



Project Summary

Effect of Wastewater Disinfectants on Survival of R-Factor Coliform Bacteria

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This investigation was undertaken to assess the problem of antibiotic resistance among total and fecal coliform bacteria in wastewaters. Of particular concern is the potential for dispersal of antibiotic resistance from enteric bacteria derived from the human intestinal tract to other bacteria in environments that receive effluent from wastewater treatment facilities. It is conceivable that environmental strains of opportunistic pathogens such as *Pseudomonas aeruginosa* could acquire multiple-antibiotic resistance borne on plasmids originating directly from enteric bacteria. This could complicate antibiotic therapy for patients exposed to environmental strains of potential pathogens.

The objectives of the study were to determine the incidence of antibiotic resistance among coliform bacteria in a secondary wastewater treatment facility and to determine whether various alternative disinfection procedures would select for or against antibiotic resistant coliform bacteria. In addition, experiments were designed to determine if some of the coliform bacteria that were resistant to several antibiotics carried their resistance features on plasmids (R-Factors) and, if so, to determine whether they could transfer these plasmids to other organisms in the environment.

Widespread antibiotic resistance was found among total and fecal

coliform bacteria. Levels of 20% resistance were commonly encountered for the various antibiotics tested including streptomycin, chloramphenicol, tetracycline, and kanamycin. Higher levels (up to 80% of total coliform bacteria) were resistant to ampicillin, but this resistance was associated primarily with *Klebsiella pneumoniae* and thought to be chromosomal-borne (all strains of this commonly encountered total and fecal coliform species that we analyzed were resistant to ampicillin whether or not they contained plasmids)

All wastewater treatment procedures tested (chlorination, ozonation, and ultraviolet disinfection) resulted in significant decreases both in antibiotic-resistant as well as antibiotic-sensitive total and fecal coliform bacteria. No dramatic selection for antibiotic-resistant types was apparent during treatment although occasionally some specific types were found at somewhat higher levels in secondary and disinfected effluent than in raw wastewater. The high diversity of total and fecal coliform bacteria found in the wastewaters, and the high diversity of antibiotic-resistant patterns and plasmid compositions among the coliform bacteria complicated the resolution of this question.

Attempts were made to determine whether naturally occurring plasmid-containing, multiple-antibiotic resistant *Escherichia coli*

could transfer resistance to laboratory strains and vice versa. Though this could be demonstrated in the laboratory under conditions simulating that of the natural environment, the transfer rates were low using bacterial concentrations that were much higher than would be normally encountered in the environment. This suggests that transfer may not occur in natural environments unless there is some unknown mechanism for concentration of *E. coli* in receiving waters.

Non-coliform heterotrophic bacteria from wastewaters were found to carry equally high proportions of antibiotic resistance. Since these organisms occur at about 100-fold higher concentrations than coliform bacteria, they may prove to be a more important source of R-Factors for environmental bacteria.

This Project Summary was developed by EPA's Water Engineering Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

For public health reasons there is concern about the widespread dispersal of antibiotic resistance among bacteria in the environment. Antibiotic-resistance traits are sometimes borne on plasmids which can be transferred from one species of bacterium to another. Thus, by this mechanism, antibiotic-resistance traits could be disseminated from enteric bacteria of human origin through wastewater treatment facilities to an organism in the environment that are sensitive to the antibiotics of concern. Some of these environmental organisms, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, are potential pathogens. Their acquisition of antibiotic resistance could complicate antibiotic therapy if they were subsequently responsible for an infectious disease.

Wastewater is a possible major source of antibiotic-resistance traits. Patients receiving antibiotic therapy are known to harbour large numbers of antibiotic-resistant enteric bacteria in their intestinal tracts. These enter wastewater treatment facilities and are discharged into receiving waters. The overall goal of this project was to learn more about the incidence of antibiotic resistance among

total and fecal coliform bacteria in raw and treated wastewaters. Specific objectives included: (a) a determination of the extent to which antibiotic-resistant total and fecal coliform bacteria exist in (i) raw wastewater, (ii) treated secondary effluent from an activated sludge facility, and (iii) secondary effluent disinfected by chlorination, ozonation, and ultraviolet light; (b) assessment of whether some of the resistance is due to R-Factor plasmids; and, (c) determination of whether antibiotic resistance can be transferred from one bacterium to another in wastewater.

Materials and Methods

During 18 surveys of a secondary activated sludge wastewater treatment facility, fifty total and fecal coliform bacterial isolates were randomly chosen at each stage of treatment from primary enumeration media (mEndo medium for total coliforms and mFC medium for fecal coliform bacteria). In early chlorination surveys, a comparison was made between direct selection of coliform bacteria on the mEndo and mFC media by incorporating antibiotics individually in the media at 20 µg/mL of each [ampicillin (A), streptomycin (S), chloramphenicol (C), tetracycline (T), kanamycin (K), and naladixic acid (N)] versus indirect selection on mEndo and mFC media containing no antibiotics followed by streaking for isolation on MacConkey agar into which the antibiotics were individually incorporated. The indirect selection procedure in which 50 randomly chosen isolates were tested for resistance, generally provided higher recoveries of antibiotic-resistant strains, so it was used in all subsequent work.

In six of the surveys, chlorination was used in the laboratory at two concentrations to disinfect the secondary clarified effluent. In another six surveys, ultraviolet light (UV) was used at two levels as a disinfectant, and in the final six surveys ozone was used at low and high levels as a disinfectant.

In one survey the 50 fecal coliform bacteria isolated from each stage of treatment (i.e. raw wastewater, secondary effluent and ultraviolet-treated effluent at low and high levels of disinfection) were identified to species using the API-20E system (Analytab Products, Plainview, N.Y.)* and their antibiotic resistance determined as described above. Strains were lysed and plasmids were characterized using gel electrophoresis.

The antibiotic-resistance patterns of total heterotrophic bacteria isolated from standard plate count agar were determined in raw wastewater, secondary clarified effluent and UV-treated effluent.

Experimental Results

Direct selection procedures resulted in lower recoveries of antibiotic-resistant total and fecal coliform bacteria than with indirect selection (Table 1) suggesting that coliform organisms are more susceptible to antibiotic effects upon primary isolation. Thus, in all subsequent studies, indirect selection procedures were used to obtain isolates.

Antibiotic resistance was common among total and fecal coliform bacteria. The most common resistance was ampicillin. Frequently, greater than 80% of the total coliform bacteria were resistant to this antibiotic throughout the various treatment stages regardless of the disinfectant used. Generally low levels of resistance to this antibiotic were found among the fecal coliform isolates. This may be explained by the results of one survey (October 9, 1980), when the identity of all fecal coliform isolates was determined, that showed most strains of *Escherichia coli* were sensitive to the ampicillin (Table 2). In contrast, the other common fecal coliform bacterium was *pneumoniae*, and all of the strains of this organism were resistant to this antibiotic. Because *E. coli* was the predominant fecal coliform bacterium, the percentage of fecal coliforms resistant to A was reduced in comparison to total coliforms where *K. pneumoniae* and related resistant species may be predominant. Strains of *K. pneumoniae* that are singly resistant to this antibiotic probably do not carry this resistance on a plasmid because plasmids were not detected in some strains of this species and some strains were resistant to this antibiotic (Table 2).

Lower levels of resistance were usually found for antibiotics other than ampicillin. However, levels of 20% resistance or greater were often encountered for the other antibiotics (Table 1). Tables 3 and 4 show the results for another survey. Table 3 indicates the percentage of strains showing resistance to each antibiotic. Table 4 shows the antibiotic resistance patterns of each strain from that survey.

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use

Table 1. Comparison of Recovery of Antibiotic-Resistant Bacteria by Direct and Indirect Selection Procedures (6-13-80)

Wastewater	Percentage of Antibiotic-Resistant Coliforms									
	Ampicillin		Chloramphenicol		Kanamycin		Streptomycin		Tetracycline	
	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
<i>Total Coliforms</i>										
Raw	10	80.4	0.80	15.2	11.8	10.9	11.9	52.2	4.5	8.7
Secondary	47	76.0	1.1	24.0	1.5	54.0	1.5	70.0	1.7	20.0
Low Chlorination*	5.7	87.5	9.6	20.8	14.3	31.3	6.8	50.0	21.1	2.1
High Chlorination*	6.1	81.3	0.70	22.9	11.9	16.7	4.9	29.2	5.2	14.6
<i>Fecal Coliforms</i>										
Raw +	1.3	48.9	0.85	27.7	1.6	42.6	13.9	55.3	12.2	21.3
Secondary	7.4	46.0	0.20	26.0	1.5	30.0	3.2	38.0	5.3	40.0
Low Chlorination	9.6	66.0	0.24	24.0	4.3	38.0	8.2	56.0	8.6	32.0
High Chlorination	19.0	60.0	0	30.0	0.33	14.0	0	24.0	12.7	0

* 48 strains checked rather than 50.

+ 47 strains checked rather than 50.

Table 2. Identification and Antibiotic Resistance of Fecal Coliform Bacteria from Raw Wastewater and Secondary Effluent from the Renton Wastewater Treatment Plant (October 9, 1980)

Species	Antibiotic Resistance Pattern (No. of strains/No. of plasmids)*	
	Raw Wastewater	Secondary Wastewater
<i>Escherichia coli</i>	0 (16/0)	0 (6/0)
" "	0 (12/1-4) +	0 (16/1-6)
" "	A (2/6-8); T (1/2); AT (1/3); KS (1/2); ST (1/3); ACS (2/1); CST (1/1); KST (1/3); AKST (1/2); ACKST (1/5)	A (1/0); S (1/3); T (1/1); AS (2/1-2)
<i>Klebsiella pneumoniae</i>	A (3/1-3)	A (4/0); A (16/1-7)
<i>Klebsiella oxytoca</i>	A (2/2-3)	
<i>Citrobacter freundii</i>	0 (2/1-6)	
" "	A (3/1-8)	

*Antibiotics tested included ampicillin (A), tetracycline (T), kanamycin (K), streptomycin (S), and chloramphenicol (C).

+ This indicates that of these 12 strains, none were resistant to any of the antibiotics tested and that 1-4 plasmids were detected in each strain.

Antibiotic resistance occurred at comparable levels among noncoliform bacteria (Standard Plate Count isolates) obtained from wastewater (Table 5) Multiple resistance is also common in

this diverse group of bacteria (Table 6). Since these are 50 to 100 times more numerous than total coliforms and 100 to 200 times more numerous than fecal coliforms in raw and treated wastewater,

they comprise an important reservoir for antibiotic resistance in wastewaters.

Plasmids were responsible in part for multiple resistance to antibiotics. For example, during one of the UV surveys

Table 3. Antibiotic-Resistant Coliform Indices of Renton Wastewater(August 20, 1980)

Wastewater	None	Ampicillin	Chloramphenicol	Kanamycin	Streptomycin	Tetracycline
<i>Total Coliforms</i>						
Raw	3.5x10 ⁷ (1.9-5.2)*	1.3x10 ⁷	7.0 x10 ⁵	2.8x10 ⁶	4.2x10 ⁶	3.5x10 ⁶
% of unselected	100%	37.0%	2.0%	8.0%	12.0%	10.0%
Secondary	4.9x10 ⁵ (3.6-6.1)	2.5x10 ⁴	ND ⁺	2.9x10 ⁴	2.9x10 ⁴	ND
% of unselected	100%	50.0%		6.0%	60%	
Secondary chlorinated‡	6.0x10 ⁴	3.7x10 ⁴	ND	2.4x10 ³	9.6x10 ³	7.2x10 ³
% of unselected	100%	62.0%		4.0%	16.0%	12.0%
Secondary high chlorinated‡	3.5x10 ² (2.3-4.6)	1.8x10 ²	7.0x10 ⁰	2.1x10 ¹	7.0x10 ¹	2.8x10 ¹
% of unselected	100%	52.0%	2.0%	6.0%	20.0%	8.0%
<i>Fecal Coliforms</i>						
Raw	4.5x10 ⁶ (3.8-5.2)	2.7x10 ⁵	2.7x10 ⁵	9.0x10 ⁴	1.4x10 ⁶	9.9x10 ⁵
% of unselected	100%	6.0%	6.0%	2.0%	30.0%	22.0%
Secondary	2.2x10 ⁴ (2.1-2.3)	1.3x10 ³	1.3x10 ³	ND	4.0x10 ³	4.8x10 ³
% of unselected	100%	6.0%	6.0%		18.0%	22.0%
Secondary chlorinated‡	1.5x10 ⁴ (0.3-1.6)	2.1x10 ³	ND	9.0x10 ²	3.9x10 ³	4.2x10 ³
% of unselected	100%	14.0%		6.0%	26.0%	28.0%
Secondary high chlorinated‡	90.0(0.0-24.6)	ND	ND	ND	ND	22.5
% of unselected	100%					25.0%

*Numbers in parentheses indicate the 95% confidence intervals of the counts, e.g., 3.5 x10⁷ (1.9-5.2) would indicate that the range was from 1.9x10⁷ to 6.1x10⁷ with a mean of 3.5x10⁷.

⁺ Not detected, antibiotic resistance levels too low to be detected by this procedure.

‡Total chlorine dosage before and after test: low dosage (0.25-0.02 mg/L), high dosage (1.80-1.70 mg/L).

one strain of *E. coli* (FH 14) resistant to AKST transferred its resistance (and plasmid) to a laboratory recipient (*E. coli* K12 C600).

There was no consistent recurring pattern indicating that treatment resulted in selection of certain antibiotic-resistant types during our surveys. However, occasionally a particular resistance pattern was selected for by the disinfection process. For example, during the UV survey of October 9, 1980, it appeared that there was an increase in resistance to chloramphenicol, streptomycin and tetracycline. When the patterns of resistance for each strain were examined it was noted that whereas there were no strains of fecal coliforms resistant to AC in secondary effluent, three were resistant following low levels of UV and four were resistant following high levels of UV. Likewise two strains of fecal coliforms were resistant to ST after

low levels of UV and three were resistant after high levels, whereas none were resistant at the preceding secondary stage of treatment. Resistance to AC was found to be due almost exclusively to *K. pneumoniae*; only one strain of *E. coli* was found to be resistant to these two antibiotics. In contrast, resistance to ST was found to exclusively reside in *E. coli* during this survey. *E. coli* strains resistant to this antibiotic following low levels of UV contained different plasmid contents, although they both had a 3.2 megadalton (Md) plasmid. The three strains of *E. coli* resistant to ST after high levels of UV also had plasmids. Two of these appeared to have plasmid compositions identical to one another; however, they differed from the plasmid composition of the strains of the low UV stage. Therefore, this indicates that one particular strain was not necessarily being selected. However, all five ST

strains contained a plasmid of 3.2 to 3. Md size. Thus, this particular plasmid type may carry UV resistance as well as resistance to ST.

Attempts were made to demonstrate in situ conjugation in raw wastewater using laboratory strains and strain selected from wastewater. Although conjugation could be demonstrated, the frequency was very low using conditions more favorable than that of actual wastewater. Although this does not rule out the possibility that in situ conjugation might occur, it does suggest that, if occurs, it would be uncommon in treated wastewater.

Conclusions

Disinfection by chlorination, ultraviolet light, and ozonation is effective in reducing not only the numbers of total and fecal coliform bacteria, but also the numbers of those resistant to antibiotic

Table 4. Number of Isolated Coliform Bacteria and Their Antibiotic Resistance Patterns from Survey of 8/20/80 (see Table 3)

Antibiotic Resistance Pattern				Total Raw*	Total Sec- ondary	Total Cl	Total High Cl	Fecal Raw	Fecal Sec- ondary	Fecal Cl	Fecal High Cl
				49 ⁺	48	50	50	50	50	50	4
A				13 [‡]	23	23	24	-	-	1	-
	K			1	1	-	-	-	-	-	-
		S		1	-	-	7	5	1	2	-
			T	1	-	-	-	2	2	3	1
A	K			—	-	1	-	-	-	-	-
A		N		-	-	2	-	-	-	-	-
A			S	1	1	3	-	1	-	-	-
A				T	1	-	1	-	1	-	-
A	C		N	1	-	-	-	-	-	-	-
A		K	S	-	3	-	-	-	-	-	-
A			S	T	1	-	1	-	1	3	-
	C		S	T	-	-	-	2	2	-	-
		K	S		1	-	-	-	-	-	-
		K	S	T	2	-	-	-	1	-	-
			S	T	-	-	3	-	4	5	-
A	C	K	N	S	-	1	-	-	-	-	-
A		K		S	T	-	1	1	-	3	-
A	C		N	S		-	1	-	-	-	-
A	C			S	T	-	-	1	1	-	-
Total				23	29	36	33	17	12	17	1

*Total: Total coliform bacteria obtained by standard methods on membrane filters. Fecal: Fecal coliform by bacteria obtained by standard methods on membrane filters. Raw: Raw wastewater sample, Renton. Secondary: Secondary wastewater sample, Renton. L-Uv: Low ultraviolet light dosage to secondary wastewater

H-Uv: High ultraviolet light dosage to secondary wastewater.

⁺Total number of isolates obtained.

[‡]Number of isolates with that antibiotic-resistant pattern.

[—]Indicates a value of < 1 coliform bacterium isolated

Thus, current treatment practices have a major impact on reducing the total numbers of antibiotic-resistant bacteria that reach receiving waters. Furthermore, there was no evidence of a major increase in the proportion of any antibiotic-resistant total or fecal coliform bacteria during wastewater treatment or disinfection. Therefore, we conclude that current treatment and disinfection practices are reasonably nonselective and effective in reducing the widespread dispersal of antibiotic-resistant bacteria.

Total and fecal coliform bacteria comprise only one reservoir for antibiotic resistance among bacteria in raw wastewater. Standard Plate Count (SPC) bacteria occur in higher concentrations and have similar levels of antibiotic resistance. Therefore it would be desirable to know the source of the antibiotic-resistant SPC bacteria and also determine where they acquire their resistance. It seems reasonable that the intestinal tract of humans carries many antibiotic-resistant noncoliform bacteria, and these may serve as a more

important source for these properties in the natural environment receiving wastewater. Thus we recommend examining the role of noncoliform bacteria as environmental sources of antibiotic resistance.

It seems unlikely that *E. coli* is able to transfer antibiotic resistance in raw wastewaters based upon the results of the conjugation experiments performed. Their numbers are lower in raw wastewater than expected for successful conjugation and are reduced even further at each successive stage of treatment

Table 5. Antibiotic-Resistance Heterotrophic Bacteria Isolated from Renton Wastewater Treatment Facility (1/14/81)

Wastewater	Numbers of Unselected*	No. of Isolates‡	% Antibiotic Resistant					
			Ampicillin	Chloramphenicol	% Antibiotic Kanamycin	Resistant Nalidixic Acid	Streptomycin	Tetracycline
Raw	$3.4 \times 10^8 (\pm 2.8 \times 10^8)^+$	32	69	9	<3	16	12	3
Secondary effluent (2°)	$3.2 \times 10^7 (\pm 2.2 \times 10^7)$	27	70	4	<4	4	30	4
2° low UV	$4.8 \times 10^5 (\pm 3.0 \times 10^5)$	20	50	5	10	30	5	5
2° high UV	$1.9 \times 10^3 (\pm 1.3 \times 10^3)$	15	67	<7	20	40	20	27

* Mean bacterial density, from three plates, per 100 mL.

+ 95% confidence limits.

‡ Isolates were obtained by standard plate count procedures. Thus, they were selected from Plate Count Agar plates incubated for 48 h at 35°C. This number refers to the number of strains.

and disinfection. Therefore, unless some unknown and unexpected concentration mechanism occurs, it seems doubtful that antibiotic resistance will be transferred commonly by enteric bacteria in wastewater or following release of wastewater into receiving waters. Thus, additional treatment of wastewaters beyond disinfection appears unnecessary to control dispersal of antibiotic resistance carried by enteric bacteria.

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Table 6. Numbers of Isolated Heterotrophic Bacteria* and Their Antibiotic Resistance Patterns from Survey of 1/14/81

Antibiotic Resistance Pattern					Raw + (32)‡	2° (27)	Low-UV (20)	High-UV (15)
A					17**	13	5	5
	C				1	-	-	-
		N			1	-	3	1
			S		1	-	-	-
A	C				††	-	1	-
A		N			1	-	1	1
A			S		1	6	1	-
A				T	1	-	1	1
	C		S		-	1		-
		K	N		-	-	1	1
			N	S	1	-	-	-
A	C		N		1	-	-	-
A	C			S	1	-	-	-
A		K	N		-	-	1	-
A			N	T	-	-	-	1
A			S	T	-	-	-	1
	C		S	T	-	1	-	
		K	N	S	-	-	-	1
A		K	N	S	T	-	-	1
Total resistant					26	21	14	13

*Obtained by standard method procedures (see Table 5).

† Raw: Raw wastewater sample, Renton wastewater treatment facility.

2°: Secondary wastewater sample, Renton wastewater treatment facility.

L-UV: Low ultraviolet light dosage to secondary wastewater.

H-UV: High ultraviolet light dosage to secondary wastewater.

‡Total number of isolates obtained.

**Number of isolates with that antibiotic resistant pattern.

††Indicates a value of < 1 bacterium isolated.

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Albert D. Venosa is the EPA Project Officer (see below).
The complete report, entitled "Effect of Wastewater Disinfectants on Survival of R-Factor Coliform Bacteria," (Order No. PB 88-112 339/AS; Cost \$19.95, subject to change) will be available only from:
National Technical Information Service
5285 Port Royal Road
Springfield, VA 22161
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