CHAPTER V

RESULTS

1. Determination of the sensitivity of MP-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. First-step PCR with MP-1 and MP-2 primers generated a 466-bp fragment was prepared. By running with electrophoresis in 1.5% agarose gel, the product from the lowest template, 10 fg were detected (Fig 1). After using 5 μl of each of first PCR product as a template for nested PCR, it was found that sensitivity increased. The product (342-bp) from only 1 fg of template was detected (Fig 2).

2. Determination of the sensitivity of 16S rDNA PCR

The sensitivity of the PCR method was also tested by using serial dilutions of *M. pneumoniae* DNA. Under optimal conditions, primers 16S-1 and 16S-2 (first-step PCR, 16S rDNA PCR) detect about 10 fg of DNA, 277-bp product was generated (Fig 3). Sensitivity was increased to 0.1 fg of DNA by 16S rDNA nested PCR with primers 16S-1S and 16S-2S, generated 205-bp product (Fig 4).

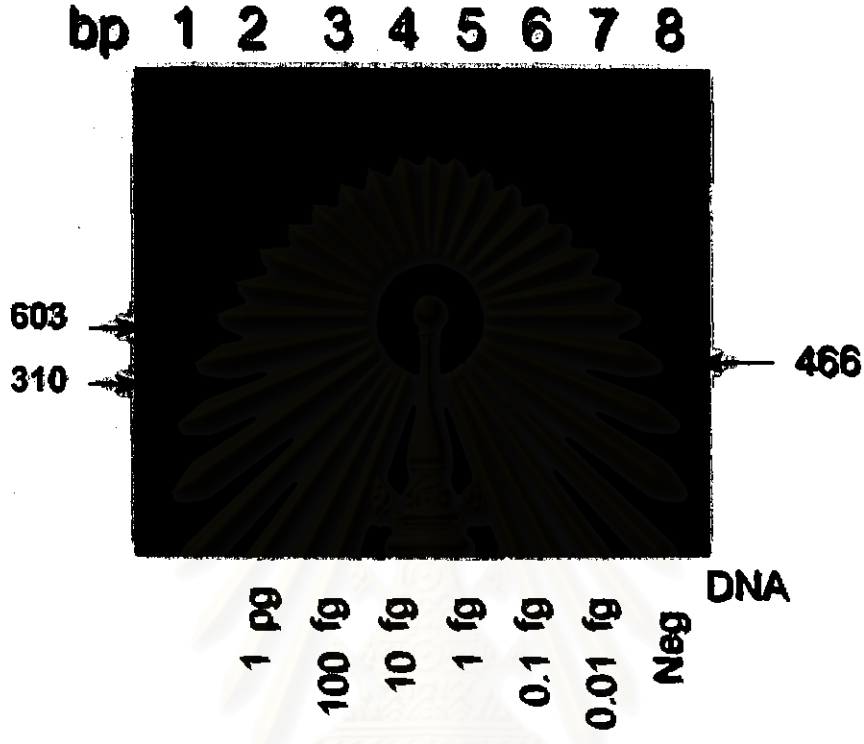

Fig 1 : Determination of MP-PCR sensitivity analysed by agarose gel electrophoresis

Amplication was performed in standard reaction mixture with 0.5 μM of each primer (MP-1 and MP-2) and varied the amount of *M. pneumoniae* DNA. Lanes 2-7, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, 0.01 fg, respectively; lane 1, HaeIII-digested ϕX174 DNA as molecular weight marker; lane 8, negative control. Amplified products were detected by agarose gel electrophoresis.

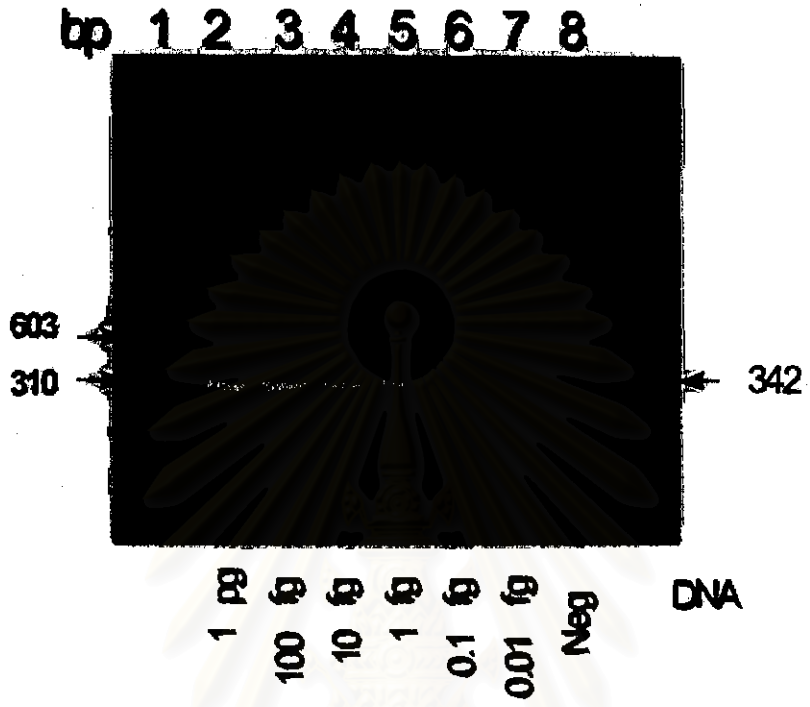

Fig 2 : Determination of MP-nested PCR sensitivity, using and analysed by agarose gel electrophoresis

Amplication was performed in standard reaction mixture with 0.5 μM of each nested primer (MP-1S and MP-2S) and using 5 μl of first PCR products as a template. Lanes 2-7, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, 0.01 fg, respectively; lane 1, HaeIII-digested ϕX174 DNA as molecular weight marker; lane 8, negative control. Amplified products were detected by agarose gel electrophoresis.

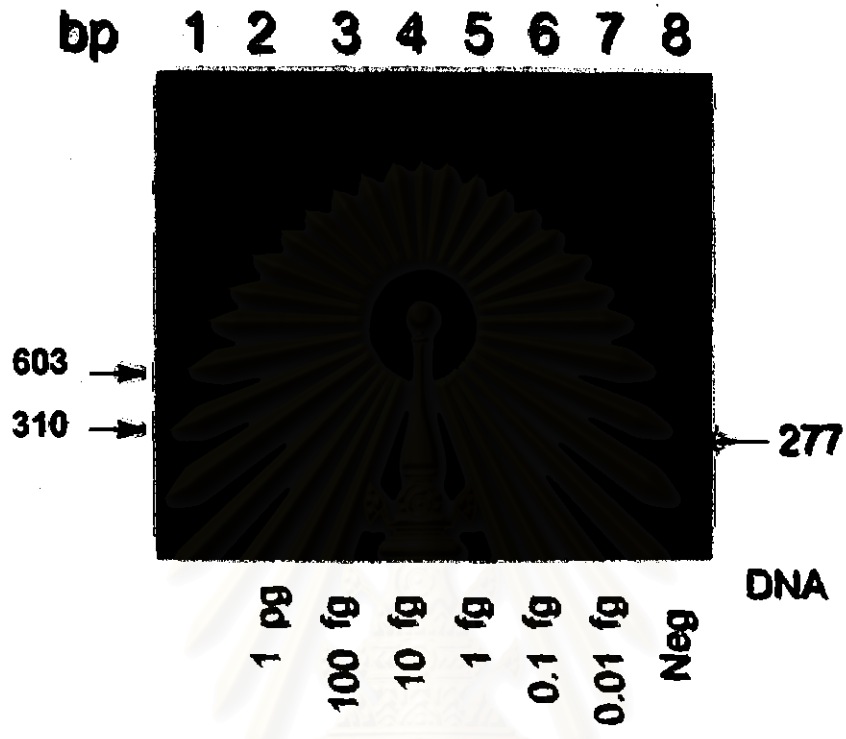

Fig 3 : Determination of 16S rDNA PCR sensitivity analysed by agarose gel electrophoresis

Amplication was performed in standard reaction mixture with 0.5 μM of each primer (16S-1 and 16S-2) and varied the amount of *M. pneumoniae* DNA. Lanes 2-7, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, 0.01 fg, respectively; lane 1, HaeIII-digested ϕX174 DNA as molecular weight marker; lane 8, negative control. Amplified products were detected by agarose gel electrophoresis.

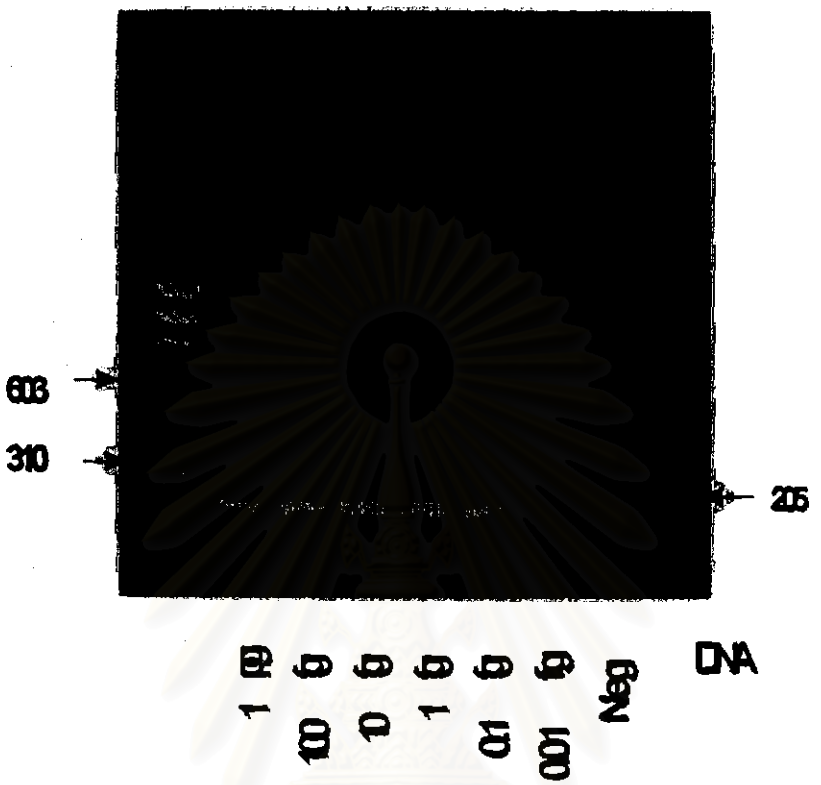

Fig 4 : Determination of 16S rDNA nested PCR sensitivity, using and analysed by agarose gel electrophoresis

Amplication was performed in standard reaction mixture with 0.5 μM of each nested primer (16S-1S and 16S-2S) and using 5 μl of first PCR products as a template. Lanes 2-7, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg, 0.01 fg, respectively; lane 1, HaeIII-digested ϕX174 DNA as molecular weight marker; lane 8, negative control. Amplified products were detected by agarose gel electrophoresis.

3. Serology, culture and PCR for *M. pneumoniae* in healthy volunteers.

From 100 controls of healthy volunteer, 81 gave titer of < 1:40, 9 gave titer of 1: 40 and 10 gave titer of 1:80 as shown in Table2. No growth for *M. pneumoniae* was observed and PCR was negative for all control samples.

Table 2 : Detection of *M. pneumoniae* by culture, serology by MAG assay and PCR in 100 healthy volunteers

No. of samples (n)	Serology (titer) in samples	Culture	PCR
81	<40	-	-
9	40	-	-
10	80	-	-

-, negative result

4. PCR of clinical samples

Specimen from 7 patients (7%) were positive by nested PCR (Table 3), 6 samples were positive by MP-nested PCR and 16S rDNA nested PCR, and in the other one sample (patient no.7) positivity was increased by 16S rDNA nested PCR (Fig 5-11). Inhibition of PCR was observed in 40 samples (20%), equally distributed among samples obtained from patient and controls. Diluting the lysates 20-fold before PCR resolved this inhibition in all cases.

Table 3 : Correlation among results obtained by amplification of *M. pneumoniae* DNA in clinical samples

Group of patients (n)	No.(%) of patients positive by PCR :			
	MP	MP-nested	16S rDNA	16S rDNA nested
I. (23)	0	2	2	3
II. (77)	2	4	3	4
Total (100)	2 (2)	6 (6)	5 (5)	7 (7)

Group I , children , Group II, adults

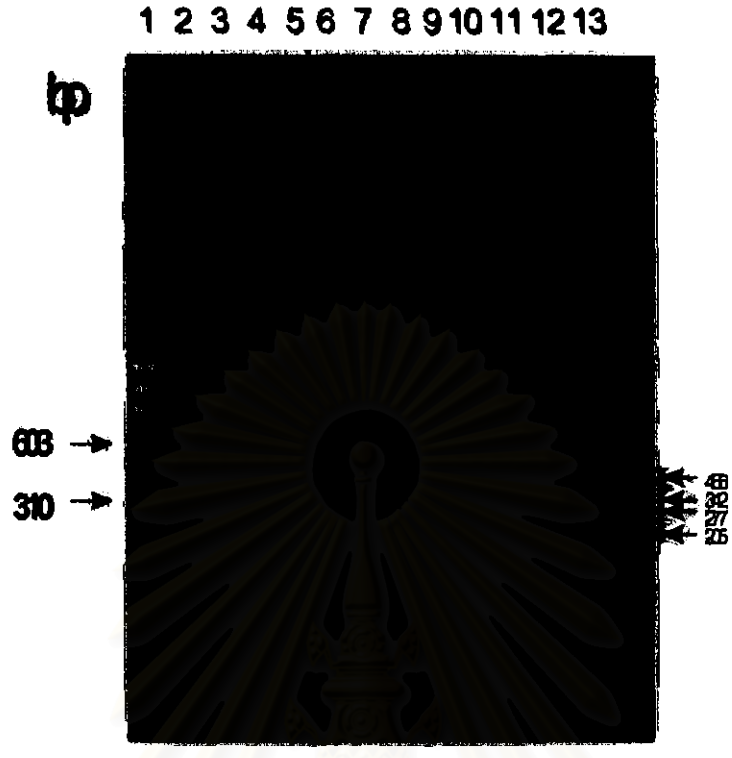

Fig 5 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.1

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

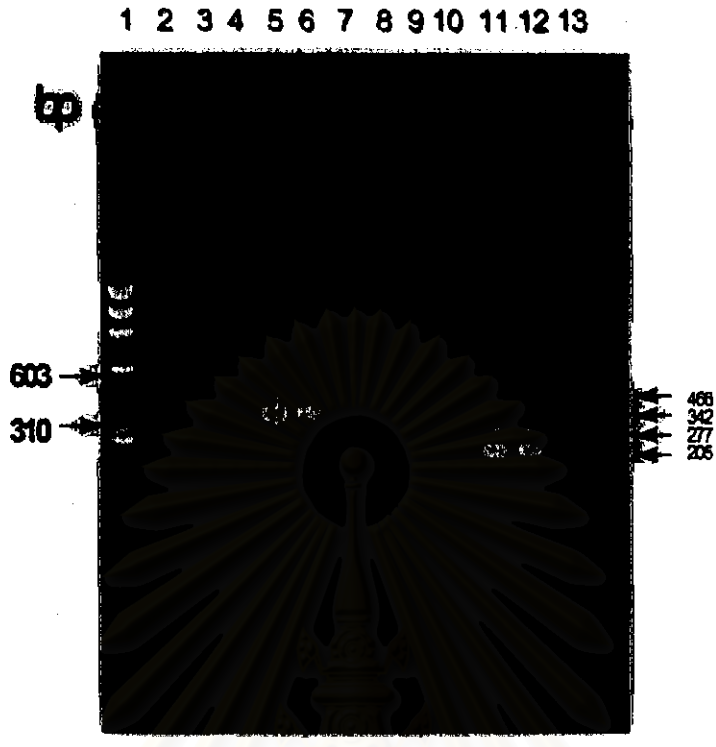

Fig 6 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.2

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

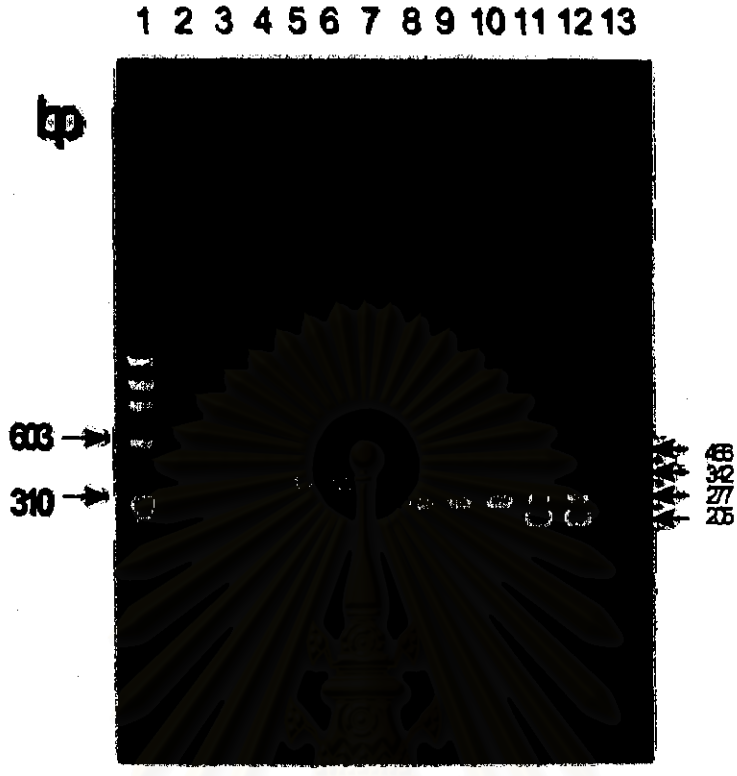

Fig 7 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.3

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

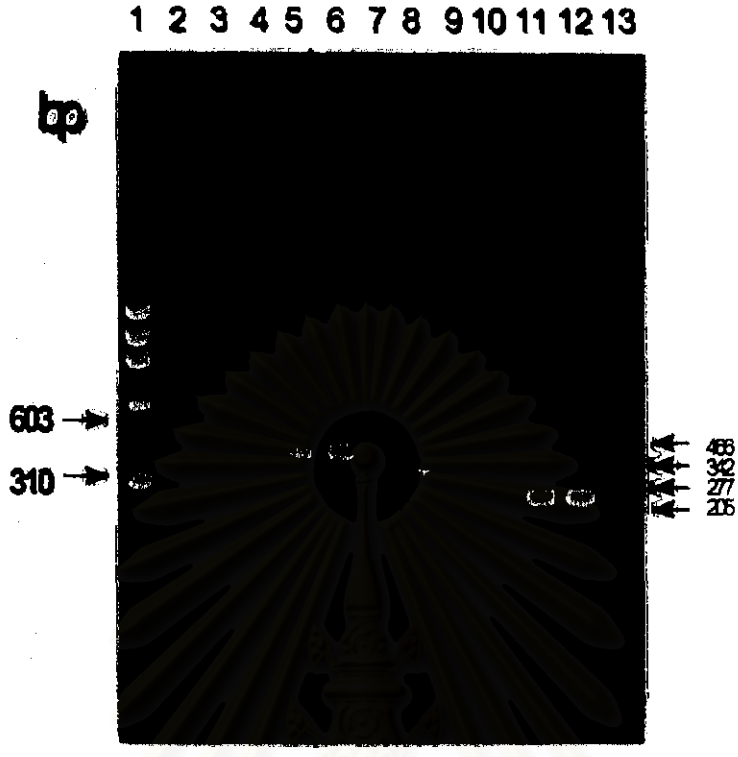

Fig 8 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.4

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

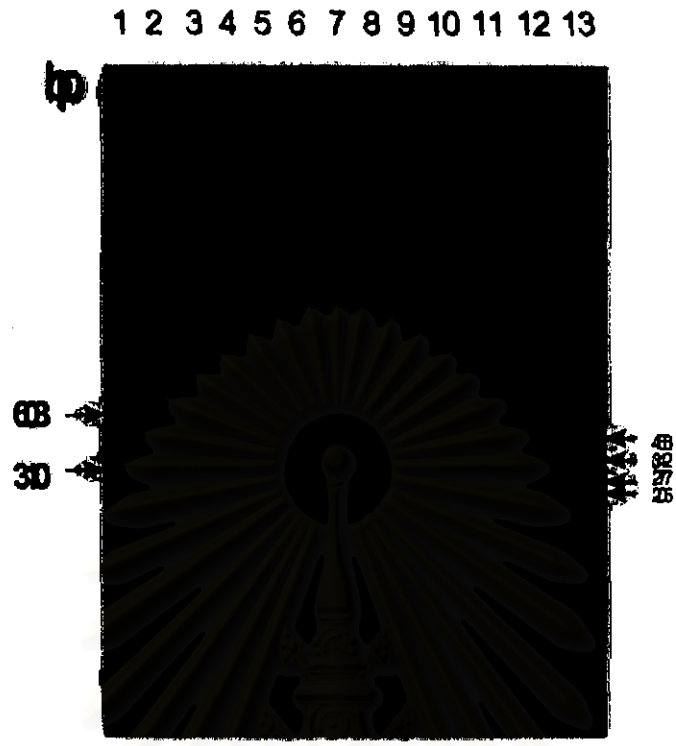

Fig 9 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.5

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

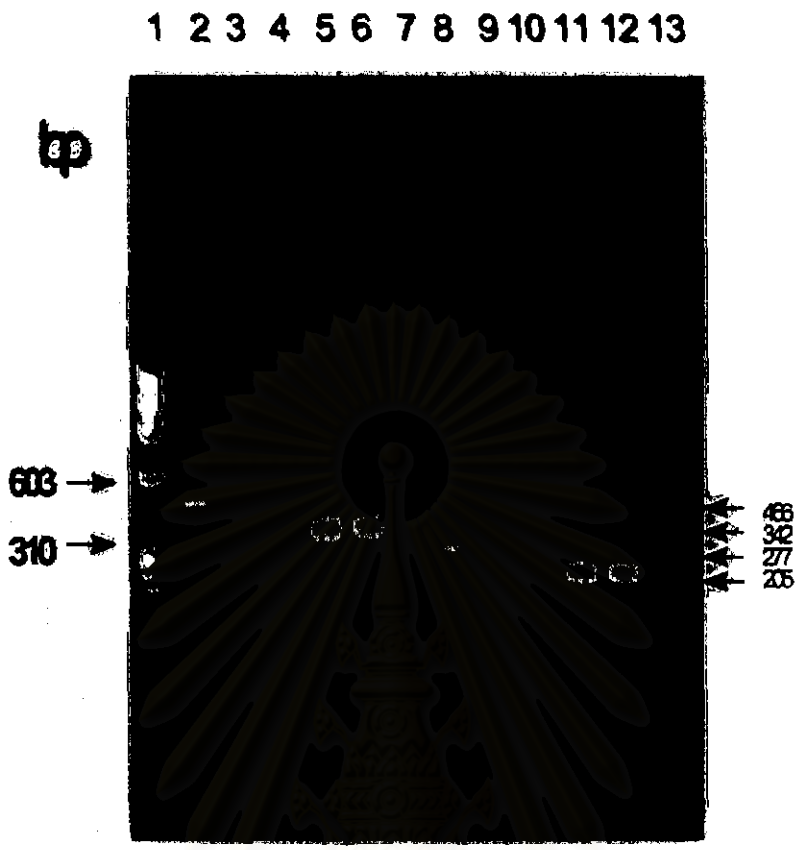

Fig10 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.6

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

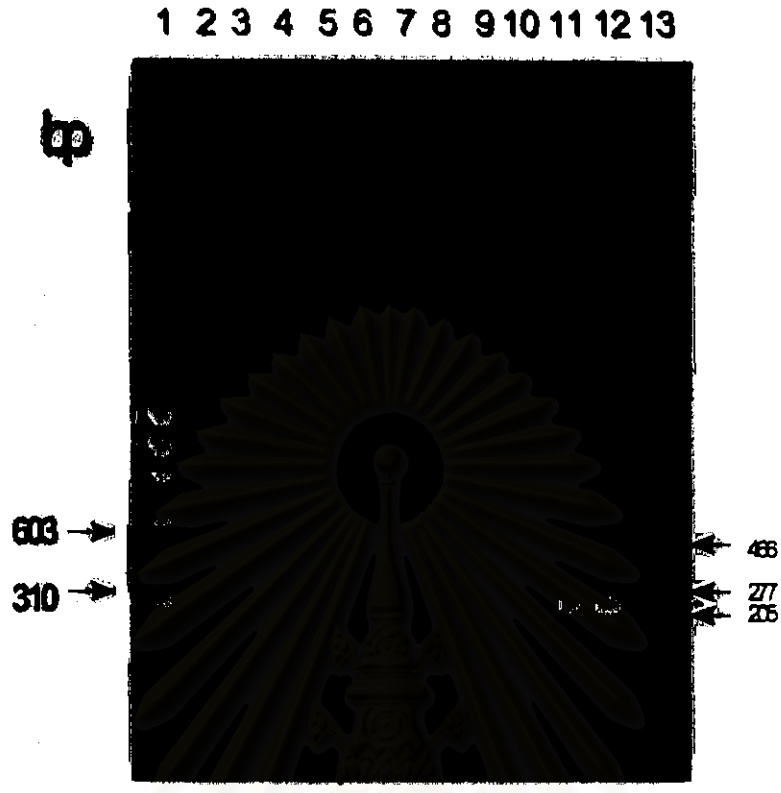

Fig 11 : Detection of *M. pneumoniae* by PCR in throat swab obtained from patient No.7

Lane 1 : ϕx174/HaeIII fragments

Lanes 2,8 : positive control amplification of MP-and 16S rDNA-PCR from treated sample spiked with 100 fg of *M. pneumoniae* DNA

Lanes 3,4 : MP-PCR products from throat swab

Lanes 5,6 : MP-nested PCR products

Lane 7 : negative control of MP-PCR

Lanes 9,10 : 16S rDNA PCR products from throat swab

Lanes 11,12 : 16S rDNA nested PCR products

Lane 13 : negative control of 16S rDNA PCR

5. Comparison of nested PCR with serology and culture for clinical specimens

PCR detected *M. pneumoniae* DNA in specimens from 7 patients (MP-nested PCR did not detect one sample), whereas serology detected anti-mycoplasma antibodies in first serum from 4 of these 7 samples. However, all of them were positive in second serum excepted for patient No.4 whose second serum was not available. Of the seven patients whose samples were positive by PCR and / or serological test, all samples were found to be negative by culture, as shown in table 4.

Table 4 : Correlation among positive results obtained by amplification of *M. pneumoniae* DNA (MP-nested PCR and 16S rDNA nested PCR) with those by serology test and culture

Group Of patients	Sample No.	Result of: PCR on throat swab (nested PCR) MP	PCR on throat swab (nested PCR) 16S rDNA	MP Antibody titer in sample 1	MP Antibody titer in sample 2	Culture
II	1	+	+	1280	5120	-
II	2	+	+	<40	10240	-
II	3	+	+	<40	1280	-
II	4	+	+	<40	NA	-
I	5	+	+	5120	NA	-
I	6	+	+	1280	1280	-
I	7	-	+	5120	20480	-

NA, not available ; Group I, children ; Group II, adults

PCR did not detect *M. pneumoniae* DNA in specimens from 93 patients whose culture results were also negative. Of these, specimens from 2 children had stable titers of 1:80 and single titer of 1:160 and specimens from 5 adults had single titers of 1:80, 1:160, 1:320 and 1:640 as shown in Table 5.

Table 5: Correlation among negative results obtained by amplification of *M. pneumoniae* DNA (MP- and 16S rDNA-nested PCR) with those by serology test and culture

Group of patients	Sample no.	Result of: PCR on throat swab (nested PCR) MP	PCR on throat swab (nested PCR) 16S rDNA	MP Antibody titer in sample 1	MP Antibody titer in sample 2	Culture
I	8	-	-	1:80	1:80	-
I	9	-	-	1:160	NA	-
II	10	-	-	1:80	NA	-
II	11	-	-	1:160	NA	-
II	12	-	-	1:160	NA	-
II	13	-	-	1:320	NA	-
II	14	-	-	1:640	NA	-
I	15-26	-	-	<1:40	<1:40	-
I	27-32	-	-	<1:40	NA	-
II	33-100	-	-	<1:40	NA	-

-, negative result; NA, not available ; Group I, children ; Group II, adults

The results of the comparison of serological and PCR results were summarizes as shown in Table 6. Corresponding negative results were obtained for 86 (86%) of the patients, and corresponding positive results were obtained for 6 (6%) of patients. Discrepant results were obtained for samples from 8 of 100 patients, only 7 of which were serological positive and 1 only of which was PCR positive for two targets. More detailed results for the patients whose samples were positive by any of the tests are summarize in Table 7 and are described more extensively in the Discussion section.

Table 6: Results of *M. pneumoniae* PCR compared with that of serology by the MAG assay in patients with respiratory complaints

PCR results for		Serology result (no. [%] of samples)		Total
P1	16S rDNA	positive	Negative	
+	+	5 (5)	1 (1)	6 (6)
-	+	1 (1)	0	1 (1)
-	-	7 (7)	86 (86)	93 (93)
Total		13	87	100

-, negative result; +, positive result

Table 7: Results for patients with respiratory complaints and whose samples were positive for *M. pneumoniae* by any of the diagnostic tests

Patient no.	Group	Clinical status[a]	PCR result		Culture	Titer by agglutination[b]	
			MP	16S rDNA		AP	CP
4	II	Pneumonia	+	+	-	<1:40	NA
8	I	RAD	-	-	-	1:80	1:80
9	I	Pneumonia	-	-	-	1:160	NA
10	II	URI	-	-	-	1:80	NA
11	II	URI	-	-	-	1:160	NA
12	II	URI	-	-	-	1:160	NA
13	II	URI	-	-	-	1:320	NA
14	II	URI	-	-	-	1:640	NA

[a] RAD, Reactive airway disease: URI, Upper respiratory infection

[b] AC, acute phase; CP, convalescent phase; NA, not available