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Occurrence of multiple antibiotic resistance and R-plasmids in gram-negative bacteria isolated from faecally contaminated fresh-water streams in Hong Kong

By G. L. FRENCH, J. LING,

Department of Microbiology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N T, Hong Kong

K. L. CHOW and K. K. MARK.

Department of Biology, The Chinese University of Hong Kong, Shatin, N T, Hong Kong

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SUMMARY

The bacterial populations of six freshwater streams in populated areas of the Hong Kong New Territories were studied. There is considerable faecal contamination of these streams, with coliform counts as high as 10^5 c.f.u./ml and the contaminating organisms show a high prevalence of antibiotic resistance and multiple resistance. With direct plating of water samples onto antibiotic-containing media, an average of 49 % of the gram-negative bacteria were ampicillin-resistant, 3 % chloramphenicol-resistant and 1 % gentamicin-resistant. At individual sites resistance to these three drugs was as high as 98 %, 8 % and 3 % respectively. More than 70 % of strains were resistant to two or more antibiotics, 29 % to five or more and 2 % to eight or more. A total of 98 patterns of antibiotic resistance were detected with no one pattern predominating. Twenty-eight gram-negative bacterial species were identified as stream contaminants. *Escherichia coli* was the commonest bacterial species isolated and other frequent isolates were *Enterobacter* sp., *Klebsiella* sp. and *Citrobacter* sp., but no enteric pathogens were detected. The greatest prevalence of resistance and multiple resistance was associated with the heaviest contamination by *E. coli*. Analysis of selected stream isolates revealed multiple plasmid bands arranged in many different patterns, but multiple antibiotic resistances were shown to be commonly mediated by single transferable plasmids. Faecally-contaminated freshwater streams in Hong Kong may be reservoirs of antibiotic resistance plasmids for clinically-important bacteria.

INTRODUCTION

Work by many authors worldwide has shown that most multiple antibiotic resistance in clinical isolates of gram-negative bacteria is plasmid-borne (Anderson *et al.* 1977; Rowe & Threlfall, 1984). Animal and human faeces have been recognized as reservoirs of resistance plasmids (R-plasmids) (Anderson, Humph-

reys & Willshaw, 1975), and faecally contaminated water and sewage may also harbour enteric bacteria and R-plasmids (Smith, 1970; Sturtevant, Cassell & Feary, 1971; Grabow, Prozesky & Smith, 1974, 1975; Bell, MaCrae & Elliott, 1981; Walter & Vennes, 1985). In Hong Kong, multiple antimicrobial resistance associated with transferable R-plasmids is common amongst clinical isolates of enteric pathogens (Chau *et al.* 1981, 1982), the faecal flora of man and domestic animals and untreated sewage (Dhillon & Dhillon, 1981). Local streams, reservoirs and beaches are heavily contaminated by coliforms (Mark & Wan, 1975). We have therefore surveyed a number of freshwater streams in Hong Kong to identify the coliforms responsible for contamination, to assess their resistance to common antimicrobial agents and to study their plasmid profiles.

MATERIALS AND METHODS

Sampling sites

Six streams in the New Territories were selected for study. These were the Hung Shui Kiu, Kam Tin, Shing Mun, Ng Tung, Sheung Yu and Tai Po Rivers (Fig. 1). The Hung Shiu Kiu, Kam Tin, Ng Tung and Sheung Yu Rivers run through rural areas used for cultivation and containing ponds for fish or poultry-raising. The Shing Mun River originates from the Lower Shing Mun Reservoir and runs through Tai Wai and Shatin, two densely populated urban areas. The Tai Po River runs through the Lam Tsuen Valley. The upper profile of this river is frequently visited by swimmers and the middle profile runs through cultivated land where domestic animals such as chickens or pigs are reared. The river ends in Tai Po Market, another densely populated urban area. The discharge of industrial and farming waste is under very limited government control in Hong Kong, and all these rivers receive unknown amounts of animal and human faeces, and toxic industrial waste. The rural rivers receive mainly animal and human faecal material; the Shing Mun River mainly human and industrial effluent; and the Tai Po River all forms of discharge. The rural Kam Tin River also received industrial waste from a nearby leather tanning factory.

Collection of water samples

Two water samples were collected from each stream between October, 1983 and January, 1984 at the sites marked on the map (Fig. 1). The water was collected in 125 ml sterilized bottles immersed at least one foot below the water surface. Samples were transported on ice to the laboratory for analysis within 3 h.

Isolation of bacteria

Enteric pathogens. Water samples from each site were centrifuged at 3000 rpm and the sediment was plated on appropriate media to isolate *Salmonella*, *Shigella*, *Vibrio* and *Campylobacter* spp. according to standard microbiological techniques (Lennette *et al.* 1980). Desoxycholate agar (Oxoid, Basingstoke, England) was used to isolate salmonella and shigella, thiosulphate citrate bile salt agar (Oxoid) to isolate vibrio and Skirrow's medium (Oxoid) incubated microaerophilically at 42 °C to isolate campylobacter. Selenite F (Oxoid) broth was also used as an enrichment medium for salmonella.

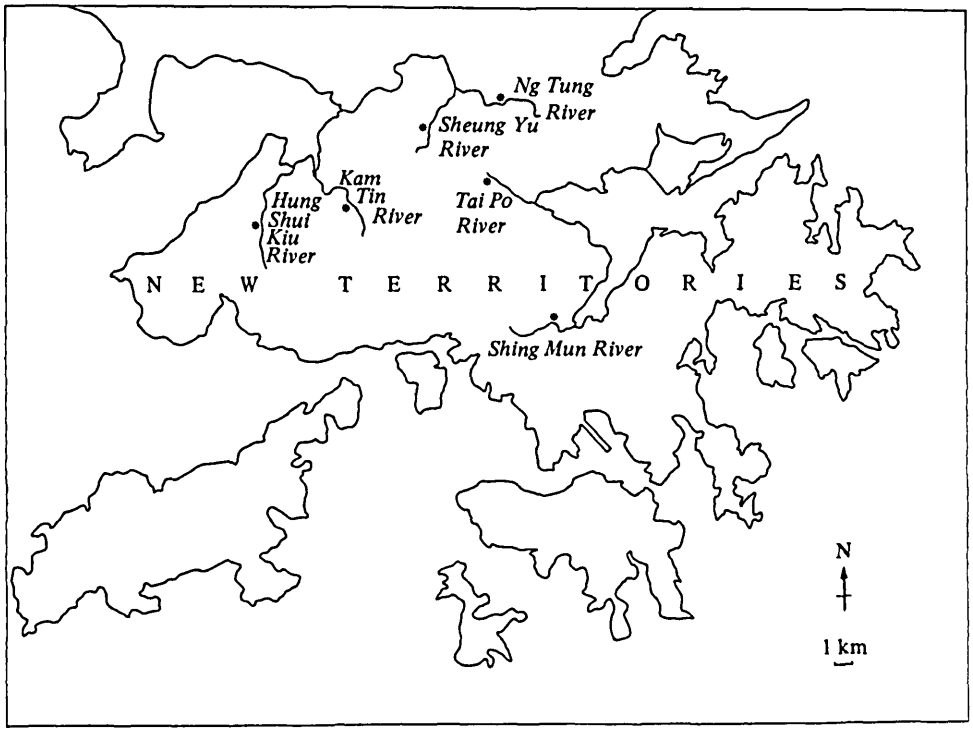

Fig. 1. Location of the six streams in the New Territories.

Faecal coliform count. Faecal coliforms from each stream were isolated in duplicate by passing 100 ml of water, suitably diluted in 0·9% sterile saline, through membrane filters of pore size 0·45 μm (HAWG 047 S1) (Millipore Corp., Bedford, Mass.). The membrane filters were placed on mFC agar (Difco Laboratories, Detroit, Michigan) and incubated at 44·5 °C for 24 h. Faecal coliforms appeared as bluish-green colonies on the membrane filters. These were counted and an appropriate calculation was performed to give the colony forming units (c.f.u.) per ml.

Gram-negative bacterial count and overall percentage of antibiotic resistance to three antibiotics (*Method 1*). Each water sample, 0·1 ml suitably diluted in 0·9% sterile saline, was plated in duplicate onto each of the following four agar plates in order to isolate single colonies: plain MacConkey agar (Oxoid), and MacConkey agar containing ampicillin (25 mg/l), chloramphenicol (20 mg/l) or gentamicin (4 mg/l) (all from Sigma Chemical Co., St. Louis, Missouri). The plates were incubated at 37 °C for 16–18 h. The numbers of gram-negative bacillary colonies growing on the plates were counted and the appropriate calculation was performed to give the c.f.u./ml. The level of resistance to ampicillin, chloramphenicol or gentamicin was taken as the c.f.u./ml on MacConkey agar containing the respective antibiotic expressed as a percentage of the number of c.f.u./ml growing on plain MacConkey agar.

Antibiotic susceptibility tests of individual strains to eight antibiotics (*Method 2*)

At least 50 well-isolated colonies of gram-negative bacilli were randomly picked

from each of the four MacConkey media and each was inoculated into 3 ml nutrient broth. These were incubated at 37 °C for 3–4 h. The cultures were diluted to match the turbidity of a MacFarland 0·5 standard, (giving a bacterial concentration of approximately $1{\cdot}5 \times 10^8$ c.f.u./ml). Tenfold dilutions of these were made and used to test their susceptibility to eight antibiotics by the microdilution method using the MIC2000 (Dynatech Laboratories, Alexandria, Va.) (Gavan & Barry, 1980). The antibiotics tested and their breakpoints for resistance were as follows: ampicillin (25 mg/l); tetracycline (10 mg/l); chloramphenicol (20 mg/l); kanamycin (10 mg/l); sulphamethoxazole (100 mg/l); trimethoprim (2 mg/l), gentamicin (4 mg/l) (all from Sigma), and streptomycin (10 mg/l) (Glaxo Co., Greenford, England).

Identification of bacteria

Isolates were initially tested for the production of indole and acetoin, the utilization of citrate, and reactivity in the methyl red test, according to the methods of Cowan (1974) (these are the IMViC tests). Organisms that failed to show the typical reactions of *Escherichia coli* were identified by the API20E system (API System, S.A., France) or the MB24E system (Microbact System, Disposable Products Pty. Ltd., South Australia).

Plasmid studies

Detection of transferable resistances. Transfer experiments according to the method of Anderson & Lewis (1965*a*, *b*) were performed on 17 *E. coli* strains which were resistant to four or more antibiotics. The rifampicin-resistant *E. coli* K12 strain (Jp995) was used as the recipient. Each resistant *E. coli* strain (donor) was incubated in 3 ml nutrient broth at 37 °C until it reached the logarithmic phase. The recipient strain was similarly incubated in nutrient broth. The conjugation mixture was prepared in duplicate by mixing equal amounts of donor and recipient cultures. One duplicate was incubated at 28 °C and the other at 37 °C, each for 18 h without aeration. Then 0·05 ml of the 10^{-3}, 10^{-2}, 10^{-1} and 10^{0} dilutions of the overnight conjugation mixtures were plated onto selective media for the isolation of transconjugants. The selective media were MacConkey agar containing rifampicin plus an antibiotic to which only the donor was resistant. From the 10^{-6}, 10^{-7} and 10^{-8} dilutions of the conjugation mixture 0·05 ml were plated on MacConkey plates containing only rifampicin to detect recipient colonies. The transfer frequency was taken as the number of transconjugants per recipient cell.

Ten colonies from each selective plate were tested for sensitivity to the antibiotics to which the respective donors were resistant using Method 2 as described previously.

Plasmid profile analysis by agarose gel electrophoresis. Plasmid DNA was extracted by the method of Kado & Liu (1981) and electrophoresed in a 0·7 % agarose gel (Sigma) with Tris-borate-EDTA buffer pH 8·3 (Meyers *et al.* 1976) at 45 mA for 2 h at room temperature using an LKB vertical electrophoresis apparatus (LKB, Bromma). The gels were stained with ethidium bromide (Sigma) for 20 min and then photographed with Polaroid 665 film under short wave UV light through a red filter.

Plasmid profile analysis was performed on 32 strains of *E. coli* (including the

Table 1. *Levels of bacterial pollution and resistance to ampicillin, chloramphenicol and gentamicin of six streams*

Site	Faecal coliform count, c.f.u./ml	Gram-negative bacteria: bacillary count* c.f.u./ml	(%) 'resistance'† to amp	cm	gm
Hung Shui Kiu River	$1{\cdot}0 \times 10^5$	$8{\cdot}2 \times 10^5$	97·5	5·6	2·7
Kam Tin River	$2{\cdot}0 \times 10^3$	$3{\cdot}0 \times 10^3$	5·3	0·0	0·7
Shing Mun River	$4{\cdot}0 \times 10^3$	$1{\cdot}8 \times 10^5$	40·4	7·7	3·4
Ng Tung River	$3{\cdot}0 \times 10^4$	$7{\cdot}3 \times 10^4$	67·1	3·3	0·2
Sheung Yu River	$1{\cdot}0 \times 10^5$	$2{\cdot}5 \times 10^6$	56·0	0·4	0·01
Tai Po River	$1{\cdot}0 \times 10^4$	$5{\cdot}6 \times 10^5$	25·0	1·6	0·05
Mean	$4{\cdot}1 \times 10^4$	$6{\cdot}9 \times 10^5$	48·6	3·1	1·2

* The gram-negative bacillary counts of water sample from the six different sites were taken as the gram-negative bacillary colony forming units per ml (c.f.u./ml) growing on plain MacConkey agar.

† The percentage of bacterial strains resistant to amp (ampicillin 20 mg/l), cm (chloramphenicol 20 mg/l) and gm (gentamicin 4 mg/l) was calculated from the ratio of the c.f.u./ml growing on MacConkey agar containing the respective antibiotic compared with the count of organisms growing on plain MacConkey agar.

Table 2. *Percentage of resistance of selected gram-negative bacterial colonies from plain MacConkey agar to eight antibiotics*

Site	Total no. tested	Resistance to (%*): A (20)	S (10)	T (10)	C (20)	K (10)	Su (100)	Tm (4)	G (4)	2 or more antibiotics
Hung Shui Kiu River	44	50	23	14	5	27	70	41	0	66
Kam Tin River	4	50	0	0	0	0	50	100	0	50
Shing Mun River	42	24	52	71	26	31	76	40	0	83
Ng Tung River	37	51	38	51	16	27	86	30	0	78
Sheung Yu River	34	32	6	15	12	3	32	3	0	59
Tai Po River	48	88	0	6	15	0	92	17	0	96
Total	209	51	23	30	14	17	73	28	0	77

* The percentage of bacterial strains resistant to ampicillin (A), streptomycin (S), tetracycline (T), chloramphenicol (C), kanamycin (K), sulphonamide (Su), trimethoprim (Tm) and gentamicin (G) (concentration in mg/l in parentheses) was calculated from the number of isolates resistant to the individual antibiotics when tested by Method 2 compared with the total number tested.

17 strains used for transfer studies), 17 *Citrobacter* sp., 14 *Enterobacter* sp., 9 *Klebsiella* sp., 4 *Proteus* sp. and 1 *Serratia* sp.

RESULTS

No *Salmonella*, *Shigella*, *Vibrio* or *Campylobacter* species were detected but all six streams contained high levels of faecal coliforms. Four of the streams showed colony counts ranging from 10^4–10^5 c.f.u. per ml (Table 1), and two (the Kam Tin and Shing Mun Rivers) had levels of 10^3 c.f.u. per ml. In three streams total gram-negative bacillary counts were of the same order of magnitude as the faecal

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Table 3. *Percentage of resistance of selected gram-negative bacterial colonies from MacConkey agar containing ampicillin, chloramphenicol or gentamicin to eight antibiotics*

Site	Total no. tested	Resistance to A* (20)	S (10)	T (10)	C (20)	K (10)	Su (100)	Tm (4)	G (4)	2 or more antibiotics
Hung Shui Kiu River	99	45	37	54	42	40	69	38	6	73
Kam Tin River	11	100	0	36	0	27	82	27	0	100
Shing Mun River	112	35	53	60	50	56	69	44	12	71
Ng Tung River	108	69	77	72	64	52	95	52	15	84
Sheung Yu River	97	39	51	44	59	32	76	22	27	75
Tai Po River	112	80	3	21	29	4	41	46	0	86
Total	539	55	43	50	47	37	70	41	11	79

* As in Table 2.

coliform counts, but in the other three, they were 10–100 times higher (Table 1).

The percentage of strains of all species resistant to ampicillin (20 mg/l), chloramphenicol (20 mg/l) or gentamicin (4 mg/l) by Method 1 is shown in Table 1. Ampicillin resistance was common, the highest rate being 97·5% in the Hung Shui Kiu River, and the lowest, 5·3%, in the Kam Tin River. Resistance to chloramphenicol was less common, not exceeding 8% at any site. Resistance to gentamicin was even less frequent, being found in about 3% of the isolates from the Hung Shui Kiu and Shing Mun Rivers, and in less than 1% of isolates from other sites. The Kam Tin River, in addition to having the lowest level of faecal contamination, also had the lowest percentages of ampicillin and chloramphenicol resistance, although gentamicin resistance was found in 0·7% of strains from this site.

A total of 948 strains from the six sites were randomly selected from plain and selective MacConkey agar cultures. Some strains failed to survive so that only 748 were tested for their resistance to eight antibiotics by Method 2, and the results are shown in Tables 2 and 3. Fifty per cent or more of strains isolated on plain MacConkey agar were multiply-resistant (Table 2). MacConkey agar containing ampicillin, chloramphenicol or gentamicin selected more resistant populations of organisms: more than 70% of strains isolated with these selective media were resistant to two or more antibiotics (Table 3), 29% to five or more and 2% to eight (Table 6). Although the plain MacConkey agar yielded no gentamicin-resistant isolates (Table 2), the selective media revealed a small population of such strains (Table 3).

Twenty-eight different species were identified amongst 609 fully characterized strains. In all streams except the Kam Tin and Tai Po Rivers which will be dealt with separately, *E. coli* was the commonest bacterial species isolated comprising 38% of the total strains studied (Table 4). *Enterobacter* sp., *Klebsiella* sp. and *Citrobacter* sp. were the next most prevalent species isolated, making up 23% of isolates. Non-fermenting species such as *Pseudomonas*, *Acinetobacter*, *Alkaligenes* and *Flavobacterium* accounted for only 7% of isolates, but 21% of isolates could not be identified (Table 4).

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Table 4. *Distribution of gram-negative bacteria from the six streams (percentages in parentheses)*

		Site 1*					
Organism	Total	HS	KT	SM	NT	SY	TP
Escherichia coli	285 (38·1)	50	1	101	67	60	6
Enterobacter aerogenes	19 (2·5)	19	—	—	—	—	—
Ent. cloacae	44 (5·9)	9	—	2	2	5	26
Ent. agglomerans	2 (0·3)	—	—	—	—	2	—
Ent. sakazakii	2 (0·3)	—	—	—	—	1	1
Klebsiella pneumoniae	48 (6·4)	7	1	1	—	2	37
K. oxytoca	10 (1·3)	—	—	—	6	—	4
K. ozanae	6 (0·8)	—	—	—	—	6	—
Citrobacter freundii	34 (4·5)	2	—	—	18	11	3
C. diversus	4 (0·5)	—	—	—	3	1	—
Serratia rubidaea	11 (1·5)	11	—	—	—	—	—
S. liquefaciens	12 (1·6)	—	—	—	—	6	6
S. odorifera	1 (0·1)	—	—	—	—	1	—
Proteus vulgaris	11 (1·5)	—	—	9	1	1	—
P. rettgeri	3 (0·4)	—	—	1	—	2	—
P. stuartii	1 (0·1)	—	—	1	—	—	—
P. alcalifaciens	2 (0·3)	—	—	2	—	—	—
Morganella morganii	2 (0·3)	—	—	—	2	—	—
Arizona sp.	1 (0·1)	—	—	—	—	—	1
Hafnia alvei	2 (0·3)	—	—	—	—	—	2
Pseudomonas aeruginosa	7 (0·9)	—	—	—	1	—	6
Ps. putida	7 (0·9)	1	—	—	—	—	6
Ps. cepacia	1 (0·1)	1	—	—	—	—	—
Acinetobacter lwoffi	3 (0·4)	—	—	—	—	1	2
A. anitratus	31 (4·1)	—	—	—	—	1	30
Aeromonas hydrophila	37 (4·9)	1	8	9	19	—	—
Alcaligenes sp.	2 (0·3)	—	2	—	—	—	—
Flavobacterium sp.	2 (0·3)	1	—	—	—	—	1
Not identified	158 (21·1)	41	3	28	26	31	29
Total number	748	143 (19·12)	15 (2·00)	154 (20·59)	145 (19·39)	131 (17·51)	160 (21·39)

* HS, Hung Shui Kiu River; KT, Kam Tin River; SM, Shing Mun River; NT, Ng Tung River; SY, Sheung Yu River; TP, Tai Po River.

Table 5. *Percentage of resistance to eight antibiotics of bacteria isolated on plain MacConkey agar*

Organism (total no. tested)	Resistance to A* (20)	S (10)	T (10)	C (20)	K (10)	Su (100)	Tm (4)	G (4)	2 or more antibiotics
E. coli (52)	33	54	71	29	35	79	25	0	83
Enterobacter sp. (33)	58	6	9	3	15	76	24	0	70
Klebsiella sp. (22)	86	0	5	5	0	86	0	0	86
Citrobacter sp. (13)	61	8	31	23	15	69	0	0	54
Proteus/Morganella sp. (6)	17	33	67	33	50	67	83	0	67
Aeromonas sp. (11)	73	18	27	9	18	91	64	0	91
Other (16)	69	6	0	31	6	75	50	0	81
Unidentified (56)	39	14	18	13	11	52	30	0	54
Total (209)	51	22	30	14	18	72	28	0	72

* As in Table 2.

The bacterial contamination was lowest at the Kam Tin River, where *Aeromonas hydrophila* was the commonest species isolated. The Tai Po River yielded a large proportion of *K. pneumoniae* (23 % of total isolates from that river) and *Ent. cloacae* (16 %), but relatively few *E. coli* (4 %). *Acinetobacter anitratus* was the most common of the non-enterobacteriacae strains from this river constituting 19 % of the isolates from this site (Table 4).

The proportion of different species resistant to one or more of the eight antibiotics tested by Method 2 are shown in Tables 5 and 6. All species had a proportion of their strains resistant to the antibiotics tested, although resistance and multiple resistance were most frequent in *E. coli*, *Pseudomonas* sp., *Acinetobacter* sp. and *Aeromonas* sp. Ninety-eight types of antibiotic resistance pattern were detected, and the more common patterns and their relation to species are listed in Table 7. *E. coli* had the highest proportion of multiply-resistant strains, including those resistant to five or more antibiotics.

The correlation coefficients for the relationships between resistance to streptomycin and tetracycline, streptomycin and kanamycin, tetracycline and chloramphenicol, and tetracycline and kanamycin were 0·9577, 0·9392, 0·8425 and 0·8075 respectively for organisms isolated on plain MacConkey agar; and 0·8957, 0·9076, 0·8017 and 0·9061 respectively for organisms isolated on antibiotic-containing MacConkey agar. No predominant pattern of resistances was observed, and there was no obvious association of resistance pattern with either specific species or specific geographical location.

Agarose gel electrophoresis revealed that usually more than one and up to eight plasmids were present within each strain (Fig. 2). Molecular sizes of the plasmids varied widely from 0·87 to 110 MDa. No common plasmid patterns were detected, although plasmids of similar molecular size were present in different strains.

Thirteen of the 17 multiply-resistant *E. coli* strains tested could transfer part or all of their resistances at frequencies ranging from 10^{-2} to 10^{-6} per recipient cell. Two of these strains transferred their resistances more efficiently at 28 °C than at 37 °C. Agarose gel electrophoresis of plasmid DNA from transconjugants usually

Table 6. *Percentage of resistance to eight antibiotics of gram-negative bacterial species isolated on MacConkey agar containing ampicillin, chloramphenicol or gentamicin*

Organism (no of isolates)	Resistance to: A* (20)	S (10)	T (10)	C (20)	K (10)	Su (100)	Tm (4)	G (4)	2 or more antibiotics
E. coli (233)	55	72	86	77	64	89	48	19	95
Enterobacter sp. (34)	62	15	15	9	9	56	0	0	53
Klebsiella sp. (42)	74	10	10	7	2	60	7	0	60
Citrobacter sp. (25)	20	52	64	68	36	92	20	4	76
Proteus/Morganella sp. (13)	46	38	38	38	23	62	15	0	54
Aeromonas sp. (26)	81	35	54	8	23	77	42	4	85
Other (64)	78	10	17	38	8	49	78	3	89
Not identified (102)	37	22	36	29	22	32	32	12	59
Total (539)	55	43	54	49	37	68	40	11	80

* As in Table 2.

showed only one plasmid band representing a transferable resistance plasmid (Fig. 2). There were only slight differences in molecular size between plasmids that conferred the whole spectrum of antibiotic resistances (in the donor strains) and those which conferred only part of the resistances (in the recipient strains) showing that the resistance determinants occupied only a small portion of the plasmids.

DISCUSSION

All the streams examined in this study contained high concentrations of gram-negative bacteria and faecal coliforms. The serious faecal pollution of the Tai Po River has been noted previously by Mark and his colleagues in 1975 and 1983 (Mark & Wan, 1975; Mark & Chow, unpublished data). These streams are used for the disposal of human and animal wastes, and may be regarded as untreated sewage, but we were not able to determine the relative contribution of man and animals to this pollution. Because of the relatively small number of samples taken from each site in this study, and the unknown contribution of uncontrolled industrial and farming wastes discharged into each river, it is difficult to explain the differences in the distribution of organisms and their antimicrobial resistances at the different sites. However, the strikingly low bacterial counts and unusual flora of the Kam Tin River may have been due to the presence of a leather tanning factory which discharged presumably toxic industrial waste into this river. At all other sites antimicrobial resistance and multiple resistance were common, and usually associated with normally sensitive faecal organisms such as *E. coli*. Since this phenomenon was seen in both rural and urban sites, it is likely that these resistant organisms are derived from both animal and human reservoirs.

The assessment of the level of antibiotic resistance in an environment is difficult, and subject to sampling bias. Other workers have detected the presence of resistance by performing antibiotic sensitivity tests on selected colonies (Bell, Macrae & Elliott, 1980; Niemi, Sibakov & Niemela, 1983). In the present study,

Table 7. *Resistance patterns of coliforms and other gram-negative bacteria isolated from streams: the table gives the number and percentage of strains showing the indicated resistance pattern*

No. of resistances	Resistance pattern	Ec	En	Kl	Ci	Se	P/M	Ps	Ac	Ae	Other	No ID	Total No.	Total (%)
0	Sensitive	16	15	11	4	2	6	—	—	4	—	46	104	(14%)
1	A	1	6	8	1	1	—	—	—	—	6	23		
	Su	2	5	—	6	1	1	1	—	1	—	5	22	
	Others	1	—	1	1	—	1	1	4	1	—	7	17	
Total		4	11	9	8	2	2	2	4	2	—	18	62	(8%)
2	ASu	5	25	35	1	8	—	—	—	5	2	12	93	
	ATm	—	—	—	—	—	—	1	17	—	—	9	27	
	Others	13	2	—	—	1	—	—	2	2	1	19	40	
Total		18	27	35	1	9	—	1	19	7	3	40	160	(21%)
3	STSu	6	—	1	2	—	—	—	—	1	—	4	14	
	ASuTm	2	—	1	—	6	—	1	1	4	—	11	26	
	TCSu	8	—	—	3	—	—	—	—	—	—	—	11	
	ACTm	—	—	—	—	—	—	—	6	—	—	4	10	
	Others	18	7	2	4	—	2	1	1	2	2	2	41	
Total		34	7	4	9	6	2	2	8	7	2	21	102	(13%)
4	ASTSu	5	—	—	1	—	—	—	—	1	—	2	9	
	STCSu	15	—	1	1	1	—	—	—	1	—	—	19	
	TCKSu	5	—	—	—	—	—	—	—	—	1	1	7	
	ACSuTm	1	—	1	—	—	—	8	2	—	—	—	12	
	Others	11	4	3	5	3	4	2	—	9	—	9	50	
Total		37	4	5	7	4	4	10	2	11	1	12	97	(13%)

5	ASTCSu	12	—	—	2	—	1	—	—	—	—	1	16	
	ATCKSu	17	—	—	2	—	—	—	—	—	—	1	20	
	ACKSuTm	7	—	—	—	—	—	—	—	—	—	—	7	
	Others	29	1	—	1	—	—	—	—	3	1	7	42	
Total		65	1	—	5	—	1	—	—	3	1	9	85	(11%)
6	ASTCKSu	21	2	—	—	—	1	—	—	1	—	—	25	
	STCKSuTm	21	—	—	3	—	1	—	—	—	—	—	25	
	Others	10	—	—	—	—	1	1	1	2	—	5	20	
Total		52	2	—	3	—	3	1	1	3	—	5	70	(9%)
7	ASTCKSuTm	34	—	—	—	1	1	—	—	—	—	5	36	
	ASTCSuTmG	7	—	—	—	—	—	—	—	—	—	—	7	
	Others	6	—	—	—	—	—	—	—	—	—	—	6	
Total		47	—	—	—	1	1	—	—	—	—	5	54	(7%)
8	ASTCKSuTmG	12	—	—	1	—	—	—	—	—	—	2	15	(2%)
TOTAL		285	67	64	38	24	19	15	34	37	7	158	748	

Ec, *E. coli*; En, *Enterobacter* sp.; Kl, *Klebsiella* sp.; Ci, *Citrobacter* sp.; Se, *Serratia* sp.; P/M, *Proteus* sp. and *Morganella* sp.; Ps, *Pseudomonas* sp.; Ac, *Acinetobacter* sp.; Ae, *Aeromonas hydrophila*; other, includes *Flavobacterium* sp.; *Alcaligenes* sp.; *Hafnia* sp. and *Arizona* sp.; NO ID, not identified or unidentified.

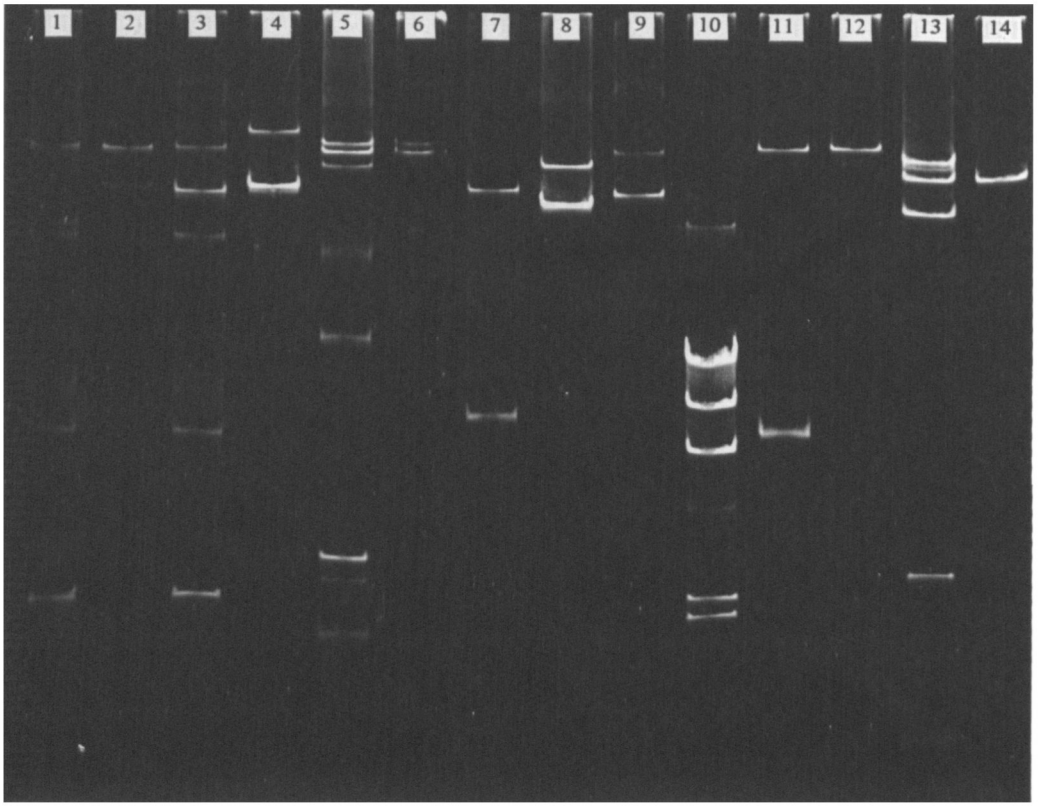

Fig. 2. Agarose gel electrophoresis of plasmid DNA extracted from multiply-resistant *Escherichia coli* and their respective transconjugants. Lanes: 1, *E. coli* (ATCKSuTm); 2, Transconjugant of 1 (ATC); 3, *E. coli* (ASTCKSu); 4, Transconjugant of 3 (ATC); 5, *E. coli* (TCKSuTm); 6, Transconjugant of 5 (TCKSuTm); 11, *E. coli* (ASTSuG); 12, Transconjugant of 11 (AT); 13, *E. coli* (ATKSuTm); 14, Transconjugant of 13 (ATm). Plasmids of known molecular weight are contained in lanes: 7, 40R268 (64 Mdal.), 48R626 (40 Mdal.); 8, RT641 (59·4 Mdal.), 34R193 (31·7 Mdal.); 9, 40R448 (77·6 Mdal.), 40R646 (37·8 Mdal.); 10, 40R660 (25·9 Mdal.), *Hind* III digest of phage λ. The faint lower band in lane 2 which is not present in lane 1 is probably the nicked form of the covalently-closed-circular (CCC) plasmid DNA formed during the extraction process. In lane 4 the top band corresponds to the top band in lane 3, which is faintly visible, probably owing to the low yield after extraction.

antibiotic resistance was assessed in two ways. Firstly (Method 1), water samples were plated onto plain MacConkey agar in duplicate for measurement of the overall numbers of gram-negative bacteria per ml (these include coliforms and non-coliform bacilli). Similar samples were plated onto MacConkey agar containing ampicillin, chloramphenicol or gentamicin at concentrations that would allow the survival of only resistant strains. The level of resistance to these three antibiotics was expressed as the percentage of colonies surviving on the selective agar compared with those surviving on the antibiotic-free medium. Secondly (Method 2), approximately 50 colonies were selected at random from each of the plain and antibiotic-containing MacConkey plates, and tested for their resistance against eight antibiotics by a microbroth dilution method.

The plating of water samples onto antibiotic-containing media (Method 1) revealed the presence of small populations of especially resistant organisms that would have been missed by culture on unselective medium alone. For example, no gentamicin-resistant strains were detected at any site by culture on plain MacConkey agar but four sites yielded gentamicin-resistant strains when samples were cultured on a gentamicin agar. Jones and his colleagues (1986*b*) have also emphasized that the methods and media used in detecting antibiotic-resistant bacteria affect the results. The level of antibiotic resistance in the environment is also affected by the location of the samples taken and the species of bacteria isolated (Jones *et al.* 1986*a*, *b*). In the present study, differences in the overall proportions of antimicrobial resistance at the sampling sites may be partly explained by differences in the proportions of different bacterial species isolated (Table 4). However, it is noteworthy that human and animal faecal organisms that are normally thought of as sensitive to antibiotics, in particular *E. coli*, made important contributions to the observed antimicrobial resistance.

Our techniques revealed a wide range of antibiotic resistance patterns amongst stream isolates with no one pattern predominating. There were significant correlations between resistance to tetracycline, streptomycin, kanamycin and chloramphenicol suggesting that these resistances are genetically linked. Examination of selected strains showed that multiply-resistant isolates usually contained several plasmid bands, and that the resistances were usually coded on a single plasmid which could be transferable. There were no common resistance patterns and no obvious common resistance plasmids. Although we were able to examine only a limited number of strains for plasmid content and resistance transfer, the correlations between resistances, the common finding of transferable resistances and plasmid bands in organisms such as *E. coli*, and the similar reports by other workers, (Linton *et al.* 1974) all suggest that a significant proportion of the resistances we have observed in Hong Kong stream organisms is plasmid-mediated, and that there is a great variety of such resistance plasmids in the environment.

Dhillon & Dhillon (1981) have previously reported a high prevalence of transmissible antibiotic resistance and multiple resistance in faecal organisms isolated from healthy humans, domestic animals and untreated sewage in Hong Kong. These authors found a significantly higher proportion of antibiotic-resistant bacteria in the faeces of chickens and pigs (80% of isolates resistant to two or more drugs) compared with cows (12% multiply-resistant). Dhillon & Dhillon related the high proportion of resistance in avian coliforms to the common use of antibiotic-containing feeds in the chicken farming industry in Hong Kong, and quote the use of chlortetracycline, sulfanilamide and bacitracin as additives. The addition of antibiotics in feedstuffs is uncontrolled in Hong Kong, and the exact type and extent of use of these drugs is unknown. It is likely that resistant coliforms from animals fed with antibiotic supplements contributed to the antibiotic resistance of stream contaminants seen in this study, but the extent of this contribution could not be determined.

Whether or not the clinical and veterinary use of antibiotics contributes to these resistances, the present study shows that environmental coliforms in Hong Kong exhibit numerous patterns of antibiotic resistance, some of which are borne on a variety of different plasmids widely distributed amongst many bacterial species.

This is in contrast to the common clinical finding of a limited number of resistance plasmid/organism combinations spreading epidemically through a hospital or community population in the presence of antibiotic pressure. Environmental studies such as the one described here suggest that plasmid-mediated multiple antibiotic resistance is common in environmental organisms even in the absence of specific antibiotic pressure, and represents a common form of bacterial variety.

There is considerable evidence in the literature, and in this study, that resistance plasmids of great variety are widespread in environmental organisms. Several authors have noted the occurrence of R-factors in sewage organisms (Sturtevant & Feary, 1969; Sturtevant, Cassell & Feary, 1971; Bell, 1978; Bell, MaCrae & Elliott, 1981; Linton *et al.* 1974; Mach & Grimes, 1982; Walter & Vennes, 1985). In the present study, faecal pollution of these Hong Kong rivers was such that they might be regarded as sewers. A high prevalence of multiple resistance amongst coliforms and other gram-negative bacteria from other less polluted river waters has been reported by workers in Britain (Smith, 1970) and elsewhere (Kelch & Lee, 1978; Bell, Macrae & Elliott, 1980; Niemi, Sibakov & Niemela, 1983; Al-Jebouri & Al-Meshhadani, 1985). In the report by Bell and his colleagues (1983), the prevalence of multiple resistance in faecal coliforms in a river where organisms originated from wildlife was lower than that in a river close to urbanized areas where exposure to antibiotics would be greater. All the Hong Kong streams in the present study run through populated areas, and there was no obvious relationship between the levels of antibiotic resistance and geographical location. The relative contributions of human and animal coliforms to stream pollution in Hong Kong, the origin of antibiotic resistance in these environmental isolates and its relation to plasmid-borne antibiotic resistance in clinical isolates are deserving of further study.

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