# Methods for the isolation of *Aeromonas hydrophila* and *Plesiomonas shigelloides* from faeces

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## SUMMARY

Two solid selective media, xylose deoxycholate citrate agar (XDCA) and bile salts brilliant green agar (BBG) and an enrichment broth–alkaline peptone water, were evaluated for the isolation of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Alkaline peptone water and XDCA are useful for recovery of *Aeromonas* but not *Plesiomonas*, whereas BBG is satisfactory for both organisms.

## INTRODUCTION

There have been numerous reports throughout the world of the isolation of *Aeromonas* and *Plesiomonas* from cases of diarrhoea in humans (Lautrop, 1961; Rosner, 1964; Cooper & Brown, 1968; von Graevenitz & Mensch, 1968; Chatterjee & Neogy, 1972; Bhat, Shanthakumari & Rajan, 1974; Gracey, Burke & Robinson, 1982). Usually the organisms have been present as the predominant aerobic faecal flora and have been isolated on Salmonella–Shigella or other 'enteric' media containing bile salts and lactose. It has been stated that these organisms are not part of normal human faecal flora (Davis, Kane & Garagusi, 1978) and several large screening series (Lautrop, 1961; Catsaras & Buttiaux, 1965; Paučková & Fukalová, 1968) found isolation rates of less than 1 % for *Aeromonas*. However, von Graevenitz & Zinterhofer (1970) found a 3·2 % carrier rate of *Aeromonas hydrophila* in persons without gastrointestinal symptoms using a specially designed DNase agar. More recently an overall isolation rate of 5 % for this organism was obtained from a large random series of faecal specimens using alkaline peptone water enrichment and xylose deoxycholate citrate agar (XDCA) (Shread, Donovan & Lee, 1981).

The aim of the present study was to evaluate XDCA and a modification of inositol brilliant green bile salts agar (BBG) (Schubert, 1977) as solid selective media for *Aeromonas* and *Plesiomonas*, and alkaline peptone water as an enrichment broth.

## MATERIALS AND METHODS

Three strains of *A. hydrophila*, one toxigenic, one non-toxigenic and one anaerogenic, and two strains of *Plesiomonas shigelloides*, one agglutinating with *Shigella sonnei* antiserum and one non-agglutinating, were kindly supplied by Dr

A. L. Furniss of the Maidstone Public Health Laboratory, Preston Hall Hospital, Kent.

Xylose deoxycholate citrate agar (XDCA) was prepared according to a protocol from the Maidstone Laboratory. The following were added per litre of distilled water: nutrient broth (No. 2 Oxoid CM 67) 12·5 g, sodium citrate 5·0 g, sodium thiosulphate 5·0 g, ferric ammonium citrate (brown) 1·0 g, sodium deoxycholate (Koch Light 84274) 2·5 g, agar (Davis) 12·0 g, xylose 10·0 g and neutral red (1 % solution in distilled water) 2·5 ml. The pH was adjusted to 7·0 and the mixture gently heated with constant mixing to 100 °C. It was simmered for 20 s, removed from the heat and allowed to cool to 50 °C before pouring plates.

Bile salts brilliant green agar (BBG) was made up by adding proteose-peptone No. 3 (Difco) 10·0 g, lab-lemco powder (Oxoid) 5·0 g, bile salts (Oxoid L 55) 5·0 g, sodium chloride 5·0 g, brilliant green 0·05 % w/v 1·0 ml to one litre of distilled water. The pH was adjusted to 7·0 and the mixture heated as for XDCA.

Horse blood agar was supplied by Difco. MacConkey agar (Oxoid) was made up according to the manufacturer's instructions. Alkaline peptone water (APW) pH 8·6, as used for vibrio isolation, was supplied by the Central Public Health Laboratory, Colindale Avenue, London N.W. 9.

All test organisms were grown overnight at 37 °C in nutrient broth and tenfold dilutions made for a Miles and Misra plate count (Stokes & Ridgway, 1980) on blood agar, or MacConkey agar in the case of *Proteus* sp. Test stool specimens were pools of 3–5 normal faeces diluted 1 in 10 and emulsified in Ringer's solution. These were counted as above on MacConkey agar. Single colonies of each test strain were streaked on MacConkey agar, XDCA and BBG to examine colonial characteristics.

To test the inhibitory properties of XDCA and BBG, counts of standard strains of *Escherichia coli* (NCTC 9111), *Proteus mirabilis* (NCTC 2896), *Salmonella typhimurium* (NCTC 74) and *Pseudomonas aeruginosa* (NCTC 8056) on blood agar or MacConkey agar were compared with those on the test media after incubation overnight at 37 °C and again at 48 h. The degree of inhibition was expressed as $\log_{10}$ of the count on a non-inhibitory medium divided by the count on the test medium (Table 1). Each experiment was repeated twice and a mean taken. All strains of *Aeromonas* and *Plesiomonas* were similarly tested.

To test the detection ability of the solid media one 0·02 ml drop of each tenfold dilution of test organism was added to 1 ml of stool suspension, mixed well and streaked out using a 1 $\mu$l loop. Plates were incubated at 37 °C and examined at 18 h and 48 h. Non-xylose-fermenting colonies on XDCA were tested for cytochrome C oxidase activity by the method of Kovács using 1 % tetramethyl-*p*-phenylenediamine dihydrochloride (Cowan, 1979*a*). Subculture to nutrient agar first is not necessary (Millership, Curnow & Chattopadhyay, 1983). BBG was flooded with the reagent and positive colonies subcultured immediately. Final identification of oxidase-positive colonies was done using Hugh and Leifson's O/F test (Cowan, 1979*b*), vibrostatic agent O/129 (Furniss, Lee & Donovan, 1978) and twenty biochemical tests on API 20E trays following the manufacturer's instructions. The tests include beta-galactosidase activity by hydrolysis of ONPG, arginine dihydrolase, lysine and ornithine decarboxylase activity, citrate utilization, hydrogen sulphide production, urease activity, tryptophane deaminase, indole production, VP reaction, liquefaction of gelatin and utilization of glucose,

mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. Fermentative, oxidase-positive organisms resistant to 0/129 on a 150 $\mu$g disk with an API profile consistent with *A. hydrophila* were called *A. hydrophila*. Such strains are 80–100 % ONPG, arginine dihydrolase, indole, gelatin, glucose, mannitol and sucrose positive and 50–70 % citrate, VP, melibiose and arabinose positive.

*Plesiomonas shigelloides* is oxidase-positive, fermentative and sensitive to 0/129 on a 150 $\mu$g disk. It is 80–100 % ONPG, arginine dihydrolase, lysine and ornithine decarboxylase, indole, glucose and inositol positive.

The effect of the addition of xylose to BBG was tested by adding 10 g/l xylose and retesting the inhibitory and detection properties of the medium as above, in comparison with MacConkey agar. The effect of varying concentrations of brilliant green in BBG was tested by comparing counts of both strains of *Plesiomonas* on blood agar with those on BBG containing brilliant green at 0·01 g/l to 0·0001 g/l.

Alkaline peptone water was then examined. To evaluate the growth of small inocula, 0·02 ml drops of tenfold dilutions of test organism were added to three 1 ml volumes of APW and incubated at 37 °C for 18 h. Tubes showing growth were subcultured to check for purity. Selectivity was examined by adding 1 ml of stool suspension already inoculated with the test organism to 10 ml of APW and incubating overnight at 37 °C or room temperature. A 1 $\mu$l sample was taken from the top of the broth and streaked out on XDCA or BBG; colonies were identified as before. A similar suspension was also incubated at room temperature and plated on XDCA after 18 h, 2 days and 5 days (Table 2).

Isolation was performed from 1004 faecal specimens chosen at random from those received in the laboratory for testing for recognized pathogens from persons with gastrointestinal symptoms, healthy contacts and food handlers. No attempt was made to sample a normal population, but rather one representative of that likely to be received in a routine diagnostic laboratory. Specimens were cultured directly on XDCA, enriched in APW and subcultured to XDCA. A further 225 specimens were cultured directly on BBG, of which 198 were also subcultured to both XDCA and BBG after APW enrichment.

## RESULTS

All the standard strains of *Aeromonas* and *Plesiomonas* appeared as non-lactose fermenters on MacConkey agar. The plesiomonads were pinpoint colonies after 18 h, becoming clearly visible at 48 h. On XDCA *Aeromonas* appears as colourless colonies whereas xylose fermenters are pink, often with a surrounding pink halo. Plesiomonads are minute colourless colonies. On BBG all coliforms appear as colourless colonies. Aeromonads and plesiomonads are distinguished by their rapid oxidase reaction, the latter often being clearly visibile even at 18 h.

Table 1 compares the inhibitory properties of XDCA and BBG. XDCA reduces counts of the standard strain of *E. coli* by $10^5$, and *Pr. mirabilis* and *S. typhimurium* by $10^1$. On the other hand BBG inhibits only *E. coli* by $10^1$. Neither medium has any effect on *P. aeruginosa*. XDCA inhibits pooled normal faecal flora by $10^1$, whereas BBG has no overall effect.

Table 1. *Comparison of inhibitory properties of XDCA and BBG*

|  | XDCA | BBG |
|---|---|---|
| **Inhibited organisms** | | |
| *Escherichia coli* NCTC 9111 | 5 | 1 |
| *Proteus mirabilis* NCTC 2896 | 1 | 0 |
| *Salmonella typhimurium* NCTC 74 | 1 | 0 |
| *Pseudomonas aeruginosa* NCTC 8056 | 0 | 0 |
| **Selected organisms** | | |
| *Aeromonas* | | |
| Non-toxigenic | 0 | 0 |
| Toxigenic | 0 | 0 |
| Anaerogenic | 0 | 0 |
| *Plesiomonas* | | |
| Agglutinating | > 3 | 0 |
| Non-agglutinating | > 3 | 0 |
| **Pooled normal faeces** | | |
| A | 1 | 0 |
| B | 1 | 0 |
| C | 1 | 0 |
| D | 2 | 0 |
| E | 1 | 0 |
| F | — | 0 |

Inhibition is expressed as

$$\log_{10}\left[\frac{\text{count on non-inhibitory medium}}{\text{count on test medium}}\right]$$

Neither medium is inhibitory for *Aeromonas*, whereas XDCA reduces counts of *Plesiomonas* by more than $10^3$.

Table 2 compares the ability of the two media to detect the test strains in artificially inoculated stools. BBG is not significantly different in sensitivity from XDCA for *Aeromonas* ($\chi^2 = 0.59$, $0.5 < P < 0.8$) and is satisfactory for *Plesiomonas*.

The addition of xylose to BBG did not change the counts of any organism in pure culture. However, detection of *Aeromonas* and *Plesiomonas* in stools was markedly affected. At ratios of $1:10^1-<10^2$, only one out of 14 *Aeromonas* tests was positive and none out of nine *Plesiomonas* tests. Only at a ratio of 10 *Aeromonas* to one normal flora was isolation achieved in 90 % of tests. This was also the case using MacConkey agar.

Brilliant green at concentrations of 0·01 g/l inhibited the agglutinating strain of *Plesiomonas* by $> 10^2$. At concentrations below 0·005 g/l, colony counts of both strains were the same as those of blood agar, but the colonies were much smaller until the concentration was less than 0·001 g/l. Hence 0·0005 g/l was chosen as the optimum concentration.

Testing of APW showed that all five test strains of *Aeromonas* and *Plesiomonas* grew from an inoculum of 1–10 c.f.u./ml. Table 2 lists the results of artificially inoculating the test strains into normal faeces, enriching in APW and plating out

Table 2. *Recovery from artificially contaminated stools*

| | Number of stools positive/number of tests (%) at a test organism to normal flora $\log_{10}$ ratio of: | | | |
| --- | --- | --- | --- | --- |
| | 1–< 2 | 2–< 3 | 3–< 4 | ≥ 4 |
| Direct plating | | | | |
| XDCA | | | | |
| *Aeromonas* | 21/22 (95) | 18/22 (82) | 5/20 (25) | 0/12 (0) |
| *Plesiomonas* | 1/9 (11) | 1/9 (11) | — | — |
| BBG | | | | |
| *Aeromonas* | 16/16 (100) | 15/19 (79) | 7/15 (47) | 0/9 (0) |
| *Plesiomonas* | 8/9 (89) | 2/4 (50) | — | — |
| Alkaline peptone water enrichment | | | | |
| 37 °C | | | | |
| Subculture to XDCA | | | | |
| *Aeromonas* | 6/10 (60) | 3/10 (30) | 1/10 (10) | 0/10 (0) |
| Subculture to BBG | | | | |
| *Aeromonas* | 7/9 (78) | 14/16 (88) | 16/18 (89) | 14/19 (74) |
| Room temperature | | | | |
| Subculture to XDCA | | | | |
| *Aeromonas* | 6/9 (67) | 5/9 (56) | 6/9 (67) | 4/10 (40) |
| Subculture to BBG | | | | |
| *Aeromonas* | 6/7 (86) | 13/16 (81) | 12/18 (67) | 12/18 (67) |

on XDCA or BBG. Extending the incubation time beyond 18 h markedly reduced the isolation rate of *Aeromonas*. At *Aeromonas*-to-coliform ratios of $1:10^3$ or less, incubation of APW at room temperature significantly improves isolation on XDCA ($\chi^2 = 7\cdot8$, $0\cdot001 < P < 0\cdot01$). Enrichment at 37 °C is no better than direct plating ($\chi^2 = 1\cdot35$, $0\cdot05 < P < 0\cdot02$). At ratios of $1:10^3$ or less, enrichment at room temperature and subculture to BBG is significantly better than direct plating on BBG ($\chi^2 = 8\cdot11$, $0\cdot001 < P < 0\cdot01$). Unlike subculture to XDCA, this improvement also occurs after enrichment at 37 °C and the isolation rate is not significantly different from room temperature enrichment ($\chi^2 = 1\cdot97$, $0\cdot1 < P < 0\cdot2$).

*Plesiomonas* was not isolated from a total of 24 tests using APW enrichment, six each at ratios of $1:10^1–< 10^2$ and $1:10^2–< 10^3$ at room temperature and 37 °C. This is clearly much worse than direct plating.

Examination of real faecal specimens using APW and XDCA yielded 42 strains from 1004 samples, an isolation rate of 4·2 % (Millership, Curnow & Chattopadhyay, 1983). In 450 specimens there were 62 non-xylose-fermenting, oxidase-positive isolates which were not *Aeromonas*, giving a false positive rate of 14 %. All the false positive isolates were oxidative, non-reactive or produced an alkaline reaction in O/F medium; 43 (69 %) were *Pseudomonas* sp., identified either by production of blue-green pigment or by their API profile. The remaining organisms were non-reactive in API, and comprised a heterogeneous collection of *Alcaligenes*, *Chromobacterium* and other similar non-fermentative genera not easily identifiable in a diagnostic laboratory. Of the 198 specimens subcultured on BBG as well, 10 yielded *A. hydrophila*, an isolation rate of 5 %, which is not significantly different from the rate of 4·2 % obtained in the previous study involving 1004 specimens

($\chi^2 = 0.3$, $0.5 < P < 0.8$). However, eight isolates grew on both media and two on BBG alone. The false positive rate on BBG was also 14%, and was composed of similar organisms to those on XDCA.

From 258 specimens cultured directly on BBG, there were three isolates of *A. hydrophila* as against 13 in 1004 samples on XDCA, giving a 1% isolation rate in both cases. There was also a single isolate of *Plesiomonas shigelloides* (from a woman with acute, self-limiting diarrhoea recently returned from Australia).

## DISCUSSION

Von Graevenitz & Bucher (1983) recommended four solid media for *Aeromonas* including XDCA and a medium similar to BBG containing inositol, the latter alone for *Plesiomonas*, and APW as enrichment for both organisms. We have also shown that XDCA is unsuitable for *Plesiomonas*, and indeed Schubert (1977) described inhibition by lactose containing DCA.

The inclusion of inositol in BBG seems unnecessary. It possibly delays the oxidase reaction of *Plesiomonas* and does little to aid interpretation of mixed cultures. It might even have an inhibitory effect if other carbohydrates besides xylose and lactose behave in the same way as these do in our experiments.

The reasons why xylose in BBG and lactose in MacConkey agar inhibit *Aeromonas* and *Plesiomonas* is not clear, but presumably a by-product of carbo-hydrate metabolism is involved. XDCA would then work because it is highly inhibitory to most other coliforms. This would also partly explain the difficulty in isolating *Aeromonas* and *Plesiomonas* on 'enteric' media which all contain carbohydrates, and the apparent rarity of the latter in the literature.

Like von Graevenitz & Bucher (1983) we have found APW a useful enrichment medium for *Aeromonas*. However, unlike them we could not obtain good results incubating at 37 °C and subculturing to XDCA, which is in agreement with recommendations from Maidstone Public Health Laboratory (personal communi-cation). It is clear from our results using BBG that *Aeromonas* does grow in APW at 37 °C, and possibly the single strain tested by von Graevenitz & Bucher was unusual in this respect. This argument is strengthened by the rather low isolation rate of 1·6% from faecal specimens obtained by these authors. The discrepancy between subculture on XDCA and BBG is difficult to explain. BBG may be a slightly more sensitive medium or perhaps APW at 37 °C renders the organisms more susceptible to the inhibitory effects of XDCA.

We could find no evidence that *Plesiomonas* isolation was aided by enrichment in APW. Here again von Graevenitz & Bucher (1983) used a single strain, and we were able to obtain only two for testing. Although these two showed no numerical difference in isolation rates there were qualitative differences in the size of colonies and rate of growth. Strain variation could easily explain the discrepancy between our results and those of von Graevenitz & Bucher.

Our recommended isolation technique for *Aeromonas* and *Plesiomonas* is as in Figure 1. BBG alone is a suitable isolation medium, but the inclusion of XDCA sometimes speeds identification of presumptive strains since flooding of the plate is not required. We have not attempted to divide *Aeromonas* into more than one
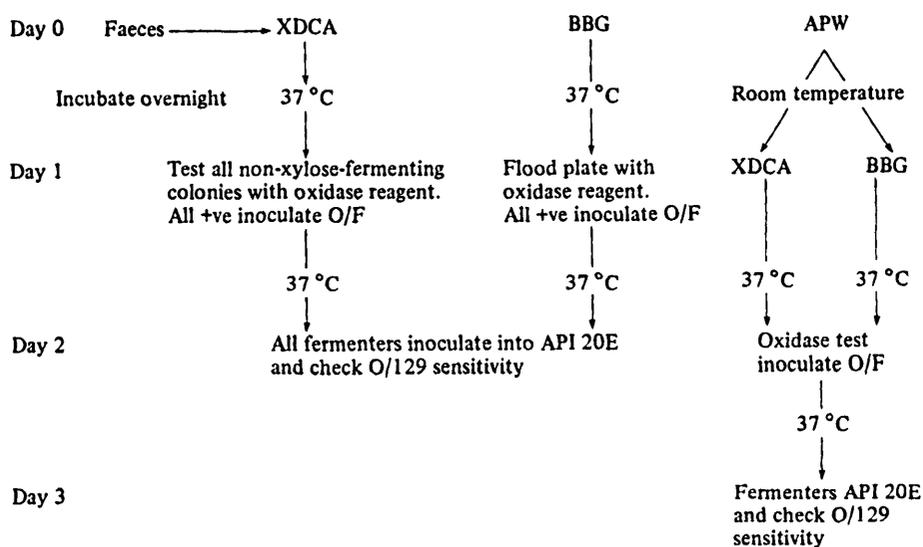
Fig. 1. The recommended isolation technique for *Aeromonas* and *Plesiomonas*.

species since the only widely accepted species is *A. salmonicida*, a fish pathogen which does not grow at 37 °C.

Our isolation rates of 4–5 % for *Aeromonas* (Millership, Curnow & Chattopadhyay, 1983) are comparable with others using a similar study population (Shread, Donovan & Lee, 1981), patients with diarrhoea (Rogol *et al.* 1979) and even in normals (von Graevenitz & Zinterhofer, 1970). Our single isolation of *Plesiomonas* seems better than other screening series (Rogol *et al.* 1979; von Graevenitz & Bucher, 1983), but statements that it is rare in faeces should be interpreted with caution since there is no effective enrichment medium.

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