INCIDENCE OF ENTERIC BACTERIAL PATHOGENS IN WATER FOUND AT THE BOTTOM OF COMMERCIAL FREEZERS IN CALABAR, SOUTHEASTERN NIGERIA

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Abstract. Bacteriological analysis of water that accumulates at the bottom of freezers in restaurants when the power was cut in Calabar, southeastern Nigeria, was carried out using standard procedures. Mean heterotrophic bacterial counts and *Escherichia coli* counts ranged from 3.1 ± 0.02 to 7.1 ± 0.30 x 10^4 cfu/ml and 0.2 ± 0.10 to 0.6 ± 0.50 x 10^4 cfu/ml, respectively, indicating heavy bacterial contamination whose source was mostly fecal. There was no significant difference ($p > 0.05, 0.01$) in bacterial counts between freezers. Some biochemically identified enteric bacterial pathogens were *Salmonella typhi, Shigella* sp, enteropathogenic *E. coli, Yersinia* sp, *Klebsiella pneumoniae, Vibrio cholerae* 01 and *Vibrio parahaemolyticus.* This reveals that the hygienic quality of the food items stored in the freezers and the hygienic status of the restaurants are in doubt. Infection could be going on unnoticed and thus endemicity maintained in the area. The pathogens showed alarming antibiotic resistance. The water in the freezers was a "soup" in which different species of the enteric pathogens were close to each other and could transfer drug resistance among themselves. Public health education of restaurant operators in southeastern Nigeria is recommended.

INTRODUCTION

Enteric bacterial pathogens are facultatively anaerobic gram-negative bacilli, whose natural habitats or reservoirs are the intestinal tracts of humans or animals. They include bacteria in the families Enterobacteriaceae, Vibrionaceae and Pasteurellaceae (Prescott *et al*, 2005). The main pathogens of interest are Enterobacteriaceae and Vibrionaceae, which are together unofficially known as enteric bacilli or enteric bacterial pathogens (Davies *et al,* 1980). Representative pathogens in Enterobacteriaceae are entropathogenic *Escherichia coli*, causing gastroenteritis or urinary tract infections; *Salmonella*, causing typhoid fever and gastroenteritis; *Shigella*, causing bacillary dysentery; *Klesbsiella*, causing pneumonia and *Yersinia*, causing plague (Cheesbrough, 1991; Prescott *et al,* 2005). Representative pathogens in Vibrionaceae are *Vibrio cholerae*, causing cholera; *V. parahaemolyticus*, sometimes causing gastroenteritis in humans following consumption of contaminated seafood, and *V. anguillarum* and others, which are responsible for fish disease (Prescott *et al*, 2005). Almost all the mentioned enteric bacterial pathogens are implicated in water-borne diseases (White *et al*, 1972, Todd, 1990; Slevenson, 1993; Tibbets, 1996; Eja, 2003) and food-borne infections and intoxications (Prescott *et al*, 2005).

Operators of restaurants in Calabar store all sorts of food, especially perishable foods, in deep freezers. The stored items may be alcoholic beverages in cans or bottles, meat, fish and shellfish, sachet water, etc. The food items are not usually washed before they are stored in the freezers. Sometimes left-over food remains in the restaurants, which is usually stored in the freezers. Consequently, water draining from these food stuffs when the power is cut, as is often the case in this part of Sub-saharan Africa, accumulates at the bottom of the freezers. As time goes by, the bottom of the freezers becomes a source of heterotrophic bacteria which become active when taken out of the freezers (White and West, 1992).

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In this study, the aim was to determine the bacteriological quality of the water found at the bottom of freezers in some restaurants in Calabar and establish the hygienic status and epidemiological significance of the restaurants in the spread of diseases in Calabar, southeastern Nigeria.

MATERIALS AND METHODS

Water collection

Water samples were collected in triplicates aseptically from 8 different restaurants (R_1-R_8) randomly selected in different areas of Calabar. Because the bottom water of the freezers was of a minimum volume, water samples were collected with sterile 250 ml sampling bottles by dipping the bottles hung on sterile strings into the water, and withdrawing the bottles filled with the water. The samples were carried in an ice box at 4ºC to the laboratory for analysis within 2 hours.

Total heterotrophic bacterial counts

Because of the high turbidity of the water found at the bottom of the freezers, ten-fold serial dilutions in the ranges of 10^{-1}-10^{-6} were prepared using sterile distilled water (Atlas and Bartha, 1992). One milliliter aliquots of samples diluted to 10^{-3}-10^{-6} were seeded into sterile disposable Petri dishes (90 mm diameter) in triplicates and the total heterotrophic bacterial count was determined by pour plate technique (American Public Health Association, 1998) using tryptone soya agar (Oxoid, England). The cultures were incubated at 37ºC for 48 hours after which bacterial colony counting was carried out.

Coliform and *E. coli* counts on membrane filter

A membrane filtration technique was applied (Anonymous, 1969; American Public Health Association, 1998) using lauryl tryptose broth (Oxoid, England) on the membrane filter. Total colifom counts were determined after incubation at 37ºC for 24-48 hours, while fecal coliforms (*E. coli*) were cultered at 44±0.5ºC for 24-48 hours in a water bath (Gallenkamp, England), using a 100 ml water sample (diluted to 10^{-4}).

Detection of enteric bacterial pathogens

A multiple-tube enrichment technique (American Public Health Association, 1998) was adopted, in which 100 ml of sample dilutions were filtered through a 0.45 µm pore-size membrane filter (Oxoid, 1985; Itah *et al*, 1996). The whole of the deposit on the filter membrane was transferred to 100 ml of a suitable enrichment medium, thoroughly mixed, and incubated at 37ºC for 24 hours. Inoculations from the tubes that showed growth turbidity were made from the enrichment medium to selective medium in order to isolate and biochemically identify the suspect organisms.

For *Salmonella*, the enrichment medium was tetrathionate broth (American Public Health Association, 1998), while the selective medium was deoxycholate citrate agar (DCA) (Oxoid, 1985) on which suspect colonies were opaque. *Salomnella* species were identified by smearing the surface of Kligler iron agar (KIA) slope and stabbing the butt with a colony picked off the DCA, and incubating at 37ºC for 18-48 hours (Oxoid, 1985: Cheesbrough, 1991). Color change of the butt and slope, and hydrogen sulfide gas production formed the basis for identification. Also, a smooth suspect colony was inoculated into a tube of motility indole urea (MIU) medium by stabbing and incubating for about 4 hours. Pink color throughout the medium indicated urease production (Cheesbrough, 1991).

The enrichment medium for *Shigella* was nutrient broth adjusted to a pH of 8.0 (a less favorable growth pH for coliforms). Cultures of 6 and 18 hours incubation at 37ºC were streaked on xylose lysine deoxycholate (XLD) agar to optimize *Shigella* recovery (Oxoid, 1985; Cheesbrough, 1991; American Public Health Association, 1998). The biochemical tests described for *Salmonella* were performed on suspected *Shigella* species.

McConkey broth was used as selective enrichment medium for enteropathogenic *E. coli*. Identification of isolates from McConkey agar was done through a lactose fermentation test and Indole-Methyl-Red-Voges-Proskauer and citrate utilization (IMVic) tests.

The selective enrichment procedure and identification described earlier for *E. coli*, applied in the case of *Klebsiella*, which does not produce gas from lactose at 44ºC.

Yersinia was selectively enriched with McConkey broth and incubated at room temperature (Cheesbrough, 1991), while the selective media was blood agar. Identification was carried out by biochemical tests and the production of a yellow butt and pink-red slope on Kligler iron agar (KIA) with no H_2S gas production or blackening (Cheesbrough, 1991).

In the isolation and identification of *Vibrio cholerae*, alkaline peptone water (pH 9.0) was used as the enrichment medium, while thiosulphate - citrate - bile - salt-sucrose (TCBS) agar was the selective medium. The yellow and green colonies on the TCBS agar were examined for biochemical and morphological characteristics using the scheme of Cheesbrough (1991). All the biochemically identified *Vibrio cholerae* were serotyped using polyvalent 01, non-specific Ogawa and Inaba antisera. Biotyping was done on the basis of production of soluble hemolysins, hemaglutination with chicken red blood cells (RBC) and resistance to 0/129 vibriostatic compound (Cheesbrough, 1991).

Antibiogram of bacterial isolates

A disc diffusion technique using the Kirby-Bauer method (Stokes and Ridway, 1980; Prescott *et al*, 2005) was applied in testing pure cultures of the isolates for their antimicrobial resistance patterns.

Statistical analysis

Differences, if any, between the restaurants with respect to bacterial counts in the freezer bottom water, were determined using the statistical method, analysis of variance (ANOVA) (Bailey, 1981; Miller and Miller, 1986).

RESULTS

Bacterial counts of water samples from freezers

Mean heterotrophic bacterial counts in water samples from the freezers of the restaurants, as shown in Table 1, ranged from 3.1 ± 0.02 to 7.1 ± 0.3 x 10^4 cfu/ml, while mean coliform counts ranged from 0.6 ± 0.1 to 2.4 ± 0.2 x 10^4 cfu/ml. *E. coli* ranged from 0.1 ± 0.1 to 0.6 ± 0.5 x 10^4 cfu/ml. There was no significant difference ($p>0.05$, 0.01) among the freezers in the restaurants with respect to bacterial counts.

Incidence of enteric bacterial pathogens

The incidence of enteric bacterial pathogens in the water samples from the freezers in the restaurants is shown in Table 2. The results indicate that e*nteropathogenic Escherichia coli* was the most frequently occurring organism (46.7%), followed by *Salmonella typhi* (20.0%), *Shigella* sp (13.3%), *Yersinia* species (6.7%), *Klebsiella pneumoniae*, *Vibrio cholerae* 01 (5.0% each) and *Vibrio parahaemolyticus* (3.3%).

Antibiotic resistance pattern

The antibiotic resistance pattern for the identified enteric bacterial pathogens is repre-

Table 1
Bacterial counts from water samples from freezers in eight restaurants studied.

Restaurants	No. of samples	Mean heterotrophic bacterial count (x 10^4 cfu/ml)	Mean coliform count (x 10^4 cfu/ml)	Mean *E. coli* count (x10^4 cfu/ml)
R_1	3	4.9 ± 0.2	1.3 ± 0.05	0.4 ± 0.5
R_2	3	6.7 ± 0.4	2.4 ± 0.2	0.6 ± 0.5
R_3	3	3.4 ± 0.2	0.6 ± 0.1	0.2 ± 0.1
R_4	3	3.6 ± 0.1	0.8 ± 0.2	0.3 ± 0.2
R_5	3	7.1 ± 0.3	1.0 ± 0.1	0.1 ± 0.1
R_6	3	3.8 ± 0.2	1.3 ± 0.4	0.3 ± 0.1
R_7	3	3.1 ± 0.02	0.9 ± 0.3	0.2 ± 0.1
R_8	3	5.7 ± 0.02	1.2 ± 0.01	0.6 ± 0.2

R_1 - R_8 represent restaurants whose freezer water was sampled

Table 2
Incidence of enteric bacterial pathogens in the water samples from the freezers of restaurants.

Sources of samples	No. of samples analysed	No. (%) of Enteric bacterial pathogens isolated							
		Entero-pathogenic *E. coli*	*Salmonella typhi*	*Shigella* species	*Klebsiella pneumoniae*	*Yersinia* species	*Vibro cholerae 01*	*Vibrio parahae-molyticus*	Total
R_1	3	4 (40.0)	2 (20.0)	1 (10.0)	0 (0)	2 (20.0)	0 (0)	1 (10.0)	10
R_2	3	6 (54.5)	2 (18.2)	2 (18.2)	1 (9.1)	0 (0)	0 (0)	0 (0)	11
R_3	3	3 (42.8)	1 (14.3)	2 (28.6)	1 (14.3)	0 (0)	0 (0)	0 (0)	7
R_4	3	4 (66.7)	2 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6
R_5	3	2 (40.0)	0 (0.0)	2 (40.0)	0 (0)	0 (0)	1 (20.0)	0 (0)	5
R_6	3	2 (40.0)	0 (0.0)	1 (20.0)	0 (0)	1 (20.0)	1 (20.0)	0 (0)	5
R_7	3	4 (40.0)	3 (30.0)	0 (0)	1 (10.0)	1 (10.0)	0 (0)	1 (10.0)	10
R_8	3	3 (50.0)	2 (33.3)	0 (0)	0 (0)	0 (0)	1 (16.7)	0 (0)	6
Total	24	28 (46.7)	12 (20.0)	8 (13.3)	3 (5.0)	4 (6.7)	3 (5.0)	2 (3.3)	60

Numbers in parenthesis represent percentage incidence.
R_1 - R_8 represent restaurants whose freezer water was sampled.

Table 3
Antibiotic resistance pattern of enteric bacterial pathogens isolated from the bottom water of freezers.

Enteric bacterial species	No. of isolates tested		Percentage of isolated strains resistant to:										No. showing multiple resistance
			AMP	C	SU	TE	ST	GN	CRO	N	CPX	SXT	
Salmonella typhi	10		20.0	10.0	30.0	30.0	60.0	10.0	70.0	40.0	50.0	0	60.0
Shigella sp	15		20.0	20.0	13.3	0	6.7	20.0	33.3	26.7	26.7	20.0	73.3
Escherichia coli	8		25.0	37.5	12.5	62.5	25.0	50.0	25.0	25.0	37.5	0	50.0
Yersinia sp	4		50.0	25.0	50.0	25.0	0	75.0	50.0	25.0	25.0	50.0	50.0
Klebsiella pneumoniae	6		83.3	66.7	16.7	50.0	16.7	33.3	66.7	33.3	16.0	66.7	66.7
Vibrio cholerae o1	25		12.0	16.0	20.0	4.0	8.9	4.0	20.0	24.0	12.0	16.0	24.0
Vibrio parahaemolyticus	30		16.6	3.3	0	0	13.3	6.7	3.3	6.7	20.0	6.6	16.7
Total	98	R	19.4	17.3	14.3	13.3	16.3	15.3	26.5	21.4	23.5	15.3	
		S	80.6	82.7	85.7	86.7	83.7	84.7	73.5	78.6	96.5	84.7	

AMP = Ampicillin (25 µg/ml); C = Chloramphenicol (25 µg/ml); SU = Sulphonamide (25 µg/ml);
TE = Tetracycline (25 µg/ml); ST = Streptomycin (25 µg/ml); GN = Gentamicin (25 µg/ml);
CRO = Ceftriazone (25 µg/ml); N = Nitrofurantoin (25 µg/m); CPX = Ciprofloxacine (25 µg/ml);
SXT = Trimethoprim-sulfamethoxazole (25 µg/ml); R = Resistant; S = Sensitive.

sented in Table 3. *S. typhi* showed the highest resistance to ceftriazone (70.0%) and ciprofloxacin (50.0%). *S. typhi* was least resistant to trimethoprim-sulfamethoxazole (0%), chloramphenicol (10.0%) and gentamicin (10.0%). *Shigella* sp (33.3%) was very resistant to ceftriazone (33.3%), nitrofurantoin and ciprofloxacin (26.7% each). *Shigella* was least resistant to tetracycline (0%). *E. coli* showed a high resistance (62.5%) to tetracycline but the least resistance (0%) to trimethoprim-sulfamethoxazole. *Yersinia* sp was resistant to gentamicin (75.0%), sulphonamide (50.0%), trimethoprim-sulfamethoxazole (50.0%) and ampicillin (50.0%), while the least resistance

(25.0%) was seen to chloramphenicol, tetracycline, nitrofurantoin and ciprofloxacin. *Klebsiella pneumoniae* showed the highest resistance to ampicillin (83.3%), chloramphenicol (66.7%) and trimethoprim-sulfamethoxazole (66.7%). *Vibrio cholerae* and *V. parahaemolyticus* showed minimal resistance to most of the antibiotics. Between 16.7% (*V. parahaemolyticus*) and 73.3% (*Shigella* sp) of the enteric bacterial strains showed multiple resistance to antibiotics. In all, 13.3 to 26.5% of the enteric bacterial pathogens were generally resistant, while 73.5 to 96.5% were sensitive to the antibiotics tested.

DISCUSSION

All the freezers in the study were heavily contaminated with bacteria. Mean heterotrophic bacterial counts ranged from 3.1 ± 0.02 to 7.1 ± 0.30 x 10^4 cfu/ml (Table 1). The presence of *Escherichia coli* up to 0.6 ± 0.50 x 10^4 cfu/ml indicates that all the freezers were fecally contaminated (Anonymous, 1969; American Public Health Association, 1998; Eja, 2003). There was no significant difference ($p>0.05$, 0.01) among the freezers with respect to heterotrophic bacterial counts and/or fecal coliform counts, indicating that freezers in all the restaurants are potential sources for the spread of disease.

The identified enteric bacterial pathogens were *Salmonella typhi, Shigella* sp, enteropathogenic *E. coli, Yersinia* sp, *Klebsiella pneumoniae, Vibrio cholerae* 01 and *Vibrio parahaemolyticus*. All these organisms occurred in different proportions in the freezers (Table 2), *E. coli* was the most frequently occurring organism (46.7%), followed by *Salmonella typhi* (20.0%) and *Shigella* sp (13.3%). The least were *Klebsiella pneumoniae and Vibrio cholerae* 01 (5.0%), followed by *V. parahaemolyticus* (3.3%). These organisms are implicated in water-related diseases (American Public Health Association, 1998; Eja, 2003). These results reveal that most freezers used in restaurants in Calabar are of epidemiological significance in the spread of waterborne and foodborne infections. Nair *et al* (1988) report that aquatic reservoirs of *V. cholerae* may be a mechanism by which cholera endemicity is maintained in a given area, and that an aquatic reservoir is probably the vehicle for primary transmission of infection. Elsewhere in Nigeria, the epidemiological patterns and prevalences of enteric bacterial pathogens and associated diseases have been noted (Agbonlahor and Odugbemi, 1982; Olanipekun, 1984).

In this study, it is inferred that unhygienic maintenance of commercial freezers is one of the means of maintaining diarrheal endemicity in the Calabar area of southeastern Nigeria. Ecological factors that are capable of stabilizing a focus of diarrhea endemicity in Calabar have been highlighted previously (Utsalo *et al,* 1992). As part of on-going research in the Calabar River Basin to identify those environmental niches that may contribute to the maintenance of cholera endemicity within the area, Udo (1993) concluded that shrimps harvested from the Cross River estuary play an important role. Shrimps are one of the meal delicacies of the people of Calabar, and freshly harvested shrimps are usually stored in freezers.

It is worrisome to note in this study that no identified enteric bacterial pathogen was 100% sensitive to the antibiotics tested for resistance pattern. Although, in general, 73.5 to 96.5% of the organisms were sensitive to the antibiotics, up to 26.5% were resistant. However, individual differences in the resistance patterns of the organisms were discernible in the number/percentage of each organism showing multiple resistance (Table 3). Multiple resistance to the antibiotics was highest in *Shigella* (73.3%), followed by *Klebsiella penumoniae* (66.7%), *Salmonella* (60.0%) and *Escherichia coli* (50.0%). Since the water at the bottom of the freezers is like a "soup", there is the possibility of drug resistance transferring from one strain to another and from one species to another (Prescott *et al,* 2005). Unless public health education of operators of restaurants is started by health authorities, the rise in drug resistance will complicate the already existing problem of endemicity of the diarrhea-related diseases in Calabar, southeastern Nigeria.

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