Diagnostic methods for intracellular pathogens

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Diagnosis of infection with *Chlamydia pneumoniae* is difficult and the optimal diagnostic procedure has yet to be established. *C. pneumoniae* is more difficult to isolate in tissue culture than *C. trachomatis*. Attention must be paid to the site from which the specimen is taken, and all specimens should be processed within 24 h or stored at 4 °C. Serologic diagnosis largely depends on microimmunofluorescence testing which, despite proposed criteria, still has a large subjective component. Serology has limitations in terms of both sensitivity and specificity and antibodies may be difficult to detect in individuals with positive *C. pneumoniae* cultures. Non-culture methods include enzyme immunoassays, fluorescent-antibody techniques and DNA probes. The most promising appears to be PCR. Co-infection of *C. pneumoniae* and other respiratory organisms seem to be common.

Key words: *Chlamydia pneumoniae*, culture, PCR, respiratory infection, serology

CHLAMYDIA PNEUMONIAE INFECTION

Chlamydia pneumoniae has emerged as a common cause of respiratory infection. Recent studies suggest that the organism is responsible for 10–20% of community-acquired pneumonia in adults and children and 10–20% of acute bronchitis in adults [1–4]. *C. pneumoniae* has also been implicated as an infectious trigger for asthma [5]. Infection with *C. pneumoniae* is frequently subclinical and may persist for prolonged periods of time [6]. Chronic infection with *C. pneumoniae* has also been suggested as a factor in the development of atherosclerotic heart disease [7].

Despite the increasing appreciation of its role as a respiratory pathogen, diagnosis of *C. pneumoniae* infection remains difficult, due in part to the limited availability of facilities capable of performing culture or serology and the lack of a commercially available rapid non-culture test. There is even controversy on which method would be the 'gold standard' for diagnosing infection. As infections with *C. pneumoniae* are treatable with antimicrobial agents, more attention has recently been directed to diagnosis.

The first isolates of *C. pneumoniae* were serendipitously obtained during trachoma studies in the 1960s [1]. TW-183 was isolated from the eye of a child with suspected trachoma in Taiwan, and IOL-207 was isolated from the eye of another child with trachoma from Teheran. Later, serologic studies of an outbreak of mild pneumonia among children in Finland in the 1970s suggested that an organism related to TW-183 was the cause [1]. Originally the organism was difficult to grow in tissue culture; it could only be propagated in eggs. On the basis of inclusion morphology and failure to stain with iodine, TW-183 was initially considered to be a strain of *C. psittaci* [1]. Following recovery of similar isolates from the respiratory tracts of college students with pneumonia and bronchitis in Seattle, Grayston and colleagues demonstrated that the organism was distinct from both *C. trachomatis* and *C. psittaci* [1]. Restriction endonuclease pattern analysis and nucleic acid hybridization studies suggest a high degree of genetic relatedness (> 95%) among the *C. pneumoniae* isolates examined so far [1].

DIAGNOSTIC METHODS

Culture of *C. pneumoniae*

As previously stated, initial studies suggested that *C. pneumoniae* was very difficult to isolate in tissue culture compared with *C. trachomatis*. Kuo et al [8] reported that HeLa cells pretreated with DEAE-dextran were superior to McCoy cells for isolation and propagation of *C. pneumoniae*. Inclusions tended to be very small, and experience in some clinical studies found that up to four serial passages, every 72 h, were needed to maximize the isolation rate [3]. Subsequent studies found that *C. pneumoniae* grows more readily in other cell lines derived from the human respiratory tract including HL, Hep-2 and H 292 cells [9–13]. Hep-2

Table 1 *C. pneumoniae* culture

1. Requires tissue culture (Hep-2, HL cells)
2. Takes 3–7 days
3. Culture confirmation by fluorescent-antibody staining with species-specific monoclonal antibody

and H 292 cells appear to be the most sensitive for isolation of the organism from clinical specimens and for propagation [11–13]. Use of DEAE-dextran is not necessary with these cell lines and actually results in smaller, less visible, inclusions [12]. Roblin et al [12] found that an initial inoculation and one passage in Hep-2 cells was equivalent to four serial passages in HeLa cells pretreated with DEAE-dextran. Culture confirmation is preferably performed by staining with a fluorescein-conjugated, species-specific monoclonal antibody (Table 1). However, these reagents are not readily available commercially. Thus, many laboratories use the more readily available genus-specific reagents [14].

Another factor that may influence the ability to isolate *C. pneumoniae* from patients is the specimen site. Most studies done so far have used throat swabs. However, the posterior nasopharynx (NP) may be a superior site. As part of a multicenter pediatric pneumonia treatment study, duplicate specimens were obtained from the throat and NP of a subgroup of the children enrolled. Of the children who had *C. pneumoniae* isolated, all the NP cultures were positive, but only 50% of the throat swabs from the same children were positive [4]. NP cultures can be obtained with Dacron-tipped, wire-shafted swabs.

The adhesive used to attach the Dacron to the swab shaft can change lot-to-lot, depending on what the manufacturer buys. Some adhesives can be toxic to the cells or inhibit the growth of *C. pneumoniae*. Thus each new lot of swabs, as well as fetal calf serum and media, should be tested to ensure that there is no inhibition. Specimens for culture should be placed in appropriate transport media, usually a sucrose-phosphate buffer with antibiotics and fetal calf serum. Specimens should be stored immediately at 4°C. Viability may decrease if specimens are held at room temperature. If specimens cannot be processed with 24 h they should be frozen at −70 °C until culture can be performed. *C. pneumoniae* has also been isolated from sputum, bronchoalveolar lavage fluids and pleural fluids [1,15,16].

Serology

To date most investigations have relied on serologic diagnosis, using modifications of the microimmunofluorescence (MIF) test. Grayston and associates [1] have proposed criteria for serologic diagnosis of *C. pneumoniae* infection that have been used by many clinicians (Table 2). For acute infection the patient should have a four-fold increase in the IgG titer, a single IgM titer ≥ 1:16, or a single IgG titer ≥ 1:512. Past or pre-existing infection is defined as an IgG ≥ 1:16 and ≤ 1:512. Rheumatoid factor may cause false-positive IgM titers in the MIF test and it has been recommended that sera, especially from older individuals, be absorbed before testing [17]. Grayston [1] further proposed that the pattern of antibody response in primary infection may differ from that seen in reinfection. In initial infection, the IgM response appears about 3 weeks after the onset of illness and the IgG response at 6 to 8 weeks. In reinfection, the IgM response may be absent and the IgG response occurs earlier, usually within 1 to 2 weeks. The MIF test is mainly available in research laboratories. It is a difficult test to perform, with a significant subjective component.

Grayston and others have also suggested that a four-fold titer rise or a titer ≥ 1:64 with the *Chlamydia* complement-fixation (CF) test could be diagnostic for *C. pneumoniae* infection (Table 2) [1,18]. The CF test is genus-specific, but is probably the most widely available serologic test for *Chlamydia* infection. It has been used for many years for the diagnosis of psittacosis. It is possible that many cases of pneumonia, especially without a history of avian exposure, diagnosed as psittacosis on the basis of the CF test were probably due to *C. pneumoniae* [19–21]. Early studies suggested that the CF test was more likely to be positive in the initial infection than during reinfection [1].

During an outbreak of *C. pneumoniae* infection at the University of Washington campus, all of eight culture- and/or polymerase chain reaction (PCR)-positive students with pneumonia or bronchitis had CF titers ≥ 1:64 [2]. There are several ELISA antibody tests that are being marketed for the diagnosis of chlamydial infections, primarily for *C. trachomatis* infection.

Table 2 Serologic criteria for diagnosis of *C. pneumoniae* infection

MIF	
Acute infection	Four-fold rise IgG
	IgM ≥ 1:16
	IgG ≥ 1:512
Pre-existing antibody	IgG ≥ 1:16, <512
Chlamydia CF[a]	
Acute/infection	Four-fold rise
	≥ 1:64

[a]Genus specific
From Grayston et al [1].

Table 3 Correlation of *C. pneumoniae* serology and culture in children with community-acquired pneumonia

	C. pneumoniae culture		
Serology	Positive	Negative	Total
Acute	8	40	48
None/pre-existing	26	186	212
Total	34	226	260

From Block et al [4].

However, these tests are genus-specific and have not been specifically evaluated for the diagnosis of *C. pneumoniae* infection.

Grayston initially suggested that serology, using these criteria with the MIF test, was more sensitive than culture. However, it was noted by Chirgwin et al that some individuals with positive *C. pneumoniae* cultures and community-acquired pneumonia did not meet the serologic criteria even with multiple serum samples obtained over several months [3]. Some culture-positive children did not develop any detectable antibody by MIF. In a multicenter pediatric pneumonia treatment study it was found that only eight of 34 (24%) of the children with culture-documented *C. pneumoniae* infection met the serologic criteria for acute infection. The majority had no detectable MIF antibody even after 3 months of follow-up (Table 3) [4]. These children were also negative with the CF test. Most of the culture-negative serologically positive children did not seroconvert or have a four-fold titer rise but had stable IgG titers ≥ 1:512. Kern et al [22] in a study of subjectively healthy adults (fire-fighters and policemen) found serologic evidence of recent *C. pneumoniae* infection (IgM ≥ 1:16 and/or IgG ≥ 1:512) in 13% of their subjects. The overall seroprevalence was 86%. They also found a strong correlation between IgM titers for *C. pneumoniae* and *C. trachomatis*. Cultures for *C. pneumoniae* were not performed in this study. However, very similar results were reported recently by Hyman et al in a study of subjectively healthy adults, where culture and PCR for *C. pneumoniae* were employed (Table 4) [23]. They studied 104 healthcare workers; two were found to have positive NP cultures and/or PCRs. One individual had a single IgG titer of 1:256, and the other was seronegative. However, 18.3% of the remaining culture-negative, PCR-negative, asymptomatic subjects met the serologic criteria for acute infection as described above. The overall prevalence of antibody to *C. pneumoniae* was 81% in this population.

These studies raise some questions about the sensitivity and specificity of MIF for the diagnosis of *C. pneumoniae* infection. Although the DNA homology between *C. pneumoniae* and *C. trachomatis* is less than 5%, some parts of the genome, specifically the *omp1* gene, are highly conserved between the three chlamydial species. Thus there may be cross-reactions in the MIF test due to cross-reactions to the major outer membrane protein (MOMP) [24]. The MOMP does not appear to be immunodominant in the serologic response to *C. pneumoniae* as it is for *C. trachomatis* [25–27]. In the MIF test, the immunodominant protein is probably the MOMP, which is an important surface antigen of both chlamydial elementary and reticulate bodies [28]. The MOMP displays genus-, species- and serovar-specific antigen determinants that are recognized during human infection and is known to cross-react with sera against either of the three species [29]. This may explain the culture-positive, MIF-negative children.

When we examined sera from these children with immunoblotting, we found that 95% reacted with several *C. pneumoniae* proteins, most frequently at 98 kDa and 35 kDa, but less than 30% reacted with the MOMP [30]. If the specific antigenic determinants for *C. pneumoniae* are not expressed on the surface of the elementary body, sera from these children may not react in the MIF test. Also, antibodies to the genus-specific lipopolysaccharide (LPS) antigen may cross-react in the MIF test with elementary bodies of the three species [28]. Additionally, it has been suggested that infections

Table 4 PCR-EIA, cultural and serologic data for 104 healthy adults who were evaluated for the presence of asymptomatic *C. pneumoniae* infection

No. individuals in the study		No. (%) of individuals with positive culture and/or PCR results	No. (%) with pre-existing antibody	No. (%) with titers indicating acute infection	Total no. (%) with positive titers
Male	71	0	48 (68)	13 (18)	61 (86)
Female	33	2 (6.1)	17 (53)	6 (19)	23 (72)
Total	104	2 (1.9)	65 (63)	19 (18)	84 (81)

From Hyman et al [23].

with any of the chlamydial species may provoke an anamnestic response to the common antigenic determinants, resulting in multiple positive reactions in the MIF test when several chlamydial antigens are used [28,29]. This may explain the similar MIF titers to *C. pneumoniae* and *C. trachomatis* seen in the healthy adults reported by Kern et al [22] and also reported by Moss et al [31] in patients seen in a sexually transmitted disease (STD) clinic.

As more studies of *C. pneumoniae* infection are being done, similarities to *C. trachomatis* infection are becoming appreciated. Like *C. trachomatis* genital infection, many respiratory infections with *C. pneumoniae* are subclinical and often of long duration [5,6,23]. Patients may remain infected for months following an acute respiratory infection [6]. This makes the demonstration of a four-fold titer rise difficult because of timing of the specimens. Serology with paired sera also only offers a retrospective diagnosis, which makes it difficult to manage patients prospectively or to assess the efficacy of treatment. Serology is not used for the diagnosis of genital *C. trachomatis* infections. One looks for the presence of the organism by culture or non-culture methods. The same may apply for *C. pneumoniae.*

Non-culture methods

Use of non-culture methods, including enzyme immunoassays (EIA), direct fluorescent antibody (DFA) and DNA probes, for diagnosis of *C. trachomatis* infection has become common. A commercial PCR has recently been approved and released in the USA. These tests have been extensively evaluated for genital specimens in men and women and generally average 80% sensitivity and > 95% specificity compared to culture, with the exception of PCR, which may be 10% more sensitive than culture. Culture itself is not 100% sensitive. Unfortunately, there is not as yet a commercially available non-culture test for *C. pneumoniae.* As all the currently marketed EIAs use either a polyclonal antibody or genus-specific monoclonal antibody, they can detect *C. pneumoniae.* However, these tests have not been extensively evaluated in respiratory specimens, and preliminary data suggest that they are not sufficiently sensitive to detect *C. pneumoniae.* Chirgwin et al [3] obtained nasopharyngeal swabs for testing with Chlamydiazyme (Abbott Diagnostics) from 91 children and adults with community-acquired pneumonia. The EIA detected only two of 15 (13.3%) culture-positive patients. There were no false-positive results, although other investigators have reported false positives when used with throat cultures. Group A *Streptococcus* can give a positive reaction with Chlamydiazyme [32].

C. pneumoniae-specific monoclonal antibodies have also been used as DFA reagents. None of these reagents has been evaluated for direct detection of *C. pneumoniae* in smears of patient specimens from any source, including pharyngeal, NP and lower respiratory tract specimens. Grayston and colleagues found DFA staining of throat specimens to be less sensitive than cell culture for identifying *C. pneumoniae* infection [33]. We have had a similar experience and also have concern about specificity due to numerous artifacts and non-specific staining which can occur with respiratory specimens that contain mucus.

The most promising non-culture methodology appears to be DNA amplification, specifically PCR. Most data available come from evaluations of in-house tests with limited numbers of patients, including culture positives. In 1992, Campbell et al [34] described a PCR assay, using genomic *C. pneumoniae*-specific primers. They found a very good correlation with culture. However, specimens were evaluated from only 36 patients, eight of whom were culture positive. This PCR assay was subsequently used in other clinical studies of respiratory infection from the University of Washington [2] and for the detection of *C. pneumoniae* DNA in atheroma specimens from patients [7]. All the atheroma specimens were culture negative, or culture was not attempted. However, these initial studies have not determined the sensitivity or specificity of PCR by testing a large number of clinical specimens in parallel with cultures from symptomatic and asymptomatic individuals.

The most extensively evaluated PCR is from Gaydos and her colleagues at Johns Hopkins University. They have developed an assay using primers from the 16S rRNA gene with the amplification products being detected by EIA, offering a method that would be useful for screening large numbers of clinical specimens [35,36]. They have compared PCR-EIA to culture in specimens from symptomatic and asymptomatic adults and children [36]. DFA staining of culture transport media was done to resolve discrepant specimens, usually secondary to the presence of non-viable organisms. They examined specimens from 56 patients with

Table 5 Comparison of culture and PCR-EIA for detection of *C. pneumoniae* in nasopharyngeal specimens

	Culture	
	Positive	Negative
PCR-EIA		
Positive	23	4
Negative	8	101
Sensitivity: 74.2%		
Specificity: 96.2%		

respiratory infection and 80 asymptomatic persons; 35 were positive by culture and/or PCR-EIA; 101 were negative by both assays; 23 specimens were culture and PCR positive (Table 5). When culture- and/or DFA-positive results were used as a reference or 'gold standard', the sensitivity and specificity of PCR-EIA were 76.5% and 99%, respectively. When PCR- and/or DFA-positive results were used as the reference, the sensitivity of culture was 87.5%. False-negative PCRs can result from the presence of inhibitors such as mucus or blood or loss of amplified DNA due to nucleases in the sample. Sometimes inhibition can be eliminated by freeze-thawing or diluting the original sample. False-positive PCR results can also occur, usually due to amplicon contamination. The development of totally enclosed test systems would minimize this possibility. When the results of PCR and culture were compared to the results of single acute sera, only eight of 35 (23%) positive patients had diagnostic antibody titers. Of the 80 asymptomatic patients, two of whom were culture and/or PCR positive, 15 (19%) had antibody titers considered to be diagnostic of acute infection. These results indicate that culture and/or PCR-EIA is more reliable for prompt diagnosis of *C. pneumoniae* infection than single-point serology alone.

PCR and related methods also offer the potential to run multiple hybridizations to look for several organisms in the same sample. Co-infection of *C. pneumoniae* and other respiratory organisms, especially *Mycoplasma pneumoniae*, appears to be very common. Almost 20% of the children with culture-documented *C. pneumoniae* infection enrolled in the multicenter pneumonia treatment study were also infected with *M. pneumoniae* [4]. Clinically, one could not differentiate these children from those infected with either organism alone. A rapid, specific method of identifying *C. pneumoniae* will enable the physician to diagnose these infections and offer appropriate therapy.

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