A single step method for rapid isolation and identification of *Mycoplasma pneumoniae*

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طريقة من خطوة واحدة للاستفراد السريع للمفطورات الرئوية وكشفها

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الخلاصة: تم تطوير تركيبة مُستَنبت بسيط وحيد الطور وثنائي الطور بضمن الاستفراد الفعَّال للمفطورات الرئوية وكشفها. وتشتمل التركيبة على مُستَنبت مائل يغطى قاعُه بميلي لتر واحد من المرق، مما يجعله وسطاً ثنائي الطور (سائل – صلب) في قاع أنبوب الاختبار محاطاً بوسط صلب وحيد الطور. تحضن العينة مباشرةً في الطور السائل وتمزج وتمال مرة أو مرتين لتغطية الجزء المائل العلوي قبل الحضن. ولهذه الطريقة فوائد عديدة على الطُرُق الأخرى تتمثل بالحصول على نتائج سريعة، والتخلص من الوسط الناقل واستخدام وسطين منفصلين لاستكمال كل من الكشف والتعرُّف على المفطورات الرئوية، ويوفر مرونة في اختبار الـمُستَنبتات في طورَيْها السائل والصلب.

ABSTRACT A simple monophasic–diphasic culture set-up was developed to provide efficient isolation and identification of *Mycoplasma pneumoniae*. The set-up consisted of a slant medium, the bottom covered with 1 mL of broth, establishing a diphasic solid-liquid environment at the bottom of the test tube surmounted by a monophasic solid one. The specimen was directly inoculated into the liquid phase, mixed, and tilted once or twice to cover the upper slanted portion prior to incubation. The method had several advantages over other techniques including rapid results, elimination of transport medium, and use of two separate environments to accomplish both the detection and identification of *M. pneumoniae*.

Une méthode en une étape pour l'isolement et l'identification rapide de *Mycoplasma pneumoniae*
RESUME Un dispositif de milieu de culture monophasique-diphasique simple a été mis au point pour permettre un isolement et une identification efficace de *Mycoplasma pneumoniae*. Ce dispositif se composait d'un milieu incliné, le fond étant couvert d'1 ml de bouillon, créant un environnement solide-liquide au fond du tube à essai surmonté par un environnement solide monophasique. L'échantillon était inoculé directement dans la phase liquide, mélangé et penché une ou deux fois pour recouvrir la portion inclinée supérieure avant incubation. La méthode présentait plusieurs avantages par rapport aux autres techniques, notamment la rapidité des résultats, l'élimination du milieu de transport, et l'utilisation de deux environnements distincts pour la détection et l'identification de *M. pneumoniae*.

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Introduction

The mycoplasmas are a class of wall-less bacteria, known as mollicutes, comprising more than 150 species [1]. At least four species are of major importance to human heath: *Mycoplasma pneumoniae* is a respiratory pathogen causing pneumonia and has been associated with joint and other pathologies [2], M. hominis occasionally causes postpartum fever, Ureaplasma urealiticum is a cause of non-gonococcal urethritis in adults and lung disease in premature infants, and *M. genitalium* is linked to urethral infections [3].

Because of the small size of the colonies, the plasticity and delicacy of the cells and the very weak staining with aniline dyes, mycoplasmas do not lend themselves easily to conventional bacteriological methods of culture and identification. However, they can be grown on complex media containing lipoprotein and sterol over long periods of incubation. Culture techniques for the diagnosis of *M. pneumoniae* require incubation of plates and diphasic medium at 35–36 °C in a CO_2 incubator with daily inspection for up to 3 weeks [4]. The alternatives to this lengthy procedure are the direct detection of antigen, enzyme immunoassay (EIA) for antibodies or specific DNA, polymerase chain reaction (PCR), or RNA nucleotide sequencing by probe hybridization [4–10]. Most of these methods are very expensive or unavailable in our country. The aim of this study therefore was to develop a simple and inexpensive method for the rapid diagnosis of *M. pneumoniae* by improving the existing diphasic medium culture technique.

Methods

A total of 243 throat swabs and 242 sputum samples were taken from 456 community-acquired pneumonia cases and 29 hospital-acquired cases during the period September 1998 to March 2000. Cases were diagnosed clinically and by chest X-ray prior to microbiological diagnosis and identification of *M. pneumoniae*.

Two types of liquid media (A and B) and two solid media (D and E) were prepared according to Marmion and Harris [4] and Baron et al. [11]. Media C and F were modifications of B and E respectively. The composition of all media is presented in Tables 1 and 2. As can be seen, the media differed with regard to the presence or absence of methylene blue, glucose, DNA or K_2HPO_4 and the replacement of phenol red by cresol red. They differed too in their application as part of a monophasic, diphasic or monophasic-diphasic system. Monophasic and diphasic cultures were prepared as described in the literature [4,11], using media A and the combination BE respectively.

Table 1 **Liquid media used for cultivation and preliminary identification of mycoplasmas from clinical specimens**

Media composition		
A	B	C
PPLO broth	PPLO broth	PPLO broth
Horse serum	Horse serum	Horse serum
Yeast extract	Yeast extract	Yeast extract
Thallous acetate	Thallous acetate	Thallous acetate
Penicillin	Penicillin	Penicillin
Glucose	Glucose	Glucose
Phenol red	Phenol red	Cresol red
Methylene blue	Methylene blue	Methylene blue
No DNA	DNA	DNA
No K_2HPO_4	K_2HPO_4	K_2HPO_4

PPLO = pleuropneumonia-like organisms.

Table 2 **Solid media used for the isolation and preliminary identification of *Mycoplasma pneumoniae***

Media composition		
D	E	F
PPLO agar	PPLO agar	PPLO agar
Horse serum	Horse serum	Horse serum
Yeast extract	Yeast extract	Yeast extract
Thallous acetate	Thallous acetate	Thallous acetate
Penicillin	Penicillin	Penicillin
Glucose	No glucose	Glucose
No methylene blue	No methylene blue	Methylene blue or sheep erythrocytes
No DNA	DNA	DNA
No K_2HPO_4	K_2HPO_4	K_2HPO_4

PPLO = pleuropneumonia-like organisms.

The monophasic-diphasic culture setup (MDCS) was a simple system in which a slant of medium F was prepared in 14 test tubes (160 mm size) followed by the introduction of 1 mL of medium C to cover the lower portion of the slant only. The lower portion of the test tube thus represents a diphasic environment (a liquid phase in contact with solid one), while the upper portion is single phase (solid only).

To cultivate and isolate *M. pneumoniae*, throat swabs and sputum samples were placed in transport medium followed by inoculation as described by Marmion and Harris [*4*]. Specimens were inoculated directly into the liquid phase of the MDCS, mixed well and then tilted once or twice to cover the upper portion of the slant prior to incubation. All inoculated media were incubated aerobically at 37 °C and observed daily for a colour change from red to yellow in the liquid phase, and from yellow to greenish-blue on the slanted solid phase. Isolated colonies appeared after the colour change. Identification and confirmation of isolated mycoplasmas was carried out by negative staining and biochemical tests [*1*]. Negative staining of pure cultures by nigrosin produced clear pictures of these pleomorphic microorganisms.

Sheep erythrocytes (7%) were added to the solid phase of the MDCS in several trials to detect blood haemolysis by *M. pneumoniae*.

Results

Table 3 shows an increase in the percentage recovery of *M. pneumoniae* in samples collected at different times, from 8.3% in medium A to 18.1% in the diphasic medium BE and 33.4% in the MDSC of media C and F. This difference was highly significant. Conversely, a sharp decrease in the percentage recovery of other bacteria is apparent (varying from 15.1% to 7.6% and 1.7% with these three methods). The recovery of non-*M. pneumoniae* mycoplasmas was highest in the MDCS of medium C and F and lowest in medium A.

The recovery profile (Table 4) of 50 other specimens was similar to that shown in Table 3. The major difference was a higher recovery of *M pneumoniae* (40%) by MDCS compared to 8% in BE diphasic medium and 6% in medium A, a statistically significant difference.

The replacement of phenol red by cresol red in medium C produced a faster colour change in this liquid medium, i.e. 24 hours instead of > 96 hours in medium B (Table 5), which was statistically significant.

The greater efficiency of MDCS compared to the monophasic and diphasic media for the detection of *M. pneumoniae* is highlighted again in Table 6. Colour

Table 3 **Recovery of *Mycoplasma pneumoniae* and other mycoplasmas from three collections of clinical specimens using three methods**

Medium	Time of sampling	No. specimens examined	No. (%) from which *M. pneumoniae* recovered	No. (%) from which other mycoplasmas recovered	No. (%) from which other bacteria recovered
A (monophasic)	1.9.98–30.1.99	205	17 (8.3)	8 (3.9)	31 (15.1)
BE (diphasic)	1.5.99–31.8.99	105	19 (18.1)	23 (21.9)	8 (7.6)
C-F (mono–diphasic)	1.9.99–31.3.2000	175	58 (33.1)	41(23.4)	3 (1.7)

$\chi^2 = 95.08$, $P < 0.0001$.

Table 4 **Recovery of *Mycoplasma pneumoniae* and other mycoplasmas from 50 clinical specimens using three methods**

Medium	No. specimens examined	No. (%) from which *M. pneumoniae* recovered	No. (%) from which other mycoplasmas recovered	No. (%) from which other bacteria recovered
A (monophasic)	50	3 (6)	6 (12)	4 (8)
BE (diphasic)	50	4 (8)	20 (40)	3 (6)
C-F (mono–diphasic)	50	20 (40)	17 (34)	1(2)

$\chi^2 = 42.64$, $P < 0.001$.

Table 5 Effect of replacement of phenol red by cresol red on colour change in liquid medium inoculated with pure culture of *M. pneumoniae* or primary isolates

Specimens for primary isolation	Inoculum time (hours) for colour change in: Cresol-containing liquid medium	Phenol red-containing liquid medium
1	24	> 96
2	24	> 120
3	48	96
5	24	120
6	24	120
7	48	120
8	24	96
9	24	96
10	24	120
Pure culture	24	120

$t = 15.46$, $P < 0.0001$.

Table 6 **Comparative recovery of *Mycoplasma pneumoniae* from clinical specimens by three culture techniques within 72 hours of incubation**

Time (hours) for colour change	Liquid phase A	Liquid phase B	Liquid phase E	Liquid phase C (slant)	Solid phase F	Species
1	Nil	Nil	Nil	24	72	*M. pneumoniae*
2	Nil	Nil	Nil	24	72	*M. pneumoniae*
3	Nil	96	Nil	24	72	*M. pneumoniae*
4	Nil	Nil	Nil	24	72	*M. pneumoniae*
5	Nil	Nil	Nil	24	Nil	*M. salivarium*
6	Nil	Nil	Nil	24	72	*M. pneumoniae*
7	Nil	Nil	Nil	24	72	*M. pneumoniae*
8	Nil	Nil	Nil	24	72	*M. pneumoniae*
9	Nil	Nil	Nil	24	Nil	*M. orale*
10	Nil	Nil	Nil	24	72	*M. pneumoniae*
11	Nil	Nil	Nil	24	Nil	*M. salivarium*
12	Nil	Nil	Nil	24	72	*M. pneumoniae*
13	Nil	Nil	Nil	24	72	*M. pneumoniae*
14	Nil	Nil	Nil	24	72	*M. pneumoniae*
15	Nil	Nil	Nil	24	Nil	*M. buccale*
16	Nil	Nil	Nil	24	72	*M. pneumoniae*
17	Nil	Nil	Nil	24	Nil	*M. salivarium*
18	Nil	Nil	Nil	24	72	*M. pneumoniae*
19	Nil	Nil	Nil	24	72	*M. pneumoniae*
20	Nil	Nil	Nil	24	Nil	*M. orale*
21	Nil	Nil	Nil	24	Nil	*M. buccale*
22	Nil	Nil	Nil	24	72	*M. pneumoniae*
23	Nil	Nil	Nil	24	Nil	*M. salivarium*
24–32	Nil	Nil	Nil	24	Nil	Other bacteria

changes appeared in the liquid phase after 24 hours, and isolated colonies became apparent on the upper portion of the slant after 72 hours. No colour change was observed in medium A or the diphasic medium BE within this period.

Application of the MDCS clearly distinguished between *M. pneumoniae* and other mycoplasmas as the former appeared on the upper portion of the slant within 72 hours but the latter did not. Of 32 samples tested, 23 (71.9%) of them contained mycoplasmas represented by *M. pneumoniae* (46.8%), *M. salivarium* (12.5%), *M. orale* (6.2%) and *M. buccale* (6.2%). All primary isolates of *M. pneumoniae* were confirmed by biochemical tests.

Discussion

M. pneumoniae is the cause of primary atypical pneumonia, which accounts for at least 20% of all pneumonia of bacterial etiology [*12*]. Various laboratory tests have been designed to diagnose mycoplasma infections [*4–10*]. The diagnostic value and applicability of any method depends on its simplicity, rapidity, reproducibility and cost. If we assess MDCS for the diagnosis of *M. pneumoniae* by these criteria it appears to be the simplest, most rapid culture procedure with the lowest cost.

This conclusion was arrived at by comparing the performance of MDCS with the current monophasic and diphasic cultures [*4*] (Tables 3–6). The higher recovery of *M. pneumoniae* by MDCS (Table 3) is a clear indication of its efficiency and selectivity. Table 4 shows that a five-fold better recovery of *M. pneumoniae* was achieved by MDCS than by diphasic culture. Thus a higher rate of recovery of mycoplasmas in general and *M. pneumoniae* in particular, combined with a lower recovery of other bacteria, is consistently shown by MDCS irrespective of whether different sets of specimens (Table 3) or the same set (Table 4) are screened.

The high performance of MDCS is due to the changes in the composition of the liquid and solid media, and the manner in which they are set up. These changes include the replacement of phenol red in liquid medium B by cresol red to make medium C, and the addition of glucose and methylene blue or sheep erythrocytes to the solid medium E to obtain medium F. Data from Tables 5 and 6 lend strong support to this reasoning, as these media show colour changes in the liquid phase of the MDCS within 24 hours instead of > 96 hours in the diphasic medium, a difference which was statistically highly significant.

Coupled to the rapid detection of *M. pneumoniae* in the liquid phase (Table 5) is its isolation on the upper portion of the slant two days later (Table 6). This means that we have a two-stages check, an early detection followed by primary isolation and confirmation. This in part explains the high sensitivity and selectivity of MDCS relative to other culture techniques. The difference in the detection time of *M. pneumoniae* between the liquid and solid phase of the MDCS can be ascribed to the shorter time needed for the collective metabolic activity of all viable cells to be expressed as colour change in the liquid than the time necessary for isolated cells on the solid phase to form visible colonies.

The advantage of the MDCS lies in its versatility, as it permits a solid phase to be in direct contact with a liquid one, both of which are below a solid phase. It also allows phases with different compositions to be prepared, as well as opposite slants of different ingredients. For example, it is possible to prepare one slant of medium F to isolate *M. pneumoniae* with an opposite slant of medium E to detect other mycoplasmas.

MDCS is inexpensive because of the small quantities of liquid and solid media consumed, the omission of transport media, and the short incubation time within one test tube.

The use of MDCS showed that *M. pneumoniae* was implicated in a high proportion of bacterial pneumonia cases, up to 46% (Tables 3–6).

Based on these findings we suggest MDCS is suitable for a wide range of medical and non-medical applications, particularly since current culture procedures and serological tests are relatively insensitive, and culture is also time-consuming and molecular techniques more expensive [*13,14*].

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