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The Pneumoplex Assays, a Multiplex PCR-Enzyme Hybridization Assay That Allows Simultaneous Detection of Five Organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and Its Real-Time Counterpart

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Respiratory disease caused by atypical bacteria remains an important cause of morbidity and mortality for adults and children, despite the widespread use of effective antimicrobials agents. Culture remains the "gold standard" for the detection of these agents. However, culture is labor-intensive, takes several days to weeks for growth, and can be very insensitive for the detection of some of these organisms. Newer singleplex PCR diagnostic tests are sensitive and specific, but multiple assays would be needed to detect all of the common pathogens. Therefore, we developed the Pneumoplex assays, a multiplex PCR-enzyme hybridization assay (the standard assay) and a multiplex real-time assay to detect the most common atypical pathogens in a single test. Primer and probe sequences were designed from conserved regions of specific genes for each of these organisms. The limits of detection were as follows: for *Bordetella pertussis*, 2 CFU/ml; for *Legionella pneumophila* (serotypes 1 to 15) and *Legionella micdadei*, 9 and 80 CFU/ml, respectively; for *Mycoplasma pneumoniae*, 5 CFU/ml; and for *Chlamydia* (*Chlamydophila*) *pneumoniae*, 0.01 50% tissue culture infective doses. Recombinant DNA controls for each of these organisms were constructed, and the number of copies for each DNA control was calculated. The Pneumoplex could detect each DNA control down to 10 copies/ml. The analytical specificity demonstrated no cross-reactivity between 23 common respiratory pathogens. One hundred twenty-five clinical bronchoalveolar lavage fluid samples tested by the standard assay demonstrated that the Pneumoplex yielded a sensitivity and a specificity of 100 and 98.5%, respectively. This test has the potential to assist clinicians in establishing a specific etiologic diagnosis before initiating therapy, to decrease hospital costs, and to prevent inappropriate antimicrobial therapy.

The recent severe acute respiratory syndrome outbreak demonstrated that one of the most challenging problems in the management of patients with atypical lower respiratory tract infections is the rapid identification of a wide array of organisms that might cause the disease. Microbiological analysis has identified atypical pathogens, such as *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, and *Legionella pneumophila*, as common causes of acute bronchitis and community-acquired pneumonia and has indicated that these organisms account for 15 to 50% of cases of atypical pneumonia (15, 17, 23). According to Ouchi (20), *M. pneumoniae* and *C. pneumoniae* were the causative organisms in a third of the cases of community-acquired pneumonia in childhood, with many patients having dual infections. In addition, many *Legionella* species other than *L. pneumophila* serotype 1 have been shown to cause human disease, and the incidence of *Legionella* disease is believed to be grossly underestimated (22). Finally, *Bordetella pertussis* has emerged as a common cause of respiratory illness in adults and atypical respiratory illness in children (4, 24).

The rapid and accurate identification of these causative agents is a key component for establishment of a proper prognosis, facilitation of early treatment, and perhaps, easing of public health concerns. Classical microbiological identification techniques, however, cannot provide an efficient means of diagnosis, because most of these organisms grow either slowly or not at all in culture, leading to delays in detection and diagnosis. These problems led to the emergence of other diagnostic methods, such as serologic testing by microimmunofluorescence or complement fixation assay (1, 6, 11). However, these tests demonstrate a lack of sensitivity and specificity, and increased titers are hard to detect in the acute phase of the disease (5). Therefore, nucleic acid amplification-based techniques have been developed to diagnose the infections caused by these pathogens. These assays are based on amplification of either isolated nucleic acid or whole-organism DNA by PCR with gene-specific primers. Individual singleplex PCR assays have been developed and accepted for *M. pneumoniae*, *Legionella* species, *Bordetella*, and *C. pneumoniae* organisms (9, 12, 13, 17–19, 21). However, to be clinically useful, it would necessitate the running of multiple singleplex PCR tests for each patient. This would be costly and inefficient. Therefore, there is a need for the development of a single diagnostic test capable of precisely detecting multiple pathogens simultaneously with exceptional sensitivity and specificity. A multiplex PCR assay was developed to detect *M. pneumoniae*, *L. pneumophila* (se-

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TABLE 1. Primer and probe sequences used in the standard and real-time Pneumoplex assays

Organism and primer or probe	Sequence
B. pertussis[a]	
Forward primer	5′-ATCGGGCATGCTTATGGGTGTTCA-3′
Reverse primer	5′-GCGGGCTAACTGTGAAGATTCAATAG-3′
Probe	5′-CTTGAGTGAACTGGGGGGTCGGCGATTTCCAGTT-3′
C. pneumoniae	
Forward primer	5′-AGGGCTATAAAGGCGTTGCT-3′
Reverse primer	5′-GTTAAGTTTAAAACAGCTGTA-3′
Probe	5′-GGAACAAAGTCTGCGACCATC-3′
L. micdadei	
Forward primer	5′-GTTGCTGACCGACGACCAGATGAAGGATGTGCTA-3′
Reverse primer	5′-TATTCCACAGTAACGACGTCGTCATCC-3′
Probe	5′-ATCCTTGAACGAGGCGATGGTGCTAAACCGACC-3′
L. pneumophila	
Forward primer	5′-CAATGTCAACAGCAATGGCTGCAAC-3′
Reverse primer	5′-CTCATAGCGTCTTGCATGCCTTTAGCC-3′
Probe	5′-CAAGGATAAGTTGTCTTATAGCATTGGTGCCGAT-3′
M. pneumoniae	
Forward primer	5′-GTTATGGAAACATAATGGAGGT-3′
Reverse primer	5′-TACAAGCTGGCGACTGTTTGTATTGGCCAT-3′
Probe	5′-TAACCGAGTGACAGGTGGTGCATGGTTGTCGT-3′

[a] *B. pertussis* was tested only by the standard Pneumoplex assay (PCR-enzyme hybridization assay).

rotypes 1 to 15) and *Legionella micdadei*, *B. pertussis*, and *C. pneumoniae* in a single test. These assays (compared to conventional techniques) provide both a qualitative and a semiquantitative means of identification of pathogens in a rapid, sensitive, specific, and cost-effective manner.

MATERIALS AND METHODS

Bacterial strains. All bacterial and viral strains used in this study were purchased from the American Type Culture Collection (ATCC), Manassas, Va. Some of the organisms, particularly *L. pneumophila*, *B. pertussis*, and *M. pneumoniae*, were supplied as lyophilized pellets and were resuspended in nuclease-free water before their DNA was extracted. All strains of bacteria were also maintained on specialized media that supported the growth of these different organisms.

DNA extraction. All ATCC strains of the different organisms were resuspended in 1.0 ml of RNase- and DNase-free H_2O. Serial 10-fold dilutions of the bacterial suspensions were prepared in M4 medium (Microtest viral transport medium; Remel, Lenexa, Kans.). DNA was extracted from 400 μl of the suspension by using a Nucleospin tissue kit (BD Biosciences, Palo Alto, Calif.) or a High Pure viral nucleic acid kit (Roche), according to the instructions of the manufacturers. Both extraction kits performed well and had similar sensitivities for the extraction of DNA from the different organisms (data not shown).

Generation of recombinant positive controls. The primers and probes used in the Pneumoplex assay were designed from highly conserved regions of different genes for each organism and are listed in Table 1. These primers specifically targeted the *mip* gene for *L. pneumophila* and *L. micdadei*, the porin gene for *B. pertussis*, the outer membrane protein gene for *C. pneumoniae*, and the 16S rRNA gene for *M. pneumoniae*.

The PCR products generated from the whole organisms were purified and cloned into plasmids by using the TOPO TA cloning system (Invitrogen, Carlsbad, Calif.), according to the instructions of the manufacturer.

An internal control that contained the same primer binding sequence for *B. pertussis* but a different probe binding sequence was generated in-house. This internal control DNA was used in experiments to determine if the PCR was inhibited in the presence of clinical specimens.

All positive controls were sequenced to verify the amplicon sequences and the possibility that any mismatches were generated during cloning. The positive controls were further amplified to generate large quantities, which were purified by a standard plasmid midi preparation protocol (Invitrogen). The quantity of DNA extracted was measured by determination of the absorbance at 260 nm and the A_{260}/A_{280} ratio to ensure the purity of the DNA.

To determine the approximate copy number present in the maxi prep, the absorbance (A_{260}) for the positive control was used in combination with Avogadro's number (6.022×10^{23}) and the plasmid molecular mass (established by using an average molecular mass per base pair of 660 Da and the total number of base pairs of the plasmid plus the total number of base pairs of the amplicon). Serial dilutions of the plasmids were used to help establish the analytical sensitivity and specificity of the assay.

Standard assay. The methodology used for the standard assay was that described previously (2). Briefly, 10 μl of extracted DNA was amplified by adding 39.5 μl of Multiplex Supermix, which contained five pairs of primers (the 3′ ends of the primers were biotinylated), and appropriate buffers to support a PCR with 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer). Amplification was performed in a thermocycler (2700; Perkin-Elmer), which included a hot-start step with *Taq* polymerase, followed by a total of 40 cycles of PCR under the following conditions: 2 cycles of 95°C for 60 s, 55°C for 30 s, and 72°C for 45 s, followed by 38 cycles of 94°C for 60 s, 60°C for 30 s, and 72°C for 30 s. The final cycle included an extension step at 72°C for 7 min. Following amplification the PCR products were purified with a Clontech Nucleospin extraction kit (BD Biosciences). Hybridization solutions (65 μl), each of which contained a horseradish peroxidase-labeled probe corresponding to a different bacterial species (*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *L. micdadei*, and *B. pertussis*), were added to 96-well streptavidin-coated microtiter plates; and the purified PCR products (5 μl) were then added to each well. Following hybridization, the plates were washed and tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well. After 10 min the reaction was stopped by adding 1 N H_2SO_4, and the optical density (OD) at 450 nm of each well was measured on a spectrophotometer. The positive cutoff value for this assay was chosen to be four times greater than the value for the negative control and greater than or equal to an OD value of 0.400 (2, 3, 7, 14).

Real-time assay. All organisms except *B. pertussis* were tested by the real-time assay in a manner similar to that described above for the standard assay. *B. pertussis* was not included in the real-time assay. All primer and probe sequences for the different organisms (except *B. pertussis*) were similar to those used in the standard assay format and targeted the same genes. The real-time probes were dual labeled with a reporter dye at the 5′ end and a quencher dye at the 3′ end. *L. pneumophila* and *L. micdadei* were labeled with 6-carboxyfluorescein (FAM), *C. pneumoniae* was labeled with Texas Red, *M. pneumoniae* was labeled with hexachloro-6-carboxy-fluoroscein (HEX), and the internal control was labeled with Cy5.

TABLE 2. Specificity of the Pneumoplex assay with 47 different microorganisms

Microorganism[a]	OD at A_{405} obtained with probe specific for[b]:				
	B. pertussis	*C. pneumoniae*	*L. micdadei*	*L. pneumophila*	*M. pneumoniae*
L. pneumophila serotype 1	0	0	0	>3.0	0
L. pneumophila serotypes 2 to 15	0	0	0	>3.0	0
L. micdadei	0	0	>3.0	0	0
Legionella anisa	0	0	0	0	0
Legionella birminghamensis	0	0	0	0.7	0
Legionella bozemani	0	0	0	0	0
Legionella dumoffic	0	0	0	0.6	0
Legionella hackeliae	0	0	0	0.3	0
Legionella longbeachae	0	0	0	0.5	0
Legionella maceachernii	0	0	0	0	0
Legionella wadsworthii	0	0	0	0	0
M. pneumoniae	0	0	0	0	>3.0
Mycoplasma fermentas	0	0	0	0	0
Mycoplasma genitalium	0	0	0	0	0
Mycoplasma hominis	0	0	0	0	0
Ureaplasma urealyticum	0	0	0	0	0
B. pertussis	>3.0	0	0	0	0
Bordetella parapertussis	0	0	0	0	0
Septococcus pneumoniae	0	0	0	0	0
Moraxella catarrhalis	0	0	0	0	0
Haemophilus influenzae	0	0	0	0	0
C. pneumoniae	0	>3.0	0	0	0
Chlamydia trachomatis	0	0	0	0	0
Adenoviruses A to F	0	0	0	0	0
Parainfluenza virus 2	0	0	0	0	0
Parainfluenza virus 3	0	0	0	0	0
Influenza virus A	0	0	0	0	0
Influenza virus B	0	0	0	0	0
Respiratory syncytial virus type A	0	0	0	0	0

[a] All bacteria were tested at greater than 1,000 colonies/ml. All viruses were tested at 100 $TCID_{50}$/ml.
[b] Values for *L. birminghamensis*, *L. dumoffi*, *L. hackeliae*, and *L. longbeachae* indicate minimal cross-reactivity, but only at concentrations of >1,000 colonies/ml.

Real-time PCR amplification was performed with an iCycler iQ instrument from Bio-Rad Laboratories (Hercules, Calif.). Ten microliters of purified DNA was added to 39.5 μl of a multiplex real-time Supermix (Invitrogen) that contained five pairs of primers, probes, and appropriate buffers to support PCR. Platinum *Taq* polymerase (2.5 U; Invitrogen) was added to each reaction mixture, and the reactions were run under the following cycling conditions: 5 min at 95°C, followed by 50 cycles at 95 for 15 s and 55°C for 1 min. Data analysis was performed by analyzing the real-time amplification plots with the iCycler iQ software for detection.

Analytical sensitivity. The analytical sensitivities of the standard and real-time Pneumoplex assays were determined by using serial dilutions of both recombinant DNA controls and whole organisms. Tenfold serial dilutions (1,000 to 1 copy/ml) of the DNA controls were made in M4 medium and tested. A 0.5 McFarland standard of the bacterial cultures was serially diluted ($1{:}10^6$ and $1{:}10^7$), and the DNA was extracted to assess the sensitivities of these assays.

Analytical specificity. The specificities of both Pneumoplex assays were evaluated by testing many of the common respiratory pathogens that could be present in clinical specimens for potential cross-reactivity. All ATCC bacterial and viral strains were diluted to 1:1,000 CFU/ml and 1:100 50% tissue culture infective doses ($TCID_{50}$s)/ml, respectively, and further tested by the Pneumoplex assays.

Clinical sensitivities and specificities of the standard Pneumoplex assay. A total of 125 clinical bronchoalveolar lavage (BAL) fluid specimens previously determined to be negative by PCR were used in the Pneumoplex assay. We spiked 20 samples with each of the five organisms mentioned, and 25 were left unspiked as negative controls. Of the specimens that were spiked, 50% were spiked at 5,000 CFU/ml and the other 50% were spiked at 500 CFU/ml. A total of 3.0 ml of the sample was spiked and split for blind testing by either culture (Milwaukee Health Department, Milwaukee, Wis.) or the standard Pneumoplex assay. Each 1.5-ml sample was spun at 5,220 × *g* to pellet any particulate material or bacteria. Then, all except 400 μl of the supernatant was removed; the 400 μl was used to resuspend any pellet prior to DNA extraction. Because the Milwaukee Health Department had difficulties culturing *M. pneumoniae*, 10 μl of frozen DNA from each of the samples was tested by a singleplex PCR-based assay (Viromed, Minnetonka, Minn.).

Evaluation of clinical samples. Three clinical specimens from a local hospital were simultaneously tested by both culture and the standard Pneumoplex assay format (enzyme hybridization assay).

RESULTS

Analytical specificities of Pneumoplex assays. The specificities of the primers and probes used in the Pneumoplex assays were first assessed by amplifying DNA from all five organisms with each of the primer pairs individually (singleplex assays). The amplicons were screened against all five probes to determine whether any cross-reactivity existed within the standard and the real-time Pneumoplex assays. All primers and probes efficiently amplified the specific target and demonstrated no cross-reactivity (data not shown).

To further assess the integrity of our primers in the Supermix, DNA was extracted from a panel of 47 different respiratory viruses and bacteria commonly found in the respiratory tract. The amplified products were analyzed against each probe to determine the specificity of the assay. A low level of cross-reactivity of the *L. pneumophila*-specific probe with four other pathogenic species of *Legionella* was observed, although this was evident only at higher concentrations ($>10^3$ CFU/ml) (Table 2). None of the other organisms tested demonstrated cross-reactivity by either the standard or the real-time assay.

TABLE 3. Analytical sensitivities of standard and real-time Pneumoplex assays

Organism	Standard Pneumoplex assay		Real-time Pneumoplex assay		
	No. of DNA copies/ml vs OD[a]	No. of whole-organism CFU/ml vs OD	Fluorophore/quencher[b]	No. of DNA copies/ml vs C_T[c]	No. of whole-organism CFU/ml vs C_T
B. pertussis	1:2.7	2:2.2			
C. pneumoniae	10:0.7	0.01 $TCID_{50}$/ml:0.4	Texas Red/BHQ2	10:34.9	0.1 $TCID_{50}$/ml:34.8
L. micdadei	10:1.3	1:1.4	FAM/BHQ1	100:32.6	80:37.5
L. pneumophila	1:2.9	9:1.0	FAM/BHQ1	10:37.0	90:34.8
M. pneumoniae	10:1.5	5:1.9	HEX/BHQ1	100:35.0	50:33
Internal control	1:3.0		Cy5/BHQ2	10:36.5	

[a] Serial 10-fold dilutions of DNA controls and whole organisms starting with a 0.5 McFarland standard of each organism were tested to determine the limit of detection of each of these strains by the standard and real-time Pneumoplex assay. ODs at *A*450 were determined on a spectrophotometer.
[b] BHQ2, black hole quencher 2; BHQ1, black hole quencher 1.
[c] C_T, cycle threshold; number of amplification cycles at which a signal was determined to be above the background by an internal algorithm of the real-time PCR machine.

Analytical sensitivities of standard and the real-time Pneumoplex assays. The analytical sensitivities of recombinant DNA controls and DNA extracted from whole organisms were tested by the standard and the real-time Pneumoplex assays. A comparative analysis of the limits of detection of both assays are shown in Table 3. Both assays could detect DNA to levels less than 100 copies/ml and colony counts to levels of less than 100 CFU/ml. The standard assay demonstrated increased sensitivity compared with that of the real-time assay.

Analytical sensitivities of the standard Pneumoplex assay with clinical samples compared to those of tissue culture and another singleplex PCR assay. The standard Pneumoplex assay was compared to the "gold standard" assay, i.e., cell culture, for all organisms except *M. pneumoniae*, which was tested by a *Mycoplasma*-specific PCR assay directed to a genomic target different from that to which the Pneumoplex assay is directed. A total of 125 clinical BAL fluid specimens were tested blindly to assess the validity of our assay. The Pneumoplex assay was positive for *L. micdadei* in 20 of 20 *L. micdadei*-positive specimens. No false-positive results were reported. Similar results were observed by culture, indicating a sensitivity and a specificity of 100% each for the Pneumoplex assay. By the Pneumoplex assay with *L. pneumophila*, one sample was found to be false positive for *B. pertussis* (on retesting, the sample was negative for *B. pertussis*). Therefore, the calculated sensitivity and specificity of the Pneumoplex assay for the detection of *L. pneumophila* were 100 and 99%, respectively. Twenty specimens positive for *C. pneumoniae* were tested by both methods, and culture provided a negative result for one specimen. The Pneumoplex assay successfully detected *C. pneumoniae* in all 20 positive specimens, demonstrating a sensitivity and a specificity of 100% each. Additionally, two specimens tested false positive for *B. pertussis* by the Pneumoplex assay but not by culture. Twenty *M. pneumoniae*-spiked BAL fluid specimens and five negative specimens were sent to another commercial laboratory for testing by a singleplex PCR. The commercial laboratory failed to detect the organism in 4 positive specimens, but the Pneumoplex assay provided the correct results for all 20 positive specimens and the 5 negative specimens. Even when these known spiked samples are counted as false positive, the Pneumoplex assay had a sensitivity and a specificity of 100 and 96%, respectively. Two clinical specimens that were culture positive for *B. pertussis* and *L. micdadei* were also positive by the Pneumoplex assay (Table 4), with an overall sensitivity of 100% (confidence interval [CI], 96 to 100%) and a specificity of 98.5% (CI, 97 to 99%).

Detection of dual infections by the standard Pneumoplex assay. Samples containing various combinations of the organisms used in this study were evaluated by the Pneumoplex assay. Clinical specimens were spiked with medium concentrations (10^6 dilution and 10^3 $TCID_{50}$s/ml) or low concentrations (10^7 dilution and 10^2 $TCID_{50}$s/ml) of organisms to determine if the presence of more than one organism in a sample could be detected efficiently. The results of the dual infectivity assays are shown in Table 5. The assay had no trouble identifying both organisms in samples with mixtures of organisms present at equal or unequal concentrations of each organism.

Evaluation of internal control spiked directly into positive samples containing whole organism or purified DNA. The accurate interpretation of a PCR-based assay requires the inclusion of sufficient controls for validation of each clinical run. These include specific PCR controls (positive and negative) and detection controls (probe controls). The clinical evaluation of PCR-based assays to monitor for the presence of potential inhibitors was considered critical. However, with the recent advancements in column-based DNA and RNA extraction methods, inhibitors are primarily eliminated. For some clinical specimens or in some situations, monitoring of a specific specimen for inhibition is needed or desired. Therefore, a series of experiments was performed to evaluate the usefulness of the inclusion of an internal control during the development of the Pneumoplex assay (the standard and real-time assays).

In a series of experiments, a fixed amount of the internal control (10^3 copies/ml) was added to serial dilutions of an organism prior to nucleic acid extraction. The DNA was amplified by the real-time and standard assays to determine if the organism in the test sample as well as the internal control was detected independently by both assays. The real-time Pneumoplex assay was able to detect the specific organism along with the internal control efficiently; however, there seemed to be some competition (only with low copy numbers) between the internal control and the genomic DNA during amplification by the standard Pneumoplex assay (data not shown). Additional experiments demonstrated that both assays performed well independently and efficiently in detecting a positive control DNA mixed with an internal control DNA.

Inhibition testing of the standard Pneumoplex assay. Four different specimen types (sputum, nasopharyngeal swab, se-

TABLE 4. Comparison of Pneumoplex (multiplex PCR), tissue culture, and singleplex PCR for detection of *B. pertussis*, *C. pneumoniae*, *L. micdadei*, *L. pneumophila*, and *M. pneumoniae* in spiked BAL fluid samples

Organism and Pneumoplex assay result	No. of samples with the following culture and singleplex PCR[a] result:		Sensitivity (% [95% CI])	Specificity (% [95% CI])
	Positive[b]	Negative		
B. pertussis				
Positive	21	2		
Negative	0	104	100 (84–100)	98 (93–100)
C. pneumoniae				
Positive	19	1		
Negative	0	107	100 (82–100)	99 (95–100)
L. pneumophila				
Positive	20	1		
Negative	0	106	100 (83–100)	99 (95–100)
L. micdadei				
Positive	21	0	100 (84–100)	100 (97–100)
Negative	0	106		
M. pneumoniae				
Positive	16	4	100 (79–100)	96 (91–99)
Negative	0	107		
Total (n = 127)			100 (96–100)	98.5 (97–99)

[a] The singleplex PCR was performed by a commercial laboratory (ViroMed) only with *M. pneumoniae*.
[b] Fifty percent of the samples (n = 10) were spiked with 500 CFU/ml (low positive), and 50% (n = 10) were spiked with 5,000 CFU/ml (medium positive).

rum, and cerebrospinal fluid specimens) were spiked with DNA controls and whole organisms to test for inhibition of the Pneumoplex assay (Table 6). PCR inhibition was determined by comparison of the results for samples spiked in M4 medium and those for different clinical specimens. The results demonstrated no difference in the OD values for the clinical specimens and spiked M4 medium (viral transport medium), indicating no inhibition of the PCR for this assay.

Evaluation of clinical samples. We blindly tested three clinical specimens (two BAL fluid specimens and one nasopharyngeal swab specimen) from patients from a local hospital suspected of having pneumonia. The Pneumoplex standard assay correctly detected *B. pertussis* in samples from two patients and *L. micdadei* in a sample from one patient. All three patients were determined to have high-positive values by the assay. We have contacted numerous laboratories around the country but have been unsuccessful in securing positive clinical specimens for further evaluation.

Although the results presented here are for only a few patients, they validate the ability of this assay to detect atypical bacteria. Studies with large numbers of patients will further confirm the usefulness of this assay for the diagnosis of complicated infections.

TABLE 5. ODs for dual infections by Pneumoplex assay

Organisms spiked in M4 medium[a]	Dilution or $TCID_{50}$s/ml[b]	OD at A_{450}[c]
BP + *CP*	$10^6 + 10^4$	*BP*, >3.0; *CP*, >3.0
CP + *LM*	$10^4 + 10^6$	*CP*, >3.0; *LM*, >3.0
LM + *LP*	$10^6 + 10^6$	*LM*, >3.0; *LP*, >3.0
LP + *MP*	$10^6 + 10^6$	*LP*, >3.0; *MP*, >3.0
MP + *BP*	$10^6 + 10^6$	*MP*, >3.0; *BP*, >3.0
BP + *CP*	$10^7 + 10^4$	*BP*, >3.0; *CP*, >2.3
CP + *LM*	$10^3 + 10^6$	*CP*, >3.0; *LM*, 2.18
LM + *LP*	$10^7 + 10^6$	*LM*, 2.40; *LP*, >3.0
LP + *MP*	$10^7 + 10^7$	*LP*, >3.0; *MP*, 2.7
MP + *BP*	$10^7 + 10^7$	*MP*, 1.3; *BP*, >3.0

[a] Abbreviations: *BP*, *B. pertussis*; *CP*, *C. pneumoniae*; *LM*, *L. micdadei*; *LP*, *L. pneumophila*; *MP*, *M. pneumoniae*.
[b] The spiked concentrations of all organisms were based on the numbers of dilutions for all organisms except *C. pneumoniae*; for which the concentrations are $TCID_{50}$s per milliliter.
[c] ODs at A_{450} were determined on a spectrophotometer.

DISCUSSION

In this study we report for the first time a comprehensive test based on a patented technology for the simultaneous detection of five pathogens that cause atypical pneumonia and respiratory illness in adults and children. Recent advances in diagnostic technologies have not resulted in satisfactory tools for the diagnosis of pneumonia. These include nonspecific biologic markers and clinical assessments of signs, symptoms, erythrocyte sedimentation rate, and C-reactive protein levels to predict the presence of pneumonia (8, 16). According to Reimer and Carroll (22), it is difficult to establish an etiologic diagnosis in 50% of cases of community-acquired pneumonia.

Specific diagnostic tests, such as a kit for the rapid detection of *M. pneumoniae*-specific immunoglobulin M (IgM) antibody with an ImmunoCard (Mycoplasma Test; Meridian Biosciences, Cincinnati, Ohio) and traditional bacterial culture, have demonstrated low sensitivities and/or prolonged times to detection (1, 5, 6, 13, 15).

Further review of the diagnostic tests available to clinical laboratories reveals that most of these tests also lack sensitivity or provide only a retrospective (delayed) diagnosis. Direct fluorescent-antibody (DFA) assays are specific but have poor

TABLE 6. Testing of various clinical specimens spiked with DNA controls or organisms for inhibition of PCR

Specimen	No. of samples tested	OD at A_{450}[a]			
		DNA controls + specimen	DNA controls + transport medium[b]	Organism + specimen	Organism + transport medium
Nasopharyngeal swab	20	2.9	2.9	2.8	2.8
Sputum	21	2.8	2.7	2.6	2.9
Cerebrospinal fluid	20	3.0	2.9	2.9	2.9
Serum	1	3.0	3.0		

[a] The ODs at A_{450} were determined on a spectrophotometer.
[b] M4 viral transport medium (Microtest; Remel).

sensitivities; therefore, use of DFA assays to detect antigens in respiratory specimens is neither sensitive nor specific enough to warrant their general use. Radioimmunoassay-based tests are used to detect bacterial antigens in urine and may be satisfactory for the detection of some bacterial strains (e.g., *L. pneumophila* serotype 1), but they miss serotypes 2 to 15 and other *Legionella* species detected by the Pneumoplex assay (10). Serologic (IgG and IgM) diagnosis is considered moderately sensitive and reasonably specific, but its application is restricted as an adjunct to diagnosis by culture except in the case of mycoplasmas, for which commercial IgM assays have demonstrated good sensitivities, although their ability to detect an existing infection is poor. A major disadvantage is that accurate serologic diagnosis requires paired acute- and convalescent-phase serum samples for testing. Furthermore, serologic assays demonstrate cross-reactions among species and serogroups, rendering species-specific diagnosis difficult.

Therefore, there is a need for more sophisticated molecular biology-based diagnostic tests for the rapid, accurate, and specific diagnosis of pneumonia. Molecular diagnostic techniques that use PCR have proven to be promising tools for the rapid etiologic diagnosis of many infections. PCR offers potential advantages over conventional tests for the detection of *M. pneumoniae*, *Legionella* species, and *C. pneumoniae*. These PCR-based assays must go through stringent evaluation, standardization, and validation before they can be used to test clinical samples. Nucleic acid amplification-based techniques do not depend on the viability of the target microbe, whereas conventional microbiological techniques do. With the added sophistication and modernization of amplification processes like multiplex PCR and real-time PCR, technology has enabled testing to be more efficient and precise. As with all molecular biology-based amplification methods, contamination and false-positive results are always a risk. Good molecular biology practices in the laboratory and experience reduce this to a very low level.

Recently, several real-time PCR assays have been developed for detection of *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* and are specific and sensitive. However, all of these assays are designed to detect only a single pathogen at one time (11, 17, 19, 20, 23, 25). In another study, Welti et al. (26) have described a multiplex real-time PCR that was developed. That assay detected three pathogens, *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*, and thus is similar to the Pneumoplex assay except for the detection of *B. pertussis* and *L. micdadei*. However, they reported a sensitivity of 95.8%, whereas our standard and real-time Pneumoplex assays had sensitivities of 100% each.

Using our unique technology, we developed a multiplex PCR-enzyme hybridization assay (the standard assay) and a real-time assay that were capable of detecting very small amounts of nucleic acid from five different atypical respiratory pathogens in a single assay. Our results demonstrated that the Pneumoplex assay is extremely sensitive (100%) and specific (98.5%). In addition to its speed (approximately 2 and 5 h for the real-time and standard assays, respectively) and modest use of technician time (approximately 2 h [range, 1 to 2 h, depending on the number of samples tested]), this assay should be an efficient tool for the detection of these pathogens in patients.

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