testing of final product															
Microbiology testing of final product															
Chemical testing of final product															
Distribution 1	√	√	!	√											
Ice washing		!	!												
Downsizing of ice 1	!	!	!							!	!			!	!
Distribution 2			!			√									
Downsizing of ice 2			!	!	!	!	!	!	!						
Ice crusher	√	√	√	√	√	√	√	√	√						
Storage 2	!	!	!	!	!		!			√	√	√	√	√	√
Mixing	!	!	!	!	!	!	!	!	!	!	!	!		!	!
Presentation	√	√	√	√	√	√	√	√	√	!	!	!	!	!	!

√ Control point; blank: Not tested; ! Critical control point; * Process started in vendor.

Table 3. Microbiology testing results on iced beverage processing lines in Jakarta.

Sampling points	Bacterial number														
	Line 1					Line 2					Line 3				
	Col	*E.c*	*S*	*V. c*	*S. a*	Col	*E.c*	*S*	*V.c*	*S. a*	Col	*E.c*	*S*	*V.c*	*S. a*
Manufacturer															
Water for ice raw material	< 3	< 3	-	-	NT	< 3	< 3	-	-	NT	< 3	< 3	-	-	NT
Hands of workers	1.4×10^1	< 3	-	-	1.9×10^1	4.3×10^0	+(< 3)	-	-	1.6×10^2	+(< 3)	< 3	-	-	< 10
Water filtration results	NT	NT	NT	NT	NT	< 3	< 3	-	-	NT	< 3	< 3	-	-	NT
Ice cubes	4.5×10^0	< 3	-	-	NT	< 3	< 3	-	-	NT	2.2×10^1	< 3	-	-	NT
Rinsing of ice cube plastic packaging	1.1×10^3	2.1×10^1	-	-	NT	< 3	< 3	-	-	NT	NT	NT	NT	NT	NT
Distributor															
Surface of transport equipment	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	2.3×10^2	1.8×10^1	-	-	2.4×10^2
Hands of workers	NT	NT	NT	NT	NT	4.7×10^1	1×10^1	-	-	< 10	+(< 3)	< 3	-	-	3.7×10^2
Ice washer	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	7.5×10^0	< 3	-	-	NT
Ice cube	NT	NT	NT	NT	NT	< 3	< 3	-	-	NT	< 3	< 3	-	-	NT
Vendor															
Water for ice cubes raw material	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Ice cube	+ (< 3)	< 3	-	-	NT	+(< 3)	< 3	-	-	NT	< 3	< 3	-	-	NT
Hands of workers	+ (< 3)	< 3	-	-	< 10	7.3×10^0	5.5×10^0	-	-	< 10	7.7×10^1	2.2×10^1	-	-	< 10
Water for iced beverages raw material	< 3	<3	-	-	NT	3.8×10^1	< 3	-	-	NT	1.2×10^1	< 3	-	-	NT

Col: coliform; *E. c*: *E. coli*; *S*: *Salmonella*; *V. c*: *V. cholerae*; *S. a*: *S. aureus*; NT: not tested; -: negative qualitative test results; +(< 3): positive qualitative test results; *Salmonella* & *V. cholerae* test: qualitative; nits for coliform & *E. coli*: MPN/g or mL; units for *S. aureus*: colonies/hands of workers or 100 cm^2 surface equipment.

Table 4. Microbiology testing results on iced beverages processing lines in East Java.

Sampling points	Bacterial number											
	Line 1				Line 2				Line 3			
	E. c	*S*	*V. c*	*S. a*	*E. c*	*S*	*V. c*	*S. a*	*E. c*	*S*	*V. c*	*S. a*
Manufacturer												
Water for ice raw material	< 3	-	-	NT	< 3	-	-	NT	< 3	-	-	NT
Water filtration results	< 3	-	-	NT	< 3	-	-	NT	< 3	-	-	NT
Ice cubes	< 3	-	-	NT	< 3	-	-	NT	< 3	-	-	NT
Hands of workers	NT	NT	NT	< 10	NT	NT	NT	< 10	NT	NT	NT	< 10
Packaging surfaces in direct contact with ice	NT	NT	NT	< 10	NT	NT	NT	< 10	NT	NT	NT	< 10
Distributor												
Ice cubes	2.1×10^1*	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hands of workers	NT	NT	NT	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Surface of saw	NT	NT	NT	3.5×10^2	NT	NT	NT	NT	NT	NT	NT	NT
Vendor												
Ice cubes	< 3	-	-	NT	< 3	-	-	NT	< 3	-	-	NT
Water for iced beverages raw material	< 3	-	-	NT	< 3	-	-	NT	< 3	-	-	NT
Hands of workers	NT	NT	NT	< 10	NT	NT	NT	< 10	NT	NT	NT	< 10

E.c: *E. coli*; *S*: *Salmonella*; *V. c*: *V. cholerae*; *S. a*: *S. aureus*; NT: not tested; *: ETEC detected; -: negative qualitative testing results; Units for coliform & *E. coli*: MPN/g or mL; units for *S. arureus*: colonies/100 cm^2 surface equipment.

These results were confirmed by PCR, which also gave negative results for both bacteria. Although all tests of *E. coli*, *Salmonella* spp., and *V. cholerae* in the iced beverage processing lines in City A showed negative results, the risk of contamination may still exist and can be determined by the presence *S. aureus* on workers' hands at the production and distribution lines, which indicated improper sanitation.

City B

In City B, microbiological testing was performed on samples taken from three types of iced beverage supply chains, namely those that produce beverages with pieces of ice blocks (line 1), beverages with ice crystals (line 2), and frozen iced beverages (line 3). The test results in City B are shown in Table 4. Among these three processing lines, only one line distributed ice through a distributor. Testing results of the ice cubes from the distributor showed *E. coli* as high as 2.1×10^1 MPN/g. The PCR assay demonstrated that ice cube samples were positive for ETEC. *S. aureus* testing of workers' hands and the packaging surface in contact with ice cubes were negative, but the bacteria were found in the line 1 distributor as high as 3.5×10^2 colonies/100 cm^2 of the tool surface. *Salmonella* spp. and *V. cholerae* were not found in all lines of iced beverage processing in City B, which confirmed the negative PCR results.

City C

Microbiological testing in City C was conducted for three lines of iced beverage processing, namely beverages with crushed ice blocks (line 1), beverages with ice cubes in a plastic package (line 2), and beverages with ice crystals (line 3). The results of microbiological testing are shown in Table 5.

Conventional tests found *E. coli* in the ice cubes used by vendors in line 2, but the PCR results for the bacteria were negative. This indicates that the *E. coli* found in the sample was not ETEC or EPEC. Water as raw material for ice-making and the ice block taken from the manufacturer in line 1 were positive for *S.* Typhimurium in the PCR assay. Testing for the presence of *V. cholerae* gave positive results at several points. In the PCR assay, *V. cholerae* (toxin co-regulated pilus) was detected in the filtration apparatus used by the manufacturer in line 3, as well as the hook tool and the icebreaker used by the distributor in line 1. Furthermore, *V. cholerae* (toxin regulator) was detected on the distribution equipment of the manufacturer in line 1 and water as raw materials used by iced beverage vendors in line 2. *Staphylococcus aureus* was not found

Table 5. Microbiology testing results on iced beverages processing lines in south Sulawesi.

Sampling points	Bacterial number											
	Line 1				Line 2				Line 3			
	E. c	*S*	*V. c*	*S. a*	*E. c*	*S*	*V. c*	*S. a*	*E. c*	*S*	*V. c*	*S. a*
Manufacturer												
Water for ice raw material	< 3	+	-	< 10	< 3	-	-	< 10	< 3	-	-	< 10
Filtration equipment	NT	NT	NT	NT	NT	NT	NT	NT	< 3	-	+	< 10
Production equipment	< 3	-	-	< 10	NT	NT	NT	NT	< 3	-	-	< 10
Hands of workers	< 3	-	-	< 10	< 3	-	-	< 10	< 3	-	-	< 10
Distribution equipment	< 3	-	+	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Ice cubes	< 3	+	-	< 10	< 3	-	-	< 10	< 3	-	-	< 10
Distributor												
Ice block before being crushed	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Ice block after being crushed	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Hands of workers	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Ice storage	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Water of rinsing results	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Hook and ice breaker	< 3	-	+	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Vendor												
Ice cubes	< 3	-	-	< 10	2.2×10^1	-	-	< 10	< 3	-	-	< 10
Water for ice raw material	NT	NT	NT	NT	+ (< 3)	-	+	< 10	< 3	-	-	< 10
Water washers	< 3	-	-	< 10	NT	NT	NT	NT	NT	NT	NT	NT
Production equipment	< 3	-	-	< 10	< 3	-	-	< 10	< 3	-	-	< 10
Hands of workers	< 3	-	-	< 10	< 3	-	-	< 10	< 3	-	-	< 10
Chisel/ice hammer	NT	NT	NT	NT	< 3	-	-	< 10	NT	NT	NT	NT

E. c: *E. coli*; *S*: *Salmonella*; *V. c*: *V. cholerae*; *S. a*: *S. aureus*; NT: not tested; + (<3): the average result positive test; -: negative qualitative test results; +: positive qualitative test results; *Salmonella* & *V. Cholerae* test: qualitative; units for Coliform & *E. coli*: MPN/g or mL; units for *S. aureus*: colonies/mL.

on workers' hands in manufacturing, distribution, and vendor lines.

Discussion

Critical points have been identified along iced beverage processing lines. These points indicate the need for good processing control along the line, by ice cube manufacturers as a source of raw material, by the distributor, and by the vendor who directly serves the product to consumers. The end product (iced beverages) will not be safe to consume unless one of the critical points is controlled properly.

The test results demonstrate the presence of pathogenic bacteria in iced beverage processing lines. This suggests the possibility that iced beverages are unsafe for consumption. The presence of pathogenic bacteria may contaminate the end product and bring risks of illness due to consumption of contaminated iced beverages. The contamination is likely to occur unless appropriate control measures are carried out at critical points identified here.

ETEC was found in ice blocks from the line 1 distributor in City B. ETEC is a Gram-negative rod-shaped bacterium that can cause gastroenteritis in humans. ETEC often comes from contaminated water and food handlers infected with the bacteria [13]. ETEC found in ice blocks from the distributor likely come from contaminated water used as the raw material in ice making. Bacteria were not found at the producer level because samples taken from manufacturers and distributors did not necessarily come from the same batch, and thus test results could not be directly compared to detect changes in contamination levels at each step of the processing and distribution lines. Another possibility is that ice was washed by the distributor using contaminated water, as bacteria were not found in workers who handle distribution.

In City C, *V. cholerae* was found in production equipment and in distribution, as well as in water used for making ice blocks. *V. cholerae* exists naturally in aquatic environments. The presence of *V. cholerae* in the filtration apparatus can contaminate water used for making ice blocks; bacteria will be present in the iced beverage, rendering it unsafe for consumption. Waturangi *et al*. [3] found iced beverages in Jakarta contaminated with *V. cholerae*. The infectious dose of *V. cholerae* is 10^6 cells [13].

S. Typhimurium was found in water used as a raw material for making ice blocks and in the ice blocks produced by one manufacturer in City C. Another study found *S*. Typhimurium in ice cubes and iced beverages sold in Jakarta [4]. *S*. Typhimurium is a *Salmonella enterica* serovar that can cause non-typhoidal salmonellosis in humans [13]. The infectious dose of these microbes is < 10 cells [14]. *S.* Typhimurium is able to survive at low temperatures [15], even growing on melting ice cubes stored at room temperature [16]. This indicates potential hazards due to the presence of *S.* Typhimurium in ice cubes and iced beverages, similar to those found in this study.

S. aureus was found on the hands of workers in the ice production and distribution lines in City A, as well as on tools at ice distributors in City A and City B, but its potency to cause disease is considered relatively low. *S. aureus* bacteria can produce toxins and cause gastroenteritis in humans [13]. However, direct contact by food handlers with *S. aureus* wound infection (reaching 10^5 CFU/cm^2) is not sufficient to cause infection or toxin formation because *S. aureus* cannot produce sufficient toxins in this exposure period [17]. In addition, it requires appropriate conditions for growth. *S. aureus* is a mesophyll bacterium that generally grows between 7.0°C and 47.8°C [13]. The low number of *S. aureus* found in this study and the iced beverage as a low-temperature food product of concern may indicate a low possibility for *S. aureus* to cause disease due to consumption of iced beverages, but it still must be controlled.

The availability of safe food requires collaboration among government, industry, and community. Standards concerning microbiological quality of iced beverages in Indonesia have been regulated by the government by the head of the NADFC [18]. According to the regulation, food in the form of ice to be consumed (edible ice) shall meet a maximum limit of 10^4 colonies/g for total plate count (TPC) (30°C, 72 hours), < 3 MPN/g for coliform MPN, and negative for *Salmonella*/25 g. The Health Minister's regulation sets a maximum limit of 100/cm^3 for TPC and negative for *E. coli* in equipment that comes in contact with food [19].

Conclusions

Microbiological tests on iced beverage processing lines indicate the presence of ETEC, *V. cholerae*, and *S.* Typhimurium, which potentially cause disease in humans. Steps in the iced beverage processing line that are critical points include water as raw material for making ice, water boiling, water filtration, the mixing of other ingredients in ice production (manufacturer), water filling into the mold, ice sorting, packaging, ice storage in the factory, distribution of ice from the factory to the depot, ice washing, ice downsizing, distribution to the location of vendors, ice downsizing

on the location of vendors, ice storage during selling, mixing of ice with other ingredients used to make beverages, and serving of iced beverage. Manufacturers, distributors, and vendors must apply food safety practices to prevent microbial contamination in ice.

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