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Antibiotic Resistance in *Acinetobacter* spp. Isolated from Sewers Receiving Waste Effluent from a Hospital and a Pharmaceutical Plant

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The possible increase of antibiotic-resistant bacteria in sewage associated with the discharge of wastewater from a hospital and a pharmaceutical plant was investigated by using *Acinetobacter* species as environmental bacterial indicators. The level of susceptibility to six antimicrobial agents was determined in 385 *Acinetobacter* strains isolated from samples collected upstream and downstream from the discharge points of the hospital and the pharmaceutical plant. Results indicated that while the hospital waste effluent affected only the prevalence of oxytetracycline resistance, the discharge of wastewater from the pharmaceutical plant was associated with an increase in the prevalence of both single- and multiple-antibiotic resistance among *Acinetobacter* species in the sewers.

Previous studies have shown that waste effluents from hospitals contain higher levels of antibiotic-resistant enteric bacteria than waste effluents derived from other sources (5, 6, 9, 16). To our knowledge, no investigations have been conducted concerning the effects on the indigenous bacterial microflora of sewage caused by the release into sewers of effluents from hospitals or other potential sources of antibiotic-containing effluent such as pharmaceutical plants. In this study, we have used *Acinetobacter* spp. as bacterial indicators of antibiotic resistance to investigate whether the discharge of wastewater from a hospital and a pharmaceutical plant may increase the numbers of antibiotic-resistant bacteria in the receiving sewers. *Acinetobacter* spp. were chosen as the target group of bacteria due to their ubiquitous distribution in the environment (1, 3, 7, 8) and their remarkable ability to develop resistance to antimicrobial agents (2, 14), which makes these microorganisms particularly suitable for monitoring antibiotic resistance in the environment.

Samples were collected from sewers receiving wastewater from a hospital and a pharmaceutical plant. During the period of sampling, products containing penicillin, tylosin, tetracyclines, and to some extent sulfonamides and streptomycin were the principal products prepared at the pharmaceutical plant. Sampling sites were situated upstream (site A) and downstream (sites B and C) from the sewage discharge points of the hospital and the pharmaceutical plant. At the hospital, site A was situated 350 m upstream from the discharge point, site B was 60 m downstream, and site C was 500 m downstream. At the pharmaceutical plant, site A was located 70 m upstream from the discharge point, site B was 30 m downstream, and site C was 250 m downstream. Samples were collected from each of the sites four times between June and September 1997. Samples of sewage mixed with sediment particles were aspirated from the bottom of the sewers with sterile 150-ml syringes and catheters and transported to the laboratory within 1 h. Serial 10-fold dilutions of samples were prepared in physiological saline, and 0.1-ml aliquots were streak plated on Luria-Bertani agar (LBA) (10). Plates were incubated for 24 h at 30°C before bacteriological counts were performed. At the hospital sewers, total bacterial counts were below 5×10^3 CFU/ml in samples from site A and varied from 1.07×10^6 to 7×10^7 CFU/ml in samples from sites B and C. Among samples collected from the sewers at the pharmaceutical plant, total bacterial counts varied from 5×10^5 to 1.22×10^7 CFU/ml and no significant differences were detected between upstream and downstream samples. At the last two sampling times only, relative numbers of *Acinetobacter* spp. among total heterotrophic bacteria were determined in samples collected from the sewers at the pharmaceutical plant. For this purpose, plates for bacteriological counts containing an adequate number of colonies (100 to 150) were replicated on nylon membranes (Hybond-N; Amersham International, Little Chalfont, Buckinghamshire, England), and the membranes were hybridized with a genus-specific probe as described below. At both sampling times, the prevalence of *Acinetobacter* among total culturable heterotrophic bacteria was higher in samples from sites B (23 and 42%) and C (9 and 31%) than in those from site A (<1% and 4%).

Bacteria were isolated by using a selective enrichment procedure for *Acinetobacter* spp. (1). From each sample, 20 to 30 subsamples were enriched by inoculating 1 ml of sewage into 9 ml of Baumann medium (Bie and Berntsen, Aarhus, Denmark); this was followed by incubation at 30°C with vigorous shaking. After 48 h of incubation, enrichment cultures were streaked on LBA and incubated at 30°C overnight. Subsequently, two or three colonies showing different colony morphology were selected and subcultured on LBA to obtain pure cultures. *Acinetobacter* isolates were identified by colony hybridization with a genus-specific 16S rRNA-targeted alkaline phosphatase-labeled oligonucleotide probe (DNA Technology, Aarhus, Denmark), the specificity of which had been evaluated previously by dot blot analysis (15). Colonies from overnight cultures were transferred to nylon membranes and treated for hybridization as recommended by the manufacturer. *Acinetobacter calcoaceticus* ATCC 23055 and *Escherichia coli* 39R 861 (13) were included on all membranes as positive and negative controls, respectively. Hybridization was performed at 46°C by the protocol described by Wright et al. (17).

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TABLE 1. Numbers of *Acinetobacter* strains isolated at each sampling time and site

Sample source	Sampling time	No. of *Acinetobacter* strains			
		Total	Site A	Site B	Site C
Pharmaceutical plant	1	43	13	13	17
Pharmaceutical plant	2	30	13	17	—[a]
Pharmaceutical plant	3	70	26	20	24
Pharmaceutical plant	4	62	24	22	16
Hospital	1	27	11	16	—
Hospital	2	33	12	12	9
Hospital	3	63	15	23	25
Hospital	4	57	—	27	30

[a] —, sample not collected due to technical problems.

Among the 770 isolates obtained, 385 were identified as *Acinetobacter* by hybridization with the probe, including 180 strains from the sewers of the hospital and 205 strains from the sewers of the pharmaceutical plant. The numbers of *Acinetobacter* isolates obtained in relation to sampling times and sites of sampling are shown in Table 1.

Six antimicrobial agents were selected as representatives of important classes of antibiotics: amoxicillin (AM), oxytetracycline (OT), chloramphenicol (CHL), sulfamethoxazole (SU), gentamicin (GE), and ciprofloxacin (CIP). The level of susceptibility was measured by disc diffusion following the recommendations of the Swedish Reference Group for Antibiotics (12). Discs were purchased from Oxoid (Basingstoke, England), and the following disc concentrations were used: AM, 10 μg; OT, 30 μg; CHL, 30 μg; SU, 25 μg; GE, 30 μg; and CIP, 5 μg. Inhibition zone diameters were measured after 18 to 24 h of incubation at 30°C. Strains were defined as resistant when zone diameters were smaller than 14 mm for AM (MIC ≥ 32 μg/ml), 17 mm for CHL (MIC ≥ 32 μg/ml), 23 mm for CIP (MIC ≥ 2 μg/ml), 24 mm for GE (MIC ≥ 8 μg/ml), 19 mm for OT (MIC ≥ 16 μg/ml) and 13 mm for SU (MIC ≥ 512 μg/ml). Zone diameter breakpoints and corresponding MICs specific for environmental *Acinetobacter* had been determined previously by testing a subset of 200 strains by both disc diffusion and agar dilution methods (data not shown). The agar dilution assay was performed as described by the National Committee for Clinical Laboratory Standards (11) with the following ranges of concentrations of antimicrobial agents: AM, 2 to 32 μg/ml; CHL, 8 to 32 μg/ml; CIP, 1 to 2 μg/ml; GE, 2 to 4 μg/ml; OT, 4 to 16 μg/ml; and SU, 256 to 512 μg/ml. The relationship between inhibition zone diameters and MICs was studied by using scattergrams and Statistix Analytical Software (Tallahassee, Fla.). Each MIC was plotted against the corresponding inhibition zone diameter, and the zone diameter breakpoints were selected by visual examination as the zone sizes which best separated between populations of resistant and sensitive strains. For each antimicrobial agent two analyses were carried out to determine whether the discharge of wastewater from the hospital or the pharmaceutical plant was statistically associated with an increase in the prevalence of antibiotic resistance (comparison between sites A and B) and to provide information concerning variations dependent on distance from the discharge points (comparison between sites B and C). In order to establish associations between frequencies of antibiotic resistance and sites, logistic regression analysis including sampling time as a factor with four levels (Statistix Analytical Software) was performed. When such an analysis was not appropriate due to excessive variability of data, chi-square analysis was performed separately for each sampling time.

The highest prevalence of resistance among the total number of *Acinetobacter* isolates was shown for CHL (36.9%). This result confirms data reported previously by other authors indicating that *Acinetobacter* spp. are frequently resistant to CHL (2, 4). In comparison with the levels of antibiotic resistance reported in the literature for clinical isolates (2, 14), *Acinetobacter* spp. from sewage were generally more susceptible to antimicrobial agents (Table 2). The relatively low incidence of antibiotic resistance observed in our study suggests that these microorganisms are not inherently resistant to any particular class of drugs but more likely have a predisposition to develop resistance under conditions of antibiotic selective pressure (e.g., hospital environments).

Among strains isolated from the sewers receiving the effluent from the hospital, OT-resistant bacteria were isolated only from samples collected at sites B and C and represented 37.2 and 12.5% of the total isolates from those sites, respectively (Table 2). For each sampling time the prevalence of OT resistance observed at site B was significantly higher than that observed at site A (chi-square, $P < 0.01$). Although not restricted to site A, the prevalence of CHL-resistant strains was significantly higher at this site than at site B (logistic regression, $P < 0.01$). Since the sampling site situated upstream from

TABLE 2. Prevalences of antibiotic resistance among *Acinetobacter* strains isolated from the sewers at the hospital and the pharmaceutical plant

Drug	Prevalence (%) of resistance to drug[a]						
	Total (n = 385)	Hospital (n = 180)			Pharmaceutical plant (n = 205)		
		Site A (n = 38)	Site B (n = 78)	Site C (n = 64)	Site A (n = 76)	Site B (n = 72)	Site C (n = 57)
AM	13.0	2.6	2.6	3.1	10.5	26.4[b]	31.6
CIP	0.8	0	2.6	0	1.3	0	0
GE	6.2	0	0	3.1	0	20.8[c]	12.3
CHL	36.9	55.3	16.7[d]	26.6	25.0	51.4[b]	61.4
OT	28.8	0	37.2[b]	12.5[e]	5.3	48.6[b]	61.4
SU	19.5	0	2.6	4.7	0	51.4[c]	57.9

[a] n, number of isolates.
[b] Statistically significant increase of antibiotic resistance at site B in comparison with site A ($P < 0.01$).
[c] Statistically significant increase of antibiotic resistance at site B in comparison with site A ($P < 0.01$) (first three sampling times only).
[d] Statistically significant decrease of antibiotic resistance at site B in comparison with site A ($P < 0.01$).
[e] Statistically significant decrease of antibiotic resistance at site C in comparison with site B ($P < 0.01$).

TABLE 3. Prevalences of multiple-antibiotic resistance among *Acinetobacter* strains isolated from different sites in the sewers at the hospital and the pharmaceutical plant

Phenotype	% of isolates with phenotype[a]					
	Hospital (n = 180)			Pharmaceutical plant (n = 205)		
	Site A (n = 38)	Site B (n = 78)	Site C (n = 64)	Site A (n = 76)	Site B (n = 72)	Site C (n = 57)
Sensitivity	44.7	44.9	64.1	68.4	31.9	8.8
Resistance to one antibiotic	52.6	51.3	29.7	21.1	12.5	28.1
Resistance to two antibiotics	2.6	2.6	1.6	10.5	19.4	24.6
Resistance to three or more antibiotics	0	1.3	4.7	0	36.1[b]	38.6

[a] n, number of isolates.
[b] Statistically significant increase of multiple-antibiotic resistance at site B in comparison with site A ($P < 0.001$) (first three sampling times only).

the hospital discharge point received sewage originating from both households and factories, the cause of the high prevalence of CHL resistance detected among upstream isolates was unknown. The lower prevalence of CHL-resistant strains observed at sites situated downstream from the hospital discharge point could represent a side effect caused by the selection of OT-resistant bacteria, since all CHL-resistant strains isolated from upstream samples were susceptible to OT (data not shown). No other significant increases in the prevalence of antibiotic resistance were observed at the two sampling sites situated downstream from the discharge point and, above all, multiple-antibiotic-resistant strains were rarely isolated from these sites (Table 3).

Acinetobacter strains isolated from the two sites situated beyond the discharge point at the pharmaceutical plant demonstrated sharply increased levels of antibiotic resistance compared with those isolated from site A (Table 2). When temporal variations were taken into account, higher prevalences of CHL, AM, and OT resistance were statistically associated with site B than with site A (logistic regression, $P < 0.01$). SU and GE resistances were observed only among isolates from sites B and C. Except for the last sampling time, when resistance to these two antibiotics was not detected at any site, a significant increase of SU and GE resistance was observed at site B in comparison with site A (chi-square, $P < 0.01$). For all antimicrobial agents, no statistically significant differences were detected between the two sites situated downstream from the sewage discharge point. Resistance to three or more antibiotics was frequently observed among isolates from sites B (36.1%) and C (38.6%), whereas it was not detected among isolates from site A (Table 3). At the first three sampling times, a statistically significant increase in the prevalence of multiple-antibiotic resistance was shown at site B in comparison with site A (chi-square, $P < 0.001$). Among multiple-antibiotic-resistant strains isolated from the sewers at the pharmaceutical plant, SU and OT resistances were often associated, since 47.2 and 54.4% of the isolates exhibited resistance to these two antibiotics at sites B and C, respectively (data not shown). This resistance pattern was observed in association with CHL resistance in 34.7% of the strains isolated from site B and in 29.8% of those from site C. Resistance to all antimicrobial agents tested, with the exception of CIP, was demonstrated in 15.3 and 17.5% of the strains isolated from sites B and C, respectively. None of these multiple-antibiotic-resistance patterns was detected among isolates from site A. A certain degree of variability in resistance patterns was shown among strains isolated from the same sampling sites (data not shown), demonstrating that most antibiotic-resistant bacteria from downstream samples were truly different strains and did not arise as the progeny of a single strain.

From the present investigation we can conclude that the release of wastewater from the hospital under study was associated with an increase in the prevalence of OT resistance. However, 500 m beyond the discharge point this increase was significantly reduced from what it was at the site immediately downstream from the discharge point. The waste effluent derived from the pharmaceutical plant was likely to result in selection of antibiotic-resistant *Acinetobacter* spp. in the sewers. In fact, although influenced by temporal variation, the prevalences of both single- and multiple-antibiotic resistance were significantly higher at the two sites situated downstream from the pharmaceutical plant. Such an increase in the prevalence of antibiotic resistance was shown to persist 250 m after the discharge point. At the pharmaceutical plant, the tanks where products containing antimicrobial agents have been prepared are washed, and the residual water is released directly into the sewage system. Therefore, the presence in the waste effluent of residues derived from the production of antibiotics and/or antibiotic-resistant *Acinetobacter* spp. selected inside the pharmaceutical plant could represent a factor increasing the prevalence of antibiotic resistance downstream from the discharge point.

It is generally agreed that the selection and dissemination of resistant bacteria in nature should be avoided in order to ensure effective treatment against infectious disease in humans and maintain an ecological balance that favors the predominance of a susceptible bacterial flora in nature. According to the results of this study, factors other than the indiscriminate use of antibiotics in human medicine, animal husbandry, and agriculture may disrupt the microbial balance in favor of resistant bacteria. In particular, wastewater from pharmaceutical plants could play a role in the selection of antibiotic-resistant bacteria in sewage. In order to assess the ecological impact of residues derived from the production of antimicrobial agents, further studies should be performed to evaluate the persistence of antibiotic-resistant bacteria selected at these sites after sewage treatment and the possible dissemination of antibiotic resistance genes caused by their eventual release into the aquatic environment.

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