TWO ANTIBIOTIC-RESISTANT STRAINS OF *ESCHERICHIA COLI* FOR TRACING THE MOVEMENT OF SEWAGE IN GROUNDWATER

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ABSTRACT

Two antibiotic-resistant derivatives of the bacterium *Escherichia coli* K-12—*E. coli* J6-2 (lactose negative, nalidixic acid resistant) and *E. coli* PB 922 (rifampycin resistant)—were evaluated as tracers of sewage movement in groundwater. Both strains appear to meet certain safety criteria pertaining to non-pathogenicity, absence of plasmid-borne (non-transferable) antibiotic resistance factors and inability to become established in the human gastro-intestinal tract. The organisms were recovered from water samples by membrane filtration and incubated on MacConkey agar impregnated with the appropriate antibiotic. A nalidixic acid concentration of 75 mg/litre for the recovery of *E. coli* J6-2 and a rifampycin concentration of 50 mg/litre for the recovery of *E. coli* PB 922 caused total suppression of background faecal coliform bacteria in sewage without reducing counts of the tracer organisms. Both *E. coli* J6-2 and *E. coli* PB 922 exhibited survival rates in laboratory-stored groundwater samples that were similar to an *E. coli* neotype. Both strains were successfully used to trace the movement of sewage-polluted groundwater over a distance of 920 m.

INTRODUCTION

It is not always possible to identify the origins of microbial contaminants in extensive groundwater systems such as those of the New Zealand Canterbury Plains, particularly where potential pollution sources such as septic tank soak pits, effluent irrigation schemes and leaking sewer mains exist in close proximity. In these areas, specific micro-organisms are required to label suspected contamination sources, and to accurately trace subsequent movement of the microbial pollutants in the groundwater.

The idea of using specific laboratory-cultured micro-organisms to trace groundwater movement is not new. Ditthorn and Luerssen (1909) used the red-pigmented bacterium *Serratia marcescens* to demonstrate groundwater movement between two wells 19 m apart. Mallman and Mack (1961) referred to an unpublished study in the early 1920s in which *S. marcescens* was used to monitor the movement of septic tank effluent in groundwater over a distance of 3 m. Fournelle *et al.* (1957) recorded the movement of *S. marcescens, Chromobacterium violaceum* and *Bacillus subtilis* over a distance of 15.2 m from a well in shallow

groundwater. Martin and Thomas (1974) used a bacteriophage as a groundwater tracer and were able to follow it over a distance of 680 m. Wood and Ehrlich (1978) used baker's yeast *(Saccharomyces cerevisiae)* to monitor the penetration of micro-organisms in a sand and gravel aquifer.

In New Zealand, Pyle and Thorpe (1979), using a hydrogen sulphide positive strain of *E. coli,* were able to trace groundwater movement over distances of up to 100 m at two sites in the Heretaunga Plains. At Burnham, *E. coli* (H_2S+) and the thermophile *Bacillus stearothermophilus* were used to monitor the movement of groundwater over 920 m (Sinton, 1980). However, both *B. stearothermophilus* and E. coli (H_2S+) are unsuitable for use in sewage-polluted groundwater. *B. stearothermophilus* is consistently found in sewage and hydrogen sulphide producing strains of *E. coli* are present intermittently.

The advantage of using antibiotic-resistant strains as tracers (thereby facilitating the suppression of non-resistant background organisms), has been demonstrated in a number of surface water studies. For example, the strains of *S. marcescens* used by Robson (1956), Putman *et al.* (1956) and Rippon (1963), and the *S. marcescens* and *B. subtilis* strains compared by Pike *et al.* (1969) as tracers of sewage movement in marine and freshwaters, were all resistant to certain antibiotics. More recently, Hagedorn *et al.* (1978) used antibiotic-resistant *E. coli* and *Streptococcus faecalis* strains isolated from sewage, to monitor the percolation of septic tank effluent into underlying groundwater. In a similar study, Rahe *et al.* (1978) compared antibiotic-resistant *E. coli* from sewage and fluorescein dye as tracers of soil water movement.

This paper describes the selection (principally to meet certain safety criteria) of two antibiotic-resistant strains of *E. coli* and their evaluation as tracers of sewage movement in groundwater systems.

TRACER SELECTION

To be suitable for monitoring the movement of sewage-polluted groundwater, a microbial tracer must meet the following criteria:

1 It should be safe to use.
2 It should not be present naturally in groundwater or in sewage.
3 It should be readily distinguished from normal sewage biota and the method of detection should, preferably, be totally selective for the tracer species or strain.
4 It should have a reasonable rate of survival in sewage and in groundwater but should not reproduce in either medium.
5 Where the behaviour (survival and penetration) of a particular species is under study, the tracer organism should be a similar species or, preferably, another strain of the same species.

Whenever laboratory-cultured micro-organisms are introduced into natural waters, particularly where there is any possibility of downstream extraction for human consumption, the safety criterion becomes paramount. There are two safety aspects to be considered when selecting a microbial tracer. The first pertains to the likely pathogenicity of the species. For example, *S. marcescens* has been implicated in cases of

septicaemia and other infections by Davis *et al.* (1970). *B. subtilis* has ben implicated as the primary or secondary infective agent in eye and urinary tract infections, pneumonia, septicaemia and other ailments (Cox *et al.,* 1959). Sattar *et al.* (1972), in reviewing the safety record of different micro-organisms as tracers of air movements in hospitals, maintained that, of the suitable tracer species, *E. coli* was the most frequent cause of infection among hospital patients. Therefore, positive identification of the potential tracer species or strain as a non-pathogenic serotype is important.

The second safety consideration concerns the use of antibiotic-resistant bacterial strains. The genetic material that determines antibiotic resistance may be either incorporated into the main bacterial chromosome or it may be carried on plasmids—extra-chromosomal genetic elements. In the former situation, transfer of the antibiotic-resistance character is unlikely to occur. However, plasmid-borne resistance characters or "R-factors" may be transferred to other strains. For example, *E. coli* (R+) cells (possessing R-factors), if ingested, may colonise the human gastro-intestinal tract and transfer their resistance to already colonised bacteria or to any normally antibiotic-sensitive pathogens with which their host may become infected (Smith, 1969). This may significantly affect subsequent treatment of an infected person.

R-factors among coliform and faecal coliform bacteria appear to be widespread in nature. They have been found in sewage by Sturtevant and Feary (1969), in rivers and marine waters by Smith (1970, 1971) and in shellfish by Cooke (1976). For this reason, the random isolation of antibiotic-resistant faecal coliform bacteria from sewage for laboratory culture as tracers may be unwise. Apart from the possibility of selecting an R+ strain, the danger also exists of isolating a pathogenic serotype.

The tracer organisms selected for evaluation as sewage tracers in this study were *E. coli* J6-2 and *E. coli* PB 922. *E. coli* J6-2 is a spontaneous nalidixic acid-resistant mutant and is also unable to ferment lactose in contrast to normal *E. coli.* The strain was supplied by Mr W J Kelly, Biochemistry Department, Lincoln College. *E. coli* PB 922 is a spontaneous rifampycin-resistant mutant and was supplied by Dr Lane, Department of Cell Biology, University of Auckland. Both strains are derivatives of *E. coli* K-12 (Bachmann, 1972).

Both *E. coli* J6-2 and *E. coli* PB 922 appear to meet the necessary safety requirements for tracer studies. Both strains were tested by the New Zealand National Health Institute and were confirmed as non-pathogenic serotypes. This was expected from the genealogy outlined by Bachmann (1972). Similarly, neither strain appears to possess R-factors (W J Kelly, Biochemistry Department, Lincoln College). As derivatives of the *E. coli* K-12 strain which has been laboratory-cultured for over 50 years, neither *E. coli* J6-2 nor *E. coli* PB 922 are likely to have retained the competitive capacity necessary for colonisation of the human gastro-intestinal tract (Gorbach, 1978). Attempts to implant E. coli K-12 strains in human volunteers have consistently failed, the strains being excreted within a few days (Anderson, 1975).

The experimental programme described in this paper was designed to

evaluate the selected tracer strains in relation to the last four criteria listed earlier. This entailed (1) The determination of a suitable concentration of each antibiotic for the recovery of the appropriate strain from sewage, (2) Comparison of their survival rates with that of a "typical" *E. coli* strain (*E. coli* Neotype 01:K1:H7—supplied by the New Zealand National Health Institute) in laboratory-stored groundwater samples and (3) A field experiment in which their performance in tracing the movement of sewage-polluted groundwater was assessed.

METHODS AND MATERIALS

Culture Procedure

The tracer strains and *E. coli* neotype were cultured separately in brain heart infusion broth (BBL). The culture media were aerated and incubated at 37°C for 36 hours. The cultures were then centrifuged and the precipitated cells were washed twice and resuspended in sterile 0.85% w/v NaCl. For the laboratory survival experiments, the cells were resuspended in sterile groundwater.

Recovery and Enumeration

The bacterial cells were recovered from water samples by membrane filtration in all experiments, using Gelman GN-6 0.45μm filters. Dilution series of 0.1% peptone water were prepared when required. The filters were incubated on inverted plates of MacConkey agar (BBL) at 30°C ± 0.1°C for 4 hours followed by a further 14 hours at 44.5°C ± 0.05°C. A solution of the appropriate antibiotic (nalidixic acid or rifampycin) was mixed with the medium prior to pouring at an agar temperature of approximately 45°C. *E. coli* J6-2 cells produced yellow (lactose negative) colonies on the membranes. *E. coli* PB 922, *E. coli* neotype 01:K1:H7 and faecal bacteria produced pink-purple (lactose positive) colonies.

The Effects of Antibiotic Concentration

The effects of a range of antibiotic concentrations (from 0 to 400 mg/*l*) on the tracer strains, the *E. coli* neotype and faecal coliform bacteria from oxidation pond effluent were determined. Each MacConkey agar plate contained only one of the two antibiotics (one concentration per plate).

Survival in Groundwater Samples

The comparative survival rates of the tracers and the *E. coli* neotype were studied using six 5 litre laboratory-stored groundwater samples (one strain per sample, two replicates per strain). The samples were held in flasks and stored in the dark at 12.5°C (mean annual Canterbury groundwater temperature). A measured aliquot of washed and resuspended cells of the appropriate *E. coli* strain was added to each flask. The amount was calculated to ensure an initial concentration in the groundwater sample of between 1.0×10^8 and 1.0×10^9 cells/100 ml. The flasks were sampled 11 times over a period of 22 days. All three strains were recovered on MacConkey agar containing no antibiotics.

Field Evaluation

The field work was conducted at the Ministry of Works and Development Burnham groundwater study area (Fig. 1). The underlying aquifers are part of the extensive Canterbury groundwater systems and are composed of a network of alluvial gravel strata. The site consists of seven investigation wells located adjacent to a border-dyked oxidation pond effluent disposal area. The wells are positioned along the line of groundwater flow, estimated from regional piezometric surveys.

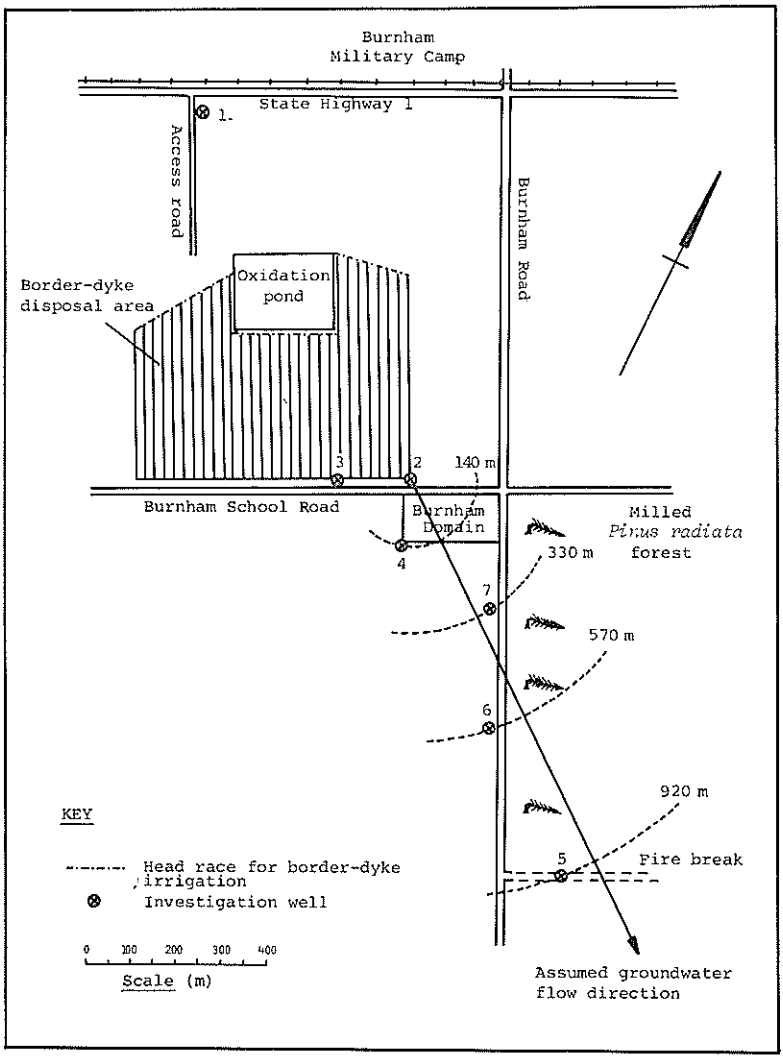

FIG. 1—Burnham effluent disposal area, associated investigation wells and radial distances of wells 4, 7, 6 and 5 from well 2.

Each well consists of a 75 mm diameter PVC bore sunk to a depth of 20 m and screened over the lower 6 m. Static well water level varies seasonally from 5.5 to 10.0 m below ground level.

The border dyke strips in the south-eastern sector of the disposal area were irrigated for 3 to 4 days to establish background levels of faecal coliform bacteria in the downstream wells of between 50 and 300/100 ml. A mixture of approximately 8.6×10^{11} *E. coli* J6-2 cells and 8.8×10^{11} *E. coli* PB 922 cells suspended in 20 litres of 0.85% w/v sterile NaCl was injected into well 2. The injection depth was 17 m below ground level (approximately in the middle of the screened section of the well and 12 m below static well water level).

Wells 2, 4, 6 and 5 were sampled for a period 7 days after injection. Sample collection—from a depth of 17 m—was by means of sterile "jerk"-samplers (Sinton, 1979). Samples were packed in ice for transport to the laboratory. Two replicates of each sample or dilution aliquot were filtered and the membranes were placed on separate plates of McConkey agar. One plate contained 75 mg/*l* of nalidixic acid and the second contained 50 mg/*l* of rifampycin. After incubation, all (yellow) colonies growing on the nalidixic acid plates were counted as *E. coli* J6-2 and all (pink-purple) polonies on the rifampycin plates as *E. coli* PB 922.

RESULTS AND DISCUSSION

The Effects of Antibiotic Concentration

The data in Table 1 indicate that both *E. coli* J6-2 and *E. coli* PB 922 meet the second and third criteria for a sewage tracer, ie, absence from sewage and total selectivity of the detection method. For the recovery, on MacConkey agar, of *E. coli* PB 922, any rifampycin concentration between 50 and 75 mg/*l* was satisfactory, totally suppressing all sewage species, as well as the *E. coli* neotype and *E. coli* J6-2, without reducing the *E. coli* PB 922 count. A rifampycin concentration of 50 mg/*l* was subsequently selected for the isolation of *E. coli* PB 922. A wider range of nalidixic acid concentrations of between 50 and 200 mg/*l* were suitable for the recovery of E. coli J6-2. However, the selected concentration of 75 mg/*l* was high enough to ensure total suppression of all background organisms including *E. coli* PB 922 and the neotype.

Survival in Groundwater Samples

E. coli J6-2, *E. coli* PB 922 and *E. coli* Neotype 01:K1:H7 all conformed to $\log_{10}$ "die-off" curves. Correlation coefficient (r) values were, respectively, 0.93, 0.94 and 0.92. The T 90 value (time taken for 90% of the cells to die-off) for *E. coli* J6-2 was 2.6 days, for *E. coli* PB 922—3.0 days and for *E. coli* neotype 01:K1:H7—3.2 days. These data indicate that *E. coli* J6-2 and *E. coli* PB 922 comply with the fourth and fifth criteria for a microbial tracer, i.e, both exhibited reasonable survival rates in groundwater samples and these rates were similar to that of "typical" *E. coli* cells. However, although the laboratory-stored groundwater samples provide useful comparative survival information, they probably do not accurately represent natural

TABLE 1—THE EFFECTS OF ANTIBIOTIC CONCENTRATION ON *E. COLI* J6-2, *E. COLI* PB 922, *E. COLI* NEOTYPE O1:K1:H7 AND FAECAL COLIFORM BACTERIA

— Data expressed as a percentage of the number of colonies on the control (no antibiotic) plates

Antibiotic	Organism	Milligrams antibiotic/litre MacConkey Agar											
		0	25	50	75	100	125	150	200	250	300	350	400
Rifampycin	*E. coli* J6-2	100	0	0	0	0	—	—	—	—	—	—	—
	E. coli PB 922	100	106	92	98	36	21	0	0	0	0	0	0
	E. coli Neotype	100	0	0	0	0	—	—	—	—	—	—	—
	Faecal Coliform Bacteria	100	1	0	0	0	—	—	—	—	—	—	—
Nalidixic Acid	*E. coli* J6-2	100	98	112	94	89	106	88	87	14	1	0	0
	E. coli PB 922	100	0	0	0	0	—	—	—	—	—	—	—
	E. coli Neotype	100	0	0	0	0	—	—	—	—	—	—	—
	Faecal Coliform Bacteria	100	2	0	0	0	—	—	—	—	—	—	—

groundwater conditions. In particular, the effects on survival of adsorption to soil and gravel particles cannot be readily simulated in flask-stored samples. The T 90 values may not therefore typify natural survival rates in groundwater strata.

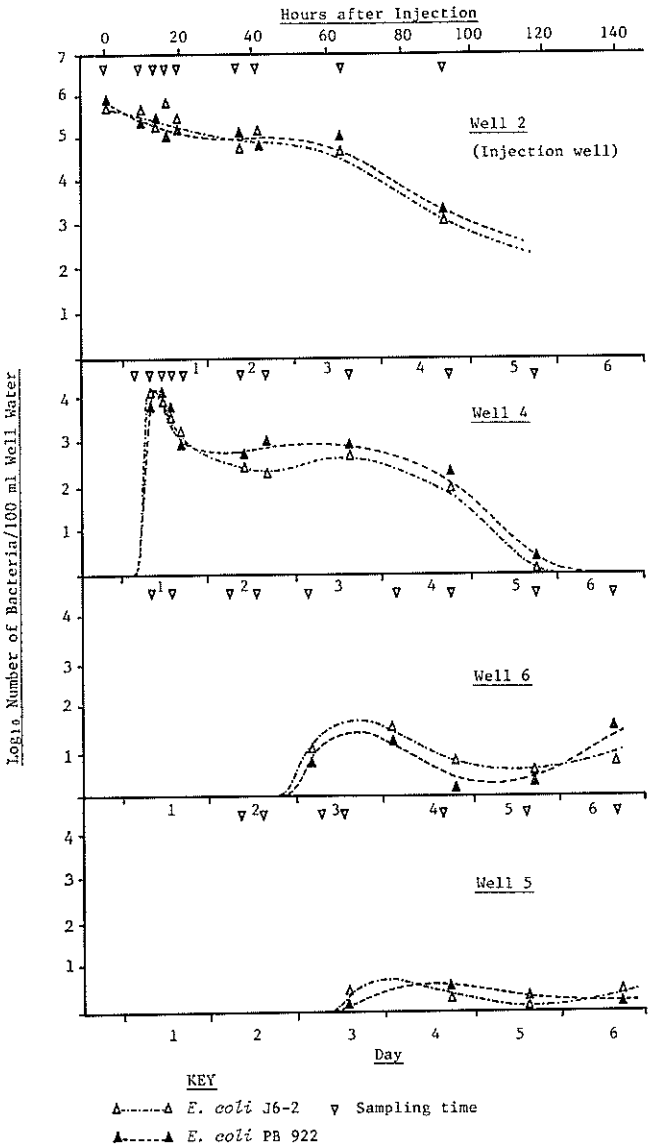

FIG. 2—Numbers of *E. coli* J6-2 and *E. coli* PB 922 cells in investigation wells at Burnham following injection into well 2.

TABLE 2—ESTIMATED TRAVEL TIMES AND VELOCITIES FOR *E. COLI* J6-2 AND *E. COLI* PB 922 IN GROUNDWATER AT BURNHAM

Well No.	Radial distance from well 2 (m)	Estimated time to first arrival (hr)	Estimated time to peak concentration (hr)	Estimated Velocity					
				To first arrival			To peak concentration		
				m/hr	m/day	mean	m/hr	m/day	mean
4	140	9	14	15.6	373	340 m/day	10.0	240	224 m/day
6	570	48	66	11.9	285		8.6	207	
5	920	61	*	15.1	362		—	—	

* Indistinguishable

Field Evaluation

Both *E. coli* J6-2 and *E. coli* PB 922 were recovered from the three downstream wells at the Burnham site (Fig. 2). They exhibited almost identical recovery patterns and the rates of travel were the same for both strains. The rate of decrease of cell numbers in the injection well indicates that both strains appeared to survive considerably longer in well water than in laboratory-stored groundwater samples. The presence of soil particles and/or organic material in the wells may have enhanced their survival rate.

The field experiment was designed solely to test the efficacy of the strains as groundwater tracers and was not intended to provide hydrological information on the Burnham site. However, some general conclusions may be drawn from the data (Table 2). In each case, arrival time was assumed to have occurred mid-way between the time of first detection and the previous sampling occasion. The figures indicate a first arrival velocity (mean of wells 4, 5 and 6) of approximately 340 m/day and a velocity to peak concentration (mean of wells 4 and 6) of approximately 215 m/day. These velocities were considerably higher than the 200 m/day to first arrival and 128 m/day to peak concentration previously recorded at the site (Sinton, 1980). The differences were probably due to the greater injection depth (17 m below ground level as compared to 13 m in the previous study). Subsequent investigations in the Burnham area (unpublished data, MWD, Christchurch) have confirmed the presence of a higher permiability aquifer at the greater depth.

CONCLUSIONS

The bacterial strains *E. coli* J6-2 and *E. coli* PB 922 appear to have considerable potential as tracers of sewage-polluted groundwater movement. Studies by previous workers indicate that both strains appear to meet the safety criteria of non-pathogenicity and inability to transfer their resistance characters or to become established in the human gastro-intestinal tract. Both strains exhibited survival rates in groundwater samples that were similar to that of a non-mutant *E. coli* strain. Both *E. coli* J6-2 and *E. coli* PB 922 were readily isolated from sewage-polluted groundwater on McConkey agar containing the appropriate antibiotic at a concentration that totally suppressed background species without reducing tracer organism counts. Both strains were successful in monitoring sewage-polluted groundwater over a distance of 920 m throughout a six-day sampling programme.

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