ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1976, p. 885-888 Vol. 9, No. 6

Antibiotic Resistance Among Coliform and Fecal Coliform Bacteria Isolated from the Freshwater Mussel *Hydridella menziesii*

MARYLYN D. COOKE
Cawthron Institute, Nelson, New Zealand

Received for publication 18 December 1975

Freshwater mussels (*Hydridella menziesii*) collected from Lakes Rotoroa, Rotoiti, and Brunner, South Island, New Zealand, contained coliform and fecal coliform bacteria. The majority of these bacteria were resistant to one or more antibiotics, but none transferred streptomycin, tetracycline, or kanamycin resistance to an antibiotic-susceptible strain of *Escherichia coli* K-12.

The use of antibiotics in agriculture and medicine favors the retention of antibiotic-resistant bacteria as components of the intestinal microbial flora, and, although R factors have been isolated from presumably healthy individuals (3), it has been shown that antibiotic-resistant bacteria are infrequently isolated from persons not exposed to modern antibiotic therapy (5, 9). It has been suggested that domestic and wild animals may also serve as reservoirs of R factors (7).

The aim of this study was to determine the incidence of antibiotic resistance among coliform and fecal coliform bacteria isolated from shellfish collected from areas known to be remote from important sources of domestic and agricultural wastes likely to be contaminated with antibiotics. This was to provide base line data to compare with results obtained from waters and shellfish polluted with human fecal material (2). The freshwater mussel *Hydridella menziesii* grows up to 5 to 10 cm long in mud at the bottom of lakes, streams, and rivers and was formerly much favored by the Maoris as a special food for the young and sick. Filter-feeding shellfish tend to concentrate bacteria from the overlying waters (12) and may retain such bacteria for some weeks (8).

The salient geographical characteristics of the three lakes have been reported (1a); the following information is abstracted from their paper.

Two of the lakes, Rotoroa and Rotoiti, are situated in the Nelson Lakes National Park and are therefore subject to only slight population pressure from hikers, shooters, and recreational fishermen. Lake Rotoroa, 447 m above sea level, is 14.4 km long and has an area of 24 km^2, a maximum depth of 145 m, and a calculated volume of 2,250 million m^3. There is no agricultural development or human settlement within the whole catchment area, and the lake is surrounded by steep hills that are completely covered in natural beech forest vegetation to the water's edge. Lake Rotoiti, 617 m above sea level, is 8 km long and has an area of 9.6 km^2, a maximum depth of 80 m, and a volume of about 403 million m^3. At the southern end of the lake is a farm grazed by sheep and cattle, and one feeder stream at the northern end of the lake passes through the small township of St. Arnaud (population, approximately 30, increased to approximately 500 at peak holiday times) and receives drainage from septic tanks and farmland. Lake Brunner is situated 83 m above sea level on the West Coast of the South Island and has an area of 39 km^2 and an average depth of 108 m. About 20% of the lake catchment is farmed, and there are one small township (population, 84) and many holiday cottages within the catchment area.

MATERIALS AND METHODS

Shellfish collection. Samples of *H. menziesii* were collected by an Ekman grab sampler from the mussel beds situated about 5 to 9 m below the water surface. The shellfish were placed in plastic bags for transport to the laboratory and prepared for testing in accordance with methods recommended by the American Public Health Association (1).

Isolation of bacteria. Diluted shellfish homogenates were inoculated into a five-tube three-dilution series of MacConkey broth (Oxoid, CM5a) and incubated at 37 ± 0.5 C for 48 ± 2 h for estimation of most probable number (MPN) presumptive coliforms.

Methods for confirmation of the presence of coliform and fecal coliform bacteria and methods for testing the susceptibility of pure isolates to various antibiotics are described in the accompanying paper (2). Techniques for demonstrating transfer of resistance determinants to an antibiotic-susceptible strain of *Escherichia coli* K-12 are described in the same paper.

RESULTS

Coliform bacteria were isolated from all samples of the freshwater mussel *H. menziesii* (Table 1). Duplicate samples from the same lake site showed only slight differences in MPN values, although samples taken on different days showed variation in MPN values for coliform and fecal coliform bacteria perhaps related to the degree and timing of pollution over the mussel beds. Greater variation was found from site to site within one lake, associated with proximity of the mussel beds to feeder streams.

MPN values obtained from Lake Rotoroa samples were higher than those from Lake Rotoiti. This was an unexpected result, as Lake Rotoiti is apparently more subject to human and animal pollution from the St. Arnaud settlement at the north end and a small farming area at the south end of the lake.

The incidence of antibiotic resistance among the coliform and fecal coliform isolates is presented in Tables 2 and 3. All samples showed a very similar incidence of antibiotic resistance among the coliform isolates, ranging from 76.9% among the isolates from Lake Rotoiti to 80% among isolates from Lake Rotoroa. This result was unexpected, since it was thought that Lakes Rotoiti and Brunner, being more subject to pollution from farming and human sources, would exhibit a higher incidence of antibiotic-resistant isolates. No isolates were resistant to chloromycetin, gentamicin, or paramomycin. Resistance to ampicillin, cephalothin, and rifampin was prevalent amongst coliform isolates, whereas few of the fecal coliform isolates from Lake Rotoroa were resistant to cephalo-

TABLE 1. *MPN determinations of coliforms and fecal coliforms per 100 g of the freshwater mussel Hydridella menziesii from three South Island, New Zealand, freshwater lakes*

Organisms	MPN per 100 g of *H. menziesii* from:					
	Lake Rotoroa (11 samples)		Lake Rotoiti (5 samples)		Lake Brunner (4 samples)	
	Mean	Range	Mean	Range	Mean	Range
Presumptive coliforms	1,200	350–2,500	1,150	250–2,500	480	250–800
Confirmed coliforms	990	250–2,500	370	50–1,300	370	250–800
Confirmed fecal coliforms	250	<10–1,300	70	<10–250	20	20
Confirmed *Escherichia coli*	210	<10–1,300	70	<10–250	20	20

TABLE 2. *Incidence of antibiotic resistance among coliform bacteria isolated from the freshwater mussel Hydridella menziesii*

Source	No. tested	No. resistant	% Resistant	% Resistant isolates exhibiting resistance to:									% Multiply resistant
				amp[a]	rif	tri	sul	tet	cep	nal	str	kan	
Lake Rotoroa	120	96	80.0	72.9	74.0	14.6	9.4	7.3	65.6	2.1	5.2		68.8
Lake Rotoiti	39	30	76.9	80.0	66.7	23.3		3.3	96.7				80.0
Lake Brunner	39	31	79.5	64.5	NT[b]				83.9			3.2	51.6
Total	198	157	79.3	72.6	72.2	13.4	5.7	5.1	75.2	1.3	3.2	0.6	67.5

[a] amp, Ampicillin; rif, rifampin; tri, trimethoprim; sul, sulfafurazole; tet, tetracycline; cep, cephalothin; nal, nalidixic acid; str, streptomycin; kan, kanamycin.

[b] NT, Not tested.

TABLE 3. *Incidence of antibiotic resistance among fecal coliform bacteria isolated from the freshwater mussel Hydridella menziesii*

Source	No. tested	No. resistant	% Resistant	% Resistant isolates exhibiting resistance to:				% Multiply resistant
				amp[a]	rif	sul	cep	
Lake Rotoroa	43	28	65.1	32.1	71.4	32.1	7.1	39.3
Lake Rotoiti	10	5	50.0			60.0	40.0	0
Lake Brunner	3	0	0					
Total	56	33	58.9	27.3	60.6	36.4	12.1	33.3

[a] See footnote *a*, Table 2.

thin. A higher incidence of resistance to sulfafurazole was found among the fecal coliforms than among the coliform isolates. No antibiotic-resistant fecal coliforms were isolated from mussels collected from Lake Brunner, but this may not be statistically significant since only four samples were collected from this lake.

More than 50% of coliform isolates exhibited multiple resistance, that is, simultaneous resistance to more than one antibiotic; incidence of multiple resistance was highest in coliform bacteria isolated from Lake Rotoiti mussels (80%) and lowest among those from Lake Brunner (52%).

One set of mussel samples from Lake Rotoroa was taken from an area at the head of the lake where there was extensive blackening of the bottom mud and a noticeable odor of sulfide. Moreover, 6/40 coliform and 3/10 fecal coliform isolates produced H_2S in triple sugar iron agar and were confirmed as *E. coli* using API 20 cards (Analytab Products Inc., New York) for identification. H_2S production has been shown to be determined by transmissible plasmids in some H_2S^+ *E. coli* strains (10), and these isolates have been retained for further study. No other samples from this lake or the other lakes showed the same phenomenon.

None of the isolates resistant to streptomycin, tetracycline, or kanamycin was able to transfer these resistances to an antibiotic-susceptible recipient strain of *E. coli* K-12.

DISCUSSION

The results from this study indicate that freshwater mussels collected from areas remote from important known pollution sources contain coliform bacteria. Many of the isolates were found to be resistant to one or more of the antibiotics used in the screening tests. The incidence of pollution, as measured by mean MPN values, appeared to be higher with samples from Lake Rotoroa, although coliforms were also isolated from all mussel samples from other lakes. It would seem probable that coliform bacteria, not confirmed as being of fecal origin by the elevated-temperature lactose fermentation test, have entered the lake from land runoff. It is possible that contamination with fecal coliform bacteria was due to wild animals, such as opossums, deer, or birds, since Stuart et al. (13), studying open and closed watersheds, found similar indicator bacteria in animal droppings and water samples from mountain streams.

The incidence of antibiotic resistance among the coliform bacteria was unexpectedly high in comparison with reports from other countries, where widespread usage of antibiotics has been established as an important selective force favoring the retention of antibiotic-resistant bacteria in the environment. Coliform bacteria isolated from marine shellfish taken near a sewage outfall also show a high incidence of antibiotic resistance (2). However, the high incidence reported here may be partially a reflection of the number of antibiotics used in the screening program. Maré (9) studied Kalahari bushmen and wild animals in antibiotic-free South African communities using five antibiotics: ampicillin, chloromycetin, streptomycin, sulfafurazole, and tetracycline. Although 57/582 (10%) of the specimens contained gram-negative antibiotic-resistant bacteria, the majority of these were resistant to ampicillin alone. None was resistant to streptomycin, and none of the isolates transmitted resistance. Gardner et al. (5) screened soil and human fecal specimens from an antibiotic-free community in the Solomon Islands. Specimens were cultured directly on plates containing one of five antibiotics: chloromycetin, kanamycin, nalidixic acid, streptomycin, or tetracycline, all at higher concentrations than used in this study. One isolate from soil and one from feces transmitted resistance to streptomycin and tetracycline, but no indication was given of the overall incidence of antibiotic resistance in the specimens. Huber et al. (7) found that only 3% of *E. coli* isolates from wild animals subject to minimal antibiotic exposure were resistant to ampicillin or chloromycetin, whereas 8% were resistant to dihydrostreptomycin, and 11% were resistant to oxytetracycline.

Selective pressure from antibiotic-like substances naturally present in soil may favor persistence of antibiotic-resistant bacteria (4, 14). Therefore, although curtailment of antibiotic usage may reduce the incidence of coliform bacteria carrying R factors (11), it is likely that resistant bacteria will naturally occur in the environment and may have a selective advantage once disseminated in natural waters (M. D. Cooke, N.Z. J. Mar. Freshwater Res., in press). Grabow et al. (6) have recently shown that strains isolated from river water transmitted resistance determinants at low frequency in dialysis bags immersed in the water concerned at 37 and 20 C.

ACKNOWLEDGMENTS

I am indebted to the Medical Research Council of New Zealand for a grant to purchase equipment, to E. Chittenden, P. Gillespie, and J. Stanton for the collection of samples, and to Joy M. Lamb for excellent technical assistance.

LITERATURE CITED

1. **American Public Health Association.** 1970. Recommended procedures for the examination of sea water and shellfish, 4th ed. American Public Health Associ-

ation, New York.

1a. **Chittenden, E., C. Childs, and R. Smidt.** 1976. N.Z. J. Mar. Freshwater Res. 10:61-76.

2. **Cooke, M. D.** 1976. Antibiotic resistance among coliform and fecal coliform bacteria isolated from sewage, seawater, and marine shellfish. Antimicrob. Agents. Chemother. 9:879-884.

3. **Datta, N.** 1969. Drug resistance and R factors in the bowel bacteria of London patients before and after admission to hospital. Br. Med. J. 2:407-411.

4. **Dowding, J., and J. Davies.** 1975. Mechanisms and origins of plasmid-determined antibiotic resistance, p. 179-186. *In* D. Schlessinger (ed.), Microbiology-1974. American Society for Microbiology, Washington, D.C.

5. **Gardner, P., D. H. Smith, H. Beer, and R. C. Moellering.** 1969. Recovery of resistance (R) factors from a drug-free community. Lancet 2:774-776.

6. **Grabow, W. O. K., O. W. Prozesky, and J. S. Burger.** 1975. Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. Water Res. 9:777-782.

7. **Huber, W. G., D. Korica, T. P. Neal, P. R. Schnurrenberger, and R. J. Martin.** 1971. Antibiotic sensitivity patterns and R factors in domestic and wild animals. Arch. Environ. Health 22:561-567.

8. **Janssen, W. A.** 1974. Oysters: retention and excretion of three types of human waterborne disease bacteria. Health Lab. Sci. 11:20-24.

9. **Maré, I. J.** 1968. Incidence of R factors among Gram negative bacteria in drug-free human and animal communities. Nature (London) 220:1046-1047.

10. **Ørskov, I., and F. Ørskov.** 1973. Plasmid-determined H_2S character in *Escherichia coli* and its relation to plasmid-carried raffinose fermentation and tetracycline resistance characters. J. Gen. Microbiol. 77:487-499.

11. **Richmond, M. H.** 1975. R factors in man and his environment, p. 27-35. *In* D. Schlessinger (ed.), Microbiology-1974. American Society for Microbiology, Washington, D.C.

12. **Slanetz, L. W., C. H. Bartley, and K. W. Stanley.** 1968. Coliforms, fecal streptococci and *Salmonella* in seawater and shellfish. Health Lab. Sci. 5:66-78.

13. **Stuart, D. G., G. K. Bissonnette, T. D. Goodrich, and W. G. Walter.** 1971. Effects of multiple use on water quality of high-mountain watersheds: bacteriological investigations of mountain streams. Appl. Microbiol. 22:1048-1054.

14. **Watanabe, T.** 1971. Infectious drug resistance in bacteria. Curr. Top. Microbiol. Immun. 56:43-98.