MEDIA FOR ISOLATION OF *AEROMONAS* SPP. FROM FAECES

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SUMMARY. Five solid media were evaluated for isolation of *Aeromonas* spp. from faeces: desoxycholate citrate agar (DCA), MacConkey's agar (MAC), xylose-desoxycholate-citrate agar (XDCA), Rogol's medium (ROG), which contained ampicillin 20 mg/L and *p*-nitrophenyl-glycerine 25 mg/L as inhibitors, and blood agar (BA) with ampicillin 10 mg/L. False negative oxidase tests limited the usefulness of DCA and MAC and, although the use of XDCA avoided the problem of fermentation of lactose, some *Aeromonas* spp. failed to grow on XDCA or produced minute colonies unsuitable for oxidase tests. BA yielded the highest rate of isolation for *Aeromonas* spp. from 323 faecal samples—15·2% for all *Aeromonas* spp. and 9·3% for enterotoxigenic (ENT⁺) strains. This compares with 10·8% for all strains and 6·5% for ENT⁺ strains isolated on DCA, 7·1% for all strains and 4% for ENT⁺ strains on MAC and 4% for all strains and 1·5% for ENT⁺ strains on ROG. Blood agar with ampicillin is recommended for isolation of *Aeromonas* spp. from faeces.

INTRODUCTION

Since 1961, when *Aeromonas* spp. were isolated from faecal specimens during an outbreak of diarrhoea (Martinez-Silva, Guzmán-Urrego and Caselitz, 1961), it has been suggested that they may be enteric pathogens (Trust and Chipman, 1979). Recently we found that enterotoxigenic *Aeromonas* spp. were present in faeces from 10% of children with diarrhoea, but in less than 1% of control children (Gracey, Burke and Robinson, 1982). This is strong evidence that enterotoxigenic *Aeromonas* spp. are enteric pathogens.

It is important to determine whether *Aeromonas* spp. can be isolated on media in routine use for faecal specimens and, if not, which additional medium should be chosen. Ideally the medium should avoid changes in pH likely to affect oxidase testing (Overman, D'Amato and Tomfohrde, 1979; Havelaar *et al.*, 1980), the essential step in separating *Aeromonas* spp. from the Enterobacteriaceae. In the present investigation we compared the isolation rate of *Aeromonas* spp. from faecal specimens on MacConkey's agar and on desoxycholate citrate agar, media in general use for isolation of enteric pathogens, with the isolation rates on the selective medium

described by Rogol *et al.* (1979) as modified by Mouldsdales (1983) and on xylose-desoxycholate-citrate agar (Shread, Donovan and Lee, 1981). We also included blood agar containing ampicillin because of its advantages for direct oxidase tests (Overman *et al.*, 1979).

MATERIALS AND METHODS

Faecal specimens. All faecal samples submitted to the Department of Microbiology, Princess Margaret Hospital, Perth, and to the South-West Clinical Laboratories, Bunbury, during a period of 3 weeks in late summer 1983 (323 in all) were cultured in parallel on the media being evaluated. The specimens were either plated directly or stored in Cary Blair transport medium without agar (CB) (Cary and Blair, 1964) for a maximum of 3 days. In addition, other faecal specimens from children with diarrhoea, which had previously been submitted to this laboratory, shown to yield *Aeromonas* spp. and stored in CB, were used to evaluate the media.

Bacterial strains. *Aeromonas hydrophila* (80 strains) and *Aeromonas punctata* ss. *caviae* (66 strains) were isolated from faecal specimens in Thailand, Indonesia, New York and Western Australia. These strains were stored at 20°C in minimal medium (MM) (Burke *et al.*, 1982).

Isolation media. Ampicillin blood agar (BA) consisted of Columbia base agar (Oxoid) with horse blood 5% and ampicillin 10 mg/L; MacConkey's agar (MAC) and sodium desoxycholate citrate agar (DCA) were prepared according to the manufacturer's instructions (Oxoid). Modified Rogol's medium (ROG) containing *p*-nitrophenyl glycerine (PNPG) 25 mg/L and ampicillin 20 mg/L was prepared as described by Mouldsdales (1983). Desoxycholate xylose agar (XDCA) was prepared according to Shread *et al.* (1981). All media were examined after incubation at 37°C for 18–20 h. The characteristics of the media are summarised in table I.

Identification. Oxidase-positive colonies were identified by the method of Kovacs (1956); haemolytic and non-haemolytic colonies on BA and non-lactose fermenters from DCA and MAC and non-xylose fermenters from XDCA were tested for oxidase production. The oxidase reaction of isolates from DCA, MAC or XDCA was confirmed after subculture on blood agar. *Aeromonas* spp. were further identified with the medium of Kaper *et al.* (1979). *Aeromonas* spp. were classified according to the scheme of Schubert (1974), with minor modifications. All Voges-Proskauer (VP)-positive strains were regarded as *A. hydrophila* and all VP-negative strains as *A. punctata*. VP-negative strains were classified as *A. punctata* ss. *caviae* if they failed to produce gas from glucose or oxidise gluconic acid. The methods for these tests are described by Cowan (1974).

Tests for exotoxins. Enterotoxin was detected by the suckling mouse method (Burke *et al.*, 1981) and haemolysins assayed as described previously (Burke *et al.*, 1982). In our experience, results of enterotoxin and haemolysin assays are concordant in 97% of *Aeromonas* strains (Burke *et al.*, 1982).

Table I
Media investigated for isolation of Aeromonas spp. from faeces

Medium (abbreviation)	Inhibitor	Basis for recognition of <i>Aeromonas</i> spp.	Suitability for oxidase tests
Blood-agar ampicillin (BA)	ampicillin 10 mg/L	haemolysis*	yes
Modified Rogol's medium (ROG)	PNPG† 25 mg/L ampicillin 20 mg/L	non-xylose fermenter	yes
Xylose-desoxycholate-citrate-agar (XDCA)	sodium desoxycholate citrate	non-xylose fermenter	yes
MacConkey's agar (MAC)	bile salts	non-lactose fermenter‡	no
Desoxycholate citrate agar (DCA)	sodium desoxycholate citrate	non-lactose fermenter‡	no

* Haemolysis is more easily recognised on layered blood agar plates.

† *p*-nitrophenyl-glycerine.

‡ Unsuitable for lactose-fermenting strains.

Evaluation of media. All faecal specimens received during the weeks of the study were plated at the same time from samples stored in CB on to each of the test media. Other faecal specimens that had been stored in CB and from which *Aeromonas* spp. had previously been isolated, were also plated in parallel on to the test media. Strains of *A. hydrophila* and *A. punctata* ss. *caviae* previously isolated from faeces and stored in MM were inoculated directly on to the test media. In all cases, presence or absence of growth was recorded, but not extent of growth or colony size.

RESULTS

Table II shows the result of inoculating strains of *A. hydrophila* and *A. punctata* ss. *caviae* on to BA, DCA, MAC and ROG. All strains grew on BA and all *A. hydrophila* grew on MAC, but only 79 (98.7%) of 80 strains of *A. hydrophila* grew on DCA and 37 (82.2%) of 45 strains grew on ROG. Of strains which grew on DCA, 27.2% of *A. punctata* ss. *caviae* and 11.3% of *A. hydrophila* strains fermented lactose. With MAC, 25.7% of *A. punctata* ss. *caviae* and 8.8% of *A. hydrophila* strains were lactose-fermenters.

Although ROG seemed unsuitable as an isolation medium because it failed to support growth of 44.6% of *A. punctata* ss. *caviae* and 17.8% of *A. hydrophila* strains, this medium was included in the investigation of faecal specimens. The isolation rates of *Aeromonas* spp. from the 323 faecal specimens on the four media: BA, DCA, MAC and ROG, are shown in table III. On BA, *Aeromonas* spp. were isolated from 15.2% of

TABLE II
Growth of Aeromonas strains on the media investigated

Medium	<i>A. punctata</i> ss. <i>caviae</i>			<i>A. hydrophila</i>		
	Number of strains tested	Number (%) that grew	Number (%) that fermented lactose	Number of strains tested	Number (%) that grew	Number (%) that fermented lactose
BA	66	66 (100)	...	80	80 (100)	...
DCA	66	63 (95.5)	18 (27.2)	80	79 (98.7)	9 (11.3)
MAC	66	65 (98.5)	17 (25.7)	80	80 (100)	7 (8.8)
ROG	65	36 (55.4)	...	45	37 (82.2)	...

BA = blood agar with ampicillin; DCA = desoxycholate-citrate-agar;
MAC = MacConkey's agar; ROG = modified Rogol's medium.

TABLE III
Isolation from 323 faecal samples of Aeromonas spp. on the media investigated

Medium	Number of specimens (%) yielding growth	
	All <i>Aeromonas</i> spp.	<i>A. hydrophila</i> (enterotoxigenic)
BA	49 (15.2)	30 (9.3)
DCA	35 (10.8)	21 (6.5)
MAC	23 (7.1)	13 (4.0)
ROG	13 (4.0)	8 (1.5)

For abbreviations see table II.

samples on DCA from 10.8%, on MAC from 7.1% and on ROG from 4.0%. Isolates were either *A. hydrophila* or *A. punctata* ss. *caviae*; no *A. punctata* ss. *punctata* were isolated.

All *A. hydrophila* isolates were enterotoxigenic, as defined by a haemolysin titre of > 4, or a positive suckling mouse assay with a haemolysin titre of 3 or 4. None of the *A. punctata* ss. *caviae* produced haemolysins. Only four strains of *A. hydrophila* produced borderline values for haemolysin and required confirmation of enterotoxigenicity by the suckling mouse assay. When only the enterotoxigenic *A. hydrophila* strains are considered, these were isolated from 9.3% of faecal specimens on BA, from 6.5% on DCA, from 4% on MAC and from 1.5% on ROG (table III). When the results with all four media were evaluated, 34 faecal specimens were found to contain enterotoxigenic *A. hydrophila*. Thirty of them were positive on BA, but two were positive on DCA only and two were positive on MAC only.

Faecal specimens known to contain *Aeromonas* spp. and stored in CB were also used for evaluation of BA, MAC, ROG, and XDCA. *Aeromonas* spp. were isolated on BA from all 17 faecal specimens tested but only 14 strains were recovered on MAC, and only 10 on ROG (table IV). Sixteen strains were isolated on XDCA, but only 12 strains produced colonies large enough to be recognised easily and used for direct oxidase testing. Four other specimens yielded colonies < 0.5 mm in diameter, unsuitable for oxidase testing of individual colonies.

TABLE IV

Isolation of A. hydrophila from 17 known positive faecal samples stored in Cary-Blair medium

Medium	Number (%) of samples that yielded <i>A. hydrophila</i>
BA	17 (100)
MAC	14 (82)
XDCA	16 (94)
ROG	10 (59)

XDCA = xylose desoxycholate citrate agar;
for other abbreviations see table II.

DISCUSSION

Blood agar containing ampicillin 10 mg/L was the most satisfactory medium for isolation of faecal *Aeromonas* spp. The presence of β -haemolysis on BA (most readily seen on layered plates) often, but not always, indicates those strains which produce exotoxin haemolysin, the marker of enterotoxigenicity in 97% of strains.

ROG was inferior to the other media with all three methods of evaluation; only 8 of the 34 enterotoxigenic *A. hydrophila* strains in faeces were recovered on ROG. Although most *A. hydrophila* are not sensitive to ampicillin in a concentration of 20 mg/L, some *A. punctata* ss. *caviae* are susceptible to concentrations of ampicillin as low as 3 mg/L (Richardson *et al.*, 1982). It seems unlikely that susceptibility to ampicillin should account for a much lower isolation rate of *A. hydrophila* as well as *A. punctata* ss. *caviae* from ROG. PNPG may be inhibitory, as we found that blood agar containing PNPG 43 mg/L caused a decrease both in colony size of *Aeromonas* spp.

and in the amount of haemolysis (unpublished data) but this concentration is in excess of the 25 mg/L used in ROG.

DCA allowed recovery of 61% of *A. hydrophila* and 66% of all *Aeromonas* spp. isolated from 323 faecal samples, but is unsuitable for the oxidase-testing that is necessary to detect the strains of *Aeromonas* that ferment lactose. In our study of 146 strains from different geographical sources (table II), 11% of *A. hydrophila* which grew on DCA fermented lactose. For this reason, DCA modified by replacing lactose with xylose, which is not fermented by *Aeromonas* spp., was investigated. Twelve of the 17 faecal samples known to contain *Aeromonas* spp. produced easily recognisable colonies of *Aeromonas* on XDCA. One specimen was negative and the remaining four produced minute colonies that were difficult to recognise and were not suitable for direct oxidase testing.

We have previously found that it is unnecessary to pick multiple similar colonies of *Aeromonas* spp. for biotyping and toxin testing, whereas with toxigenic *Escherichia coli* multiple colonies should be tested for adequate recognition of enterotoxigenic strains (Morris *et al.*, 1976). On the other hand, when colonies of different morphology are present, differences in biotype and exotoxin assays are common.

Others (Shread *et al.*, 1981; Mouldsdales, 1983; Von Graevenitz and Bucher, 1983) have recommended enrichment, particularly with alkaline peptone water, for isolation of faecal *Aeromonas* spp. We have previously evaluated several liquid media for enrichment (unpublished data). These were strontium hydrogen selenite and strontium chloride B enrichment broth (Iveson, 1973), trypticase soy broth and tetrathionate broth (Bailey and Scott, 1974), potassium tellurite and alkaline peptone water (Furniss, Lee and Donovan, 1978), and nutrient broth (Cowan, 1974). Nutrient broth was the most satisfactory and produced excellent enrichment after incubation of faecal samples for 24 h. In a large epidemiological study of children with and without diarrhoea (Gracey *et al.*, 1982) we found that *Aeromonas* spp. isolated from our direct culture on blood agar showed good correlation with the presence of diarrhoea, whereas isolation only after enrichment did not do so. We believe that enrichment is too sensitive for clinical application.

On the basis of the ability of pure cultures of *Aeromonas* spp., incubated overnight in TSB, to grow on the media tested, and of the recovery of *Aeromonas* spp. added to faecal samples, Von Graevenitz and Bucher (1983) recommended dextrin fuchsin-agar (DFS), inositol-brilliant green-bile salts (IBBS), Pril-xylose-agar (PXA) and XDCA as solid media suitable for isolation of *Aeromonas* spp. from faeces. They also examined faecal specimens, but only four strains of *Aeromonas* spp. were isolated, and these results cannot be compared with our investigation. Our initial studies with DFS showed it to be unsuitable for oxidase-testing, as well as being complex and time-consuming to prepare for use in a routine microbiology laboratory. IBBS also had the disadvantage of complexity, and we did not have access to the commercially prepared DFS and IBBS used by Von Graevenitz and Bucher (1983).

Because Pril was unobtainable, we used PXA (Rogol *et al.*, 1979) modified as described by Mouldsdales (1983) so that PNPG replaced Pril. This modified medium was the least satisfactory of those tested. XDCA, the other solid medium recommended by Von Graevenitz and Bucher (1983), was in our study, less suitable than BA. Our previous experience agrees with that of Von Graevenitz and Bucher (1983) who

considered that the medium of Shotts and Rimler (1973) and that of Rippey and Cabelli (1979) are both unsuitable for isolation of faecal *Aeromonas* spp.

Although some strains of *Aeromonas* spp. can be isolated on media such as MAC and DCA used for routine examination of faecal samples, not all faecal *Aeromonas* spp. will be detected in this way. We believe it is more satisfactory to include a plate of layered blood agar with ampicillin, a medium which is simple to prepare, in routine investigation of faecal samples. Oxidase-positive colonies may be identified by multitest systems such as API20E (Analytab Products, La Balme-Les-Grottes, Montalieu-Vercieu, France) or Kaper's medium (Kaper *et al.*, 1979), a medium which is inexpensive, reliable and particularly suitable for processing large numbers of presumptive *Aeromonas* isolates.

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