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This research has been conducted in compliance with all applicable Federal Regulations governing the protection of human subjects in research, performed under NHRC IRB-approved protocol 2000.0002.

Abstract

A multiplex PCR was developed that is capable of detecting four of the most important bacterial agents of atypical pneumonia, *Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila,* and *Bordetella pertussis* in uncultured patient specimens. These organisms cause similar symptomologies and are often not diagnosed because they are difficult to identify with classical methods such as culture and serology. Given this, the overall impact of these pathogens on public health may be grossly underestimated. The molecular test presented here provides a simple method for identification of four common, yet diagnostically challenging, pathogens.

1. Introduction

1.1. Atypical vs. typical pneumonia

Pneumonia, which is caused by a wide variety of different pathogens, is characterized by an infection of the lung parenchyma [1]. Acute pneumonias are those with a recent and sudden onset, and are commonly classified into two groups, community-acquired pneumonia (CAP) and nosocomial pneumonia. Nosocomial pneumonias are usually acquired in the hospital setting and are typically caused by different pathogens than CAP [1].

CAP is often sub-divided into typical and atypical pneumonias. *Streptococcus pneumoniae* is the primary causative agent of typical pneumonia, and causes two thirds of all diagnosed cases of bacterial pneumonia [2]. PCR detection of *S. pneumoniae* from throat swab or sputum samples may indicate colonization rather than illness, as it is often found in nonsterile sites in healthy individuals. For this reason, serum or urine samples are optimal for diagnosis of infections caused by this organism [3].

Other less common agents of bacterial pneumonia are responsible for pneumonias that are categorized as atypical. In an effort to enable more comprehensive determination of pneumonia etiology, the atypical pneumonia agents *Mycoplasma pneumoniae, Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis* were considered in this paper.

1.2. Mycoplasma pneumoniae

M. pneumoniae may be second only to *S. pneumoniae* as a causative agent of CAP, with associations occasionally rising as high as 50% during outbreaks [4, 5]. Symptoms are generally mild but in some instances can lead to hospitalization or even death [6, 7]. Identification methods include culture, serology, and PCR. Culture is very time consuming, taking up to 5 weeks for results, and is less sensitive than serology [8]. Serology samples must be collected at two specific

points in the illness, at onset and 2 to 3 weeks later, and the sensitivity of serology is dependent on the precise timing of collection. Clearly, neither culture nor serology is rapid enough to assist in patient treatment. In contrast, PCR allows for rapid detection of *M. pneumoniae*, and has been identified as the most promising diagnostic method for this organism [4].

The P1 cytadhesion gene was chosen as a target for detection of *M. pneumoniae* in the multiplex. The P1 protein facilitates attachment to host cells [9] and plays a direct role in pathogenicity. *M. pneumoniae* strains can be broken down further into two main groups based on variability of the P1 gene [10, 11]. Our primers were designed to match sequences conserved in both major variants, type 1 and type 2. P1 sequences used to design these primers included the type 1 strains M129, MP4817, and MP22 and type 2 strains Mac and MP1842 [11], as found using Entrez (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) and BLAST (<http://www.ncbi.nih.gov/BLAST/>) [12] in GenBank® sequence records. These were all of the sequences available for the P1 region of *M. pneumoniae*, but they represent a broad spectrum of isolates and hence the sequences conserved within this group should be broadly conserved among *M. pneumoniae*.

1.3. Chlamydophila pneumoniae

C. pneumoniae (formerly *Chlamydia pneumoniae*) is an obligate intracellular pathogen [4, 13]. *C. pneumoniae* was recognized as an agent of respiratory tract infection in 1986 [14]. It is estimated that *C. pneumoniae* infections account for up to 10% of CAP [15]. Isolation from tissue is very difficult and much of the known epidemiology has been learned through microimmunofluorescence serology testing [15]. Serological evidence suggests that 50-60% of adults have had a *C. pneumoniae* infection during their lives, making it a very prevalent infectious agent [16].

All *Chlamydophila* (and the formerly conspecific *Chlamydia* species) cause persistent infection in their appropriate host tissues, but differ widely in symptomology and epidemiology. The ability to differentiate *C. pneumoniae* from closely related species is extremely important [15]. PCR amplification of the *Pst*I fragment allows for this specificity and offers broad sensitivity among CAP-associated strains of *C. pneumoniae* [15, 16].

1.4. Legionella pneumophila

L. pneumophila, an opportunistic bacterial pathogen, is most commonly identified as a cause of disease among people whose health is already compromised. Examples include cigarette smokers, the elderly, people receiving immunosuppressive therapy, and organ transplant recipients [17]. If a healthy person contracts *L. pneumophila* there will often be no symptoms, and titers of *Legionella*-specific antibodies will be low [18]. However, *L. pneumophila* is now believed to be the cause of 3-8% of all CAP [19]. The importance of *L. pneumophila* is magnified by its potential virulence; 5-30% of patients who develop legionellosis will die from the disease [17]. Over forty species are currently identified as belonging to the genus Legionella [19, 20], but most clinical cases are attributed to *L. pneumophila* [4].

The macrophage infectivity potentiator (*mip*) gene was chosen as a PCR target for *L. pneumophila*. The *mip* gene is associated with intracellular invasion and survival [20, 21]. Aside from a recognized hypervariable region, the *mip* gene sequence is highly conserved within the genus Legionella [20]. *L. pneumophila* causes approximately 90% of Legionnaires' disease cases, and the vast majority of these involve serotypes 1, 4, and 6 (A4). *L. micdadei* is the second most common cause [22]. *L. pneumophila* and *L. micdadei* both cause Legionnaires' disease by colonizing alveolar macrophages [22]. Generally, *L. micdadei* is less virulent and is typically

seen in immunocompromised patients, but there are forms such as 31b that appear to be just as virulent as *L. pneumophila*.

Our primers were designed with reference to *L. pneumophila* and *L. micdadei* sequence in GenBank, though our initial tests suggest that they do not amplify from *L. micdadei* (see Results and Discussion). Positive samples may be further identified by sequence analysis of the 16S RNA gene [23].

1.5. Bordetella pertussis

B. pertussis has been identified as a cause of atypical pneumonia [4]. *B. pertussis* infections are most common in unvaccinated infants and are usually characterized by a persistent cough, sometimes with a unique symptomology called "whooping cough"; pneumonias and occasional deaths are also reported [24]. Almost all Americans are vaccinated as children against *B. pertussis*. As antibodies wane with time, adolescents and adults may become infected with *B. pertussis* and experience milder symptomology with persistent (non-whooping) cough of 2 or more weeks and only occasional pneumonia, whooping cough, apnea, and/or vomiting [25, 26]. In unvaccinated or partially vaccinated populations, outbreaks of pertussis may occur in both adults and children with little or no classic “whooping” symptomology, making the disease difficult to distinguish from other agents of atypical pneumonia [26]. *B. pertussis* appears to cause approximately 13% of persistent cough illness in adults and adolescents in the United States, approximately one million cases of pertussis per year [27].

Culture and direct fluorescent antibody (DFA) tests for *B. pertussis* are notoriously insensitive, owing to either fragility, low titer, or both; hence PCR is the method of choice for detection of this organism [28, 29]. To maximize sensitivity, the IS*481* insertion sequence was used as a target. This element is present at 50-100 copies/per cell in *B. pertussis* [30], and greatly

outperforms single-copy targets such as the pertussis toxin gene in sensitivity comparisons on clinical specimens [28]. This sensitivity comes at a small price, since the IS*481* sequence is conserved in the closely related *B. holmesii* [31], a less understood pathogen usually seen in septicemia among compromised patients, and occasionally in respiratory samples from patients with pertussis-like symptoms [31]. The primers used here were chosen for broad surveillance in adult populations, among which tests for *B. pertussis* are particularly insensitive [29, 32]. Positive results should be followed by species-discriminating tests [33, 34] to achieve diagnostic levels of specificity [28, 31].

This test will not amplify sequence from *B. parapertussis* [33], and should not amplify from other known *Bordetella* species based on the apparent absence of the IS*481* element outside of *B. pertussis* and *B. holmesii* [28, 33].

1.6. Primer design.

The value of comparative sequence analysis, whether it is directed at the primer sites alone (as with the simple PCR/agarose gel analysis used here) or involves the sequencing of intervening regions as well, is in great part determined by the availability of existing sequence data. In this work we deliberately targeted genomic regions that have been previously used as marker regions, allowing us to address specificity among species, as well as breadth of coverage within species, through comparative sequence analysis in GenBank. This methodology maximizes utilization of readily obtainable knowledge related to the target regions, minimizes the testing needed to address issues like sequence conservation, and maximizes the potential for comparative analysis of sequenced amplicons if information beyond presence/absence is needed. Sequence data from GenBank is also used to prevent nonspecific amplification by checking for

matches to nontarget sequences, especially other respiratory pathogens, commensal microbes, viruses, or human genomic DNA.

Primer choices were made manually. Primer selection programs are available, but inevitably focus on a small subset of the criteria used here because the complexity of analyzing several interacting variables in a large aligned set of divergent sequences is computationally prohibitive. One of the authors (D.M.) has designed hundreds of primers for use in complex PCR reactions (see [35] for example), and has found that thoughtful application of a set of simple criteria (see Materials and Methods) will yield sensitive and specific sets of multiplex primers 90% of the time. Furthermore, primers designed in this fashion are almost always compatible with a standardized set of PCR reagents and cycling conditions. The time spent choosing primers by hand is more than compensated for by the time and frustration saved in the later process of primer testing and optimization.

1.7. Validation

Four sets of primers for each target were initially chosen using GenBank. Two forward and two reverse primers were ordered from each collection. A set of primers were then chosen for each organism so that all four amplicons in the multiplex could be easily distinguished from one another when run together. All four sets of primers were optimized together at all times. Optimization considerations included, but were not limited to, most appropriate annealing temperature, use of Q solution (Qiagen, Valencia, Calif), the percent gel used and time for which the gel was run.

Ten-fold dilution series of standard titered American Type Culture Collection (ATCC) bacteria were tested and the results were used to determine the quantitative sensitivity of the multiplex. Patient specimens were used to evaluate the sensitivity and specificity of the test.

ATCC controls were diluted with throat swab samples in 1X Tris-EDTA buffer (Sigma-Aldrich, St. Louis, Mo) from healthy individuals to check for inhibition. Sensitivity was compared with different monoplex primers already used in our lab [30, 36, 37]. These published monoplex tests were also used to verify our results, and in some cases, to choose specimens for initial testing. A broad range of healthy patient specimens, blank media, and negative controls from ATCC were used to verify that nonspecific amplification would not generate false-positive signals.

2. Materials and Methods

2.1. Primer choice

The name of each target organism (Table 1) was used to search GenBank. The genetic targets for which the greatest number of sequences had already been determined were identified. The papers referenced in these sequences' annotations were obtained and read, and the associated genes or genomic regions (Table 1) were therein identified as favored targets for PCR-based identification of the target pathogen.

All available sequences for the most promising of these targets, as defined by existing literature, were downloaded and aligned on Lasergene® software (DNASTAR, Madison, Wis). Well-conserved regions were identified that would include all previously sequenced isolates and were of appropriate genetic distances to yield PCR amplicons of a wide range of sizes, all compatible with simultaneous analysis on the same gel (100 to 600 bp).

Within these regions, primers were chosen on the basis of several criteria. The following rules were applied rigorously: All primers must be between 18 and 25 bp in length. All primers must have between 9 and 11 G or C bases (this, and the previous rule, are designed to generate primers with compatible annealing temperatures). Primers will have at least one, but not more than 3, G or C bases anchoring the 3' end. 3' ends of 5 bp or more will not match (in reverse complement)

internal sequences of other primers or the same primer. This is critical, because these kinds of matches cause 3' primer-templated extension, often misidentified as "primer dimers."

The following additional criteria were also taken into consideration (and balanced) within the limits of the available sequence: Primers will not contain (hairpins) internal matches between sequences in the same primer of more than 5 bp. Primers will not contain G/C runs of >4 bp (G/C bases will be as evenly spaced as possible), and primers will not contain repetitive sequences (AAAAA, GAGAGA, etc). It is especially important to avoid repetitive sequences because they are far more common in eukaryotic genomes than sequences of greater complexity [38], and will therefore be more likely to misanneal to host sequences.

Chosen sequences were screened for mismatches to other pathogens, commensals, and the human genome. Mismatches to the human genome were the most common, as would be predicted given its length.

Sequences with >17 bp mismatches to non-target sequences were rejected, and those with long perfect mismatches to the 3' end were moved far enough to introduce an unmatched base in the last 5 bp of the 3' end. This region approximates the polymerase-binding region, where a mismatch is most likely to exclude or suppress mispriming and elongation.

This was done for all target organisms, choosing four forward/reverse pairs with predicted amplicons of varying lengths and including at least two forward and two reverse primers. The sets were compared, and from them two multiplexes, including compatible pairs for all four targets were chosen. Initial testing of one multiplex suggested two of the primer sets were suboptimal (the amplicons were too close in size), and these two primer sets were replaced with two more sets producing amplicons of complementary sizes. This multiplex worked well, and it includes the primers shown in Table 1.

2.2. Multiplex PCR.

The Multiplex PCR Kit (Qiagen) was used to make 25 µl reactions per the manufacturer's instructions (except all reagents were halved, since the instructions were designed for 50 µl reactions). Reactions contained: 12.5 µl 2X Qiagen Multiplex solution, 2.5 µl 10X primer mix (primers were obtained from Integrated DNA Technologies, Coralville, Iowa), 2.5 µl Q solution, 2 µl DNA, and 5.5 µl water. The final primers that were chosen and used for further testing, and the expected amplicon sizes from those primers, are shown in Table 1.

Primers were diluted to 100 µM concentration. From this stock a 100-fold dilution of each primer was made into the same 1 mL tube and diluted with water to make a 10X stock containing 1 µM of each primer in the multiplex. The primers were added to the PCR for a final concentration of 100 nM each.

2.3. Verifying PCR

Verifying PCRs (previously published monoplexes) were used for two purposes, first for identification of throat swab specimens containing *B. pertussis, C. pneumoniae,* and *M. pneumoniae,* and second for sensitivity comparisons with the multiplex. Verifying PCRs were generally performed using the procedures outlined in their original papers, including cycling times, temperature, Mg concentration, dNTP concentration, polymerase concentration and primer concentration [30, 36, 37]. We used Promega Taq DNA Polymerase (Promega Madison, Wis) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif) for all verifying PCRs. In the verifying *B. pertussis* PCR 35 cycles of amplification were used instead of 30 cycles as stated in the paper in order to increase band intensity. For other verifying PCRs, cycle numbers were retained from the original studies. To accurately compare sensitivity using dilution

series, 2 µl samples of extracts were tested in 25 µl reactions. This is the same amount of extract and total volume used in the multiplex.

2.4. Sensitivity comparisons

The sensitivity of the multiplex was compared with the sensitivity of published monoplex PCRs (the verifying PCRs used here for identification and verification of *B. pertussis, M. pneumoniae,* and *C. pneumoniae* patient specimens [30, 36, 37]). Both PCRs were performed on the same dilution series of the target organisms and run side by side on the same gel. For all targets, 10-fold dilution series were tested. These were generated by serial dilution of the original culture resuspension and subsequent independent extraction of all dilutions. *M. pneumoniae* was also tested by PCR of serial 2-fold dilutions of the initial dilution extract to provide greater resolution and to address the effects of cell aggregation on sensitivity (see Results and Discussion).

2.5. PCR cycling conditions

PCR amplification was performed using an iCycler Thermal Cycler (Bio-Rad). Denaturation was performed for 15 min at 95ºC followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 90 s, and primer extension at 72ºC for 60 s, and a final product extension at 72ºC for 7 min.

Agarose gel electrophoresis was performed as follows: 10 µl of reaction product and 2 µl of Gel Loading Solution (Sigma-Aldrich) were electrophoresed for 90 min at 120 V on a 1.5% gel using Agarose NA (Amersham Biosciences, Piscataway, NJ) in 1X TBE buffer with ethidium bromide (Sigma-Aldrich). A 100 bp DNA ladder (New England Biolabs, Inc., Beverly, Mass) was used as a standard, as was a "positive control ladder" (see positive and negative

controls). The gel was visualized and recorded by Polaroid photography and with a Gel Doc 2000 (Bio-Rad).

2.6. Positive and negative controls, dilution series

Initial testing was performed with ATCC isolates. *L. pneumophila* strain 33152, *C. pneumoniae* strain Cm-1 VR-1360, *M. pneumoniae* strain 15531, and *B. pertussis* strain 9797 were used to create dilution series by 10-fold serial dilution in water. These dilutions were tested with the multiplex. Titer information was provided by ATCC and used to quantitate the dilution curve results. For *B. pertussis* only a range could be given and sensitivity calculations are shown with its highest possible titer. A positive control ladder, which displays bands from all targets in the multiplex PCR, was generated by choosing the concentration of each positive control that was two dilutions above the detection limit and mixing them, in order to have a clear and consistent ladder that also served as a universal PCR and primer control. Other ATCC bacteria and yeast served as negative controls (Table 2). Many of these bacteria were chosen because they included agents of respiratory disease likely to be collected in throat swabs or to be found in a patient's natural surroundings.

2.7. Patient specimens

B. pertussis, M. pneumoniae, and *C. pneumoniae* positive patient specimens from the Naval Health Research Center archives were collected during routine surveillance from individuals with pneumonia as throat swabs in 1X Tris EDTA buffer (Sigma-Aldrich). These were originally tested using PCR tests described in the existing literature. Tests included *M. pneumoniae* [37], *C. pneumoniae* [36], and *B. pertussis* [30]. Samples were thereafter stored at –80ºC without further processing or stabilization. Samples having originally tested positive were

re-extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and retested by the original method to control for sample degradation. Samples that retested positive for the target organism were used as experimental samples to test the described multiplex PCR. *L. pneumophila* original patient specimens were generously provided by the Centers for Disease Control and Prevention (CDC). The CDC had previously identified these as positive for *L. pneumophila*.

3. Results

3.1. Positive controls (sensitivity)

Results are shown in Figures 1 and 2. *C. pneumoniae* detection extended to one tissue culture infectious dose per reaction (TCID/rxn) with the multiplex and also with the verifying PCR, though the verifying PCR yielded a very weak band (Fig. 2B). *L. pneumophila* amplification gave a strong band for 20 colony-forming units per reaction (CFU/rxn) and a weak band for 2 CFU/rxn with the multiplex (Fig. 2C). Verifying tests for *L. pneumophila* were performed by the CDC and sensitivity information for those tests was not available. *B. pertussis* amplification was clearly visible to 0.2 CFU/rxn with the multiplex and 0.2 CFU/rxn with the verifying PCR, and both PCRs yielded a weak band at 0.02 CFU/rxn (Fig. 2A). The extreme sensitivity of the *B. pertussis* PCRs is attributable to both the use of a high-copy insertion sequence as the genetic target of the PCR (this element is present at 50-100 copies per cell) and the generally low plating efficiency of *B. pertussis*.

When using a 10-fold dilution series of a resuspended ATCC culture that was extracted after dilution, *M. pneumoniae* was visible to 200 color-changing units per reaction (CCU/rxn) with the verifying PCR while it was visible to 20 CCU/rxn with the described multiplex (Fig. 2B). Faint bands were visible for both PCRs at the next lowest dilution point as well, though they

do not show well in this photograph. When using a 2-fold dilution series of the extract obtained from the 20 CCU dilution, *M. pneumoniae* was visible to 20 CCU/rxn with the verifying PCR and to less than 1 CCU/rxn with the multiplex (Fig. 2D). The difference between the 10-fold and the 2-fold dilution series comparisons probably results from cell aggregation. *Mycoplasma* cells are known to stick very tightly to one another in liquid suspension. When dilution series are made at the specimen level (as in the 10-fold dilution series in Fig. 2B), aggregation will result in high variance of copy number in the process of dilution, and loss of all copies in many dilutions. When dilutions are from pre-extracted specimens (as in the 2-fold dilution series in Fig. 2D), consistent dilution of copy number is expected. Aggregation also explains the apparently unrealistic sensitivity seen in the 2-fold dilution series (one CCU corresponds to 10-100 cells).

All positive controls (ATCC strains) of the target species gave positive results. As expected, the *B. pertussis* primers also amplified a specific product from the closely related *B. holmesii*. The two most common *L. pneumophila* serotypes both yielded positive results.

3.2. Negative controls

All ATCC negative controls tested negative with the multiplex. Control strains, negative controls, healthy specimens, and blank media, along with the associated results, are listed in Table 2.

3.3. Patient specimens

Patient specimens used to test the multiplex, along with the associated results, are listed in Table 3. Fourteen original patient specimens that previously tested positive for *B. pertussis* by PCR were chosen from our archives and tested again to control for sample degradation. Ten of these fourteen retested positive for *B. pertussis* by the verifying PCR. All 10 of these tested

positive by the multiplex. Three original patient specimens that had previously tested positive for *M. pneumoniae* retested positive with the verifying PCR. All three of these samples tested positive with the multiplex. Two newly collected original patient samples tested positive for *C. pneumoniae* by the verifying PCR, and both of these samples were also positive with the multiplex PCR. The CDC provided three *L. pneumophila* samples for testing. All these samples tested positive with the multiplex PCR. It should be noted that all patient specimens were throat swabs in TE buffer except for the *L. pneumophila* samples, which included two lung samples and one sputum sample in unknown buffer.

4. Discussion and Conclusions

The multiplex PCR worked well for all four targeted pathogens. Specificity was 100%, with no nonspecific amplifications from healthy patient specimens, commensal organisms, other (nontarget) agents of respiratory disease, blanks, or other negative controls. Sensitivity was also 100% within the set of 18 verified patient specimens tested in this study. The sensitivity of the new multiplex PCR was equal to or greater than the sensitivity of previously published monoplex PCR tests targeting the same organisms. There were no cross reactions between primers (primer dimers). The chosen primers produced a clear band of the expected size for each target organism, and worked effectively on both cultured control specimens and original patient specimens. The test was able to detect the targeted pathogens across a wide range of concentrations, and was able to identify multiple organisms in mixtures as demonstrated by amplification of the positive control ladders.

Given the considerable increase in sensitivity of PCR over previously used methods, it is critical that future epidemiological studies using PCR techniques include matched healthy controls. This, along with strict adherence to clinical case definition requirements, will allow

determination of the clinical significance of positive test results and help resolve the possibilities of asymptomatic carriage state, inadequate specificity, or persistence of the organism after infection. While these organisms have been clearly linked to outbreak phenomena and fatalities, the rate of passive carriage has not been extensively documented.

This multiplex was developed as a research diagnostic tool. However, given the ability of the test to identify the targeted organisms in a limited number of routine throat swab specimens and its sensitivity as compared with other accepted tests, we expect that continued testing of the described primer sets (using existing mono- or multiplex primer sets for independent verification) will allow validation and use of this test for clinical diagnosis.

PCR tests rely on sequence conservation of the regions targeted by the relevant primers. It is always possible that new strains may exist for which the primers do not match. In fact, if treatment is chosen on the basis of PCR results, then selective pressure will be created that favors divergence of the primer-targeted sequences. For use in diagnostics, we believe independently targeted PCR tests should always be used in tandem (two tests for each organism) so that divergence of a single sequence will not prevent identification of new strains. This approach also greatly decreases the chance of false positives based on nonspecific amplification, something that cannot be completely prevented given the (essentially infinite) potential sequence diversity in nature. Given paired PCR tests, an ambiguous result (one positive and one negative) can indicate either a false positive or a new strain. These possibilities can be distinguished by sequence analysis of the positive test amplicon or by querying with a third primer set.

In general, the use of multiplex PCR reactions for groups of organisms causing similar syndromes provides an efficient way to ask several related epidemiological questions simultaneously. Multiplex PCR tests can be developed easily using archived sequences and

references from public databases to identify appropriate target genes and primer sequences. Furthermore, complementary sets of primers can be efficiently chosen manually by application of a few intuitively consistent rules and guidelines designed to maximize compatibility and standardize reaction conditions.

The described multiplex offers a simple method for surveillance and epidemiological investigations designed to identify causative agents of atypical pneumonia. The primers presented here were designed for use in public health research and are unprotected by patents or other limitations. One commercial PCR-EHA (enzyme hybridization assay) is available that allows testing for the same set of pathogens in a single molecular procedure, the Prodesse Pneumoplex assay [39]. However, this test is patented and requires specialized reagents (including horseradish peroxidase labeled probes, biotinylated PCR primers, strepavidin-coated microtiter plates, and a proprietary and apparently necessary PCR buffer) and related expertise and equipment, all of which make it more expensive and less universally accessible than a traditional PCR/agarose gel electrophoresis method. The method presented here is deliberately simplified. Four primer pairs target species-specific sequences in the four organisms and generate products of clearly distinguishable sizes, allowing identification of one or more of the targeted pathogens using only a PCR machine, nonproprietary standard PCR reagents, unmodified oligonucleotide primers, and an agarose gel electrophoresis apparatus.

The four bacteria targeted by the described multiplex are difficult to culture and are rarely tested for on the scale necessary to determine their impact and epidemiological characteristics. We intend to use this test to more clearly define the role that these organisms play in the etiology of military disease, and we hope that others will use the test for similar pursuits in other populations.

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Table 1. Primers used in the multiplex PCR for atypical pneumonia agents.

Primer	Sequence	Target	Organism	Product
M.p.F	5' gtttgctgctaacgagtacgag	P1	*M. pneumoniae*	360 bp
M.p.R	5' gtaatcatcgtctgactgcc	P1	*M. pneumoniae*	
B.p.F	5' gttgtatgcatggttcatccg	IS*481*	*B. pertussis*	122 bp
B.p.R	5' cgacgtaggaaggtcaatcg	IS*481*	*B. pertussis*	
L.p.F	5' caatggctgcaaccgatgc	*mip*	*L. pneumophilia*	487 bp
L.p.R	5' gggataacttgtgaaacctg	*mip*	*L. pneumophilia*	
C.p.F	5' cggctagaaatcaattataagactg	*Pst*I	*C. pneumoniae*	283 bp
C.p.R	5' ggtgtgtttctaatacctgtcc	*Pst*I	*C. pneumoniae*	

Table 2. Negative control strains used to test specificity of the multiplex PCR.

Organism	ATCC #	Multiplex result	Source
Staphylococcus aureus	29213	negative	ATCC
Haemophilus influenzae	49247	negative	ATCC
Haemophilus influenzae	10211	negative	ATCC
Streptococcus pneumoniae	49619	negative	ATCC
Streptococcus pneumoniae	6303	negative	ATCC
Streptococcus pyogenes	19615	negative	ATCC
Streptococcus salivarius	13419	negative	ATCC
Streptococcus agalactiae	13813	negative	ATCC
Haemophilus parainfluenzae	7901	negative	ATCC
Pseudomonas aeruginosa	27853	negative	ATCC
Escherichia coli	25922	negative	ATCC
Enterococcus faecalis	29212	negative	ATCC
Corynebacterium	49676	negative	ATCC
Candida albicans	10231	negative	ATCC
Neisseria lactamica	23970	negative	ATCC
Bacillus subtilis	6633	negative	ATCC
Bordetella parapertussis	15311	negative	ATCC

Table 3. Multiplex PCR results from amplifications of archived patient specimens.

Sample[a]	*M.p.*	*C.p.*	*L.p.*	*B.p.*
*M.p.*1	+	-	-	-
*M.p.*2	+	-	-	-
*M.p.*3	+	-	-	-
*C.p.*1	-	+	-	-
*C.p.*2	-	+	-	-
*L.p.*1	-	-	+	-
*L.p.*2	-	-	+	-
*L.p.*3	-	-	+	-
*B.p.*1	-	-	-	+
*B.p.*2	-	-	-	+
*B.p.*3	-	-	-	+
*B.p.*4	-	-	-	+
*B.p.*5	-	-	-	+
*B.p.*6	-	-	-	+
*B.p.*7	-	-	-	+
*B.p.*8	-	-	-	+
*B.p.*9	-	-	-	+
*B.p.*10	-	-	-	+

[a]Samples previously identified as containing *M. pneumoniae (M.p.), C. pneumoniae (C.p), B. pertussis (B.p.)* (identified by verifying PCR) and *L. pneumophila (L.p.)* (identified by the Centers for Disease Control and Prevention).

Figure Legends.

Fig. 1. Positive controls, negative controls, and patient specimens amplified by multiplex PCR. Positive (+) control ladders are template mixtures of all four positive controls. Control strains used are from ATCC and are listed in Table 2. Patient specimens are representatives from the set shown in Table 3, and were amplified from extracted throat swab specimens, except for *L. pneumophila*, for which we extracted a sputum sample from the CDC. Healthy control is an extracted throat swab specimen from asymptomatic laboratory personnel.

Fig. 2. Dilution series test results. +CL = positive control ladder (all four positive controls together). Numbers represent TCID (*C. pneumoniae*), CFU (*L. pneumophila, B. pertussis*) or CCU (*M. pneumoniae*) per reaction, as calculated from ATCC titer estimates.

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14. ABSTRACT (maximum 200 words)

A multiplex PCR was developed that is capable of detecting four of the most important bacterial agents of atypical pneumonia, *Mycoplasma pneumoniae, Chlamydophila* (formerly *Chlamydia) pneumoniae, Legionella pneumophila,* and *Bordetella pertussis,* in uncultured patient specimens. These organisms cause similar symptomologies and are often not diagnosed because they are difficult to identify with classical methods such as culture and serology. Given this, the overall impact of these pathogens on public health may be grossly underestimated. The molecular test presented here provides a simple method for identification of four common, yet diagnostically challenging, pathogens.

15. SUBJECT TERMS
Multiplex PCR, Atypical pneumonia, Primer design, Legionnaires' Disease, Pertussis, Bacterial pneumonia, *Mycoplasma pneumoniae, Chlamydophila* (formerly *Chlamydia) pneumoniae, Legionella pneumophila, Bordetella pertussis*

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