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Measurement of Sputum Antibodies in the Diagnosis of Acute and Chronic Respiratory Infections Associated with *Chlamydia pneumoniae*

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The aim of this study was to develop methods for the measurement of sputum antibodies in the laboratory diagnosis of acute and chronic lower respiratory tract infections caused by *Chlamydia pneumoniae*. Paired serum specimens, sputum specimens, and pharyngeal or nasopharyngeal swabs were obtained from 97 patients; 51 of them had community-acquired pneumonia, and 46 had chronic obstructive pulmonary disease (COPD). *C. pneumoniae*-specific serum immunoglobulin G (IgG), IgA, and IgM antibodies were measured by the microimmunofluorescence (micro-IF) test. For sputa, specific IgA and IgG antibodies were measured by the micro-IF test and secretory IgA (sIgA) was measured by enzyme immune assay (EIA) with *C. pneumoniae* elementary bodies as the antigen. Sputum IgA and sIgA antibodies to *C. pneumoniae* were found, respectively, in 52 and 51% of the COPD patients. Elevated levels of stable serum IgG and IgA antibodies (IgG titer of ≥128 and IgA titer of ≥40), suggesting chronic infection, were found in 54% of the COPD patients. The sensitivity for the sputum IgA micro-IF test compared with elevated serum antibody levels was 87.5%, and that for the sputum sIgA EIA was 88%; the respective specificities were 90 and 95%. Acute *C. pneumoniae* infection was diagnosed in seven pneumonia patients, and two (29%) of these patients were positive by sputum EIA antibody measurements. Two pneumonia patients without acute infection had stable elevated IgG and IgA levels in their sera, and both of them were sputum antibody positive. We conclude that the measurement of IgA antibodies to *C. pneumoniae* in sputum is a useful additional diagnostic tool for chronic *C. pneumoniae* infections.

Chlamydia pneumoniae, one of the most recently discovered human pathogens, has been associated not only with acute respiratory infections, pneumonia, pharyngitis, and acute bronchitis but more recently also with chronic respiratory diseases, such as adult-onset asthma and chronic obstructive pulmonary disease (3, 12, 31). In acute infections, laboratory diagnosis has traditionally been based on serology; a fourfold rise in titer in the microimmunofluorescence (micro-IF) test between paired sera or the presence of immunoglobulin M (IgM) antibodies has widely been used as the "gold standard" to indicate acute infection. A single serum sample has been shown to be of limited value because IgM antibodies frequently are not present in reinfections (6, 10). No diagnostic criteria have yet been established for serological tests for chronic infections. In a pilot study with asthma patients, an elevated IgG antibody level with a concomitant elevated IgA antibody level proved to be a useful marker of chronic *C. pneumoniae* infection (13). Unlike for *Chlamydia trachomatis* infections, culture and direct antigen detection with clinical specimens have proven to be unsuccessful in both acute and chronic *C. pneumoniae* infections (6, 9, 17, 27). The PCR test is promising, but it is expensive and not yet available for routine analysis. The increasing weight of evidence that chronic *C. pneumoniae* infection may lead to severe sequelae (12, 25, 26) underscores the need for a rapid, inexpensive, and reliable method for detecting chronic *C. pneumoniae* infections.

It has been suggested that the locally produced secretory IgA (sIgA), which appears in secretions after a natural infection and is short-lived, is a more reliable indicator of chlamydial infection than serum antibodies (19). Unlike many other agents associated with pneumonia and exacerbations of chronic bronchitis, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Branhamella catarrhalis*, *C. pneumoniae* does not normally reside in the nasopharynx, and therefore the presence of *C. pneumoniae*-specific sIgA in secretions may be evidence of a current chlamydia infection.

This preliminary study was designed to evaluate the usefulness of measurement of antibodies in sputum as a diagnostic tool in addition to serology for patients with acute and chronic lower respiratory tract diseases associated with *C. pneumoniae.*

MATERIALS AND METHODS

Specimens. Specimens were collected from 97 patients with lower respiratory tract disease at several hospitals in Finland between September 1992 and May 1994. Fifty-one of these patients had radiographically and clinically verified community-acquired pneumonia (32 military trainees and 19 mostly middle-aged patients; mean age, 30 years; range, 19 to 91 years), and 46 had been diagnosed as having chronic obstructive pulmonary disease (COPD) as determined either from lung function tests (2) or, in the case of some severely ill patients, from clinical evidence (11 ambulatory and 35 hospitalized patients, with or without exacerbation; mean age, 68 years; range, 45 to 85 years). Paired sera taken 2 weeks apart (or, for COPD outpatients, 3 months apart) were frozen at −20°C, and expectorated sputum samples obtained on admission were frozen at −70°C (if not homogenized immediately) within 6 h of collection. Pharyngeal or nasopharyngeal (from COPD inpatients) swabs immersed in Chlamydia Transpocult medium (Orion, Espoo, Finland) were refrigerated for 3 to 4 h before being frozen at −70°C.

Treatment of sputum specimens. Sputum specimens were homogenized with an equal volume of 1:10-diluted Sputolysin Reagent (Calbiochem Behring, La Jolla, Calif.) and vortexed for 3 min, and the homogenate was allowed to stand at room temperature for 15 min. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C to remove cellular debris and mucus, and the supernatant was stored at −70°C until assayed for *C. pneumoniae*-specific IgA, IgG, and sIgA antibodies.

Sputum micro-IF test. *C. pneumoniae*-specific IgA and IgG in treated sputum were measured by the method of Wang and Grayston (32) with elementary

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bodies of *C. pneumoniae* Kajaani 6 (6) as the antigen. Fluorescein isothiocyanate-conjugated anti-human IgA (Sigma Chemical Co., St. Louis, Mo.) and IgG (Kallestad Diagnostics, Ghaska, Minn.) diluted 1:40 with phosphate-buffered saline (PBS) (10 mM sodium phosphate with a salt concentration of 0.9%, pH 7.3) were used as conjugates. Serial twofold dilutions of sputum were tested, with 1:1 to 1:8 dilutions. Antibody titers were read with an Axiophoto microscope (Zeiss, Oberkochen, Germany) at a magnification of ×400. A sputum antibody titer of ≥4 was considered positive.

Sputum enzyme immunoassay (EIA). Ninety-six-well microtiter plates (Maxisorp Immunoplates; Nunc, Rosklide, Denmark) were coated with 50 μl of elementary body antigen (strain TWAR; Washington Research Foundation, Washington, D.C.) at a concentration of 2×10^7/ml in PBS. The plates were incubated at room temperature overnight, washed three times with PBS–0.5% Tween 20, postcoated with heat-inactivated 10% fetal calf serum (Gibco, Paisley, United Kingdom), and washed. The samples were diluted 1:20 in 10% fetal calf serum and added to the wells in a volume of 50 μl. The plates were incubated for 2 h at 37°C. After the plates were washed, mouse monoclonal antibody to human secretory component (Sigma) diluted 1:5,000 was added. The plates were incubated overnight at room temperature and washed, and alkaline phosphatase anti-mouse conjugate (Jackson Immuno Research, West Grove, Pa.) was added at a dilution of 1:1,000. The plates were incubated for 2 h at 37°C and washed with PBS–0.5% Tween 20 and distilled water. Dinitrophenyl phosphate substrate (Sigma) at a concentration of 1 mg/1 ml of diethanolamine-$MgCl_2$ buffer (Orion) was added. After 30 min of incubation at 37°C, the A_{405} was read with an enzyme-linked immunosorbent assay reader (Multiscan MCC 340; Labsystems, Helsinki, Finland). A positive and a negative control were included in every run. All of the samples were tested in duplicate. The cutoff points were defined by calculating the mean of the results for 30 negative sputum samples measured by the micro-IF test and adding the standard deviation, multiplied by three, to the mean, resulting in a value of 1.8 for sIgA. Between absorbances of 0.2 and 3.0, the value increased linearly with the sample dilution ($r = 0.9$) (data not shown).

Isolation of *C. pneumoniae*. The pharyngeal and nasopharyngeal swab specimens were cultured in HL cells as described in detail by Kuo and Grayston (16). Briefly, each sample was inoculated into three vials with HL cell monolayers, centrifuged at 800 × *g* at room temperature for 1 h, and incubated in the presence of CO_2 at 35°C for 72 h or 7 days. After 3 and 7 days of incubation, one vial was used for the detection of *C. pneumoniae* inclusions by staining the coverslip with a genus-specific monoclonal antibody (Kallestad Diagnostics). If any inclusions were seen in either of these samples, a second passage was performed with the remaining vial, which was stained after 3 days of incubation. The specimens were considered negative if no inclusions were detected in any of the stained samples.

Serum micro-IF test. *C. pneumoniae*-specific IgG, IgA, and IgM were measured by the micro-IF test (32) with elementary bodies of *C. pneumoniae* Kajaani 6 as the antigen. Sera were tested in serial twofold dilutions from 1:8 (IgG) or 1:10 (IgA and IgM) to the end point. Fluorescein isothiocyanate-conjugated anti-human IgG (Kallestad) or anti-human IgA (Sigma), each at 1:40, or anti-human IgM (Dako Immunoglobulins, Copenhagen, Denmark) at 1:20 was used as the conjugate. Before IgA and IgM measurements, the sera were treated with goat antibody to human IgG antibody (Gullsorb; Gull Laboratories, Salt Lake City, Utah) to remove IgG. All serum samples were also tested for *C. trachomatis* and *Chlamydia psittaci* antibodies by using *C. trachomatis* elementary bodies in three pools (immunotypes CHIJ, GFK, and BED [Washington Research Foundation]) and a mixture of *C. psittaci* OA and 6BC strain (Slovak Academy of Sciences, Bratislava, Slovakia) elementary bodies as antigens. The positivity criterion for an acute *C. pneumoniae*, *C. trachomatis*, or *C. psittaci* infection was defined as a fourfold rise in the titer of any of the immunoglobulin classes or an IgM titer of ≥20 for a single serum (gold standard) (10). For chronic *C. pneumoniae* infection, the criterion of stable elevated IgG (titer ≥ 128) and stable elevated IgA (titer ≥ 40) was used.

Statistical analysis. Results for sputum IgA measured by micro-IF and for sputum sIgA measured by EIA were compared with serum antibody findings with the micro-IF test. Sensitivities, specificities, and positive and negative predictive values were determined by standard methods (11). Confidence intervals were measured at the 95% level. Pearson's correlation coefficient was calculated to measure the strengths of the correlations.

RESULTS

Patients with COPD. By the criterion of stable elevated serum IgG and IgA for chronic *C. pneumoniae* infection, 25 (54%) of the 46 patients were positive (in five cases the assessment was based on a single serum sample, and four of these samples fulfilled the positivity criterion). With the sputum micro-IF test, diagnostic levels of specific sputum IgA antibodies were detected in 23 (52%) of the 44 patients tested, and with the sputum EIA, sIgA antibodies were detected in 23 (51%) of the 45 patients tested (Table 1). Twenty-one of the 24 serum antibody-positive patients were also sputum IgA positive by the micro-IF test, and by EIA, 22 of the 25 seropositive patients were also sputum sIgA positive (Table 2). Thus, the sensitivity (compared with serology) for chronic infection was 87.5% for the sputum IgA micro-IF test and 88% for the sputum sIgA EIA, with specificities of 90 and 95%, respectively. The positive and negative predictive values for the sputum IgA micro-IF test were 91 and 86%, respectively, and those for the sputum sIgA EIA were 96 and 86%, respectively. Two patients who had positive sputum antibodies but did not fulfill the criterion for chronic serum antibodies had IgG titers of 32 and 64 and IgA titers of 40 and 80, respectively.

TABLE 1. Overall prevalence of diagnostic *C. pneumoniae* antibody levels in the four different tests on patients with COPD and pneumonia

Test	% Positive[a] (no. positive/total no.)	
	COPD patients	Pneumonia patients
Serum micro-IF		
Chronic	54 (25/46)	4 (2/51)
Acute	2 (1/46)	14 (7/51)
Sputum IgA micro-IF	52 (23/44[b])	8 (4/51)
Sputum sIgA EIA	51 (23/45[c])	10 (5/49[b])
Sputum IgG micro-IF	24 (11/45[c])	14 (7/51)

[a] Percentage with diagnostic *C. pneumoniae* antibody levels.
[b] Data not available for two patients.
[c] Data not available for one patient.

Sputum IgG antibodies were detected by the micro-IF test in 11 (24%) of the 45 patients, all of whom were also positive in the sputum IgA micro-IF test (Table 1). In comparison with serology, the sensitivity for the sputum IgG micro-IF test was 44%, and the specificity was 100%. One COPD patient showed seroconversion with IgG antibodies between paired sera (titer rise from 32 to 128), suggesting acute infection, while no seroconversions were detected with IgA antibodies. For this patient, both sputum tests showed a negative result.

The correlation between sputum IgA levels measured by the micro-IF test and sputum sIgA levels measured by EIA is shown in Fig. 1 (Pearson's correlation coefficient = 0.72). When serum IgA levels were compared with sputum IgA levels, a correlation coefficient of 0.6 was obtained (Fig. 2).

Patients with pneumonia. Acute *C. pneumoniae* infection was detected serologically in 7 (14%) of the 51 pneumonia patients. The diagnosis was based on single serum sample in

TABLE 2. Comparison of serum antibody findings suggesting chronic *C. pneumoniae* infection with measurements of sputum IgA, sputum sIgA, and sputum IgG antibodies in patients with COPD

Test and result	No. of patients:		Correlation (%)
	With elevated serum antibody levels[a]	Without elevated serum antibody levels	
Sputum IgA (micro-IF)			
Positive	21	2	77
Negative	3	18	
Sputum sIgA (EIA)			
Positive	22	1	83
Negative	3	19	
Sputum IgG (micro-IF)			
Positive	11	0	51
Negative	14	20	

[a] IgG titer of ≥128 and IgA titer of ≥40.

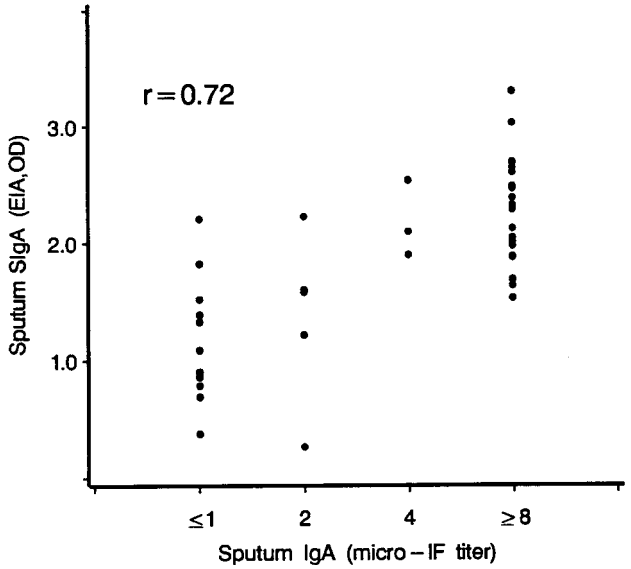

FIG. 1. Correlation between *C. pneumoniae*-specific sputum IgA measured by micro-IF and sputum sIgA measured by EIA in patients with COPD. OD, optical density.

one case (IgM titer of 160). Four patients had primary infection demonstrated by the presence of IgM antibodies. Two pneumonia patients had chronic antibodies (stable elevated IgG and IgA levels) in their sera. Specific sputum IgA antibodies were found in only 4 (8%) of the 51 patients by the micro-IF test, and sIgA antibodies were found in 5 (10%) of the 49 patients tested by EIA (Table 1). When the positive sputum test results were compared with the serum micro-IF test results, we found that two patients with positive sputum IgA and/or sIgA tests had acute *C. pneumoniae* infection (primary infection in both cases), while two other patients fulfilled the criterion for chronic infection (stable elevated IgG and IgA antibody levels).

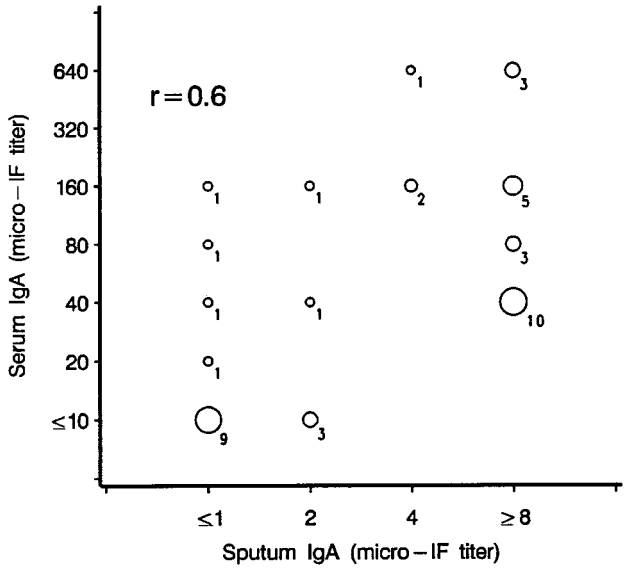

FIG. 2. Correlation between *C. pneumoniae*-specific sputum IgA and serum IgA measured by micro-IF in patients with COPD. The numbers next to the circles represent the number of subjects at each point.

Age stratification performed for both COPD patients (age 45 to 64 and 65 to 85 years) and pneumonia patients (age 19 to 34 and 35 to 91 years) revealed no significant differences in sputum sIgA prevalence between the older and younger patients in either group.

No diagnostic *C. trachomatis* or *C. psittaci* antibody levels were found in any of the patients. All *C. pneumoniae* cultures from pharyngeal or nasopharyngeal swabs were negative.

DISCUSSION

Our results indicate that IgA and sIgA antibodies to *C. pneumoniae* can be demonstrated in sputum specimens by the micro-IF and EIA methods in patients with suspected chronic *C. pneumoniae* infection and that sputum antibody measurements obtained from these tests correlate well with serological findings when the criterion of elevated serum IgG and IgA levels for chronic *C. pneumoniae* infection is used. Only two of the seven patients with acute *C. pneumoniae* infection had IgA antibodies in sputum, suggesting that the method is not sensitive enough for diagnosis of acute *C. pneumoniae* infections.

Local antigenic stimulation of mucosal surfaces results in the local production of antibodies, predominantly of the IgA class, which appear in mucosal secretions. In humans, only marginal amounts of IgA are transported from the circulation into external secretion. The secretory and systemic immune systems are separate and largely independent of each other (21, 30). Following a natural mucosal infection, specific antibodies are formed locally within a few days, but serum antibodies are not usually detected until about a week later (22). sIgA is short-lived. The half-life of sIgA on mucosal surfaces seems to be much shorter than that of serum IgA, because sIgA is soon carried away in mucosal secretions (22). In addition, dimeric sIgA is more rapidly catabolized than monomeric serum IgA, because sIgA is subject to additional specific clearance mechanisms, such as secretory-component-mediated transport (28). Because of this rapid turnover rate, sIgA may be a potential marker of an ongoing and especially chronic *C. pneumoniae* infection. The fairly good correlation between levels of sputum IgA (micro-IF) and sIgA (EIA) against *C. pneumoniae* found in this study shows that sputum IgA is primarily secretory, locally produced IgA.

The sputum IgG micro-IF test was positive in fewer than half of the cases positive by the sputum IgA micro-IF test. Sputum IgG antibody, evidently appearing in secretions by passive transudation from the circulation (20), does not seem to be suitable for diagnostic purposes. The good overall accordance between elevated levels of serum IgG and IgA antibodies and the sputum IgA and sIgA antibodies in COPD patients with suspected chronic *C. pneumoniae* infection may reflect the shift from a cell-mediated Th1 immune response toward an antibody-mediated Th2 response with mucosal antibodies, as has been suggested to occur in chronic disease (23).

In contrast to the situation with chronic infections, sputum antibody measurements were not useful in diagnosing acute *C. pneumoniae* infections in pneumonia patients. The sensitivities for both the sputum micro-IF and sputum EIA were low. There are several reasons for the poor sensitivities of sputum tests. Pneumonia caused by *C. pneumoniae* is often associated with a nonproductive cough, especially at early stages of the disease (15). Any respiratory tract specimens obtained in these cases frequently consist mostly of saliva. Our measurements of specific antibodies in saliva have similarly shown poor sensitivity in acute *C. pneumoniae* infections (unpublished data), which may partly be explained by the degrading and inactivating properties of saliva enzymes, which can rapidly reduce the

antibody levels in the saliva sample even during the sample treatment procedure. Second, since the kinetics of sputum antibodies is not known, it is possible that IgA antibodies in secretions have already disappeared during the incubation period or that they develop later in the course of infection. These problems are not generally encountered in chronic infections; thus, the presence of antibodies in secretions in patients with suspected chronic *C. pneumoniae* infections may indicate continuous mucosal stimulus by the agent.

The diagnosis of chronic *C. pneumoniae* infection is difficult. The inadequacy of serological tests in chronic infections is well known (24, 27), but there are few alternatives. In this study, all attempts to isolate chlamydiae were unsuccessful. The difficulty of isolating *C. pneumoniae* in both acute and chronic infections is recognized. The relevance of pharyngeal or nasopharyngeal swabs for isolation in, for example, lower respiratory tract infections has been questioned (24). The PCR test for sputum has not yet been fully evaluated, and there is as yet no agreement on what sample to use in the case of chronic infection. In this study we compared sputum antibodies with serum antibodies. The sensitivity and specificity of the serum micro-IF test in the diagnosis of chronic *C. pneumoniae* infections have not been evaluated because there is no available gold standard for chronic infections. In this study, however, a good correlation was observed between the presence of sputum antibodies and elevated levels of serum antibodies suggestive of chronic infection.

The presence of short-lived sIgA antibodies has been shown to indicate recent or current infection (1, 4, 19), and the diagnostic value of sIgA in *C. trachomatis* infections has been propounded (5, 14, 19, 29). The most commonly occurring agents involved in pneumonia and COPD are, unlike chlamydiae residents of the normal nasopharyngeal flora (7, 8, 18). The presence of specific local antibodies in sputum suggests that *C. pneumoniae* is involved in these infections. The sputum EIA test had a slightly higher sensitivity and specificity than the sputum micro-IF test when compared with serum antibody findings. EIA has the advantages that it gives an objective reading, can be automated, and is relatively easy to perform. The sputum EIA test may therefore be more suitable than the micro-IF test for clinical use.

Our results suggest that locally produced specific sIgA is a sensitive and specific marker of chronic but not acute *C. pneumoniae* infection. The role of suspected chronic *C. pneumoniae* infection in the etiopathogenesis of COPD remains to be studied.

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