Use of a triplex polymerase chain reaction for the detection and differentiation of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in the presence of human DNA

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PCR primers corresponding to the adhesin genes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* were shown to detect the corresponding organisms specifically. Absence of cross-reaction with seven other mollicute species and six unrelated bacterial species commonly found in humans was demonstrated. Positive control primers directed against human mitochondrial DNA could be mixed with the *Mycoplasma* primers without loss of specificity or sensitivity. A detection level of 10 c.f.u. of either *Mycoplasma* species could be readily obtained, even in the presence of 10⁴ human cells. The triplex PCR method developed is very simple and does not require hybridization or the use of radioisotopes and allows detection and differentiation of these mycoplasmas against the background of human DNA found in clinical specimens.

Introduction

Mycoplasma pneumoniae is the aetiological agent of many respiratory infections among which primary atypical pneumonia is the most important (Couch, 1990). This micro-organism is presently responsible for 10–20% of all radiographically-proven pneumonias and up to 25% of all paediatric primary atypical pneumonias (Cherry, 1987). Erythromycin or a tetracycline is generally used to treat these infections and such treatment significantly reduces the duration of illness (Shames *et al.*, 1970). Thus, the main problem with *M. pneumoniae* pneumonia is not treatment, but diagnosis which is presently inadequate.

Culture of *M. pneumoniae* is expensive, time consuming, labour intensive and generally of retrospective value only, as it can take up to 6 weeks before becoming positive (Tully & Razin, 1983). Serological methods are more extensively used than culture because they are easier to perform and more affordable. However, they are also generally non-specific (Clyde *et al.*, 1984),

insensitive and retrospective. Diagnosis is further complicated by the possible presence of *M. genitalium* which shares many antigens with *M. pneumoniae* (Lind, 1982; Lind *et al.*, 1984; Tully, 1989) and cannot be differentiated from it by culture. Since *M. genitalium* was first isolated from men with non-gonococcal urethritis (Tully *et al.*, 1981), its cross-reactivity with *M. pneumoniae* was initially of little concern in the diagnosis of *M. pneumoniae* pneumonia. However, *M. genitalium* may be isolated from respiratory tract samples (Baseman *et al.*, 1988), and its possible presence should always be considered when trying to diagnose a potential *M. pneumoniae* pneumonia (Tully, 1989); previously, many *M. genitalium* isolates might have been wrongly identified as *M. pneumoniae*.

Recently, DNA probes have been used for the diagnosis of *M. pneumoniae* infections (Hyman *et al.*, 1987; Göbel *et al.*, 1987; Dular *et al.*, 1988; Hata *et al.*, 1990; Kleemola *et al.*, 1990), but they are usually insensitive (Bernet *et al.*, 1989) and hard to handle in a diagnostic laboratory because they are generally radio-labelled, which introduces problems of safety and limited shelf life (Dular *et al.*, 1988; Kleemola *et al.*, 1990). Furthermore, they are often unable to differentiate *M. pneumoniae* and *M. genitalium*, unless specifically designed for this purpose (Dular *et al.*, 1988; Hata *et al.*, 1990; Kleemola *et al.*, 1990).

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Presently, the polymerase chain reaction (PCR) is the most promising method for the diagnosis of *M. pneumoniae* pneumonias, as it is rapid, extremely sensitive and can be designed to be specific (Peter, 1991). It can also be used without radioisotopic labelling. It is, however, susceptible to artifacts, of which the most serious with regards to diagnostic tests are the occurrence of false positives, usually through sample contamination, or false negatives, through the presence of inhibitors that prevent DNA amplification (Erlich, 1989).

The main goal of our study was to design a rapid, sensitive and specific PCR test that could eventually be used to detect and differentiate *M. pneumoniae* and *M. genitalium* directly in clinical specimens, without the need for hybridization or radioisotopes. We also investigated the inclusion of primers in the assay procedure that could amplify a fragment of human DNA as an internal positive control to validate both the PCR process and the sampling procedure (Keller *et al.*, 1990). Finally, we wanted to optimize the PCR conditions to perform the entire test in the same tube; it would then be a triplex PCR.

Methods

Bacterial strains and culture procedure. The following mycoplasma strains were used and were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA): *Mycoplasma pneumoniae* ATCC 15531, *M. pneumoniae* ATCC 29342, *M. genitalium* ATCC 33530, *M. buccale* ATCC 23636, *M. salivarium* ATCC 23064, *M. orale* ATCC 23714, *M. fermentans* ATCC 19989, *M. hominis* ATCC 23114, *Acholeplasma laidlawii* ATCC 23206 and *Ureaplasma urealyticum* ATCC 27618. All mycoplasma strains were cultured in diphasic SP-4 medium (Tully *et al.*, 1977) containing 2000 units penicillin G ml⁻¹, 500 units polymyxin B ml⁻¹ and 2.5 µg amphotericin B ml⁻¹, as described by Razin & Tully (1983). Thallium acetate was not added to the medium since it can inhibit growth of *M. genitalium* (Razin & Tully, 1983). The medium was further supplemented with glucose (0.5%), arginine (0.25%) or urea (0.5%) depending on the nutritional needs of the species being cultivated. Unrelated bacterial species, obtained from Ste-Justine's hospital (Montreal, Quebec, Canada), were: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. A fresh *Streptococcus pneumoniae* clinical isolate was used because no ATCC strain was available at the time of study. Additional bacterial species, *Branhamella catarrhalis* ATCC 25238 and *Haemophilus influenzae* ATCC 33391, were obtained from the Biotechnology Research Institute (Montreal, Quebec, Canada). *E. coli* and *P. aeruginosa* were grown in 2×YT broth (Sambrook *et al.*, 1989), *Staph. aureus* and *B. catarrhalis* were grown in trypticase soy broth (BBL) and *Strep. pneumoniae* and *H. influenzae* were grown in brain heart infusion with haemin (Difco).

Clinical samples. Throat swabs were collected in 1 ml SP-4 broth and immediately sent to the laboratory, on ice. They were processed upon arrival, or after storage at -70 °C. A portion of the sample (100 µl) was inoculated onto a SP-4 agar plate which was regularly observed microscopically, over 3 weeks, for the appearance of typical mycoplasma colonies. At the same time, half of the remaining volume was inoculated into a vial of diphasic SP-4 medium which was regularly observed for pH change for 6 weeks, and subcultured on to SP-4 agar

every week. Potential *M. pneumoniae* or *M. genitalium* colonies were tested by haemadsorption and haemolysis (Razin & Tully, 1983). In parallel, the other half of the remaining volume was prepared for PCR, as described below. Other clinical specimens were processed as described for throat swabs except that 10⁻¹ and 10⁻² dilutions were also inoculated into diphasic SP-4 medium, to prevent overgrowth of contaminating micro-organisms or the inhibition of mycoplasma growth by components of the clinical sample.

DNA extraction and sample preparation for PCR. DNA from different mycoplasma strains and clinical samples was prepared for PCR as follows: the sample was centrifuged at 14000 r.p.m. for 20 min, washed in PBS (0.1 M-NaCl, 2.5 mM-KCl, 10 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.4) and resuspended in 60 µl distilled water. The sample was then boiled for 5–10 min and stored at 4 °C.

Primers. The three pairs of primers used in this study were synthesized on an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method. The pair P4 (P4A: 5' AGG CTC AGG TCA ATC TGG CGT GGA 3' and P4B: 5' GGA TCA AAC AGA TCG GTG ACT GGG T 3') was specific to the *M. pneumoniae* P1 adhesin gene and amplified a 345 bp fragment from nucleotides 3947 to 4291 (GenBank accession no. M18639). The pair G3 (G3A: 5' GCT TTA AAC CTG GTA ACC AGA TTG ACT 3' and G3B: 5' GAG CGT TAG AGA TCC CTG TTC TGT TA 3') was specific to the *M. genitalium* adhesin gene and amplified a 507 bp fragment from nucleotides 3754 to 4260 (GenBank accession no. M31431). The pair H6 (H6A: 5' ATG ACC CAC CAA TCA CAT GCC TAT CA 3' and H6B: 5' ACT AGT TAA TTG GAA GTT AAC GGT ACT A 3') was specific to the human mitochondrial cytochrome oxidase subunit 3 gene (GenBank accession nos J014125, M12548, M58503, M63932 and M63933) and amplified a 828 bp fragment from nucleotides 9207 to 10034 which was used as an internal positive control. The primers were chosen to have a high but equal normalized length (L_n) (Wu *et al.*, 1991) to allow the use of a high annealing temperature which would reduce the possibility of obtaining unwanted bands originating from non-specific amplification.

PCR amplification and electrophoresis. PCR reactions were performed in a total volume of 50 µl containing 5 µl 10×PCR buffer (100 mM-Tris/HCl pH 8.3, 500 mM-KCl, 27 mM-MgCl₂, 0.1% gelatin), 250 µM of each dNTP (Pharmacia), 0.5–1.5 µM (depending on the pair of primers used) of each primer, 2 units of *Taq* DNA polymerase (Pharmacia), and an appropriate volume (5 or 25 µl of a clinical sample or a volume corresponding to 10⁴ bacterial or human cells) of the sample to be amplified. The reaction volume was overlaid with 50 µl paraffin oil to prevent evaporation. The samples were placed in a thermal cycler (Perkin Elmer-Cetus model TC-1) and submitted to 30 cycles of amplification. One such cycle consisted of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and elongation at 72 °C for 1 min. After the last cycle, the samples were kept at 72 °C for an additional 10 min to ensure that the polymerization of every fragment was complete. The samples were then kept at 4 °C until analysed on a 1.5% (w/v) agarose gel (SeaKem LE, FMC BioProducts). The gel was run in TAE buffer at 10 V cm⁻¹ for 1–1.5 h and then stained with a solution of 500 µg ethidium bromide l⁻¹ before being photographed with a Polaroid MP-4 camera.

Southern blotting and hybridization. To verify that the fragments amplified by the pairs of primers P4, G3 and H6 were the ones expected, oligonucleotides specific to internal regions of those fragments were synthesized and used as probes to hybridize Southern blots. Probe 4A4B was specific to the 345 bp fragment of *M. pneumoniae* DNA amplified by the P4 primers and had the sequence 5' GAT GTT GAT GGT ATT GAT C 3'. Probe 3A3B was specific to the 507 bp fragment of *M. genitalium* DNA amplified by the G3 primers and had the sequence 5' CCT TTG ATT GTA ACT GTT C 3'. Finally, probe

6A6B was specific to the 828 bp fragment of human DNA amplified by the H6 primers and had the sequence 5' AGA AAA CAA CCG AAA CCA A 3'. The probes were radiolabelled at their 5' end with T4 polynucleotide kinase (Pharmacia) and [γ - 32 P]ATP. The capillary transfer of the DNA on to nitrocellulose membranes (Ba-85, Schleicher & Schuell) was done by the Southern method (Sambrook *et al.*, 1989) except that the denaturing and neutralizing solutions contained 0.15 M-NaCl. Prehybridizations and hybridizations were performed as follows. The nitrocellulose membranes were prehybridized in 6 $\times$ SSC, 0.5% Triton X-100, 1 $\times$ Denhart's reagent, 3% (w/v) dextran sulphate at 42 °C for 1 h. The probe was then added and hybridization performed at 42 °C for 2 h. After three washes in 3 $\times$ SSC, 0.5% Triton X-100 at 42 °C for 15–30 min, the membranes were autoradiographed on X-ray film (Kodak) in a cassette with amplifying screens at –80 °C for about 4 h.

Results

Optimization of the PCR conditions

In amplifying *M. pneumoniae*, *M. genitalium* and human DNA together in the same tube, the most important variable was the relative concentrations of the different pairs of primers. The longest fragment (828 bp from human DNA) needed the highest concentration of primers (H6), whereas the shortest fragment (345 bp from *M. pneumoniae* DNA) needed the lowest concentration of primers (P4) to be amplified efficiently. The *M. genitalium* DNA fragment (amplified by the G3 primers) was of intermediate length (507 bp). When an equal concentration of all six primers was used, only the smallest fragment was amplified, whilst the others were undetectable (data not shown). Reliable amplification of all three bands was obtained when the relative concentrations of the different primers were adjusted to 0.5 μ M for each of the P4 primers, 1.0 μ M for each of the G3 primers and 1.5 μ M for each of the H6 primers. Other variables studied were the MgCl₂ concentration, which was established at 2.7 mM for optimal results (data not shown) and the number of amplification cycles, where it was found that 30 cycles were sufficient for maximum sensitivity.

Specificity of the primers

To determine the specificity of the experimental design, amplification was performed on *M. pneumoniae* DNA (10^4 c.f.u.) with the P4 primers, the G3 primers and the H6 primers separately (Fig. 1a, lanes 2–4). Only the P4 primers amplified the *M. pneumoniae* DNA and the fragment obtained was 345 bp long, as expected. The same experiment was then performed with 10^4 c.f.u. of *M. genitalium* (Fig. 1a, lanes 5–7) and 10^4 human cells (Fig. 1a, lanes 8–10). It was found that only the G3 primers amplified the *M. genitalium* DNA yielding the expected 507 bp fragment and only the H6 primers amplified the human DNA giving rise to the expected

828 bp fragment. We also performed the triplex PCR where *M. pneumoniae*, *M. genitalium* and human DNA were placed in a single tube, mixed with the three pairs of primers and submitted to 30 cycles of PCR. The results show that the three fragments of the expected size were amplified simultaneously (Fig. 1a, lane 11), although the yield of each fragment was somewhat reduced when compared to the result where only one pair of primers was used.

To prove that the three fragments obtained were the ones expected, we hybridized them with oligonucleotides specific to their internal regions. The first probe we used

Fig. 1. (a) Specificity of the three pairs of primers tested with *M. pneumoniae*, *M. genitalium* and human DNA. Lane 1: 100 bp ladder (Pharmacia). Lanes 2–4: DNA from 10^4 c.f.u. *M. pneumoniae* ATCC 15531 with 0.5 μ M P4 primers (lane 2), 1.0 μ M G3 primers (lane 3) and 1.5 μ M H6 primers (lane 4). Lanes 5–7: DNA from 10^4 c.f.u. *M. genitalium* with 0.5 μ M P4 primers (lane 5), 1.0 μ M G3 primers (lane 6) and 1.5 μ M H6 primers (lane 7). Lanes 8–10: DNA from 10^4 human cells with 0.5 μ M P4 primers (lane 8), 1.0 μ M G3 primers (lane 9) and 1.5 μ M H6 primers (lane 10). Lane 11: all three types of DNA with all three pairs of primers (triplex positive control). Lane 12: no DNA with all three pairs of primers (negative control). (b) Southern blot of the gel from (a) hybridized with the probe 4A4B. Lanes 2 and 11: the probe hybridized to the 345 bp *M. pneumoniae*-specific fragment only.

Fig. 2. Specificity of the triplex PCR tested with various mollicute species. Lane 1: 100 bp ladder. Lanes 2–12: 0.5 μ M P4 primers, 1.0 μ M G3 primers and 1.5 μ M H6 primers with DNA from 10^4 c.f.u. of: *M. pneumoniae* ATCC 15531 (lane 2), *M. pneumoniae* ATCC 29342 (lane 3), *M. genitalium* (lane 4), *M. buccale* (lane 5), *M. orale* (lane 6), *M. salivarium* (lane 7), *M. fermentans* (lane 8), *M. hominis* (lane 9), *Ureaplasma urealyticum* (lane 10), *Acholeplasma laidlawii* (lane 11) and 10^4 human cells (lane 12). Lane 13: *M. pneumoniae* ATCC 15531, *M. genitalium* and human DNA with all three pairs of primers (triplex positive control). Lane 14: no DNA with all three pairs of primers (negative control).

was 4A4B which hybridized to the 345 bp fragment from *M. pneumoniae* that was amplified by the P4 primers (Fig. 1*b*, lanes 2 and 11). Similar results were obtained with the probes 3A3B and 6A6B, specific to internal regions of the 507 bp fragment from *M. genitalium* and the 828 bp fragment from human DNA, respectively (data not shown).

To assess the specificity of our three pairs of primers, we used them together (Fig. 2), to try to amplify the DNA from the other mollicutes and cell-walled species described in Methods. As expected, fragments were amplified only when *M. pneumoniae*, *M. genitalium* or human DNA was present in the sample (Fig. 2, lanes 2, 3, 4, 12 and 13) and there was no amplification when the DNA from other mycoplasma species (Fig. 2, lanes 5–11) or cell-walled bacteria (data not shown) was used.

Sensitivity of the primers

To determine how sensitive the primers were, we used them to amplify 10-fold dilutions of *M. pneumoniae*, *M. genitalium* and human DNA to determine the detection limit. In the case of *M. pneumoniae* dilutions amplified with the P4 primers, the DNA from as little as 10 c.f.u. was easily detected (data not shown). On some occasions, DNA from 1 c.f.u. was apparently detected, but that result was not generally reproducible and thus not considered as being the detection limit. Such variability in the results obtained with samples containing DNA from 1 c.f.u. was to be expected. As described by Saiki *et al.* (1988), the actual number of target sequences in such a dilution will vary according to a Poisson distribution.

Such a distribution predicts that the probability of a sample containing one or more target sequences is 0.632 when the average number of copies present is 1 per sample. The sensitivity of the G3 primers with dilutions of *M. genitalium* DNA was similar. However, when human DNA was used, the H6 primers were slightly less sensitive than the P4 and the G3 primers and about 100 human cells were needed for a visible band to be obtained.

Next, we wanted to see if the detection limit was the same when a fragment was amplified individually or within the triplex PCR. At the same time, we wanted to determine if a very low concentration of one of the targets could be amplified effectively if the two other targets were present at high concentrations. To answer these questions, we tried to amplify 10-fold dilutions of *M. pneumoniae* DNA in the presence of 10^4 c.f.u. of *M. genitalium* and 10^4 human cells (Fig. 3*a*, lanes 10–16). To be able to make an adequate comparison, we also amplified the same *M. pneumoniae* dilutions individually (Fig. 3*a*, lanes 2–8). As shown in Fig. 3*a*, the detection limit for *M. pneumoniae* DNA was 10 c.f.u. whether the amplification was performed individually or in the triplex PCR (Fig. 3*a*, lanes 5 and 13). Fig. 3*a* also shows that the sensitivity was not affected by the presence of the two other targets in much higher concentrations, even though the intensity of the *M. pneumoniae*-specific band was a little weaker in the triplex PCR.

Finally, we wanted to determine if the same level of sensitivity could be achieved in a clinical sample. For this purpose, we used nasopharyngeal secretions that were negative for *M. pneumoniae* DNA with the P4 primers, negative for *M. genitalium* DNA with the G3 primers and positive for human DNA with the H6 primers. Ten-fold dilutions of *M. pneumoniae* DNA were added and amplification with the P4 and the H6 primers performed (Fig. 3*b*, lanes 10–16). Once again, to be able to make an adequate comparison, the same *M. pneumoniae* dilutions were amplified with the P4 primers only (Fig. 3*b*, lanes 2–8). As determined in the other cases, the detection limit was 10 c.f.u. (Fig. 3*b*, lanes 5 and 13) whether they were amplified individually or in the clinical sample. Amplification of the human DNA present in the clinical sample was not hampered by the presence of the *M. pneumoniae* DNA (Fig. 3*b*, lanes 10–16).

Clinical samples

A preliminary study of 30 clinical samples (8 expectorations, 14 throat swabs, 3 nasopharyngeal secretions, 2 bronchoalveolar lavages, 1 bronchial secretions, 1 bronchial lavage and 1 cerebrospinal fluid) was performed. It was sometimes necessary to dilute the sample 1/5 to obtain an amplification because of the presence of

Fig. 3. (a) Sensitivity of the P4 primers with 10-fold dilutions of *M. pneumoniae* DNA tested in the triplex PCR. Lanes 1 and 9: 100 bp ladder. Lanes 2–8: 0.5 μ M P4 primers with DNA from 10^4 (lane 2) to 10^{-2} (lane 8) c.f.u. *M. pneumoniae* ATCC 15531. Lanes 10–16: 0.5 μ M P4 primers, 1.0 μ M G3 primers and 1.5 μ M H6 primers with DNA from 10^4 c.f.u. *M. genitalium*, 10^4 human cells and DNA from 10^4 (lane 10) to 10^{-2} (lane 16) c.f.u. *M. pneumoniae* ATCC 15531. Lane 17: no DNA with all three pairs of primers (negative control). (b) Sensitivity of the P4 primers with 10-fold dilutions of *M. pneumoniae* DNA added to a clinical sample. Lanes 1 and 9: 100 bp ladder. Lanes 2–8: 0.5 μ M P4 primers with DNA from 10^4 (lane 2) to 10^{-2} (lane 8) c.f.u. *M. pneumoniae* ATCC 15531. Lanes 10–16: 5 μ l of the clinical sample with 0.5 μ M P4 primers, 1.5 μ M H6 primers and DNA from 10^4 (lane 10) to 10^{-2} (lane 16) c.f.u. *M. pneumoniae* ATCC 15531. Lane 17: no DNA and no clinical sample with 0.5 μ M P4 primers and 1.5 μ M H6 primers (negative control).

inhibitors despite the boiling of samples during preparation. Thus, samples were always tested undiluted by triplex PCR and then diluted with each pair of primers individually and also in triplex PCR.

Out of the 30 clinical samples so tested, none were positive for *M. pneumoniae* or *M. genitalium* DNA. However, only two were negative for human DNA, a cerebrospinal fluid and a bronchoalveolar lavage which contained little cellular material if any. When cultured, only two samples out of 30 yielded a mycoplasma but neither were *M. pneumoniae* or *M. genitalium*.

The lack of positive results in the limited number of samples available to us is not surprising in view of the low success of previous researchers in detecting *M. pneumoniae* even under epidemic situations in high risk groups (Kleemola *et al.*, 1990).

Discussion

The methods presently used to diagnose *M. pneumoniae* infections are inadequate because they lack speed, sensitivity and specificity. Since PCR technology can overcome these problems, it is a promising candidate to replace current diagnostic methods. The goal of our

study was to design a PCR test that was sensitive, specific, differentiated between *M. pneumoniae* and *M. genitalium*, included an internal positive control, and could be performed in a single tube and used directly on clinical samples.

Several protocols of DNA preparation for PCR amplification use combinations of organic solvent extraction (e.g. chloroform and/or phenol extraction) or treatment with harsh detergents such as SDS. Although these methods may ensure removal of inhibitors, the organic reagents used may themselves interfere with the amplification reaction and the extra manipulations involved in removing reagents increase the potential for sample contamination and loss of DNA through adsorption on tubes or pipettes. Since PCR can be performed on relatively impure DNA, samples used in this study were simply boiled, thus inactivating nucleases and potential inhibitors of the polymerase. Furthermore, the boiling step efficiently liquified viscous clinical samples such as expectorations that would otherwise have been difficult to process without any additional treatment.

In optimizing the conditions for the triplex PCR the relative concentration of the three pairs of primers was

the most important variable. It appeared that polymerization or annealing of the primers to the largest DNA fragment was inhibited by the presence of the smaller fragments or their primers. This problem is probably at the annealing level because increasing the concentration of primers specific to the longest fragment allows it to be efficiently amplified, whilst amplification of the smallest fragment is unaffected. Other publications have also mentioned this type of problem in multiplex PCR (Bej *et al.*, 1990, 1991). Indeed, when fragments of different lengths are amplified simultaneously, the shorter ones are advantaged and varying the amount of primers can take care of that problem.

To assess the specificity of the primers was a very important part of this study. First, we had to make sure that our primers were specific to their target sequence and did not amplify DNA from other sources, including the targets of the other primers. Next, we also had to verify that their specificity was as satisfactory in the triplex PCR as it was individually. It was possible that, when used together, primers from different pairs could form a new pair and give rise to unwanted secondary bands with *M. pneumoniae*, *M. genitalium* and human DNA or give bands with DNA from other sources. Several pairs of primers had to be tested before the set used in this study was decided upon.

The next important step in our study was to evaluate the sensitivity of the different pairs of primers. When 10-fold dilutions of *M. pneumoniae* and *M. genitalium* were amplified with their respective primers, as little as 10 c.f.u. could be detected, which constitutes a very high level of sensitivity. However, when the same experiment was done with dilutions of human DNA, at least 100 cells were needed for an amplification to occur. This lack of sensitivity achieved with the human primers could be due to the formation of secondary structures in the target DNA itself, which could then affect the annealing of the primers to their specific target sequence. However, we did not consider this lower level of detection for the human DNA as constituting a problem, because it reinforced its role as an internal positive control. Indeed, *M. pneumoniae* and *M. genitalium* adhere strongly to epithelial cells and a sample of good quality that has been harvested correctly will contain a considerable number of epithelial cells (Clyde *et al.*, 1984).

Since one of our goals was to use all the primers together in the same tube, we had to see if the sensitivity level achieved with an individual pair of primers would be reduced in the triplex PCR. However, the detection limit was unchanged (with the exception that the intensity of the signal was reduced in the case of the highest DNA dilutions); it seems that as long as the relative concentrations of the different pairs of primers have been adjusted to allow an efficient amplification of all the

fragments in the triplex PCR, the initial quantity of each of the targets has very little importance, if any.

Finally, we have shown that the sensitivity of the assay remains unchanged when one of the targets is added to and amplified directly in a clinical sample, which shows that our method of sample preparation by simply boiling does yield DNA suitable to be amplified directly without any further treatment.

A few groups have already demonstrated the ability of PCR technology to detect *M. pneumoniae* added to clinical samples. Bernet *et al.* (1989) detected between 100 and 1000 c.f.u. in bronchoalveolar lavages and Jensen *et al.* (1989) less than 40 c.f.u. in throat swabs. Sasaki *et al.* (1992) were also able to differentiate *M. pneumoniae* and *M. genitalium* in a test that detected 100 *M. pneumoniae* and 1000 *M. genitalium*. Two groups have recently addressed the internal control issue in detecting *M. pneumoniae* by PCR. The first group, Ursi *et al.* (1992), cloned their PCR fragment in a plasmid and then inserted a piece of foreign DNA in that fragment, to use it as a positive control. The control was important in avoiding false-negative results, which would have otherwise reached a level of 37%. Assay specificity was not addressed but sensitivity was good in that they were able to detect 1 fg of purified *M. pneumoniae* DNA (approximately one genome), but this decreased to 5 fg when their positive control was included. This phenomenon was also observed in our work where the intensity of the amplified bands was less in the triplex than in the simplex PCR. The second group, Skakni *et al.* (1992), used the primers published by Bernet *et al.* (1989) to amplify *M. pneumoniae* DNA directly in clinical samples. To achieve maximum sensitivity they followed the PCR amplification by a Southern blot and hybridization with a radiolabelled oligonucleotide. The most interesting part of their study was the use of the human β -globin gene as a positive control. Such a control is superior to the one used by Ursi *et al.* (1992) because it indicates if the sample is of good quality. The main drawback is that they tested for the presence of the human β -globin gene and *M. pneumoniae* in separate tubes, which does not prevent false-negative results due to technical problems present in the sample tube only. Also, while the use of Southern blotting and radiolabelling increases sensitivity, it takes away some of the advantage of the speed and convenience of the PCR method.

In conclusion we have succeeded in establishing a triplex PCR method to determine the presence of *M. pneumoniae* and/or *M. genitalium* in a clinical sample, with an internal positive control designed to verify both the performance of the PCR process and the validity of the sample collected. The protocol reliably detects 10 c.f.u. of either organism without the need for hybridization and radioisotopes.

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