

Isolation and Identification of *Legionella* species – An Overview

Although the first outbreak of what has become known as Legionnaires' Disease occurred in mid 1976, it was not until 1977 that the first isolations of the causative agent were made. Early attempts to isolate the causative agent using conventional bacteriological methods were unsuccessful. The initial isolation of the **Legionella** bacterium was made using methods more applicable to the culture of Rickettsiae. This first isolation was made by infecting guinea-pigs and fertile hen eggs. In 1978, the causative agent was first cultivated on conventional bacteriological culture media, although growth was poor.

Isolation

In 1979, Charcoal Yeast Extract agar (1) was first described for the routine culture of *Legionella* spp.. The performance of this medium was further enhanced by the addition of ACES Buffer (N-2-acetamido-2-aminoethane-sulphonic acid) in 1980 (2) and alpha ketoglutarate in 1981 (3).

Since 1981 no significant modifications have been published concerning this basic growth medium, and although other media

have been described, this medium has become the medium of choice (4).

Despite the development of

Table 1. Basic composition of Charcoal Yeast Extract Agar.

Charcoal Yeast Extract Agar

Basic Formulation

Charcoal (Norit A)	2gm/litre
Yeast Extract	10gm/litre
ACES Buffer	10gm/litre
Agar	13gm/litre

Additives

Ferric Pyrophosphate	0.25gm/litre
L-Cysteine	0.40gm/litre
Alpha-Ketoglutarate	0.10gm/litre

Notes

Charcoal: This is commonly incorporated into culture media as a detoxifying agent either to neutralise the effects of toxic short chain fatty acids which may be present in agar or to detoxify waste products of bacterial growth which may be potentially inhibitory.

ACES Buffer: Added to ensure that the pH of the medium is maintained within a narrow range around 6.9.

Ferric Pyrophosphate: Acts as a source of ferric ions which are essential growth factors. No details of its exact role have yet been determined.

Cysteine-HCl: This amino acid is an essential growth factor. Its exact role has not been determined but two possible roles may be:

- As a reducing agent, neutralising the toxic effects of superoxides which may be present in the medium.
- *Legionella* spp. may be unable to synthesise this amino acid which in itself is essential for the synthesis of purine and pyrimidine bases required for DNA synthesis.

Alpha-Ketoglutarate: This has been established as a growth stimulating additive, particularly for *L.pneumophila* (5).

suitable bacteriological culture media, guinea-pig inoculations were often required to isolate **Legionellae** from both clinical and environmental samples. These specimens were often heavily contaminated with other bacteria many of which were capable of out growing the slower growing **Legionellae** despite its low nutritional value.

To counter this contamination with naturally occurring commensal or environmental bacteria, a range of selective isolation media have subsequently been developed:

BMPA (5)

Cephmandole 4ug/ml
 Polymyxin B 80IU/ml
 Anisomycin 80ug/ml

Notes:

CYE Agar supplemented with this antibiotic combination provides a good selective isolation medium for all **Legionella spp.** although it was originally developed for the isolation of **L. pneumophila**.

Today in excess of 30 different species have been described, the growth of a number of which may be inhibited to some degree by this antibiotic combination. We have found that the following strains may be inhibited on this medium.

- L. birminghamensis**
- L. bozemanii** (serogroup 1 + 2)
- L. cincinnatiensis**
- L. erythra**
- L. feeleeii** (serogroup 1 + 2)
- L. micdadei**
- L. quinlivianii**
- L. longbeachae** (serogroup 1)

Investigations undertaken by the author have shown that these organisms may be susceptible to concentrations of cephamandole as low as 1ug/ml, the recommended concentration being 4ug/ml. However, in practice **L. quinlivianii** and **L. longbeachae** (serogroup 1) are frequently isolated on this medium. Polymyxin B exhibits no obvious inhibitory effect on any of the species we have examined.

MWY (6)

Glycine 0.3%
 Vancomycin 1ug/ml
 Polymyxin B 50IU/ml
 Anisomycin 80ug/ml

Notes:

Media supplemented with this antibiotic combination were also developed primarily for the isolation of **L. pneumophila**. We have also examined the growth and recovery rates of a wide range of species on media supplemented with these antibiotics and have found only a few species to be significantly inhibited, those being **L. erythra**, **L. cherrii** and **L. hackeliae** serogroup 2.

Although this antibiotic supplement is considerably less inhibitory for the species mentioned above, it does not contain any antibiotic as effective as cephamandole against members of the family **Enterobacteriaceae**. In practice, plates containing this supplement often have less overgrowth and contamination, than do plates containing BMPA. However, it is recommended that when using this medium, suitable

specimen decontamination procedures are employed.

Also included in this supplement, are Bromocresol Purple and Bromothymol Blue both at a final concentration of 10ug/ml. The inclusion of these two indicators is reported to assist in the differentiation of different species based on the development of colonies with differing colours.

GVPC (7)

Glycine 0.3%
 Vancomycin 1ug/ml
 Polymyxin B 80IU/ml
 Cycloheximide 80ug/ml

Notes:

This selective supplement is reported to be the most efficient medium for the isolation of **L. pneumophila**. Due to the low level of selectivity it imparts, it is recommended that it be used in conjunction with either heat or acid decontamination. Cycloheximide is employed as it has greater antifungal activity than Anisomycin which is more effective against yeasts. This makes this medium more effective for use with environmental samples.

Identification

The identification and differentiation of **Legionella spp.** cannot be achieved using the same conventional biochemical test methods employed with other organisms. Members of the genus do not ferment sugars, although some species hydrolyse starch, hippurate and gelatin, they are catalase

Table 2. Characteristics of Legionella species.

Characteristic	<i>L.pneumophila</i>	<i>L.bozemanii</i>	<i>L.dumofii</i>	<i>L.micdadei</i>	<i>L.longbeachae</i>
Growth on:					
BCYE	+	+	+	+	+
Blood Agar	-	-	-	-	-
Colony colour on dye containing media	White/green	Blue - grey	Green	Green	White/ Green
Catalase	+	+	+	+	+
Oxidase	+	-	-	+	+
Hippurate	+	+	+	-	+
Gelatin	+	-	-	-	-

positive and oxidase variable (Table 2.)

To efficiently and accurately identify *Legionella* spp, serological typing is the simplest method and is suitable for the differentiation of individual serotypes of species. *L. pneumophila* is by far the most common environmental isolate from water samples and clinically, the most common cause of Legionellosis (7). Currently there are 16 recognised serogroups of *L. pneumophila* and in excess of 30 other species of which only a few have been reported as the causative agent of human disease (8). *L. pneumophila* Serogroup 1 is the most common environmental isolate and the most common causative agent of human disease.

Environmentally, *Legionella* spp. are associated almost exclusively with wet surfaces and potable waters. The routine examination of water supplies for *Legionella* spp. is now a routine procedure within all water testing

laboratories (9,10). Due to the common occurrence of *L. pneumophila* serogroup 1, these testing methods focus specifically on the isolation and enumeration of *L. pneumophila* and its confirmation and differentiation being based on serology using latex agglutination.

Microgen Bioproducts Ltd has broadened the specificity it's Microgen® Legionella Latex Agglutination Test Kit through the inclusion of *L. pneumophila* Serogroup 15 (Lansing 3) in its *Legionella pneumophila* typing panel. As Serogroup 16 cross-reacts with Serogroup 6, the 2-15 reagent is likely to detect this new serogroup. However this yet to be verified This kit now comprises three latex reagents, *L. pneumophila* serogroup 1 and *L. pneumophila* serogroups 2 – 15 (Lansing 3) and a reagent for 9 clinically non-*L. pneumophila* species and a positive control for quality control purposes. The excellent specificity of the Microgen® antibodies avoids the need to use a control latex

and makes the test quicker and easier to use. For added convenience, individual reagents are available for purchase.

References:

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- 8.Murray P.R. (Ed) 1999 Manual of Clinical Microbiology 7th Edition. American Society for Microbiology, Washington, DC
- 9.Waters – Examination for legionellae including *Legionella pneumophila* AS/NZ 3896:1998
- 10.Waters quality – Detection and enumeration of Legionella ISO 11731:1988

Reagent	Product Code	Detects	Quantity
Reagent 1	M45ACE	L.pneumophila serogroup 1	2.5mL
Reagent 2	M45BCE	L. pneumophila serogroup 2-15	2.5mL
Positive Control	M45CCE	Positive control, reactive with test reagents 1, 2-15 and species.	1.0mL
Species Reagent	M45DCE	10 commonly isolated clinically significant species	2.5mL

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