Isolation of *Plesiomonas shigelloides* from nutrient broth with brilliant green: its use in screening stool samples from an African population

P NAIR, S E MILLERSHIP

From the Department of Bacteriology, Royal Postgraduate Medical School, London

SUMMARY The suitability and selectivity of nutrient broth with bile salts and varying concentrations of brilliant green as an enrichment medium was tested for the isolation of *Plesiomonas shigelloides* from faeces. The broth was most effective at a concentration of 0.001 g/l brilliant green, using faeces deliberately contaminated with *Plesiomonas*. Two hundred and nineteen faecal samples from Nigeria were then examined for the presence of *Plesiomonas* and other bacterial pathogens. One yielded *Plesiomonas* following enrichment in bile salts brilliant green broth (BBG broth) whereas no samples were positive on direct plating.

In 1947 Ferguson and Henderson¹ described an organism with the somatic antigen of *Shigella sonnei*, which was later placed in a new genus *Plesiomonas* of the family *Vibrionaceae*, having a single species *shigelloides*.^{2,3} It is present in soil and surface water^{4,5} and has been found in drinking water in association with an outbreak of diarrhoea (B Chattopadhyay, personal communication). Most isolates from man have been from faeces or the gastrointestinal tract,⁶ and reports from several parts of the world have implicated *Plesiomonas* as a cause of gastroenteritis.^{3,7-9}

This organism may well be more common than the few published reports would suggest. Investigation of its role in human disease, however, has been hampered by the lack of suitable primary isolation and enrichment media. Previous studies^{1,5,10,11} have shown that although it will grow well in pure culture on many media, recovery is poor when it is in mixed culture. We report our findings on the selectivity of nutrient broth combined with bile salts and varying concentrations of brilliant green as an enrichment medium, and the use of this to examine a series of stool samples from an African population.

Material and methods

Four strains of *Plesiomonas shigelloides* were used in laboratory tests; two were supplied by Dr AL Furniss of Maidstone Public Health Laboratory comprising one agglutinating with *S sonnei* antiserum (a) and one

non-agglutinating (b). The remaining strains (c and d) were isolated from patients with diarrhoea; neither had *S sonnei* phase I antigen.

Bile salts brilliant green agar (BBG) was prepared as previously described.¹¹ Horse blood agar was supplied by Gibco. MacConkey agar (Oxoid) was made up according to the manufacturer's instructions. Four broths were tested: nutrient broth (Oxoid No 2) alone, and three nutrient broths with bile salts (Oxoid L55, 5 g/l) and 0.0005 g/l, 0.001 g/l, or 0.005 g/l brilliant green, respectively. In all cases the broth was dispensed in 10 ml aliquots and autoclaved at 15 lb for 15 minutes. The final pH was 7.17 to 7.2 at 21°C.

All test organisms were grown overnight at 37°C in nutrient broth and 10-fold dilutions made for a Miles and Misra plate count¹² on blood agar. Test stool specimens were pools of two to five random faecal specimens diluted 1:10 and emulsified in Ringer's solution. These were counted as above on MacConkey agar.

To evaluate the growth of small inocula in the test broths 0.02 ml drops of 10-fold dilutions of test organism were added to three 1 ml aliquots of the relevant broth and incubated at 37°C for 18 hours. Tubes showing growth were subcultured to check for purity. A medium was considered to be satisfactory if growth occurred in at least two of the three tubes to which 1-10 colony forming units/ml had been added.

Selectivity was examined by inoculating 0.02 ml of 10-fold dilutions of each test strain into 1 ml aliquots of faecal suspension, mixing well, and plating out 0.001 ml on BBG agar before adding 10 ml of test broth. All broths were incubated overnight at 37°C. A

sample of 0.001 ml was then streaked out on BBG agar. Plates were incubated overnight at 37°C, flooded with Kovac's oxidase reagent¹³ and positive colonies subcultured immediately on to blood agar. Identification was confirmed using API 20E trays according to the manufacturer's instructions. Uninoculated control samples of each faecal pool were also treated in the same way. One experiment was continued for 48 hours and another for 72 hours.

Two hundred and nineteen stool samples were collected from a Nigerian population. Specimens were frozen at -70°C and thawed on the day of culture. They were plated out on BBG agar and about 1 g faeces added to each of 100 ml of BBG broth with 0.001 g/l brilliant green and alkaline peptone water. On the second day BBG broth and alkaline peptone water were subcultured to BBG agar. All media were incubated overnight at 37°C, except alkaline peptone water, which was incubated at room temperature.

Oxidase positive colonies were identified as described previously. All stool specimens were also examined from *Salmonella*, *Shigella*, and *Campylobacter* spp using standard methods.

Results

All four test strains of *Plesiomonas* grew in nutrient broth and the three bile salts brilliant green (BBG)

broths from an inoculum of 1-10 colony forming units/ml. Table 1 shows the results of artificially contaminated faeces in BBG broth compared with those obtained with direct plating. All faecal coliform counts were in the range $10^6 - 2.5 \times 10^8$. Nutrient broth and BBG with 0.0005 g/l were abandoned after one experiment as no isolations of any of the test strains could be made even at concentrations of 10 *Plesiomonas* to one of faecal flora. In one experiment a stool specimen had a heavy growth of *Ps aeruginosa*. *Plesiomonas* could not be recovered either on direct plating or on enrichment from any sample and the results were omitted from the analysis.

Both enrichment broths with brilliant green were significantly better than direct plating at *Plesiomonas* to coliform ratios of $1: > 10^4$ ($p < 0.001$, $\chi^2 = 13.19$ and 15.31 , respectively). There was no significant difference between the two broths ($\chi^2 = 0.09$, $0.80 > p > 0.50$). Increasing the incubation time to 48 hours considerably reduced the positive isolations and, by 72 hours, 13 of 20 positive test results were all negative.

There was also variation in the recovery rates of different strains (table 2). Strain (d) was more readily isolated than strain (a) (direct plating $p = 0.015$, 0.001 g/l broth $p = 0.0008$, 0.005 g/l broth $p = 0.00004$; Fisher's exact test). In no case was either broth significantly worse than direct plating; in par-

Table 1 Recovery from artificially contaminated stools

No of stools positive/No of tests (%) at test organism: normal flora $\log_{10}$ ratio of:

	0- < 1	1- < 2	2- < 3	3- < 4	> 4
BBG agar	8/8 (100)	3/8 (38)	1/8 (13)	3/16 (19)	0/46 (0)
BBG broth (0.001 g/l)*	3/8 (38)	3/8 (38)	2/8 (25)	1/12 (8)	7/27 (26)
BBG broth (0.005 g/l)*	5/8 (63)	4/8 (50)	4/8 (50)	2/12 (17)	8/27 (30)

*Concentration of brilliant green.

Table 2 Comparison of isolation rates of different test strains

Strain	Direct plating	BBG broth (0.001 g/l)*	BBG broth (0.005 g/l)*	Totals (%)
a	3/22	0/16	1/16	4/54 (7)
b	3/22	2/16	3/16	8/54 (15)
c	2/22	5/16	9/16	16/54 (30)
d	7/20	8/15	12/15	27/54 (50)

*Concentration of brilliant green.

Table 3 Isolation of enteric pathogens from 219 stool samples tested: comparison of different media used

Organism	BBG solid medium	BBG broth	Campylobacter medium	Alkaline peptone water	Selenite F	DCA	MacConkey
<i>Plesiomonas shigelloides</i>	—	1	—	—	—	—	—
<i>Yersinia enterocolitica</i>	—	1	—	—	—	1	—
<i>Salmonella</i> spp	—	—	—	—	5	2	2
<i>Shigella sonnei</i>	—	—	—	—	—	—	2
<i>Campylobacter</i> spp	—	—	4	—	—	—	—

ticular, the probability of obtaining the results for strain (a) in enrichment medium with 0.001 g/l brilliant green was 0.25 (Fisher's exact test).

Table 3 shows the results of examining 219 specimens of faeces from Nigeria. There was one isolate of *Plesiomonas shigelloides* from BBG broth alone. There were nine strains of *Pseudomonas* spp isolated from BBG broth which gave a positive oxidase reaction; six of these were also isolated from alkaline peptone water.

Discussion

Several studies have shown that *Plesiomonas* grows on a variety of conventional enteric media—for example, MacConkey, Salmonella-Shigella, and deoxycholate citrate agars.^{15 11} Schubert suggested that this was not entirely satisfactory for primary isolation from mixed culture¹⁴ and devised a new medium, inositol brilliant green bile salts agar (IBB agar). This, or a modification of it, seems to be the solid medium of choice.^{11 15}

No satisfactory enrichment medium, however, has been described. Alkaline peptone water was effective in one study but not in others.^{11 15} Our results show that brilliant green at the correct concentration is, as suggested by Winton,¹⁶ a useful selective agent. Nutrient broth alone is not useful so the effect is not simply one of dilution, also bile salts alone do not make any difference as, in the presence of a very low concentration of brilliant green, the broth is still ineffective. All the broths have a pH that is nearly neutral, ruling this out as the selective mechanism.

The interference with isolation caused by other oxidase positive colonies, particularly *Ps aeruginosa*, has been noted with alkaline peptone water but usually does not affect more than a few specimens,¹¹ and our findings confirm this. The pronounced differences between test strains of *Plesiomonas* may explain previous discrepant results with alkaline peptone water enrichment.^{11 15} For screening purposes it would be wise to use two enrichment broths—for example, brilliant green and alkaline peptone water.

In most of the prospective studies, workers have either failed to isolate *Plesiomonas*, or isolated it only in small numbers from the faecal samples tested.^{10 15 17} Unfortunately, it was impractical to use a better preservation technique for the faecal samples examined in this study, and the relatively low isolation rates of other pathogens suggest that this may have been a factor in the failure to recover more

Plesiomonas. Our finding of a single isolate therefore suggests that BBG broth is useful as an enrichment medium, although further confirmation is needed.

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Requests for reprints to: Dr P Nair, Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Duane Road, London W12 6HS, England.