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A multiplex PCR for detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis* in clinical specimens

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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.

Keywords: Multiplex PCR; Atypical pneumonia; Primer design; Legionnaires' disease; Pertussis; Bacterial pneumonia

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 non-sterile 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 2–3 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*,

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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 40 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 (4). *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 Sections 3 and 4). 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