The genus Aeromonas with particular reference to human enteric strains

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The genus Aeromonas (aeromonads) encompasses a number of species. No specific media for the genus have been described. Several other species may yet be added to the list. However, it is appreciated that the group comprises obligate parasites (and phytophiles).

The Natural habitat of A. salmonicida is usually considered to be fresh-water-dwelling fishes (particularly salmonids). These may be asymptomatic carriers of the bacterium or may exhibit symptoms of the disease furunculosis. Fish furunculosis occurs world-wide, except in Australia and New Zealand. Recently, however, A. salmonicida-like bacteria were isolated from diseased goldfish in Australia. For descriptions of furunculosis, and aspects of the natural history of the disease, the reviews of McGraw and McCarthy are recommended.

Virulence factors

A. salmonicida produces a number of extracellular products which might play a role in its virulence. These include a leucolytic factor, tissue necrotizing proteases, and haemolysins.

In addition to extracellular products, virulent A. salmonicida produce a surface layer of protein which confers virulence upon strains possessing it. Some strains also produce adhesins which may play a part in the organism's virulence. Thus, A. salmonicida is endowed with an array of virulence factors, which, acting in concert, confer a considerable pathogenic propensity for what is 'potentially the most destructive of all bacterial fish diseases'.

Hydrophila-punctata complex

Natural habitats

Hydrophila-punctata aeromonads are ubiquitous in fresh-water environments throughout the world. Lehmann and Neumann, in 1901, reported that Bacterium (Aeromonas) punctatum is one of the most common of water bacteria. These bacteria even find their way into municipal water-supplies in various parts of the world including Australia, Canada, and England. Many water-dwelling animals and plants are frequently associated with, or colonized by, aeromonads of this group. Some reported examples include zooplankton, healthy fish, medicinal leeches, and frogs.

Aquatic animals are not the only ones to harbour these bacteria. Flies have been shown to carry them, and asymptomatic carriage in the human alimentary tract has been reported to occur with a frequency ranging from less than 1 per cent to 4.7 per cent.

Disease associations: non-human. Hydrophila punctata aeromonads have long been recognized as causative agents of disease in poikilothermic animals. In 1890, Banarel described a disease in frogs caused by motile aeromonads. This disease, later named 'red-leg', is the result of a complex set of interactions involving bacterial endotoxin, haemolysin(s), and stress-producing environmental factors.

Homeothermic animals are also susceptible to infection. Scheirag, in 1937, described an epizootic septicaemia in guinea pigs, and the disease 'black rot' in hen's eggs was described in the same year.

Figure 1: Representative colonies of Aeromonas hydrophila-punctata strains grown on horse-blood agar prepared from Oxoid tryptone soya agar-base medium.

(a) Typical A. hydrophila (wild type) haemolysis. Note beta-haemolysis surrounded by a zone of precipitation (double-zone haemolysis). Also note the typical green colouration in the area of confluent growth.
(b) Atypical A. hydrophila (ex. ATCC 15467) haemolysis. Note lack of any haemolytic activity around isolated colonies.
(c) Typical A. caviae (wild type) haemolysis. Note precipitation zone around isolated colonies. Haemolysis is not evident.
(d) Typical A. caviae (ex. ATCC 15468) haemolysis. Note precipitation zone around isolated colonies. (J-haemolysis has occurred only in areas of confluent growth.)
monads, isolated from diarrhoeal faeces, have features that suggest strongly that they are the cause of human diarrhoeal disease. In fact, in some situations cited above, the result is little surprise that A. hydrophila can be isolated from the list of bacterial enteropathogens. Therefore, it would seem to be appropriate to include an isolation and identification protocol for Aeromonas in all investigations of diarrhoeal disease.

Isolation and identification
Hydrophila punctata aeromonads grow well on blood agar at 37°C, and this medium is usually used for their isolation from clinical material of a non-enteric nature. On this medium, they often produce a distinctive β-haemolysis (Figure 1). Typical A. hydrophila (Figure 1a) produce a double-zone haemolysis (not unlike that produced by Cladostium perfringens), with a characteristic green colouration in areas of confluent growth. However, a typical A. hydrophila may be non-haemolytic (Figure 1b). A sobria typically produces a single zone of β-haemolysis without greening of the confluent growth areas on the plate (Figure 1c), while A. caviae, which do not cause β-haemolysis around isolated colonies typically, may lyse the blood on confluent areas of the plate (Figures 1d and 1e). There have been several attempts made to produce selective media which will allow the ready recognition of hydrophila-punctata strains. Those include Rimler-Schotts and ampicillin-plr strains. Unfortunately, these media are considered by some workers to be less than satisfactory. On the other hand, aeromonads grow well on 5% ox blood agar, usually producing non-lactose fermenting colonies (occasionally lactose fermenting). However, they may be difficult to recognize when they do grow on such media. They frequently do not grow on TCBS medium.

To aid the isolation and recognition of aeromonads from faeces, blood agar media selected by the addition of ampicillin, should be used; in addition to other enteric selective media such as Bacteroides and Salmonella-Shigella agars. However, while such a medium in increased isolation rates for Aeromonas, it should be appreciated that not all aeromonads are haemolytic (Figure 1b), and that some strains are sensitive to ampicillin. In spite of the shortcomings of ampicillin-seeded blood agar, the medium does have some advantages over other enteric selective media. For instance, it is possible to perform oxidase tests directly on the medium; something which cannot be done with confidence on other selective media. Oxidase activity is not a property by which they are haemolytic, should be tested to determine their identity using conventional identification media or commercial miniature test kits.

Table 1 lists those characteristics useful in the speciation of hydrophila-punctata aeromonads.

Summary
Aeromonads are widely distributed in nature and can be pathogens of aquatic and amphibian as well as human faeces. They cause disease in humans and are frequently isolated from diarrhoeal faeces. However, the role of Aeromonas in human enteric disease is still open to question. Despite this, an aeromonad isolated from diarrhoeal faeces should be viewed as a probable cause of diarrhoea. Therefore, until the relationship of Aeromonas to human enteric disease is established unequivocally, it is recommended that diagnostic laboratories attempt to isolate and identify aeromonads from specimens of diarrhoeal faeces.

To accomplish this, in the presence of an ideal selective medium for Aeromonas, it is appropriate that laboratories include an ampicillin-seeded blood agar plate, as the existing battery of enteric plating media, when culturing human faeces for bacterial pathogens.

References
Clostridium difficile — isolation, identification and significance

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Clostridium difficile is now firmly established as the principal causal agent of pseudomembranous colitis (PMC). Moreover, few workers would dispute the assertion that the organism is a major cause of a spectrum of antibiotic-associated conditions ranging from non-specific diarrhea to life-threatening self-limiting diarrhoea. It has also become apparent that outbreaks of diarrhoea may occur as a result of cross-infection with C. difficile.

Isolation

The presence of C. difficile in faecal material may be established by the demonstration of the organism or its extracellular products. The isolation of C. difficile is made easier by the use of an efficient selective medium, but invariably requires a minimum of 48-hours incubation. The development of more rapid detection methods is discussed below.

A variety of selective agents have been employed for the isolation of C. difficile, including p-creol, clindamycin, ampicillino-glycyrin, cefoxitin and polymyxin B. The most widely used selective media contain cycloserine and cefoxitin.14 A medium containing egg yolk, fructose, neutral red, cycloserine (500mg/l) and cefoxitin (16mg/l) was devised by George et al., However, some strains of C. difficile are inhibited by these concentrations, and most workers now use medium with reduced cycloserine and Bng/l or 10mg/l cefoxitin.15

Both blood and egg-yolk agar have advantages in this context. A suitably chosen blood-agar base such as brain-heart infusion, Columbia or Iso-Sensitest agar permits the rapid presumptive detection of C. difficile colonies by their yellow-green fluorescence under long-wave (365nm) UV light (Figure 1).16 Fluorescence cannot be used for presumptive identification on egg-yolk CCFA, since all clostridia growing on this medium will reduce the neutral red indicator and fluoresce brilliant yellow when viewed under UV light. However, the use of an egg-yolk agar makes it easier to differentiate C. difficile from commonly-occurring organisms such as C. bifermentans, C. perfringens, C. sordelli and C. sporogenes, which produce other lecinthinase or lipase, and which are not infrequently recovered from faeces on CCFA medium. There are several species of egg-yolk-negative clostridia which exhibit colonial morphologies similar to that of C. difficile on CCFA, however, these organisms can rapidly be differentiated from C. difficile by the use of gas-liquid chromatography.18

The inclusion in the selective medium of pure, synthesized sodium taurocholate (0.1 per cent) or sodium chloride (0.05 per cent) may stimulate spore germination and enhance the recovery of the organism when present in low numbers. A similar phenomenon may occur when egg-yolk agar, which contains lysocyme, is used. This material may be plated directly onto the selective medium or subjected to alcohol shock prior to plating on to both selective and non-selective media. Alcohol shock increases the isolation rate of C. difficile by up to 50 per cent.19 A 50 per cent suspension of faecal material in alcohol is prepared. It is unnecessary to use absolute alcohol; industrial methylated spirit (74 OP) is satisfactory. After incubation at room temperature for 1 hour, a few drops of the supernatant are plated on to both selective and non-selective media. All plates are incubated at 37°C for a minimum of 48 hours. If an anaerobic jar is employed it should not be opened after overnight incubation. Enrichment cultures are of value in detecting low numbers of bacteria. An aliquot of the faecal suspension in 0.85 per cent saline is inoculated into a cooked-meat broth, and incubated at 37°C for 3-5 days. The broth is then subcultured onto blood agar and CCFA medium as described above.

Identification

C. difficile is an obligately anaerobic, Gram-positive rod, which forms oval, sub-terminal (or occasionally central) spores. When cultured on CCFA medium the bacilli become elongated and there is marked inhibition of spore production. Colonies on blood agar after 48 hours are 2-3mm in diameter, haemolytic, greyish-white, or greenish-white. They have a low convex surface and are roughly circular in shape, with a sharp regular margin (Figure 2). Most strains also exhibit a rough colonial form, the colonies being highly irregular with rhizoidal edges occurring in the medium of p-hydroxyphenylacetic acid and nor­uleine stimulates C. difficile to produce p-creol and capric acid, respectively.21 The detection of clostridial acid, capric acid and p-creol, by gas-liquid chromatography of agar plugs, enables C. difficile to be identified directly from the primary isolation medium. C. difficile may also be identified by the detection of pre-formed enzymes.22 Commercially available enzyme detection kits have an advantage over conventional identification methods since they have a much reduced incubation time (Figure 3).

Toxin detection

C. difficile is now known to produce two protein exotoxins. The cytotoxin produced by most strains was designated toxin B and the enterotoxin, toxin A. The biological methods available for the detection of these toxins include ligated gut loops, vascular permeability and the suckling-mouse assay. Both toxins exhibit cytotoxic activity, but that of toxin B is much greater than that of toxin A. Cytotoxin is present in the stools of patients with PMC, and there is a highly significant association between the presence of cytotoxin and C. difficile in the stools of patients with colitis. The most widely used method of detecting cytotoxin is the tissue-culture assay, in which the cytotoxin, present in either a faecal extract or culture supernatant, is neutralized by C. sordelli antitoxin. A portion of the stock is suspended in an appropriate equal volume of phosphate-buffered saline containing penicillin (1000mg/l), streptomycin (1000mg/l) and metronidazole (100mg/l). After centrifugation inclusion in the medium of p-hydroxyphenylacetic acid end nor­uleine stimulates C. difficile to produce p-creol and capric acid, respectively. The detection of clostridial acid, capric acid and p-creol, by gas-liquid chromatography of agar plugs, enables C. difficile to be identified directly from the primary isolation medium. C. difficile may also be identified by the detection of pre-formed enzymes. Commercially available enzyme detection kits have an advantage over conventional identification methods since they have a much reduced incubation time (Figure 4).

Figure 1: Heavy growth of Clostridium difficile on Columbia blood agar showing yellow-green fluorescence under long-wave UV light.

Figure 2: Smooth surface colonies of Clostridium difficile after 48-hour incubation on blood agar.

Figure 3: Rough surface colonies of Clostridium difficile after 48-hour incubation on blood agar.

Figure 4: Typical colonies of Clostridium difficile on egg yolk CCFA medium after 48-hour incubation.
at 2000g for 20 minutes the supernatant is removed and four drops (100µl) added to each of two tubes containing a tissue-culture monolayer (MRC-5 cells are preferred, but a number of cell lines are equally sensitive to the cytotoxin). C. perfringens, are available commercially and have the advantage over other methods that a result is available within minutes of removing the specimen. However, it has been demonstrated recently that one commercial latex-agglutination assay for toxin A detects not toxin A, but an antigen produced by both toxigenic and non-toxigenic C. difficile strains.50 Several attempts have been made to develop techniques for the rapid detection of the organism or its metabolites in faeces. These include the use of Gramstaining, immune-fluorescence staining, counter-immunoelectrophoresis and gas-liquid chromatography. None of these approaches has yet been successful.

Typing

Following the demonstration of clustering of cases of C. difficile infection several approaches to typing that schemes have been adopted. The simplest of these is the use of antibiotic resistance patterns. While this may be of some value if a strain is isolated which has a particularly uncommon resistance pattern, the majority of strains of C. difficile differ little in their sensitivity to antibiotics. Biochemical tests have also been used.22,23 However, such schemes lack the requisite sensitivity. Several schemes have been described which show considerable promise. A bacteriophage- and bacteriocin-type typing scheme was evaluated using a random model26 and more recently was applied to the study of cross-infection in human neonates.50 A number of methods based upon the separation of cellular proteins by polyacrylamide gel electrophoresis have been developed. Of these, the methods described by Poxton et al and Tabachniki et al show the most potential. More recently a serotyping scheme has been described.4 This approach has the merit of being the simplest and most rapid technique yet applied.

The application of typing schemes to isolates of C. difficile recovered from clusters of cases will permit the assessment of the significance of close relationships. It is apparent that in some instances clustering is not the result of cross-infection.4 However, in cases where cross-infection does occur a reliable typing scheme is of great value in tracing the likely source of the outbreak and the resulting spread of the organism.

Significance of isolation of C. difficile

The detection of C. difficile and its toxigenicity in the stools of infants may be of some significance. Asymptomatic carriage of the organism and excretion of its toxins is common in the first year of life, and excretion rates of up to 60 per cent have been recorded in neonatal populations. However, carriage of the organism is intermittent, and it is probable that all infants are colonized by C. difficile at some time during their first year. The incidence of carriage of C. difficile by adults is much lower (2-5 per cent) and, though the detection of cytotoxin in the stools of a diarrhoeal patient may be considered significant, asymptomatic carriage is more common among elderly adults and, thus, isolation of the organism and/or detection of its toxins requires careful interpretation than is the case in younger age groups.

C. difficile can be recovered from the stools of patients suffering acute symptomatic relapses of chronic inflammatory bowel disease, but the significance of this association has been challenged.22,23 Similarly, the involvement of C. difficile in the pathogenesis of Hirschsprung's enterocolitis26 and sudden infant-death syndrome has been questioned.

Conclusion

The isolation of C. difficile is now within the scope of most diagnostic laboratories. Detection of cytotoxin presently requires tissue culture facilities, but the introduction of rapid immunological methods making use of specific antibodies will eliminate this necessity. Further development of typing schemes will enable the epidemiology of infection with C. difficile to be better elucidated. This in turn will permit the accurate assessment of the relative importance of C. difficile as a pathogen of the gastrointestinal tract.

References