CHAPTER IV

RESULTS

1. Selection of primers

The selected primers were corresponded to nucleotides 3,133 to 3,151 and 3,580 to 3,598 (Fig. 1) and were named MP-1 (5'-GTG AAT GGG TTG TTG AAT C-3') and MP-2 (5'-TTG TTG CGG TGG TGG TAG A-3'), respectively.

The nested primers were corresponded to nucleotides 3,202 to 3,221 and 3,524 to 3,543 (Fig. 2) and were named MP-1S (5,-TCC AAC ACG ACC AGT TCA CC-3,) and MP-2S (5,-CCC CTT CAA ATC CCA CAC AC-3').

2. Isolation of the DNA from *M. pneumoniae* Mac strain

M. pneumoniae was grown in modified Hayflick medium. The DNA was isolated by the method described in chapter III. The optical density (OD) at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 ug/ml. The ratio between the reading at 260nm and 280 nm (OD260/OD280) provides an estimate for the purity of nucleic acid. Pure preparation of DNA has OD260/OD280 of 1.8 to 2.0 (87). The concentration of the DNA extracted by this method was 912 ng/ul and the OD260/OD280 of the prepared DNA was 1.9.

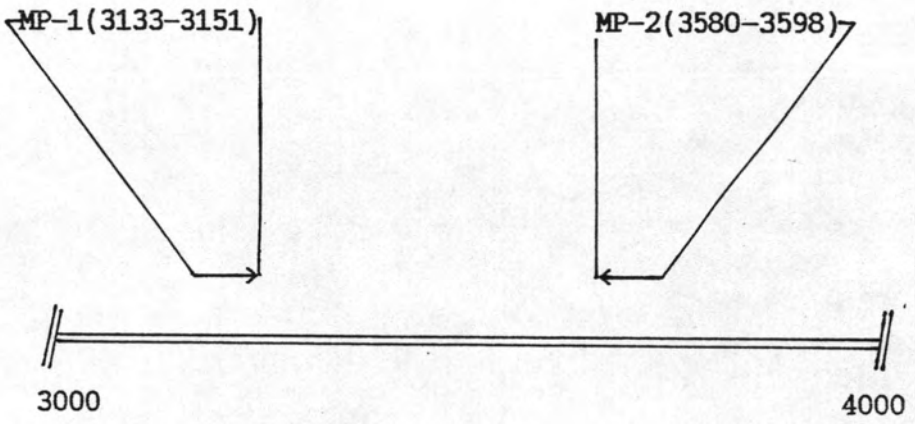

Fig.1 Oligonucleotide primers on P1 gene of *Mycoplasma pneumoniae* used for the detection of *Mycoplasma pneumoniae* by PCR.

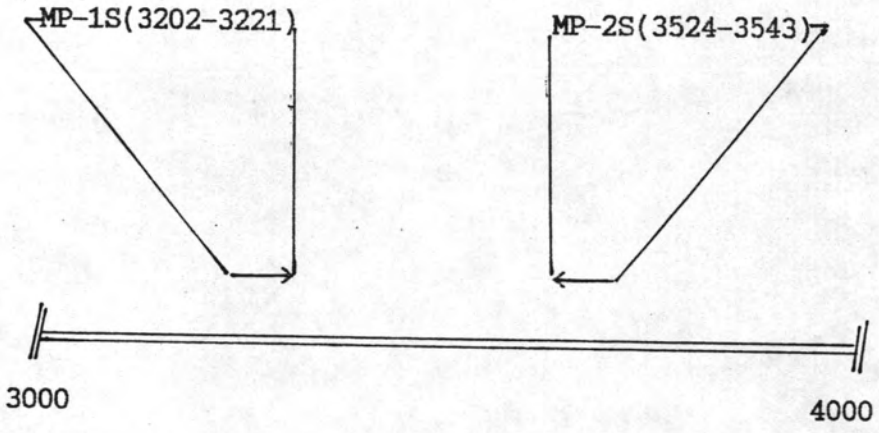

Fig.2 Nested oligonucleotide primers on P1 gene of *M. pneumoniae* used for preparing the probe and nested PCR.

3. Amplification of *M. pneumoniae* DNA by PCR

3.1 Setting up the PCR reaction mixture

In the initial setting of the PCR reaction mixture for amplification with primers MP-1 and MP-2, the optimal concentration of $MgCl_2$ was determined by varying the concentration of $MgCl_2$ at 0.5, 1.0, 1.5, 2.0, and 2.5 mM in the standard PCR reaction as described in chapter III with 0.5 uM of each primer in 50 ul volume. The cycling of three-step reaction was 35 cycles of 1 min denaturation at 94°C, 1 min of annealing at 56°C, and 1 min of primer extension at 72°C and then extended at 72°C for 10 min in order to complete the extension. It was found that the optimal concentration of $MgCl_2$ was 1.5 mM (Fig. 3).

After the optimal $MgCl_2$ concentration was known, the optimal concentration of *Taq* polymerase was determined by varying the amount of *Taq* polymerase (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 unit per 50 ul volume of the reaction mixture)in the PCR reaction mixture. The reaction was run as described above. The lowest amount of *Taq* polymerase which gave a satisfactory result was 1.25 unit per 50 ul volume of reaction mixture (Fig. 4).

3.2 Determination of the optimal PCR cycles

To determine the least number of PCR cycles that gave a satisfactory product, the cycling was repeated approximately 25 cycles,30 cycles, 35 cycles, 40 cycles and 45 cycles, respectively.

Using the PCR standard reaction with 1.5 mM $MgCl_2$ and 1.25 unit *Taq* polymerase. The amplification products were analysed on 1.5 % Tris-acetate agarose gel electrophoresis. The optimal number of PCR cycles for this experiment was 35 cycles (Fig. 5). A 466-bp amplified product was seen on ethidium bromide-stained agarose gel.

4. Determination of the sensitivity of PCR

In order to determine the PCR sensitivity for detection of *M. pneumoniae*, serial dilutions of template DNA were applied to PCR with further analysis of amplification products by electrophoresis and dot blot hybridization. First PCR with MP-1 and MP-2 primers generated a 466-bp fragment was prepared. By runing with electrophoresis in 1.5% agaros gel, the product from the lowest template, 10 fg were detected (Fig.6). The PCR sensitivity was increased to 1 fg when using the dot blot hybridization with 342-bp fluorescein-labelled probe (Fig.7). After using 10 ul of each of first PCR product as a template for nested PCR, it was found that sensitivity increased. The product from only 1 fg of template was detected (Fig.8).

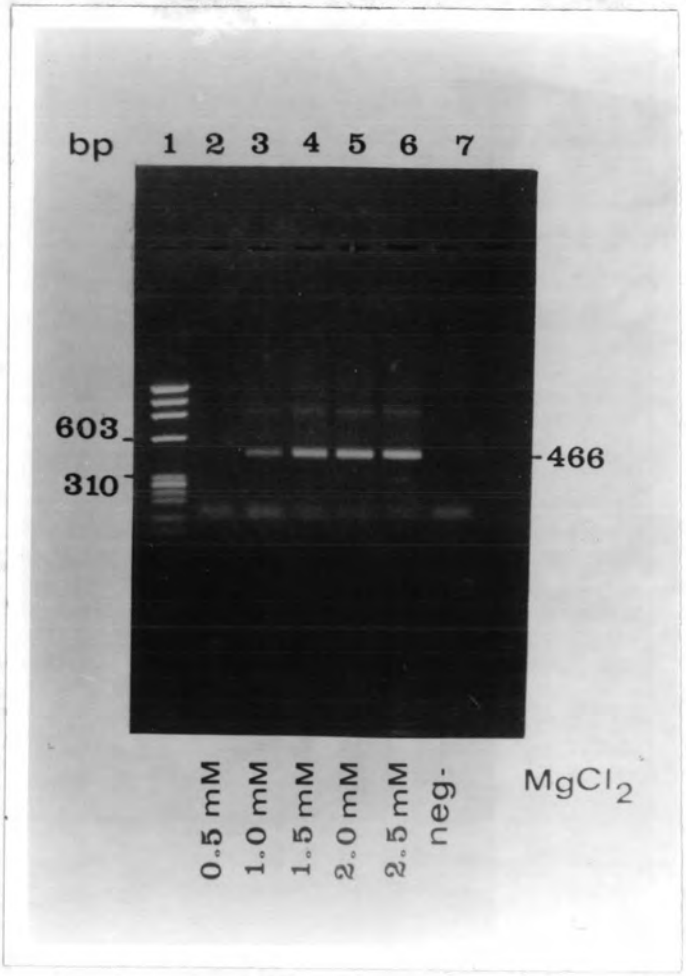

Fig. 3 Effect of $MgCl_2$ concentration on yield of the amplified product.

Amplification was performed in standard reaction mixture with 0.5 uM of each of primer (MP-1 and MP-2) and varied concentration of $MgCl_2$. Lanes. 2-6, 0.5, 1.0, 1.5, 2.0 mM, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker; lane 6, negative control. Amplified products were detected by agarose gel electrophoresis.

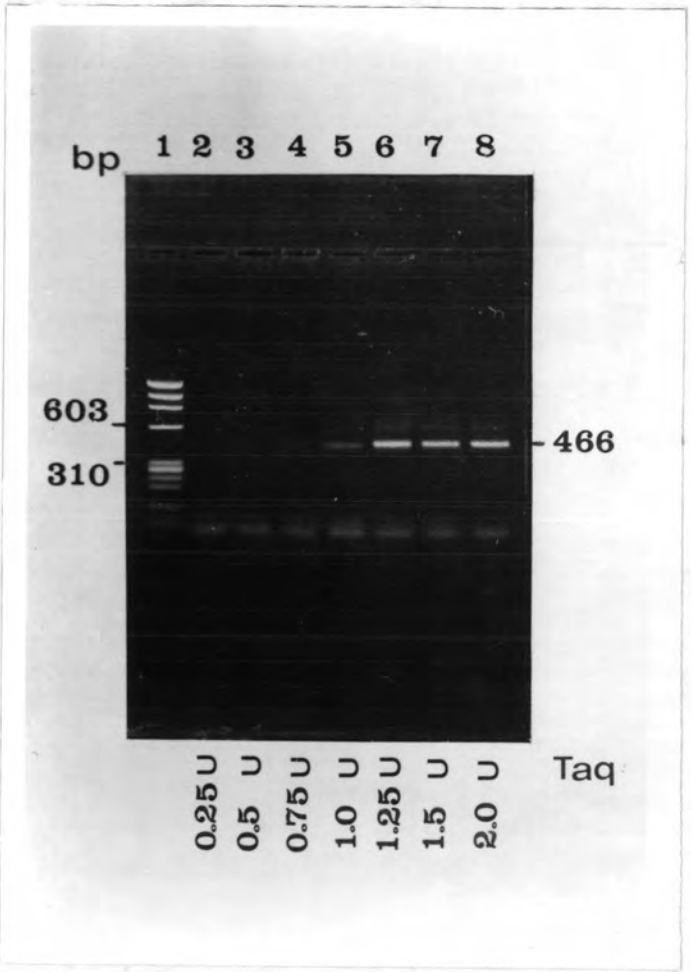

Fig. 4 Effect of *Taq* polymerase on yield of the amplified product. Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2) and varied concentration of *Taq* polymerase. Lanes. 2-8, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 U, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker. Amplified products were detected by agarose gel electrophoresis.

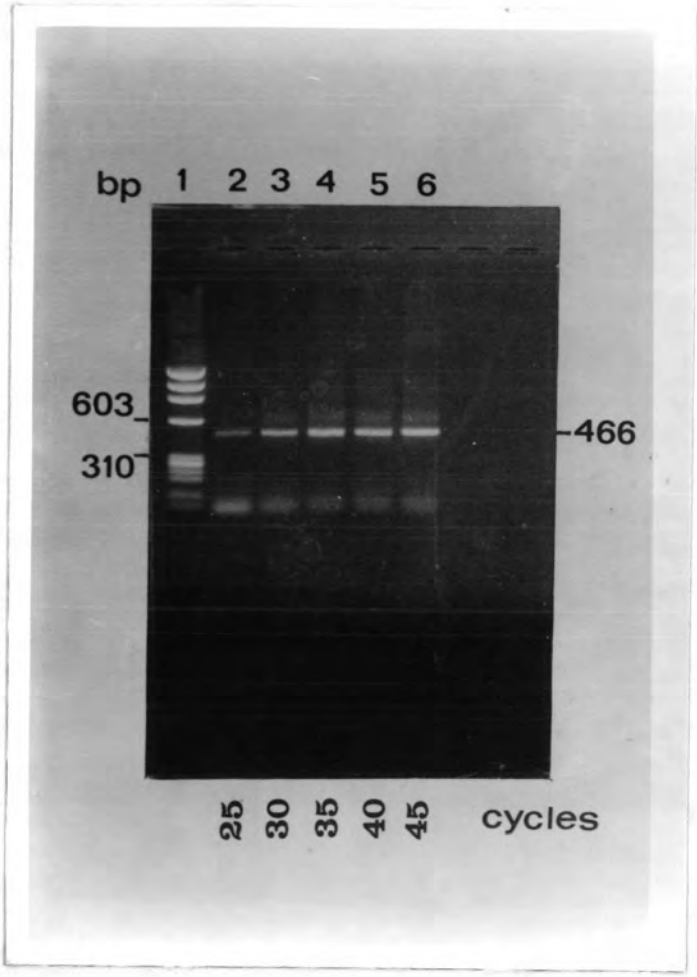

Fig. 5 Effect of the number of PCR cycles on yield of the amplified product.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer(MP-1 and MP-2) and varied the number of PCR cycles. Lanes. 2-6, 25, 30, 35, 40, 45 cycles, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker. Amplified products were detected by agarose gel electrophoresis.

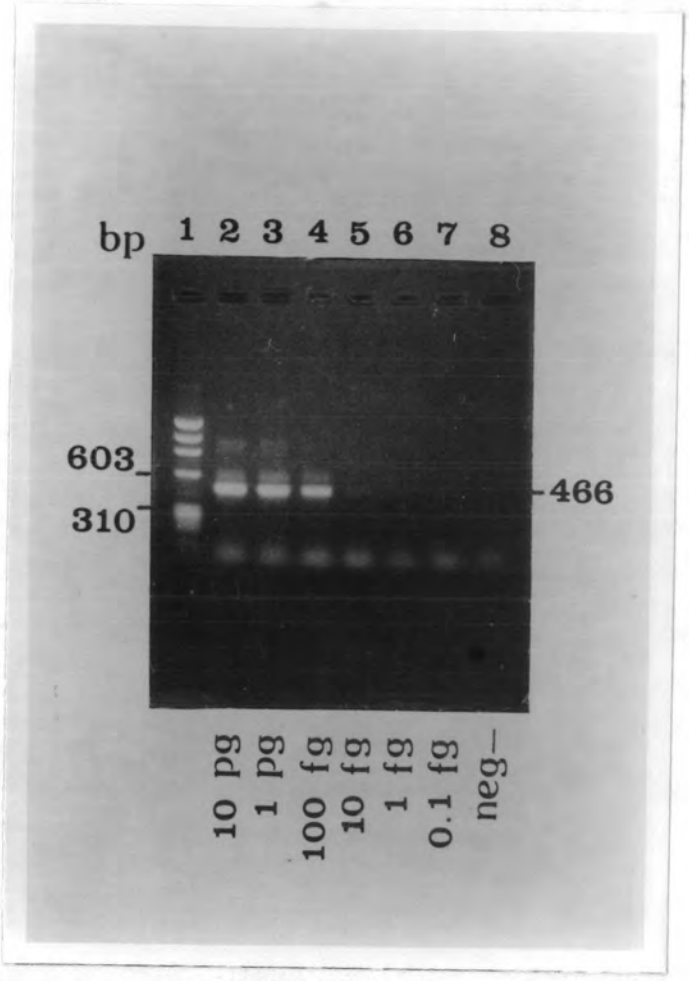

Fig. 6 Determination of PCR sensitivity analysed by agarose gel electrophoresis.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer(MP-1 and MP-2) and varied the amount of *M. pneumoniae* DNA. Lanes 2-7, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, respectively; lane 1, HaeIII-digested OX174 DNA as molecular weight marker; lane 8, negative control. Amplified products were detected by agarose gel electrophoresis.

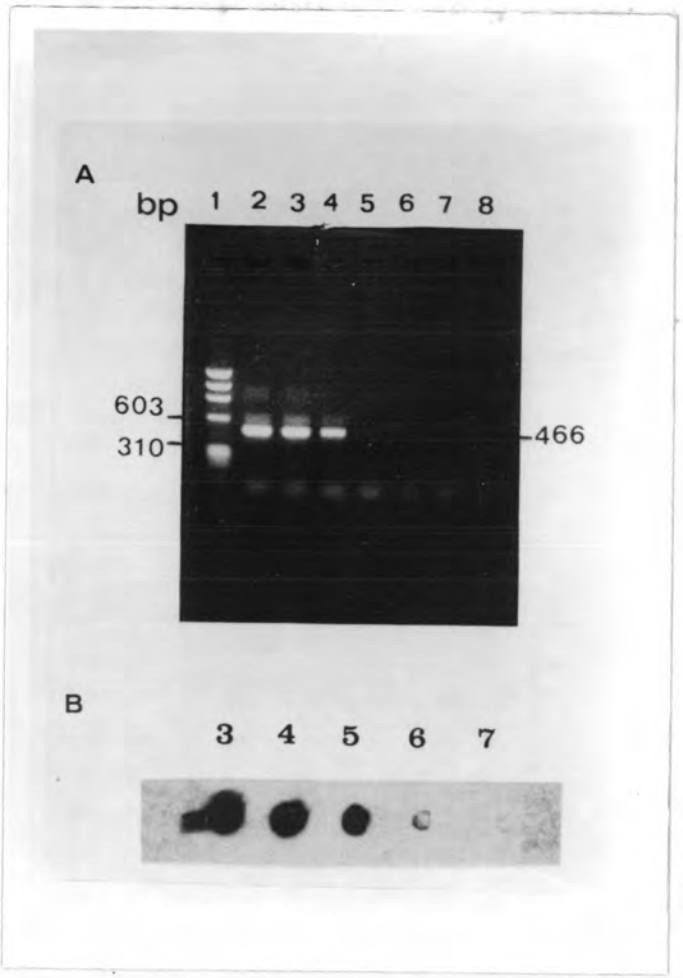

Fig. 7 Determination of PCR sensitivity analysed by agarose gel electrophoresis and dot blot hybridization.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer(MP-1 and MP-2) and varied the amount of *M. pneumoniae* DNA. Lanes 2-7, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker; lane 8, negative control.

(A). A total of 10 ul of amplified products was analysed by agarose gel electrophoresis.

(B). A 1 ul of amplified products was hybridized with specific fluorescein-labelled probe. Dot blot analysis of the fragments in panel A from lane 3 - lane 7.

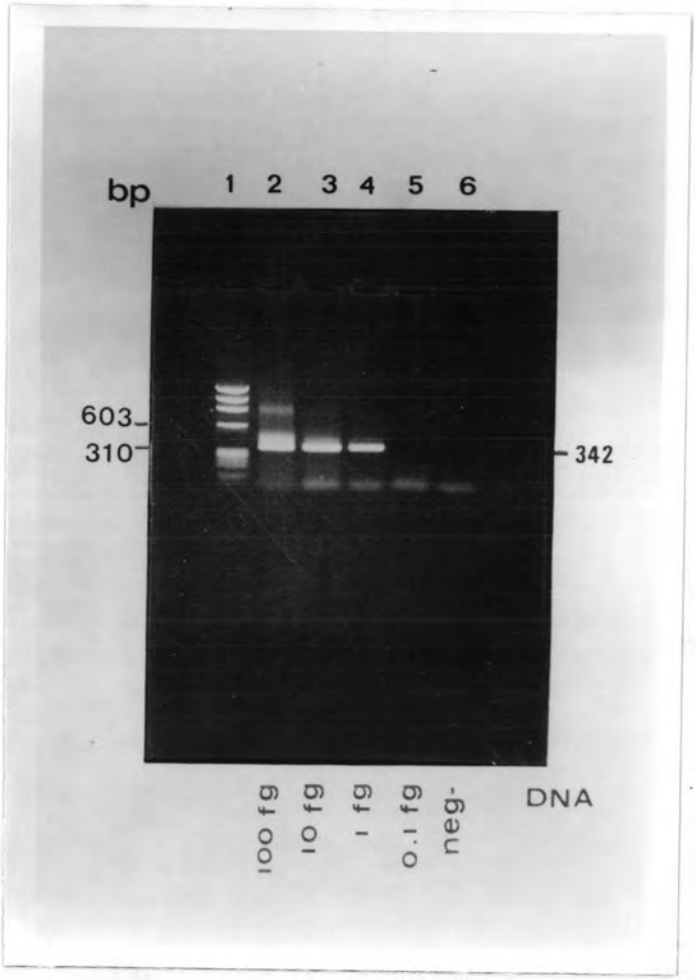

Fig. 8 Determination of PCR sensitivity, using nested PCR and analysed by agarose gel electrophoresis.

Amplification was performed in standard reaction mixture with 0.5 uM of each nested primers (MP-1S and MP-2S) and using 10 ul of first PCR products as a template. Lanes 2-5, 100 fg, 10 fg, 1 fg, and 0.1 fg, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker; lane 6, negative control. Amplified products were detected by agarose gel electrophoresis.

5. Determination of the specificity of PCR

The 466-bp segment was successfully amplified from pure cultures of all five of the American Type Culture Collection strains of *M. pneumoniae* but not from other mycoplasma tested (Fig. 9). These strains represent the most likely mycoplasma found as normal flora in human respiratory specimens.

Negative results were obtained from PCRs with chromosomals DNAs from a variety of different bacterial genera (Fig. 10) and also from that with human leukocytes DNA (Fig. 11).

6. Optimization of the dUTP concentration

In order to prevent amplicon carryover, the dUTP was used instead of dTTp and 0.1 unit of UNG was added in the reaction mixture to cleave the amplicons into small fragments prior to PCR. The initial denaturation step in the first PCR cycle was extended to 10 min and the amplified products were soaked at 72°C (at this temperature the UNG was inactivated). Because of the amplification may be somewhat less efficient when incorporation of dUTP in place of dTTP resulting in slightly decreased yields of products. The concentration of dUTP was optimized for satisfactory results by varying the dUTP concentration. The PCR was run 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. It was showed that the optimal concentration of dUTP used in the reaction mixture was 2.0 mM (Fig.12).

Because of the high concentration of dUTP was used in the PCR, the $MgCl_2$ concentration was adjusted in order to receive the highest yield by varying $MgCl_2$ concentration. The result shows that the optimal concentration of $MgCl_2$ was 3.0 mM (Fig.13).

7. Determination of PCR sensitivity by using dUTP

The sensitivity of PCR after setting up the condition by using the standard PCR reaction with 2.0 mM dUTP, 3.0 mM $MgCl_2$ was showed in Fig. 14A. The sensitivity was not affected by the incorporation of dUTP instead of dTTP, even though the intensity of the *M. pneumoniae*-specific band was slightly weaker. The first dU-PCR products was observed at 10 fg as same as dTTP PCR. Nested PCR was also run by nested primers (MP-1S, MP-2S), specific band was also able to detect at 1 fg (Fig. 15) as well as dot blot hybridization (Fig. 14B).

To demonstrate the gravity of amplicon contamination we tested 10 ul of 1,000-fold serial dilution of a 15 ng/ul 466-bp amplicon solution in PCR (Fig.16). Fig. 16A shows that 10 ul of a 15 ng/ul amplicon solution can still be amplified in PCR, even following a 10^6-fold dilution. This solution contains 150 fg of amplicon (~2,500 amplicons).

Panels B and C of Fig. 16 show that 0.1 U of UNG was able to break down 150 pg of 466-bp amplicons. The use of UNG did not

affect the sensitivity of PCR, as shown in Fig. 16A, and 16B where the amplicon band in lanes 9,10,11 are of equal intensity. Since PCR was with 1 pg, 100 fg, 10 fg and 1 fg of *M. pneumoniae* DNA in the absence and presence of UNG. Both PCRs had the same sensitivity of detection 100 fg and 10 fg of *M. pneumoniae* DNA by gel electrophoresis and dot blot hybridization, respectively.

8. Evaluation of the PCR in simulated samples

To determine the detection limit of the PCR in simulated samples, PCRs were performed with 10 ul of the extracted sample. Negative results were found in all specimens (Fig.17). To detect the presence of inhibitors, negative samples were tested again in the presence of 10 pg of *M. pneumoniae* DNA. In all cases the spiked DNA can be detected (Fig. 18) however the intensity of the band was not equal indicating that PCR was not fully inhibited and each specimen contained different amount of undefined inhibitors.

In addition,determination of the sensitivity of PCR in pooled specimens showed that the PCR can detect at least 100 fg of *M. pneumoniae* DNA analysed by gel electrophoresis and dot blot hybridization (Fig. 19).

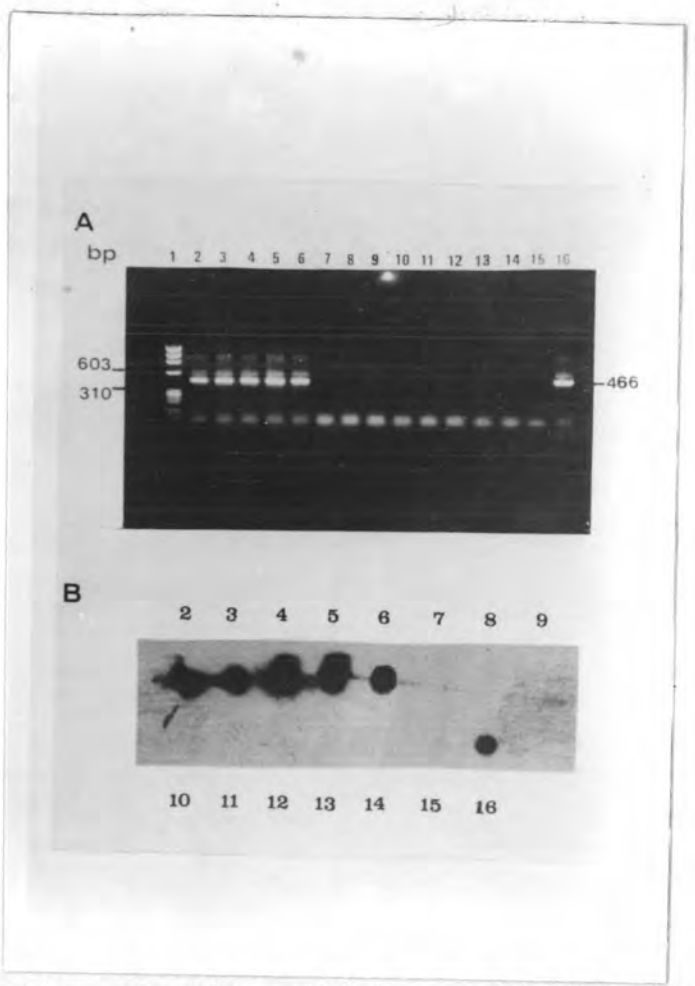

Fig . 9 Determination of PCR specificity tested with *Mycoplasma* species and analysed by gel electrophoresis and dot blot hybridization.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2).

(A). Electrophoretic analysis of PCR fragments.

(B). Hybridization analysis of fragments in panel A.

Lane.1	HaeIII-digested ΦX174 DNA	Lane.10	*M. baccale* ATCC 23636
Lane.2	*M. pneumoniae* ATCC 15293	Lane.11	*M. fermentans* ATCC 15474
Lane.3	*M. pneumoniae* ATCC 29085	Lane.12	*M. salivarium* ATCC 14277
Lane.4	*M. pneumoniae* ATCC 15531	Lane.13	*M. fecium* ATCC 25293
Lane.5	*M. pneumoniae* ATCC 15372	Lane.14	*Acholeplasma laidlawii* ATCC 29804
Lane.6	*M. pneumoniae* ATCC 29342		
Lane.7	*M. hominis* ATCC 27545	Lane.15	*Ureaplasma urealyticum* ATCC 27613
Lane.8	*M. orale* ATCC 15544		
Lane.9	*M. genitalium* ATCC 33530	Lane.16	*M. pneumoniae* Mac strain

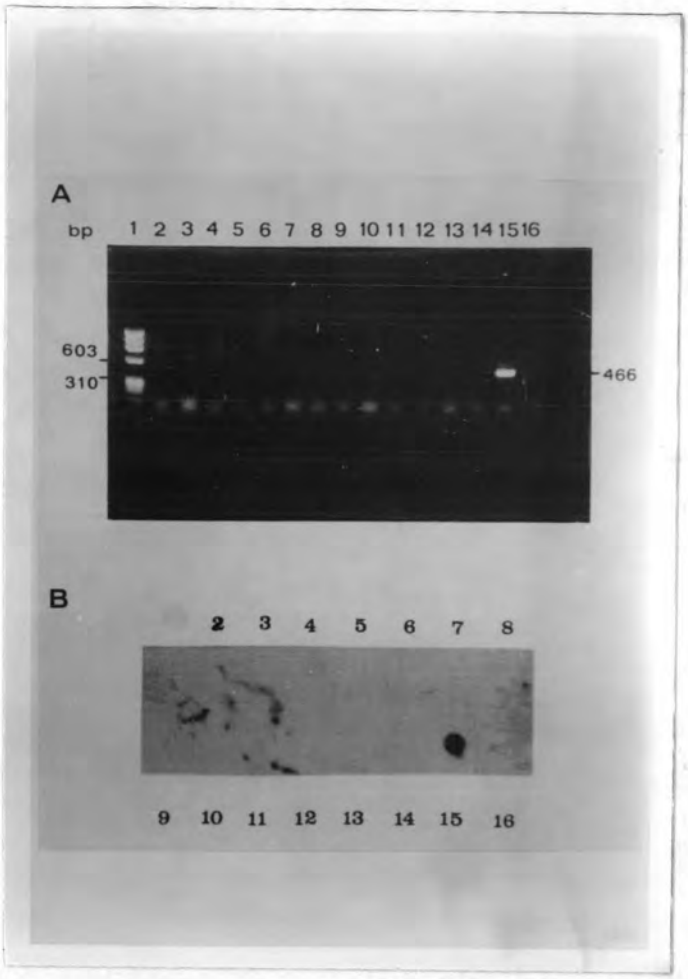

Fig. 10 Determination of PCR specificity tested with bacterial DNAs and analysed by gel electrophoresis and dot blot hybridization.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2).

(A). Electrophoretic analysis of PCR fragments.

(B). Hybridization analysis of fragments in panel A.

Lane.1	HaeIII-digested 0X174 DNA	Lane.9	B-Streptococcus group A
Lane.2	*Klebsiella spp.*	Lane.10	*S. pneumoniae*
Lane.3	*Enterobacter spp.*	Lane.11	α-Streptococcus
Lane.4	*Pseudomonas spp.*	Lane.12	*Neisseria spp.*
Lane.5	*Haemophilus influenzae*	Lane.13	*Staphylococcus aureus*
Lane.6	*Haemophilus parainfluenzae*	Lane.14	*Staphylococcus spp.*
Lane.7	*Acinetobacter spp.*	Lane.15	*M. pneumoniae* Mac strain
Lane.8	*Corynebacterium spp.*	Lane.16	Negative control

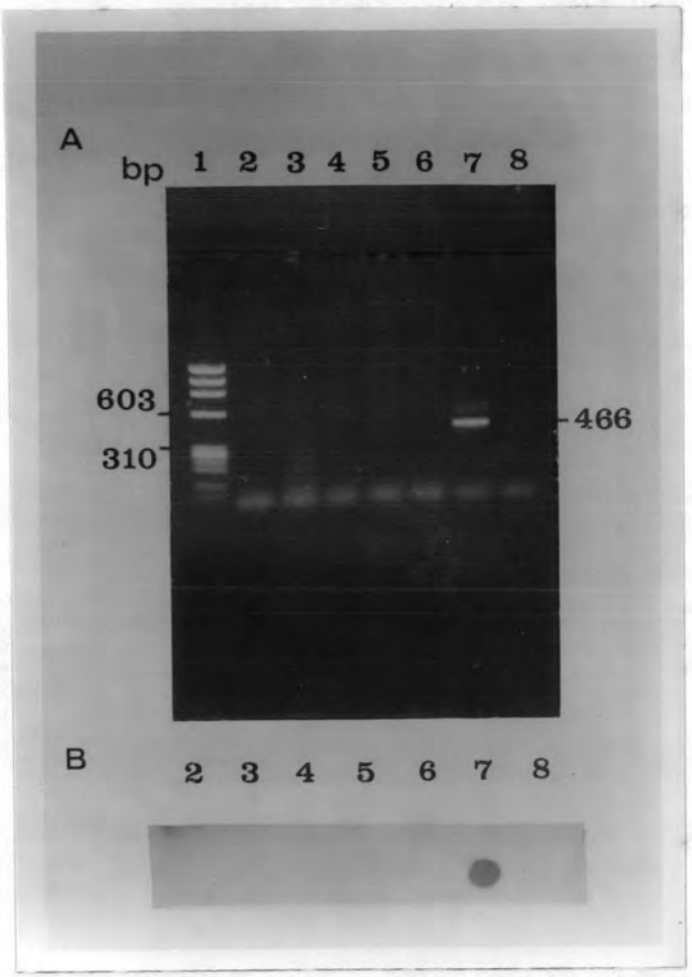

Fig. 11 Determination of PCR specificity tested with human leukocytes DNAs and analysed by gel electrophoresis and dot blot hybridization.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2).

(A). Electrophoretic analysis of PCR fragments.

(B). Hybridization analysis of fragments in panel A.

Lane 1 HaeIII-digested OX174 DNA

Lane 2 to 6 PCR products from 10 ul of extracted samples

Lane 7 Positive control

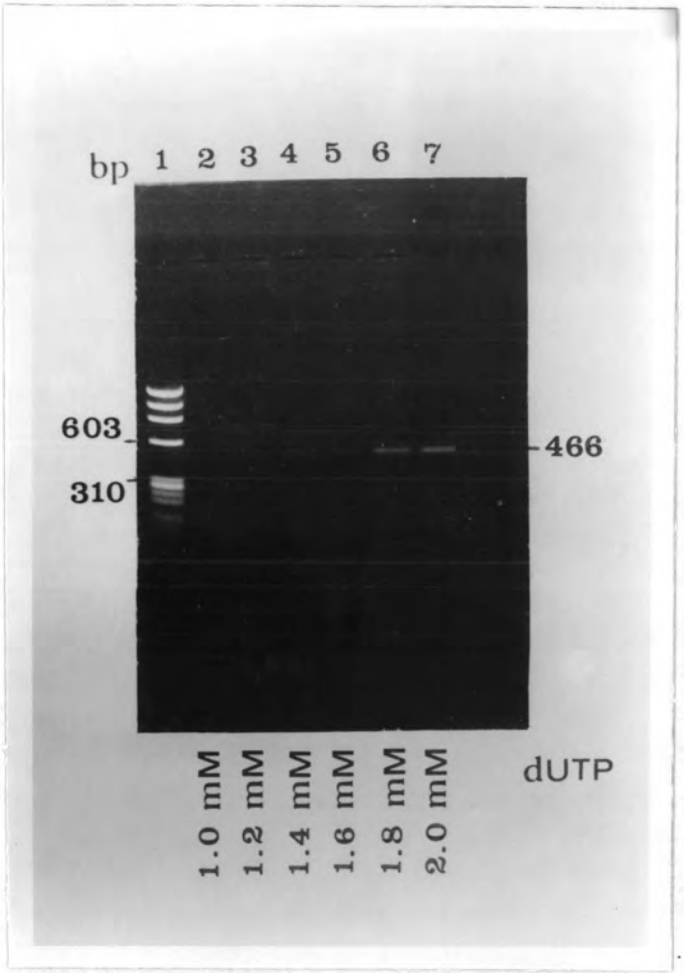

Fig.12 The PCR results of using dUTP instead of dTTP in the PCR reaction.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2), 0.1 unit of UNG in 1XPCR buffer, and varied the concentration of dUTP. Lanes 2-7, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mM, respectively; lane 1, HaeIII-digested OX174 DNA as molecular weight marker. Amplified products were detected by agarose gel electrophoresis.

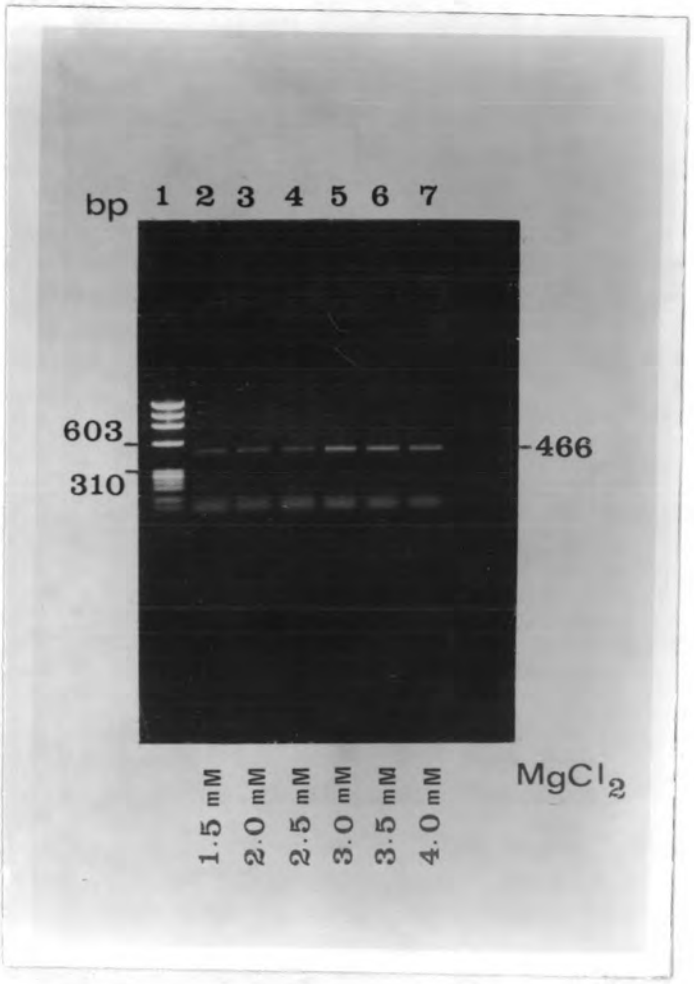

Fig. 13 Effect of $MgCl_2$ concentration on the yield of amplified product when concentration of dNTP was changed.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2), 0.1 unit of UNG in 1XPCR buffer, 2.0 mM of dUTP and varied the concentration of $MgCl_2$. Lanes 2-7, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mM, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker. Amplified products were detected by agarose gel electrophoresis.

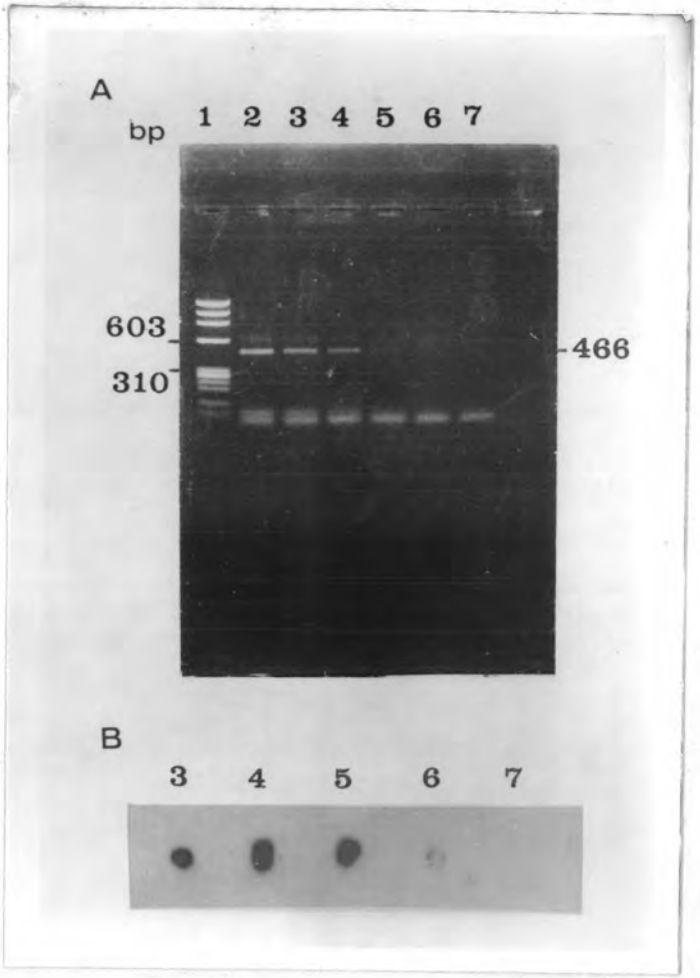

Fig. 14 Determination of dU-PCR sensitivity analysed by gel electrophoresis and dot blot hybridization.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2), 0.1 unit of UNG in 1XPCR buffer, 2.0 mM of dUTP, 3.0 mM of $MgCl_2$, and varied the amount of *M. pneumoniae* DNA. Lanes 2-7, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker.

(A). A total of 10 ul of amplified products was analysed by agarose gel electrophoresis.

(B). A 1 ul of amplified products was hybridized with specific fluorescein-labelled probe. Dot blot analysis of the fragments in panel A from lane 3 - lane 7.

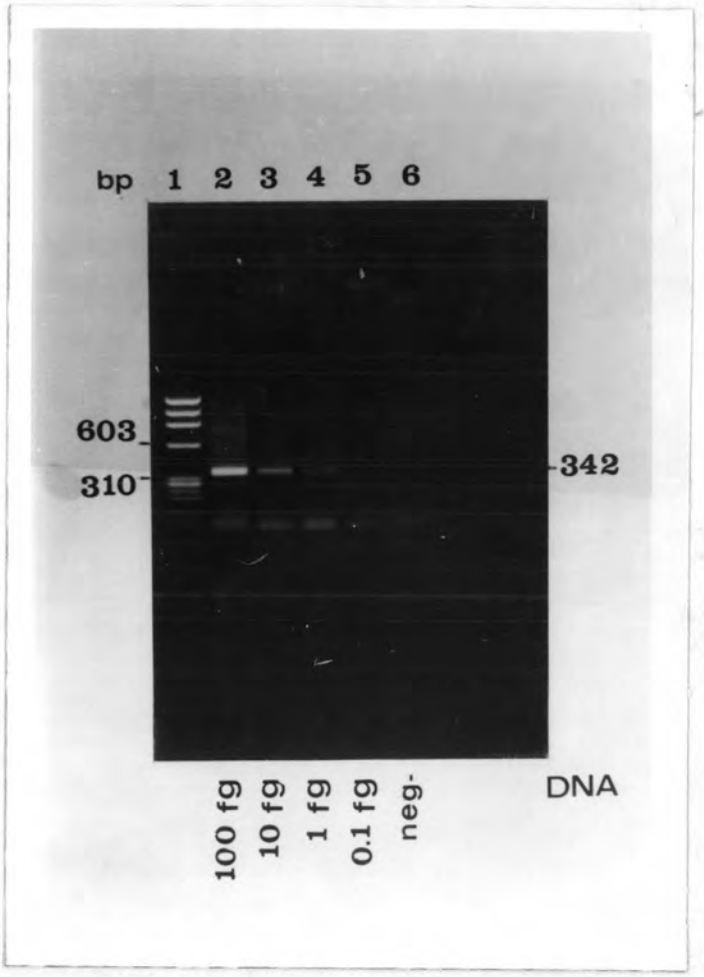

Fig. 15 Determination of PCR sensitivity, using nested PCR and analysed by agarose gel electrophoresis.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2), 0.1 unit of UNG in 1XPCR buffer, 2.0 mM of dUTP, 3.0 mM of $MgCl_2$, and using 10 ul of first PCR products as a template. Lanes 2-5, 100 fg, 10 fg, 1 fg, and 0.1 fg, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker; lane 6, negative control. Amplified products were detected by agarose gel electrophoresis.

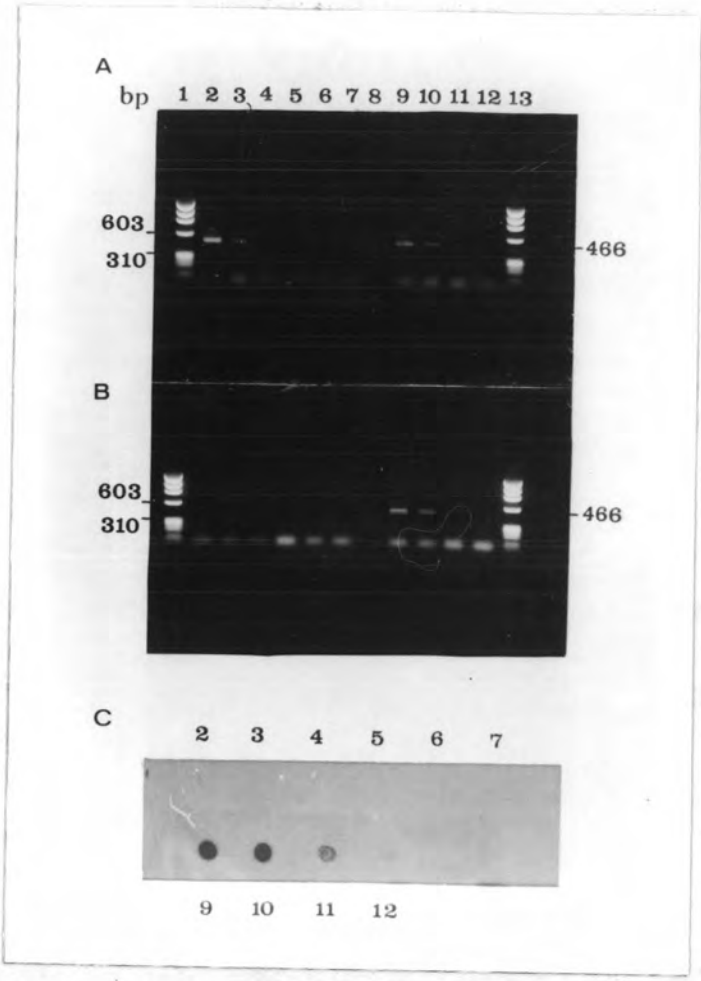

Fig. 16 Determination of UNG ability.

Ability of UNG to break down dUTP-contaminating amplicons. dUTP-contaminating amplicons and *M. pneumoniae* DNA were used as template in PCR in absence (A) and presence (B and C) of 0.1 U of UNG.

Lanes 2 to 6 : 10^3, 10^6, 10^9, 10^{12}, and 10^{15}-times diluted 15 ng/ul of amplicon solution

Lanes 9 to 12 : 1 pg, 100 fg, 10 fg, and 1 fg of *M. pneumoniae* DNA

Lane 7 : Negative control

Lanes 1 and 13: ΦX174/HaeIII fragments

Amplification products were detected by agarose gel electrophoresis (A and B) and by dot blot hybridization (C)

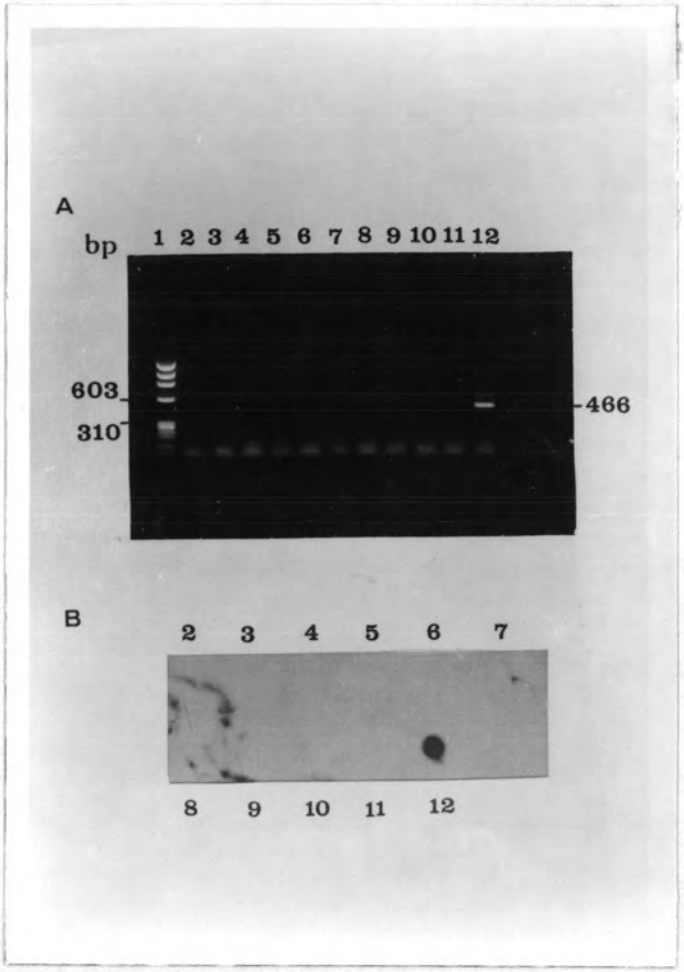

Fig.17 Determination of dU-PCR specificity by testing with simulated samples (10 specimens).

Lane 1 ΦX174/HaeIII fragments

Lane 2 to 11 PCR products from 10 ul treated samples

Lane 12 Positive control

Amplification products were detected by agarose gel electrophoresis (A) and by dot blot hybridization (B).

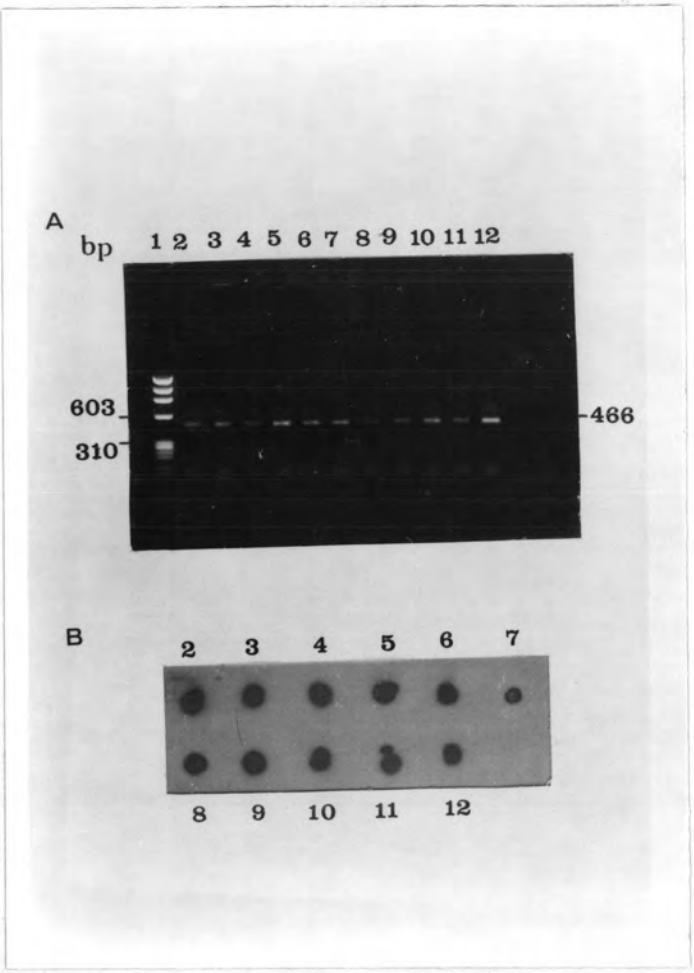

Fig. 18 Determination of dU-PCR specificity by testing with simulated samples (10 specimens) spiked with *M. pneumoniae* DNA.

Lane 1 : OX174/HaeIII fragments

Lane 2 to 11: PCR products from 10 ul treated samples spiked with 10 pg of *M. pneumoniae* DNA

Lane 12 : Positive control

Amplification products were detected by agarose gel electrophoresis (A) and by dot blot hybridization (B).

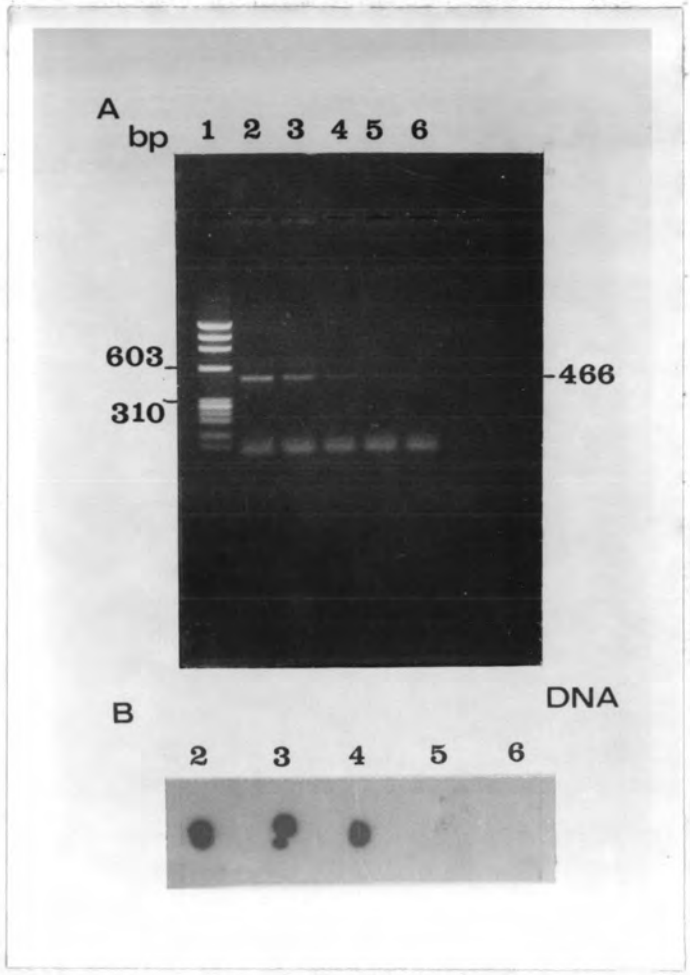

Fig. 19 Determination of dU-PCR sensitivity in pooled simulated specimens.

Amplification was performed in standard reaction mixture with 0.5 uM of each primer (MP-1 and MP-2), 0.1 unit of UNG in 1XPCR buffer, 2.0 mM of dUTP, 3.0 mM of $MgCl_2$, and varied the amount of *M. pneumoniae* DNA. Lanes 2-6, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg, respectively; lane 1, HaeIII-digested ΦX174 DNA as molecular weight marker.