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Evaluation of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* as Etiologic Agents of Persistent Cough in Adolescents and Adults

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***Chlamydia pneumoniae* and *Mycoplasma pneumoniae* were evaluated as agents of persistent cough in adolescents and adults ($n = 491$). Tests of 473 respiratory specimens by culture or PCR or both identified four episodes (0.8%) of *M. pneumoniae*-associated illness and no episodes of *C. pneumoniae* illness, suggesting that these bacteria do not frequently cause persistent cough.**

In the United States, adults frequently seek medical attention for a persistent cough (9), a symptom often associated with *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* infections. This study evaluated the frequency with which these bacteria cause persistent cough in adolescents and adults by using molecular, culture, and serologic techniques.

Healthy subjects ($n = 491$) were enrolled at seven centers in the United States between September 1997 and December 1998 as part of an acellular pertussis vaccine efficacy study approved by an internal review board. The subjects were monitored for up to 2 years for a nonimproving cough lasting 5 days or longer. The subjects had a median age of 35.4 years (range, 15 to 65 years); 64.4% were females, 66.4% were Caucasians, and 28.9% were African-Americans. Throat swab specimens were obtained at the baseline visit and at each visit during illness (illness visit) and a nasopharyngeal aspirate was obtained at each illness visit. The specimens were suspended in transport medium (6, 7, 12) and stored at −80°C. Cultures for *M. pneumoniae* and *C. pneumoniae* were done as described previously (6, 7, 12).

A multiplex PCR assay was designed using primers for amplification of a 438-bp target (internal control) in the β-actin gene of human DNA (16) and primer sets MP1-MP2 and P4A-P4E (1) for amplification of 124- and 230-bp targets, respectively, within the P1 cytadhesion gene of *M. pneumoniae*. Probes HAC7, MP7, and P4C are complementary to the respective targets. Primers CP10 and CP11 were designed to amplify a 323-bp target in the *C. pneumoniae*-specific 474-bp *Pst*I restriction fragment (2); probe CP13 is complementary to this target. The oligonucleotides unique to this study and their 5′ → 3′ nucleotide sequences were as follows: HAC7, GCCA TGTACGTTGCTATCCAGGCTGTGCTATCCCTG; MP1, GTGGGACACTTCACAAGTACC; MP2, GATACGTTCAC GGGGTTAAGC; MP7, AATTTAGCTACACCCGCCCTGA CGAGGTCGC; P4A, AGGCTCAGGTCAATCTGGCGTG GA; P4C, TGGGGCAGTTACCAAGCACGAGTGACGG AA; CP10, CAGATTACGAAACGGCATTAC; CP11, TGCT GCATAACCTACGGTGTG; and CP13, GGTGTCATTCGC CAAGGTTAAAGTCTACG. Sample lysates were prepared as described previously (11). DNA was amplified in 100-μl reaction mixtures containing a 200 μM concentration (each) of dATP, dUTP, dCTP, and dGTP, 2.0 U of AmpliTaq DNA polymerase, 1.0 U of uracil-*N*′-glycosylase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 25 μl of sample. The reaction mixtures for the multiplex PCR assay contained 3.5 mM $MgCl_2$, 1 ng of CEM cell DNA (16), a 0.25 μM concentration (each) of primers HAC3 and HAC5, and a 0.1 μM concentration (each) of primers MP1, MP2, P4A, and P4E. Reaction mixtures for amplification of *C. pneumoniae* DNA included 3.75 mM $MgCl_2$ and a 0.25 μM concentration (each) of primers CP10 and CP11. The thermal cycling conditions used were described previously (14), except that a 55°C annealing temperature and 40 PCR cycles were used in the *C. pneumoniae* assay. PCR products were detected by a liquid hybridization gel retardation assay using ^{32}P-labeled DNA probes, a highly sensitive platform (15) that avoids the use of cumbersome washing steps needed with colorimetric systems.

The PCR assays were initially evaluated by testing purified DNAs (16). Single copies of *M. pneumoniae* DNA (strain ATCC 15531) and *C. pneumoniae* DNA (strain ATCC 2282-VR [TW-183]) were detected in PCRs with 1 μg (approximately 300,000 copies) of human DNA (CEM cell line). Tests of the human DNA (i.e., 1 μg/reaction) did not yield any *M. pneumoniae* or *C. pneumoniae* PCR products or any nonspecific PCR products as determined by agarose gel electrophoretic analysis. None of the DNAs from two panels ($n = 65$ and $n = 85$) of nontarget microorganisms (Table 1), mostly isolated from the respiratory tract, yielded PCR products in the multiplex or *C. pneumoniae* assays, respectively. These results indicate that primers for *M. pneumoniae* and *C. pneumoniae* are sensitive, specific, and do not react with human

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TABLE 1. Microorganisms used in the specificity studies

Microorganism	Origin[a]	No. of strains tested in assay	
		Multiplex PCR	*C. pneumoniae* PCR
Acinetobacter baumannii	CI	1	
Candida albicans	CI	5	7
Chlamydia trachomatis	CI		1
Corynebacterium species	CI	5	7
Enterobacter cloacae	CI	1	
Enterococcus species	CI		1
Escherichia coli	CI	1	
Haemophilus influenzae	CI	5	14
Haemophilus parainfluenzae	CI	3	8
Klebsiella pneumoniae	CI	1	
Legionella pneumophila	CI	1	
Moraxella catarrhalis	CI	5	6
Mycoplasma fermentans	ATCC 19989	1	1
Mycoplasma genitalium	ATCC 33530	1	
Mycoplasma orale	ATCC 23714	1	
Mycoplasma salivarium	ATCC 23064	1	
Neisseria mucosa	CI	2	2
Neisseria species other than *N. meningitidis* and *N. gonorrhoeae*	CI	3	4
Proteus vulgaris	CI	1	
Pseudomonas aeruginosa	CI	1	
Salmonella enterica subsp. I	CI	1	
Staphylococcus aureus	CI	5	6
Staphylococcus species, coagulase negative	CI	5	6
Streptococcus pneumoniae	CI	5	7
Streptococcus pyogenes	CI	5	7
Streptococcus species, β-hemolytic group C	CI		1
Streptococcus species, viridans group	CI	5	7

[a] CI, clinical isolate.

DNA, results which are consistent with those of previous studies demonstrating high specificity of the *C. pneumoniae Pst*I fragment (2).

The detection limits of the PCR assays were compared to those of culture assays (6, 7, 12) by testing swabs which had been seeded with suspensions of *M. pneumoniae* and *C. pneumoniae* and processed as described previously (14). The detection limits of the two assays were nearly identical (Fig. 1 and 2). Because *M. pneumoniae* and *C. pneumoniae* survive poorly outside the human host, in situations where specimens are not processed promptly and carefully, the PCR assays may be more sensitive than the culture assays.

During the subject follow-up period, 516 episodes of persistent cough were identified (incidence rate = 0.75 episode/person year). *M. pneumoniae* was detected by PCR in 4 of 473 (0.8%) throat swab specimens collected at illness visits but not in any of 437 throat swab specimens collected at baseline visits and by culture in 2 of 350 (0.6%) throat swab specimens collected at illness visits but not in any of 457 throat swab specimens collected at baseline visits. Two of four subjects with an *M. pneumoniae*-associated cough illness had ≥4-fold increases in anti-*M. pneumoniae* immunoglobulin G antibody titer measured by microimmunofluorescence (3), and *M. pneumoniae* was detected in the nasopharynges of these two subjects by PCR (Fig. 3). Totals of 54 and 458 throat swab specimens

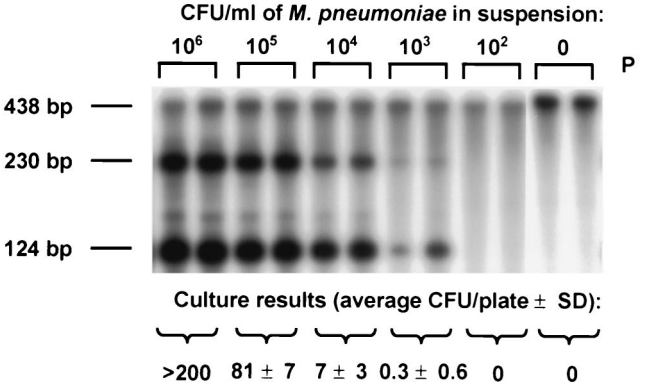

FIG. 1. Comparison of the results of culture assay and the multiplex PCR assay for the detection of *M. pneumoniae* in seeded swab samples. The β-actin PCR product (i.e., internal control) (438 bp) and the *M. pneumoniae* PCR products (230 and 124 bp) are indicated. P, probe control; SD, standard deviation.

obtained at baseline visits, 416 and 411 throat swab specimens obtained at illness visits, and 414 and 396 nasopharyngeal aspirate specimens obtained at illness visits were evaluated for *C. pneumoniae* by the PCR and culture assays, respectively. All of the specimens yielded negative results. A limitation in funding precluded the testing of all of the collected specimens by the different assays.

Initially, 17% of specimens (n = 910) tested in the multiplex PCR yielded 438-bp control bands with 50 to 75% reductions in intensity. However, repeat testing yielded bands with maximum intensities. Because earlier studies in our laboratory showed that the β-actin amplification control system had an adverse effect on amplification of the *C. pneumoniae* DNA target, this internal control was not included in the *C. pneumoniae* PCR assay. Instead, inhibition was assessed by random testing of 150 specimens in PCR mixtures containing 1,000 copies of *C. pneumoniae* DNA. Only one of these samples was negative, but repeat testing yielded a *C. pneumoniae* band of maximum intensity. Technical variability in sample processing or in probe hybridization may have accounted for the different results observed in the initial and repeat assays. Overall, these studies confirmed that the specimen processing procedure (11) provides material suitable for PCR studies.

Paired sera from the first cough illness visits of 99 subjects with negative results from PCR and culture studies were eval-

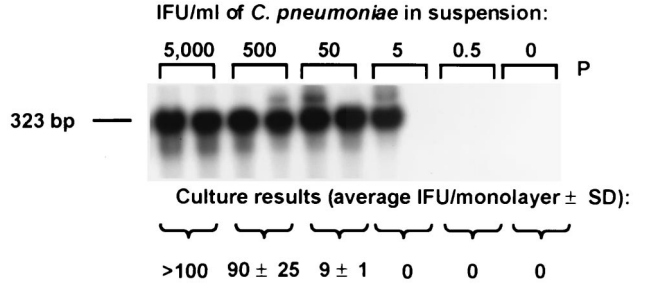

FIG. 2. Comparison of the results of the PCR and culture assays for the detection of *C. pneumoniae* in seeded swab samples. IFU, inclusion-forming units. The *C. pneumoniae* PCR product (323 bp) is indicated. P, probe control; SD, standard deviation.

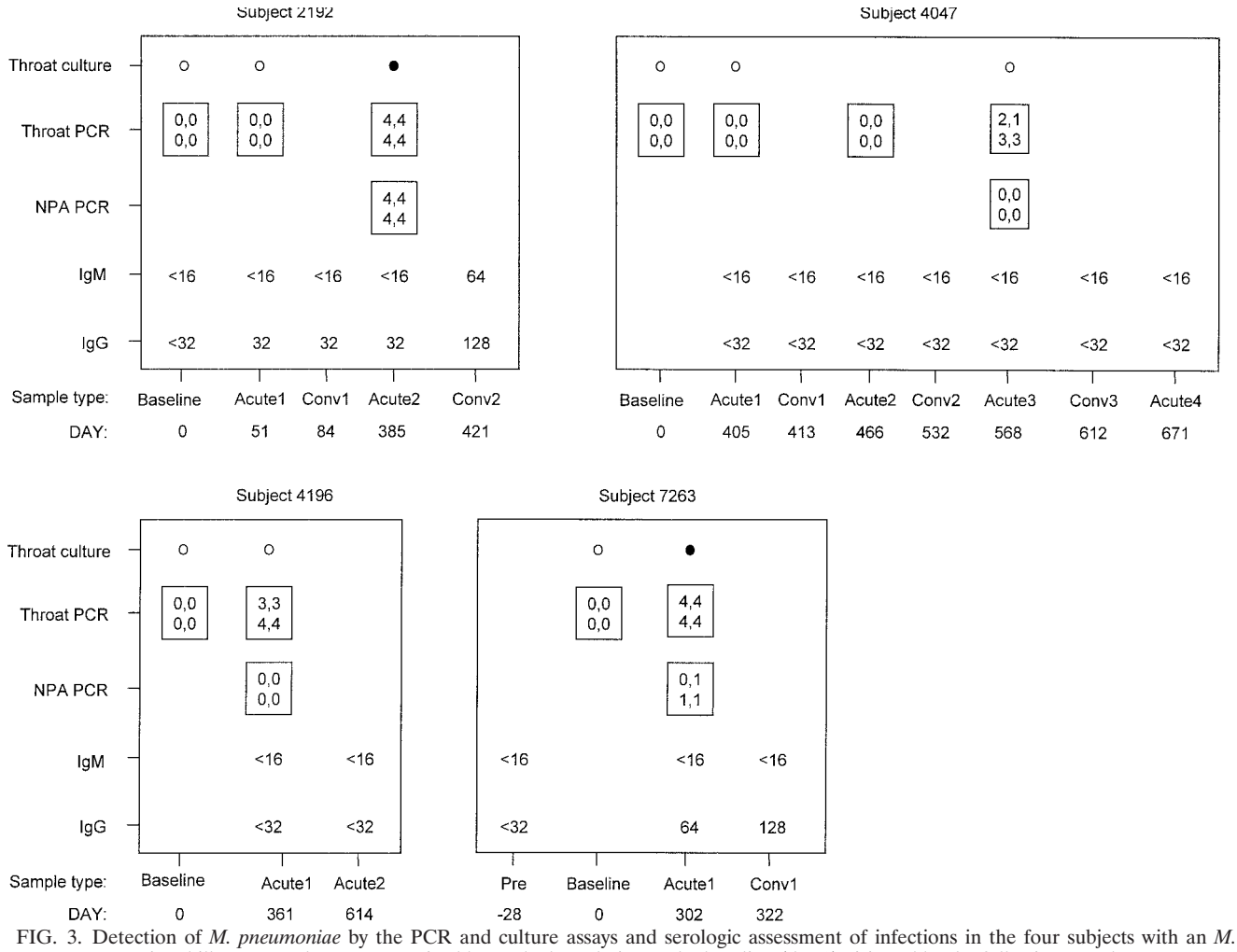

FIG. 3. Detection of *M. pneumoniae* by the PCR and culture assays and serologic assessment of infections in the four subjects with an *M. pneumoniae*-associated illness. Results are categorized by study day relative to the baseline (day 0) visit and by the following sample types: acute, sample obtained at a visit during an acute illness; conv, sample obtained at a visit during convalescence. The number following the sample type refers to the number of the consecutive illness visit for each subject. NPA, nasopharyngeal aspirate; ○, negative culture result; ●, positive culture result. Boxes with four numbers are intensity results for the PCR-based assay: the values in the upper row correspond to the intensities of the 230-bp *M. pneumoniae* band in the duplicate assays, and the values in the lower row correspond to the intensities of the 124-bp *M. pneumoniae* band in the duplicate assays. Band intensity ratings: 0, not visible; 1, barely visible; 2, dim but definite; 3, dark (approximately 75% of maximum intensity); and 4, maximum intensity.

uated by microimmunofluorescent-antibody tests (3, 8). There were no increases in anti-*M. pneumoniae* IgG antibody titers of ≥4-fold or any positive anti-*M. pneumoniae* IgM titers. There were no increases in anti-*C. pneumoniae* IgA or IgG antibody titers of ≥4-fold or any detectable anti-*C. pneumoniae* IgM antibody titers. The seroprevalence rates of anti-*M. pneumoniae* IgG antibody (i.e., titer of ≥32) and anti-*C. pneumoniae* IgG antibody (i.e., titer of ≥8) were 38 and 74%, respectively, and the coseroprevalence rate of the two antibodies together was 27%.

Sensitive, specific, and robust PCR assays, as well as culture and serologic techniques, were utilized to assess *M. pneumoniae* and *C. pneumoniae* infections in adolescents and adults. The frequency of *M. pneumoniae*-associated cough illness was 0.8%, and no episodes of *C. pneumoniae*-associated illness were detected. The high seroprevalence rates of IgG antibodies to *M. pneumoniae* and *C. pneumoniae* identified in a subgroup of the study subjects suggest that many of the subjects had acquired some measure of protective immunity prior to the development of their first episode of persistent cough (10, 13). Accordingly, differences in *M. pneumoniae* and *C. pneumoniae* infection rates may be expected in other populations where the seroprevalence rates are lower or at times when the agents are more common in the community (4, 5).

In conclusion, the results of this study suggest that *M. pneumoniae* is an infrequent agent of cough illnesses that are present for 5 days or longer in adolescents and adults in the United States and that *C. pneumoniae* is of even less importance.

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