DIAGNOSTIC METHODS

Media for the detection and recognition of the enteropathogen *Providencia alcalifaciens* in faeces

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A medium (PAM: *Providencia alcalifaciens* medium) is described that enables the presence of the enteropathogen *P. alcalifaciens* in faeces to be detected with ease and simplicity. This organism is probably the only oxidase-negative organism likely to be present in tetrathionate broth cultures of faeces that is unable to ferment the mannitol, xylose or galactose present in the medium. Thus the red colonies of *P. alcalifaciens* appeared quite distinct from the lemon-yellow acid-forming colonies of all the other bacteria that ferment one or more of these sugars. Extensive tests showed the medium to be both highly specific and sensitive in detecting *P. alcalifaciens*. Two additional media are described that enable the identity of presumptive *P. alcalifaciens* isolates to be confirmed unequivocally and with ease.

Introduction

Over many years, several groups [1–5] have believed the Providence group of bacteria to cause diarrhoea in man, and particularly in young infants. On the other hand, Graber and Lincoln [6] found only one such isolate in 607 stools of infants aged < 1 year in Denver with diarrhoea. It has been thought by others that *Providencia* spp. are normal intestinal commensals that flourish only during infections caused by bacterial or viral enteropathogens.

The significance of many of these old studies is difficult to evaluate because examinations were not then made for the presence of bacteria such as *Campylobacter jejuni* and *Yersinia enterocolitica* and viruses such as rotavirus, which are now known to cause diarrhoea. Moreover, the identification of an isolate as *Providencia* sp. as opposed to another member of the *Proteaeae* was not so clearly defined then as it is now.

More recent studies have shown that *P. alcalifaciens* was found seven-to-eight times more frequently in the stools of British adults with diarrhoea who had travelled abroad than in those who had not [7]. Albert *et al.* [8, 9] have shown that *P. alcalifaciens* strains

have the ability to invade intestinal mucosa and other cell types, cause diarrhoea in rabbits, and bring about actin condensation in a manner similar to that caused by *Shigella flexneri*. Guth and Perrella [10] have confirmed that isolates of *P. alcalifaciens* from cases of diarrhoea have the ability to invade cells and cause actin condensation whereas other isolates of *P. alcalifaciens* from non-diarrhoeal stools do not. The genetic determinant(s) for invasiveness appeared to be different from those of invasive *Shigella* spp. and *Escherichia coli* and were not plasmid-borne [10]. As a preliminary to studies on the enteropathogenicity of *P. alcalifaciens*, a medium which facilitates the detection of this organism in specimens of faeces is described here.

Materials and methods

Providencia alcalifaciens medium (PAM)

The medium contained 10 g of either Lab Lemco powder (Oxoid L29) or Bacto Tryptone (Difco), sodium deoxycholate 5 g, anhydrous disodium hydrogen orthophosphate 0.8 g, phenol red dye 80 mg and agar 12 g in 1 L of distilled water. The medium was sterilised in 500-ml volumes at 121°C for 15 min. Before use, xylose 1.5 g, mannitol 1.5 g and galactose 1.5 g were added to 500 ml of solidified sterile medium and steamed for *c.* 30 min to sterilise the sugars and melt the agar. When molten and cool the complete medium was poured into petri dishes.

Sugar-supplemented phenylalanine deaminase agar

This contained Tryptone (Oxoid CM 87) 1.5 g, L-phenylalanine 1 g, agar 1.3 g and bromocresol purple dye (0.08%) 2.5 ml in 100 ml of distilled water. After sterilisation at 121°C for 15 min and when molten but cool, the medium was aseptically supplemented with trehalose, maltose and inositol, each to a final concentration of 0.5% from sterile (steamed for 1 h) stock sugar solutions (10% w/v in water) and dispensed either in 200- μ l amounts into the wells of a microtitration plate or in 2-ml amounts into screw-capped bottles and left to solidify as a slope.

Adonitol peptone water

This contained Peptone (Oxoid CM 9) 1.5 g and bromocresol purple dye (0.08%) 2.5 ml in 100 ml distilled water. After sterilisation at 121°C for 15 min and when cool, the medium was aseptically supplemented with adonitol to 0.5% from a sterile (steamed for 1 h) stock solution (10% w/v) in water.

Analysis of faeces for P. alcalifaciens

A specimen of faeces (about the size of a small pea) from patients under investigation for infection with intestinal microbial pathogens was inoculated into 10 ml of Tetrathionate Broth (Oxoid). Separate tetrathionate broths were also inoculated with pure cultures of clinical isolates of *P. alcalifaciens*. After overnight incubation at 37°C, the tetrathionate broth cultures of faeces were plated out on PAM (and other media for detection of other bacterial enteropathogens). In some instances, and blind to the operator, a known volume of a faecal tetrathionate broth culture was supplemented with 0.1 volume of a tetrathionate broth culture of *P. alcalifaciens* before plating out on PAM.

After incubation of the PAM plates overnight at 37°C, they were examined for the presence of red colonies. Red colonies were tested for oxidase activity and those that were oxidase-negative were picked off and subcultured in 2 ml of nutrient broth for a few hours at 37°C. The sugar-supplemented phenylalanine deaminase agar and a tube of adonitol peptone water were then inoculated with the nutrient broth subculture and both were incubated overnight at 37°C.

If the bromocresol purple dye in the sugar-supplemented phenylalanine deaminase medium remained blue, it indicated that none of the sugars trehalose, maltose or inositol present in the medium had been fermented to acid. Formation of phenylpyruvic acid was then demonstrated by the development of a dark green colour within a few moments of the addition to the slope of a few drops of ferric chloride solution (10% w/v) in water. Only isolates of *P. alcalifaciens* form both phenylpyruvic acid from phenylalanine and

acid from adonitol but not from trehalose, maltose or inositol.

Results

The specificity of PAM for detecting *P. alcalifaciens* was investigated initially by culturing on it a variety of organisms likely to be present in tetrathionate broth cultures of faeces from man. These included isolates of *Salmonella* spp., *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Ent. aerogenes*, *Hafnia alvei*, *Proteus mirabilis*, *Pr. vulgaris*, *Morganella morganii* and all species of *Providencia*. All gave rise to lemon-yellow or white colonies except for those of *P. alcalifaciens* which were red.

An examination by culture on PAM of 80 coded (code unknown to operator) pure cultures showed that, in every instance that a culture gave rise to red colonies on PAM, subsequent secondary tests proved these colonies to be *P. alcalifaciens*. The cultures examined comprised 16 isolates of *P. rettgeri*, 12 isolates of *P. rustigianii*, six isolates of *P. heimbachae* (kindly donated by Professor H.E. Muller), 9 isolates of *M. morganii*, and 14 isolates of *P. stuartii* – all organisms thought to be likely to cause confusion with *P. alcalifaciens* by similarity or ability to acidify only one of the sugars in PAM – and 23 strains of *P. alcalifaciens* acidifying none. All the 23 *P. alcalifaciens* strains tested gave rise to red colonies on PAM and all 57 isolates of the other organisms gave rise to lemon-yellow or white colonies.

Of 200 faecal specimens examined from patients either with symptoms of diarrhoea or under investigation for infection with enteropathogenic micro-organisms, 12 were found to be infected with *Campylobacter* spp. and seven with *Salmonella* spp. Bacterial enteropathogens were not detected in the remainder. Of the 200 tetrathionate broth cultures plated out on PAM and incubated overnight at 37°C, 14 gave rise to red colonies that were oxidase-positive. API 20NE tests subsequently confirmed all these as being isolates of *Pseudomonas aeruginosa*.

Four tetrathionate broth cultures gave rise on PAM to oxidase-negative colonies that were peach-coloured rather than red. Nevertheless, these were examined and the secondary tests confirmed that none was *P. alcalifaciens*. Further studies showed that after incubation for a further 24 h on PAM, the colonies of all four cultures had become more yellow and API 20E tests showed that all four were isolates of *M. morganii*.

Of the 47 tetrathionate broth cultures seeded blind with *P. alcalifaciens* before culture on PAM, red colonies that were oxidase-negative were detected in

all 47 of them (i.e., 100% sensitivity). One representative colony from each specimen on subsequent testing on sugar-supplemented phenylalanine deaminase agar and adonitol peptone water sugar medium gave reactions which, in every instance, showed unequivocally that the isolate was *P. alcalifaciens*. Thus PAM appears to be a sensitive and specific medium for detecting the enteropathogen *P. alcalifaciens* in faeces.

Discussion

There is now good evidence that some strains of *P. alcalifaciens* cause diarrhoea in man [8, 10]. Therefore, a method is needed that will enable this organism to be detected reliably with ease, sensitivity and accuracy. The media and methods described appear to fulfil these criteria. Because *P. alcalifaciens*, when found in stools, is not always the predominant organism [10], the starting material was a tetrathionate broth culture of the faeces to maximise the sensitivity of isolation of the organism. *P. alcalifaciens* isolates grow well in this medium [11].

The PAM culture medium distinguishes *P. alcalifaciens* from almost all other bacteria capable of growing in tetrathionate broth because unlike the latter organisms it is unable to form acid from any of the constituent sugars of the medium. Therefore, *P. alcalifaciens* colonies appeared red (alkaline) against a background of lemon-yellow or white (acid-forming) colonies of other bacteria.

PAM contains three sugars: xylose, mannitol and galactose. These sugars were chosen, because they are not fermented by *P. alcalifaciens* and because two or more of them are fermented by virtually all other faecal bacteria capable of growth in tetrathionate broth. Thus possible confusion that could arise by detection of variants unable to acidify a sugar was minimised and was not experienced. The exceptions are non-fermentative organisms, *M. morganii* and all *Providencia* spp. apart from *P. rettgeri*. These latter organisms ferment only one of the sugars in the medium, galactose, yet they did not give rise to false positive red colonies. Although *Proteus* spp. do not swarm on Lab-Lemco agar-based media, sodium deoxycholate was nevertheless a necessary ingredient in the medium to make it selective. The medium was buffered with phosphate to an alkaline pH (c. 7.8) to counteract the high acidity formed by the organisms other than *P. alcalifaciens*.

Despite examination of 200 clinical faecal samples (only 13 were from children under 1 year) for *P. alcalifaciens* by conventional methods, both directly and also after tetrathionate broth enrichment, and also by examination of tetrathionate broth cultures on PAM, this organism was not detected. This shows that

P. alcalifaciens is not a common enteropathogen of adults and also confirms the importance of the need for the use of a medium such as PAM to facilitate its detection.

However, because of this, in order to determine the specificity of the medium, it was necessary to seed blind some of the tetrathionate broth cultures with *P. alcalifaciens*. The PAM medium appears to be very sensitive because in every instance when *P. alcalifaciens* was added to broths, even in small amounts (routinely at 1 in 10, but also on occasions at up to 1 in a 1000 dilution in faecal tetrathionate broth cultures), the organism was detected.

Although several of the samples of faeces tested were found to contain other enteropathogens such as *Campylobacter* and *Salmonella* spp., the only occasion that red colonies were found on PAM was when *P. aeruginosa* (oxidase positive) or *P. alcalifaciens* (oxidase negative) was present. Thus the medium is also highly specific and the detection of a red, oxidase-negative colony on PAM is almost certainly indicative of *P. alcalifaciens*. This was readily confirmed by the additional supplementary tests.

The sugar-supplemented phenylalanine deaminase agar together with the adonitol peptone water were devised to permit the most important confirmatory tests for *P. alcalifaciens* to be made on the red, oxidase-negative colonies from PAM in the simplest way. The sugars in these media were chosen not only to be discriminatory for *P. alcalifaciens* but those in the phenylalanine deaminase agar were also selected as sugars that are acidified by most of the organisms listed above, which fermented only galactose in PAM, in order to avoid confusion through the detection of sugar-negative variants. It should be noted that if one or more of the sugars in the sugar-supplemented phenylalanine deaminase agar is fermented, not only is the isolate not *P. alcalifaciens*, but the acid conditions prevent further testing of the medium for phenylalanine deaminase activity [12].

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