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Effect of Chlorination on Antibiotic Resistance Profiles of Sewage-Related Bacteria

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A total of 1,900 lactose-fermenting bacteria were isolated from raw sewage influent and chlorinated sewage effluent from a sewage treatment plant, as well as from chlorinated and neutralized dilute sewage, before and after a 24-h regrowth period in the laboratory. Of these isolates, 84% were resistant to one or more antibiotics. Chlorination of influent resulted in an increase in the proportion of bacteria resistant to ampicillin and cephalothin, the increase being most marked after regrowth occurred following chlorination. Of the other nine antibiotics tested, chlorination resulted in an increased proportion of bacteria resistant to some, but a decrease in the proportion resistant to the remainder. Multiple resistance was found for up to nine antibiotics, especially in regrowth populations. Identification of about 5% of the isolates showed that the highest proportion of *Escherichia coli* fell in untreated sewage. Some rare and potentially pathogenic species were isolated from chlorinated and regrowth samples, including *Yersinia enterocolitica*, *Yersinia pestis*, *Pasteurella multocida*, and *Hafnia alvei*. Our results indicate that chlorination, while initially lowering the total number of bacteria in sewage, may substantially increase the proportions of antibiotic-resistant, potentially pathogenic organisms.

The occurrence of multiply antibiotic-resistant (MAR) bacteria in both drinking water and wastewater has been demonstrated in many studies (2, 3, 22, 23, 25) and is considered an important potential health problem. Antibiotic resistance in pathogens causes difficulty in effectively treating human infections, but antibiotic resistance in organisms which are not considered primary pathogens is also important because of the ability of these organisms to transmit resistance to other organisms by means of transmissible resistance factors (R-factors) (8, 9, 21, 24).

In a survey of 193 healthy adults and children who were not attending hospital and who had not recently received antibiotics, 53% were found to carry antibiotic-resistant coliforms in their feces, and in 61% of these coliforms transmissible R-factors were demonstrated (15). Another similar study indicated that 52% of patients entering hospital for nonurgent surgical operations carried antibiotic-resistant *Escherichia coli*. Approximately 60% of the resistant bacteria possessed R-factors, with multiple resistance patterns being more frequent than single ones (8). Other studies have supported the view that intestinal bacteria carrying R-factors are widespread in the human population (16, 19).

Wastewater treatments have been found to increase the proportion of bacteria which carry R-factors (5, 6, 11, 14, 18). Furthermore, Shuval et al. (20) have shown that extensive growth of both fecal and nonfecal coliforms occurred in chlorinated samples, even though no coliforms were detected immediately after chlorination. Under field conditions the regrowth of coliforms in chlorinated effluent that had been held for 3 days was inversely correlated with both the residual chlorine in the reservoir and the initial number of surviving coliforms. Laboratory experiments showed that regrowth occurred after initial exposure to 11 ppm (11 μg/ml) of chlorine, even in the absence of chlorine neutralization (20).

Other workers (14) found that the proportion of antibiotic-resistant coliforms increased from those in fecal material (0.1 to 1% of total coliforms being resistant) through urban wastewater (10% resistant) to river water (50%) and finally to potable water, where 80% of any coliforms present were antibiotic resistant. The increase in the proportions of antibiotic-resistant bacteria has been attributed to R-factor transfer (20). Multiple resistance in bacteria isolated from chlorine-treated and untreated drinking water has been studied, with the conclusion that treatment of raw water and its subsequent distribution selected for bacterial populations resistant to several antibiotics (2, 3). The chlorination step was thought to be involved in the selection of antibiotic resistance.

The purpose of the present research was to determine the extent which chlorination plays in the development of antibiotic resistance. We studied the changes in antibiotic resistance patterns after wastewater chlorination at a municipal plant and also after laboratory chlorination and regrowth of sewage-contaminated drinking water. The antibiotic resistance patterns to 11 antibiotics were determined and analyzed. A preliminary identification of a sample of the surviving organisms was made.

MATERIALS AND METHODS

Description of bacterial populations studied. Four populations of bacteria were used in this study. The following terms are used to describe them. (i) The effluent population was isolated from samples of chlorinated effluent obtained from the Green Creek Sewage Treatment Plant, Ottawa, Ont., Canada. (ii) The influent population was isolated from raw sewage influent above the treatment plant. (iii) The chlorinated influent population was isolated from the influent raw sewage which had been chlorinated in the laboratory. (iv) The regrowth population was isolated from a sample of chlorinated influent, following 24-h recovery (regrowth) after laboratory chlorination.

Sample collection. Influent or effluent samples were collected at 10:00 a.m. by plant personnel and held at 4°C during transportation to the laboratory. The bacterial populations were isolated from samples collected over the period February through March 1981.

Wastewater treatment in the sewage treatment plant. The influent remained in holding tanks for 1.5 h to allow gravity sedimentation of particulate matter; coagulation was aided

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TABLE 1. Antibiotic disks used

Antibiotic	Disk potency (μg)	Inhibition zone diam (including disk diam) (mm)		
		Resistant	Intermediate	Sensitive
Ampicillin	10	<12	12–13	>13
Cephalothin	30	<15	15–17	>17
Chloramphenicol	30	<13	13–17	>17
Gentamicin	10	≤13		>13
Kanamycin	30	<14	14–17	>17
Nalidixic acid	30	<14	14–18	>18
Nitrofurantoin	300 U	<15	15–16	>16
Polymixin B	300 U	<9	9–11	>11
Sulfonamides	300	<13	13–16	>16
Tetracycline	30	<15	15–18	>18
Trimethoprim	25	<11	11–15	>15

by the addition of 0.25 ppm (0.25 μg/ml) of Percol 727 (Allied Colloids, Rexdale, Ont., Canada). The influent was then dosed with chlorine, such that after 20 min the residual available chlorine was 0.5 mg/liter.

Isolation of effluent and influent populations. The samples were shaken mechanically for 30 min at room temperature to disrupt clumps of particulate material. Serial dilutions of both influent and effluent samples were made with chlorine-free, filter-sterilized tap water. Samples of these dilutions were passed through membrane filters (0.45 μm; Nuclepore Corp., Pleasanton, Calif.) which were incubated on eosin methylane blue agar (Difco Laboratories, Detroit, Mich.) at 37°C for 18 h, after which the colonies were counted and the bacterial count in the original samples was calculated. Eosin methylene blue agar was chosen as the initial medium on which to isolate the sewage bacteria because of its selectivity for lactose-fermenting strains (1). Such lactose-fermenting (metallic sheen) colonies were then purified by streaking for single colonies onto MacConkey agar (Difco).

Laboratory chlorination of influent. To part of the initial 10-fold dilution of influent, chlorine was added from a standardized stock solution to give an initial dose of 1 mg of total chlorine per liter in tap water (25). The sample was allowed to stand at room temperature in the dark for 1 h, a portion was withdrawn for chlorine measurement, and free chlorine was neutralized in the remainder by the addition of 3 ml of 1% sodium thiosulfate (J. T. Baker Chemical Co., Phillipsburg, N.J.) per 100 ml of bacterial suspension. Target bacteria survival varied from 10^{-3} to 10^{-4} in these chlorinated samples. There was no detectable killing in an identical nonchlorinated control sample.

Laboratory determination of chlorine levels. Total available chlorine levels were measured by the *N,N-diethyl-p*-phenylenediamine procedure, using the Hellige hand colorimeter, in accordance with method 409F of the American Public Health Association (1). Total available chlorine was 0.8 to 0.9 mg/liter in all experiments, the difference from that added presumably being due to organic matter in the samples.

Regrowth population. The regrowth population was isolated as above, from chlorinated influent which had been allowed to stand at room temperature for 24 h after neutralization of the chlorine by sodium thiosulfate. After this recovery period the viable count increased 100-fold.

Determination of antibiotic resistance profiles. Antibiotic resistance profiles were obtained for approximately 1,900 isolates. A single colony of each of the purified isolates from the MacConkey agar plates was inoculated into 5 ml of sterile broth and incubated at 37°C for 2 to 10 h to give an optical density visually equal to a MacFarland no. 3 standard (ca. 10^9 cells per ml). Mueller-Hinton agar (Difco) plates were confluently seeded by swabbing with cotton (Q-tips; Johnson & Johnson, Cheeseborough-Ponds, Markham, Ontario) moistened with the culture. After the plates were dried for 1 h, a semiautomatic dispenser was used to place up to 12 different antibiotic-impregnated disks on each plate. Diameters of the inhibition zones surrounding the disks were recorded after 18 h of incubation at 37°C. Bacterial isolates were characterized as "resistant," "intermediate," or "sensitive" according to the specifications for the disks provided by the manufacturer (Pfizer Inc., New York, N.Y.) and given in Table 1.

Identification of strains. After determination of antibiotic resistance profiles, the strains were stored on the MacConkey agar plates until all strains were thus characterized. One isolate of every 10 from these MacConkey plates was selected at random and provisionally identified by using the Analytical Profile Index system (Analytab Products, Ayerst Laboratories, Plainview, N.Y.).

Statistical methods. The date of sample collection was treated as a blocking factor in the analysis. The difference between the proportions of isolates which were resistant in the different populations was examined for each antibiotic, using the Mantel-Haenszel test for proportions in a blocked experiment (17). Comparisons of proportion were made between (i) influent and chlorinated influent, (ii) influent and regrowth, (iii) influent and effluent, and (iv) regrowth and

TABLE 2. Comparison of antibiotic resistance in bacterial populations[a]

Antibiotic[b]	Influent (%)				Effluent (%)				Chlorinated influent (%)				Regrowth (%)			
	R	I	S	No. of isolates	R	I	S	No. of isolates	R	I	S	No. of isolates	R	I	S	No. of isolates
AMP	68	6	25	692	73	6	20	190	77	8	14	723	83	5	12	258
CEPH	45	12	43	692	53	3	44	189	60	5	35	722	75	6	19	257
KAN	4	5	91	692	3	2	95	190	6	5	89	723	2	3	95	258
POL	1	8	90	692	0.5	0.5	99	190	2	8	91	723	0	1	99	258
GEN	1		99	692	1		99	188	2		98	723	1		99	258
TET	8	11	81	692	4	10	86	190	11	8	80	722	18	6	76	258
CHLOR	3	1	96	690	0	4	96	190	2	2	97	723	2	0.4	97	258
NIT	8	13	79	691	2	3	95	190	3	7	90	723	4	1	95	258
NAL	1	6	93	691	0.5	2	98	190	2	2	96	723	3	1	96	258
SUL	20	10	70	690	18	4	77	190	21	11	69	723	34	6	59	258
TRI	4	1	94	691	5	3	92	189	4	2	93	722	10	1	88	258

[a] R, Resistant; I, intermediate; S, sensitive.

[b] AMP, Ampicillin; CEPH, cephalothin; KAN, kanamycin; POL, polymixin B; GEN, gentamicin; TET, tetracycline; CHLOR, chloramphenicol; NIT, nitrofurantoin; NAL, nalidixic acid; SUL, sulfonamides; TRI, trimethoprim.

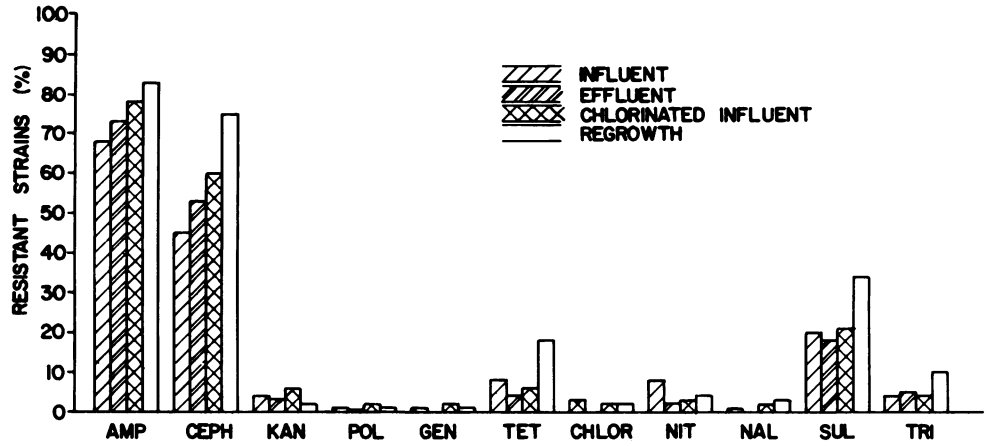

FIG. 1. Percentage of strains resistant to each antibiotic.

chlorinated influent. Because of the exploratory nature of the study, no attempt was made to control overall error rate in the experiment. Significance was tested at $P < 0.05$.

RESULTS

About 84% of the 1,900 isolates were resistant to at least one of the antibiotics tested: in particular, 79.5% in the influent, 88.2% in the chlorinated influent, 87.5% in the regrowth, and 81.2% in the effluent. Table 2 shows the proportion resistant to each antibiotic for the different populations, and Fig. 1 summarizes this information graphically. The statistical significance of these results is shown in Table 3.

The proportion of bacteria resistant to ampicillin and cephalothin increased significantly in the order influent, effluent, chlorinated influent, and regrowth. For tetracycline, nalidixic acid, sulfonamides, and trimethroprim the regrowth population showed the highest incidence of resistance. Relatively little resistance to other antibiotics appeared in any of populations studied. At these low levels, however, decreased resistance to kanamycin, chloramphenicol, nitrofurantoin, gentamicin, polymixin, and nalidixic acid was observed after chlorination or regrowth or both (Fig. 1; Table 3).

Occurrence of multiple resistance. Table 4 shows the proportion of each population resistant to one to nine antibiotics. The mean value of multiple antibiotic resistance was significantly higher in the regrowth population at 2.34 antibiotics per isolate than it was in the chlorinated effluent (1.89), influent (1.64), and effluent (1.61).

Resistance to certain antibiotics appeared to be linked. For example, over 90% of the isolates resistant to four or more antibiotics were resistant to ampicillin, cephalothin, sulfonamides, and tetracycline or to ampicillin, cephalothin, sulfonamides, and trimethoprim. In the group multiply resistant to four or more antibiotics, resistance to nitrofurantoin and nalidixic acid was associated with one another in 88% of the isolates where resistance to one of them was detected.

Strains identified in the populations. Table 5 shows the proportions of bacterial species identified in each of the populations. This information applies to the 70% of strains which survived storage after determination of their antibiotic resistance profiles. Thirty-five percent of these failed to key out on the Analytical Profile Index system. Consequently, the species distribution we found may differ somewhat from the environmental distribution. However, as expected, most species were coliforms or related species in the family *Enterobacteriaceae*. Most interesting is the decrease in the proportion of *E. coli* strains identified in the chlorinated influent, regrowth, and effluent populations compared with the nonchlorinated influent.

DISCUSSION

The proportions of bacteria resistant to at least one antibiotic ranged from 79.5% in the influent to 87.5% in the

TABLE 3. Patterns of incidence of resistance

Antibiotic	Significant differences ($P < 0.05$)[a]
Ampicillin	CI > I R > I R > CI
Cephalothin	CI > I R > I R > CI
Kanamycin	CI > R I > R
Polymixin B	CI > R I > R
Gentamicin	I > E
Tetracycline	CI > I R > I R > CI
Chloramphenicol	I > E I > R
Nitrofurantoin	I > CI
Nalidixic acid	R > CI
Trimethoprim	R > CI
Sulfonamides	R > CI R > I

[a] I, Influent; CI, chlorinated influent; R, regrowth; E, effluent.

TABLE 4. Percentage of strains resistant to zero, one, or more antibiotics

No. of antibiotics to which bacteria exhibited resistance	% of strains resistant in given population			
	Influent	Chlorinated influent	Regrowth	Effluent
0	20.5	11.8	12.5	18.8
1	30.9	26.5	10.9	30.6
2	26.3	35.3	34.6	29.0
3	13.8	17.1	24.5	15.6
4	5.4	6.5	13.2	4.3
5	1.6	1.5	1.6	1.6
6	1.2	1.1	1.2	0.0
7	0.1	0.0	0.8	0.0
8	0.1	0.0	0.8	0.0
9	0.1	0.1	0.0	0.0
Population (*n*)	689	720	254	186
Mean resistance	1.65	1.90	2.33	1.61

TABLE 5. Identified strains[a]

Species	Frequency in populations			
	Influent	Chlorinated influent	Regrowth	Effluent
Klebsiella ozaenae	0	2	0	0
Klebsiella oxytoca	7	8	2	2
Klebsiella pneumoniae	8	7	5	2
Aeromonas hydrophila	6	11	3	2
Escherichia coli	12	1	0	1
Enterobacter cloacae	1	0	1	0
Enterobacter aglomerans	0	2	1	1
Yersinia pestis	0	1	0	0
Yersinia enterocolitica	0	0	0	1
Pasteurella multocida	0	1	1	0
Serratia liquifaciens	0	1	0	0
Hafnia alvei	0	0	0	2
Citrobacter freundii	0	1	1	0
Shigella boydii	1	1	0	0
Unidentifiable	20	19	2	3

[a] Strains identified were selected at random (every 10th numbered isolate) from isolates that survived storage after determination of antibiotic resistance profiles.

regrowth samples. These values are comparable to those obtained by Armstrong et al., who found proportions of up to 87% in river water and 84.5% in clear well water resistant to one or more antibiotic (2), and with the findings of LeClerc and Mizon (14), who found that up to 80% of coliforms in potable water were antibiotic resistant.

While our study was in progress, Armstrong et al. (2) published comparisons between standard plate count isolates from source river water and drinking water produced by flash mixing with chlorine. They found a significant increase in the proportions of MAR bacteria. They also studied antibiotic resistance profiles of isolates that survived laboratory chlorination of river water, resulting in an 800-fold decrease in the standard plate count population, and found no increase in the proportion of MAR strains in these isolates. In our study we found a significant increase in the percentage of strains multiply resistant to two or three antibiotics when influent was chlorinated in the laboratory and a marginal increase when influent was compared with effluent which had been treated at the sewage treatment plant (Table 3).

Differences between our results and those of Armstrong et al. (2) may be more apparent than real: in our study only lactose-fermenting bacteria isolated from eosin methylene blue plates were studied, so our populations are more representative of coliforms, and in our laboratory chlorination procedures we observed a 10-fold-greater degree of bacterial death by chlorination than that observed by Armstrong et al. (2, 3). It is possible that chlorinated coliform populations are more likely to develop MAR strains than other bacteria or that increase in the number of MAR strains appears only after the survival rate falls to 10^{-3} or less. The difference in the incidence of MAR strains arising in our laboratory studies compared with those found after wastewater treatment at the sewage treatment plant probably reflects differences between chlorination and recovery which occur in the two environments. Despite such differences, both treatments increase the incidence of antibiotic resistance in survivors, but the effect on MAR incidence is more pronounced in laboratory-chlorinated samples.

Though increased resistance, notably to ampicillin, cephalosporin, tetracycline, and sulfanilimide, was the most striking finding, resistance to some antibiotics decreased on chlorination (see Results). There is, thus far, no clear relation between mode of antibiotic action and pattern of resistance development. Among the antibiotics to which resistance increased after chlorination, ampicillin and cephalothin act on cell wall synthesis and sulfanilamide and trimethoprim act on folic acid synthesis and metabolism, respectively. Tetracycline, to which resistance increases, and chloramphenicol, gentamicin, and kanamycin, to which it decreases, act on different aspects of ribosomal function (10). Nitrofurantoin, to which resistance decreases after chlorination, has been reported to affect translation initiation and to cause DNA damage (13).

It is not clear whether chlorination selects or induces changes in antibiotic resistance in bacterial populations. Several workers have suggested that the fecal coliforms, which are generally more antibiotic resistant than other coliforms, may have a survival advantage in natural and treated wastewaters (4, 5, 7, 11).

Armstrong et al. (2) suggested, without specifying the mechanisms, that stress-tolerant strains selected by chlorination would be more antibiotic resistant. It is uncertain whether specifically chlorine-resistant coliforms exist (12), and certainly no physiological linkage between resistance to chlorine and that to antibiotics suggests itself. However, the possibility that resistant or MAR strains survive chlorination better than sensitive strains could be tested directly. Another possibility, that chlorination helps in the transfer of antibiotic resistance plasmids to the surviving population of bacteria, is also open to experimental investigation.

Although most coliforms are usually considered as harmless indicators of water quality, strains of MAR bacteria that colonize the intestinal tract of humans or animals could transfer their resistance to intestinal commensals and in turn to drug-sensitive pathogens (9, 21, 24). The maximum removal of MAR bacteria from sewage before discharge to the environment and prevention of their contamination of drinking water are obviously highly desirable. However, whereas chlorination initially lowers the total number of bacteria, it may substantially increase the proportions of those resistant to one or more antibiotics and thus facilitate the transfer of resistance to other, possibly pathogenic, strains.

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