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COMPARISON OF FOUR PCR TESTS FOR THE DETECTION OF *MYCOPLASMA PNEUMONIAE*

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ABSTRACT

Recently, PCR is being used more frequently as a diagnostic method to detect *M. pneumoniae*. We used primer pairs reported by Van Kuppeveld,[13] Leng,[5] Lunerberg,[7] and Bernet[1] targeting 16s rRNA, P1 protein, *tuf* genes, and a short DNA sequence (MP5) to evaluate the sensitivity among different PCRs. Reoptimization experiments showed that *tuf* PCR had the highest sensitivity amongst these four PCRs, detecting 10 organisms. Detection limit for the rest of the PCRs was 100 copies of DNA.

This study confirmed that 92°C would be the best dissociation temperature rather than higher temperatures that are still being used frequently in other studies. Besides, accurate optimizing of the annealing temperature and extension time had important roles on the sensitivity as well as using milli-Q distilled water rather than double distilled water. Experiments done on MP5 PCR proved that the non-specific products mentioned in previous studies[2] were not eliminated by increasing the annealing temperature, although they disappeared on gel electrophoresis after careful optimization of extension time.

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INTRODUCTION

Polymerase chain reaction promises to revolutionize diagnostic microbiology, particularly for problematic atypical pathogens such as *Mycoplasma pneumoniae*. At present, the diagnosis of *M. pneumoniae* infection is not satisfactory, because culturing the organism takes up to 3 weeks. CF test has low sensitivity and is non-specific. Measuring the increase in IgM titers has no advantage during acute infection, because specific IgM appears in patient's sera about 7 days after the onset of symptoms.[3]

There have been a number of studies on the detection of *M. pneumoniae* in clinical samples by PCR targeting of different genes and also various primer pairs for each one with different amplification targets. Amongst the different parameters affecting PCR, the choice of the target gene and primers is crucial to be carefully studied. Four mycoplasma genes have been used as targets for PCR amplification to date. These genes are P1 protein, 16s rRNA, *tuf* gene encoding the elongation factor, and a short DNA sequence (MP5) reported by Bernet[1] as a target for PCR amplification.

In this study primer pairs reported by Van Kuppeveld,[13] Leng,[5] Lunerberg,[7] and Bernet[1] have been used for targeting 16s rRNA, P1 protein and *tuf* genes, respectively. The primer pair designed by Van Kuppeveld[13] was only used by Tjhie[12] to detect clinical specimens. Despite no reports concerning the use of the primer pair reported by Leng[5] and Lunerberg,[7] the primer pair reported by Bernet[1] has been used frequently[2,6,11] while applying various conditions in each work. By this comparison we wanted to know which test has the highest detection limit. We also wanted to evaluate the optimized amplification conditions used in

these four PCRs and the effect of parameters influencing PCR.

MATERIALS AND METHODS

Strains and culture

M. pneumoniae strain 10119 and *Legionella pneumophila* strain 11150 were obtained from National Culture Type Collection. *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis,* and *Klebsiella pneumoniae* were obtained from the Manchester University Collection of Bacteria. Three chlamydia spp., *C. pneumoniae* IOL-207, *C. trachomatis* (T181), and *C. psittaci* 6BC, were obtained from Clinical Virology, Manchester Royal Infirmary, U.K. (Dr. Storey). Human DNA was extracted from lung fibroblast cells (Clinical Virology, Manchester Royal Infirmary). Selective mycoplasma agar and broth base with supplement-p (Oxoid) were used for mycoplasma culture.

DNA extraction

DNA was extracted with phenol-chloroform as follows. The organisms were centrifuged at 14,000g for 30 min, and the pellet resuspended in 500 µL TE buffer (10 mM tris/HCl pH 8.0, 1 mM EDTA) containing 250 µg/mL proteinase K and then incubated for 2h at 56°C. DNA was extracted using phenol: chloroform, and then precipitated with ethanol.

Primer pairs, PCR amplification, and electrophoresis

The sequences of the four primer pairs used in this study are mentioned in Table I. PCR reactions were performed in a total volume of 50 µL in 10 mM tris/HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 M of each primer, and 0.5 U of Taq DNA polymerase. Different amplification programs (Table II) and a total of 40 PCR cycles were used for each PCR set. PCR products were analyzed by agarose gel electrophoresis in TBE buffer (89 mM boric acid, 89 mM tris pH 8.0, and 10 mM EDTA), and DNA visualized by ethidium bromide.

Quantitation methods

Chromosomal DNA was quantified by spectrophotometry at 260-280 nm and by an ethidium bromide fluorescent quantitation method.[10]

RESULTS AND DISCUSSION

These four PCRs were first optimized, and optimization experiments showed that some conditions could be improved for each of these PCRs (Table II). The concentration of each primer was 0.3 µM and $MgCl_2$ was 2.5 mM. A total of 40 PCR cycles was run for each PCR set. The primer pair

Table I. List of primer pairs.

Primer Pair	Primer sequence (5'-3')	Primer size	Tm	GC%	Product size
tuf gene					950
MP 38	TACTCGTTACGACCAAATCGATAAG	25	55	40	
MP 39	GTTCAACTGTAATCGAGGTATTG	23	49	39	
P1 gene					631
P1-178	CAATGCCATCAACCCGCGCTTAACC	25	56	55	
P1-809	CAGATCCAGATGTGGTTGAAG	21	45	45	
16s rRNA gene					277
M. pnl	AAGGACCTGCAAGGGTTCGT	20	68	56	
M. pn2	CTCTAGCCATTACCTGCT	20	50	48	
Mp5					144
Mp5-1	GAAGCTTATGGTACAGGTTGG	21	57	42	
Mp5-2	ATTACCATCCTTGTTGTAAGG	21	52	42	
MP5					152
MP5-a	GAAAAGAAGCTTATGGTACAGGTTGG	26	49	48	
MP5-b	GTGATTACCATCCTTGTTGTAAGG	24	46	38	

Table II. Amplification program and improved conditions of the PCR tests used in this study.

	tuf gene	16s rRNA	P1 gene	MP5
Reagents				
dNTP (mM of each)	0.2	0.2	0.1	0.2
Taq DNA polymerase (U)	0.5	0.5	0.75	0.5
Amplification program(°C/min)				
Denaturation	92/1.5	92/1	92/1	92/1
Annealing	60/1.5	60/1	55/1	60/1
Extension	72/2	72/1.5	72/2	72/1.5

The concentration of primers and $MgCl_2$ were 0.3 µM and 2.5 mM for all PCR tests.

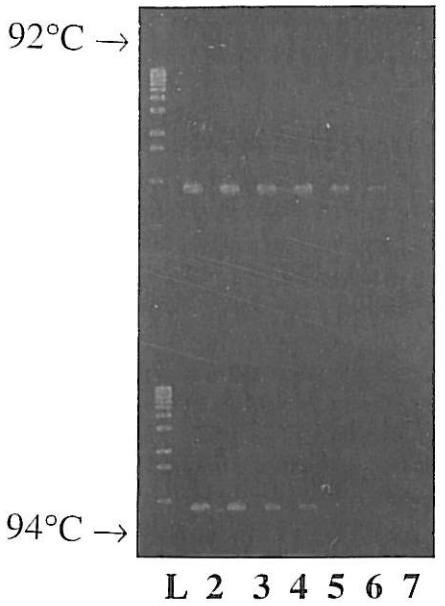

Fig. 1. Optimizing the denaturation temperature for *tuf* PCR. *M. pneumoniae* DNA was diluted in ten-fold steps from 10^5 to 1 copies and amplified at two different temperatures, 92°C and 94°C. The intensity of the product bands was better at 92°C for all dilutions.

targeting the *tuf* gene was shown to be the most sensitive primer pair, after titration of *M. pneumoniae* DNA using each of the four PCRs.

Results of the sensitivity test showed that the 10 organisms recovered from pure DNA can be detected by the *tuf* PCR, although a better intensity of product bands was observed after modifying the denaturation temperature (Fig. 1). By changing the amplification conditions it was possible to detect down to 100 copies of bacterial DNA for the other mycoplasma PCRs (Fig. 2).

MP-5 PCR

The primer pair targeting the unknown fragment MP5

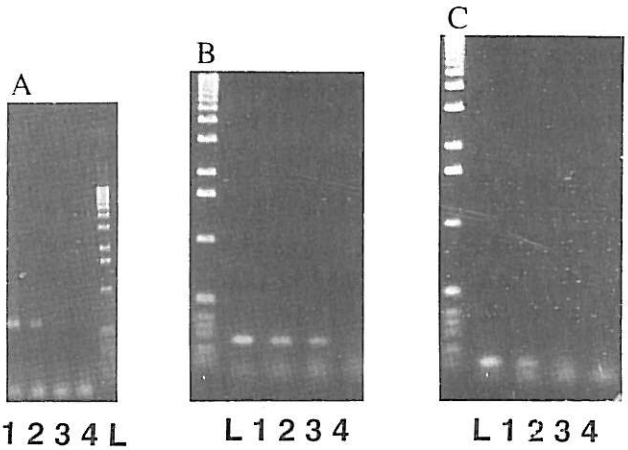

Fig. 2. Detection limit of the P1 PCR(A), 16s rRNA PCR(B), and MP5 PCR(C). The copy numbers of mycoplasma DNA in lanes 1,2,3, and 4 are 10^4, 10^3, 10^2, and 10, respectively.

has been used in different studies.[1,2,6,11] This fragment was scanned with PC/Gene (CD-ROM released October 1995) to try to determine its possible gene sequence, but without success.

A non-specific product has been reported for this PCR by Garret (1993). Increasing the annealing temperature could actually reduce the likelihood of such a product being produced. Computer analysis of the physico-chemical specificity of MP5 primers revealed that Tm was low. Therefore, to achieve a higher annealing temperature, increasing the Tm by upstream extension of the length of each primer was thought to be necessary[4] (Table I). The amplified PCR product with the new extended primers and predicted length was shown to be produced using 60°C as annealing temperature rather than 55°C. Results showed a high sensitivity for the test which detected 100 chromosomal copies of the purified DNA. Results also showed that non-specific products which were hoped to be eliminated or at least decreased in quantity at the higher annealing temperature were still produced. In another experiment the

effect of extension time was evaluated for elimination of non-specific products. When the extension time was increased for an additional 30 seconds the unwanted product was no longer observed on the agarose gel. Thus, extension time seems to have an important role in the amplification of mycoplasma DNA.

PCR targeting of the P1 protein gene

Leng[5] in an evaluation of mycoplasma PCRs, reported that primer pair 'p 178' and 'p 807' were the best for targeting the P1 gene and obtained 19 positive PCR results from a total of 21 known positive specimens. The highest sensitivity which Leng reported was 1 pg corresponding to 1200-1300 genome copies, although Leng estimated that each pg contains 100 genomes, which is an obvious miscalculation. However, by modifying some parameters in the amplification procedure 100 copies of DNA were detected. This increase in sensitivity was due to better optimization of the cycle number and denaturation temperature. Sensitivity dropped one log with the use of double-distilled water compared with milli-Q water. Experiments on optimizing the $MgCl_2$ concentration showed that this primer pair was more sensitive than the other mycoplasma primer pairs to variations in $MgCl_2$ concentration.

PCR detecting the 16s rRNA gene

Another PCR used in this study was based on the detection of the 16s rRNA gene using a primer pair designed by van Kuppeveld.[13] Using computer analysis they predicted a primer pair which could specifically detect *M. pneumoniae* among other mycoplasmas. Tjhie[12] actually used this primer pair for detection of *M. pneumoniae* in throat and nasopharyngeal swabs and reported a sensitivity of 1.5 CFU for direct PCR on clinical specimens, which corresponds to 140 chromosomal copies. Reoptimization experiments showed nearly the same results except for the amount of taq polymerase (0.5 U rather than 1 U) and the denaturation temperature (92°C instead of 95°C). The detection limit attained in this study was the same as MP5 and P1 PCRs (100 copies), which was near to that previously reported.[12]

Specificity tests showed cross reactions with *H. influenzae*, *H. parainfluenzae*, and *S. pneumoniae*. The intensity of the product bands was very low, indicating a poor match of the primers to those templates. Although Van-Kuppeveld[13] reported that their primer pair is species-specific for *M. pneumoniae*, they included only mycoplasma spp. in their computer analysis in an attempt to evaluate the specificity of the species-specific *M. pneumoniae* primers.

Parameters influencing PCR

PCR as a diagnostic microbiology method should have the highest sensitivity possible, and this will not be achieved unless all parameters are perfectly optimized and the test is used under the best conditions of amplification.

Optimization of the different PCR sets confirmed 92°C would be the best dissociation temperature, rather than higher temperatures which are being used frequently in other studies. All experiments confirmed not only that this temperature led to clearer product bands, but also it permitted use of a lower concentration of taq polymerase which is therefore more cost effective.

The annealing temperature should also be optimized carefully. A perfect match of primer with the target takes place when both primers are balanced in G/C content and have the same Tm. The detection limit will fall if a temperature above or below the optimum is used.

Experience with *tuf* and MP5 PCRs indicated that extension time also has a special role. In the *tuf* PCR the sensitivity dropped one log (ten times) when the extension time was reduced to 1 min for the standard 100 µL reaction mixture. Using a lower volume of reaction mixture permits amplification through a more rapid cycle including a shorter extension time. However, using too short an extension time may yield non-specific products. Rolf[9] has discussed the possible effect of extension time on production of non-specific products. In this current study with the MP5 PCR the non-specific products disappeared on gel electrophoresis when the extension time was increased from 1 to 1.5 minutes.

Basically it is best to run the PCR as rapidly as possible by choosing the minimum essential amplification cycle. The minimum number of amplification cycles yielding the highest amount of product under optimized conditions would be the best cycle number for the test. All investigations indicated that 40 cycles was the best cycle number to use.

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