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A modification of bile salts brilliant green agar for isolation of motile *Aeromonas* from foods and environmental specimens

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SUMMARY

Aeromonas has recently been recognized as an important enteropathogen. In faecal specimens the organism is readily identified and differentiated from coliforms by the positive oxidase reaction. However, few media have proved useful for foods and environmental specimens. Bile salts brilliant green starch agar (BBGS) was studied for this purpose by testing artificially contaminated foods as well as natural potential sources. The results indicate the suitability of the medium for the isolation of *Aeromonas* from foods and from environmental sources.

INTRODUCTION

The genus *Aeromonas* has been considered autochtonous inhabitants of aquatic environments. During the past two decades, the role of motile *Aeromonas* in human disease has come under scrutiny. It has been found that the organisms were isolated significantly more often from individuals with diarrhoea than from those without diarrhoea (Holmberg & Farmer, 1984). However, neither the enteropathogenicity nor the epidemiology has yet been clarified. Indeed the possibility that *Aeromonas* sp. can merely be recovered more readily from stools of patients with diarrhoea is still unsettled. Although the organisms seem to be isolated with increased frequency during summer months when maximum concentrations of aeromonads occur in aquatic habitats (von Graevenitz & Zinterhofer, 1970; Burke *et al.* 1984), sources and routes of the infection are still obscure. The need is therefore to identify outbreaks and sporadic cases and to compare strains recovered from stools with those from foods and the environment.

From faecal specimens, aeromonads can be readily isolated using bile salts brilliant green agar (BBG), because oxidase-positive bacteria, except pseudomonads, hardly grow at all on the medium (Millership & Chattopadhyay, 1984).

On the other hand, for environmental specimens, few media are available to recover, quantitatively, aeromonads and readily differentiate them from other background organisms. The purpose of the present study was to modify BBG for the isolation of aeromonads in foods and environment samples and to assess its suitability for the detection of the organisms from natural sources.

MATERIALS AND METHODS

Strains

Aeromonas hydrophila (10 strains), *A. sobria* (10 strains) and *A. caviae* (10 strains) were studied. Five strains of each had been originally isolated from faecal specimens and the other five from river water.

Media

Bile salts brilliant green starch agar (BBGS) was prepared by adding proteose peptone (Difco) 10·0 g, lab-lemco powder (Oxoid) 5·0 g, bile salts (Oxoid L55) 5·0 g, sodium chloride 5·0 g, soluble starch 10·0 g, agar 15·0 g, brilliant green 0·05% w/v 1·0 ml to one litre of distilled water. The pH was adjusted to 7·2 and the mixture was heated until completely dissolved. Alkaline peptone water pH 8·8 (APW) was prepared according to the manufacturer's instruction (Nissui, Tokyo).

Growth of Aeromonas

Strains were grown overnight in nutrient broth, diluted to yield a colony count of between 50 and 300 and plated in 0·1 ml on Trypticase soy agar (TS) and BBGS. Recovery rate on BBGS was expressed by adding the number of colonies of all the strains on BBGS, dividing by the total number of colonies on the corresponding TS plates and multiplying by 100.

Field studies

Water samples from rivers and the sea were collected in sterile polypropylene 500 ml bottles land refrigerated until processed. The interval between collection and assay of the samples did not exceed 24 h. Food samples were obtained from the Laboratory of Food Hygiene, Osaka Municipal Wholesale Market. 0·1 ml of a tenfold dilution of the sample was spread on the medium. Plates were incubated at 30 °C and examined at 24–48 h. To test the inhibitory properties on a background flora of BBGS, total viable aerobic heterotrophic counts were determined on standard methods agar (Nissui, Tokyo) and compared with those on the BBGS. Then, BBGS plates were flooded with about 0·5 ml of Lugol solution (iodine 1·0 g, potassium iodide 2·0 g, distilled water 100 ml) and amylase-positive colonies with a clear zone surrounding the colony were identified as presumptive *Aeromonas*. For those samples yielding *Aeromonas* counts of fewer than 10 cells per ml, enumeration was accomplished by a most-probable number procedure in which APW was used for enrichment.

Identification

Amylase-positive colonies were picked and inoculated into the multiple test medium (Kaper *et al.* 1979). Strains which yielded an alkaline surface and an acid butt and were motile were considered to be motile *Aeromonas* and were tested further for Gram-negativity by the KOH method (Gregersen, 1978), cytochrome oxidase, catalase, indole production, gelatin hydrolysis (Smith & Goodner, 1958), and sensitivity to 0/129. Strains were identified to species level according to the scheme given in Bergey's manual (Popoff, 1984).

Table 1. *Recovery rates** (%) *of* Aeromonas *strains on BBGS*

Species	Strains from faeces	Strains from river water
A. hydrophila	92	88
A. sobria	104	87
A. caviae	94	92

* Recovery rate (%) is expressed as

$$\frac{\text{counts on BBGS}}{\text{counts on trypticase soy plates}} \times 100.$$

† Each species contains 5 strains from faeces and 5 strains from river water.

Table 2. *Inhibition** *of background flora in 64 foods and 20 water samples by BBGS*

	No. of samples showing inhibition of				
	< 1 log	1 – < 2 log	2 – < 3 log	3 – < 4 log	> 4 log
Foods	40	17	4	1	2
River water	2†	16	2	—	—

* Colony counts on Standard Methods Agar were compared with those on BBGS.
† Major parts of standard plate counts of these two specimens consisted of aeromonads.

Detection of Aeromonas *sp. in the presence of other organisms*

Bacterial flora, which did not include aeromonads, from ten different kinds of foods were grown overnight in nutrient broth and diluted 1 in 100. A preparation of *A. hydrophila* was added to the bacterial flora in varying amounts such that the proportions in the different mixtures extended from 1 part *Aeromonas* to 10 parts flora to 1 part *Aeromonas* to 10^4 parts flora. Samples of the mixture were inoculated onto BBGS medium, which was then incubated for 48 h at 30 °C. When APW was used as pre-enrichment medium, the proportions started at $1:10^4$ and 1·0 ml aliquots of APW, which were incubated overnight at 30 °C and then streaked onto BBGS.

RESULTS

Inhibition of Aeromonas

Compared with colony counts on TS, BBGS were not inhibitory for *Aeromonas*. Regardless of the species and origin either from faeces or environment, BBGS was suitable for quantitative recovery (Table 1).

Inhibition of background flora

Results for 64 foods and 20 water specimens are shown in Table 2. BBGS inhibited river flora by 10; in foods the inhibitory effect was observed in 40% of the samples.

Table 3. *Recovery of* A. hydrophila *in 10 artificially contaminated foods*

	No. of foods positive at an *Aeromonas*-to-background flora ratio of					
	$1:10-<10^2$	$1:10^2-<10^3$	$1:10^3-<10^4$	$1:10^4-<10^5$	$1:10^5-<10^6$	$1:10^6-<10^7$
Direct plating	10	8	2	ND	ND	ND
Enriched with APW	—	—	—	10	7	4

ND, not detected; —, not done

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Field studies

A total of 350 amylase-positive colonies from 52 river water specimens were picked and examined further. Of these, 196 strains (56%) were identified as motile *Aeromonas*.

Recovery of Aeromonas *strains from artificially contaminated foods*

The results are shown in Table 3. The sensitivities of BBGS decreased from aeromonas-to-background flora proportions of 1 to 10^3. Enrichment with APW was more effective than direct plating at low *Aeromonas*-to-background flora ratios.

DISCUSSION

For isolation of *Aeromonas* and *Plesiomonas* from faeces, Millership & Chattopadhyay (1984) modified inositol brilliant green bile salts (Schubert, 1977) and developed BBG agar. Xylose deoxycholate citrate agar is also useful for detection of faecal aeromonads (Shread, Donovan & Lee, 1981). *Aeromonas* can be readily differentiated from coliforms on these media by the oxidase positive reactions or the absence of xylose fermentation. In the examination of environmental specimens, however, neither of these characteristics could be used as a differential marker as many of the background flora are oxidase positive and do not ferment xylose either. Palumbo *et al.* (1985) reported that in gram-negative species associated with foods starch hydrolysis is largely restricted to *Aeromonas* and *Vibrio* species, and recommended the use of starch ampicillin agar (SA) for the examination. Although we had used SA according to their recommendation, the plates were very often obscured by the swarming of proteus in samples. Furthermore, Rahim *et al.* (1984) have pointed out that 50% of the strains of *A. hydrophila* were susceptible to 12.5 μg of ampicillin per ml. *A. sobria* and *A. caviae* seem to be more susceptible than *A. hydrophila* to antibiotics (Motyl, McKinley & Janda, 1985). Therefore, in this study starch was added into BBG agar, and assessed as to its suitability for examinations of foods and environmental specimens.

Clearly BBGS has proved a suitable medium for quantitative recovery of motile *Aeromonas* and the species could be readily differentiated from other organisms. Most of amylase-positive colonies from food samples were *Aeromonas* which is in agreement with the observations of Palumbo *et al.* (1984). Even in the direct plating of river water, 56% of amylase-positive colonies on the medium were motile *Aeromonas*. Satisfactory sensitivity can also be obtained by the combined use of APW and BBGS. Adequate separation of colonies on the plate is essential for the differentiation of those colonies surrounded by the halo caused by starch hydrolysis. Serial inoculation of several plates is preferable to overcrowding.

Although motile *Aeromonas* are now recognized as important enteropathogens, the sources and routes of the infection are not clear. *Aeromonas* is isolated with increased frequency during summer months both from the environment and from human faeces. However, to date there has been no clear description of the characteristics that distinguish presumably enteropathogenic clinical isolates from the environmental isolates. In differentiating strains recovered from stool from those recovered from foods and environment, it is ideal to compare the strains isolated from the media which contain same selective substances. It is possible that use of BBG for faecal and BBGS for environmental specimens satisfied this prerequisite.

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