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Nalidixic acid as a selective agent for the isolation of enterobacteria from river water

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SUMMARY

Enterobacteria are more resistant to nalidixic acid than the majority of other Gram-negative organisms isolated from river water, so allowing their selection on MacConkey agar containing nalidixic acid. Selection is further improved by anaerobic incubation which, with nalidixic acid, virtually eliminates oxidase-positive strains such as *Pseudomonas* or *Aeromonas*.

INTRODUCTION

Following studies on the incidence of antibiotic-resistant Gram-negative rods in the River Stour, Kent (Hughes & Meynell, 1974), heavy rain and flooding resulted in enterobacteria like *Escherichia coli* becoming outnumbered by other organisms of less obvious public health importance like *Pseudomonas* and *Aeromonas*. A selective medium has therefore been devised for the isolation of enterobacteria which depends on their relatively greater resistance to low concentrations of nalidixic acid.

MATERIALS AND METHODS

Culture media

Strains were isolated on Oxoid MacConkey Agar No. 3 (CM115). Subcultures were made to Oxoid Blood Agar Base (CM55) and to Oxoid Nutrient Broth No. 2 (CM67). Buffer pH 7·2, contained (g./l.), gelatin (0·01), KH_2PO_4 (3), $Na_2HPO_4.12H_2O$ (7), NaCl (5).

Bacterial strains

'River' strains were isolated between January 1974 and January 1975 from six different river sites in East Kent (National Grid references TR138677, TR157676, TR143577, TR174599, TR324588 and TR338618), and were isolated on MacConkey agar either as supplied or containing sodium ampicillin, 20 μg./ml. 'Standard' strains were obtained from the National Collection of Type Cultures, National Collection of Industrial Bacteria, National Collection of Plant Pathogenic Bacteria and the American Type Culture Collection.

Examination of strains

The following tests were used. River strains were incubated at 37° C.; standard strains at their appropriate temperature (30 or 37° C.).

(i) Lactose fermentation, assessed on MacConkey agar or by subculture to lactose-deoxycholate agar (Meynell & Meynell, 1970).

(ii) Catalase formation, assessed both immediately and 5 min. after adding 3% (v/v) H_2O_2 to cultures grown overnight on blood agar base.

(iii) Oxidase reaction (Kovacs, 1956).

(iv) Oxidation or fermentation of glucose (Hugh & Leifson, 1953).

(v) Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-di-isopropyl pteridine; Bain & Shewan, 1968).

(vi) Flagellar morphology, as determined by electron microscopy. Strains were grown overnight in broth or in sucrose peptone broth to increase flagella formation (Fuerst & Haywood, 1969). They were then fixed in glutaraldehyde and negatively stained with uranyl acetate.

Antibiotic sensitivity tests

(*a*) Preliminary screening of 96 river strains was carried out using impregnated disks. Plates of blood agar base were spread with 0·1–0·2 ml. of growing broth cultures containing *ca.* 10^5 colony-forming units (c.f.u.)/ml. and overlaid with Oxoid Multodisks containing chloramphenicol (10 μg.), tetracycline (10 μg.), nalidixic acid (30 μg.), streptomycin (10 μg.) and kanamycin (10 μg.). Plates were incubated for 15 hr. precisely at 37° C. Diameters of inhibition zones were measured using a viewing box and callipers, the results being recorded to the nearest mm.

(*b*) Minimum inhibitory concentrations of nalidixic acid were determined for 47 river strains and 28 standard strains by plating on nalidixic acid agar. Initially, overnight broth cultures were diluted in buffer to contain 2–5 $\times 10^3$ c.f.u./ml. and 0·02 ml. samples inoculated on MacConkey agar containing 0, 0·5, 1·25, 2·5, 5·0, 7·5, 10·0, 12·5, 15·0, 20·0 or 25 μg. nalidixic acid/ml. The tests were then repeated using lower concentrations: 0, 0·06, 0·13, 0·25, 0·5, 0·75, 1·25 and 2·5 μg./ml. Plates were incubated for 20 hr. precisely at 37° C. and the presence or absence of colonies on each plate then recorded.

Colony counts on river waters

Samples of about 200 ml. river water were collected in sterile bottles opened about 6 in. below the surface and about 2 ft. from the bank, using standard precautions (Report, 1969). Counts were made within 3 hr. of collecting samples from the River Stour, Site I, downstream of Canterbury sewage works (TR174599). Dilutions in buffer were spread on MacConkey agar as supplied or supplemented with nalidixic acid, 0·13, 0·25, or 0·5 μg./ml. Colonies were counted after 20 hr. incubation at 37° C.

A subsequent series of counts, using 0·4 μg./ml. nalidixic acid, were made on

samples taken from site II (upstream of the sewage works at TR164598) in addition to site I. These plates were incubated both aerobically for 20 hr. at 37° C. and anaerobically for 48 hr. at 37° C. in jars fitted with cold catalysts.

RESULTS

Since the purpose of this investigation was to select enterobacteria, rather than to identify all the species isolated from river water, the following empirical classification was used, based on the phenotypes of 47 river strains summarized in Table 1.

(*a*) Oxidase-negative (Ox^-), lactose-fermenting or non-fermenting ($Lac^{+/-}$), with peritrichous or no flagella. All fermented glucose (G^f). These are presumed to be enterobacteria.

(*b*) Ox^+Lac^- with polar or no flagella. Some oxidized glucose (G^o) and presumably included *Pseudomonas*. Others fermented glucose and presumably included genera such as *Aeromonas*.

(*c*) Ox^+Lac^+ G^f with polar or no flagella. These presumably included other *Aeromonas* since this genus may be either Lac^+ or Lac^-.

River strains

All 47 river strains were Gram-negative catalase-positive asporogenous rods which utilized glucose. None were susceptible to the compound 0/129, and were therefore not vibrios (Shewan, Hodgkiss & Liston, 1954).

Preliminary sensitivity tests with disks

Fig. 1 shows the distribution of inhibition zone diameters for 96 river strains, 69 of which were isolated on ampicillin agar, tested against Oxoid Multodisks. In the case of kanamycin, streptomycin, tetracycline and chloramphenicol, the sensitivities of the three phenotypic classes of organisms overlapped to varying degrees and with each of these drugs, a proportion of strains were not inhibited. However, the distribution for nalidixic acid showed two interesting features. First, the $Ox^-Lac^{+/-}$ class (presumptive enterobacteria) were all more resistant than the Ox^+Lac^+ class. Secondly, the distribution for the Ox^+Lac^- class was bi-modal, some strains being as sensitive as the Ox^+Lac^+ class but others being almost wholly resistant.

Minimum inhibitory concentrations of nalidixic acid

Table 1 shows the M.I.C. for 47 river strains. They showed first, that the $Ox^-Lac^{+/-}$ class could grow on MacConkey agar containing nalidixic acid at concentrations between 0·13–0·5 μg./ml.,whereas the Ox^+Lac^+ class was inhibited; and second that, as in the disk tests, the Ox^+Lac^- class contained two types of strain, one being at least as resistant as the $Ox^-Lac^{+/-}$ group and the other as sensitive as the Ox^+Lac^+ group.

Table 1. *Minimum inhibitory concentrations of nalidixic acid for 47 river strains*

Phenotype					Nalidixic acid μg./ml. MacConkey agar No. 3							
Oxidase	Lactose*	Glucose†	Flagella‡	No. tested	0·06	0·13	0·25	0·50	0·75	1·25	2·5	>2·5
−	+/−	F	Peri	15 (5)	—	—	—	—	1	8 (4)	6 (1)	—
+	−	O	Polar	8 (3)	—	—	—	—	—	—	—	8 (3)
		F	Polar	12 (5)	5 (3)	7 (2)	—	—	—	—	—	—
+	+	F	Polar	12 (5)	6 (3)	6 (3)	—	—	—	—	—	—

M.I.C. were determined by inoculating 50–100 c.f.u., followed by overnight incubation at 37° C.
* Fermented/non-fermented.
† Fermented/oxidized in Hugh & Leifson's test (1953).
‡ Peri/polar; peritrichous/polar, when present, determined by electron microscopy.
The numbers of strains isolated on ampicillin agar are shown in parentheses.

Table 2. *Colony counts of river water on MacConkey agar containing nalidixic acid*, 0–0·5 μg./ml.

	Nalidixic acid μg./ml. MacConkey agar No. 3.											
	0			0·13			0·25			0·5		
Phenotype	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.	c.f.u./ml.	% Ox⁻	Ox⁻/ml.
Lac⁺	6·6	26	1·7	2·0	56	1·1	2·3	97	2·2	1·4	100	1·4
Lac⁻	4·0	8	0·3	1·3	31	0·4	1·0	35	0·4	1·1	39	0·4
Total	10·6	19	2·0	3·3	46	1·5	3·3	79	2·6	2·5	73	1·8

c.f.u./ml.: colony-forming units expressed as thousands/ml. river water as determined after aerobic incubation at 37° C.
% Ox⁻: percentage of oxidase-negative colonies of a total of 60–80 colonies tested at each concentration.
Ox⁻/ml.: oxidase-negative colonies/ml. (= c.f.u./ml. × % Ox⁻/100).

Table 3(*a*). *Colony counts from river water plated on MacConkey agar No. 3 containing nalidixic acid* (0·4 *μg./ml.*)

		Aerobic count c.f.u./ml.			Anaerobic count c.f.u./ml.			
Site	Phenotype	−Nal	+Nal	%	−Nal	%	+Nal	%
I	Lac+	2100	630	(30)	1700	(81)	560	(27)
	Lac−	1100	210	(19)	580	(53)	66	(6)
	Total	3200	840	(26)	2280	(71)	626	(20)
II	Lac+	1600	290	(19)	960	(60)	280	(18)
	Lac−	1300	240	(19)	410	(32)	62	(5)
	Total	2900	530	(18)	1400	(48)	340	(12)

% = colony count expressed as percentage of aerobic count without nalidixic acid.

Table 3(*b*). *Efficiency of selection of oxidase-negative organisms*

	Aerobic				Anaerobic			
	−Nal		+Nal		−Nal		+Nal	
Phenotype	% Ox−	Ox−/ml.	% Ox−	Ox−/ml.	% Ox−	Ox−/ml.	% Ox−	Ox−/ml.
Lac+	21	3·4	100	2·9	42	4·0	100	2·8
Lac−	9	1·2	34	0·8	22	0·9	88	0·6
Total	16	4·6	70	3·7	36	4·9	95	3·3

Ox−/ml. = Oxidase-negative colonies expressed as hundreds/ml., calculated as in Table 2. Values calculated as in Table 2, from counts on site II (in Table 3*a*).

Subclasses of the Ox+Lac− class

Biochemical tests on a total of 20 such river strains, summarized in Table 1, showed that of the eight strains resistant to nalidixic acid, $\geqslant 2{\cdot}5$ *μg.*/ml., none could ferment glucose. These were therefore presumed to be oxidative Pseudomonads. Of the 12 strains sensitive to nalidixic acid, $\leqslant 0{\cdot}13$ *μg.*/ml., all fermented glucose. These were therefore thought likely to be Lac− *Aeromonas*.

Standard strains

Tests on 28 standard strains confirmed the previous conclusions, within the limits of the number of species available. Some species which might be expected to occur in river water were unable to grow on MacConkey agar No. 3 as supplied. These were the *Pseudomonas* strains *P. phaseolicolor* (ATCC 11365), *P. fluorescens* (NCIB 9494), *P. putida* (NCIB 9034), *P. sp.* (NCIB 8858) and *Xanthomonas compestris* (NCPPB 528), *Zymomonas mobilis* (NCIB 8938), *Achromobacter lwoffi* (NCIB 9020), *Erwinia caratavora* (NCPPB 312).

The following all grew and had M.I.C.s of nalidixic acid within the range 0·75–2·5 *μg.*/ml.: the coliform organisms *Escherichia coli* B1, *E. coli* K12 (2 strains), *Klebsiella aerogenes* type I, *K. aerogenes* type II (NCIB 5938), *Enterobacter aerogenes* (NCIB 10102 and NCTC 10006), *Citrobacter freundii* (NCTC 9750), and the non-lactose fermenting enterobacteria *Proteus vulgaris* (NCTC 4175), *Serratia marcescens* (NCTC 1377 and NCIB 2847).

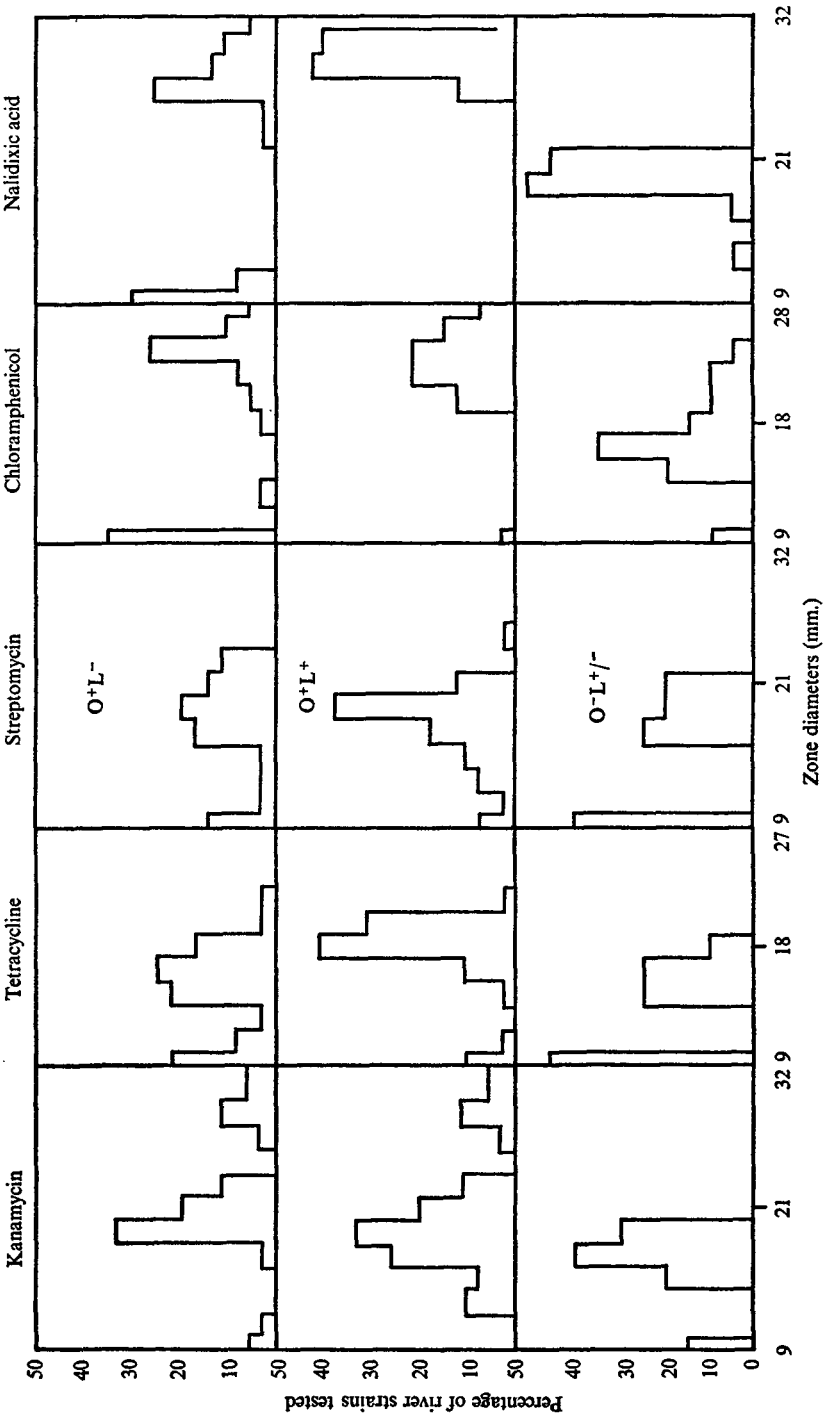

Fig. 1. Susceptibility of 96 river strains to five anti-bacterial agents, as measured by the diameters of inhibition zones surrounding Oxoid Multodisks. The diameter of each disk was 9 mm. The strains were divided into three phenotypic classes, according to their oxidase reaction (O) and their ability to hydrolyse lactose (L), as described in the text.

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Strains of *Pseudomonas aeruginosa* (NCTC 10332, NCIB 8295 and NCIB 0950), *Pseudomonas alcaligines* (NCIB 9398) and *Xanthomonas phaseoli* all had M.I.C.s greater than 2·5 μg./ml. *Alcaligines faecalis* (NCIB 8156) had an M.I.C. of 1·25 μg./ml. while of three non-lactose fermenting strains of *Aeromonas* (*A. caviae* NCIB 9671, *A. formicans* NCIB 9232 and *A. liquefaciens* NCIB 9233), two were inhibited by 0·13 μg./ml. and one by a concentration between 0·25 and 0·5 μg./ml.

Colony counts on river water

The preceding results suggested that nalidixic acid at 0·13–0·5 μg./ml. MacConkey agar would selectively inhibit the growth of the Ox^+Lac^+ class and the G^f subclass of Ox^+Lac^- strains. Colony counts were therefore made on water from site I, downstream from a sewage works, using concentrations of either 0, 0·13, 0·25 or 0·5 μg./ml. (Table 2).

The selective power of the medium was indicated by the increasing percentage Ox^- colonies obtained on nalidixic agar. Moreover, the colony count/ml. of Ox^- organisms remained constant, showing that they were not inhibited by this range of concentrations. Inhibition of Ox^+Lac^+ organisms was complete at 0·5 μg./ml., since all Lac^+ colonies tested proved to be Ox^-. At this concentration, 61% of Lac^- colonies tested were still Ox^+, as expected from the sensitivity tests of Fig. 1 and Table 1, and of 20 tested, all were G^o. This remaining fraction of Ox^+Lac^- G^o would therefore be expected to be obligate aerobes and anaerobic culture was therefore tested as an additional means of contraselection.

Colony counts were made from sites I and II using aerobic and anaerobic culture on MacConkey agar containing either no nalidixic acid or 0·4 μg./ml. (Tables 3*a*, *b*). The counts are shown in Table 3(*a*) and their details are given in Table 3(*b*). The results were consistent with those obtained previously. In both aerobic and anaerobic culture using nalidixic acid, 0·4 μg./ml., 100% of Lac^+ colonies were Ox^- compared to 21% (aerobic) and 42% (anaerobic) without nalidixic acid selection. Of the Lac^- colonies on nalidixic acid agar, only 34% were Ox^- in aerobic culture whereas the value rose to 88% in anaerobic culture. These values compare with only 9% and 22% Ox^-, respectively, in the absence of nalidixic acid. Thus, nalidixic acid and anaerobiosis together had almost eliminated the unwanted $Ox^+Lac^{+/-}$ group.

DISCUSSION

MacConkey agar No. 3 containing nalidixic acid, 0·4 μg./ml. when incubated aerobically, will inhibit all oxidase-positive lactose-fermenters and a high proportion of oxidase-positive non-lactose-fermenters. It therefore allows the direct counting of typical coliforms in river water contaminated with other organisms from sewage effluent, soil and vegetation. This is sufficient for most studies because the majority of oxidase-negative strains found in such samples are lactose-fermenters. However, if non-lactose-fermenting enterobacteria are sought then the persisting oxidase-positive non-lactose-fermenters may be further inhibited by anaerobic incubation (Table 3).

Other possible applications of these findings are to the culture media used for

colony counts on membrane filters, and also to the liquid media used for counts by the dilution method where organisms such as *Aeromonas* may produce false positive results in the presumptive coliform test (Holden, 1970).

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