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Detection of pulmonary *Mycoplasma pneumoniae* infections in HIV-infected subjects using culture and serology

Esaki Muthu Shankar [a], Nagalingeswaran Kumarasamy [b], Pachamuthu Balakrishnan [b], Shanmugam Saravanan [b], Suniti Solomon [b], A. Vengatesan [c], K.G. Murugavel [b], Usha Anand Rao [a,*]

[a] *Department of Microbiology, Dr ALM PG Institute of Basic Medical Sciences, University of Madras, Chennai 600 113, India*
[b] *YRG Center for AIDS Research and Education, Voluntary Health Services, Taramani, Chennai 600 113, India*
[c] *Clinical Epidemiology Unit, Government Stanley Medical College and Hospital, Chennai 600 001, India*

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Summary
Objective: The true prevalence of *Mycoplasma pneumoniae* infections involving the respiratory tracts of HIV-infected individuals is still unclear. This study examined the prevalence of *M. pneumoniae* in 100 HIV-infected individuals at an AIDS care center in Chennai, India, using conventional laboratory techniques and interpretation criteria.
Methods: Diagnosis was based on culture, cold agglutination test, and commercial enzyme-linked immunosorbent assay (ELISA) for the qualitative determination of IgM antibodies against *M. pneumoniae*. The efficacies of the different diagnostic procedures used in the study were analyzed.
Results: The prevalence of *M. pneumoniae* was 31% by culture and 21% by IgM ELISA. Cough ($p = 0.03$, OR 3.8, 95% CI 1—17.8), myalgia ($p = 0.04$, OR 2.5, 95% CI 1—6.6), rales ($p = 0.04$, OR 2.4, 95% CI 1—6.6), and cervical adenopathy ($p = 0.03$, OR 2.7, 95% CI 1—7.1) were the symptoms that significantly corroborated culture positivity. Patients positive for *M. pneumoniae* by culture or IgM antibody had significantly greater CD4+ T-cell depletion and anemia than those without any evidence of infection.
Conclusions: This study provides the means to diagnose *M. pneumoniae* infection and information on the prevalence of the pathogen in HIV-infected individuals in resource constrained settings. Although modern molecular techniques may provide more insight into the prevalence of *M. pneumoniae* in HIV-infected individuals, conventional methods can still be used in diagnosis.

* Corresponding author. Tel.: +91 22 24925317; fax: +91 44 24927609.
E-mail address: drushaanand@yahoo.com (U.A. Rao).

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Introduction

Mycoplasma pneumoniae is recognized as an important pathogen in the human respiratory tract, where it can persist for months after an initial infection in the absence of a measurable immune response. It has recently been associated with exacerbation of chronic conditions including bronchial asthma and atypical cold agglutinin pneumonia.[1–3] *M. pneumoniae* infection occurs endemically and occasionally epidemically in all age groups.[1,4] Bronchopneumonia develops in 3–10% of infected persons, accounting for 20% or more of community-acquired pneumonias (CAP) overall.

Gnarpe et al. detected *M. pneumoniae* in 13.5% of healthy individuals.[5] While Lockman et al. reported a 17% association of *M. pneumoniae* with lower respiratory disease in Botswana, Park et al. reported a 7% prevalence in HIV-infected adults[6,7] suggesting that *M. pneumoniae* is common among HIV-infected adults. *M. pneumoniae* is also a significant cause of severe pneumonia requiring hospitalization in elderly patients, often resulting in death.[8]

Current diagnostic methods primarily rely on serology, as symptoms vary a lot. Culture is time-consuming, although it is the gold standard for diagnosis.[9] IgM ELISA has been reported to be the most sensitive and specific test for diagnosis of *M. pneumoniae* infections[10–13] and specific IgM antibody detection allows the diagnosis of acute or recent infection using a single serum sample.[14–16]

Very few reports are available globally on the prevalence of *M. pneumoniae* in HIV-infected patients,[6,7] and data on *M. pneumoniae* in HIV-infected individuals along the Indian subcontinent is entirely lacking.[17] Therefore, a study was designed to determine the prevalence of *M. pneumoniae* in HIV-infected patients with pulmonary complaints at a tertiary AIDS care center in Chennai, India using conventional diagnostic procedures.

Patients and methods

Study design

This study was conducted between August 2004 and July 2005, after prior approval from the Institutional Review Board (IRB) of the YRG-Center for AIDS Research and Education, University of Madras. Written, informed consent was obtained from all the patients, or their legal representatives, before study enrolment. The study was carried out in compliance with good clinical practice, including the International Conference on Harmonization Guidelines and the Declaration of Helsinki.

Patients

A total of 100 inpatients aged $\geq$18 years were eligible for inclusion. HIV infection was confirmed in the patients using Western blot assay (positive for HIV-1 antibody) (Immunetics, Inc., MA, USA) against *gp160 env, gp120 env, p66 pol*, *p55 gag*, *gp41 env, p24 gag*, and *p17 gag*. All patients with acute X-ray-verified upper and lower pulmonary complaints were enrolled. The body temperature, pulse rate, blood pressure, and pulse oximetry were recorded from all the patients upon admission. Patients with evidence of nosocomial pneumonia, lung cancer, and those terminally ill were excluded from the study. HIV-infected patients for the present study were classified based on the absolute CD4+ lymphocyte counts (Guava Technologies, CA, USA) and other indicator conditions suggestive of AIDS as per the CDC, 1993.[18]

Respiratory and blood specimens

Sputum specimens were obtained according to a standard protocol by respiratory therapists. Briefly, the subjects were hydrated orally prior to induction. Patients inhaled nebulized 3% saline for 20 min from a DeVilbiss Ultra-Neb 99 ultrasonic nebulizer in a closed system via a mouthpiece. Respiratory specimens were collected in sterile containers and a single blood specimen (3 mL) was collected aseptically from all the patients. The sera were separated and stored at $-20\ ^{\circ}C$ until tested for analysis. The criteria for diagnosis of *M. pneumoniae* infection were a positive culture or a positive IgM ELISA.

Bedside cold agglutination test (BCAT)

Bedside cold agglutination tests were carried out as previously described by Griffin.[19] Briefly, 1 mL of the patient's blood collected in a BD vacutainer® (BD, Franklin Lakes, NJ, USA) with anticoagulant, was incubated in an ice pack, and after several minutes, the red blood cells could be seen to agglutinate on the sides of the tube. When warmed in the hand and examined, dissociation of the hemagglutination could be observed in the tube.

Cold agglutination test (CAT)

Cold agglutinin (CA) titration was performed as earlier described by Griffin.[19] A positive agglutination titer of $\geq$32 was taken as suggestive of recent or current *M. pneumoniae* infection. A positive CA titer together with a positive IgM ELISA supports the diagnosis of a current *M. pneumoniae* infection.

Culture

The respiratory specimens were cultured on classical Hayflick's diphasic medium with 0.001% methylene blue (Himedia, Mumbai, India) and agar plates incubated at 37 °C in a Gas-pak container (BBL Inc., ML USA) for up to three weeks. Preliminary biological identification was carried out using the hemadsorption test.[20] Briefly the positive plates were flooded with a suspension of 0.5% guinea pig erythrocytes and incubated at 37 °C for 30 min before washing with phosphate-buffered saline (PBS), pH 7.2. The plates were then observed under 10 $\times$ magnification. The isolates were later confirmed by growth inhibition tests using high titer sera against *M. pneumoniae*.[21] Following identification, the organism was stored at $-70\ ^{\circ}C$ for future analysis.

Mycoplasma pneumoniae qualitative IgM-ELISA

Specimens were tested using a commercial qualitative ELISA (IBL-GmbH, Hamburg, Germany) according to the

manufacturer's instructions. Results were expressed as the cut-off index, which is equal to the absorbance of the patient's serum sample at 450 nm divided by twice the absorbance of the negative control at 450 nm. A cut-off index of >12 U/mL was considered as positive and a value <8 U/mL was considered negative. Values between 8 and 12 U/mL were considered borderline.

Other investigations

Immunological (CD4+ count and CD4%) and hematological parameters (total leukocyte count (TC), total lymphocyte count (TLC), total platelet count (TPC), erythrocyte sedimentation rate (ESR), and hemoglobin of the HIV-infected patients were analyzed.

Statistical analysis

The prevalence of *M. pneumoniae* is presented as a percentage with the 95% confidence interval (95% CI). The association between the symptoms and signs, clinical complaints, and culture positivity were analyzed using Pearson's Chi-square test and odds ratio (OR) with 95% CI. The relationship between *M. pneumoniae* by culture positivity and anemia in the HIV-infected patients screened were analyzed using Student's *t*-test and the difference in mean values using Fisher's 95% CI. Sensitivity, specificity, correct classification rate (concurrent value), misclassification rate, positive predictive value, negative predictive value, false positivity and false negativity rates, likelihood ratio positive test, likelihood ratio negative test, Cohen's kappa (κ), and McNemar's test were calculated to compare different diagnostic tests with respect to sputum, the gold standard for diagnosis of Mycoplasma infections in limited resource settings. A p value ≤ 0.05 was considered significant.

Results

A total of 100 HIV-infected patients were screened in the present study. The age range of the HIV-infected patients was 18–60 years and the mean age was 40 years. The age mean ± SD of the male and female HIV-infected patients were 37.29 ± 7.87 years (range 19–60 years) and 35.7 ± 7.63 years (range 25–53 years), respectively. The mean values of TC were 6.05×10^9/L, TLC 0.94×10^9/L, ESR 78.3 mm, hemoglobin 9.71 g/dL, and TPC was 242.6×10^9/L. Definitive diagnosis of *M. pneumoniae* was made in 31 (31%) HIV-infected patients by culture and 21 (21%) by IgM ELISA. Cold agglutinins were detected in 34 (34%) HIV-infected patients. All patients positive for CA titers were concurrently positive for BCAT.

The clinical status of patients with an etiologic diagnosis of respiratory complaints due to *M. pneumoniae* is presented in Table 1.

Among the various methods used for the detection of *M. pneumoniae* infection, culture (sputum and throat swab combined) has been compared with other methods as it has been reported to be the gold standard. The diagnostic profile of *M. pneumoniae* infection in HIV-infected individuals is shown in Table 2. A definitive diagnosis was made in 31 cases as per the criteria for diagnosis while a non-specific diagnosis was made in an additional three patients.

Compared to culture, the sensitivity of CAT was 96.5% and specificity 94.2% and there was no significant difference in the hematological and immunological parameters among the patients who were positive by culture. Nevertheless, a significant depletion ($t = 2.01$, $p = 0.05$) in the levels of absolute CD4+ counts was evident among patients who were positive by IgM ELISA (75 ± 69.1 cells/μL) compared to those who were negative (119 ± 135.2 cells/μL). Subjects who were culture positive for *M. pneumoniae* had significantly lower hemoglobin levels (8.8 ± 1.7 g/dL, OR 2.4, $t = 3.05$, 95% CI 0.79–4.01, $p = 0.01$) than culture negative subjects

Table 1 Clinical status of patients with an etiologic diagnosis of pulmonary complaints due to *Mycoplasma pneumoniae*

Symptoms and signs	*Mycoplasma pneumoniae* culture		x^2	p value[a]	OR with 95% CI
	Positive ($N = 31$) n (%)	Negative ($N = 69$) n (%)			
Anemia	19 (61.3)	46 (66.7)	–	NS	–
Smoking	17 (54.8)	36 (52.2)	–	NS	–
Cough	28 (90.3)	49 (71.0)	4.50	0.03	3.8 (1–17.8)
Malaise	22 (71.0)	41 (59.4)	–	NS	–
Headache	16 (51.6)	30 (43.5)	–	NS	–
Chills	8 (25.8)	13 (18.8)	–	NS	–
Sore throat	7 (22.6)	13 (18.8)	–	NS	–
Chest discomfort	17 (54.8)	34 (49.3)	–	NS	–
Nasal symptoms	4 (12.9)	5 (7.2)	–	NS	–
Myalgia	20 (64.5)	29 (42.0)	4.33	0.04	2.5 (1–6.6)
Skin lesions	5 (16.1)	9 (13.0)	–	NS	–
Fever	26 (83.9)	48 (69.6)	–	NS	–
Rales	18 (58.1)	25 (36.2)	4.16	0.04	2.4 (1–6.6)
Pharyngeal erythema	7 (22.6)	13 (18.8)	–	NS	–
Cervical adenopathy	15 (48.4)	18 (26.1)	4.81	0.03	2.7 (1–7.1)
Crepitations	13 (41.9)	23 (33.3)	–	NS	–

[a] Test of significance was Pearson's Chi-square test. NS, not significant ($p \leq 0.05$ considered significant).

Table 2 Diagnosis of *Mycoplasma pneumoniae* in HIV-infected patients[a]

No.	Age (years)	Culture		Serology		
		Induced sputum	Throat swab	BCAT	CA titer	IgM ELISA
1	40	+	+	+	32	+
2	37	+	+	+	64	+
3	35	+	+	+	32	+
4	30	+	+	+	32	+
5	28	+	—	+	32	—
6	28	+	—	+	32	+
7	37	+	—	+	32	—
8	24	+	—	+	32	—
9	45	—	+	+	32	—
10	35	+	+	+	32	+
11	37	+	—	+	32	—
12	30	+	—	+	32	—
13	42	+	+	+	32	+
14	47	—	+	+	32	—
15	59	+	+	+	32	+
16	33	+	—	+	64	—
17	60	+	+	+	32	—
18	31	+	+	+	64	+
19	31	—	+	+	32	+
20	33	+	+	+	32	+
21	29	—	+	+	32	+
22	33	+	—	+	64	+
23	38	+	+	+	64	+
24	60	+	+	+	32	+
25	41	+	+	+	32	+
26	35	+	+	+	64	+
27	35	+	+	+	32	+
28	36	+	+	+	64	+
29	32	+	+	+	32	+
30	40	+	+	+	32	+
31	35	—	—	+	32	—
32	45	+	—	+	32	—
33	34	—	—	+	32	—
34	35	—	—	+	32	—

[a] The criteria for diagnosis were a positive culture or a positive IgM ELISA irrespective of a diagnostic CA titer (CAT is a non-specific test for *M. pneumoniae* infections). Accordingly 31 patients were found to meet the criteria. All the patients positive by CAT (cold agglutination test) were concurrently positive by BCAT (bedside cold agglutination test).

Table 3 Association of HIV status with *Mycoplasma pneumoniae* infection in HIV-infected patients screened for *M. pneumoniae* by culture (sputum and throat swab) and IgM ELISA

HIV status[a]	Culture		IgM ELISA	
	Positive ($N = 31$)	Negative ($N = 69$)	Positive ($N = 21$)	Negative ($N = 79$)
A1	0	1	0	1
A2	1	4	0	5
A3	5	15	5	15
B2	2	2	2	2
B3	4	7	1	10
C1	2	0	1	1
C2	1	4	1	4
C3	16	36	11	41

[a] Classified based on CDC criteria, 1993.[18]

(11.2 ± 2.7 g/dL). The association of HIV status with *M. pneumoniae* infection in HIV-infected patients screened by culture and IgM ELISA is shown in Table 3.

Discussion

Our study has shown that *M. pneumoniae* is prevalent among HIV-infected patients, viz., 31% by culture (induced sputum and throat swab combined) and 21% by IgM ELISA. Recent studies have demonstrated that HIV-infected adults may never reveal measurable IgG against *M. pneumoniae*.[22] Occasionally, patients continue to produce detectable levels of IgM due to incomplete clearance of the organisms from the respiratory tract.[22,23] Furthermore, immunocompromised patients such as those with HIV infection might not mount a detectable antibody response. Culture and serology are still the standard laboratory methods for the diagnosis of *M. pneumoniae* infections,[16,24] and therefore both methods have been used in our resource-limited setting. The complement fixation test (CFT) is reported to give false positive results[24–27] and so was not considered. Park et al. documented a 7% prevalence of *M. pneumoniae* in HIV-infected individuals using immunofluorescence methods, while Lockman et al. reported a 17% prevalence.[6,7] Our study has shown that culture (combined) was found to be 100% sensitive and 87% specific compared to IgM ELISA (95% CI 84—100). Diagnostic titers of cold agglutinins were evident among 34% of cases by CAT, the conventionally used serological test for diagnosing *M. pneumoniae* infections. Cold agglutinins are the first non-specific indicators of acute *M. pneumoniae* infection whose association has been described in 34—86% of *M. pneumoniae* infections.[19] Moreover, since cold agglutinins are not found usually in normal individuals, their detection aids in *M. pneumoniae* diagnosis. In addition, the interpretation of a negative result is critical because a negative result does not exclude infection.[19] Our study showed that CA titer was 96.8% sensitive and 94.2% specific compared to culture, albeit being a non-specific test, and could be used as an adjunct to other expensive procedures in resource-limited settings. However, more studies are required to substantiate our findings.

Although rapid methods for the detection of mycoplasmas are developing, the choice of the best specimen and thorough sampling are essential for optimal performance.[24] *M. pneumoniae* frequently causes lower respiratory tract infections (LRT) and specimens obtained from the LRT may be more sensitive than throat swabs. Some authors have shown that induced sputum is an important sampling technique for the assessment of lower airway secretions.[28] Throat swabs may be used in preference to other respiratory specimens in *M. pneumoniae* infection, as patients do not normally produce sufficient sputum. However, our previous pilot study data supported induced sputum as the best specimen.[17] The present study reports that among the two culture methods employed, sputum culture was found to be more sensitive (90%) while throat swab was more specific (94.9%) compared to IgM ELISA. Therefore, sputum samples could be more sensitive for rapid detection of *M. pneumoniae* in clinical specimens. Throat swab culture has 81.8% positive predictive value compared to 70% by sputum culture. If culture were not used as a diagnostic test, diagnosis would not have been established in 10 cases, as ELISA proved only 20 cases positive for IgM against *M. pneumoniae*. Culture has been shown to give a false positivity rate of 13% (95% CI 6—22) and a false negativity rate of 0% (95% CI 0—16), the likelihood ratio of a positive test being 7.90 (4.4—14.1). However, CAT should never be compared for diagnostic usage to culture, as the former is only ~50% specific.[19] Therefore, comparison was made as the test was used in the study.

Clyde found that Mycoplasma was responsible for approximately 30% of cases of community-acquired pneumonia in the general population, and that the role of *M. pneumoniae* in adults with HIV infection needs to be defined.[11] Studies in other geographical areas have shown different prevalence rates in HIV-infected adults and our report differs substantially from others; Lockman et al. reported 17% from Botswana and Park et al. a 7% prevalence from Seattle, USA.[6,7] We have reported earlier that mycoplasmas are more frequent in the sputum of HIV-infected patients (36%) than from that of HIV-uninfected individuals (16.6%).[29] Teel et al., reported that *M. pneumoniae* may be more common in HIV-infected than HIV-negative patients.[30] Studies by some authors have shown that *Mycoplasma spp* colonize 12.5% of HIV-infected cases compared to <0.9% HIV-negative patients,[31] whilst others have documented *Mycoplasma spp* in 87% of HIV-positive patients compared to only 20% from HIV-negative controls.[32]

Detection of *M. pneumoniae* specific IgM by commercial ELISA does not correlate with culture (and CA values) in the present analysis, as 10 cases positive by culture have failed to demonstrate IgM against *M. pneumoniae*. This could be attributed to compromised antibody responses in AIDS patients.[22] *M. pneumoniae* might continuously be liberated in respiratory secretions with no detectable antibody response, and therefore measurements of antibody levels are of limited value and diagnosis cannot be based on the demonstration of specific IgM alone as reported by others,[33–37] hence culture was attempted. In addition, since adult patients in particular are known to develop weak IgM responses during primary infection or reinfection[15,37,38] it was important to examine the diagnostic yields of testing using culture and CA assay. Therefore, we chose culture along with direct ELISA instead of a capture immunoassay described by others[39] to avoid false-negative IgM reactions caused by IgG antibodies occupying the epitopes of the solid-phase antigens.[40]

Foy et al. reported several clinical parameters that positively or negatively correlated with *M. pneumoniae* infection.[41] Our study has shown that cough (OR 3.8, 95% CI 1—17.8, $p = 0.03$), myalgia (OR 2.5, 95% CI 1—6.6, $p = 0.04$), rales (OR 2.4, 95% CI 1—6.4, $p = 0.04$), and cervical adenopathy (OR 2.7, 95% CI 1—7.1, $p = 0.03$) were found to corroborate *M. pneumoniae* culture positivity. However, in general it is difficult to discriminate *M. pneumoniae* from other pathogens causing respiratory tract infection on clinical parameters only, and this emphasizes the need for laboratory confirmation.

Our data suggest there may be an association between AIDS stage and *M. pneumoniae* infection (Table 3) but this warrants a larger sample size.

An association between cold agglutinins and anemia during *M. pneumoniae* infection was also evident in our study and is in agreement with reports by others.[42] Among the 31 culture positive cases, 19 cases (8.8 ± 1.7 g/dL) showed a

significant depletion in HgB levels (t = 3.05, p = 0.01, OR 2.4, 95% CI 0.79—4.01) compared to those without anemia (11.2 ± 2.7 g/dL). The finding corroborated the reports that *M. pneumoniae* infection may lead to hemolytic anemia that is related to cold agglutination.[41]

Interestingly, our study has disclosed a 34% diagnostic CA titer with 96.8% sensitivity and 94.2% specificity compared to culture, suggesting that CA titer could still be used in limited resource settings and third-world nations for prompt diagnosis.[43] Comparison has been made between culture and CAT because the latter was positive in all the cases that were positive by specific tests viz. culture. However, while using CAT, false positivity cannot be ruled out, the probable reason being cold agglutinins are also produced during other underlying conditions such as pregnancy, staphylococcemia, trypanosomiasis, Raynaud's disease, etc. Moreover, Khoo et al. reported that HIV-infected adults may carry *M. pneumoniae* for months after illness[44] and therefore, detection does not necessarily indicate infection but rather may reflect respiratory carriage. Studies by Beersma et al. have shown that serology may be non-specific.[45]

In conclusion, culture has a low positive predictive value and should therefore be used in conjunction with CAT in resource-limited settings in particular as the test has more sensitivity and specificity in HIV-infected patients. Sputum culture was found to be more sensitive (90%) while throat swab was more specific (94.9%) compared to IgM ELISA.

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Conflict of interest: The authors declare no conflict of interest for the present study.

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