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Development and Evaluation of Real-Time PCR-Based Fluorescence Assays for Detection of *Chlamydia pneumoniae*

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***Chlamydia pneumoniae* is an important respiratory pathogen recently associated with atherosclerosis and several other chronic diseases. Detection of *C. pneumoniae* is inconsistent, and standardized PCR assays are needed. Two real-time PCR assays specific for *C. pneumoniae* were developed by using the fluorescent dye-labeled TaqMan probe-based system. Oligonucleotide primers and probes were designed to target two variable domains of the *ompA* gene, VD2 and VD4. The limit of detection for each of the two PCR assays was 0.001 inclusion-forming unit. Thirty-nine *C. pneumoniae* isolates obtained from widely distributed geographical areas were amplified by the VD2 and VD4 assays, producing the expected 108- and 125-bp amplification products, respectively. None of the *C. trachomatis* serovars, *C. psittaci* strains, other organisms, or human DNAs tested were amplified. The amplification results of the newly developed assays were compared to the results of culturing and to two nested PCR assays, targeting the 16S rRNA and *ompA* genes. The assays were compared by testing *C. pneumoniae* purified elementary bodies, animal tissues, 228 peripheral blood mononuclear cell (PBMC) specimens, and 179 oropharyngeal (OP) swab specimens obtained from ischemic stroke patients or matched controls. The real-time VD4 assay and one nested PCR each detected *C. pneumoniae* in a single, but different, PBMC specimen. Eleven of 179 OP specimens (6.1%) showed evidence of the presence of *C. pneumoniae* in one or more tests. The real-time VD4 assay detected the most positive results of the five assays. We believe that this real-time PCR assay offers advantages over nested PCR assays and may improve the detection of *C. pneumoniae* in clinical specimens.**

Chlamydia pneumoniae is an intracellular bacterium implicated in upper and lower respiratory tract infections in humans. It has been reported to be responsible for ~10% of cases of community-acquired pneumonia and to be an etiologic agent of bronchitis, sinusitis, and other respiratory tract illnesses (15, 17, 18, 23). Recently, *C. pneumoniae* was associated with several chronic diseases, including multiple sclerosis, Kawasaki's disease, and Alzheimer's disease (2, 34, 43), although these associations have been disputed by other studies (14, 19, 41). More importantly, data from numerous studies have suggested a possible link between *C. pneumoniae* infections and atherosclerotic vascular diseases. The reports of the association between *C. pneumoniae* and atherosclerosis are based on serologic and animal model studies, direct detection of the organism in atherosclerotic lesions, and preliminary clinical trials showing improved outcome among patients treated with antibiotics (16, 22, 33, 38, 40). The accumulating data demonstrating an association between *C. pneumoniae* and atherosclerosis are not entirely consistent; some studies show a significant association (9, 26, 31), but others do not (39, 48, 49). Moreover, it must be emphasized that evidence proving a causal role of *C. pneumoniae* in the pathogenesis of atherosclerosis is still lacking.

The isolation and propagation of *C. pneumoniae* from clinical specimens by using cell cultures is relatively labor-intensive and insensitive, and interpretation requires technical expertise (8). Serologic analysis, particularly microimmunofluorescence tests, has been extensively used; however, interpretation is problematic, since a large part of the population has preexisting immunoglobulin G antibodies from a previous exposure(s) (47). In addition, serologic methods are subjective, and there is considerable cross-reaction with other species of *Chlamydia* and with *Bartonella* (24, 30, 35, 47). Due to the difficulties with culturing and serologic analysis, a number of nucleic acid amplification assays for detecting *C. pneumoniae* have been developed (6). Current PCR methods are based on the amplification of a cloned *Pst*I fragment (7), genes encoding 16S rRNA (3, 11, 28, 32), or the gene for the major outer membrane protein, known as *omp1* or *ompA* (45). There is no commercially available PCR assay for *C. pneumoniae*, and results have varied widely, especially for detection of this microorganism in atheromatous lesions. For instance, the reported rate of *C. pneumoniae* DNA detection within atherosclerotic lesions by PCR varies from 0 and 80% (20, 44, 49), indicating a critical need for standardized assays. In an attempt to standardize the currently available *C. pneumoniae* diagnostic assays, an international meeting was convened by the U.S. Centers for Disease Control and Prevention (CDC) and the Canadian Laboratory Centre for Disease Control (LCDC) (8). Four PCR methods

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TABLE 1. *C. pneumoniae* isolates tested by real-time PCR based fluorescence assays designed to detect VD2 and VD4 of the *ompA* gene

Isolate	Site of isolation	Geographic location	*Mycoplasma* contamination[a]	Source[b]
TW-183	Conjunctiva	Taiwan	No	ATCC (VR2282)
CM-1	Sputum	Ga.	No	ATCC (VR1360)
CWL-029	Pharynx	Ga.	No	ATCC (VR1310)
TW-2043	Nasopharynx	N.Y.	Yes	ATCC (VR1355)
TW-2023	Nasopharynx	N.Y.	Yes	ATCC (VR1356)
AR-39	Pharynx	Wash.	Yes	ATCC (535920)
CWL-011	Pharynx	Ga.	No	C. Black
CWL-050	Pharynx	Ga.	No	C. Black
A-03	Coronary atheroma	Ky.	Yes	J. Summersgill
BAL-15	BAL[c]	N.Y.	Yes	M. Hammerschlag
BAL-16	BAL	N.Y.	Yes	M. Hammerschlag
UL-029	Pharynx	Ky.	No	J. Summersgill
AR-388	Pharynx	Wash.	Yes	T. Grayston
BR-393	Pharynx	Unknown	Yes	C. Gaydos
W2	Pharynx	Wis.	No	B. MacDonald
W3	Pharynx	Wis.	Yes	B. MacDonald
W4	Pharynx	Wis.	No	B. MacDonald
W5	Pharynx	Wis.	No	B. MacDonald
W6	Pharynx	Wis.	No	B. MacDonald
IOL-207	Conjunctiva	Iran	No	P. Nicolini
FML-7	Nasopharynx	Norway	No	B. P. Berdal
FML-12	Nasopharynx	Norway	No	B. P. Berdal
FML-16	Nasopharynx	Norway	No	B. P. Berdal
FML-19	Nasopharynx	Norway	No	B. P. Berdal
H-12	Pharynx	Finland	Yes	P. Saiku
K-6	Pharynx	Finland	Yes	P. Saiku
K-66	Pharynx	Finland	Yes	P. Saiku
P1 (parola)	Pharynx	Finland	Yes	P. Saiku
UZG1	Pharynx	Belgium	Yes	J. Ossewaarde
12N	Pharynx	Finland	No	B. P. Berdal
19N	Pharynx	Finland	No	B. P. Berdal
YK-41	Pharynx	Japan	Yes	Y. Kanamoto
U172	Unknown	Sweden	Yes	J. Boman
U1271	Unknown	Sweden	Yes	J. Boman
U1272	Unknown	Sweden	Yes	J. Boman
U1273	Unknown	Sweden	Yes	J. Boman
T45953	Unknown	Sweden	Yes	J. Boman
AL-1	Unknown	Sweden	Yes	J. Boman
IOL-1515	Unknown	Sweden	Yes	J. Boman

[a] Mycoplasma contamination was tested by an in-house PCR designed to detect the 16S rRNA gene.
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] BAL, bronchoalveolar lavage.

(all conventional gel-based assays) met the proposed criteria for a validated assay (7, 11, 28, 45).

The recently introduced real-time PCR-based fluorescence technologies have many advantages: (i) high sensitivity; (ii) high specificity due to binding of two primers and one probe; (iii) usefulness as quantitative assays; (iv) operation in a closed system, avoiding contamination; and (v) ability to provide results faster than gel-based PCR assays, allowing rapid intervention (25, 46). We have developed two real-time PCR assays for *C. pneumoniae* by using a fluorescent dye-labeled TaqMan probe-based system (Applied Biosystems, Foster City, Calif.) (25). Two pairs of primers and two fluorescent probes were designed based on the nucleotide sequences of two regions of the *ompA* gene corresponding to variable domains VD2 and

VD4. In contrast to the situation for *C. trachomatis* and *C. psittaci*, the *ompA* VD4 of *C. pneumoniae* is highly conserved and is therefore a good target for a species-specific PCR (12). Here we describe the development and validation of VD2 and VD4 PCR assays.

MATERIALS AND METHODS

Bacterial isolates. *C. pneumoniae* isolates and other bacterial species are described in Tables 1 and 2, respectively.

Animal tissues. Two 8-week-old C57BL/6J female mice were inoculated intranasally with 40 μl of either sterile saline (control mouse) or sterile saline containing 2×10^6 inclusion-forming units (IFU) of *C. pneumoniae* strain BR-393 (infected mouse). The animals were sacrificed 3 days postinoculation. Liver, lung, and spleen tissues from each animal were tested by PCR to determine if the

TABLE 2. Microorganisms ($n = 84$) used to test the cross-reactivity of the real-time PCR assays designed to detect VD2 and VD4 of the *ompA* gene of *C. pneumoniae*

Genus	Species, serovar, serotype, or serogroup	No. tested
Chlamydia	*C. trachomatis* serovars A, Ba, C, D, E, F, G, H, I, J, K, and L2; *C. psittaci* serovars A, B, D, and E	17
Mycoplasma	*M. pneumoniae, M. salivarium, M. fermentans, M. hominis, M. orale, M. faucium, M. buccale,* and *M. penetrans*	16
Legionella	*L. pneumophila, L. jordanis, L. micdadei,* and *L. longbeachae*	4
Streptococcus	*S. pneumoniae, S. pyogenes, S. agalactiae,* group C, *S. oralis, S. mitis, S. crista, S. gordonii, S. sanguis, S. parasanguis,* and *S. vestibularis*	22
Haemophilus	*H. influenzae* serotypes a, b, c, d, e, and f; nontypeable *H. influenzae; H. parainfluenzae*	8
Neisseria	*N. meningitidis* serogroups A, B, C, Y, and W135; nongroupable *N. meningitidis, N. sicca; N. flavescens*	8
Bordetella	*B. pertussis*	2
Branhamella	*B. catarrhalis*	1
Staphylococcus	*S. aureus*	1
Corynebacterium	*C. diphtheriae*	1
Mycobacterium	*M. tuberculosis*	1
Eikenella	*E. corrodens*	1
Pseudomonas	*P. aeruginosa*	1
Proteus	*P. mirabilis*	1

real-time PCR assays could detect *C. pneumoniae* DNA in animals following acute-phase infection.

Clinical specimens. Specimens were collected from 317 subjects (age, ≥ 60 years) enrolled in an ongoing study designed to determine if *C. pneumoniae* infection increases the risk of first ischemic stroke. Informed written consent was obtained from all subjects, and procedures were carried out in accordance with the institutional review boards of the CDC and Columbia-Presbyterian Medical Center of New York. Specimens were collected from October 1999 to January 2001. These included 228 samples of peripheral blood mononuclear cells (PBMC) and 179 oropharyngeal (OP) swab samples. Because the specimens are part of an ongoing study, laboratory personnel remained blinded to the identities of cases and controls. Three atheromatous plaques and one lung tissue specimen obtained from a person free of *C. pneumoniae* and submitted to the CDC for reference diagnostic testing were also included in order to check for PCR inhibition. The PBMC specimens were analyzed by PCR, and the OP specimens were analyzed by PCR and culturing.

For PBMC fractionation, approximately 8 ml of whole blood was drawn from subjects at the Columbia-Presbyterian Medical Center of New York and transferred to Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, N.J.) within 2 h after collection. The blood samples were centrifuged at $1,500 \times g$ for 1 h, and the mononuclear cell layer was aspirated, divided into two aliquots of approximately 200 μl each, and frozen at $-70°C$. PBMC preparations were shipped to the CDC in batches on dry ice. The samples collected with the OP swabs, with Dracon tips and plastic shafts (Remel Inc., Lenexa, Kans.), were placed in 3 ml of Multi-Microbe Medium M4-3 (Remel) and transported at 4°C, generally within 24 h. Although unknown for *C. pneumoniae*, the percentages of *C. trachomatis* recovery in M4-3 transport medium are reported by the manufacturer to be 82% after 8 h, 43% after 24 h, and 33% after 48 h at 2 to 8°C. Upon arrival, the swabs were vortexed in the transport medium for 1 min and pressed against the side of the tube to extract all of the liquid. One milliliter of this fluid was either stored at $-70°C$ or immediately centrifuged at $20,000 \times g$ for 20 min, and the pellet was used for DNA extraction. Frozen samples were thawed later for testing, centrifuged, and processed in the same manner as the fresh samples. A total of 350 μl of the original fluid was used for the cell culture inoculum.

Cell culturing. *C. pneumoniae* isolates (Table 1) were propagated in HEp-2 cells as previously described (50). Briefly, the cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, and 10% fetal calf serum. Monolayers were grown in 150-cm² culture flasks at 36°C in a 5% CO_2 atmosphere. Prior to infection, HEp-2 cells were used to seed eight 25-cm² culture flasks and were incubated under the same conditions for 48 to 72 h, depending on monolayer confluence. The *C. pneumoniae*-infected HEp-2 cells were centrifuged at $1,000 \times g$ for 1 h at 25°C, and the inoculum was removed and replaced with IMDM supplemented with cycloheximide (1 μg/ml). The cells were incubated at 36°C in a 5% CO_2 atmosphere for 72 h. The monolayers were harvested and sonicated at 60 Hz for 30 s, and cellular debris was removed by centrifugation ($500 \times g$, 10 min, 25°C). Elementary bodies were pelleted at $30,000 \times g$ for 45 min at 4°C, resuspended in 0.5 ml of sucrose-phosphate-glutamate medium supplemented with 10% fetal calf serum, and stored in aliquots at $-70°C$ until used.

The titration of *C. pneumoniae* frozen stock cultures was performed with 96-well microtiter plates containing HEp-2 cells. Fifty-microliter quantities of 10-fold dilutions of *C. pneumoniae* stock cultures were used to inoculate triplicate wells. After incubation, the cells were fixed with methanol and stained with a *Chlamydia* genus-specific monoclonal antibody (Pathfinder chlamydia culture confirmation system; Bio-Rad S.A., Redmond, Wash.). The inclusions were counted by using an inverted fluorescence microscope, and the number of IFU per milliliter was calculated for each stock. Contamination with another *Chlamydia* species was excluded by staining the inclusions with a *C. pneumoniae*-specific monoclonal antibody by using a chlamydia cel Pn IF test (Cellabs, Brookvale, Australia) and PCR analysis with genus- and species-specific primers (32). Contamination with *Mycoplasma* was checked by a PCR assay designed to detect the 16S rRNA gene (36).

OP specimens were cultured in HEp-2 cells by using multiple centrifugations as described by Pruckler et al. (37). OP specimens (350 ml) were sonicated for 30 s, and 50 μl was used to inoculate, in triplicate, 96-well microtiter plates containing HEp-2 cells. Two plates were used for each experiment; one was stained, and the other subcultured. After centrifugation at $1,000 \times g$ for 1 h at 25°C, the inoculum was removed and replaced with IMDM supplemented with cycloheximide (1 μg/ml), gentamicin (10 μg/ml), vancomycin (25 μg/ml), and amphotericin B (2 μg/ml). Cultures were incubated at 36°C in a 5% CO_2 atmosphere. On day 3, cultures were centrifuged, and the medium was replaced. On days 4 and 5, cultures were centrifuged again, but the medium was not replaced. After the total of 7 days of incubation, the cultures were blindly passaged twice and incubated for another 3 days per passage before being considered negative. Cultures were fixed, stained, and visualized as describe before. If a potential inclusion was seen, the HEp-2 cells were scraped from the three wells, and the presence of *C. pneumoniae* was tested by the real-time PCR assay targeting the VD4 region. If the PCR assay was positive, the culture was considered positive, and up to five additional passages were performed in order to propagate the isolates.

DNA extraction. DNAs from *C. pneumoniae* purified elementary bodies, other bacterial isolates, clinical specimens, and animal tissues were extracted with a QiaAmp DNA mini kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions, eluted in 100 μl of Qiagen elution buffer, and stored at $-20°C$. One negative no-template control was included for every five processed specimens; in the control, sterile distilled water was added instead of specimen. The concentrations of DNAs extracted from *C. pneumoniae* isolates were measured with a spectrophotometer (A_{260}).

Real-time PCR assays. Two pairs of primers and two fluorescent probes were designed based on the nucleotide sequences of VD2 and VD4 of the *ompA* gene of *C. pneumoniae*. They were designed by using ABI Primer Express software (Applied Biosystems) and generated PCR products of 108 bp (VD2) and 125 bp (VD4). The fluorogenic probes were synthesized with a 6-carboxy-fluorescein (FAM) reporter molecule attached at the 5′ end and a 6-carboxy-tetramethyl-rhodamine (TAMRA) *N*-hydroxysuccinimide (NHS) ester quencher dye linked to a linker arm nucleotide phosphoramidite (Glen Research, Sterling, Va.) close to the 3′ end (25). The probes were synthesized with a 3′-terminal phosphate group to prevent extension during PCR. Primers and probes were synthesized in
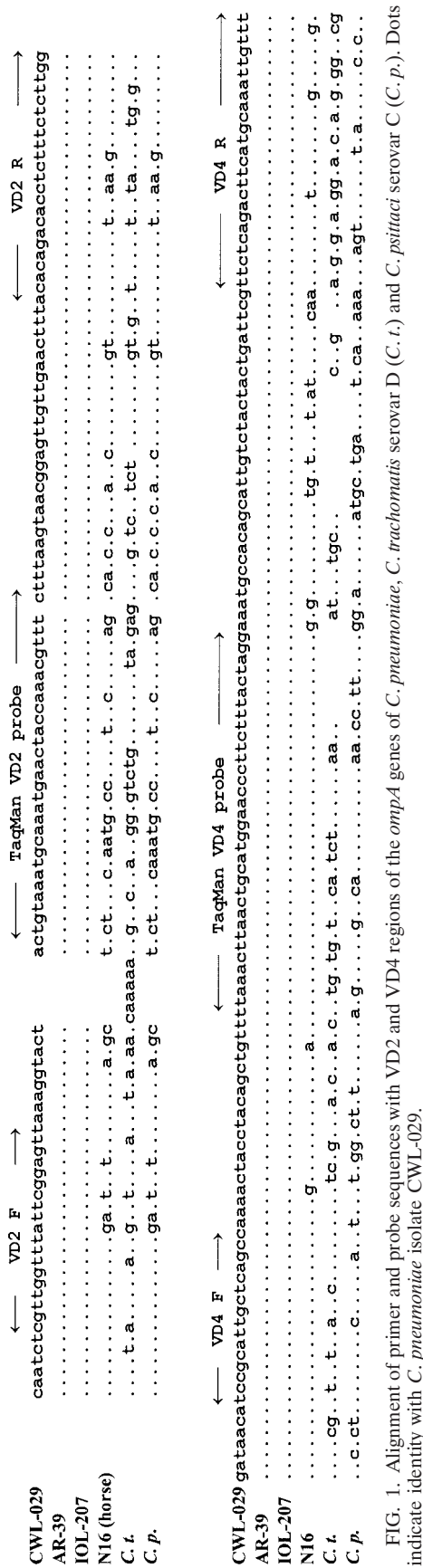
FIG. 1. Alignment of primer and probe sequences with VD2 and VD4 regions of the *ompA* genes of *C. pneumoniae*, *C. trachomatis* serovar D (*C. t.*) and *C. psittaci* serovar C (*C. p.*). Dots indicate identity with *C. pneumoniae* isolate CWL-029.

the Biotechnology Core Facility at the CDC by using a model 394–8 DNA synthesizer (Applied Biosystems) and standard phosphoramidite chemistries.

A BLAST search was performed to check the specificity of the DNA sequences of the VD4 and VD2 primer and probe sets. In addition, the targeted sequences of four isolates of *C. pneumoniae* (GenBank accession numbers: CWL-029, AE001652; AR-39, M69230; IOL-207, M64064; and N16, L04982) were aligned with the VD2 and VD4 sequences of *C. trachomatis* (GenBank accession number X77364) and *C. psittaci* (GenBank accession number L25436) in order to verify the species specificity of both assays (Fig. 1).

Reactions were prepared with a 96-well MicroAmp optical plate (Applied Biosystems) by the addition of a 5-μl aliquot of extracted DNA to 20 μl of a PCR master mixture consisting of 1× TaqMan universal PCR master mix (Applied Biosystems), 200 nM each primer, and 100 nM fluorescent probe. Primers and probes had been previously titrated to check for amplification efficiency. Amplification and detection were performed with an AB Prism 7700 sequence detection system (Applied Biosystems) by using the manufacturer's standard protocols. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard procedures for the operation of the model 7700 system were followed in this study, including the use of all default program settings, with the exception of reaction volume, which was changed from 50 to 25 μl. Cycle threshold (C_T) values, defined as the fraction of a cycle number at which the measured fluorescence generated by the released reporter molecule during cleavage exceeds a fixed threshold value above the baseline, were automatically calculated by the instrument for each reaction. Target gene copy values were derived from a standard curve generated by plotting the C_T values for 10-fold serial dilutions of 10^9 to 10^0 copies of the VD4 PCR product. Each run contained at least six no-template controls to establish the baseline emission intensity of the quenched reporter dye. Negative controls (one for every five extracted DNA samples) were included, as was a positive control consisting of 1 ng of DNA extracted from *C. pneumoniae* isolate ATCC VR1360. The VD4 PCR product was obtained by amplification of the DNA extracted from the same isolate, following by purification with a QIAquick PCR purification kit (Qiagen) and precipitation with 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate. The pellet was washed twice with ethanol, dried, and resuspended in 50 μl of Qiagen elution buffer. The DNA concentrations were determined by using a spectrophotometer (A_{260}), and the number of copies was calculated. The 10-fold serial dilutions were dispensed in single-use amounts to avoid freeze-thaw and were stored at −20°C until needed.

The sensitivities of both real-time PCR assays were evaluated by testing approximately 1 ng of DNA from each of 39 *C. pneumoniae* strains (Table 1) and by testing 10-fold serial dilutions of DNA extracted from purified elementary bodies of *C. pneumoniae* ATCC VR1360. The specificity was determined by testing DNA extracted from other bacterial species commonly found in the human respiratory tract (Table 2) and human placental DNA (Sigma, St. Louis, Mo.). In order to identify potential inhibitors in the samples or in the PCR itself, three pools of repeatedly negative DNA extracts from clinical specimens (five PBMC specimens, five OP specimens, and three atheromatous plaques) and one lung tissue specimen were spiked with 10-fold serial dilutions of purified *C. pneumoniae* DNA (ATCC VR1360) ranging from 10^6 to 10^2 copies. Amplification plots were compared to those obtained by using only the serial dilutions (10^{-1} to 10^{-5}) of the *C. pneumoniae* isolate as DNA templates. In addition, amplification of the human β-globin gene was used as a control for PCR inhibition. A 5-μl aliquot of DNA extracted from each clinical specimen was tested in a separate reaction for the presence of amplified DNA by using the real-time PCR protocol described by Tucker et al. (46).

Comparison to nested PCR assays. The performances of the VD2 and VD4 real-time PCR assays in detecting *C. pneumoniae* DNA were compared to those of two nested PCRs, one targeting the 16S rRNA gene (32) and the other targeting a different region of the *ompA* gene (45). The latter PCR uses the technique of "touchdown," in which the annealing temperature is lowered 1°C every two cycles from 65 to 55°C. The four assays were compared by (i) determining the sensitivity for detecting *C. pneumoniae* DNA, (ii) testing tissues from animals infected with *C. pneumoniae*, (iii) testing OP and PBMC specimens obtained from subjects enrolled in the stroke case control study, and (iv) examining the ability of the assays to detect *C. pneumoniae* in OP specimens relative to that of cell culturing. The sensitivities of the four methods were compared by using DNA extracted from *C. pneumoniae* ATCC VR1360 (titer, 7×10^5 IFU/ml). The stock culture was 10-fold serially diluted in culture medium containing HEp-2 cells to keep the level of the DNA background constant. DNA was extracted from each dilution. Assuming 100% DNA isolation efficiency, the amount of IFU used for amplification ranged from 1.4 IFU to 0.00014 IFU per PCR. For all four PCRs, the amplification reactions were run in a volume of 50 μl containing 5 μl of DNA in triplicate and on the same day to avoid freeze-thaw

TABLE 3. PCR assays for *C. pneumoniae* detection

Assay format	Target	Primer or probe designation	Primer or probe sequence (5′ → 3′)	Amplicon size (bp)	*Taq* DNA polymerase	DNA/PCR (μl)	Source or reference
Real-time VD2	*ompA*	VD$_2$F	CGT TGG TTT ATT CGG AGT TA	108	Hot start	5/25	This study
		VD$_2$R	CCA AGA GAA AGA GGT GTC TGT				
		VD$_2$ probe	FAM-TGT AAA TGC AAA TGA ACT ACC AAA CGT TTC-TAMRA				
Real-time VD4	*ompA*	VD$_4$F	TCC GCA TTG CTC AGC C	125	Hot start	5/25	This study
		VD$_4$R	AAA CAA TTT GCA TGA AGT CTG AGA A				
		VD$_4$ probe	FAM-TAA ACT TAA CTG CAT GGA ACC CTT CTT TAC TAG G-TAMRA				
Nested touchdown	*ompA*	CP1	TTA CAA GCC TTG CCT GTA GG	333	Regular	10/50	45
		CP2	GCG ATC CCA AAT GTT TAA GGC				
		CPC	TTA TTA ATT GAT GGT ACA ATA	207			
		CPD	ATC TAC GGC AGT AGT ATA GTT				
Nested	16S rRNA	OF	ACG GAA TAA TGA CTT CGG	436	Hot start	10/50	32 (Modified)
		OR	TAC CTG GTA CGC TCA AAT				
		IF	CGG AAT AAT GAC TTC GGT TGT TAT TTA G	224			
		IR	TCA TCG CCT TGG TGG GCT T				

of DNA extracts. The nested assays were performed according to published protocols, with a slight modification made for the inner primer sequences of the 16S rRNA nested PCR (32) in order to decrease the difference in melting temperatures between the primers (Table 3). The concentrations of the PCR components and cycling conditions were the same, except that 1.5 mM MgCl$_2$ and a 62°C annealing temperature were used in the 16S rRNA inner PCR. For testing of clinical specimens or animal tissues, three reactions were carried out for each DNA extract (undiluted and 1:5 and 1:25 dilutions) (M. Zhang, B. P. Holloway, W. L. Thacker, S. B. Schwartz, and D. F. Talkington, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. G18, 1999), except for the nested PCR targeting the *ompA* gene (45), used only for undiluted clinical specimens. Positive amplification visualized by 1.5% agarose gel electrophoresis was confirmed by sequencing of the PCR product. A positive result in a single PCR determination was considered a positive test. Several measures were applied to all PCRs to avoid carryover contamination of specimens by amplified products. Separate rooms were used for preparing specimens, setting up PCRs, and analyzing prod-

ucts. The samples and the PCR master mixtures were prepared in two dedicated class II laminar safety cabinets. A separate set of pipettes was devoted to each step of the reaction, and aerosol barrier pipette tips as well as disposable gloves and gowns were used all the time.

Statistical analysis. The VD2 PCR, VD4 PCR, 16S rRNA nested PCR, *ompA* nested PCR, and culture results for *C. pneumoniae* detection were compared by using Fisher's exact test. Statistical significance was defined as a *P* value of <0.05.

RESULTS

Analytical sensitivity and specificity. The real-time VD2 and VD4 assays successfully amplified DNAs from all *C. pneumoniae* isolates (Table 1). The C$_T$ values ranged from 16 to 24 cycles. The lowest level of detection of purified DNA extracted
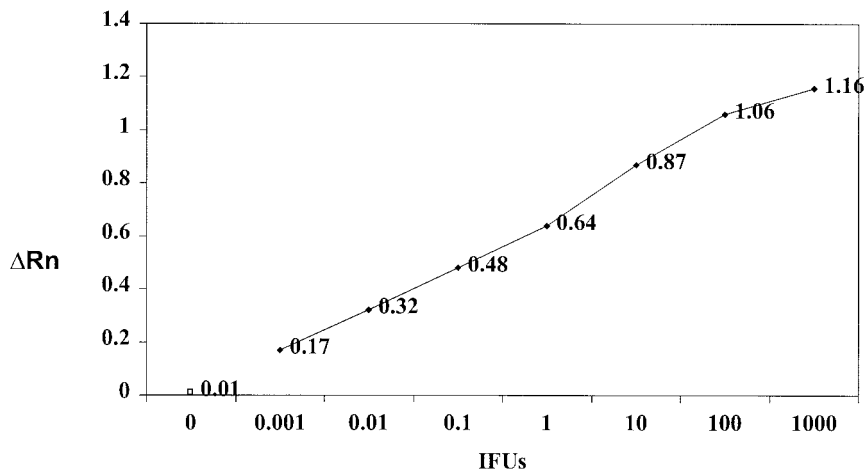

FIG. 2. Sensitivity limits of the *ompA* VD4 real-time PCR assay. Tenfold dilutions of DNA extracted from *C. pneumoniae* isolate ATCC VR1360 and with a known titer (IFU per milliliter) were used in the assay. The number of IFU per dilution, fluorescence measurement, and data analysis were determined as described in Materials and Methods. The limit of detection of the real-time PCR assay was 0.001 IFU per PCR. ΔRn, fluorescence emission values. Open square, negative control.

TABLE 4. Comparison of real-time and nested PCR assays with cell culturing for detection of *C. pneumoniae* in 179 oropharyngeal specimens

No. (%) of oropharyngeal specimens	Result[a] of:				
	Culturing[b]	Real-time TaqMan		16S rRNA nested PCR	*ompA* nested touchdown PCR
		VD2	VD4		
2 (1.1)	+	+	+	−	−
2 (1.1)	+	−	−	−	−
1 (0.6)	−	+	+	−	−
2 (1.1)	Overgrowth[c]	−	+	−	−
1 (0.6)	−	−	+	−	−
1 (0.6)	Overgrowth	−	−	+	−
2 (1.1)	−	−	−	+	−
6 (3.3)	Overgrowth	−	−	−	−
162 (90.5)	−	−	−	−	−

[a] +, positive; −, negative.
[b] One to three chlamydial inclusions were visualized in three wells. Only one of the positive cultures could be propagated after three blind passages. The other three positive cultures could not be propagated, and results were confirmed by PCR analysis of the infected monolayers.
[c] Overgrowth by respiratory flora.

from *C. pneumoniae* isolate ATCC VR1360 was 0.001 IFU per PCR when either VD2 (data not shown) or VD4 (Fig. 2) was targeted, corresponding to less than 10 copies of *C. pneumoniae* DNA. No amplified DNA products were detected when DNAs extracted from *C. psittaci*, *C. trachomatis*, or the other bacterial species listed in Table 2 or human DNA was tested for specificity.

Evaluation of PCR inhibition in spiked specimens. The pools of negative clinical specimens (PBMC, OP swabs, or atheromatous plaques) and the lung tissue spiked with serial dilutions of *C. pneumoniae* DNA ranging from 10^6 to 10^2 copies did not show any inhibition when VD4 was targeted. In a similar manner, the VD2 assay was positive at all concentrations tested, except for 100 copies of *C. pneumoniae* DNA in the atheromatous plaque tissue pool. The human β-globin gene was detected in all 14 specimens tested as a control for inhibition. The *C. pneumoniae* DNA-spiked specimens showed C_T values similar to those obtained for reactions containing only purified DNA up to the dilution 10^{-3}, corresponding to 10,000 copies. Some variability in C_T values was seen for the higher dilutions in both VD2 and VD4 assays (data not shown). It is possible that this variability occurred because of some degree of inhibition in the presence of a low copy number or some other unknown limiting factor.

Comparison to nested PCR assays. The sensitivity limits of the VD2 and VD4 real-time assays were compared to those of two nested PCR assays by using DNA extracted from dilutions of *C. pneumoniae* cultures with known titers. The limit of detection for all four assays was 0.001 IFU per PCR when 10-fold serial dilutions in Tris buffer of DNA extracted from the stock culture were tested. The sensitivities of the VD4 and VD2 assays were 0.014 and 0.14 IFU of *C. pneumoniae* per PCR, respectively, when a *C. pneumoniae* culture was serially diluted in HEp-2 cells (to mimic eukaryotic DNA levels in clinical samples) and the DNA was extracted from each dilution. With a standard curve, the average minimal amounts of *C. pneumoniae* DNA detected in the three replicates of the VD4 and VD2 real-time PCR assays were 12 and 83 copies, respectively. The detection limit for the nested PCR targeting the 16S rRNA gene was 0.014 IFU per PCR, and that of the nested PCR targeting the *ompA* gene was 0.14 IFU per PCR.

The performances of the VD2 and VD4 assays were compared to those of nested PCR assays by using DNAs extracted from lung, liver, and spleen tissues of a mouse infected with *C. pneumoniae*. All four assays detected *C. pneumoniae* only in the lung tissue of the infected animal. The three DNA extracts obtained from the lung tissue (undiluted and 1:5 and 1:25 dilutions) were found positive by all PCR assays. The tissues from the sham-inoculated control mouse were PCR negative.

A total of 228 PBMC specimens were analyzed by the four PCR assays. The real-time VD4 PCR assay and the 16S rRNA nested PCR assay each detected *C. pneumoniae* in a single, but different, PBMC specimen. All PBMC specimens were found negative by the VD2 and *ompA* PCR assays. The identity of the *C. pneumoniae* PCR product detected by the 16S rRNA nested PCR was confirmed by DNA sequencing. The discrepancy between the VD4 and 16S rRNA nested PCR assays was not resolved because both specimens were found negative by the other assays. The C_T and relative copy number values determined for the PBMC specimen found positive by the VD4 assay were 38 cycles and 1.3 copies, respectively. Two of five repeated VD4 reactions were positive with undiluted DNA extracts only, confirming the small amount of *C. pneumoniae* DNA in this sample. In contrast, the human β-globin gene was successfully amplified from all 228 PBMC specimens.

Among the 179 OP specimens tested by PCR, 3 (1.7%) were found positive by both the VD2 and the VD4 assays (Table 4). An additional three samples (1.7%) were found positive by the VD4 assay only. Three samples (1.7%) were found positive by the 16S rRNA nested PCR but were not reactive in any other assay (Table 4). The DNA sequences of the 16S rRNA PCR products were confirmed to be those of *C. pneumoniae*. The nested PCR targeting the *ompA* gene did not detect *C. pneumoniae* DNA in any of the specimens. Interestingly, two OP specimens found positive by the 16S nested PCR and one of the six specimens found positive by the VD4 PCR were found positive only when 1:5 dilutions of the DNA extract were used. This result could have been caused by the presence of PCR inhibitors in the undiluted samples. Attempts to repeat the reactions with diluted DNA extracts were not successful, possibly because of DNA degradation during storage. All OP specimens found positive by the VD2 or VD4 assay had C_T

values of 35 cycles or higher, suggesting a low DNA copy number in those specimens. The relative copy number calculated by the standard curve for *C. pneumoniae* DNA in those specimens was between 17 and 47 copies. The human β-globin gene was successfully amplified in all but two OP specimens, suggesting the presence of PCR inhibitors. Those two specimens were found negative by the four PCR assays and culturing.

Comparison to culturing. The PCR and culture results for *C. pneumoniae* detection in OP specimens were compared (Table 4). Four (2.2%) of the 179 OP specimens were culture positive. Very few chlamydial inclusions were observed in positive cultures (one to three per culture). They were confirmed as *C. pneumoniae* inclusions by VD4 PCR analysis of the infected HEp-2 cell monolayers. The inclusions of one culture were successfully propagated by serial passage. Two of four culture-positive samples were also found positive by the VD2 and VD4 real-time PCR assays but not by either nested PCR assay (Table 4). Successful recovery of *C. pneumoniae* was not possible for nine OP specimens due to overgrowth of the respiratory flora. Three of those samples were found PCR positive, two by the VD4 assay and one by the 16S rRNA nested PCR assay (Table 4). Overall, 6.1% (11 of 179) OP specimens showed evidence of the presence of *C. pneumoniae* in one or more of the five methods. The VD4 assay detected the most positive results (3.3% positives), followed by culturing (2.2% positives) and then VD2 and 16S nested PCR assays (1.7% positives). The nested touchdown PCR targeting the *ompA* gene was the least sensitive test.

We compared the proportions of OP specimens found positive by the VD4 assay with the proportions of OP specimens found positive by any of other four assays and found no statistically significant difference among the assays, except for the comparison of the VD4 PCR assay results (6 of 179 positive) with the *ompA* nested PCR assay results (0 of 179 positive), which was statistically significant ($P = 0.01$).

DISCUSSION

A large number of PCR assays have been developed to detect *C. pneumoniae* infection. This large number is partly due to the potential impact of demonstrating a causal role for *C. pneumoniae* in atherosclerosis and partly due to the difficulty in isolating the bacteria by culturing (6, 8). Current PCR assays are noncommercial assays that use various primers, reaction conditions, and methods of product visualization. In a multicenter comparison of PCR methods for the detection of *C. pneumoniae* in endarterectomy specimens, the positivity rates varied between 0 and 60% for the different methods (1).

Real-time PCR-based fluorescence assays have advantages over nested or single-step gel-based assays. First, fluorescent probes make the assays more specific than a non-probe-based PCR. Second, they require less manipulation, reducing the potential for amplification product carryover. This second point is an enormous advantage, especially over nested PCR technology. Finally, by use of a standard curve for the target of interest, relative copy number values can be determined for any unknown sample. Quantitative assays may be useful for determining the chlamydial load in *C. pneumoniae* carriers,

especially for epidemiologic studies or for monitoring the effect of therapy in treatment trials.

This study describes the optimization of two real-time PCR assays by using the validation criteria established during a meeting for the standardization of *C. pneumoniae* diagnostic assays (8). The CDC-LCDC recommendations stated that all new PCR assays should be compared to at least one of the four selected assays that met the proposed validation criteria (three single-step PCR assays [7, 11, 28] and one nested touchdown PCR assay [45]). Nested PCR assays are usually more sensitive than single-step PCR assays for the detection of *C. pneumoniae* in respiratory and PBMC specimens (3, 4, 29, 42). The sensitivities of the VD2 and VD4 assays were compared to those of the nested PCR assays described by Tong and Sillis (45) and Messmer et al. (32). Using purified *C. pneumoniae* DNAs as templates, the limits of *C. pneumoniae* detection of the real-time PCRs were equivalent to those of the nested PCRs (0.001 IFU per PCR). Similar results for an *ompA* nested PCR were reported by Mahony et al. (29) in a comparison of five PCR methods. Conversely, the lower limit of sensitivity for the four methods was different when a *C. pneumoniae* culture was serially diluted in medium containing HEp-2 cells. The limit of detection of the VD4 PCR assay was the same as that of the 16S rRNA nested PCR assay and 10-fold lower (more sensitive) than that of the VD2 or *ompA* PCR assay (0.014 versus 0.14 IFU).

PCR is a technique theoretically capable of detecting a single copy of purified target DNA, but the procedure often lacks sensitivity, reproducibility, and specificity when applied to direct testing of clinical material (10). This unsatisfactory performance of PCR is believed to be due to the presence of inhibitors of the polymerase in clinical material, low numbers of DNA molecules in clinical specimens, degradation of DNA molecules (false negative), or contamination by previously amplified DNA (false positive). The sensitivities of the four PCR assays were similar when acutely challenged animal tissues were used as DNA templates. This result was partly due to the high concentration of *C. pneumoniae* DNA in the lungs of the intranasally inoculated mouse. In contrast, performances were slightly different when clinical specimens from stroke patients or controls subjects were tested. The VD4 real-time PCR assay and the 16S rRNA nested PCR assay were both able to detect *C. pneumoniae* in a single but different PBMC specimen, but the results of the VD2 assay and the *ompA* nested PCR assay were negative. These results were consistent with the higher sensitivity of the VD4 and 16S rRNA PCR assays than of the VD2 and *ompA* PCR assays when mixtures of *C. pneumoniae* elementary bodies and HEp-2 cell DNA were tested. In contrast to our findings, the *ompA* nested PCR assay (45) was found to be the most sensitive assay in another study of five gel-based PCR assays for *C. pneumoniae* detection in PBMC specimens (29). In that study, the results of three single-step PCR assays (7, 11, 28) were all negative for 148 PBMC specimens tested, even though 11 specimens were found positive by an *ompA* PCR assay ($P < 0.001$).

The detection of *C. pneumoniae* DNA in PBMC specimens is the subject of some controversy. While some investigators have reported a high prevalence of *C. pneumoniae* DNA in patients with cardiovascular disease and in middle-aged blood donors (5, 27), others have found *C. pneumoniae* DNA in

PBMC specimens from only a small proportion of patients with coronary artery disease (29, 51). It has been hypothesized that some of the differences in the performances of PCR assays for detecting *C. pneumoniae* in PBMC are attributable to differences between assays, the presence of PCR inhibitors in the blood, sampling variability, or low concentrations of *C. pneumoniae* DNA in PBMC (29, 42). Smieja et al. (42) found that performing 5 or 10 replicates considerably increased sensitivity and reproducibility by demonstrating that the proportion of replicates that were positive would increase with the concentration of *C. pneumoniae* in the PBMC sample. We confirmed the low amount of *C. pneumoniae* DNA in the positive PBMC sample by using a standard curve in the VD4 real-time assay. Only two of five replicates of the VD4 reaction were positive, consistent with the observations for low amounts of DNA reported by Smieja et al. (42).

Among the 179 OP specimens tested by PCR and culturing, two of four culture-positive specimens were found positive by the VD2 and VD4 real-time PCR assays. In contrast, all four culture-positive samples were found negative by both nested PCR assays. Although nucleic acid amplification techniques can detect the presence of *C. pneumoniae* in clinical specimens and have been considered more sensitive than culturing (4), discrepant results for culturing and PCR have been reported. In a study to assess the utility of PCR for the diagnosis of acute-phase infection with *C. pneumoniae* in symptomatic and asymptomatic patients (13), only 23 of 31 culture-positive specimens were PCR positive. The authors suggested that one possible explanation for this discrepancy was the presence of PCR inhibitors in some samples. We tested PCR inhibition by amplification of the β-globin gene and found complete inhibition in only two OP specimens, which did not correspond to any of the culture-positive specimens. Some level of inhibition may be present in all specimens. For some specimens, the inhibition may be limited so that higher concentrations of human DNA are amplified but low levels of *C. pneumoniae* DNA are not detected. This notion is consistent with the low number of inclusions visualized in the positive cultures of OP specimens, as well as by the low DNA copy numbers or C_T values of ≥ 35 detected in all positive OP specimens by the real-time PCRs. We found a better correlation between the real-time PCR assays and culturing than between the nested PCR assays and culturing.

All positive specimens were repeat tested at least once by using undiluted extracts and 1:5 dilutions of the same DNA extracts. A few specimens were not repeatedly positive, suggesting DNA degradation due to thaw-freeze cycles or sampling variability (42). Another possibility for samples being found positive by a single assay is a false-positive reaction. However, the presence of contamination is not likely, as none of the negative controls was positive.

Several parameters were optimized to enable the VD2 and VD4 real-time assays to detect *C. pneumoniae* with relatively high sensitivity and specificity. The novel primer sets generate short PCR products (smaller than 200 bp); in contrast, products are larger than 400 bp in a majority of PCR methods previously described for *C. pneumoniae* detection (6). Therefore, enzyme and other reaction components do not become limiting factors, and poor template integrity in crude DNA preparations will not compromise the yield of amplified prod-

ucts. In addition, the primers are designed to target a species-specific region, avoiding cross-reactions with other *Chlamydia* species. The *ompA* PCR target is more species specific than the 16S rRNA target. The rRNA genes are some of the most highly conserved genes in nature, making them poor specific targets for PCR. In contrast to those of some bacterial species, the 16S rRNA gene of *C. pneumoniae* is present only as a single copy in the genome (21); therefore, its use as a target does not increase the possibility of detection of small numbers of *C. pneumoniae*.

The VD4 assay appears to be sensitive and specific for *C. pneumoniae* detection. Our results suggest that testing several replicates of recently extracted DNA before freeze-thaw cycles by using a sensitive test such as the VD4 real-time PCR is the best approach for detecting *C. pneumoniae* in clinical specimens. This approach is particularly relevant when low copy numbers of *C. pneumoniae* DNA are present. In addition, the practice of diluting DNA extracts will increase the chances of positive reactions if PCR inhibitors are present.

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