MAJOR ARTICLE

Standardizing *Chlamydia pneumoniae* Assays: Recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada)

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***Chlamydia pneumoniae* has been associated with atherosclerosis and several other chronic diseases, but reports from different laboratories are highly variable and "gold standards" are lacking, which has led to calls for more standardized approaches to diagnostic testing. Using leading researchers in the field, we reviewed the available approaches to serological testing, culture, DNA amplification, and tissue diagnostics to make specific recommendations. With regard to serological testing, only use of microimmunofluorescence is recommended, standardized definitions for "acute infection" and "past exposure" are proposed, and the use of single immunoglobulin (Ig) G titers for determining acute infection and IgA for determining chronic infection are discouraged. Confirmation of a positive culture result requires propagation of the isolate or confirmation by use of polymerase chain reaction (PCR). Four of 18 PCR assays described in published reports met the proposed validation criteria. More consistent use of control antibodies and tissues and improvement in skill at identifying staining artifacts are necessary to avoid false-positive results of immunohistochemical staining. These standards should be applied in future investigations and periodically modified as indicated.**

Chlamydia pneumoniae is a fastidious bacterium that was first established as a cause of acute respiratory infection >15 years ago; more recently, it has been associated with certain chronic diseases, including atherosclerotic cardiovascular disease. The ever-expanding spectrum of diseases associated with *C. pneumoniae* infection has led a sizable influx of new investigators and laboratories to become involved in *Chlamydia*-related research. Test results are often contradictory and difficult to interpret; dramatic findings from one laboratory are unconfirmed by others. As a result, researchers in the field and external reviewers have called for validated and standardized diagnostic techniques to promote research applications and improve the recognition and care of patients infected with *C. pneumoniae* [1, 2].

A meeting was recently convened by the Centers for Disease Control and Prevention (Atlanta) and the Laboratory Centre for Disease Control (Ottawa, Ontario, Canada) to review current diagnostic tests for *C. pneumoniae* and provide recommendations for standardized approaches.

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SEROLOGICAL TESTING

There are no wholly satisfactory serological methods for diagnosis of *C. pneumoniae* infection. Problems arise from the difficulty in obtaining appropriately paired serum samples, the high background of IgG antibody prevalence in certain adult populations, the lack of standardized testing methods, and a shortage of high-quality reagents.

For patients with acute *C. pneumoniae* infection, it is important to take into consideration the kinetics of the antibody response. In patients with primary infection, IgM antibody appears ~2–3 weeks after the onset of illness and is generally undetectable after 2–6 months. IgG antibody may not reach high titer until 6–8 weeks after the onset of illness. *C. pneumoniae* infection does not induce good protective immunity, and reinfection may occur. In cases of reinfection, IgM antibody may not appear and the level of IgG antibody titer increases quickly, within 1–2 weeks. Serological testing most often provides only a retrospective diagnosis of acute infection, because a convalescent serum specimen is needed to show a 4-fold increase in titer, so it is not optimum for patient management. Nevertheless, serological testing is the most useful means of determining the cause of an outbreak or the prevalence of infection in epidemiologic studies.

Review of Currently Used Tests: Serological Testing

Complement fixation (CF), whole-inclusion fluorescence, and EIA cannot currently be endorsed. CF has objective end points and can detect increases in antibody levels in specimens obtained at intervals as close as 1 week apart, but the test cross-reacts with other *Chlamydia* species and other enteric bacteria, the sensitivity for detecting reinfection is low, and reagents are not readily available [3–5]. Whole-inclusion fluorescence tests are available as commercial kits, but they also are not species specific and have not been widely evaluated [4]. The EIA holds the most promise, and several kits are commercially available, although none has been approved by the US Food and Drug Administration (FDA) for use in the United States. Advantages of the EIA include high throughput, objective end points, technical accessibility, and an electronic record of the results. However, the limited published evaluations of these kits that have appeared to date have included reports of problems with both sensitivity and specificity [6]. Therefore, no currently available assay can be recommended because of a lack of peer-reviewed evaluations to document that the specificity is adequate when compared with that of microimmunofluorescence (MIF). In addition, use of EIA as a screening method is not endorsed because the high sensitivity required by this approach has not been demonstrated.

The MIF test is the serological testing method of choice for diagnosis of acute *C. pneumoniae* infection. It was the use of the MIF that led to the identification of *C. pneumoniae* as a distinct species of *Chlamydia* [7]. It is the only species-specific antibody test available that can measure isotype-specific antibody titers to all *Chlamydia* species simultaneously. The specificity of the MIF test can be attributed to the use of purified elementary bodies of all 3 species of *Chlamydia* rather than reticulate bodies that express predominately genus-specific epitopes.

The test format uses purified formalinized elementary bodies from *C. pneumoniae, Chlamydia trachomatis,* and *Chlamydia psittaci* that have been fixed onto glass slides as distinct dots of antigen. Dilutions of sera are placed over the antigen dots and incubated. However, the assay is technically complex, interpretation is subjective, and neither reagents nor diagnostic criteria have been standardized [8]. Kits based on the MIF format are commercially available. Their performance characteristics require further evaluation and peer-reviewed publication before endorsement.

Specific Recommendations for Serological Testing

A standardized approach to performing and interpreting the MIF assay is presented in table 1. Quality assurance procedures are particularly important to emphasize because of the subjective nature of the interpretation; they are included in table 1.

Several caveats regarding MIF serological testing are important to emphasize. Diagnosis of acute infection based on a single IgG titer cannot be routinely recommended; if single IgG titers are reported, they should be interpreted with caution. Serum samples obtained from elderly patients and from patients with chronic obstructive pulmonary disease have had persistently high IgG titers in the absence of clinically apparent disease. Failure to adsorb serum before IgM testing may lead to false-positive IgM results due to the presence of rheumatoid factor in the sera. The quality of the IgA conjugates has been found to vary, and their use requires careful evaluation and further standardization. Finally, the absence of MIF antibodies in persons with culture-confirmed infection has been reported. This is rare in adults but may be more common in young children [9–11].

One of the most challenging aspects of *C. pneumoniae* testing is the identification of persons with persistent or chronic infection by means of serological testing. Persistently elevated IgG or the presence of IgA antibodies have been frequently used [12–14]. There is no reference test for validating persistent infection, and studies that have attempted to correlate single-sample IgG or IgA antibody levels with disease status have produced equivocal results, owing in part to the different methodologies and titer cutoff points that were used in different studies [15, 16]. It has been proposed that high IgA titers may be a better marker of chronic *C. pneumoniae* infection than are IgG titers because serum IgA has a half-life of 5–7 days, whereas IgG has a half-life of weeks to months. However, there is at present no validated serologic marker of persistent or chronic

Table 1. Recommendations for use of the microimmunofluorescence test.

Assay component	Recommendations
Antigen	Renografin-purified elementary bodies resuspended in phosphate-buffered saline that contain 0.02% formalin, combined with 0.5% yolk sac and fixed in acetone
Serum samples	Paired serum samples, obtained 4–8 weeks apart
Testing	Screen at 1:8 or 1:16 and titer at 2-fold dilutions to end point
	Preabsorb serum samples with anti-IgG before IgM and IgA testing
	Add Evan's blue stain (0.05%) or rhodamine-conjugated bovine albumin stain (at 1/15 volume) as counterstain to fluorescein-conjugated second antibody
Results	Read slides using ×10 eyepiece and ×40 plan achromatic objective
Interpretation	Acute infection, IgM of ⩾1:16 or 4-fold increase in IgG
	Possible acute infection, IgG of ⩾1:512
	Presumed past infection, IgG of ⩾1:16
Quality assurance	Positive and negative control serum samples in each run
	Check titer of positive control serum sample for reproducibility between runs
	Determine optimal conjugate dilution by titrating with a high-titered serum
	Aliquot undiluted conjugate in small quantities and store at −20°C until use
	Technician blinded to case/control and acute/convalescent status

infection, and the use of serological testing to define patients as "persistently infected" must await further validation.

CULTURE

C. pneumoniae is an obligate intracellular bacterium and must be cultivated within a eukaryotic host cell. The specificity of culture is dependent on the ability of the laboratory worker to distinguish true *Chlamydia* inclusions from artifacts on microscopic examination of the cell monolayer after fluorescent antibody staining has been performed. Tests that are not based on culture, such as PCR, have become widely used for detection of *Chlamydia* infection, in part because of the technical complexity and low yield of culture protocols. Problems with low yields may also be related to the contamination of culture with *Mycoplasma* species [17, 18]. However, culture remains essential to document the viability of the organism, to provide isolates of the organism for biological characterization and antimicrobial susceptibility testing, and to assess microbiologic efficacy in treatment trials.

Review of Currently Published Protocols for Culture

All currently accepted culture procedures involve inoculation of a specimen onto a human cell line via centrifugation. The inoculated cells are incubated and are later stained with a fluorescent-labeled antibody specific to *Chlamydia* to visualize the bacteria that are multiplying within the host cells.

There are a number of modifications to these procedures that remain controversial. These include simultaneous centrifuging of the cell line and inoculum onto the culture vessel [19], the use of serum-free cell culture medium [20], prolongation of culture times, and increases in the number of times the cell cultures are centrifuged after inoculation [21, 22]. Host-cell monolayers have been pretreated with polyethylene glycol, trypsin, and diethylaminoethyl dextran to improve the recovery of isolates of *C. pneumoniae* from either true or mock specimens [22, 23]. Other researchers, however, have reported that diethylaminoethyl dextran pretreatment actually decreased the size of inclusions [24] or failed to document improved recovery by use of either technique [21]. None of these modifications has been sufficiently tested to warrant their routine recommendation.

There is a good deal of controversy regarding the number of times a culture should be passaged before the results are determined [25–27]. Most laboratories agree that ⩾2 passages after the primary culture step is performed are needed to maximize the recovery of *C. pneumoniae* isolates from respiratory specimens. Other reports have successfully used greater numbers of passages, particularly for isolation from tissue specimens, although no systematic comparison of passage numbers has been attempted. Increased passages may result in a concentration of cell debris that may contribute to nonspecific staining of the monolayer.

We recommend that respiratory specimens should be cultured by means of primary isolation procedures plus 2 additional passages. Tissue specimens should be cultured by means of primary isolation procedures plus 4–6 additional passages.

Specific Recommendations for Culture

Specimen types. Specimens obtained for detection of *C. pneumoniae* respiratory tract infection by use of culture include swabs of the nasopharynx or oropharynx, sputum specimens, bronchoalveolar lavage specimens, and tissue biopsy specimens.

Table 2. Recommendations for use of culture for *Chlamydia pneumoniae*.

Assay component	Recommendations
Cell type	HEp-2 or HL cells in 6-, 12-, 24-, or 96-well tissue culture plates or shell vials
Media	Eagle's MEM or IMDM supplemented with fetal calf serum (10%), l-Glutamine (2 m*M*), MEM nonessential amino acids, HEPES buffer, gentamicin (10 μg/mL), vancomycin (25 μg/mL), and amphotericin B (2 μg/mL)
Inoculation	Centrifuge the homogenized specimen onto the monolayer at 900–3000*g* for 60 min; after centrifugation, replace culture medium with cycloheximide-supplemented medium
Incubation	35°C with 5% CO_2
Passages	Examine cultures for *C. pneumoniae* on day 3, homogenize duplicate wells, and pass to a fresh cell monolayer twice
Identification of inclusions	Monolayers should be fixed and stained with a genus-specific monoclonal antibody and then with a species-specific monoclonal antibody for confirmation; inclusion-forming units per milliliter should be used for quantifying the number of infectious organisms in the specimen
Quality assurance	Positive controls (cells infected with *C. pneumoniae*) and negative controls (uninfected human cells) should be used in each run
	New lots of swabs, fetal calf serum, and MEM medium should be tested by mock infection and titrated to ensure that they support the growth of *C. pneumoniae*
	Controlling for well-to-well contamination is especially important when using microtiter plates and multiple passages
	Laboratory workers should have sufficient experience and training in interpretation of *C. pneumoniae* microscopic evaluation to differentiate specific staining from the variety of artifacts
	Cell stocks should be routinely tested for *Mycoplasma* contamination by use of a commercially available test or PCR

NOTE. HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulfonic acid; IMDM, Iscove's modified Dulbecco's medium; MEM, minimal essential medium.

C. pneumoniae has not been successfully cultured from blood samples, although the DNA can be detected in samples of peripheral blood mononuclear cells, and the organism has been recovered from a limited number of vascular tissue specimens.

Specimen collection. Swab specimens should be collected only on swabs with a Dacron tip and an aluminum or plastic shaft. Swabs with calcium alginate or cotton tips and wooden shafts may inhibit the growth of the organism, depending on the adhesive used, and are unacceptable. Swabs should be placed in 2SP transport medium (sucrose, 68.4 g; potassium phosphate dibasic, 2.01 g; potassium phosphate monobasic, 1.01 g; gentamicin, 10 μg/mL; amphotericin B, 100 μg/mL; vancomycin, 25 μg/mL; and 10%–20% fetal calf serum, made up to a final volume of 1000 mL, pH 7.2) and not removed before transport. Bronchoalveolar lavage, sputum, and pleural fluid samples should be collected in 2SP at a ratio of specimen to medium of 1:2.

Specimen transport. All specimens that can be processed in the laboratory within 24 h should be held and shipped at 4°C (on wet ice). Samples that cannot be processed within 24 h should be frozen and held at −70°C.

Specimen processing. Swabs should be vortexed in the transport medium for 15–20 s and pressed against the side of the tube to extract all of the liquid. One hundred to 200 μL of this fluid is used as the inoculum. The specimen is then centrifuged at 8000–10,000*g,* resuspended in cell culture medium, and homogenized. It should be noted that sputum frequently inhibits cell growth and may be toxic to the monolayer. Calcified areas of vascular tissue specimens should be removed and the tissue resuspended in cell culture medium before homogenization.

Assay procedures and quality control. A standardized approach to culture procedures and quality assurance testing is presented in table 2. The use of methanol as a fixative for the monolayers before staining should be avoided or evaluated in advance, because there is some evidence that the antigenicity of certain proteins may be destroyed [28].

Whether a few inclusions that fail to propagate in subsequent passages should be considered a "true-positive" result of culture has been an area of debate. For all results of culture for *C. pneumoniae,* we recommend that the detection of an average of ⩾1 inclusions per well or tube be considered a "presumptive positive." Only if the strain can be propagated by means of subsequent passage or confirmed by use of an additional test, such as PCR, should it be reported as a "confirmed positive." It should also be recognized that *C. pneumoniae* has been cultured from specimens of the upper respiratory tract obtained from asymptomatic persons.

PCR

The description of the clinical spectrum of *C. pneumoniae* disease has been hampered by the lack of sufficiently sensitive diagnostic methods. Nucleic acid–based amplification tech-

niques, such as PCR, have identified *C. pneumoniae* in clinical samples ranging from respiratory specimens [29–31] to samples of vascular tissue [32–35], serum [36], and peripheral blood mononuclear cells [37]. Despite significant improvements in the development of molecular methods for the detection of *C. pneumoniae,* some laboratories report consistent detection of the organism in specimens of vascular tissue [32, 33, 38, 39], whereas others do not [40–42]. This variation may be related to differences in means of specimen collection and processing, primer design, nucleic acid extraction, amplification product detection, or prevention and identification of false-positive and false-negative results.

Review of Current Tests: PCR

Table 3 summarizes 18 reports regarding PCR assays, published as of 1 May 2000, with the target regions, product sizes, and methods for detection of the products. Although many in-house PCR methods are available for detection of *C. pneumoniae,* the sensitivity and specificity of the majority of these tests remain unknown. More studies need to be conducted using proper controls and a large number of clinical specimens obtained from patients to compare and evaluate more adequately the usefulness of different PCR tests for the diagnosis of *C. pneumoniae* infection. In addition, comparison of PCR results with those of a sensitive culture system and at least 1 other validated PCR assay that targets a different gene or a different sequence of the same gene is necessary to validate any newly proposed PCR assay.

Among the assays described in table 3, only 4 widely used protocols, which are highlighted at the top of the table, satisfy the optimal criteria for a validated assay. First, they have been validated for sensitivity and specificity in $\geqslant 2$ outside laboratories using both calibrated artificial specimens and true clinical specimens. Second, sensitivity has been documented to a level of detection of $\leqslant 1$ inclusion-forming unit (IFU). Finally, for each of these assays, specificity has been documented against other *Chlamydia* species as well as a wide range of other prokaryotic and eukaryotic DNA. We emphasize that all of these assays are research tools and none have been commercially standardized or cleared by the FDA.

Each PCR type has advantages and disadvantages that should be carefully considered before new assays are designed or evaluated. Seven PCR approaches and the advantages and disadvantages of each approach are briefly reviewed.

Table 3. PCR assays for detection of *Chlamydia pneumoniae* in clinical specimens.

Type of report or assay	Target region	Product size, bp	Method of detection	Year of study [reference]
Published reports regarding assays that meet validation criteria[a]				
S + R	Cloned *Pst*I fragment	437	AGE	1992 [43]
S	16S rRNA gene	463	AGE	1992 [44]
N + T	MOMP	Outer, 333; inner, 207	AGE	1993 [29]
S + T + HS + M	16S rRNA gene	195	AGE	2000 [45]
Published reports regarding assays				
S	16S rRNA gene	463	EIA	1993 [46]
N	16S rRNA gene	Outer, 1397; inner, 858	AGE	1994 [47]
S	53-kDa protein coding gene	499	AGE	1996 [48]
N	16S rRNA gene	Outer, 317; inner, 178	EIA	1996 [49]
N + M	16S rRNA gene	Outer, 436; inner, 221	AGE	1997 [50]
N	Cloned *Pst*I fragment	Outer, 437; inner, 128	AGE + SBH	1997 [51]
N	16S rRNA gene	Outer, 463; inner, 269	AGE	1997 [39]
S	60-kDa protein coding gene	183	EIA	1998 [52]
S	16S rRNA gene	465	EIA	1998 [53]
S + T + HS	16S rRNA gene + MOMP	165	AGE + SBH	1998 [54]
N	MOMP	Outer, 496; inner, 189	TRF	1998 [55]
S + IC	16S rRNA gene	463	EIA	1998 [56]
S + R + IC	16S rRNA gene	465	AGE	1999 [57]
N	16S rRNA gene	Outer, 492; inner, 304	AGE + DBH	1999 [58]

NOTE. AGE, agarose gel electrophoresis; DBH, dot-blot hybridization; HS, hot-start PCR; IC, internal control; M, multiplex PCR; MOMP, major outer membrane protein; N, nested PCR; R, restriction enzyme digestion; S, single-step PCR; SBH, Southern blot hybridization; T, touchdown PCR.

[a] See the section on PCR.

Nested PCR is, in general, more sensitive than is single-step PCR because of the 2-step amplification and the use of 2 sets of primers. The disadvantages of nested PCR are the increased risk of contamination and reamplification of the products, which makes the assay more time-consuming and expensive.

A multiplex PCR amplifies >1 target sequence in the same assay. For the *Chlamydia* genus, this has been used to discriminate among species. Multiplex reactions decrease sensitivity and specificity if the annealing temperatures for the individual primers are not identical. In general, these assays are not as sensitive as single-target PCR.

Internal or amplification controls allow the monitoring of PCR assay inhibition, which may be caused by a number of factors. These controls can have the disadvantage of competing for primers when identical primers are used for both target genes and internal controls. If a different set of primers is used to detect the internal control, differences in amplification conditions may decrease sensitivity.

Hot-start PCRs increase specificity by preventing nonspecific primer binding at temperatures that are less than the optimum temperature. Such assays are not recommended unless special hot-start *Taq* polymerases are used, because for all others, the need to open the tube to add more polymerase increases the potential for contamination.

Touchdown PCRs increase specificity by allowing the initial primer-template hybridization events to occur at annealing temperatures that are greater than the optimum annealing temperature. However, this type of PCR requires more cycles, which increases the duration of the PCR run.

Hybridization probe methods should routinely be used to ensure the specificity of the PCR product. They may also increase sensitivity, compared with standard visualization of PCR products in agarose gel after electrophoresis and ethidium bromide staining. These methods have the disadvantage of increased cost of reagents and are, in general, more time-consuming than are nonprobe methods.

The fluorescent probe–based assays that are currently being developed have the advantages of a closed system that avoids contamination by PCR product carryover. They can have real-time or end point readings, depending on the system that is used. These assays may be more sensitive and are inherently more specific than is single-step PCR because of dual primer and probe binding. However, the equipment that is required is very expensive.

Specific Recommendations for PCR

Specimen collection and processing. Specific recommendations regarding collection, transport, and processing of clinical specimens are similar to those described in the culture section. One aliquot (1 mL) of the transport medium should be centrifuged at ~18,000*g* for 15 min and the pellet should be processed for DNA extraction. Commercially available cell-preparation tubes facilitate separation of mononuclear cells from whole blood. Tissue samples should be cut into small pieces (~25 mg) and processed for DNA extraction. Specimens should be formed into aliquots to avoid >1 freeze-thaw cycle for optimal yield.

Specimens, controls, and PCR mixture reagents should be handled with dedicated pipettes in physically separated areas to avoid contamination. Aerosol barrier pipette tips, dedicated laboratory coats, and gloves are strongly recommended. Benchtop and equipment should be monitored routinely for DNA contamination by conducting swipe tests.

Assay procedures and quality control: DNA extraction. *C. pneumoniae* DNA should be extracted from clinical samples by use of a highly efficient and reliable protocol. Any new extraction method should be validated before routine use and should be assessed for problems with inhibitors of DNA polymerases.

Assay procedures and quality control: positive and negative controls. These should be included in all runs, in parallel with the clinical samples throughout the extraction and detection procedures. The positive controls should consist of small and very small amounts of DNA (titrations down to <10 ng of DNA) from a culture with <10 IFU. Each laboratory should prepare titrations of their *C. pneumoniae* stocks and form a large number of low and very low positive controls into aliquots. At least 1 negative control, consisting of water in place of the clinical specimen, should be run every fifth DNA extraction.

Assay procedures and quality control: amplification controls. The use of spiked nonrelated DNA adds validity to results by identifying potential inhibitors in the samples or in the PCR reaction itself. Such controls should be included in newly developed assays. Several different internal controls have been used, such as λ phage DNA [56], MIMICS or competitive primers [59, 60], and cloned fragments into the pUC19 vector [48]. Because of the potential for competition of the control with the target sequence, a low copy number of control DNA is important.

Development and quality control of new PCR assays. There is a critical need for commercially standardized, FDA-cleared assays. In the meantime, researchers should design primers and probes that are based on a highly conserved gene sequence by using sequence databases. The targeted DNA sequences should be searched by use of Basic Local Alignment Sequence Tool (available at http://www.ncbi.nlm.nih.gov/BLAST/) to check for specificity. Any newly developed primers or probes should be validated for analytic sensitivity and specificity.

Sensitivity should be determined by performing titrations of designated isolates to determine the lowest level of detection of the target gene. At a minimum, both *C. pneumoniae* type strain TW-183 (ATCC VR-2282) and CM-1 (ATCC VR-1360)

should be titrated with each newly designed assay and the number of IFUs quantified as described in the culture section above. Ideally, all available *C. pneumoniae* strains should be tested for contamination with *Mycoplasma* species by use of a genus-specific PCR and with stock cultures that are similarly titrated. The specificity of new primers and probes should be tested with a bank of DNA preparations from *C. psittaci, C. trachomatis,* and other bacteria and viruses commonly found in the respiratory tract, with human DNA, and with at least 1 of the 4 recommended PCRs in table 3 by the laboratory developing the assay and by an independent laboratory.

Interpretation and reporting of results. For increased specificity, positive samples are often re-extracted and reanalyzed, but such selective repeat testing of only positive specimens introduces deliberate bias toward decreased sensitivity while increasing specificity. Whenever feasible, those persons who conduct the assays and interpret the results for research studies should be blinded to the patient status (case patient or control) and results of other testing (antibody status, culture or tissue diagnostic results). The resulting publications should specify how blinding was ensured or why it was not done.

TISSUE DIAGNOSTICS (IMMUNOHISTOCHEMISTRY)

C. pneumoniae has been detected in tissue specimens by use of a variety of methods [15, 61]. Of those methods, immunofluorescence, immunohistochemistry (IHC), and in situ hybridization offer the advantage of preserving tissue morphology and permitting localization of the infectious agent to specific areas and cells. Cell types reported to be susceptible to infection include macrophages, endothelium, and smooth muscle [61]. Of the tissue diagnostic methods, IHC has been the most frequently used in studies of *C. pneumoniae,* and we will focus on this method. For IHC, careful interpretation is the critical challenge, because true-positive results of staining (figure 1) and false-positive results of staining (figure 2) can be very difficult to distinguish.

As of 1 May 2000, >20 publications have reported the detection of *C. pneumoniae* by use of IHC in atheromatous plaques obtained from diverse sites in human [32, 35, 38, 61–73] and animal subjects [74–76]. Detection rates in human atheromata have varied widely (21%–71%) between various laboratories [15, 38, 61, 70]. Studies that have reported detection of *C. pneumoniae* in the same atheromatous plaque by use of both IHC and other methods, such as culture or PCR, have shown poor correlation between the different methods [40, 61, 70]. Typically, detection rates that are determined by use of IHC are higher than those determined by use of PCR. This is attributed to several factors, including faster degradation of DNA (compared with antigens), difficulty in extraction of DNA from atheromas (mostly due to calcifications), and the presence of PCR inhibitors [61, 70, 77]. It is possible that these discrepancies may be due, in part, to false-positive and false-negative results of IHC. However, this has been difficult to evaluate in the absence of an accepted and standardized approach for both the methodology and interpretation of IHC results.

Figure 1. Positive results of staining of smooth muscle cells for *Chlamydia pneumoniae* in an atheroma *(arrow)* by use of the TT-401 antibody, an avidin-biotin complex (ABC) method, and horseradish peroxidase. Reprinted with permission from [63].

Review of Currently Used Tests and Recommendations for IHC

Procedure. The most widely used IHC assay is the avidin-biotinylated immune-complex method [78]. However, there are interlaboratory variations at different steps in the method, including pretreatment of the tissues, tyramide signal amplification, and colorimetric detection. Because the effect of variation in the aforementioned procedural steps has not been evaluated, we cannot yet recommend a standardized approach. An IHC study that compares these methodological issues in control tissues is recommended.

Antibodies. The majority of published studies have used CF-2, a monoclonal antibody directed against the lipopolysaccharide of all *Chlamydia* species [71, 75, 77, 79], or RR-402 and TT-401, 2 antibodies that are specific for *C. pneumoniae* [61, 80, 81]. There is some evidence of differences in reactivity of the various antibodies [82]. Use of CF-2 is a reasonable compromise for initial screening because no other *Chlamydia* species has been found in atheromas, but patients positively identified by use of this antibody should be further tested with specific antibodies for *C. pneumoniae* and *C. trachomatis* [34, 62, 63, 68, 72, 73].

Negative control antibodies must be used for every specimen to assess background staining of the tissue. Most reported studies have used the antibodies in mouse ascitic fluid as the only negative control. However, the best negative antibody control should be an antibody of the same isotype as the *Chlamydia*

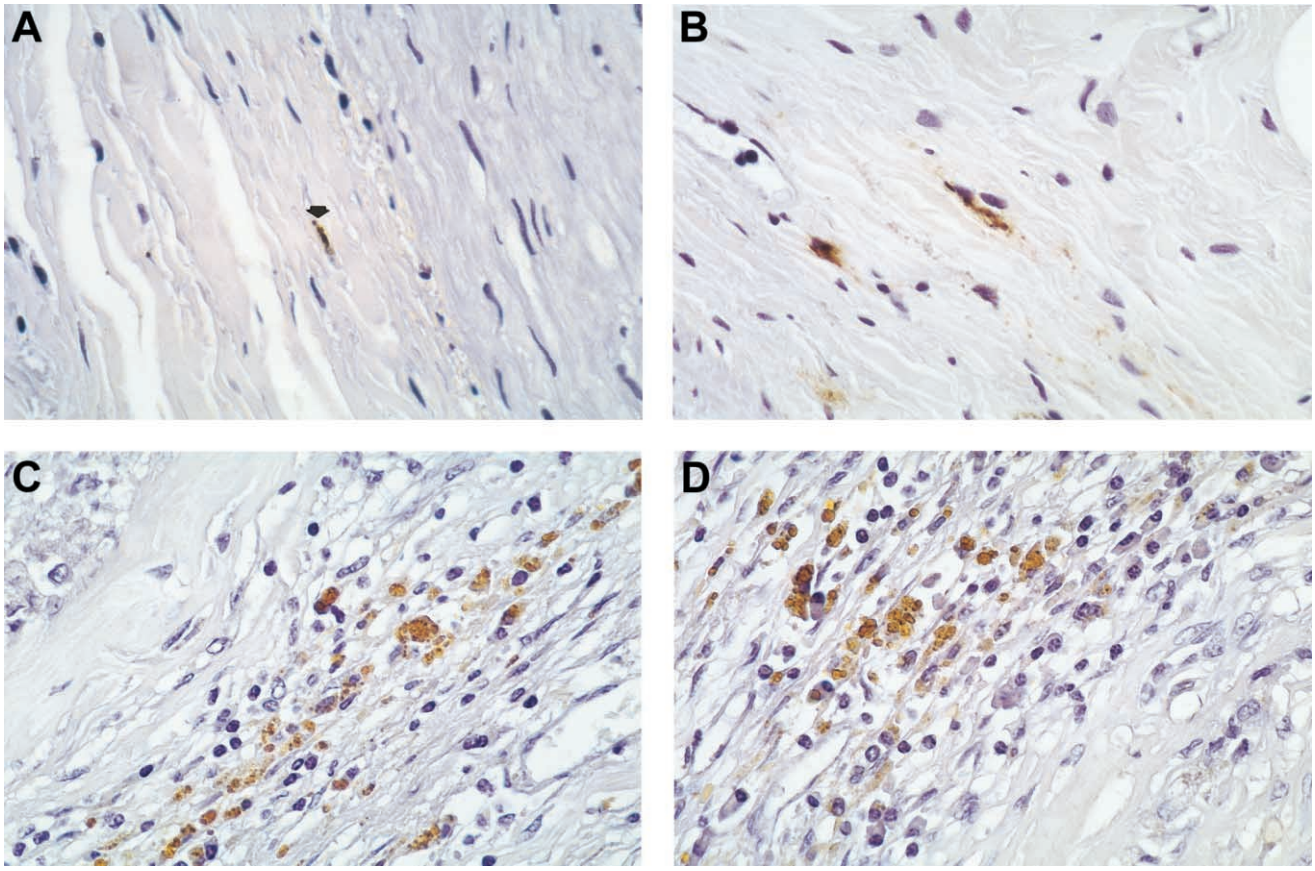

Figure 2. *A,* False-positive *Chlamydia* staining of smooth muscle cell in an atheroma *(arrow)* by use of the avidin-biotin complex (ABC) method, horseradish peroxidase, and a monoclonal antibody specific for *Chlamydia* (CF-2). *B,* The same tissue block showing similar staining with a nonspecific monoclonal antibody of the same isotype (IgG2a antibody to Lassa fever virus). *C,* False-positive *Chlamydia* staining of inflammatory cells (macrophages) by use of ABC, horseradish peroxidase, and a monoclonal antibody specific for *Chlamydia* (CF-2). *D,* The same tissue block showing similar staining with a nonspecific monoclonal antibody of the same isotype (IgG2a antibody to Lassa fever virus).

antibody being used in the assay. For example, if CF-2, which is an IgG2a antibody, is used, the negative control antibody should be an IgG2a antibody directed against a different infectious agent that does not cross-react with *Chlamydia* species. Therefore, we recommend the use of 2 negative control antibodies for each tissue block. The first should be either normal mouse ascitic fluid or hyperimmune serum, and the second should be a non-*Chlamydia* antibody of the same isotype.

Tissues and controls. Both fresh and formalin-fixed tissues have been used for IHC *Chlamydia* testing. One positive and one negative tissue control should be carefully selected and used consistently with each experimental run. Theoretically, infected human specimens should be the best positive tissue control. However, IHC staining for *C. pneumoniae* in human atheromas has been described as very focal and scanty, and it has not been possible to identify tissue blocks that consistently yield a positive result. Other positive control tissues that are currently used include infected tissue culture cells and tissue from experimentally infected animals. Negative tissue controls included in each run should either be uninfected cells of the same type as the *Chlamydia*-infected cells, or they should be specimens of normal artery, brain, lung, or other tissues (as appropriate for the experimental tissue). In addition, at least 2 sections from the same paraffin block as the case being studied should be incubated with negative antibody controls and included in each run. In summary, each staining run should include 1 positive and 1 negative tissue control incubated with the 2 positive and 2 negative antibodies that are used on the specimen of interest.

Interpretation of results. Establishing the difference between signal and background is the crucial issue. Interpretation of IHC for infectious diseases tends to rely more heavily on correlations between signal morphology (granular staining, intact bacteria) and histopathological context (type of cell associated with signal). Accurate interpretation requires specialized training and, at a minimum, the ability to consistently identify and distinguish the major inflammatory cells (polymorphonuclear cells, mast cells, and plasma cells) and pigments (lipofuscin, hemosiderin) in tissue sections. Only intracyto-

Table 4. Recommendations for standardizing *Chlamydia pneumoniae* diagnostic assays.

Assay type	Major recommendations
Serological testing	Microimmunofluorescence remains the only currently acceptable approach
	Acute infection is defined by a 4-fold rise in IgG or an IgM titer of ⩾16; use of a single elevated IgG titer is discouraged
	Past exposure is indicated by an IgG titer of ⩾16
	Neither elevated IgA titers nor any other serologic marker are validated indicators of persisting infection
Culture	Documentation of a positive culture result requires propagation of the isolate or PCR confirmation
	In the absence of propagation or PCR confirmation, an average of ⩾1 inclusion per culture well should be considered a presumptive positive culture
	The use of serum-free media, multiple centrifugations, or pretreatment of cells is not endorsed
PCR	Four of 18 currently published assays met proposed criteria for optimal validation
	Each PCR run should include low positive controls (⩽1 inclusion-forming units), and water controls every fifth extraction
Immunohistochemistry	Each tissue block should be tested with 2 *Chlamydia* antibodies and 2 control antibodies
	Each staining run should include 1 positive and 1 negative tissue control, each incubated with the 4 antibodies used on the specimen of interest
	Intracytoplasmic staining of macrophages, endothelial cells, or smooth muscle cells in a granular pattern may be considered positive; interpretation of a homogenous staining pattern is controversial

plasmic IHC staining of macrophages, endothelial cells, and smooth muscle cells should be considered a positive result [61]. An intracytoplasmic granular staining pattern in the correct location and in the absence of artifact (as assessed by means of control slides) constitutes a true-positive result. There is disagreement regarding whether a homogenous staining pattern in the proper context can be considered a true-positive result or whether such staining should always be regarded as artifact.

The key conclusions and recommendations that resulted from this meeting are summarized in table 4 and should provide immediate guidance to those working with current *C. pneumoniae* assays. In addition, meeting participants emphasized the need for concentrated efforts on several future research priorities, all of which should benefit from a standardized approach to assay use, interpretation, and quality control.

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