A simple disc technique for the presumptive identification of *Legionella pneumophila*

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SUMMARY A method is described for the presumptive identification of *Legionella pneumophila* by the formation of satellite colonies around filter paper discs impregnated with ferric pyrophosphate and L-cysteine hydrochloride on a deficient basal medium. This technique simplifies the differentiation of picked colonies of *L. pneumophila* from other organisms in mixed cultures from environmental and contaminated clinical samples.

Several culture media have been developed for the cultivation of *L. pneumophila* and with the addition of antibiotics to produce semiselective media, the isolation of *L. pneumophila* from mixed cultures has been facilitated.¹⁻⁴ Until now *L. pneumophila* has been initially identified by colonial appearance, morphology when stained and failure to grow on non-specialised media such as blood agar.⁵ A method has now been devised which simplifies the identification of isolates when dealing with large numbers of suspect colonies from mixed cultures.

Material and methods

The basal medium for this identification technique is a modified charcoal yeast extract medium (BCYE α -base) without ferric pyrophosphate and L-cysteine hydrochloride.⁴ Filter paper discs impregnated with ferric pyrophosphate and L-cysteine hydrochloride provide the source of the *Legionella* growth supplements.

BCYE α -BASE

This consisted of 10 g yeast extract (Difco), 1.5 g Norit A activated charcoal (Sigma) 10 g ACES (Sigma) 1 g α -ketoglutarate-monopotassium salt (Sigma), 12 g Agar No 3 (Oxoid) distilled water to one litre, adjusted to pH 6.9 with 8 M potassium hydroxide and autoclaved at 121°C for 15 min. Culture plates were dried before use.

FILTER PAPER DISCS

Discs (5 mm diam) were punched from Postlip filter paper sheets (Evans, Adlard & Co Ltd,

Winchcombe, UK) and decontaminated in a hot air oven at 65°C for 24 h.

GROWTH SUPPLEMENTS

A filter sterilised stock solution of ferric pyrophosphate 12.5 mg/ml, L-cysteine hydrochloride 20 mg/ml was prepared. The sterile discs were saturated with 10 μ l of stock solution each, dried at 37°C and stored with silica gel at 4°C for up to three months.

BCYE α -base was inoculated by streaking individual suspect colonies across the culture plate using sterile cotton applicator swabs, each streak being at least 70 mm in length. Standard 90 mm Petri dishes will accommodate five streaks and 100 mm square dishes will accommodate 10 streaks. The impregnated discs were placed in the centre of each streak and the plates incubated for up to three days at 35°C-37°C in a humidified incubator. A known *L. pneumophila* culture was included on each plate as a control.

Results

See Fig. 1. Typical satellite growth was produced around the discs by stock cultures of *L. pneumophila* serogroups 1 to 6, eight wild isolates of serogroup 1, one wild isolate of serogroup 4 and one wild isolate ungrouped.

All non-*Legionella* organisms examined grew across the streaks and did not produce satellite growth. These organisms included *Haemophilus influenzae* from a clinical sample as well as *Pseudomonas* spp, *Flavobacterium* spp and other unidentified water organisms from 56 environmental samples.

Fig. 1 Upper disc with satellite colonies of *L. pneumophila*; lower disc with non-*Legionella* organism (in this case *Haemophilus influenzae*) failing to produce satellite colonies.

Fig. 2 Flow pattern for the use of the disc technique.

Discussion

When this laboratory investigated a recent case of hospital-acquired Legionnaires' disease, large numbers of environmental samples were processed using semiselective media. These samples on primary isolation yielded mixed cultures of *L. pneumophila* and environmental organisms. Many of these environmental organisms resembled *L. pneumophila* colonially and grew slowly on blood agar. At first, time-consuming immunofluorescent serogrouping

was employed to identify isolates. However, using the flow pattern shown in Fig. 2, the disc technique described was found to be much simpler when dealing with the large numbers of potential *Legionella* isolates encountered.

Most of the *L. pneumophila* cultures tested produced satellite growth in 18–24 h with a few requiring 24–72 h, this variation was due to inoculum size. All the non-*Legionella* organisms which grew slowly on blood agar (more than three days), grew well on BCYEα-base in less than three days and growth was not improved by the impregnated discs so they were easily differentiated from *L. pneumophila*.

The BCYEα-base and the impregnated discs were simple to make from the ingredients already used in current *Legionella* culture media. It was found that Oxoid *Legionella* growth supplement (SR94) could be used as a source of ferric pyrophosphate and L-cysteine hydrochloride.

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