Simultaneous Use of Direct and Indirect Diagnostic Techniques in Atypical Respiratory Infections From *Chlamydomphila pneumoniae* and *Mycoplasma pneumoniae*

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In 2008, 50 samples (BAL), coming from hospital patients, with acute respiratory symptoms have been investigated using two real-time PCR methods: one assay for the single detection of *Chlamydomphila pneumoniae* and *Mycoplasma pneumoniae* DNA and one commercially available real-time duplex PCR assay for the detection of *C. pneumoniae* and *M. pneumoniae* DNA. Both techniques used here showed

compliant results, with 100% concordance for detection of *C. pneumoniae* and 98% for detection of *M. pneumoniae*. The positive results obtained agreed with the clinical suspicion of such infections in some cases and with the presence of IgM specific for *C. pneumoniae* and *M. pneumoniae* in all cases of acute infection. J. Clin. Lab. Anal. 23:206–209, 2009. © 2009 Wiley-Liss, Inc.

Key words: *C. pneumoniae*; *M. pneumoniae*; CAP; RT-PCR

INTRODUCTION

Community-acquired respiratory infections constitute a high cause of morbidity and mortality (1). Although *S. pneumoniae* remains the bacterium most commonly implicated in this infection and in particular in community-acquired pneumonia (CAP), atypical respiratory pathogens are being found with increasing frequency. The most common atypical respiratory infections are caused by three nonzoonotic pathogens, *Chlamydomphila pneumoniae*, *Mycoplasma pneumoniae* and *L. pneumophila*, and three zoonotic pathogens, *C. psittaci*, *F. tularensis*, and *C. burnetii*. These atypical agents, unlike the typical pathogens, often cause extrapulmonary manifestations. *C. pneumoniae* and *M. pneumoniae* account for frequent causes of community acquired respiratory infections (1,2). Diagnosis of these pathogens is normally performed through direct (cultures and NAATs) and indirect (serology) techniques with different sensitivity and specificity and different turn-around-time. In particular, molecular biology techniques such as real-time PCR (RT-PCR) show a higher specificity and sensitivity for the detection of *C. pneumoniae* and *M. pneumoniae* in comparison to other diagnostic techniques (3,4).

In this study we used two RT-PCR techniques (LightCycler Real-Time PCR Roche for the single detection of *C. pneumoniae* and *M. pneumoniae* DNA

and affigene Cp/Mp tracer Alfa Wassermann Diagnostics duplex PCR assay for the simultaneous detection *C. pneumoniae* and *M. pneumoniae* DNA) and two serological techniques (Microimmunofluorescence IgG and IgM for *C. pneumoniae* and ELISA Novagnost *M. pneumoniae* IgG and IgM Dade Behring Siemens technique) for the diagnosis of acute infection from *C. pneumoniae* and *M. pneumoniae*.

METHODS

In 2008, 50 selected and not consecutive samples (BAL) by patients with acute respiratory symptoms, have been investigated using two RT-PCR to detect genetic material of *C. pneumoniae* and *M. pneumoniae* and the sera of these patients have been investigated for presence of IgM and IgG specific for *C. pneumoniae* and *M. pneumoniae*. Two weeks later another blood test was performed for each patient to check seroconversion.

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The BAL were selected by patients with acute respiratory symptoms (nonproductive cough and low-grade fever) and willing to submit two samples of blood.

Extraction Using NucliSens easyMag Biomerieux

After preparing the cellular pellet, samples have been extracted using NucliSens easyMag Biomerieux, according to the manufacturer's instructions (5).

Amplification Using LightCycler Real-Time PCR Roche Technique

The extracted material has been amplified with RT-PCR technique using LightCycler II with *M. pneumoniae* LCSet Kit and *C. pneumoniae* LCSet kit (TIB molbiol for Roche, Berlin, Germany). The method consisted in the use of glass capillaries where 15 µl of master mix and 5 µl of DNA extracted from the sample have been put into contact. The amplification mix has been prepared adding to primers and probes the hot start Taq DNA polymerase present in the LightCycler FastStart DNA Master plus Hybridization Probes (Roche). During the reaction, a genomic fragment (target gene) of the pathogen (polymorphic membrane protein G family of 140 bp for *C. pneumoniae* and cytoadhesin P1 gene of 342 bp for *M. pneumoniae*) has been amplified using FRET probes and specific primers (6) and detected using fluorescence coming from the couple of specific hybridization probes. The detection has been performed with a real-time reading of the fluorescence from amplification products, directly inside the capillary with a detection limit of five genomic equivalents/PCR reaction in samples for both pathogens. The reading is performed at each cycle on six channels, using probes labelled with different fluorophores (ex.: LC-Red 640). The use of this test is for scientific research only.

Amplification Using Affigene Cp/Mp Tracer Alfa Wassermann Diagnostic Technique

The extracted material has been amplified with a RT-PCR multiplex technique that allows for the simultaneous detection of *C. pneumoniae* (Hex) and *M. pneumoniae* (Fam) and an Internal Control DNA IC Carrier (Rox), certified CE-IVD, using the Stratagene Mx3000P system for the amplification with the affigene Cp/Mp tracer kit, which allows results analysis using the affigene analysis software. The affigene Cp/Mp tracer kit implies the use of scorpions primers (7) that act through a unimolecular mechanism amplifying the signal and preventing aspecific signals from happening. These aspecific signals come from the amplification reaction of the genomic fragment (target gene) of the pathogenic

agent major outer membrane protein for *C. pneumoniae* and P1 adhesion protein for *M. pneumoniae*. During the reaction, strips with eight optical caps and strips with eight microtubes are used. Inside them 25 µl of master mix and 25 µl of DNA extracted from the clinical sample are put into contact. The limit of detection in samples is 3.7 genomic equivalents/PCR reaction of *C. pneumoniae* (interval 2.5–8.7) and 4.2 genomic equivalents/PCR reaction of *M. pneumoniae* (interval 2.5–11.1). The use of this test is for diagnostic as well as scientific research, because it is CE-IVD certified.

MIF Assay for Detection Antibodies Anti-*C. pneumoniae*

The MIF assay was used to measure *C. pneumoniae* antibodies. The MIF assay, used in this study, was home made with crude lysate of EBs purified of *C. pneumoniae*, as described in other studies (8). Specific chlamydial antibodies were detected by using fluorescein isothiocyanate-conjugated anti-human IgM and IgG (Dako A/S, Glostrup, Denmark). Serological diagnosis of a recent *C. pneumoniae* infection was based on the following criteria: the presence of *C. pneumoniae*-specific IgM (titer 1:16) in sera or four-fold increase in IgG. The positive IgG, associated to IgM <1:16 and negative RT-PCR are presumed past infection.

ELISA Novagnost *Mycoplasma pneumoniae* IgG and IgM Dade Behring Siemens Technique

The semi-quantitative immunoenzymatic determination of IgG/IgM-class antibodies to *M. pneumoniae* is based on a ELISA commercial kit, as described by the manufacturer (9).

Serological diagnosis of a recent *M. pneumoniae* infection was based on the following criteria: the presence of *M. pneumoniae*-specific IgM in sera or four-fold increase in IgG. The positive IgG, negative IgM, and negative RT-PCR are presumed past infection.

RESULTS

Though both techniques of RT-PCR showed a different sensitivity for the presence of *C. pneumoniae* and *M. pneumoniae* genetic material, by according to the instructions of the two manufacturers (Tables 1 and 2), both techniques used here showed compliant results except for one sample, positive for *M. pneumoniae* only with the RT-PCR affigene Cp/Mp tracer Alfa Wassermann Diagnostics method (Table 2). Such a result has been confirmed by serology performed for the research of IgM and IgG anti-*M. pneumoniae* antibodies with an immunoenzymatic ELISA technique, showing seroconversion in comparison to a previous sample.

TABLE 1. Simultaneous use of Diagnostic Techniques in Suspicion of Infection From *C. pneumoniae*

No. patients	RT-PCR single	RT-PCR duplex	IgM/IgG 1° sample	IgM/IgG 2° sample	Interpretation
10/50	P	P	N ^a /N	32 ^a /N	Acute Infection
30/50	N	N	N/32 ^a	N/32 ^a –64 ^a	Past Infection
10/50	N	N	N/N	N/N	Not Infection

^aReferring to the mode (statistically the most frequent value).

TABLE 2. Simultaneous Use of Diagnostic Techniques in Suspicion of Infection From *M. pneumoniae*

No. patients	RT-PCR single	RT-PCR duplex	IgM/IgG 1° sample	IgM/IgG 2° sample	Interpretation
1/50	P	P	N/N	P/N	Acute Infection
1/50	N	P	N/N	P/N	Acute Infection
6/50	N	N	N/P	N/P	Past Infection
42/50	N	N	N/N	N/N	Not Infection

The serological results show prevalence of the following antibodies: IgM anti-*C. pneumoniae* 20%, IgG anti-*C. pneumoniae* 60%, IgM anti-*M. pneumoniae* 4% and IgG anti-*M. pneumoniae* 12%. (Tables 1 and 2).

The positive results obtained in RT-PCR agreed with the clinical suspicion of such infections in 12.0% of cases and with the presence of IgM specific for *C. pneumoniae* and *M. pneumoniae* in all cases of acute infections.

CONCLUSIONS

As opposed to previous beliefs, *C. pneumoniae* and *M. pneumoniae* are capable of causing severe as well as mild-to-moderate illness. Moreover, they can affect all age groups. Indeed, atypical pathogens are implicated in 15–20% of CAP cases and commonly occur as copathogens in mixed-infection CAP. They are associated with particularly high mortality in old and immunocompromised patients (1,10). Moreover the importance of an accurate and early diagnosis of the atypical CAP could avoid their nonresponsiveness to beta-lactam therapy, to the potential role of *C. pneumoniae* in coronary artery disease and multiple sclerosis and to the role of *M. pneumoniae* and *C. pneumoniae* in causing or exacerbating asthma (10). Good laboratory methods for detecting of atypical pathogens are very important, for their implications in public health (10) and because accurate prediction of etiology cannot be made purely on clinical or radiologic grounds (11).

RT-PCR techniques, used in this study, were easy to perform and analyzing the results obtained was simple. Both were very sensitive and therefore very useful for laboratory diagnosis of respiratory infections caused by *C. pneumoniae* and *M. pneumoniae*. The multiplex techniques of RT-PCR, which also included the

identification of DNA of *L. pneumophila*, are very interesting, because they allow the same clinical sample to diagnose the aetiology of respiratory infections by nonzoonotic atypical bacteria (12). Finally it is interesting to note the high concordance between the serological test and molecular biology test used in this study in diagnosis of acute atypical respiratory infections and a higher prevalence of *C. pneumoniae* than *M. pneumoniae*.

In conclusion, the simultaneous use of a direct test and an indirect test (serological, better if conducted on two samples) in the diagnosis of atypical respiratory infections allows greater etiology diagnostic accuracy.

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