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SIMULTANEOUS DETECTION OF *CHLAMYDIA PNEUMONIAE* AND *MYCOPLASMA PNEUMONIAE* BY PCR

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ABSTRACT

Both *C. pneumoniae* and *M. pneumoniae* are common causes of respiratory tract infection. At present, both are still diagnosed in the laboratory retrospectively by serology. This is despite many publications which indicate that PCR, which is not retrospective, is extremely good at detecting these organisms. We thought that a single PCR test which could detect both organisms simultaneously in a routine diagnostic laboratory would be more economic than using two separate PCR tests.

Chlamydia PCR was developed and optimized to detect *C. pneumoniae* using primer CpnA and CpnB which targets the MOMP gene. This test was very sensitive and could detect 10 organisms. To detect *M. pneumoniae*, the *tuf* PCR reported by Luneberg[6] was selected. After optimization of the duplex test, it was found that the sensitivity of the test for mycoplasma PCR was 100 times less than the sensitivity of the single tests, due to the inhibitory role of *C. pneumoniae* primer CpnB. Reducing the concentration of this primer helped but we decided to redesign it instead. The final form of the duplex has sufficient sensitivity, detecting 10 copies of each organism. The new primer $CpnB_2$ was a great improvement. The test was then developed to detect the product by hybridization rather than analysis with agarose gel electrophoresis.

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INTRODUCTION

Respiratory tract infections are important burdens on the health service, and pneumonia is probably the most common serious infection seen in general practice. Our understanding of the etiological agents of lower respiratory tract infection (LRTI) is incomplete in the community. *Streptococcus pneumoniae* has been found to be the most common cause of LRTI, but in nearly 50% of cases a pathogen was not identified. Part of this gap in knowledge is due to the inadequacy of currently used tests in diagnostic laboratories, especially for *Chlamydia pneumoniae*. Studies in the USA and Scandinavian countries have suggested that about 10% of adult cases of community-acquired pneumonia (CAP) are caused by *C. pneumoniae* or *M. pneumoniae* infection.

Recently, PCR has been applied to a number of infectious agents and generally has proved to be more sensitive than traditional methods. Reports on mycoplasma and chlamydia have usually shown that is suited to detect these agents,[1,9] but still needs to be developed before being accepted as a routine clinical laboratory method. Duplex PCR would be one direction in which PCR could be developed and recently tests have been described for various pathogenic organisms.[2,7,8,16] In the present study, we have combined primers targeting the chlamydia MOMP gene and *M. pneumoniae tuf* gene, simultaneously detecting *C. pneumoniae* and *M. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and culture

The following strains were used; *C. pneumoniae* IOL-207, N16, VR 1310; Avian *C. psittaci* strains 6BC, N352, Par; *C. trachomatis* serotype E/T181; *M. pneumoniae* strain 10119 and *Legionella pneumophila* strain 11150 were obtained from the National Culture Type Collection. Some micro-organisms were obtained from the Manchester University Collection of Bacteria, and used for specificity testing. These were adenovirus, *Streptococcus pneumoniae, Haemophilus influenzae*, *Neisseria meningitidis*, and *Klebsiella pneumoniae*. Human DNA obtained from lung fibroblast cells was also used for specificity testing.

Selective mycoplasma agar and broth base with supplement-p (Oxoid) were used for mycoplasma. Chlamydiae were propagated in McCoy cells by standard methods.[10] Chlamydial inclusions were detected by indirect immunofluorescence using a monoclonal antibody to LPS.

DNA extraction

M. pneumoniae and *Chlamydia* were centrifuged at 20,000 g 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 for 2h at 56°C. DNA was extracted using phenol: chloroform, and was then precipitated with ethanol. DNA was finally suspended in 50 µL TE buffer.

PCR amplification and electrophoresis

Mycoplasma primer pairs targeting the *tuf* gene reported by Luneberg[6] were selected. Chlamydia primer pairs were designed by computer analysis of the MOMP gene. PCR reaction mixtures were prepared in a total volume of 50 µL containing 0.2 mM of each dNTP, 0.3 µM of each primer (Table I), 2.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase for the single PCRs and 1 U for the Duplex PCRs and PCR buffer (10 mM tris HCl pH 8.3, 50 mM KCl). After amplification (1.5 minutes at 92°C, 1 minute at 60°C, 1 minute at 72°C, repeated 40 times), 10 µL of the 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 was stained with ethidium bromide.

Quantitation methods

The numbers of chlamydial organisms were estimated by counting the number of inclusion forming units in infected McCoy cells, stained by indirect immunofluorescence using a *C. pneumoniae*-specific mAb 'A3'.[14] Mycoplasmal DNA was quantified by spectrophotometry at 260 nm and by an ethidium bromide fluorescent quantitation method, using different concentrations of an E3 fragment of chlamydiaphage DNA.[13] After electrophoresis, the concentration of mycoplasma products were then estimated by visual comparison with the intensity of fluorescence emitted by the chlamydiophage DNA dilutions.

Hybridization

To detect the amplified products by hybridization, a mycoplasma probe was prepared by cloning a 321 bp fragment of *tuf* production in the pUC18 plasmid using *E. coli* (JM 105) as a plasmid vector.[4] Random labelling method was used to label the selected fragment.[4] Chlamydial probes were prepared and labelled by PCR DIG labelling mix method (Boehringer Mannheim).

RESULTS

Chlamydia PCR

By careful analysis of the *omp1* DNA sequence, it was

Table I. Primers used in this study and the length of product bands.

Primer pair		Primer sequences	Primer size (bp)	Product size
Chlamydia set 1:	CpnA	5' CTCCTTACAAGCCTTGCCTGAGTTT 3'	25	C. pneumoniae 336 bp
	CpnB	5' GCGATCCCAAATGTTTAAGGC 3'	21	C. psittaci 351 bp
Chlamydia set 2:	CpnA	5' CTCCTTACAAGCCTTGCCTGAGTTT 3'	25	C. pneumoniae 545 bp
	Cpn B_2	5' CATTCCCATAAGGCTCCACG 3'	20	C. psittaci 572 bp
Mycoplasma tuf:	Mpn-38	5' TACTCGTTACGACCAAATCGATAAG 3'	25	950 bp
	Mpn-39	5' GTTCAACTGTAATCGAGGTATTG 3'	23	
MP5	MP5-a	5' GAAAAGAAGCTTATGGTACAGGTTGG 3'	26	152 bp
	MP5-b	5' GTGACCATCCTTGTTGTAAGG 3'	24	

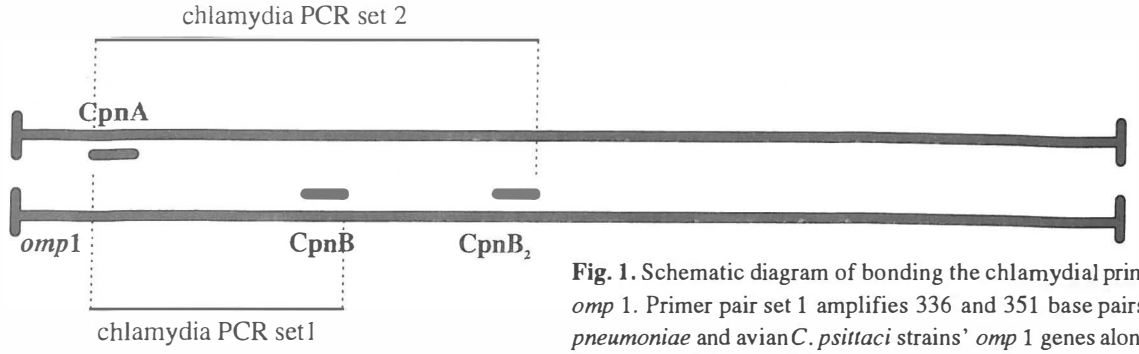

Fig. 1. Schematic diagram of bonding the chlamydial primers to *omp* 1. Primer pair set 1 amplifies 336 and 351 base pairs of *C. pneumoniae* and avian *C. psittaci* strains' *omp* 1 genes along with the length of the amplification target in *C. pneumoniae* and *C. psittaci* which primers of set 2 will anneal to, that is 545 and 572 base pairs, respectively.

possible to design a primer pair that would potentially bind to all *C. pneumoniae* types. It could also bind to avian *C. psittaci* giving PCR products with a slightly different length than *C. pneumoniae* products (Table I, Fig. 1). In practice the primers bound to the two most divergent *C. pneumoniae* types, IOL-207 and N16 giving a PCR product with the predicted length. Avian *C. psittaci* type A and E, which are the most common isolated types, gave a PCR product which was slightly longer than that found with *C. pneumoniae*. Avian type C. produced a PCR product (339) that closely resembled a *C. pneumoniae* product.

After optimization the chlamydial PCR proved to be very sensitive and could detect 10 organisms. The chlamydia PCR was also very specific, as it did not react with a range of micro-organisms or with human DNA extracted from embryo lung fibroblasts. Results also showed that C. trachomatis strain E/T181 can be detected at a very low level of 10^5 copies. Cross reactions observed with PCR set 1 do not seem to be important, because *C. trachomatis* is mainly encountered as a causative agent of oculo-genital infections.

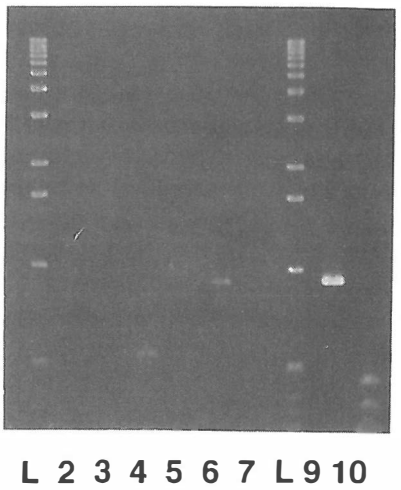

Fig. 2. Left: Titration of *C. pneumoniae*, *C. psittaci* and *M. pneumoniae*. Right:, Mycoplasma *tuf* product and selected fragment for preparation of probe.
Lanes 2,4, and 6 present 100 copies and lanes 3,5, and 7 show 10 copies of *C. pneumoniae*, *C. psittaci* and *M. pneumoniae*, respectively. Lane 9 presents mycoplasma *tuf* product, and lane 10 shows tuf fragments after digestion with restriction enzyme *Alu* 1.

Duplex PCR

Comparison of four mycoplasma PCRs showed that *tuf* PCR, reported by Luneberg,[6] was the most sensitive, detecting 10 organisms and was also quite specific among the micro-organisms tested.[4,5] Therefore, this primer pair was selected to combine with the chlamydia PCR. Optimization experiments showed that chlamydial primer 'CpnB' had an inhibitory role for the amplification of mycoplasma DNA. Although its inhibitory role was reduced at lower concentrations and the mycoplasma products showed better intensity of product bands, it still caused a 100-fold reduction of mycoplasma amplification at 0.1 μM concentration. Glycerol or dimethyl sulfoxide did not effect the sensitivity. In the next experiment, MP5 PCR was chosen for future experiments. The sensitivity of the MP5 PCR also dropped two log units (100 times) even when the concentration of chlamydia primers was reduced to 0.1 μM. The sensitivity of the chlamydia PCR remained unaffected.

A new chlamydia primer '$CpnB_2$' was designed to

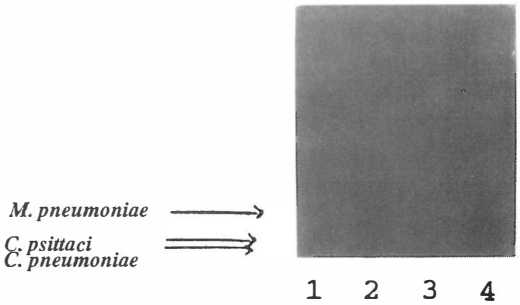

Fig. 3. Analysis of the PCR product with hybridization. The DNA of the three species were diluted ten-fold, and the last dilution containing 1-10 chromosomal DNAs was used for PCR amplification. All three bands in lanes 1 to 4 were detected after hybridization using specific probes, while no product bands were observed after gel electrophoresis stained with ethidium bromide.

replace primer CpnB by amplifying a longer product length (Table I). It has been reported that chlamydia PCR using primer $CpnB_2$ and CpnA could specifically amplify *C. pneumoniae* and *C. psittaci* with differentiable product lengths, except for avian *C. psittaci* type C. But types A and E are the types most usually encountered and type C is very rare and participates in infection only infrequently.[12,15] These two species can easily be distinguished by agarose gel electrophoresis, but using PAGE and positive control provides enough ensurance of the accuracy of the results.[3] It has also been reported that this new chlamydial PCR set is quite specific among micro-organisms encountered in respiratory tract infections.[3]

In the third experiment of our duplex PCR, this new chlamydia PCR was combined with the mycoplasma PCR targeting the *tuf* gene. Results showed that by using 0.3 μM of each primer, PCR sensitivty of both mycoplasma and chlamydia again were reduced. To decrease the inhibition, we reduced the concentration of all primers to 0.2 μM. Titration of DNA from these micro-organisms showed the same sensitivity (Fig. 2), although the intensity of the product bands was still slightly less in comparison with the single PCRs.

Evaluation of the duplex PCR with clinical samples

Mock infected clinical samples were used to evaluate the test and to compare the sensitivity of the duplex PCR with both single PCRs. Each negative sample was infected with 10 copies of DNA from all three species. Analysis of the samples proved that all specimens were positive as expected. Comparison of the product bands with positive controls proved only a slight reduction in intensity for bands 1,3, and 4 of *M. pneumoniae*, *C. pneumoniae* and *C. psittaci* respectively.

Hybridization

All probes were prepared to detect the duplex products by the hybridization. It has been reported that the mycoplasma probe prepared for *tuf* PCR is quite specific.[4] Specificity testing proved that the chlamydia probe (*C. pneumoniae* and *C. psittaci*) was specific among all microorganisms tested but both species would be detected by either of chlamydia probes. Therefore, the *C. pneumoniae* probe and the *M. pneumoniae* probe were mixed to detect the amplified products by the hybridization. Using southern blot rather than dot blot enabled us to differentiate *C. pneumoniae* and *C. psittaci* products from each other as well as differentiation from *M. pneumoniae* products.

Titration of both *C. pneumoniae* and *M. pneumoniae* DNA showed a sensitivity one log higher than analysis with agarose gel electrophoresis (Fig. 3). These experiments were repeated several times and the same results were obtained each time.

DISCUSSION

Duplex PCR is the way forward in diagnostic laboratories, but it has some problems. A number of points need to be discussed, such as the role of primer concentration and the choice of primers for the test for optimization of the duplex PCR.

Primer concentration

Optimization experiments on chlamydia and mycoplasma PCRs showed that the sensitivity of the single PCR is not very dependent on the concentration of primers. It remains similar for a wide range of concentrations from 0.1 to 0.5 μM or more. Either side of this range the sensitivity decreases. The preferred concentration is therefore that which gives the most clear product band. In contrast, experiments on duplex or triplex PCRs indicated that primer concentration is an important factor and that this concentration should be as low as possible to ensure the highest sensitivity. Thus, for these later PCRs, primers must be used at an accurate concentration.

Choosing the primers for the test

The importance of the role of primer concentration suggests that primer-primer or unwanted primer-template interaction is an influential factor. To develop a duplex PCR in this study, two main steps were considered:

1) Choice of a primer pair requiring similar amplification conditions such as annealing temperature and $MgCl_2$ concentration and which yield products of different size from each other.

2) Optimization of the primers, Taq polymerase concentrations and other possible affecting factors as were discussed before by the authors.[5]

Computer analysis played an important role in ensuring the accuracy of the annealing temperature used in the study and for scanning the primers against other microbial genes for possible homologies which might cause problems in clinical samples.

In spite of these precautions the results of the first duplex PCR were not satisfactory since there was a reduction in the sensitivity of the PCR. It would be very beneficial if a more developed computer program were available. This should facilitate evaluation, scanning, and design of suitable primers which would yield more reliable results. Such a goal is dependent upon having more information and gene sequences on the database.

Sensitivity of the PCR hybridization method

Chlamydia and mycoplasma DNA were titrated to determine the detection limits of the developed test.

Sensitivity results of PCR-hybridization were compared with the sensitivity results of gel electrophoresis and indicated a one log increase in sensitivity for both chlamydia

and mycoplasma. The lowest amount of amplicon detectable by agarose gel electrophoresis is approximately 1-10 ng[11] which corresponds to 1 to 10 copies for both mycoplasma and chlamydia DNA. In contrast, the lowest amount of DNA detectable by the hybridization method is 30 fg (Boehringer Mannheim's detection kit manual) under the most favourable conditions and more probably something between 30-300 fg. Thus hybridization has the potential to increase the sensitivity by four log dilutions.

The following conclusions may be drawn from this comparison:

1) Any possible inhibition due to the sample materials, unwanted primer interaction, etc. will obviously not affect the sensitivity of the test since very low amounts of the product can be detected by hybridization.

2) The test will be able to detect one DNA, a level which would be impossible by gel electrophoresis stained with ethidium bromide.

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