Development and clinical validation of a real-time PCR using a uni-molecular Scorpion-based probe for the detection of *Mycoplasma pneumoniae* in clinical isolates

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SUMMARY

Mycoplasma pneumoniae (Mp) is a frequent cause of Community Acquired Pneumoniae (CAP). The etiological role of Mp is usually suspected using serological assays, but the detection of specific anti-Mp antibodies becomes possible only 1-2 weeks after the primary infection. On the contrary, direct diagnosis using real-time PCR allows an efficient detection of Mp DNA in all the phases of the infection and particularly during early serum negative periods. In this study, we developed a novel Scorpion-probe real-time PCR-based assay. The probe's uni-molecular structure offers thermodynamic advantages owing to its kinetic reaction, providing faster performances compared to a TaqMan-based assay, but maintaining the same sensitivity and specificity. The Scorpion-based assay was employed on 388 clinical samples and compared with conventional qualitative PCR and serological tests. It was found more sensitive because it also allowed the detection of Mp in specimens found negative using classic qualitative PCR, but displaying seropositivity or a later seroconversion.

KEY WORDS: Quantitative PCR, Scorpion probe, TaqMan probe, Mycoplasma pneumoniae, Agglutination test

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INTRODUCTION

Mycoplasma pneumoniae (Mp), together with *Legionella pneumophila* and *Chlamydia pneumoniae,* are among the major etiological agents of Community-Acquired Pneumonia (CAP) (Blasi *et al.,* 2005), causing between 15 to 50% of these pathological conditions; among them, Mp alone accounts for 50% of cases. Infections due to Mp are frequently under-diagnosed because the microorganism is difficult to culture, requiring a long incubation period and thus confining the technique to specialized microbiology laboratories (Razin and Tully, 1983). For these reasons, the majority of etiological diagnoses are usually performed by serological assays, such as complement fixation test, indirect agglutination test and enzyme immunoassay (Beersma *et al.,* 2005; Drasbek *et al., 2004*).

Although these tests are simple and reproducible, a period ranging from 1 to 2 weeks (a "non-diagnosis" window between the day of the infection and the day of serum conversion) is required to detect a specific anti-Mp antibody titre that is representative of active infection (Talkington *et al.,* 2004). Therefore, a rapid and specific approach for microbiological identification that enables an accurate etiological diagnosis, and in turn, the selection of appropriate medical treatment, is warranted.

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The use of real-time PCR in microbial molecular diagnostics has proven enormously beneficial in terms of specificity and sensitivity. The technique has recently been included among the routine detection methods for a number of pathogens (Mackay, 2004; Welti *et al.,* 2003; Stralin *et al.,* 2005), yielding clinically relevant results in a short time. Different chemistries are employed to monitor the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle. These utilize non-sequence specific intercalating fluorophores or fluorescent probes that target the complementary sequence in the amplified products. Probe-based real-time PCR chemistry usually entails complex reaction kinetics (TaqMan, Molecular Beacon, Linear Oligoprobes) (Wong and Medrano, 2005). Real-time PCR using the Scorpion uni-molecular probe with its intra-molecular probing mechanism affords a number of important advantages, chief of which is a faster reaction kinetics thanks to its structure that ensures the near proximity of probe and target. For this reason, Scorpion probes are faster than TaqMan probes and usually display a higher signal to noise ratio. In addition, Scorpion probes do not need the fluorochrome enzymatic cleavage required by double-dye TaqMan probes, thus reducing the time for signaling (Whitcombe *et al.,* 1999). Such technical properties result in rapid-cycling that reduces the overall procedure time by more than 30%.
In this study we developed and compared real-time PCR based on TaqMan chemistry with the faster kinetics of the uni-molecular Scorpion probe. The latter was also validated in clinical samples and proved to be faster and more sensitive at detecting Mp DNA than conventional qualitative PCR procedures.

MATERIALS AND METHODS

Qualitative PCR
Mp was detected by qualitative PCR using P4A and P4B (unmodified) primers specific for P1 cytoadhesin target gene (Kong *et al.,* 2000; Sharma *et al.,* 1998).

Real-time PCR primers and probes design
Real-time PCR was developed by targeting the P1 cytoadhesin type 1 and 2 gene of the Mp genome, since it has been reported to be as sensible and sensitive as nested PCR targeting the 16S rRNA gene (Hardegger *et al.,* 2000; Ieven *et al.,* 1996) but faster. Primers and TaqMan probe were designed using both Primer Express (PE Biosystem, Foster City, CA) and Oligo 4.1 primer analysis software (National Biosciences Inc. Plymouth MN) to select the best thermodynamically performing sequences. TIB Molbiol (Genova, Italy) performed the synthesis of the chosen primers. In detail, a 72 bp fragment was amplified using the 19 bp Mycpn P1-F forward primer (5'- GCC G-CA AAG ATG AAY GAC G-3'), in combination with the 23 bp Mycpn P1-R reverse primer (5'-TCC TTC CCC ATC TAA CAG TTC AG-3'). In real-time PCR the amplicon was detected by a 27 bp TaqMan probe (5' FAM - TTG ATG GTA TTG TAC GCA CCC CAC TCG - 3' TAMRA) complementary to an internal region 3 bp downstream of the forward primer.
Uni-molecular Scorpion real-time detection was obtained utilizing the forward primer used in the TaqMan reaction modified in the ScoMycpn frw Scorpion probe of 48 bp (5'-(FAM)-CGG CGG G-GT GCG TAC AAT ACC ATC CGC CG- (BHQ1)-(blocker)-G CCG CAA AGA TGA AYG ACG in combination with the same reverse primer used in the TaqMan assay. The 17-mer DNA probe sequence, underlined above, is organized within the Scorpion probe in a hairpin loop conformation by the presence of a self-complementary 6 bp stem sequence at the 5' and 3' ends. This hairpin loop structure was covalently linked to the 5' end with the reporter dye 6-carboxy-fluorescein (FAM ™) and to the 3' end with the non fluorescent dye Black Hole Quencer-1 (BHQ1), directly linked to the 5' end of the PCR forward sequence P1 cytoadhesin-specific primer via a PCR blocker. This blocker prevents polymerase-mediated read-through, and consequently the hairpin loop opening in the absence of the specific target sequence, thereby avoiding the detection of non-specific PCR products like primer dimers or mispriming events. In this closed ("off") conformation, where the 17-mer probe does not hybridize with the complementary sequence, the fluorophore and the quencher dye are held in close physical proximity (loop structure). This ensures an efficient suppression of natural fluorescence via fluorescence resonance energy transfer (FRET) prior to thermo cycling. Upon polymerase-mediated ex-

tension of the primer, the specific probe sequence binds to its complementary sequence within the same strand of DNA. Thus, the hairpin loop structure opens ("on conformation") and fluorescence is no longer quenched by FRET, resulting in an increased real-time detection of the fluorescence signal. Hairpin design and amplicon probing, optimized with Primer Express and Oligo 4.1 primer analysis software, were modeled using the DNA *mfold* suite on the Michael Zuker web site (Zuker, 2003), according to the thermodynamic parameters established by John Santalucia (Santalucia, 1998). In particular, the thermodynamic analysis of the uni-molecular hairpin-loop in "off" conformation requires a differential free energy of at least -2.0 kcal/mole in favor of the extended and hybridized probes ("on conformation"). This particular Scorpion probe has a $\triangle G_{55°C}$"off" =-2.9 kcal/mole and a $\triangle G_{55°C}$ "on"= -10.9 kcal/mole with a differential free energy between "off" and "on" state of -8.0 kcal/mole in favor of the latter. The synthesis of the chosen Scorpion probe was performed by Proligo France SAS (Paris, France).

Real-time PCR assays conditions

The TaqMan reaction was performed in a final volume of 25 µl containing template DNA, Platinum Quantitative PCR SuperMix-UDG Master Mix (Invitrogen, Milano, Italy) reaction buffer 1x (200 µM dATP, dCTP, dGTP, 400 µM dUTP, 3 mM magnesium chloride, 0.75 U Platinum Taq DNA polymerase, 0.5 U UDG, 100 mM KCl, 40 mM Tris/HCl pH8.4 and stabilizers), primers at 300 nM, probe at 200 nM and ROX reference dye at 100 mM if needed. The TaqMan PCR cycling conditions were 2 min of degradation of pre-amplified templates at 50°C, followed by 2 min denaturation at 95°C and then 40 cycles of denaturation at 95°C for 20 s. and annealing/extension at 58°C for 60 s. Scorpion PCR amplification was performed under the same volume and reaction conditions reported above, but with the probe at 300 nM. In addition, a faster amplification profile of 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 55°C for 35 s was used for the Scorpion probe. Real-time amplifications were performed on both ABI 7500 (Applied Biosystems Europe, Belgium) and Rotor Gene 3000 (Corbett Research, Diatech S-RL, Italy) instruments. The normalized fluorescent signal ($\triangle$Rn) was automatically calculated by a computer algorithm that normalizes the reporter emission signal. In particular, using the ABI 7500 instrument the signal was first divided by the emission of the control dye (ROX) present in the PCR reaction mix; the background signals generated at the first cycles of the PCR reaction were then subtracted. The algorithm that calculates the threshold cycle (Ct) at which each PCR amplification reaches the fixed threshold value was maintained identical in all the experiments performed, and the resulting Ct was inversely proportional to the log number of target copies present in the sample.

Sensitivity and specificity of the real-time PCR assays

To compare the relative sensitivities of TaqMan and Scorpion primer and probe sets serial dilution of *Mycoplasma pneumoniae* genomic DNA (NCTC 010119 Minerva biolabs GmbH, Berlin, Germany) at different concentrations ranging from 60000 to 6 genome copies were tested on both ABI 7500 and Rotor Gene 3000 instruments. Organisms used to assess assay specificity were 4 Mycoplasmataceae species: *Mycoplasma hominis* (ATCC 23114) *Mycoplasma genitalium* (ATCC 33530D), *Mycoplasma hyorhinis* (ATCC 17981D) and *Ureaplasma urealyticum* (clinical isolate), 13 different Gram negative reference strains or clinical isolates: *Klebsiella pneumoniae* (ATCC9633), *Branhamella catarralis* (ATCC3622), *Haemophilus influenzae* (ATCC7279), *Haemophilus parainfluenzae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC9027), *Neisseria lactamica* (ATCC23971), *Neisseria meningitidis serogroup A* (ATCC13077), *Neisseria meningitidis serogroup B* (ATCC13090), *Neisseria meningitidis serogroup C* (ATCC13102), *Neisseria meningitidis serogroup Y* (ATCC35561), *Neisseria saprophita* (our laboratory collection clinical isolate) and 2 clinical isolates of *Legionella pneumophila;* and 4 different Gram positive reference strains or clinical isolates: *Streptococcus pneumoniae* (ATCC 7978), *Staphylococcus aureus* (ATCC6538), *Staphylococcus aureus serogroup 2* (ATCC8531), *Streptococcus species* (clinical isolate) and finally two fungi reference strains: *Candida albicans* (ATCC10231) and *Aspergillus niger* (ATCC16404). All microorganisms were from our laboratory collection. An additional specificity control has been performed using human genomic DNA from a healthy donor. For each

pathogen real-time TaqMan and Scorpion probe PCR assays were performed on both ABI 7500 and Rotor Gene 3000 instruments in the presence of positive (Mp quantitated DNA standard NCTC 010119 [Minerva biolabs GmbH, Berlin, Germany]) and negative controls (no DNA i.e. water in place of extracted nucleic acid for the same volume amount) of amplification. The intra- and inter-assay reproducibility were also calculated as described (Martell *et al.,* 1999).

Clinical samples

The study was performed in G. Gaslini pediatric research hospital during the winter period 2003-2004. Three hundred eightyeight swabs were collected from young patients (ranging from 2 to 13 years) admitted to the Infectious Diseases, Lung Diseases and Emergency Medicine divisions for symptomatic acute respiratory infection of unknown origin. Oropharyngeal or deep nasal swabs were collected soon after admission to the hospital, and before any antibiotic therapy was started. Swabs were stored in 1 ml of sterile saline solution at 4°C before DNA extraction.

DNA extraction

The swabs were centrifuged at 14.000 rpm for 10 minutes and the pellets were re-suspended in 200 µl of sterile saline solution and processed to extract DNA using QIAamp® DNA Mini Kit (QIAGEN spa, Milano, Italy) following the manufacturer's instructions for blood and body fluid samples.

Serological analysis

A population fraction of patients undergoing direct detection of Mp in biological samples was also studied using a commercially available, semiquantitative test for the diagnosis of Mp infection (Serodia-Myco II Fujirebio Inc. Japan). This test is based on the agglutination of gelatine particles coated with Mp antigens and it is suitable to detect both anti-Mp specific IgM and IgG.

RESULTS

Analysis of TaqMan and Scorpion-based PCR assays for Mp

Real-time amplification using the ABI 7500 instrument with Taqman probe assay gave a standard curve with a very good linearity and reproducibility (Figure 1a). The same amplification reactions, run on a Corbett Rotor Gene 3000, gave comparable results (Figure 1b). These data indicated that both assays were able to detect Mp with the same efficiency on both instruments tested. As also shown in Figure 1a and 1b, the sensitivity of the Scorpion and TaqMan probe enabled the system to detect fewer than 6 copies of pathogen on both instrument platforms. Of note, the y intercept (the cycle at which a single copy gene can be detected) corresponded to cycle 38-39 in a group of repeated experiments (Figure 1a and 1b).

Both TaqMan and Scorpion probes showed high specificity, since none of the 4 Mycoplasmataceae species tested, different from Mp, resulted positive in the performed assays. In addition no reactivity was observed also against 17 gram-positive or gram-negative bacterial pathogens and 2 fungi. Although the TaqMan probe assay was as sensitive and specific as the Scorpion probe real-time assay, the latter was faster, since, as described in the Material and Methods section, the probing mechanism was an intra-molecular event, while the TaqMan probe assay had a bimolecular kinetics. Thus, in our hands, the Scorpion probe real-time PCR reduced the entire reaction time by about 1/3 compared to TaqMan PCR on the ABI 7500 instrument.

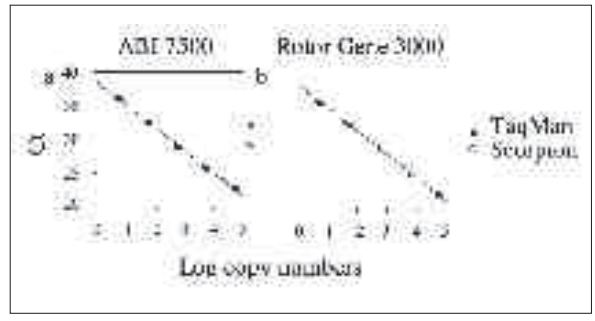

FIGURE 1 - *Standard curve of Mp DNA concentration in serial dilutions versus Ct. Each symbol represents the mean of triplicate PCR amplification for each dilution performed using either TaqMan or Scorpion probes on (a) ABI 7500 and (b) Corbett Rotor Gene 3000.*

Panel a:
ABI7500 *Taqman y = 37.58-3.32x; R2=0.999*
Scorpion y = 38.14-3.39x; R2=0.994
Panel b:
RotorGene3000 *Taqman y = 38.36-3.37x; R2=0.992*
Scorpion y = 38.46-3.37x; R2=0.994

The Inter- and intra-assay reproducibility were also evaluated. Three consecutive assays run over a 2-week period yielded an inter-assay coefficient of variation (CV% based on Ct) of 1.35% and 2.01%, respectively, for $6*10^4$ or 6 copies of standard Mp DNA with the Scorpion probe; under the same conditions, the TaqMan probe showed a CV% value of 0.73% or 2.19%. The Ct intra-assay coefficient of variation was 1.64% or 0.94% calculated on at least 5 repeats of $6*10^4$ or 6 copies each of standard DNA using Scorpion probe and 0.55% and 1.2% with TaqMan assay, respectively.

Clinical samples

As shown above, both assays have highly similar performances, except for the faster kinetics characterizing the Scorpion probe-based real-time assay. For this reason, a validation of the Scorpion real-time assay in a clinical setting was performed using 388 samples. On these isolates, the real-time PCR assay was compared with the results obtained using the qualitative one-step PCR specific for Mp. 213 samples were found positive by real time PCR, among these only 93 (44%) were found to be positive in qualitative PCR assay. In 30 samples out of 93 found positive in both assays, real-time PCR detected fewer than 6 copies of Mp.

Finally, 175 samples were found to be negative for Mp both in real-time PCR, and qualitative PCR although all these samples resulted positive for amplification of β-globin DNA used as internal control for DNA extraction and amplification (data not shown).

These findings revealed a seemingly enhanced sensitivity of real-time PCR in detecting Mp infection in oropharyngeal swabs compared to qualitative PCR. Lacking other direct evidence of Mp colonization, the detection of a positive (or negative) signal using PCR needed to be further validated. Among the 120 positive real time PCR and negative qualitative PCR samples we have access to the serological results of 38 patients, representing the 32% of the population. Twenty-four of them (63%) displayed a titre rise, while 14 out of 38 (37%) were serum negative, but later displayed a serum conversion, thus suggesting that during the acute phase of the disease the real time PCR is a very powerful tool for Mp detection in oropharyngeal swabs.

Of note, the wide range of serological titre (ranging from 40 to 1280) in the serum positive patients (data not shown) indicated that Mp PCR is able to detect the disease in the convalescent phase and thus demonstrate that Mp DNA could be detected also in patients undergoing an active immune response.

DISCUSSION

The present availability of tests allowing the detection of microorganism DNA in clinical samples has notably enhanced the diagnostic armamentarium for infectious respiratory diseases (Mackay, 2004; Welti *et al.,* 2003; Stralin *et al.,* 2005). Unlike conventional microbiological culture procedures, the detection achieved by these novel techniques is not susceptible to the pretreatment of patients with antibiotics, which are frequently recommended for CAP in the absence of (or waiting for) an etiological diagnosis.

In our hands, real-time PCR applied to the detection of Mp in clinical samples confirmed all the expected advantages (Templeton *et al.,* 2003; Morozumi *et al.,* 2004; Morozumi *et al.,* 2006). In addition we showed that both the TaqMan and the Scorpion probe-based assays resulted in highly comparable results. Indeed, the sensitivity of each method reliably detected a single copy of the Mp genome. The specificities were also comparable, since homogeneous results were obtained when the two different probes were used on the reference Mp strain, on some closely related mycoplasmataceae pathogens and on a large panel of Mp unrelated microorganisms. On these bases, it is clear that in the laboratory conditions described in this work the two different probe-based assays are interchangeable. At present, TaqMan-based assay is a widely used standard probe-based test for several real-time PCR applications. Nevertheless, the use of the uni-molecular Scorpion-based probe has significant advantages, thanks especially to its reaction kinetics that can result in a shorter cycling profile, 30% less time than that required for the TaqMan probe-based reaction. Such faster kinetics may be insignificant in small-scale studies, but they become significantly advantageous in large-scale, even centralized epidemiological or clinical studies. It is also possible that the Scorpion assay

is more robust and reliable, since the Taq enzyme undergoes a shorter thermal stress.

All the features anticipated on the basis of the thermodynamic characteristics of the Scorpion-based method were fully confirmed by use of the approach for the detection of Mp in clinical isolates. As shown in the Results section, all samples positive by qualitative PCR assays were also positive using the novel Scorpion-based real-time assay. Similarly, all negative samples using the real-time assay were also negative in the qualitative test. Interestingly, the analysis of our results indicated that real-time PCR seemingly proved more sensitive than the qualitative PCR. This finding is intriguing, since other authors (Ursi *et al.,* 2003) found no increased sensitivity of linear oligoprobe Lightcycler™-based real-time PCR assay in comparison with qualitative assays. These authors observed largely similar results, while in our hands 120 samples were positive with Scorpion-based real-time PCR and negative with qualitative PCR assays. This can be explained only by the higher specificity of the test due to the specific primers and probe recognition of the target sequence. In addition these results were confirmed using serological tests of available paired sera, as shown by other authors (Raty *et al.,* 2005). In 63% of the serological analyzed patients a positive real-time PCR associated with a negative qualitative PCR result was also linked to a high titer of anti-Mp antibodies. The serum negative patients at the time of positive real time PCR and negative qualitative Mp PCR were followed up for a longer period, and later on showed a serum conversion (not shown), thus indicating that these patients suffered from acute phase Mp infection detected only using real-time PCR. These findings suggested that real-time PCR was more sensitive than qualitative PCR in both the acute and the convalescent phases of Mp infections. Another interesting point was that a significant number of real-time PCR positive reactions were observed in patients with high titre of anti-Mp antibodies, thus demonstrating that the assay is also insensitive to the presence of an active immune response. Finally, this assay should be mandatory in all patients where a primary or drug-induced immune suppression could virtually impair the capacity to mount an efficient immune response that results in a failure of any serology-based method.

In conclusion, real-time PCR using the Scorpion-based probes offers a number of special advantages:

1) a wide range of sensitivity;
2) the insensitivity of the method to possible mispriming due to the intrinsic characteristics of the unimolecolar Scorpion-probe;
3) the possibility of detecting a great number of acute-phase patients that are negative not only on serological assay, but also on qualitative PCR;
4) the possibility of quantifying the actual number of genome copies in the biological specimen;
5) its insensitivity to previous antibiotic treatment and to the presence of an active immune response. In addition, the time required for a result to be yielded is reduced (by 30%) compared to TaqMan-based probes.

All these features suggest that real-time PCR employing the Scorpion probes could rewardingly be applied for the rapid and efficient etiological diagnosis of CAP.

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