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Chlamydia pneumoniae and Atherosclerosis: Critical Assessment of Diagnostic Methods and Relevance to Treatment Studies

Jens Boman[1] and Margaret R. Hammerschlag[2]*

Department of Virology, Umeå University, Umeå, Sweden,[1] and Division of Pediatric Infectious Diseases, Department of Pediatrics, State University of New York Downstate Medical Center, Brooklyn, New York[2]

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ASSOCIATION OF *CHLAMYDIA PNEUMONIAE* AND CARDIOVASCULAR DISEASE

Role of Inflammation and Infection in Cardiovascular Disease

Atherosclerotic heart disease is the leading cause of morbidity in the Western Hemisphere, including the United States (37). The clinical manifestations of atherosclerosis range from coronary artery disease (CAD) to cerebrovascular disease. Conventional risk factors including cigarette smoking, hypertension, and high serum lipid levels do not fully explain the incidence, prevalence, and distribution of CVD. A number of studies have found that inflammation of the vessel wall plays an essential role in both the initiation and progression of atherosclerosis, erosion, and fissure and the eventual rupture of plaques (69, 70, 85, 88, 95, 116). The hypothesis that inflammation contributes to the pathogenesis of atherosclerosis is over 100 years old, dating back to Virchow in 1859 (140a) and Osler in 1908 (102a). Recent studies have demonstrated that various markers of systemic inflammation can predict future cardiovascular events including nonfatal and fatal myocardial infarction and stroke (2, 70, 115, 116). Acute-phase reactants such as C-reactive protein (CRP) and fibrinogen have been noted to be elevated in patients with unstable angina (88, 116). A meta-analysis of 14 studies found a significant association of elevated levels of CRP and fibrinogen with CAD (24). Therapy with aspirin, in addition to affecting platelet function, reduces inflammation as measured by levels of CRP and may prevent myocardial infarction by this mechanism (115). However, only subjects in the highest quartile showed any benefit of aspirin use; 75% of men showed marginal or no benefit.

Although inflammation is present, the exact cause of this inflammation in CVD is still not known. CRP is a nonspecific marker of various stimuli including tissue damage, smoking, and infection.

Infectious agents that have been investigated as possible stimuli include viruses, specifically cytomegalovirus, human herpesviruses, and enteroviruses, and bacteria including *Helicobacter pylori*, the agents of periodontal disease, and *Chlamydia pneumoniae* (6, 20–22, 31, 33, 88, 98, 103, 118, 122, 131).

* Corresponding author. Mailing address: Department of Pediatrics, Box 49, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203-2098. Phone: (718) 245-4075. Fax: (718) 245-2118. E-mail: mhammerschlag@pol.net.

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ASSOCIATION OF *C. PNEUMONIAE* AND CARDIOVASCULAR DISEASE: BACKGROUND

C. pneumoniae was first characterized in 1986 and is now recognized as a common cause of community-acquired respiratory infection in adults and children (11, 42). The first report of a possible connection between *C. pneumoniae* and atherosclerosis came from a serologic study performed in Finland in 1988 (124). Using the microimmunofluorescence (MIF) method, Saikku et al. found that patients with proven CAD were significantly more likely to have anti-*C. pneumoniae* antibodies than were control patients selected at random. Since this initial report, almost 500 papers have been published on the association of *C. pneumoniae* and atherosclerosis; almost 300 were published in 2000. The bibliography includes a large number of reviews of the subject, some quite extensive (16, 21, 29, 37, 69, 98). Seroepidemiologic studies were followed by studies in which the organism was identified in vascular tissue from patients with CVD by electron microscopy, PCR, and immunocytochemical staining (ICC). *C. pneumoniae* has also been isolated by culture from vascular tissue in a small number of studies (4, 59, 83, 113). Animal studies, including mouse and rabbit studies, have demonstrated that *C. pneumoniae* disseminates systemically after respiratory infection and also appears to either induce or enhance the development of atherosclerosis (35, 95, 123), although these results have not been consistent (145). A recent paper by Wright et al. (150) has demonstrated that infectious agents are not necessary for initiating murine atherosclerosis. These studies have led to primary- and secondary-intervention studies using antibiotics directed at a putative intravascular *C. pneumoniae* infection.

However, no single serologic, PCR, or ICC assay has been used consistently across all studies. The assays used are also not standardized. Recent studies of serologic and PCR assays for diagnosis of *C. pneumoniae* infection have suggested that there may be substantial interlaboratory variation in the performance of these tests (5, 108, 109, 113). This could lead to sizable random measurement errors resulting in underestimation of the effect due to regression-dilution bias; alternatively, systematic measurement biases, including lack of blinding of disease status, could lead to overestimation of risk. The issue of assay performance has rarely been addressed in most studies. The major purpose of this review is to examine the impact of methods on studies of the association of *C. pneumoniae* and CVD and the relevance to treatment studies.

ROLE OF SEROLOGIC TESTING IN DIAGNOSIS OF *C. PNEUMONIAE* INFECTION

Although the potential difficulties in performance of the MIF assay, including the subjective component in reading the assay results, have been mentioned numerous times (21, 23, 37), the extent of laboratory-to-laboratory variation and interpretation of the results was not specifically addressed. Similarly, the other serologic tests used such as enzyme immunoassay (EIA) and time-resolved fluorimetry (TRF) included nonstandardized in-house assays and commercially available kits. However, none of these assays are either cleared or approved by the Food and Drug Administration for the diagnosis of *C. pneumoniae* infection. One of the major issues not addressed in any of the seroepidemiologic studies is the correlation of serology with infection as defined either by isolation of the organism in culture or by PCR. Does an immunoglobulin G (IgG) or IgA titer of 64 really mean that a person has a chronic infection with *C. pneumoniae*?

MIF Assay

Initially, as isolation of *C. pneumoniae* was felt to be difficult, emphasis was placed on serologic diagnosis using the MIF assay. The MIF assay was first developed as a seropidemiologic tool for *C. trachomatis* infection (142). Grayston et al. (42) proposed a set of criteria for serologic diagnosis of *C. pneumoniae* infection by a modification of the MIF test, specifically using *C. pneumoniae* TW183 elementary bodies instead of the 15 *C. trachomatis* type strains. Acute infection was defined as a fourfold rise in IgG titer, a single IgM titer of ≥16, or a single IgG titer of ≥512. Past or preexisting infection was defined on the basis of an IgG titer between 16 and 512. It was further proposed that the pattern of antibody response in primary infection differed from that seen in reinfection. In initial infection, the IgM response appears about 3 weeks after the onset of illness and the IgG response appears at 6 to 8 weeks. In reinfection, the IgM response may be absent and the IgG occurs earlier, within 1 to 2 weeks. Because of the relatively long period until the development of a serologic response with the MIF test in primary infection, the antibody response may be missed if convalescent-phase sera are obtained too soon, i.e., earlier than 3 weeks after the onset of illness. The criteria for use of a single serum sample have not been correlated with the results of culture and are based mainly on data from adults. Acute, culture-documented infection can occur without seroconversion, especially in children (11, 56, 76). However, culture-positive, MIF-negative children do develop an immune response to *C. pneumoniae* as measured by immunoblot analysis (76). The correlation between culture and serology is higher in adults, but culture-documented infection in adults with pneumonia can also occur in the absence of seroconversion or detectable antibody by MIF (52).

Background rates of seropositivity by MIF can also be very high in some adult populations, often exceeding 80% (57, 68). Hyman et al. (57), as part of a study of asymptomatic *C. pneumoniae* infection among subjectively healthy adults in Brooklyn, N.Y., also found that 17% of these healthy culture- and PCR-negative individuals had evidence of "acute infection" (IgG of ≥512 and/or IgM of ≥16). Therefore, one cannot really establish or rule out a diagnosis of *C. pneumoniae* infection on the basis of a single acute-phase serum titer by MIF. Evidence also suggests that antibody titers (IgG and IgM) may fluctuate significantly over time. Dean et al. (26) described two patients with persistent *C. pneumoniae* respiratory infection, documented by culture, who were infected for up to 7 years. During this period, IgM and/or IgG MIF serology was diagnostic for infection at only 25 to 50% of visits when the patients were symptomatic and culture positive.

Results of immunoblotting studies have also suggested that there may be antigenic diversity between the isolates of *C. pneumoniae* that may result in different results if different antigens are used in the MIF assay (76, 140). Some IgG antibody may result from a heterotypic response to other chlamydial species since there are cross-reactions with the major outer

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membrane protein (MOMP) between the four species as well as cross-reactions due to the genus lipopolysaccharide (LPS) antigen (18, 106). Other organisms that have been reported as possibly causing cross-reactions in the MIF assay are *Bartonella* and *Bordetella pertussis* (62, 87). The latter species could be significant since adults with pertussis frequently present with a chronic cough or severe bronchitis, which is a clinical presentation often ascribed to *C. pneumoniae*. Recent studies have found significant homology between human and *C. pneumoniae* HSP60 and *Escherichia coli* GroEL (101). Harkonen et al. (55) also reported recently that picornavirus proteins share antigenic determinants with HSP60/65, including the HSP60 proteins of humans and *C. pneumoniae*, which conceivably could also lead to cross-reactions in the MIF assay.

Problems with Standardization of the MIF Assay

As discussed above, the MIF test is not standardized and many laboratories use in-house assays. Reading the slides has a very large subjective component and requires a very experienced microscopist. A recent study by Peeling et al. (108) attempted to address the problem of interlaboratory variation in the performance of the MIF test by sending a panel of 22 acute- and convalescent-phase sera to 14 different laboratories. Nine of the laboratories used an in-house MIF. The isolates used in the majority of the assays were either AR-39 or TW-183. Three laboratories used one of two commercially available kits, which used either AR-39 or Kajaani 6 as the test isolate. The remaining two laboratories used the Washington Research Foundation kit, which uses TW-183. The overall agreement (to get within one twofold dilution of the "gold standard" as read at the University of Washington) of all laboratories was 80%. The range was 50 to 100% depending on the isotype. Agreement for serodiagnostic criteria were 69% for negative, 68% for "chronic," and 87% for a fourfold increase of IgG titer.

Since it was not clear if this poor reproducibility was secondary to individual subjective differences in reading the slides or to the use of different assays with different antigens, Peeling et al. (109) performed another study comparing the University of Washington assay, which uses TW183, and a commercially available kit which uses AR-39 as the antigen (MRL Diagnostics, Cypress, Calif.). A panel of 10 sera was sent to 17 laboratories in the United States and Europe. Using the same criteria for agreement as the previous study, agreement for IgM titers in individual serum samples, using the University of Washington kit, ranged from 53 to 100% and agreement for IgG titers ranged from 47 to 100%. There was 100% agreement for IgG titers in only 3 of the 10 sera. Compared to the reference standard at the University of Washington, the agreement for the remaining seven sera ranged from 47 to 81%. The titers in the discrepant samples reported by the other laboratories tended to be lower than the reference standard. The results were similar for the MRL kit; the agreement ranged from 80 to 100% for IgM and 60 to 100% for IgG, but there was 100% agreement for IgG titers in only 2 of the 10 sera.

Enzyme Immununoassays

Although EIAs offer the promise of standardized performance and objective end points, none have been adequately evaluated compared to culture or PCR (27, 75, 110, 111, 140). Most have been compared only to MIF. None have Food and Drug Administration clearance or approval for use in the United States. One commercial assay, the Medac rELISA, uses a recombinant LPS antigen; others are based on LPS-extracted elementary bodies or synthetic peptides (140). EIAs based on LPS are genus specific and thus may have a higher risk for cross-reactions not only with other *Chlamydia* species but also with other genera of bacteria. These kits can measure IgG, IgM, and IgA antibodies, but the cutoffs vary from kit to kit and the criteria for a positive result (acute infection, past infection) can be very complex (75, 110, 111, 140). Kutlin et al. (75), in a study from the United States, compared the recombinant LPS EIA to culture and found sensitivities ranging from 13% for IgM antibody in children to 78% for either IgA or IgG antibody in adults with respiratory infection. Specificities compared to culture ranged from 21 to 91%. Recently Persson and Haidl (110) reported cross-reactions with the rEIA and parvovirus, primarily for IgM, but it was also seen to a lesser extent with IgG and IgA. This cross-reaction was not seen in MIF.

Serologic Diagnosis of Chronic *C. pneumoniae* Infection

Anti-*C. pneumoniae* IgA antibody has been promoted as a marker of chronic *C. pneumoniae* infection. The rationale for this is that as the biological half-life of serum IgA is less than 7 days, compared to 23 days for IgG, the presence of this antibody implies an active or persistent infection. Determination of IgA antibody by MIF is also dependent on the IgA conjugate used. The presence of rheumatoid factor and high levels of IgG can also interfere with determination of the IgA titers (64a, 140a). Wang and Grayston (143) examined sera from young adults with acute respiratory infections and older adults with chronic obstructive pulmonary disease and found that only 17 to 42% of those with IgG and/or IgM antibody titers suggestive of acute or recent infection by MIF had detectable anti-*C. pneumoniae* IgA.

Current Recommendations for *C. pneumoniae* Serologic Testing

As part of a recent workshop on standardization of *C. pneumoniae* diagnostic methods, the Centers for Disease Control and Prevention (27) proposed some modifications of the serologic criteria for diagnosis. Although the MIF test was considered to be the only currently acceptable serologic test, the criteria were made significantly more stringent. Acute infection, using the MIF test, was defined by a fourfold rise in the IgG titer or an IgM titer of ≥16; use of a single elevated IgG titer was discouraged. An IgG titer of ≥16 was felt to indicate past exposure, but neither elevated IgA titers nor any other serologic markers were felt to be validated indicators of persistent or chronic infection. Available data support these recommendations, especially the poor predictive value of a single high IgG titer (57, 68).

SEROEPIDEMIOLOGIC STUDIES

The original investigations of the association of *C. pneumoniae* and CVD were based on serology, predominantly using the MIF test. Saikku et al. (124) reported an association of

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TABLE 1. Diagnostic criteria for *C. pneumoniae* infection used in prospective seroepidemiologic studies

Study	Yr	No. of patients	Serologic test (isolate)	Cutoff titer	Comments
Saikku et al. (125)	1992	102	MIF (TW 183) LPS IC[a]	IgG ≥ 16 IgA ≥ 8	IgA > 64 considered chronic infection
Meittinen et al (93)	1996	202	MIF (Kajaani 6)	IgG ≥ 32 IgA ≥ 10	IgG ≥ 128 and IgA ≥ 40 considered elevated
Gupta et al. (44)	1997	20	MIF (IOL-207)	IgG ≥ 8 IgG > 64	
Ossewaarde et al. (103)	1998	54	EIA (TW 183)	NS[b]	
Strachan, et al. (132)	1999	278	MIF (NS)	IgG ≥ 16 IgA ≥ 16	
Ridker et al. (117)	1999	343	MIF (NS)	IgG ≥ 16	
Ridker et al. (118)	1999	122	MIF (NS)	IgG > 16 to IgG 128	
Altman et al. (1)	1999	227	Whole-cell IF (CWL-029)	IgG ≥ 32	
Nieto et al. (99)	1999	246	MIF (TW 183)	IgG ≥ 8	IgG ≥ 8–32 considered low positive IgG ≥ 64 considered high positive
Glader et al. (41)	2000	78	IC EIA (Medac rELISA)	IgG ≥ 32 IgA (0.7 OD[c])	IgG antibodies were detected in IC by MIF (Kajaani 6)
Danesh et al. (23)	2000	496	TRF	IgG—NS	
Wald et al. (141)	2000	647	TRF	IgG—NS IgA—NS	
Choussat et al. (20)	2000	71	MIF (NS)	IgG—NS IgA—NS IgM—NS	
Siscovick et al. (131)	2000	213	MIF (TW183)	IgG ≥ 8	IgG ≥ 8 considered past infection

[a] IC, immune complex.
[b] NS, not specified.
[c] OD, optical density.

anti-*C. pneumoniae* titers, specifically an IgG titer of >128 and/or an IgA titer of >32, with acute myocardial infarction (MI) and chronic CAD in a retrospective study of men enrolled in the Helsinki Heart Study. A subsequent, larger study from the same group also found an association of chronic *C. pneumoniae* infection, as defined by the above serologic criteria, with the risk of developing CAD; the odds ratios (OR) were 2.3 (95% confidence interval [CI], 0.9 to 6.2) for elevated IgA titers (125). These studies were followed by an additional 16 seroepidemiologic studies of the association of *C. pneumoniae* and CAD and cerebrovascular disease published through 1997, involving some 2,700 patients (21). These studies, and the topic of infection and CVD in general, were subjected to a detailed review and statistical analysis by Danesh et al. in 1997 (21). The majority of these studies were consistent in finding an association of *C. pneumoniae* antibodies and CVD, with ORs ranging from 1.2 to greater than 8. In some, there was also an association of increasing ORs with increasing antibody titers. Only one of these studies failed to find an association of seropositivity to *C. pneumoniae* with an increased risk of CVD (144). However, these studies varied greatly in terms of population, criteria for defining CVD, and adjustment for possible confounding variables such as age and smoking, which may make them prone to bias. Only 3 of these studies were prospective; 15 were cross-sectional with population or other controls. Most of these studies used MIF as the serology test. Only four of the studies specified that the microscopist performing the MIF was blinded to the clinical status of the patients. Most importantly, there was little consistency from study to study in the serologic criteria used to define past, chronic, or persistent infection by the MIF test. Various combinations of IgG and IgA and different antibody titer cutoffs were used. In some cases the titers selected were determined post hoc after the data had been analyzed as those that had the greatest association, and in some cases the same investigators used different serologic criteria in different studies (24, 92, 125, 136, 137). In most studies, sera were also obtained only once, which does not take into account the possibility that antibody titers may fluctuate over time (26).

Danesh et al. (23) published the results of a large population-based prospective study of the association of anti-*C. pneumoniae* IgG titers and CAD with another meta-analysis of 14 other prospective studies published through May 2000. Three of these studies were included in the original metanalysis published in 1997 (21, 44, 93, 125). These studies now included a total of 3,169 cases of nonfatal myocardial infarction or death from CAD. All studies adjusted for smoking and other classic risk factors for CAD. In contrast to the earlier series and meta-analysis, these recent prospective follow-up studies have not generally supported the association of *C. pneumoniae* infection, as defined by the presence of IgG and/or IgA antibody, and CVD. Danesh et al. (23) found a combined OR of 15 studies of 1.15 (95% CI, 0.97 to 1.36) for IgG antibody. In nine studies that reported risk in association with IgA titers, the combined OR was 1.13 (95% CI, 0.90 to 1.41) (23, 41, 99, 125, 132, 141).

There was a great deal of heterogeneity among these studies in terms of the serologic tests and criteria used for seropositivity. In some studies, an IgG and/or IgA titer of ≥64 was used as an indicator of chronic infection; in others, the same or similar criteria were used as indicators of past infection (Table 1). The serologic tests and criteria used in 12 of the prospective studies analyzed by Danesh et al. (23) and 2 subsequently published studies (1, 20) are shown in Table 1. Three of the

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prospective studies analyzed by Danesh et al. (23) were available only as abstracts or as a Letter to the Editor; therefore, details about the serologic methods used were limited. These are not included in the table. Nine studies used MIF assays, and all were in-house tests (20, 44, 93, 99, 117, 118, 125, 131, 132). One study used a commercial whole-cell indirect immunofluorescence assay (1). The antigen used was specified in only four of the MIF assays, two used TW-183 and one each used Kajaani 6 and IOL-207. Two studies used EIAs, and one used two different in-house-developed assays, one genus specific and one purportedly species specific. TW-183 was the antigen, and the elementary bodies were treated with sodium periodate to remove the genus-specific LPS antigen (103). The other study used a commercially available EIA, Medac rELISA, which uses a recombinant *Chlamydia* genus-specific LPS antigen (41). Two studies (23, 141), including the Danesh et al. (23), used TRF, which was previously reported to correlate well with MIF (149), but the antigen and criteria used were not specified. The IgG titers used as the cutoff for seropositivity in the studies that used MIF assays ranged from ≥8 to ≥32. Three studies defined an anti-*C. pneumoniae* IgG titer of ≥64 as indicating past or chronic infection, or as being "elevated"; one study used an IgG titer of ≥128; and one study did not specify a titer. In the studies that used an EIA or TRF, the titers were frequently not specified. The antibody levels in EIAs were expressed as optical density rather than titer, which makes comparisons to the results of the MIF studies difficult.

IDENTIFICATION OF *C. PNEUMONIAE* IN VASCULAR TISSUE

Early Detection Studies

The first study suggesting the presence of *C. pneumoniae* in atherosclerotic plaques of coronary arteries was performed by Shor et al. in South Africa (128). The study was done during autopsy of young adult men 8 to 36 h after death and on hearts received for examination at the Pathology Department. One thousand coronary arteries were examined, and 10 with early lesions were selected for electron microscopy (EM). All samples showed pear-shaped structures. Seven atheroma lesions were examined both by EM and by *Chlamydia* immunoperoxidase staining with *Chlamydia* genus-specific (CF-2), *C. pneumoniae*-specific (RR-402), and *C. trachomatis*-specific (KK-12) monoclonal antibodies. By EM, all seven cases showed vesicles with similar *C. pneumoniae*-like organisms in the lipid-rich core of the fibolipid plaque. Five (71%) of seven EM-positive cases were positive by immunoperoxidase staining with *Chlamydia* genus-specific and *C. pneumoniae* species-specific antibodies but negative for the *C. trachomatis* species. Coronary artery tissues from five control cases were negative by immunoperoxidase staining.

The specimens examined were selected from over 1,000 autopsies done over a 2-year period. Consequently, the finding should not be taken to indicate a strong association of *C. pneumoniae* with coronary artery atherosclerosis. However, the study was not designed to provide population-based data but, rather, to find evidence that the organism can be detected in atherosclerotic lesions. In a subsequent study, Kuo et al. (71) reported further data on the possible physical association of *C. pneumoniae* with coronary atherosclerosis in additional South African autopsy cases. In that study, ICC, PCR, EM, and isolation attempts were performed (Table 2). A total of 16 early lesions or fatty streaks and 20 later lesions or fibrolipid plaques of coronary arteries from 31 men and 5 women were studied. For the ICC, a *Chlamydia* genus-specific monoclonal antibody (CF-2) was used for initial screening of specimens and screening-positive samples were sectioned for staining using a *C. pneumoniae*-specific monoclonal antibody (RR-402). PCR was used with *C. pneumoniae*-specific primers. By ICC, 15 (42%) of 36 tested cases were positive; corresponding figures for the PCR were 13 (43%) of 30 analyzed samples. Of the 30 samples tested by ICC and PCR, only 8 (27%) were positive by both methods, 8 (27%) were positive by one method only (5 by PCR and 3 by ICC), and 14 (47%) were negative in both assays. EM revealed *Chlamydia*-like elementary bodies in 6 (29%) of 21 atheromatous plaques, of which 2 were positive by both ICC and PCR, 1 was positive by ICC only, 1 was positive by PCR only, and 2 were negative by both ICC and PCR. All attempts to isolate *Chlamydia* organisms were negative. Two cases were selected for sequence analysis of PCR-amplified products: the DNA sequence was identical to the *C. pneumoniae* sequence. The agreement between the methods was not very high, since only 2 cases (10%) out of 21 positive by either method were positive by all three detection methods (ICC, PCR, and EM) used (Table 2). Discrepancies may arise from testing different areas of the lesions and/or poor sensitivities, specificities, or both for the methods used in the study. Cell culture was not able to support the presence of *C. pneumoniae* in the plaques in any case, but the conditions were not optimal since postmortem samples obtained many hours to days after death was used. *C. pneumoniae* was detected in the plaques of six (75%) of the eight cases that were negative by serology. This is somewhat paradoxical since the original hypothesis that *C. pneumoniae* and atherosclerotic coronary heart disease (CHD) were related was based on increased seroprevalence in patients with CHD compared with controls (124). However, the serologic conditions may have been suboptimal since hemolysed postmortem sera were used.

After these two studies, four other studies from Seattle (17, 43, 72, 73) and one from London (102) reported positive findings on atheromatous tissue. To further investigate a proposed relationship between *C. pneumoniae* and CHD. Weiss et al. (144) examined coronary atheromas by PCR and cell culture from patients undergoing percutaneous atherectomy. Fifty-eight atheroma specimens were collected from 50 patients for *C. pneumoniae* culture in HEp-2 cells: all were negative after four blind passages. Fifty-six specimens from these 50 patients were studied by a *C. pneumoniae*-specific PCR assay with primers amplifying a 463-bp sequence of the 16S rRNA gene of *C. pneumoniae*. The PCR product was detected by hybridization with a biotin-labeled RNA probe and subsequent EIA. One atheroma specimen (2%) was positive twice, but a duplicate specimen from the same lesion was negative. Since no controls were used for extraction or inhibition, one cannot exclude inhibition as the reason for negative results. Twenty-two specimens from 22 other patients were examined by EM; no structures resembling *C. pneumoniae* were found, but all specimens contained typical foam cells, smooth muscle cells, and cholesterol clefts. Nasopharyngeal specimens for *C. pneumoniae* cul-

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TABLE 2. Detection of *C. pneumoniae* in atheromatous tissue specimens

Study	Yr	No. of specimens	Type of tissue[a]	Results [no. positive/total no. (%)] for:						No. analyzed by two Cpn test[c]	No. positive by two Cpn test[c]
				ICC genus antibody	ICC species antibody	PCR	Culture	EM	ISH		
Shor et al. (128)	1992	7	Coronary	5/7 (71%)	5/7 (71%)	ND[b]	ND	7/7 (100%)	ND	0	0
Kuo et al. (71)	1993	36	Coronary	15/36 (42%)	15/36 (42%)	13/30 (43%)	0/36 (0%)	6/21 (29%)	ND	30	8
Kuo et al. (72)	1993	21	Aorta	7/21 (33%)	7/21 (33%)	ND	ND	ND	ND	0	0
Kuo et al. (73)	1995	18	Coronary	7/18 (39%)	5/18 (28%)	3/18 (17%)	ND	ND	ND	18	2
Campbell et al. (17)	1995	38	Coronary	17/38 (45%)	17/38 (45%)	12/38 (32%)	ND	2/2 (100%)	ND	38	9
Grayston et al. (43)	1995	61	Carotid	36/61 (59%)	10/10 (100%)	8/30 (27%)	0/4 (0%)	ND	ND	(10)	(6)
Ong et al. (102)	1996	43	Arteries	ND	3/12 (25%)	19/43 (44%)	ND	ND	ND	12	3
Weiss et al. (144)	1996	80	Coronary	ND	ND	1/56 (2%)	0/58 (0%)	0/22 (0%)	ND	56	0
Muhlestein et al. (94)	1996	90	Coronary	71/90 (79%)	ND	ND	ND	3/5 (60%)	ND	0	0
Blasi et al. (9)	1996	51	Aorta	ND	ND	26/51 (51%)	ND	ND	ND	0	0
Ramirez et al. (113)	1996	10	Coronary	5/10 (50%)	5/10 (50%)	5/10 (50%)	1/10 (10%)	3/10 (30%)	1/10 (10%)	10	5
Juvonen et al. (65)	1997	12	Aorta	12/12 (100%)	8/12 (67%)	6/6 (100%)	0/3 (0%)	3/4 (75%)	ND	6	6
Juvonen et al. (66)	1997	17	AVS	15/17 (88%)	9/17 (53%)	3/17 (18%)	ND	2/3 (67%)	ND	17	1
Jackson et al. (58)	1997	57	Coronary	11/57 (19%)	8/57 (14%)	9/57 (16%)	ND	ND	ND	57	1
Jackson et al. (59)	1997	25	Carotid	8/16 (50%)	ND	6/25 (24%)	1/25 (4%)	ND	ND	25	0
Kuo et al. (74)	1997	39	Periph	8/20 (40%)	ND	5/39 (13%)	0/26 (0%)	ND	ND	26	0
Maass et al. (82)	1997	61	Carotid	ND	ND	9/61 (15%)	ND	ND	ND	0	0
Chiu et al. (19)	1997	76	Carotid	30/76 (39%)	41/76 (54%)	ND	ND	ND	ND	0	0
Nyström-Rosander et al. (100)	1997	39	AVS	ND	ND	19/39 (49%)	ND	1/1 (100%)	ND	0	1
Maass et al. (83)	1998	70	Coronary	ND	ND	21/70 (30%)	11/70 (16%)	ND	ND	0	11
Yamashita et al. (151)	1998	20	Carotid	11/20 (55%)	11/20 (55%)	ND	ND	ND	ND	0	0
Petersen et al. (112)	1998	40	Aorta	ND	ND	14/40 (35%)	ND	ND	ND	0	0
Lindholt et al. (80)	1998	124	Aorta	ND	ND	0/124 (0%)	ND	ND	ND	0	0
Paterson et al. (107)	1998	49	Carotid/coronary	ND	ND	0/49 (0%)	ND	ND	ND	0	0
Shor et al. (129)	1998	35	Various	3/7 (43%)	ND	15/35 (43%)	ND	11/22 (50%)	ND	0	0
Taylor-Robinson et al. (133)	1998	117	Various	ND	ND	17/117 (15%)	ND	ND	ND	0	0
Davidson et al. (25)	1998	60	Carotid	20/60 (33%)	2/3 (67%)	14/60 (23%)	ND	ND	ND	(3)	(2)
Andreasen et al. (3)	1998	23	AVS	ND	ND	0/23 (0%)	ND	ND	ND	0	0
Ouchi et al. (104)	1998	51	Coronary/iliac	18/50 (36%)	18/50 (36%)	20/51 (39%)	0/24 (0%)	ND	ND	50	12
Maass et al. (84)	1998	158	Coronary	ND	ND	34/158 (22%)	ND	ND	ND	0	0
Maass et al. (85)	1998	238	Arteries	ND	ND	51/238 (21%)	ND	ND	ND	0	0
Wong et al. (147)	1999	68	Artery/vein	ND	ND	26/68 (38%)	ND	ND	ND	0	0
Bartels et al. (6)	1999	38	Vein	ND	ND	8/32 (25%)	5/32 (16%)	ND	ND	32	5
Nadrchal et al. (97)	1999	43	Carotid/aorta	ND	ND	11/43 (26%)	ND	ND	ND	0	0
Thomas et al. (138)	1999	187	Coronary	ND	ND	68/187 (36%)	ND	ND	ND	0	0
Blasi et al. (32)	1999	30	Carotid	ND	ND	18/30 (60%)	ND	ND	ND	0	0
Jantos et al. (64)	1999	50	Coronary	ND	ND	4/50 (8%)	ND	ND	0/50 (0%)	0	0
Bartels et al. (7)	2000	125	Coronary/vein grafts	ND	ND	23/125 (18%)	12/125 (10%)	ND	ND	125	12
Apfalter et al. (4)	2000	38	Various	ND	ND	ND	3/38 (8%)	ND	ND	0	0
Berger et al. (8)	2000	130	Various	ND	ND	33/130 (25%)	ND	ND	ND	0	0
Gibbs et al. (38)	2000	98	Carotid	ND	ND	25/98 (26%)	ND	ND	ND	0	0
Farsak et al. (33)	2000	46	Various	ND	ND	12/46 (26%)	ND	ND	ND	0	0
Ericson et al. (30)	2000	60	Coronary	37/60 (62%)	38/56 (68%)	ND	ND	ND	ND	0	0
No. analyzed		2,679		676	443	2,294	451	97	60	502	76
No. positive				336	202	558	33	38	1		
% positive				49.7%	45.6%	24.32%	7.32%	39.18%	1.67%		15.14%

[a] AVS, aortic valve sclerosis.
[b] ND, not done.
Parentheses indicate that the number of specimens analyzed was biased. The values in parentheses are numbers of samples that were prescreened and positive for an ICC genus antibody.

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ture were obtained from 50 patients and 28 controls. One specimen in each group was culture positive. The negative culture finding in this study is in agreement with the results of Kuo et al. (71), who were unable to isolate *C. pneumoniae* from any of 36 autopsy specimens. Since the samples in the study by Weiss et al. (144) were quickly frozen at −70°C in *Chlamydia* transport medium, it is unlikely that the specimens contained viable *C. pneumoniae* organisms. The significantly lower detection rate by the *C. pneumoniae*-specific PCR compared to the results obtained by Kuo et al. (71) and Campbell et al. (17) is difficult to explain but may be due to differences in sensitivity and/or specificity for the different PCR assays used, including sample preparation and DNA extraction, different sample size with smaller specimens used by Weiss et al. (144), and different study populations. Also, the negative EM results are in contrast to those reported by Kuo et al. (71); it is worth noting that the specimens were processed and examined by the same investigators in Seattle in the study by Kuo et al. (71) and in this study by Weiss et al. (144).

Subsequent Detection Studies

After these initial studies on the detection of *C. pneumoniae* in vascular tissue, a large number of studies dealing with *C. pneumoniae* and atherosclerotic disease were performed to confirm or refute these early observation (3, 4, 6, 7–9, 19, 25, 30, 32, 33, 35, 38, 59, 64–66, 74, 80, 82–85, 94, 97, 100, 104, 105, 107, 112, 113, 129, 133, 138, 147, 151). When different methods, including PCR, ICC, culture, and EM, were used, the reported prevalence of *C. pneumoniae* in atheromatous tissue varied substantially (Table 2), and we now span the spectrum from research groups who claim to detect *C. pneumoniae* in vascular tissue commonly and easily to groups who cannot detect the bacterium in atherosclerotic tissue under any circumstances. It is recommended that persons processing the specimens and interpreting the results for research studies be blinded to the patient status and results of other testing (27). Unfortunately, most reports do not specify how blinding was ensured, which is of major concern. Some reports, however, include a careful description of how the blinding was performed (129). This section will focus on these detection studies, and especially on the methods used and potential methodological limitations in the studies. Between 1992 and 2000, more than 40 studies were published on the detection of *C. pneumoniae* in vascular tissue. The results of 43 studies are summarized in Table 2; these studies include 2,644 atherosclerotic specimens from various human arteries and some veins. The most commonly used techniques are ICC and PCR; other techniques used are cell culture, EM, immunofluorescence (IF), and in situ hybridization (ISH). By immunostaining techniques using a *Chlamydia* genus-specific antibody, 336 (49.7%) of 676 examined specimens were positive; with a *C. pneumoniae*-specific antibody, 202 (45.6%) of 443 examined specimens were reported as positive. Corresponding figures are 558 (24.3%) of 2,294 for PCR, 33 (7.3%) of 451 for culture and 38 (39.2%) of 97 analyzed specimens for EM. In most reports, all positive results obtained by either of the methods have been accepted as true-positive findings, despite often substantial disagreement and lack of concordance (Table 2). Using this approach, the prevalence of *C. pneumoniae* in atheromatous tissue may be very high. However, the interpretation of the discrepant results obtained by similar and different diagnostic methods is problematic in the absence of a "gold standard" and is of major concern. If a more reliable approach is used as a gold standard to define a positive specimen, i.e., a specimen (e.g., an atherosclerotic plaque) is accepted as true positive if it is positive by two (or more) independent *C. pneumoniae*-specific techniques, the prevalence of *C. pneumoniae* in atheromatous tissue will drop significantly to 84 (3.14%) of 2,679 atheromatous specimens. However, only 502 samples (18.74%) were analyzed by two (or more) independent *C. pneumoniae*-specific techniques (Table 2). If only these studies are included in the calculation, the prevalence is 16.73% for atheromatous tissue specimens. Since some of the studies used only ICC with a species-specific antibody on screening-positive samples (screening with genus-specific antibody), this calculation is biased, resulting in a very high prevalence of *C. pneumoniae*-positive specimens. If such studies are excluded from the calculation, the prevalence of true-positives specimens is 76 (15.14%) of 502, a figure very close to that obtained by cell culture (16%) in studies performed with several passages (5, 78). The total number of control specimens (n = 637) included in the detection studies on vascular tissue is much smaller (24%), and several reports have not included control tissue at all (Table 3). In studies that examined control "normal" tissue, the reported prevalence of *C. pneumoniae* has been low (Table 3), with 10 positives (3.21%) out of 312 samples for ICC using a *Chlamydia* genus-specific test, 12 (13.33%) out of 90 using a *C. pneumoniae* species-specific ICC test, and 25 (4.56%) out of 548 for PCR. None of 22 examined by culture, none of 3 examined by EM, and none of 2 analyzed by ISH were positive. The choice of controls has been criticized because of inadequate numbers and inadequate age matching (134). Thus, a lower prevalence of *C. pneumoniae* in control tissue may simply be due to the use of younger persons in the control group, i.e., persons with a lower prevalence of vascular *C. pneumoniae* infection (73). When choosing a control population, it is also important to take into account the geographical location of the subjects as well as the time (month and year) of the samples collected since infection with *C. pneumoniae* has been documented to occur in local epidemics.

Immunohistochemical Detection Methods

Antigen detection methods with the use of genus- or species-specific antibodies labeled with a peroxidase (immunocytochemistry and immunohistochemistry) or a fluorescent (immunofluorescence) marker are commonly used to identify *Chlamydia* in vascular tissue (Table 4). These techniques may all allow localization of the organisms in specific cells and areas. However, most of these techniques require a subjective reading. Considerable difficulty has been experienced with the immunofluorescence technique because of nonspecific background staining and the morphological heterogeneity of *C. pneumoniae* elementary bodies (102, 138). Further, background staining made the interpretation of the results of ICC difficult for 28 (80%) of 35 specimens tested by Shor et al. (129), and great difficulty was experienced with the ICC test by Ong et al. (102) because of nonspecific staining. Of major concern is that these techniques may yield results of decreased

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TABLE 3. Detection of *C. pneumoniae* in normal tissue specimens

Study	Yr	Controls[a]	No. of specimens	Results [no. positive/total no. (%)]						No. analyzed by two Cpn test	No. positive by two Cpn test
				ICC genus antibody	ICC species antibody	PCR	Culture	EM	ISH		
Shor et al. (128)	1992	Non-CAD	5	0/5 (0%)	0/5 (0%)	ND	ND	ND	ND	0	0
Kuo et al. (71)	1993	NS[c]	11	0/11 (0%)	0/11 (0%)	0/6 (0%)	ND	ND	ND	6	0
Kuo et al. (72)	1993	Aorta	4	0/4 (0%)	0/4 (0%)	ND	ND	ND	ND	0	0
Kuo et al. (73)	1995	Non-CAD	31	0/31 (0%)	ND	0/31 (0%)	ND	ND	ND	0	0
Grayston et al. (43)	1995	Non-ather	13	0/13 (0%)	ND	ND	ND	ND	ND	0	0
Ong et al. (102)	1996	Non-ather	6	ND	ND	3/6 (50%)	ND	ND	ND	0	0
Muhlestein et al. (94)	1996	Non-ather	24	1/24 (4%)	ND	ND	ND	ND	ND	0	0
Blasi et al. (9)	1996	Normal	51	ND	ND	0	ND	ND	ND	0	0
Ramirez et al. (113)	1996	Non-ather	2	0/2 (0%)	ND	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2	0
Juvonen et al. (65)	1997	Non-ather	12	0/12 (0%)	0/12 (0%)	0/12 (0%)	ND	ND	ND	12	0
Juvonen et al. (66)	1997	AV	18	ND	9/18 (50%)	ND	ND	ND	ND	0	0
Jackson et al. (58)	1997	Tissue	146	9/146 (6%)	3/8 (38%)	5/146 (3%)	ND	ND	ND	8	0
Kuo et al. (74)	1997	Normal	8	0/8 (0%)	ND	0/8 (0%)	ND	ND	ND	0	0
Maass et al. (82)	1997	Non-ather	39	ND	ND	0/39 (0%)	ND	ND	ND	0	0
Chiu et al. (19)	1997	Normal	20	0/20 (0%)	0/20 (0%)	ND	ND	ND	ND	0	0
Nyström-Rosander et al. (100)	1997	AV	11	ND	ND	1/11 (9%)	ND	0/1 (0%)	ND	0	0
Maass et al. (83)	1998	Non-ather	17	ND	ND	0	ND	ND	ND	0	0
Petersen et al. (112)	1998	Aorta	40	ND	ND	2/40 (5%)	ND	ND	ND	0	0
Ouchi et al. (104)	1998	Normal	12	0/12 (0%)	0/12 (0%)	0/12 (0%)	ND	ND	ND	12	0
Maass et al. (85)	1998	Normal	17	ND	ND	0/17 (0%)	ND	ND	ND	0	0
Wong et al. (147)	1999	Artery/vein	71	ND	ND	12/71 (17%)	ND	ND	ND	0	0
Bartels et al. (6)	1999	Vein	20	ND	ND	1/20 (5%)	0	ND	ND	20	0
Bartels et al. (7)	2000	Vein	20	ND	ND	1/20 (5%)	ND	ND	ND	0	0
Farsak et al. (33)	2000	Normal	39	ND	ND	0/39 (0%)	ND	ND	ND	0	0
No. analyzed			637	312	90	548	22	3	2	60	0
No. positive				10	12	25	0	0	0		
% positive				3.21%	13.33%	4.56%	0%	0%	0%		0%

[a] AV, aortic valve; Non-ather, nonatherosclerotic specimen.
[b] ND, not done.
[c] NS, not stated.

specificity when applied to atheromatous tissue (146). This may be due to the nonspecific staining of inflammatory cells present in inflamed atheromatous vascular tissue (134). Consequently, it is important to be certain that the antigenic components identified by this technique are *C. pneumoniae* specific and not due to a change of normal tissue antigenicity in atherosclerotic tissue. The variation of type, specificity, and avidity of different monoclonal antibodies may lead to false-positive and false-negative results (27). Detection rates vary widely (from 14 to 100%), and there is often poor correlation between antigen detection methods and DNA detection methods in the same atherosclerotic plaque (Table 2). It is possible that some of these discrepancies are due to false-positive results in the ICC test because of lack of an accepted and standardized approach to the method and interpretation of results (27). Ericson et al. (30) used two different immunological tests for detecting *C. pneumoniae* in atherosclerotic tissue: direct immunofluorescence and immunoperoxidase stain. While both tests were positive in a large proportion of specimens from patients with severe atherosclerosis (36 [86%] of 42 and 32 [80%] of 40, respectively), the agreement was low for specimens from patients with mild atherosclerosis (1 [6%] of 18 and 6 [38%] of 16, respectively). Consequently, it is important to be certain that these antigens are *Chlamydia* specific and not damaged atherosclerotic tissue that may have higher affinity for these antibodies than does normal vascular tissue (146). To obtain results as reliable as possible, guidelines have recently been formulated (27). For the choice of antibodies, it is recommended that two *Chlamydia* antibodies and two negative control antibodies always be used. It is also recommended that control tissue (positive and negative) be carefully selected and used consistently with each experiment and incubated with all four antibodies. Human and chlamydial heat shock proteins have highly conserved and homologous antigen sites (101). As a consequence, such antigens may interfere in tissue diagnostics, resulting in cross-reactions and false-positive results. Since only a very small portion of the lesion are generally examined by these in situ techniques, a negative result does not exclude the presence of *C. pneumoniae* in the plaque.

Cell Culture

Culture of the organism in cell culture is the prevailing gold standard used to demonstrate current infection by *C. pneumoniae* and to establish viability and thus infectivity. However, the use of culture for the detection of *C. pneumoniae* is prob-

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TABLE 4. Description of methods for antigen detection of *C. pneumoniae* in vascular tissue

Study	Yr	Method 1		Method 2		Control method	
		Technique	Antibody[a]	Technique	Antibody[a]	Technique	Antibody[a]
Shor et al. (128)	1992	ICC	CF-2	ICC	RR-402	ICC	KK-12
Kuo et al. (71)	1993	ICC	CF-2	ICC	RR-402	ND	
Kuo et al. (72)	1993	ICC	CF-2	ICC	RR-402	ICC	KK-12
Kuo et al. (73)	1995	ICC	CF-2	ICC	TT-401	ICC	KK-12
Campbell et al. (17)	1995	ICC	CF-2	ICC	TT-401	ND	
Grayston et al. (43)	1995	ICC	CF-2	ICC	TT-401	ICC	KK-12
Ong et al. (102)	1996	IF	NS[b]	NS[c]		ND	
Muhlestein et al. (94)	1996	IF	Baxter	ND		ND	
Ramirez et al. (113)	1996	ICC	NS	ICC	NS	ND	
Juvonen et al. (65)	1997	ICC	LPS	ICC	IHP	ICC	*F. tularensis*
Juvonen et al. (66)	1997	ICC	LPS	ICC	RR-402	ND	
Jackson et al. (58)	1997	ICC	CF-2	ICC	TT-401	ND	
Jackson et al. (59)	1997	ICC	CF-2	ND		ND	
Kuo et al. (74)	1997	ICC	CF-2	ND		ND	
Chiu et al. (19)	1997	ICC	*Chlamydia*-Cel-Pn	ICC	TT-401	ND	
Yamashita et al. (151)	1998	ICC	CF-2	ICC	AY-6	ICC	Virostat 1641
Shor et al. (129)	1998	ICC	CF-2	ND		ND	
Davidson et al. (25)	1998	ICC	CF-2	ICC	RR-402	ND	
Ouchi et al. (104)	1998	ICC	CF-3	ICC	RR-402, AY-6	ICC	KK-12, Virostat 1651
Ericson et al. (30)	2000	IF	Pathfinder	ICC	RR-402	ND	

[a] *C. pneumoniae*-specific antibodies: RR-402, TT-401, *Chlamydia*-Cel-Pn, AY-6; *Chlamydia* genus-specific antibodies: CF-2, Baxter, LPS, and Pathfinder; *C. trachomatis*-specific antibodies: KK-12, Virostat 1641, and Virostat 1651.
[b] NS, not stated.
[c] ND, not done.

lematic because of the difficulty in growing *C. pneumoniae* in cell culture, especially from tissue samples (27). As a result, culture results generated by different laboratories are not always reproducible. Some investigators report regular recovery of viable *C. pneumoniae* (6, 83), whereas other groups have reported low isolation rates (71, 74, 144).

The first study providing evidence for the presence of viable *C. pneumoniae* in the atheroma of a patient with severe coronary atherosclerosis was published 1996 (113); a second isolate from atheromatous tissue was recovered from a carotid artery (59). A highly sensitive culture system was developed by Maass et al. (83), who examined 70 coronary artery specimens for the presence of viable *C. pneumoniae* bacteria and for *C. pneumoniae* DNA. Atherosclerotic lesions from 53 coronary endarterectomy and 17 restenotic bypass specimens were cultured and subjected to nested PCR. Three segments per coronary sample were used; each plaque suspension was divided for PCR and cell culture in HEp-2 cells. The cell culture was performed with at least 10 serial passages and stained with a fluorescein isothiocyanate-conjugated *C. pneumoniae*-specific monoclonal antibody. For confirmation and enhancement of sensitivity of the nested PCR, nonradioactive DNA hybridization was performed. For control purposes, 7 arteries and 10 segments from the aortic wall, without macroscopic evidence of atherosclerosis, were available for PCR testing. Replicating *C. pneumoniae* isolates were recovered by cell culture from the occluded coronary arteries of 11 (16%) of 70 patients; 6 (54%) of the 11 isolates could be permanently propagated by serial subcultures. The isolates were identified in passages 2 to 5. Isolation was successful in all three arterial segments examined for six patients, in two segments for three patients, and in one segment only for two patients. The nested PCR yielded positive results with the arterial samples of 21 (30%) of 70 patients, including all culture-positive patients. Nine (82%) of the patients with a parallel positive culture result had positive PCR results in all three separately examined coronary artery segments, and two (18%) were positive in two of three segments. Of the 10 culture-negative, PCR-positive patients, 7 (70%) were positive for *C. pneumoniae* DNA in three segments and 3 (30%) were positive in two segments. All 17 control samples without macroscopically evident atherosclerosis were PCR negative. DNA sequence analysis of the PCR products of two continuously propagated coronary isolates revealed 100% sequence identity to a *C. pneumoniae* reference isolate. Thus, these cardiovascular strains do not appear to be different from respiratory tract isolates.

In this study, it was shown that it is possible to isolate viable *C. pneumoniae* from atherosclerotic coronary artery specimens if a sensitive culture system with several passages and serum-free medium are used. All isolates except two were confirmed by culture of a parallel segment of the same artery. Further, coronary artery specimens from all isolation-positive patients were PCR positive. None of the isolation- or PCR-positive patients was seronegative for *C. pneumoniae* in a *C. pneumoniae*-specific MIF test. Contamination is very unlikely since the PCR and culture results were in agreement and all 17 negative controls were negative. The results were confirmed in another study by the same group (85) and in a study by another group (4). Since increased number of passages may result in a concentration of cell debris resulting in nonspecific staining (27) and since the risk of contamination increases with increased number of passages, it is important to include several

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negative controls on each plate. It is important to note that true-positive *C. pneumoniae* cultures produce inclusions, not just elementary bodies, and can be passaged and detected by PCR.

PCR

The expansion of *C. pneumoniae* from a respiratory pathogen to its association with chronic diseases including asthma (48, 49) and atherosclerosis (124, 128) and the shortcomings of classical diagnostic methods have created a requirement for sensitive methods to detect this organism. Nucleic acid amplification technology was employed to meet this need. The ability of the PCR method to rapidly amplify small amounts of specific DNA or RNA, often in a background mixture of nucleic acids from the host and from other microorganisms, has made this technique a potentially valuable diagnostic tool. By PCR it may be possible to detect DNA and RNA of microorganisms that are present in small numbers, nonviable or growing slowly, or present in material not suitable for culture. However, several problems remain, mostly involving sensitivity and specificity issues and validation of new assays (14). Important issues include the choice of appropriate primers, optimization of the assay, extraction of nucleic acids, detection of amplification products, and prevention and identification of false-positive and false-negative results (14). Therefore, before nucleic acid amplification techniques can be widely adopted for the detection of *C. pneumoniae*, these problems must be solved. Since a variety of different PCR-based assays have been developed, another important issue is the comparison of these methods and the standardization of protocols. At present the lack of a consensus standard makes it difficult to evaluate methods, and, thus far, few PCR tests for detecting *C. pneumoniae* DNA have been sufficiently evaluated (27). The PCR technique is commonly used, alone or in combination with other diagnostic methods (Tables 2 and 5), for the detection of *C. pneumoniae* in the cardiovascular system. *C. pneumoniae* DNA has been detected in atherosclerotic plaques in coronary arteries (17, 83, 128), carotid arteries (19, 43, 59, 82), arteries of the lower extremities (74), abdominal aortic aneurysms (65, 102, 112), sclerotic heart valves (66, 100), and pulmonary arteries (129) and in atrial tissue from heart transplant donors (133). However, the frequency of positive results varies considerably (from 0 to 100%) among the studies (3, 65, 80); the main reason for this variability may be the use of different DNA extraction procedures and PCR protocols. Consequently, even if samples from the same patients are investigated by different laboratories, the variation of positive findings may be significant (5, 113). The most important steps in identifying false-positive results include the use of several negative tissue controls in each run. These controls should be processed in parallel with the clinical specimens throughout the DNA extraction and detection of amplification product. To further increase the specificity, one may need to reextract and reanalyze all positive results.

In a multicenter investigation, Ramirez et al. (113) determined the frequency of *C. pneumoniae* in coronary arteries of the native heart of 12 heart transplant patients, 10 of whom had verified CAD. The tissue was sent to nine different laboratories; culture was performed at three laboratories, four laboratories used PCR assays to analyze coronary artery tissue for *C. pneumoniae* DNA, ICC was done on slides prepared from paraffin-embedded tissue blocks at two laboratories, EM was done in one laboratory, and another laboratory performed in situ hybridization. Of 10 specimens from patients with coronary atherosclerosis, 1 was culture positive for *C. pneumoniae* in all three of the study sites that performed culture. Of 10 patients with atherosclerosis, 5 (50%) were PCR positive in assays performed in at least one of the four participating laboratories that used PCR. The reported prevalence of *C. pneumoniae* DNA determined by PCR varied between 4 of 10 (40%) and 0 of 10 (0%). Eight samples collected from two patients in this study without atherosclerosis were negative by PCR; of 40 samples collected from 10 patients with coronary artery disease, 7 were positive by PCR. Of 10 patients with atherosclerosis, 5 (50%) were positive by ICC performed at at least one of the two sites that used ICC; however, samples from only 2 patients (20%) were ICC positive at both sites. Only one specimen was positive by in situ hybridization (a coronary artery specimen collected from the culture-positive patient). Specimens from 3 (30%) of 10 patients with atherosclerosis showed structures that were compatible with *Chlamydia* organisms by EM. Although this study was the first attempt to deal with the issue of laboratory-to-laboratory variation in detection methods, it has been criticized (14) because the laboratories received different pieces of tissue and *C. pneumoniae* may not be distributed uniformly in tissue. To clarify whether the discrepancies in positivity rates by PCR for *C. pneumoniae* in vascular tissue are due to methodological differences or whether they reflect differences in the amount of *C. pneumoniae* in the tissue samples, Apfalter et al. (5) have initiated and coordinated a multicenter study of different PCR methods for the detection of *C. pneumoniae* by PCR in vascular tissue. Nine laboratories with experience in PCR analysis of *C. pneumoniae* in vascular tissue received experimental panels consisting of aliquots of 20 samples identical in composition. The samples included 15 homogenized clinical coronary and carotid atheroma specimens and 5 porcine aorta specimens, 4 of which were spiked with dilutions of *C. pneumoniae* and 1 which was unspiked. The positivity rates ranged from 0 to 60% for the 15 clinical atheroma specimens; the maximum concordance was 25% for one specimen. There was no consistent pattern of positive results among the various laboratories, and the positivity rate for individual assays did not correlate with the sensitivity of the assays. For 3 (19%) of 16 assays, the negative control was reported as positive. These results indicate that the reported variability in *C. pneumoniae* prevalence in vascular tissue obtained by PCR may be due primarily to methodological differences rather than differences in the amount of *C. pneumoniae* in the tissue. A new study is under way in an attempt to determine whether the differences between different laboratories is due to differences in DNA extraction methods or differences in the PCR methods themselves, or both. Several important methodological issues must be considered to avoid false-positive results, such as aliquoting all reagents, using only plugged pipette tips, including buffer and DNA extraction controls, using only positive control DNA with low copy number, etc. (14).

Jantos et al. (64) have also shown that the choice of DNA detection method is important. Coronary atherectomy speci-

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TABLE 5. Description of PCR and extraction methods for detection of *C. pneumoniae* in vascular tissue[a]

Study	Yr	DNA extraction	PCR method 1	Target gene	PCR method 2	Target gene	PCR method 3	Target gene	PCR method 4	Target gene
Kuo et al. (68)	1993	IH	S + H	Cloned *Pst*I	ND[b]	16S rRNA				
Kuo et al. (73)	1995	IH or Qiagen	S + H	Cloned *Pst*I	ND					
Campbell et al. (17)	1995	IH or Qiagen	S + H	Cloned *Pst*I	ND					
Grayston et al. (43)	1995	IH or Qiagen	S + H	Cloned *Pst*I	ND					
Ong et al. (102)	1996	In house	N + H	MOMP coding	ND					
Weiss et al. (144)	1996	In house	S + EIA	16S rRNA	ND					
Blasi et al. (9)	1996	In house	N	16S rRNA	ND					
Ramirez et al. (113)	1996	ns	S	16S rRNA	S + EIA	16S rRNA	S + H	Cloned *Pst*I	MOMP coding	NS
Juvonen et al. (65)	1997	IH + Qiagen	S + H	MOMP coding	ND					
Juvonen et al. (66)	1997	IH + Qiagen	S + H	MOMP coding	ND					
Jackson et al. (58)	1997	IH	S + H	Cloned *Pst*I	ND					
Jackson et al. (59)	1997	IH	S + H	Cloned *Pst*I	ND					
Kuo et al. (74)	1997	IH	S + H	Cloned *Pst*I	ND					
Maass et al. (82)	1997	IH	N + H	Cloned *Pst*I	ND					
Nystrōm-Rosander et al. (100)	1997	IH	N	16s rRNA	ND					
Maass et al. (83)	1998	IH	N + H	Cloned *Pst*I	ND					
Petersen et al. (112)	1998	Qiagen	N	MOMP coding	ND					
Lindholt et al. (80)	1998	IH	N + TRF	MOMP coding	ND					
Paterson et al. (107)	1998	IH	N	MOMP coding	ND					
Shor et al. (129)	1998	IH	N + H	MOMP coding	ND					
Taylor-Robinson et al. (133)	1998	IH	N + H	MOMP coding	ND					
Davidson et al. (25)	1998	IH	S + H	Cloned *Pst*I	ND					
Andreasen et al. (3)	1998	IH	S + H	16S rRNA	ND					
Ouchi et al. (104)	1998	IH	N + RD	Cloned *Pst*I	ND					
Maass et al. (84)	1998	IH	N + H	Cloned *Pst*I	ND					
Maass et al. (85)	1998	IH	N + H	Cloned *Pst*I	ND					
Wong et al. (147)	1999	IH	N + H	MOMP coding	ND					
Bartels et al. (6)	1999	IH	N + H	Cloned *Pst*I	ND					
Nadrchal et al. (97)	1999	IH	N	16S rRNA	ND					
Thomas et al. (138)	1999	IH	N + H	MOMP coding	ND					
Blasi et al. (32)	1999	TriPure	N	Cloned *Pst*I	RT-PCR	16S rRNA				
Jantos et al. (64)	1999	IH + Qiagen	S + H	16S rRNA	N	MOMP coding				
Bartels et al. (7)	2000	IH	N + H	Cloned *Pst*I	ND					
Berger et al. (8)	2000	IH	Q-PCR	16S rRNA	ND					
Gibbs et al. (38)	2000	IH	N	Cloned *Pst*I	ND					
Farsak et al. (33)	2000	Ih	S	16S rRNA	ND					

[a] S, single-step PCR; N, nested PCR; H, hybridization; IH, in house; RD, restriction enzyme digestion; RT-PCR, reverse transcriptase PCR.
[b] ND, not done.

mens from 50 patients with CHD were examined for the presence of *C. pneumoniae* by two different PCR methods and by ISH. Specimens from four patients (8%) were positive by an *omp1*-based nested PCR method, specimens from two (4%) patients were positive by a single-step 16S rRNA-based PCR, and *C. pneumoniae* was not detected by ISH in any of the cardiovascular tissues tested. ISH was previously used by Ramirez et al. (113). The only culture-positive specimen in that study was also the only specimen that was positive by ISH.

Correlation of the Presence of *C. pneumoniae* with the Degree of Atherosclerosis

Some authors have reported a higher prevalence of *C. pneumoniae* in more advanced atherosclerotic lesions when using immunological antigen detection techniques (30, 73). It is not yet known if this is due to a higher prevalence of *C. pneumoniae* in advanced atherosclerotic tissue or is due to a lower specificity because of the damaged blood vessels, since other methods have shown discordant results with a similar prevalence of *C. pneumoniae* in mild and advanced atherosclerosis. By means of PCR, Thomas et al. (138) correlated the prevalence of *C. pneumoniae* with the severity of atherosclerosis based on the Stary classification. The distribution of *C. pneumoniae* did not match the extent of severity of atherosclerosis since the prevalence was as high in mild lesions as in severe ones. Davidson et al. (25) found no difference in the severity of atherosclerosis in subjects with and without *C. pneumoniae* present in coronary arteries. Shor et al. (129) reported a strong association between atheromatous lesions and *C. pneumoniae*; however, minimal lesions were *C. pneumoniae* positive as often as severe lesions were.

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Alternative Types of Specimens

Serologic analyses are not useful for predicting carriage of *C. pneumoniae* in the vascular wall (84), but several investigators have reported that it is rare with a vascular *C. pneumoniae* infection in *C. pneumoniae*-seronegative adult individuals (9, 82, 84, 112). Thus, at the best, serology may be used as a screening tool. PCR-based detection of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMCs) may prove useful for identifying *C. pneumoniae* carriers and to measure the effect of antichlamydial therapy, since *C. pneumoniae* may be harbored in circulating white blood cells (8, 10, 12, 13, 15, 67, 86, 135, 148). However, the reported prevalence of *C. pneumoniae* in PBMCs has varied significantly, ranging from 9 to 59% of patients with CAD and from 17 to 46% of healthy blood donors (12, 13, 148). Therefore, systematic efforts to optimize and standardize the tests and test procedures are urgently needed. Standardized quantitative nucleic acid amplification methods for the detection of *C. pneumoniae* in circulating white blood cells and vascular tissue may enable better identification of individuals with persistent and vascular *C. pneumoniae* infection and improve the diagnostic tools in antibiotic treatment trials. It has not yet been shown that carriage of *C. pneumoniae* DNA in circulating white blood cells in humans represents a viable infection. In only one study of *C. pneumoniae* DNA in circulating white blood cells have the PCR-generated results been confirmed by another detection method (12).

Correlation of Serology with Identification of *C. pneumoniae* in Vascular Tissue

The major assumption of many of the numerous seroepidemiologic studies is that the presence of anti-*C. pneumoniae* antibody implies the presence of the organism in the vascular system. The results of several studies have found that *C. pneumoniae* serology frequently does not correlate with identification of the organism by PCR or ICC in atheromas and *C. pneumoniae* DNA in circulating peripheral blood monocytic cells (7, 8, 17, 67, 84). Campbell et al. (17) did not observe an association between detection of *C. pneumoniae* in native or restenosed coronary arteries and MIF antibodies. Maass et al. (84) detected *C. pneumoniae* DNA by PCR in atheromas from 34 (26.6%) of 128 patients undergoing coronary surgery. There was no significant difference in the proportion of *C. pneumoniae*-positive and *C. pneumoniae*-negative patients who had detectable anti-*C. pneumoniae* IgG (100 and 81%, respectively) or IgA (38 and 46%, respectively) by MIF. An IgG titer of ≥16 was used to indicate seropositivity; the cutoff titer for IgA was not specified. The geometric mean titers also did not differ significantly. A subsequent study by the same group examined coronary arteries or occluded vein grafts from patients undergoing primary or repeat bypass surgery (7). Although *C. pneumoniae* was detected by culture and/or PCR in 19 and 18% of the native coronary arteries and grafts, respectively, there was no statistical difference between the IgG titers in the *C. pneumoniae*-positive or -negative patients.

Berger et al. (8) found *C. pneumoniae* DNA in 25% of atherosclerotic plaques and 20% of PBMCs in 130 patients who underwent surgery for carotid stenosis, aneurysm, or peripheral vascular disease. In addition to finding that the presence of *C. pneumoniae* in the plaque and PBMCs did not coincide, there was no correlation with IgG or IgA titers determined by MIF. Overall, 73% of the patients had an IgG titer of ≥64 and 68% had an IgA titer ≥ 64. There was no significant difference between the prevalence of antibody or titers of IgG or IgA in the patients who tested positive for *C. pneumoniae* DNA compared to those who were negative. Similar results were reported by Kaul et al. (67), who found *C. pneumoniae* DNA in PBMCs of 13 (43.3%) of 30 patients with CAD and 5 (26.3%) of 19 healthy age- and sex-matched blood donors. Only 8 (62%) of 13 PCR-positive patients with CAD and 3 (60%) of 5 PCR-positive blood donors were found to be seropositive by MIF (IgG ≥ 32).

Conclusion: Detection of *C. pneumoniae* in Vascular Tissue

The increasing interest in chronic *C. pneumoniae* infections has led to many new investigators and laboratories involved in *C. pneumoniae* research; the focus of most of these investigations has been on *C. pneumoniae* and atherosclerosis. Since the majority of the tests used are in-house developed, and the results from many of the studies are contradictory and confusing, standardization of the methods has been called for (14). Therefore, guidelines have recently been formulated to ensure that results as reproducible and comparable as possible are obtained (27).

Different detection methods have provided evidence that *C. pneumoniae* is present in a proportion of atherosclerotic blood vessels, but the true prevalence is still unknown because of divergent inter- and intralaboratory results, as a result of insufficient standardization of the methods used. In this paper, we suggest a gold standard that includes two independent *C. pneumoniae*-specific tests. Using this standard, we have calculated the prevalence of *C. pneumoniae* in atheromatous vascular tissue to be approximately 15% (Table 2). This percentage may be an underestimation of the true prevalence, but it may also be an overestimation as a result of poor validation of the methods used in most of the studies. With few exceptions (102, 147), the studies that have included controls indicate that the presence of *C. pneumoniae* (or some antigens of the bacterium) in atherosclerotic lesions is more frequent than in normal, nonatherosclerotic tissue, but the choice of control tissue has been criticized (134) and several major questions remain unresolved, for example, what is the true prevalence of *C. pneumoniae* in atheromatous tissue? What is the true prevalence of *C. pneumoniae* in normal, carefully matched, vascular tissue? What is the true prevalence of viable *C. pneumoniae* infection in such tissue? Is the higher prevalence detected by ICC compared with other techniques, including PCR (17, 43, 58, 66, 73), a result of higher sensitivity or lower specificity? Is the bacterium an innocent passenger, or is it involved in the initiation or progression of disease?

If *C. pneumoniae* is present in atherosclerotic vascular tissue, it should be emphasized that this suggests an association but does not establish an etiologic relationship between *C. pneumoniae* and atherosclerosis; it may simply represent a secondary event reflecting inflammation.

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TABLE 6. Comparative in vitro activities of various antimicrobials against *C. pneumoniae*[a]

Antimicrobial agent	MIC range (μg/ml)
Doxycycline	0.015–0.25
Erythromycin	0.015–0.25
Roxithromycin	0.0625–2
Azithromycin	0.05–0.25
Clarithromycin	0.004–0.03
Ciprofloxacin	1–4
Ofloxacin	0.5–2
Levofloxacin	0.25–1
Moxifloxacin	0.125–1
Gatifloxacin	0.125–0.25

[a] Data from references 39, 42, 52–54, 81, and 119–121.

CLINICAL TREATMENT INTERVENTION STUDIES

Although recent seroepidemiologic studies and evaluations of the performance of MIF and PCRs are creating considerable uncertainty regarding the existence of a causal relationship of infection with *C. pneumoniae* and CVD, several antibiotic trials have been published or are ongoing. The underlying rationale is that antibiotic treatments that are effective against *C. pneumoniae* may lower the risk for further cardiac events in patients with CVD by eradicating the organism from plaques, thus stabilizing the plaques (90). These studies fall into two categories, retrospective case-control studies of past antibiotic use and subsequent risk of MI and prospective, placebo-controlled treatment studies. However, even data on the microbiologic efficacy of treatment of respiratory infections due to *C. pneumoniae* are limited. None of the regimens being used in these trials have actually been evaluated or demonstrated to be effective in the eradication of *C. pneumoniae* from respiratory sites.

Treatment of *C. pneumoniae* Infection: Background

C. pneumoniae is susceptible in vitro to a wide range of antimicrobial agents, including macrolides, tetracyclines, and quinolones, that target either protein or DNA synthesis (Table 6) (39, 52, 53, 54, 119, 120, 121). Recently, Gieffers et al. (39) reported that the in vitro susceptibilities of five isolates of *C. pneumoniae* cultured from vascular tissue did not differ significantly from those of respiratory isolates. However, in vitro activity may not always predict in vivo efficacy. Anecdotal data suggest that prolonged therapy (i.e., at least 2 weeks) may be desirable since recrudescent symptoms have been described following 2-week courses of erythromycin and even after 30 days of tetracycline or doxycycline therapy (42, 51). Practically all pneumonia treatment studies evaluating new macrolides/azalides and quinolones presented or published to date have used serology alone for diagnosis, essentially limiting themselves to a clinical end point. In 1990, Lipsky et al. (81) described four patients with bronchitis and pneumonia, treated with a 10-day course of ofloxacin, who were retrospectively identified as having serologic evidence of *C. pneumoniae* infection (fourfold rise in IgG/IgM titer, single IgM titer of ≥16 or IgG titer of ≥512). All reportedly demonstrated marked clinical improvement. Based on the MICs of ofloxacin to three laboratory strains (1 to 2 μg/ml), the authors concluded that ofloxacin was effective in these patients since the MICs were lower than achievable serum levels. Subsequent treatment studies have followed this premise: if the patient has serologic evidence of infection and clinically improves, the organism has been presumed to be eradicated. Another example is the multicenter treatment study reported by File et al. (34) in 1997. They found a clinical cure rate of 98% among patients with serologic evidence of *C. pneumoniae* infection who were treated with levofloxacin compared to 93% of those treated with ceftriaxone and/or cefuroxime axitil, plus erythromycin or doxycycline, which was added to the treatment regimen at the investigator's discretion. In the latter group, the response rate did not differ between patients who had erythromycin or doxycycline added to their treatment regimen and those who were treated with a cephalosporin alone. There was also no difference in the response rate among patients who had "definite" serologic evidence of infection, i.e., a fourfold rise in MIF IgG or IgM titer, compared to those who had "probable" infection, i.e., a single IgG titer of ≥512 or IgM titer of ≥32. The success of the cephalosporin regimens supports concerns about the specificity of the serologic criteria since these antibiotics have no or poor activity against *Chlamydia* spp. in vitro (50). A number of subsequently published treatment studies have claimed microbiologic eradication, despite the fact that cultures were not done (54). Conversely, the results of studies that have assessed microbiologic efficacy have frequently found that patients improve clinically despite persistence of the organism.

There are only five published pneumonia treatment studies that have utilized *C. pneumoniae* culture and assessed microbiologic efficacy. Block et al. (11) found that a 10-day treatment with erythromycin or clarithromycin suspension eradicated *C. pneumoniae* from the nasopharynx of 86 and 79% of culture-positive children with community-acquired pneumonia, respectively. All these children improved clinically despite persistence of the organism. Persistence did not appear to be related to the development of antibiotic resistance since all the isolates remained susceptible to erythromycin and clarithromycin during and after treatment (119). The experience with azithromycin has been similar. In an open noncomparative multicenter pneumonia treatment study (120), adolescents and adults aged 12 years or older were given 1.5 g of azithromycin orally over 5 days. *C. pneumoniae* was eradicated from the nasopharynx of 7 (70%) of the 10 culture-positive patients with community-acquired pneumonia after treatment. Harris et al. (56) reported that after treatment, *C. pneumoniae* was eradicated from the nasopharynx of 19 of 23 (83%) culture-positive children with community-acquired pneumonia, 6 months through 16 years of age, who received azithromycin and 4 of 4 and 7 of 7 who received amoxicillin-clavulanate and erythromycin, respectively ($P = 0.9$, χ^2 test square). The MICs and MBCs for three of nine isolates obtained after treatment from two of seven persistently infected patients in both studies who were treated with azithromycin increased fourfold after treatment, although they were still within the range considered susceptible to the antibiotic (120). It is not clear if this was an isolated event or suggestive of possible development of resistance. All patients improved clinically despite persistence of the organism. The results of two pneumonia treatment studies in adults which evaluated levofloxacin and moxifloxacin found eradication rates of 70 to 80% (52, 53). As seen with the children, all the patients who were microbiologic failures im-

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TABLE 7. Summary of placebo-controlled clinical trials for secondary prevention of CVD

Study	Condition	Serologic inclusion criteria	No.	Intervention[a]	Outcome	Result (treatment vs placebo)
Gupta et al. (44)	Post-MI	MIF IgG ≥ 64	60	Azithromycin, 500 mg/day × 3 days, 500 mg/days × 6 days	Any coronary ischemic event	8% vs. 25% (P = 0.03) at 18 mo
Gurfinkel et al. (47)	Unstable angina	None	202	Roxithromycin, 150 mg bid × 30 days	Recurrent CVD (triple end point)	7% vs. 15% (P = 0.26) at 180 days
Muhlestein et al. (96)	CAD (prior MI or bypass)	MIF IgG ≥ 16	302	Azithromycin, 500 mg/day × 3 days, 500 mg/wk × 3 mo	Recurrent CVD	14.6% vs 16.4% (P = 0.74) at 24 mo
WIZARD (28)	History of MI	MIF IgG ≥ 16	7,000	Azithromycin, 600 mg qid × 3 days, 600 mg/wk × 11 wk	Death, MI, unstable angina, repeat bypass	Ongoing
ACES (60)	CAD	None	4,000	Azithromycin, 600 mg/wk × 1 yr	Any cardiac event	Ongoing
MARBLE (45)	CAD, waiting for bypass	None	1300	Azithromycin, 600 mg/wk × 3 mo	Any cardiac event	Ongoing
STAMINA (45)	Acute coronary syndrome	Seropositive for *C. pneumoniae* and *H. pylori*	600	Azithromycin or amoxicillin, dose not specified	Effect on inflammatory markers, any cardiac event	Ongoing

[a] bid, twice a day; qid, four times a day.

proved or were classified as clinical cures. The MICs and MBCs for isolates of *C. pneumoniae* from the patients who were microbiologic failures in response to both drugs remained the same before and after treatment. One possible explanation for persistence of *C. pneumoniae* in these studies was that the dose and duration of treatment were not optimal. Kutlin et al. (77), using a continuously infected HEp-2 cell line, reported that 6 days of treatment with 4 μg of ofloxacin per ml or 0.05 μg of azithromycin per ml, which exceeds achievable serum levels, reduced the concentration of *C. pneumoniae* from 10^6 to 10^3 inclusion-forming units/ml but failed to completely eradicate the organism. In a subsequent study using the same model, 30 days of treatment with azithromycin or clarithromycin, at concentrations achievable in the pulmonary epithelial lining fluid (32 to 500 times the MIC), reduced the concentrations of *C. pneumoniae* by 4 log units but, again, failed to eliminate the organism (79). Ultrastructural studies of these persistently infected cells demonstrated the presence of a subpopulation of abnormal inclusions, which were very similar in appearance to persistent forms induced after treatment with gamma interferon (78). These chlamydiae do not appear to be actively replicating; thus, antibiotic treatment may not be effective.

Retrospective Case-Control Studies

Meier et al. (89) attempted to determine whether previous use of antibiotics decreased the risk of developing a first-time acute MI by examining a population-based database from the United Kingdom in a case-control study. Records from a total of 3,315 cases and 13,139 age- and sex-matched controls were examined. They found that cases were significantly less likely to have used tetracyclines or quinolones than controls. No effect was found for prior use macrolides (primarily erythromycin), penicillins, or cephalosporins. A history of antibiotic exposure meant any antibiotic use during an arbitrarily selected 3-year period prior to the date of the acute MI. Although the dose of antibiotic was included in the analysis, there was no consideration of the duration of treatment. The finding of a protective effect with tetracyclines, but not macrolides, was inconsistent since the in vitro activities of both classes of drug are similar (Table 6). There are no data suggesting that tetracyclines are more effective than macrolides in treatment of *C. pneumoniae* infection. Only eight cases received quinolones, which included ofloxacin, ciprofloxacin, and norfloxacin. The last two drugs are relatively inactive against *C. pneumoniae* in vitro (Table 6), and ciprofloxacin has inadequate microbiologic efficacy in the treatment of genital *C. trachomatis* infection (114). A subsequent study from the University of Washington, using a database from a large health maintenance organization, found that use of erythromycin, tetracycline, or doxycycline during the previous 5 years was not associated with a lower risk of a first MI (60). The population included 1,796 patients and 4,883 controls. Unlike the study of Meier et al. (89), an attempt was also made to assess risk in relation to the duration of treatment. Patients who were treated for ≥29 days with any of the drugs had the same risk as those who were treated for a shorter duration or had received no antibiotics.

Prospective Controlled Treatment Trials

There are now three small, randomized controlled secondary prevention studies published in the literature (44, 46, 47, 96), and another five studies which are larger, are ongoing (Table 7) (24, 28, 45, 63). The rationale for these studies is that treatment of a putative, active *C. pneumoniae* infection will reduce the risk of further cardiac morbidity and mortality in patients who have had an acute MI. Azithromycin at various doses and durations of therapy has been the drug of choice in six of these trials. Azithromycin has in vitro MICs similar to those of erythromycin, but it has a half-life in tissue of ≥5 days, which allows for extended dosing regimens (28). The other macrolide used is roxithromycin, which has in vitro activity similar to erythromycin (121).

Gupta et al. (44) enrolled male MI survivors who were seropositive by MIF (IgG titer, ≥8). Patients who had IgG titers of ≥64, which was considered to indicated "persistent" infection, were randomized to receive azithromycin at 500 mg/

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day for 3 or 6 days. They found that patients receiving azithromycin demonstrated a decrease in their MIF IgG titers and had a lower risk for adverse cardiovascular events than did seropositive patients who were not treated. Subsequent studies have not found a significant effect on cardiovascular mortality or morbidity or serologic markers of *C. pneumoniae* infection. A larger, subsequent study reported that treatment with azithromycin, 500 mg/day for 3 days followed by 500 mg/week for 3 months, was not associated with reductions in ischemic events, including cardiovascular death, nonfatal MI, stroke, and unstable angina (96). Patients were enrolled if they were seropositive with an IgG of ≥16 in MIF. At the end of the 24-month follow-up period, they observed reductions in inflammatory markers, including CRP, interleukin-6, and tumor necrosis factor alpha, but no effect on anti-*C. pneumoniae* antibody titers. Gurfinkel et al. (47) also failed to find a significant difference in clinical end points (cardiac death, MI, or severe recurrent ischemia) in patients with unstable angina or non-Q-wave MI at 90 or 180 days after treatment with roxithromycin 150 mg twice a day for up to 30 days or placebo. Unlike the previous studies, *C. pneumoniae* seropositivity was not an enrollment criterion. Anti-*C. pneumoniae* IgG titers did not change with therapy and did not differ between the treatment and placebo groups. Treatment with roxithromycin also appeared to decrease the levels of CRP compared to placebo.

The effect of antibiotic treatment on anti-*C. pneumoniae* antibody and inflammatory markers has been reported in several small studies that did not specifically look at CVD end points. Jackson et al. (61) randomized 88 patients following percutaneous coronary revascularization to receive either azithromycin, 500 mg/day for 2 days then 250 mg/day on days 3 through 28, or placebo. They found that although azithromycin was well tolerated, there was no effect on either IgG or IgA antibody titers. Similar findings were also reported by Sinisalo et al. (130), who found that treatment with doxycycline, 100 mg twice a day for 4 months, had no effect on anti-*C. pneumoniae* MIF antibody titers in men with angiographically confirmed CHD and prior bypass surgery. There were no significant differences in levels of CRP, cholesterol, high-density lipoprotein, and low-density lipoprotein between patients who received doxycycline or placebo. Torgano et al. (139) randomly assigned 97 patients with CVD to receive either clarithromycin, 500 mg twice a day for 30 days (if they were seropositive for *H. pylori* regardless of *C. pneumoniae* antibody status) or 500 mg twice a day for 14 days (if they were seropositive only for *C. pneumoniae* [MIF IgG titer, ≥64]), or placebo. They found a significant reduction in the geometric mean IgG titer in the treated patients and significantly reduced serum fibrinogen levels in patients who were treated with 30-day, but not 14-day, courses of clarithromycin. Semaan et al (127) treated 40 patients who had documented CVD and an anti-*C. pneumoniae* MIF IgG titer of >16 and 20 controls who had normal coronary arteries with azithromycin, 500 mg/day for 3 days then twice weekly for 3 months, or placebo. They found that azithromycin treatment had no effect on the levels of soluble markers of endothelial activation, intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin, in plasma.

As of this writing, there are five ongoing, larger, randomized controlled secondary prevention studies, which are summarized in Table 7, although some details are not available for all the studies. Four of these studies are using weekly azithromycin regimens for periods of 2 weeks to 12 months. The remaining study is using daily gatifloxacin for 18 months (45). Gatifloxacin is a quinolone with MIC and MBC for 90% of isolates (MIC_{90} and MBC_{90}) of 0.25 mg/ml against recent clinical isolates of *C. pneumoniae* (54), but there are no data on the microbiologic efficacy of this drug. Three studies are screening patients for anti-*C. pneumoniae* IgG by MIF, using different titers as enrollment criteria. In addition, each study uses different cardiac entry criteria, including previous MI or bypass surgery, acute MI at least 6 weeks before enrollment, and severe CHD waiting for bypass surgery. The Weekly Intervention with Zithromax for Atheroscelrosis and Related Diseases (WIZARD) study is using an IgG titer of ≥16 (28). Patients will then be randomized to receive azithromycin, 600 mg four times/day for the first 3 days then 600 mg once weekly, or placebo. Patients will be enrolled regardless of their serologic status in the Azithromycin Coronary Events Study (ACES) and Might Azithromycin Reduce Bypass-List Events? (MARBLE) studies (45, 63). In the ACES study, patients will be treated with azithromycin, 600 mg weekly for 1 year, or placebo. Patients in the MARBLE study will be treated with azithromycin 600, mg/day for 3 days followed by 600 mg/week for 3 months. The South Thames Antibiotics in Myocardial Infarction and Angina study (STAMINA) proposes to treat patients who have serologic evidence of *C. pneumoniae* and *H. pylori* with azithromycin, an amoxicillin-based regimen, or placebo (45). The end points include the effect on inflammatory markers and cardiovascular events. None of these studies have reported any data as yet. The WIZARD study extended the enrollment period for another year to recruit more patients.

Reasons for failure to find an effect of antibiotic treatment on adverse cardiac outcome in two of the initial three intervention studies include low power due to small numbers, inadequate dosing and duration of treatment, or poor positive predictive value of *C. pneumoniae* serology. In terms of dosage and duration of treatment, none of the antibiotic regimens used previously or being used currently has been evaluated or demonstrated to be effective in eradication of *C. pneumoniae* from the respiratory tract or any other site. Ironically, the only study that found a significant reduction in adverse cardiac outcome used an azithromycin regimen, 500 mg/day for 3 or 6 days, that was the least likely to be effective (44). As described previously, 1.5 g of azithromycin over 5 days had only 70% efficacy in eradicating *C. pneumoniae* from the nasopharynx of adults with pneumonia (120). Furthermore, the weekly dosing regimens of azithromycin used in the ACADEMIC study and the ongoing intervention studies described previously may result in long periods of subinhibitory levels of drug, which may lead to the development of resistance. This has been demonstrated to occur with *Streptococcus pneumoniae* and *S. pyogenes* after azithromycin treatment (36) and could potentially occur with *C. pneumoniae*. Extrapolating from the persistently infected cell model (77, 79), one would also expect that these weekly azithromycin regimens would not be effective in eradicating chronic *C. pneumoniae* infection, if present. The reductions in inflammatory markers reported in several studies (47, 96) may be secondary to anti-inflammatory activity of macrolides and tetracyclines (126). Roxithromycin has been demon-

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strated to have very potent anti-inflammatory activity in vitro (126). Finally, as discussed for the seroepidemiologic studies, there are no reliable serologic markers for chronic or persistent *C. pneumoniae* infection. In reality, this means that we really cannot determine who is infected and who is not. It also means that we cannot assume that any effect seen is due to successful treatment or eradication of *C. pneumoniae*.

The MARBLE study proposes a slightly different approach. As stated above, patients with severe CAD waiting for bypass surgery will be treated with azithromycin or placebo for 3 months (45). These patients can typically wait for up to 12 months for surgery. At the time of operation, samples of coronary artery will be examined for the presence of *C. pneumoniae* by PCR. Melissano et al. (91) reported a similar pilot study where 32 patients were treated with either roxithromycin, 150 mg twice a day, or placebo for 17 to 35 days before undergoing carotid endarterectomy. Using a seminested PCR, *C. pneumoniae* DNA was detected in plaques of 5 (31.3%) of 16 treated patients compared to 12 (75%) of 16 who received placebo ($P = 0.034$). The authors claimed that these results demonstrated that treatment with roxithromycin was effective in eradicating *C. pneumoniae* from the plaques. However, since the pretreatment status was unknown and given the problem observed with performance of PCR, one cannot really assume that the lower prevalence of *C. pneumoniae* DNA in the treatment group was due to successful eradication.

Recently Gieffers et al. (40) treated two patients who had acute unstable angina with azithromycin, 500 mg/day for 3 days followed by 500 mg on days 14 and 16 after coronary angiography. PBMCs obtained before treatment were positive for *C. pneumoniae* DNA, but the organism was isolated by culture from PBMCs obtained on day 28 after angiography. These results are not totally unexpected since the dose and duration of therapy with azithromycin were probably suboptimal.

Effect of Treatment of *C. pneumoniae* in Animal Models of Atherosclerosis

Rabbit and mouse models of atherogenesis have been used to determine if infection with *C. pneumoniae* can induce plaque formation. Fong et al. (35) and Muhlestein et al. (95) have demonstrated that *C. pneumoniae* can cause atheromatous lesions in the aorta and increase intimal thickening in 30 to 40% of New Zealand White rabbits after intranasal inoculation. In mice, *C. pneumoniae* disseminates after intranasal inoculation and can be isolated from the lungs, spleen, and buffy coats (145). Infection with *C. pneumoniae* has also been demonstrated to cause increasing fatty-streak lesion formation in apolipoprotein E (ApoE)-deficient mice, which develop these lesions spontaneously (123). Other mouse models, including BALB/c mice, which develop atherosclerotic lesions when fed an atherogenic diet, have not demonstrated a consistent association of *C. pneumoniae* infection with enhancement of atherosclerosis (145). Treatment of acute *C. pneumoniae* infection in rabbits with high-dose azithromycin for 10 weeks was associated with reduction of intimal thickening but did not appear to eradicate evidence of the organism as demonstrated by ICC (35, 95). Rothstein et al. (123) reported that treatment of ApoE-deficient mice 4 to 5 weeks after *C. pneumoniae* infection with azithromycin had no effect on the size of the atherogenic aortic lesions. In addition, azithromycin treatment did not affect the size of lesions in sham-infected mice or the persistence of *C. pneumoniae* in the aorta, lung, or spleen, and it did not affect antibody titers. These results seem to support the experience with rabbits, which found an effect with treatment of acute but not established infection. One implication of these data for human secondary prevention trials is that antibiotic treatment may not be beneficial, since *C. pneumoniae* infection in patients with CVD, who are being enrolled in these studies, is most probably not acute.

CONCLUSIONS

The role of infection and specifically, *C. pneumoniae* remains controversial and unresolved. It now appears that some of the inconsistency of the results from study to study may be due, in part, to lack of standardized methods. Although initial seroepidemiologic studies demonstrated a significantly increased risk of adverse cardiac outcome in patients who were seropositive, subsequent prospective studies found either small or no increased risk. In addition to the lack of consistent serologic criteria, recent evaluations have demonstrated inherent problems with performance of the most widely used serologic methods. Most importantly, we do not have a reliable serologic marker for chronic or persistent *C. pneumoniae* infection. Similarly, the results of studies demonstrating the presence of *C. pneumoniae* have also been extremely variable. As with the seroepidemiologic studies, the PCR and ICC methods used are not standardized. The results of recent studies suggest that laboratories using the same methods with the same specimens may obtain different results. However, it is clear that *C. pneumoniae* is present in atherosclerotic plaque, since it has been isolated by culture in a limited number of studies, albeit at a relatively low frequency. It has been suggested that the results of the larger treatment studies will help to define or confirm the role of *C. pneumoniae* in CVD. Even if a reduction in adverse events is seen, one cannot assume that it was due to treatment of *C. pneumoniae*.

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