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ORIGINAL ARTICLE

Microbiological quality of ice used to refrigerate foods

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Ice used for human consumption or to refrigerate foods can be contaminated with pathogenic micro-organisms and may become a vehicle for human infection. To evaluate the microbiological content of commercial ice and ice used to refrigerate fish and seafood, 60 ice samples collected at six different retail points in the city of Araraquara, SP, Brazil, were studied. The following parameters were determined: total plate counts (37°C and 4°C), most probable number (MPN) for total coliforms, fecal coliforms and Escherichia coli*, presence of* Salmonella *spp.,* Shigella *spp.,* Yersinia *spp.,* E. coli*,* Vibrio cholerae *and* Aeromonas *spp.. Results suggested poor hygienic conditions of ice production due to the presence of indicator micro-organisms. Fifty strains of* E. coli *of different serotypes, as well as one* Y. enterocolitica *biotype 1, serogroup O:5, 27 and phage type Xz (Ye 1/O5,27/Xz) and one* Salmonella *Enteritidis phage type 1 (PT1) were isolated.* Aeromonas *spp.,* Shigella *spp. and* V. cholerae *were not detected. The presence of high numbers of coliforms, heterotrophic indicator micro-organisms and pathogenic strains suggested that commercial ice and ice used to refrigerate fish and seafood may represent a potential hazard to the consumer in our community.*

Introduction

Commercial ice should be safe to consume and of the same quality as drinking water because it is ingested directly when added to juices and soft drinks or indirectly when used to refrigerate foods as fish and seafood.

The relationship between contaminated water and human diseases emphasizes the importance of a study to gain information about the hygienic conditions of commercial ice. Water from which ice originates must not have pathogenic bacteria that could remain viable during storage (Dickens et al. 1985). When pathogenic micro-organisms enter the human body by ingestion of contaminated food or water, they can cause diarrheic illness. The most commonly found enteropathogenic organisms are Gram-negative bacilli of the *Enterobacteriaceae* (*Salmonella*, *Shigella*, *Yersinia* and enteropathogenic *Escherichia coli*).

Infections caused by pathogenic enterobacteria in Brazil are a frequent occurrence (Gomes et al. 1991, Toledo et al. 1983, Toledo and Trabulsi 1990, Trabulsi 1985, Trabulsi et al. 1985), as also is the isolation of these and other enteric bacteria from water (Falcão et al. 1993a, 1993b, 1998, Gibotti et al. 2000, Valentini et al. 1995). Ice was one of the most important vehicles of transmission during the cholera epidemic which occurred in Peru in the last decade and spread rapidly through Latin America. The ice, mixed with redcurrant syrup

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and sold by street vendors, was prepared from contaminated water (Ries et al. 1992). Freezing does not kill *V. cholerae* O1 which can survive in ice for 4–5 weeks (Ries et al. 1992). Another important genus is *Aeromonas,* considered an emerging pathogen, whose main habitat is water (Altwegg 1999) and which may also be implicated in human infection from contaminated ice.

Indicator micro-organisms are used to evaluate the hygienic condition of foods, including commercial ice, and the possible presence of pathogens. The coliform group, *E. coli* and the total count of heterotrophic micro-organisms may in some instances reflect the sanitary quality of food (Smoot and Pierson 1997). According to the World Health Organization (WHO), the water used to prepare ice must be free from solids, bacteria, flavors, odors and the minerals must be dissolved at the lowest possible level (WHO 1976). The Brazilian Microbiological Standard requires the absence of total coliforms, fecal coliforms and *E. coli* in 100 ml of potable water. In addition, heterotrophic bacteria must not exceed 500 cfu ml^{-1} (Anonymous 2001).

Published reports have demonstrated an association between contaminated ice and enteric diseases (Burnett et al. 1994, Moore et al. 1953, Murphy and Mepham 1988, Ries et al. 1992) as well as the association between infections and contaminated ice used in hospitals and elsewhere (Burnett et al. 1994, Dickens et al. 1985, Wilson et al. 1997). Dickens et al. (1985) examined the survival of *Salmonella* Typhi, *Shigella flexneri, Shigella sonnei* and enterotoxigenic *Escherichia coli* (ETEC) in thawed ice and in ice allowed to melt in various popular drinks. The counts of all organisms were markedly lowered by freezing alone, and the numbers were further decreased by exposure to some of the drinks. Nevertheless, none of the organisms were completely eliminated as a result of freezing for 24 h followed by melting in any of the test drinks. However, in Brazil, data on the microbiological contamination of commercial ice is not available.

The aim of this study was to analyse the microbiological quality of commercial ice, ice used in fish markets and ice used in street markets to refrigerate fish and seafood in Brazil. The following were determined: most probable number (MPN) of total and fecal coliforms and *E. coli*; heterotrophic (mesophile and psychrophile) cell counts; and the occurrence of *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *E. coli*, *V. cholerae* and *Aeromonas* spp.

Materials and Methods

Sampling

A total of 60 ice samples was collected from six different places (Table 1), 10 samples from each, with an approximate interval of 1 month between each sampling period at the same site. Samples were collected from four ice factories, one fish market and one street market. The ice had been prepared from fresh chlorinated water from the public water supply or from water from artesian wells. Ice was supplied in plastic bags in the form of cubes, at two sites, in the form of shavings at three sites and of bars, ground mechanically before delivery, at one. Table 1 shows the origin and characteristics of the 60 ice samples.

After collection, the ice samples were placed in sterile plastic bottles, transported to the laboratory and stored at 4°C until completely melted.

Analysis

The determination of total and fecal coliforms and *E. coli* were carried out by the MPN method (American Public Health Association 1998), using three series of five tubes with 10·0, 1·0 and 0·1 ml of melted ice. Lauryl tryptose broth (DIFCO) was used for the presumptive test. Total coliforms were confirmed using Brilliant-green-bile broth (DIFCO). Both broths were incubated for 24–48 h at 37°C. *Escherichia coli* broth (EC, DIFCO) was used for confirmation of fecal coliforms with incubation for 24 h at 44·5°C $\pm$ 0·2 in a water bath. *E. coli* was isolated on DIFCO Eosin methylene blue agar (EMB) followed by biochemical confirmation (Ewing 1986).

Heterotrophic (mesophile and psychrophile) counts were determined by the pour-plate method using Plate count agar (DIFCO), in quadruplicate, with incubation at 37°C and

Table 1. Origin and characteristics of the ice samples

Local/origin	Water origin	Type of ice	Use
Ice factory 1	Artesian well	Bars	General refrigeration
Ice factory 2	Treated[a]	Shaved	General refrigeration
Ice factory 3	Artesian well	Cubes	Drinks, soft drinks
Fish market	Treated[a]	Shaved	Fish refrigeration
Ice factory 4	Treated[a]	Cubes	Drinks, soft drinks
Fish stand	Treated[a]	Shaved	Fish refrigeration

[a]Chlorinated water.

4°C for 48 h and 10 days, respectively (American Public Health Association 1998).

To test for *E. coli*, *Shigella* spp., *Yersinia* spp., *Salmonella* spp., *V. cholerae* and *Aeromonas* spp., five 500 ml portions of melted ice were concentrated using a 0·45 μm pore membrane filter. The ice samples which had been in direct contact with fish had to be concentrated by centrifugation at 4°C and 3675*g* for 40 min.

The filter or sediment, for assay of *Shigella*, *E. coli* and *Yersinia*, was placed in peptone water pH 7·2 and incubated at 37°C for 24 h. The isolation of *E. coli* and *Shigella* was performed on MacConkey and *Salmonella Shigella* (SS) agars (DIFCO) with incubation at 37°C for 24 h. Suspected colonies were confirmed by biochemical and serological tests (Ewing 1986). A complete determination of somatic and flagellar antigens of *E. coli* was made by tube agglutination (Gross and Rowe 1985) using Probac antisera (São Paulo, Brazil).

Yersinia was isolated by direct plating and cryo-enrichment at 4°C in peptone water for 7 days (Falcão 1987), followed by KOH treatment (Aulisio et al. 1980). MacConkey agar, incubated at 25°C for 24–48 h, was used for isolation. Suspected colonies were confirmed by biochemical tests after which they were biosero-phage typed (Falcão 1987). The presence of the virulence plasmid pYV was evaluated in the *Y. enterocolitica* isolate with alkaline extraction, according to the Birboim and Doly (1979) technique. This strain was also tested for autoagglutination virulence markers (Farmer et al. 1992), calcium dependence at 37°C and Congo Red (CR) uptake using CR-Mox medium (Riley and Toma 1989) and salicin fermentation, esculin hydrolysis and pyrazinamidase activity (Farmer et al. 1992).

For enrichment of *Salmonella,* one filter or the sediment was placed in Selenite-Cystine broth (DIFCO) and another in Tetrathionate broth (DIFCO) and incubated at 42°C and 37°C, respectively. Isolations were performed on SS agar, Brilliant green agar and Bismuth sulfite agar. Suspected colonies were tested biochemically and serologically for somatic and flagellar antigens (Ewing 1986) using antisera from the Adolfo Lutz Institute (São Paulo, Brazil). *Salmonella* isolate was tested for the presence of plasmids (Birnboim and Doly 1979) and phage typed at the Center for Disease Control and Prevention (CDC), Atlanta (USA).

In the *V. cholera*e assay, one filter or the sediment was placed in alkaline peptone water, pH 8·4, and incubated at 37°C for 6 h. Isolates were obtained from the surface pellicle of the enrichment broth on DIFCO Thiosulfate–citrate–bile–salts–sucrose agar (TCBS) and incubated at 37°C for 24–48 h. Biochemical and serological identification of suspected colonies were performed (Tison 1999).

Aeromonas was assayed by placing the filter or the sediment in alkaline peptone water pH 8·6 and incubating at 37°C for 24 h. Isolation was performed on MERCK *Pseudomonas Aeromonas* selective agar base (GSP), incubated at 25°C for 24–48 h. Suspected colonies were identified as previously described (Altwegg 1999).

Results

The mean counts of heterotrophic organisms (mesophiles and psychrophiles) in 1 ml of water and of total coliforms, fecal coliforms and *E. coli* in 100 ml of water are presented in Table 2. These results showed that mesophilic and

Table 2. Means of 10 counts of heterotrophic (mesophile and psychrophile) micro-organisms, total coliforms, fecal coliforms and *E. coli* in 60 ice samples from six different sites

Collection sites	Counts of micro-organisms (means)				
	cfu ml^{-1}		MPN 100 ml^{-1}		
	Mesophiles	Psycrophiles	Total coliforms	Fecal coliforms	*E. coli*
Ice factory 1	$4{\cdot}6 \times 10^3$	$3{\cdot}1 \times 10^3$	$1{\cdot}2 \times 10^2$	3·4	2
Ice factory 2	$1{\cdot}3 \times 10^3$	$7{\cdot}4 \times 10^1$	$2{\cdot}6 \times 10^2$	<2	<2
Ice factory 3	$1{\cdot}9 \times 10^3$	$5{\cdot}0 \times 10^2$	$6{\cdot}1 \times 10^1$	2	2
Fish market	$2{\cdot}2 \times 10^4$	$1{\cdot}7 \times 10^5$	$1{\cdot}2 \times 10^3$	$4{\cdot}0 \times 10^2$	$3{\cdot}7 \times 10^1$
Ice factory 4	$2{\cdot}0 \times 10^3$	$1{\cdot}5 \times 10^1$	6·9	<2	<2
Fish stand	$6{\cdot}1 \times 10^5$	$5{\cdot}2 \times 10^5$	$1{\cdot}1 \times 10^3$	$5{\cdot}3 \times 10^2$	$2{\cdot}2 \times 10^2$

psychrophilic microorganisms were detected in ice from all six collection sites. Table 2 also shows that all the collection sites showed means of total coliforms equal to or greater than 2 MPN 100 ml^{-1}; four collection sites presented means of fecal coliforms and *E. coli* MPN equal to or greater than 2 MPN 100 ml^{-1}.

Fifty strains of *E. coli* were identified; they belonged to 33 different serotypes. One strain of *Yersinia enterocolitica* biotype 4, serogroup O:5,27 and phage type Xz (Ye 1/0:5,27/Xz) and one strain of *Salmonella* Enteritidis phage type 1 were also identified, as shown in Table 3.

The 50 *E. coli* strains isolated were from 23 ice samples, either from peptone water (15 isolates) or from EC broth (34 isolates) and Brilliant-green-bile broth (one isolate), as shown in Table 3.

Analysis of the *Y. enterocolitica* strain did not indicate the presence of the virulence plasmid and tests for autoagglutination at 37°C, calcium dependence at 37°C and Congo Red uptake were negative. However, positive results were obtained for salicin fermentation, esculin hydrolysis and pyrazinamidase activity tests. This strain was isolated from peptone water without treatment with KOH.

Serological analysis classified the *Salmonella* strain as *S.* Enteritidis. It was phage typed as PT1. Plasmid analysis showed a plasmid of 42·0 MDa. This strain was isolated from peptone water in *Shigella, Yersinia* and *E. coli* tests and not from the specific *Salmonella* enrichment broths. Contamination by *Shigella* spp., *V. cholerae* or *Aeromonas* spp. was not observed in any of the ice samples.

Discussion

The ice used in food or to refrigerate food must be of good microbiological quality in order not to become an important source of contamination.

The results of this survey show that the majority of the 60 ice samples had an undesirable hygienic condition due to the presence of fecal and heterotrophic indicator micro-organisms (WHO 1976, Anonymous 2001). Although the ice samples had distinct characteristics, all of them were used directly or indirectly to refrigerate food. Other authors have also described ice contamination with heterotrophic micro-organisms, total and fecal coliforms (Joader et al. 1981, Moore et al. 1953, Murphy and Mepham 1988).

The 50 *E. coli* bacteria that were isolated from all collection points, except site five, strongly suggest their high frequency of occurrence in different situations. The majority (68 %) of *E. coli* strains were isolated using the MPN technique. Six of the 33 serotypes found were comprised of serogroups (O114, O86, O119, O127, O142, O128), but not H types of enteropathogenic *E. coli* (EPEC). The virulence potential of the 50 *E. coli* strains will be evaluated to determine if these strains could express virulence markers.

The *Y. enterocolitica* strain isolated was of biotype 1A, serogroup O:5,27. The biotypes related to human and animal infections are 1B, 2, 3, 4 and 5 (Bottone 1997, Robins-Browne 1997). Biotype 1A is not considered pathogenic and not associated with human disease and is

Table 3. Enterobacteria found in the 60 ice samples correlated with the most probable number (MPN) of the enteric indicators, the isolation broth and site of collection

Collection site[a]	Isolate number	Enterobacteria	Isolation broth	MPN/total coliforms	MPN/fecal coliforms	MPN/ *E. coli*
1	1	*E.coli* ONT:HNT[b]	EC	345	2	2
1	12a	*Y. enterocolitica* 1A/ O:5,27/ Xz	PW[c]	<2	<2	<2
1	61	*E.coli* ONT:HNT	EC[d]	240	11	2
1	78	*E.coli* O103:H16	EC	130	2	2
2	47a	*S.* Enteritidis *phagetype* 1	PW	<2	<2	95
2	47b	*E.coli* O7:H4	PW	23	<2	<2
2	60	*E.coli* ONT:H8	PW	2	<2	<2
2	63	*E.coli* O88:H30	PW	<2	<2	<2
3	70	*E.coli* ONT:H-	EC	41	2	2
4	4	*E.coli* O114:HNT	PW	>1600	<2	<2
4	12	*E.coli* O166:H15	PW	>1600	<2	<2
4	13	*E.coli* O82:HNT	BG[e]	>1600	<2	<2
4	17	*E.coli* O127:HNT	PW	240	17	17
4	23a	*E.coli* O127:H51	PW	>1600	>1600	4
4	23b	*E.coli* O142:H43	PW	>1600	>1600	4
4	33a	*E.coli* O128ab:H35	EC	>1600	348	33
4	33b	*E.coli* O127:H21	EC	>1600	348	33
4	42	*E.coli* ONT:H15	EC	>1600	>1600	240
4	43	*E.coli* ONT:H18	EC	>1600	>1600	240
4	46	*E.coli* O8:H8	EC	240	79	79
4	64	*E.coli* O71:H-	EC	>1600	348	33
4	65	*E.coli* ONT:H-	EC	>1600	348	33
4	72	*E.coli* O128ab:H35	EC	240	79	79
4	79	*E.coli* ONT:HNT	EC	1600	8	8
6	6a	*E.coli* O86:H51	EC	>1600	1600	920
6	6b	*E.coli* O119:H45	EC	>1600	1600	920
6	15	*E.coli* ONT:H9	PW	>1600	1600	920
6	16	*E.coli* ONT:H11	EC	>1600	1600	920
6	18	*E.coli* ONT:H28	EC	>1600	1600	920
6	19	*E.coli* ONT:H-	EC	>1600	1600	920
6	20	*E.coli* ONT:H-	EC	>1600	1600	920
6	21	*E.coli* ONT:H-	EC	>1600	1600	920
6	22	*E.coli* O115:HNT	EC	>1600	1600	920
6	24	*E.coli* ONT:HNT	PW	>1600	>1600	4
6	25	*E.coli* ONT:H-	EC	>1600	1600	920
6	27	*E.coli* ONT:H-	EC	>1600	1600	920
6	34	*E.coli* ONT:H9	PW	920	221	221
6	35	*E.coli* O23:H34	EC	920	221	221
6	36	*E.coli* ONT:HNT	EC	920	221	221
6	37	*E.coli* O104:H2	EC	920	221	221
6	38	*E.coli* ONT:H23	EC	920	221	221
6	48	*E.coli* O8:H8	EC	240	33	33
6	54	*E.coli* O7:H17	PW	542	11	11
6	57	*E.coli* ONT:HNT	PW	7	<2	<2
6	62	*E.coli* O103:H43	EC	1600	920	14
6	68	*E.coli* ONT:H21	EC	>1600	920	920
6	69	*E.coli* O147:H21	EC	>1600	>1600	39
6	77	*E.coli* ONT:H21	EC	240	33	33
6	81	*E.coli* O163:H19	EC	542	11	11
6	83	*E.coli* ONT:H28	PW	>1600	23	23
6	84	*E.coli* O71:HNT	PW	>1600	23	23
6	85	*E.coli* ONT:HNT	EC	>1600	23	23

[a] Collection site: 1—ice factory 1; 2—ice factory 2; 3—ice factory 3; 4—fish market; 6—fish stand.
[b] NT= non-typable:
[c] PW= peptone water:
[d] EC= *Escherichia coli* broth:
[e] BG= brilliant-green-bile broth.

described as environmental (Bottone 1997, Robins-Browne 1997). However, in two outbreaks of hospital infection caused by *Y. enterocolitica* the biotype involved was 1A, showing its importance as opportunistic bacteria in some patients. The first outbreak occurred in Canada and the agent was *Y. enterocolitica* biotype 1A, serogroup O:5 (Ratnam et al. 1982) and the second, described by McIntyre and Nnochiri (1986) in the United Kingdom, the strain was biotype 1A, serogroup O:6,30. These two bioserogroups are usually not related to illness. This reflects the ability of what were thought to be 'innocuous' bioserogroups to cause diseases in immunocompromised patients (Bottone 1997). The serogroup O:5,27 has been associated with intestinal illness (Robins-Browne 1997), therefore the biotype 1A, serogroup O:5,27 isolated in this study, while not expressing some virulence factors, may cause illness in immunodepressed patients. For this reason its presence is undesirable in ice.

The ice from which the *Yersinia* was isolated was made from untreated artesian well water. The MPN of total coliforms, fecal coliforms and *E. coli* in this sample were lower than 2 MPN ml^{-1}, indicating that it was in a good hygienic condition and free from fecal coliforms. Our data are in agreement with previous reports, which demonstrated the isolation of pathogens from less-polluted sites, possibly to a lack of competition between micro-organisms (Falcão et al. 1993b). There are reports describing the isolation of *Y. enterocolitica* from water of various sources in Brazil and Latin America (Falcão 1987, Freitas et al. 1987), but this is the first documented isolation of *Y. enterocolitica* from ice in Brazil.

The isolation of *Salmonella* serovar Enteritidis was of significance. The disease caused by this pathogen is related to a growing global epidemic (Bopp et al. 1999, Lukinmaa et al. 1999, Rodrigue et al. 1990). A series of studies demonstrated the ability of these bacteria to survive at low temperatures (CDC 1990, Lukinmaa et al. 1999, Vought and Tatini 1998). The isolation of *S.* Enteritidis in ice samples to be sold, and used directly in beverages, or indirectly, refrigerating foods, is an unprecedented finding in Brazil. The assay to determine the plasmid profile of *S.* Enteritidis showed a band of 42 MDa. The presence of such a high molecular weight plasmid is closely related to and essential for the expression of the virulence potential (Darwin and Miller 1999). It is important to point out that the water used to make this ice was treated water and, therefore, it is possible that the source of contamination was a factory employee or improperly cleaned machines involved in the manufacturing process (as the number of fecal coliforms and *E. coli* were lower than 2 MPN 100 ml^{-1}). The presence of *S.* Enteritidis demonstrates the importance for public health of manufacturing and maintaining ice under strict hygienic conditions, as ice may be used directly or indirectly to refrigerate foods like drinks and fish products.

These results demonstrate that ice is an important potential source of pathogenic enterobacteria presenting a risk to consumers.

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