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ASSESSMENT OF ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* AND BACTERIAL CONTAMINATION OF ICE SOLD IN CAN THO CITY, VIET NAM

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Abstract. This study aimed to investigate the bacterial contamination of flake and cube ice being used daily in the community. Thirty-one ice samples were collected from different areas in the city of Can Tho, Vietnam. The enumeration of total aerobic mesophilic counts, presence of coliforms and *Escherichia coli* (*E. coli*) and determination of antibiotics resistance of *E. coli* isolates were examined. The results indicated that total aerobic mesophilic counts ranged from 2.5 to 6.2 log CFU/mL and there was significant difference between the total aerobic mesophilic counts found in flake and cube ice ($p < 0.05$). Coliforms and *E. coli* present on the flake and cube ice samples were 93.55 % and 58.06 %, respectively. A total of 39 *E. coli* isolates were tested against fifteen antibiotics, 74.36 % of which were multi-drug resistant to three to thirteen antibiotics. High prevalence of resistance was to Ampicillin (79.49 %), Cefotaxime (69.23 %), Ceftazidime (46.15 %), Tetracycline (56.41 %), Sulfamethoxazole/Trimethoprim (46.15 %), Colistin (20.51 %), etc. As *E. coli* is a hygiene indicator and a candidate vehicle for the transfer of antibiotic resistance gene, it is highly recommended using clean and potable water in ice making as well as preventing the spread of antibiotic resistant bacteria.

Keywords: antibiotic resistance, Can Tho city, *E. coli*, microbial contamination, ice.

Classification numbers: 1.2.3, 3.4.2, 3.6.2

1. INTRODUCTION

The production of ice is increasing greatly due to the high demand for ice cubes in bars, pubs, restaurants, street foods, etc. [1]. As already known, the water used to make ice should be of drinkable quality because it is used directly by adding to juices, soft drinks, smoothies or indirectly for preserving foods [2].

However, ice is sometimes contaminated with pathogenic microorganisms where a contaminated water source is used in production or where there is an unhygienic condition in handling [3]. The microbiological quality of ice used in foods and drinks could be a cause of outbreaks [4-7]. Outbreaks of gastroenteritis caused by ice contamination have been reported in some previous studies [8-10].

The microbiological risk of ice is represented by *Enterobacteriaceae*, mainly belonging to the genera *Salmonella*, *Shigella*, *Yersinia* and *Escherichia* [2]. *E. coli*, coliforms and a variety of microorganisms could be present in ice due to either the poor quality of source water used in manufacturing or a lack of hygiene in production or handling [3, 5-7]. It was reported that 43% (n = 190) of ice manufactures did not comply with the regulations due to neglect or insufficient hygiene and use of rough packaging materials during handling in Ho Chi Minh city [11]. The ice samples marketed in this city were contaminated with coliforms, *E. coli*, fecal *streptococci* and *Pseudomona*s *spp*. [11]. Therefore, ice contaminated with harmful microorganisms, especially multi-drug resistant bacteria such as *E. coli*, *Salmonella*, *Staphylococci*, etc., are capable of causing infection for customers and serious threat to global public health.

Vietnam is largely known for delicious and diverse street foods, especially in low and medium income areas in Can Tho city. At the street food stalls, ice is often used in juice or drinks such as sugarcane juice, orange juice, passion fruit juice or milk coffee, milk tea, ice tea, etc. [2, 12]. The high risk of food infection may result from poor knowledge and attitudes of food safety of vendors, poor hygiene practices including using bare hands, not enough clean water on-site, inadequate hand washing and waste disposal facilities etc. [13-15].

Previous studies have assessed the hygienic quality of the ice used in cooling drinks and foods, especially in developing countries such as in Nigeria, India, Ghana [3, 16-18]. The purpose of the present study is to determine the microbiological quality of ice sold in Can Tho city, Vietnam and further study the antibiotics resistance of *E. coli* collected from the ice samples. The results in this study will provide scientific information to assess the risk of edible ice to public health as well as to assist in setting guidelines for the hygienic production of ice.

2. MATERIALS AND METHODS

2.1. Sampling

Thirty-one samples of ice including 19 samples of small ice (flake ice) and 12 samples of big ice (cube ice with width of 4 to 5 cm and length of 7 to 8 cm) were collected randomly from retailers in four districts of Can Tho city (*i.e*. Ninh Kieu, Cai Rang, Omon and Phong Dien) from February to April 2019.

Approximately 500 g of ice was put into sterile bags (Stomacher bag, France) and transported in insulated ice boxes to the Food Technology Department of Can Tho University within two hours after collection. The samples were then allowed to melt at control temperature (2-4 ^{o}C) before microbial analysis.

2.2. Microbial analysis

2.1.1. Total aerobic mesophilic counts

After sampling, 1 mL of melted ice (*i.e*. water) was sampled and then transferred aseptically to a tube containing 9 mL of Maximum Recovery Diluent (MRD, Merck, Darmstadt, Germany). Subsequently, a tenfold serial dilution was made in MRD. Total aerobic mesophilic counts (TMC) were enumerated by pour-plating 1 mL appropriate sample dilutions on Plate Count Agar (PCA, Merck, Darmstadt, Germany) followed by incubation at 37 ^{o}C for 48-72 h. After incubation, all colonies were counted and transformed into common logarithms (log CFU/mL).

2.1.2. Isolation and purification of E. coli in ice

10 mL of melted water from ice sample was enriched in a stomacher bag containing 90 mL of Buffered Peptone Water (BPW, Merck, Darmstadt, Germany) and incubated for 18 - 24 h at 37 ^{o}C. After enrichment incubation, 0.1 ml was streaked onto Coliform Agar ES (Enhanced Selectivity, Merck, Darmstadt, Germany) and incubated for 24 h at 37 ^{o}C. Typical colonies of salmon to red color on Coliform Agar ES were coliform while that of blue color were *E. coli* [19].

E. coli colonies with different morphology (*i.e*. size, surface and shape) on Coliform Agar ES were selected, sub-cultured in Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany) for 18 - 24 h at 37 ^{o}C and then streaked on Tryptic Soya Agar (TSA, HeMedia, India) for 48 h at 37 ^{o}C to collect the pure colonies and further confirmation by biochemical tests.

To perform a confirmation test of *E. coli*, five tests were used: "IMViC" (Indole-Methyl red-Voges–Proskauer-Citrate; Merck, Darmstadt, Germany) and Kligler Iron Agar (KIA; Merck, Darmstadt, Germany). All confirmed *E. coli* of 39 isolates were then stored under -80 ^{o}C to be used for antibiotic sensitivity test.

2.3. Antibiotic resistance test

All 39 *E. coli* isolates recovered were tested for their resistance to 15 antimicrobial agents by the disk diffusion method. A strain of *Escherichia coli* ATCC 25922 was used as a control. Fifteen antibiotics commercially available and frequently used in the community, aquaculture and animals were tested on *E. coli* as recommended by the Clinical and Laboratory Standards Institute [20]. Antimicrobial agents on the disks include: ampicillin (AMP), 10 µg; meropenem (MER); 10 µg; gentamicin (GEN); 10 µg; tetracycline (TET), 30 µg; chloramphenicol (CHL), 30 µg; ciprofloxacin (CPR), 5 µg and fosfomycin (FOS), 200 µg (Abtek, United Kingdom); ceftazidime (Cz), 30 µg; cefotaxime (Ct), 30 µg; cefoxitin (Cn), 30 µg; kanamycin (Kn), 30 µg; streptomycin (Sm), 10 µg; sulfamethoxazole/trimethoprime (Bt), 23.75/1.25 µg; nalidixic acid (Ng), 30 µg and colistin (Co), 10 µg (Nam Khoa, Vietnam).

E. coli isolates were pre-cultured in TSB broth for 18-24 h at 37 ^{o}C and then tested for susceptibility to 15 antimicrobial agents as mentioned above. The culture of isolates in TSB was then suspended in 5 mL of MRD, and turbidity was adjusted to a 0.5 McFarland standard (approximately 10^8 CFU/mL). The suspension of isolates was then streaked onto Mueller-Hinton agar (MHA, Merck, Darmstadt, Germany) plates by using sterile swabs (Cotton swabs, Italy). The antibiotic discs were placed on the inoculated plates and they were incubated at 37 ^{o}C for 24 h. After incubation, the diameters of the inhibition zones were measured in millimeters. The isolates were classified as susceptible, intermediate, and resistant according to the zone diameter interpretative standards recommended by CLSI [20].

2.4. Microbiological criteria

Standard of Vietnam Ministry of Health regulations 35/2010/TT-BYT was used for non-alcoholic beverages (such as ice), which state that: total aerobic counts should not exceed 2 log CFU/mL [21], and Standard of Vietnam Ministry of Health regulations QCVN 10/2011/BYT was used for edible ice: absence of coliforms and *E. coli* in 250 mL of water [22] or in 100 mL of water according to EU Council Directive 98/83/EC [23].

2.5. Statistical analysis

The data was computed and graphed by Microsoft Excel version 2013 (Microsoft, U.S.A.). A comparison of the microbial counts between two types of ice was performed by analysis of variance at $\alpha = 0.05$ in SPSS Statistics version 20 (SPSS Inc., Chicago, U.S.A.). The results were reported as mean value ± standard deviation of all independent replicates.

3. RESULTS AND DISCUSSION

3.1. Total aerobic mesophilic counts of ice

The presence of high microbial load in ice is an indicator of unsanitary conditions or poor hygiene practices during or after production. The result of total aerobic mesophilic counts (TMC) of 31 ice samples collected from Can Tho city is shown in Figure 1A.

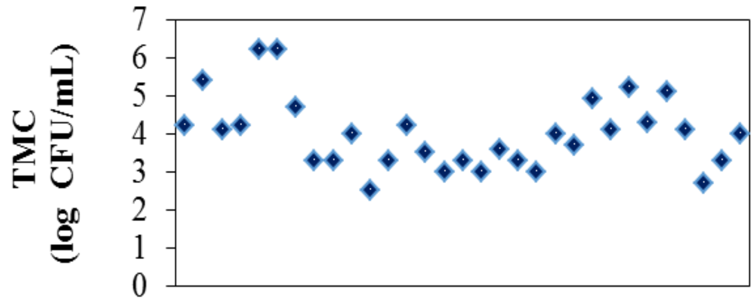

Figure 1A. The contamination of total aerobic mesophillic counts on ice (n = 31).

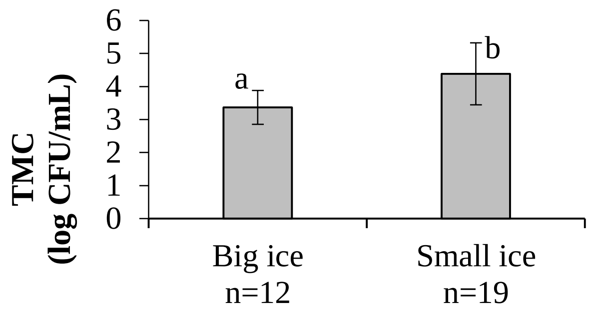

Figure 1B. Total aerobic mesophillic counts between big and small ice ($n = 31$, $p < 0.05$).

The mean of total aerobic mesophilic counts was 4.0 ± 0.96 log CFU/mL, ranging from 2.7 to 6.2 log CFU/mL. No significant difference was found among the samples collected from four different districts of Can Tho city (Ninh Kieu, n = 16; Cai Rang, n = 6; Omon, n = 3 and Phong Dien, n = 6) ($p > 0.05$). In this study, the microbial loads of ice were similar to that of ice samples collected in Brazil and Nigeria [2, 3]. However, this result obtained was higher than that of ice samples collected in Hong Kong [24]. Besides, it could be explained that poor quality of the source of water used in making ice (microbes survive in frozen conditions) and/or unsanitary hygiene practices of the vendors (ice distribution) may be closely related to the high microbial contamination in ice samples. It was reported that bacteria, pesticides, and arsenic were detected in tap water and/or water stored for drinking in Viet Nam [25, 26]. Furthermore, *Pseudomonas* spp., *Acinetobacter* spp., and *Stenotrophomonas maltophilia* were the predominant species isolated from edible ice in Viet Nam [1]. High microbial counts which ranged from 2.9 to 5.4 log CFU/mL were also detected in water samples collected from Ogbomoso Metropolis in Oyo state, Nigeria [27, 28].

Figure 1B showed that there was significant difference in TMC of big ice and small ice ($p < 0.05$). Specifically, TMC of big ice was lower than that of small ice (3.37 ± 0.51 and 4.38 ± 0.93 log CFU/mL, respectively). From the results above, all ice samples failed to conform to the standards of general hygiene (total aerobic counts of 2 log CFU/mL) according to standard of Vietnam Ministry of Health regulations 35/2010/TT-BYT [21].

3.2. Presence of coliforms and *E. coli* on ice samples

Food ice is a product obtained throughout the freezing of potable water. Thus, the presence of intestinal bacteria such as coliforms and *E. coli* is an indicator of the hygienic status of ice [3, 18]. The results in Table 1 showed the percentage of coliform and *E. coli* that was present in the ice samples. High counts of coliforms and *E. coli* i.e. 93.55 and 58.06 %, respectively was found in all the ice samples. More importantly, the highest percentage of coliforms and *E. coli* were found in small ice (100 and 68.42 %, respectively) compared to big ice (83.33 and 41.67 %, respectively). Due to the fact that the surface area of small ice is larger than that of big ice, this may be a cause for microbial contamination during handling and storage. According to Nakayama, Ha, Quoc Le, Kawahara, Kumeda, Sumimura and Yamamoto [1], many wholesalers in Vietnam transport edible ice blocks on cargo truck beds and store the ice in buckets on the floor. The study of Hampikyan, Bingol, Cetin and Colak [29] in Istanbul showed that *E. coli* was detected in 7 ice (6.7 %) and 23 ice chest (21.9 %) samples whereas *E. coli* was negative in all examined water samples. In contrast to the results of this study, *E. coli* (22 %) and coliforms (31 %) were also on ice samples in Greece [30].

According to Vietnam Ministry of Health [22], coliforms and *E. coli* should be absent in 250 g of ice. On the other hand, *E. coli* and coliforms should be absent in 100 ml of water sample as recommended by EU [23]. The presence of coliforms and *E. coli* examined in this study reflect that unsafe ice is sold in Can Tho city, especially street foods. The results may reflect poor sanitation during the preparation, storage and/or vending of these products. In addition, the observation of dirty premises and containing utensils used, the use of bare hands in preparing and vending might be the cause of *E. coli* contamination. Furthermore, the high prevalence of coliforms and *E. coli* suggested that ice used to cool drinks may present a potential hazard to street foods, especially to drinks and juice combined with ice. Hence, it is highly recommended that national regulatory guidelines should be established for the production of ice, and regular inspections should also be done to protect the consumers.

Table 1. Presence of coliforms and *E. coli* in 10 mL of ice sample (n = 31).

Size of ice	*Coliforms*	*E. coli*
Big ice	10/12 (83.33 %)[1]	5/12 (41.67 %)[2]
Small ice	19/19 (100 %)	13/19 (68.42 %)
Total	29/31 (93.55 %)	18/31 (58.06 %)

[1,2]percentage (%) of samples to be present with coliforms and *E. coli,* respectively by enrichment procedure and confirmation test.

3.3. Resistance of *E. coli* isolated from ice to antibiotics

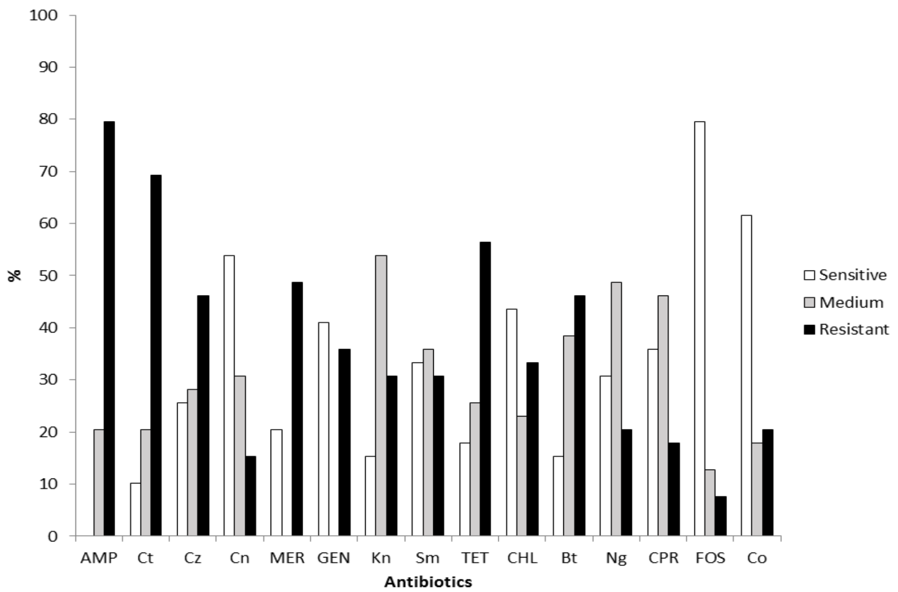

Figure 2. Percentage of antibiotic resistance of *E. coli* isolated from ice (n = 39), Ampicillin (AMP), Cefotaxime (Ct), Ceftazidime (Cz), Cefoxitin (Cn), Meropenem (MER), Gentamicin (GEN), Kanamycin (Kn), Streptomycin (Sm), Tetracycline (TET), Chloramphenicol (CHL), Sulfamethoxazole/ Trimethoprime (Bt), Nalidixic acid (Ng), Ciprofloxacin (CPR), Fosfomycin (FOS), Colistin (Co).

Fifty isolates of *E. coli* grown on selective Coliform Agar ES were confirmed by five-biochemical tests. The positive *E. coli* isolates from big and small ice was 73.91 % (17/23) and 81.48 % (22/27), respectively. Although it is reported that the diversity of bacteria contaminated on ice samples include *Bacillus subtilis*, *Streptococcus pyogenes*, *Bacillus firmus*, *Streptococcus equi*, *Staphylococcus epidermidis*, *Pseudomonas* spp., *Acinetobacter* spp., and *Stenotrophomonas maltophilia* etc.[3] [1], the present study however aimed to focus on *E. coli* and its antimicrobial resistance capacity.

The resistance of 39 isolates to different antibiotics and their patterns are as shown in Figure 2 and Table 2, respectively. The standard procedure of the CLSI [20] was strictly followed to determine susceptible, intermediate, and resistant of the bacteria to antibiotics. The degree of resistance of *E. coli* ranged from 7.69 to 79.49 %. Particularly, *E. coli* was mostly resistant to Ampicillin (79.49 %), followed by Cefotaxime (69.23 %), Ceftazidime (46.15 %), Tetracycline (56.41 %), Meropenem (48.72 %) and Sulfamethoxazole/Trimethoprime (46.15 %) while resistance to other antibiotics showed a small fraction (7.69-35.9 %) (Figure 2). Some previous studies addressed that Ampicillin was widely used in farms due to its broad-spectrum applicability and reasonable cost [31]. In addition, Sulfamethoxazole and Trimethoprim have been mostly used to treat bacterial infections in aquaculture [32]. Consequently, these antibiotics residues may persist in environmental water in Vietnam, thus contaminating it. Therefore, *E. coli* that is present in environmental water can become resistant to these antibiotics. Possibly, the ice samples studied were made from this water that have been contaminated with antibiotics resistant *E. coli* and therefore resulted in the high prevalence of resistance observed.

Table 2. Multi-antibiotic resistance of *E. coli* isolates from ice.

No.	Name	Multi-resistance pattern ($n \geq 3$)[a]	Number of resistant antibiotics	Number of multi-resistant isolates	Percentage (%)[b]
1	37ESF	AMP-GEN-TET	3	1	2.56
2	36ESF	AMP-MER-GEN	3	1	2.56
3	32ESF	Ct-TET-FOS	3	1	2.56
4	45ESF	Cz-MER-Bt	3	1	2.56
5	31ESF	AMP-Ct-MER-TET	4	1	2.56
6	5ESF	AMP-Cz-Ct-Co	4	1	2.56
7	2ESF	AMP-Cz-Ct-TET	4	1	2.56
8	28ESF	AMP-Sm-CHL-Bt	4	1	2.56
9	22ESF	Ct-Kn-TET-CHL	4	1	2.56
10	12ESF 15ESF	AMP-Cz-Ct-MER-Co	5	2	5.13
11	44ESF	AMP-Cz-MER-GEN-CHL	5	1	2.56
12	21ESF	AMP-MER-Kn-TET-CHL	5	1	2.56
13	29ESF 30ESF	AMP-Sm-TET-CHL-Bt	5	2	5.13
14	24ESF	Cz-MER-GEN-TET-Bt	5	1	2.56
15	39ESF	AMP-GEN-TET-CHL-Bt-FOS	6	1	2.56
16	20ESF	AMP-Ct-MER-Kn-TET-CHL-Bt	7	1	2.56
17	33ESF	AMP-Cz-Ct-Cn-TET-Ng-Bt	7	1	2.56
18	23ESF	AMP-Cz-Ct-Kn-TET-CHL-Bt	7	1	2.56
19	8ESF	AMP-Cz-Ct-Sm-TET-CHL-Bt	7	1	2.56
20	16ESF	AMP-Cz-Ct-Cn-MER-GEN-Sm-Kn-Ng-Bt-CPR	11	1	2.56
21	14ESF	AMP-Cz-Ct-MER-GEN-Sm-Kn-TET-CHL-Ng-Bt	11	1	2.56
22	6ESF	AMP-Cz-Ct-Cn-MER-GEN-Sm-Kn-Ng-Bt-CPR-Co	12	1	2.56
23	4ESF	AMP-Cz-Ct-Cn-MER-GEN-Sm-Kn-TET-Bt-CPR-Co	12	1	2.56
24	3ESF	AMP-Cz-Ct-MER-GEN-Sm-Kn-TET-Ng-Bt-CPR-Co	12	1	2.56
25	10ESF	AMP-Cz-Ct-Cn-MER-GEN-Sm-Kn-TET-CHL-Ng-Bt-CPR	13	1	2.56
26	1ESF	AMP-Cz-Ct-Cn-MER-GEN-Sm-Kn-TET-Ng-Bt-CPR-Co	13	1	2.56
27	9ESF	AMP-Cz-Ct-MER-GEN-Sm-Kn-TET-CHL-Ng-Bt-CPR-Co	13	1	2.56

a: Resistance pattern constructed from the antibiogram; antibiotic codes as defined under materials and methods described.
b: % Resistance obtained from the antibiogram (n = 39).

Ampicillin (AMP), Cefotaxime (Ct), Ceftazidime (Cz), Cefoxitin (Cn), Meropenem (MER), Gentamicin (GEN), Kanamycin (Kn), Streptomycin (Sm), Tetracycline (TET), Chloramphenicol (CHL), Sulfamethoxazole/ Trimethoprime (Bt), Nalidixic acid (Ng), Ciprofloxacin (CPR), Fosfomycin (FOS), Colistin (Co).

Table 2 shows the pattern of multi-antibiotic resistance of *E. coli* isolated from ice samples. Total of 29/39 isolates (*i.e*. 74.36 %) were multi-antibiotic resistant, this was to three to thirteen kinds of antibiotics, with percentage of resistance of the isolates ranging from 2.56 - 5.13 % (Table 2).

Previously, multiple-drug resistance was also found on bacterial isolates collected from water, waste water and food samples [28, 33, 34]. The problem here may be due to the quality of water sources used to produce ice, unhygienic practices, lack of neither knowledge of good hygienic practices nor safety of food products [3, 35].

The results indicated a high incidence of *E. coli* on ice samples, and its resistance to many kinds of antibiotics represent a great concern. As known, multi drug resistant strains of *E. coli* are a great matter of concern as resistance genes are easily transferable to other strains; as a result, bacterial contamination on ice might pose a potential health risk to the consumers who consumed ice on daily basis. Particularly, the ice is used daily without heating or disinfection, it could thereby constitute a risk factor for acquiring pathogenic organisms, including antibiotic resistance bacteria [1]. Therefore, regular monitoring of the quality of ice and water source used in making ice should be enforced in Can Tho city as well as other cities of Viet Nam.

4. CONCLUSIONS

The results indicated that the contamination of total aerobic mesophilic counts on the small ice was significantly higher than those of the big ice ($p < 0.05$). Coliforms and *E. coli* were present on the ice samples of 93.55 and 61.29 %, respectively. The result of testing 39 *E. coli* isolates towards their resistance to 15 different antibiotics showed that 74.36 % *E. coli* isolates were multi-drug resistant. The resistance was to Ampicillin (79.49 %), Cefotaxime (69.23 %), Ceftazidime (46.15 %), Tetracycline (56.41 %), Sulfamethoxazole/Trimethoprime (46.15 %), Colistin (20.51 %) and etc. These findings indicated that consumption of contaminated ice might pose risk to public health and may also contribute to the spread of antibiotic resistant bacteria.

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