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Blood Agar To Detect Virulence Factors in Tap Water Heterotrophic Bacteria

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Cytolytic colonies were found in 57% of tap water samples, and up to 6% of samples were found to contain bacteria having three or more virulence factors. The factors evaluated were cytotoxicity, hemolysis, cell adherence, and cell invasiveness. Overall, 17% of the samples contained cytolytic colonies that were adherent and hemolytic. Among the media tested, tryptic soy agar with sheep blood (incubated at 35°C for 48 h) was the best medium for the detection of cytolytic colonies. Of the colonies growing on this medium, 13% were cytolytic, whereas on medium R2A, less than 3% were cytolytic. Furthermore, when tryptic soy agar with blood was used, 24% of the samples contained colonies with at least three virulence factors whereas only 5% were positive with R2A. Routine monitoring by using tryptic soy agar with sheep blood is suggested as an appropriate procedure for the detection of bacteria with pathogenic potential in drinking water.

Bacterial regrowth is often a problem in water distribution systems (12, 22), as well as in domestic water filtration units (13, 23). Regulatory agencies and environmental microbiologists have suggested that the heterotrophic bacterial counts in finished drinking water should not exceed 500 CFU/ml, mainly to reduce interference with the detection of coliform bacteria (8, 9). The media used for detection of heterotrophic bacteria have been formulated to promote the growth of a large number of bacteria, including those that have been stressed or have adapted to a low-nutrient environment. R2A medium (27) and total plate count medium are widely used for the enumeration of heterotrophic bacteria. However, some researchers have recently used epifluorescence methods to detect bacteria that are viable but noncultivable (1, 2) and have detected larger numbers of bacteria in water samples. The implications of high bacterial counts for water testing are not well understood, but there is a growing belief that some heterotrophic bacteria in drinking water are opportunistic pathogens and that their growth should be controlled.

There are several approaches to detection of bacterial isolates that could have public health importance but are not known pathogens. One of these approaches is to search for virulence factors. To produce disease, bacteria must often attach to, penetrate, and multiply in epithelial cells or produce toxins or other substances that will disrupt the normal metabolism of these cells and the organ attacked. The demonstration of attachment to and penetration of the cell, the destruction of the cells (cytolysis), and the detection of enzymes or toxins are commonly used in clinical microbiology. A second approach is to attempt to cultivate bacteria in a medium such as blood agar medium that will support the growth of all clinically significant bacteria (10).

We have combined these two approaches with the methods developed by Lye and Dufour (20) to screen bacteria with cytolytic activity and have then further assayed three virulence factors: hemolysin production, cell adherence, and cell invasiveness.

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MATERIALS AND METHODS

Bacterial strains and media. Selected *Escherichia coli* strains were used as positive controls: O157:H7 (enterohemorrhagic *E. coli*) for cytotoxin assays, O127:nm (EAF$^+$ enteropathogenic *E. coli*) for cell adherence assays, and O136:nm (enteroinvasive *E. coli*) for invasion assays. They were obtained from the Quebec Laboratory of Public Health (Ste-Anne de Bellevue, Québec, Canada). Strain HB 101 was used as the negative control for all assays. Blood agar plates were obtained from Que-Lab (Québec, Canada), whereas R2A medium was prepared in the laboratory as recommended by the manufacturer (Difco Laboratories, Detroit, Mich.).

Sample collection. Water samples were collected from 75 different tap water outlets in the Montreal area. They were collected in 1-liter sterile containers containing sodium thiosulfate (5 mg/liter) to neutralize residual chlorine. All analyses were performed by membrane filtration on Millipore HAWG 047 filters. After filtration, the membranes were placed on R2A medium (Difco 0736-01), Columbia agar with sheep blood (CA-SB), and tryptic soy agar with sheep blood (TSA-SB). R2A medium was incubated for 7 days at 25°C or 48 h at 35°C, whereas blood agar plates were incubated for 48 h at 35 or 45°C.

Hemolysis assay. Hemolytic activity was determined by placing the membrane on blood agar at 35°C for 12 h. The type of hemolysis was recorded as alpha, beta, or gamma.

Cytotoxin assay. After incubation, the colonies were counted and reported as CFU and the membrane was transferred to evaluate their cytotoxic activities by the procedure described by Lye and Dufour (20). To detect cytolytic activity, the membrane was first placed on blood agar to enhance cytotoxin production and then placed over a monolayer of a HEp-2 cells covered with agar. HEp-2 cells were seeded in 60-mm tissue culture plates at a concentration of 10^5 cells per ml and incubated at 35°C in a 5% CO_2 incubator. The membranes containing bacteria were transferred onto fresh blood agar plates for 8 h at 37°C to increase expression of cytotoxins. After a short exposure to absorbent cellulose pads saturated with phosphate-buffered saline (pH 7.4), the membranes were placed on an agar overlay containing Eagle's minimal medium, 2% fetal calf serum, neutral red, and 1% sterile agar solution and covering a HEp-2 cell monolayer. The membranes were left on the agar for 14 h. They were then removed, and the monolayer was examined for the presence of

TABLE 1. Enumeration of aerobic bacteria in tap water on medium R2A and blood agar at different temperatures

Medium and temp	n^a	No. of bacteria (CFU/ml)	
		Geometric mean	Maximum
R2A			
25°C	75	365	100,000
35°C	75	145	200,000
TSA-SB			
35°C	34	2.4	98
45°C	34	0.2	0.1
CA-SB			
35°C	41	0.9	120
45°C	41	0.01	2.4

[a] *n*, number of samples.

TABLE 2. Influence of plating medium on the detection and expression of virulence factors

Plating medium and temp		No. of isolates			
Original	Secondary	Cytolytic	Hemolytic	Adherent	Invasive
R2A (25°C)	TSA-SB (35°C)	11	10	2	1
R2A (35°C)	TSA-SB (35°C)	5	2	1	1
TSA-SB (35°C)	TSA-SB (35°C)	27	17	10	6
TSA-SB (45°C)	TSA-SB (35°C)	16	12	0	0
R2A (25°C)	CA-SB (35°C)	7	2	4	2
R2A (35°C)	CA-SB (35°C)	11	3	5	1
CA-SB (35°C)	CA-SB (35°C)	4	1	1	0
CA-SB (45°C)	CA-SB (35°C)	1	1	0	0

areas of plaques of cytolysis after removal of the agar overlay with a spatula.

Cell adherence. Adherence to cells was evaluated by using Caco-2 cells (human adenocarcinoma cell line). After a 10-day incubation period, these cells have the characteristics of human enterocytes (4). They were prepared in Lab-Tek chambers (Miles Laboratories) at 8×10^4 cells per ml and incubated at 35°C in a 5% CO_2 incubator. Maintenance medium was Dulbecco's minimal essential medium supplemented with 5% fetal calf serum. Growth medium was the same medium with 20% fetal calf serum. Adherence tests were performed by a method described by Cravioto et al. (3), as follows. Isolates found cytolytic were inoculated into tryptic soy broth and incubated at 35°C for 24 h with light agitation. The number of cells was adjusted to 10^8 for a final bacterium-to-cell ratio of 100:1. The bacterial suspension was then introduced into the Lab-Tek chambers containing the formed Caco-2 monolayers and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. The monolayers were then washed three times with 0.1× phosphate-buffered saline and fixed with 100% methanol. The monolayers were stained with 0.01% acridine orange in 0.5 M acetate buffer (pH 3.5; Difco) and examined under UV fluorescence by using a 100× oil immersion objective. The index of adherence was defined as the average number of bacteria per cell and was determined by counting the number of bacteria on 100 cells. All experiments were done in triplicate.

Cell invasion. To evaluate the invasive potential of adhering bacteria, we used the method described by Donnenberg et al. (6). A 1-ml sample of Caco-2 cells at a density of 8×10^4 cells per ml was seeded in 24-well tissue culture plates and grown for 10 days postconfluence. Cytolytic isolates of bacteria were grown in tryptic soy broth at 37°C for 24 h. The concentration was then adjusted to 10^8 bacteria per ml (i.e., an optical density of 0.5 at 660 nm). The 24-well plates with Caco-2 monolayers were drained of their medium, and 1 ml of the bacterial suspension was added to each well. The plates were centrifuged at 2,000 × *g* for 15 min to increase the contact between cells and bacteria. After incubation for 3 h at 37°C in a 5% CO_2 incubator, the cells were washed three times with phosphate-buffered saline and incubated for 1 h in minimal essential medium containing 100 μg of gentamicin per ml to kill noninvasive bacteria. The monolayer was washed with phosphate-buffered saline and then lysed with 1 ml of 1% Triton X-100 for 20 min to extract bacteria that had penetrated the cells. Surviving bacteria were counted by a plate dilution method. An invasion index was obtained as follows: (number of colonies obtained/number of inoculated bacteria) × 100.

Bacterial identification. Cytolytic isolates were subcultured on nutrient agar slants until identification. API 20E kits (API Laboratory Products, St-Laurent, Québec, Canada) were used to identify gram-negative bacteria. Gram-positive microorganisms were identified to the genus level by standard biochemical tests (16).

RESULTS

Bacterial concentration in water samples. The number of bacteria detected in the water samples tested varied from less than 1 to 200,000 CFU/ml on R2A medium (Table 1). The geometric mean was 365 CFU/ml when the bacteria were incubated at 25°C (8 days) and 145 CFU/ml when the bacteria were incubated at 35°C (2 days). On TSA-SB, the geometric mean was 2.4 CFU/ml when the bacteria were incubated at 35°C and 0.2 CFU/ml when they were incubated at 45°C. Lower bacterial counts were observed on CA-SB than on TSA-SB.

Virulence factors. TSA-SB was superior to CA-SB in detecting cytolytic bacteria with virulence characteristics (Table 2). Overall, 24% of the samples tested on R2A medium contained cytolytic colonies. Among the colonies growing on this medium, 0.8% of those incubated at 25°C and 2.3% of those incubated at 35°C were cytolytic. Placing the membranes on TSA-SBA at 35°C permitted the selection of a larger number of cytolytic isolates. Incubation at 45°C to detect thermotolerant bacteria was not as useful. The percentage of samples containing at least one colony with three or more virulence factors was also higher on TSA-SB than on CA-SB (Table 3).

Data on the individual isolates grown on CA-SB (Table 4) or TSA-SB (Table 5) show that *Bacillus*, *Micrococcus* and *Pseudomonas* species were the predominant bacteria isolated on these media and that several isolates contained all the virulence factors evaluated. Two *Staphylococcus* isolates detected on CA-SB had all four virulence factors.

TABLE 3. Percentage of samples containing at least one colony with three or more virulence factors when using TSA-SB or CA-SB for the expression of virulence

Original isolation medium and temp	% of samples with ≥3 virulence factors on:	
	TSA-SB	CA-SB
R2A (25°C)	6	5
R2A (35°C)	3	5
TSA-SB (35°C)	24	2
TSA-SB (45°C)	0	0

TABLE 4. Growth characteristics of cytolytic bacteria isolated from tap water, using R2A agar or CA-SB

Isolate	n[a]	Growth on primary plating medium				Virulence factor		
		R2A (25°C)	R2A (35°C)	CA-SB (35°C)	CA-SB (45°C)	Hemolysis[b]	Adherence	Invasiveness
Actinobacillus spp.	1	+	−	+	−	γ	+	−
Aeromonas hydrophila	1	+	−	+	−	γ	+	−
Bacillus globisporus	1	+	−	−	−	γ	−	−
Bacillus lentus	1	−	−	+	−	γ	−	−
Bacillus sphaericus	1	−	+	+	−	γ	−	−
Bacillus spp.	2	−	+	+	−	γ	−	−
Bacillus spp.	4	+	+	−	+	β	−	−
Flavobacterium spp.	2	−	+	−	−	γ	−	−
Klebsiella pneumoniae	1	+	−	−	−	γ	+	−
Micrococcus sp.	1	−	+	−	−	γ	+	−
Pseudomonas cepacia	1	−	+	−	−	γ	+	−
Pseudomonas maltophilia	1	+	−	−	−	γ	+	−
Pseudomonas maltophilia	1	+	−		−	γ	−	−
Pseudomonas maltophilia	1	+	−	−	−	α	+	+
Pseudomonas maltophilia	1	−	−	+	−	β	+	−
Staphylococcus spp.	2	+	+	−	−	β	+	+

[a] n, number of isolates.
[b] Hemolysis type: α, alpha; β, beta; γ, gamma.

DISCUSSION

Our results confirm earlier reports suggesting that a small number of bacteria in drinking water possess virulence factors and could pose a significant health risk if they were allowed to proliferate (19–21). Epidemiological studies conducted a few years ago in our laboratories suggested that a significant regrowth of heterotrophic bacteria could be the source of gastrointestinal illness (24). A large number of bacterial species and strains can be detected by the current bacteriological methods of water analysis (5, 7, 17, 22, 25, 28, 29). However, these tests, whether for coliform detection or heterotrophic plate count, all suffer from the same drawback: they do not address the health significance of the bacteria detected (21, 22). Their indirect value as indicators of fecal pollution or of

TABLE 5. Growth characteristics of cytolytic bacteria isolated from tap water, using R2A or TSA-SB

Isolate	n[a]	Growth on primary plating medium				Virulence factor		
		R2A (25°C)	R2A (35°C)	TSA-SB (35°C)	TSA-SB (45°C)	Hemolysis[b]	Adherence	Invasiveness
Gram-negative, unidentified	1	−	−	+	−	β	+	ND[c]
Aeromonas hydrophila	1	−	−	+	−	β	+	−
Bacillus brevis	1	−	+	−	−	γ	−	−
Bacillus lentus	1	−	−	+	−	γ	−	−
Bacillus licheniformis	1	−	−	+	−	γ	−	−
Bacillus macerans	1	+	−	−	−	β	−	−
Bacillus megaterium	1	−	+	−	−	γ	−	−
Bacillus megaterium	1	−	−	+	−	β	−	−
Bacillus mycoides	1	−	−	+	−	γ	−	−
Bacillus pantothenticus	1	−	−	+	−	γ	−	−
Bacillus pasteurii	1	+	−	−	−	γ	−	−
Bacillus polymyxa	1	−	−	+	−	γ	−	−
Bacillus polymyxa	2	−	−	+	−	β	+	+
Bacillus polymyxa	3	+	−	+	−	β	−	−
Bacillus pumilus	1	−	−	+	−	β	−	−
Bacillus spp.	8	−	−	+	+	γ	−	−
Bacillus spp.	7	+	−	−	+	α	−	−
Bacillus spp.	11	+	+	+	+	β	−	−
Bacillus spp.	2	+	−	+	−	β	+	−
Micrococcus spp.	2	−	+	+	−	γ	−	−
Micrococcus spp.	2	−	+	+	−	α	+	+
Micrococcus spp.	3	−	−	+	−	β	+	+
Pseudomonas cepacia	3	+	−	+	−	α	−	−
Pseudomonas fluorescens	1	+	−	−	−	α	−	−
Pseudomonas fluorescens	1	−	−	+	−	α	−	−
Pseudomonas maltophilia	2	+	−	+	−	α	+	+

[a] n, number of isolates.
[b] Hemolysis type: α, alpha; β, beta; γ, gamma.
[c] ND, not done.

inadequate disinfection cannot be denied, but they are of little value in estimating the health risk to the population.

This diversity of bacterial species and strains found in water precludes the use of the usual methods of detection of pathogenic bacteria because of the large number of specific tests to be performed. The method of Lye and Dufour (20) is a relatively simple screening method applicable to numerous colonies in a sample by using a primary isolation medium poor in nutrients (e.g., R2A agar) or rich in nutrients (e.g., blood agar). Using this method, we have shown that a significant number of bacteria isolated from drinking water samples possess virulence factors that increase their disease-causing potential. There are very few data on the relationship between the number of bacteria in a sample (i.e., the infective dose) and the outcome (i.e., disease). Some bacteria can easily be qualified as pathogens of public health significance, but, even then, their pathogenicity depends on the strain. Opportunistic pathogens are more difficult to describe, and their levels of pathogenicity are still more diversified. Heterotrophic bacteria in drinking water have always been considered to be a group of nonpathogenic bacteria with little public health significance (9, 12). With the frequent detection of pseudomonads and aeromonads in drinking-water samples, there has been an increased awareness of the potential health risks associated with bacterial regrowth in drinking-water samples (14, 15).

The isolates detected from drinking water on blood agar (Tables 4 and 5) were mostly from genera that have not been traditionally associated with significant public health problems. They are, however, bacteria that have been found in the human gastrointestinal tract and have been implicated in clinically significant infections (10). They are also found in tap water, bottled water, and point-of-use filtration devices (13, 22, 23). *Bacillus* spp. in particular are often found in these waters and have been implicated in opportunistic infections as well as gastrointestinal illnesses (10). Little is known about their association with subacute diseases such as endemic gastrointestinal illness, especially for bacteria that produce toxins that are important in the development or induction of disease. When they are allowed to grow in large numbers, such as those observed in reverse-osmosis units (18), the outcome could be low-level gastrointestinal illnesses which have a significant economic impact (11), as we have observed during epidemiological studies (24, 26).

The percentage of heterotrophic bacteria that were found to be cytolytic was higher at 35°C, suggesting that these are the bacteria that should be sought. The use of blood agar allows the growth of bacteria that are potentially pathogenic. Although only a small percentage of the bacteria found in drinking water were cytolytic on R2A medium, one-quarter of the bacteria isolated on blood agar at 35°C were cytolytic and had other virulence factors. Because of the large number of species and strains found in drinking water, it would be difficult to attempt to detect specific bacterial species. The use of blood agar at 35°C could be a suitable alternative to the use of coliforms or total plate counts to detect bacteria that have disease-causing potential in humans and are of public health significance.

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