CHAPTER V

RESULTS

1. Determination of the sensitivity of *omp1*-based PCR

The sensitivity of PCR with primers CP1 and CP2 was assayed with serial dilutions of DNA extracted from elementary bodies of *C. pneumoniae*. In repeated experiments, as few as 10 IFU of *C. pneumoniae* DNA could be detected by PCR and visualized by agarose gel electrophoresis (Fig 5). After using 1 μl of 1:10 dilution of each of the first step PCR products as a template for nested PCR (Fig 7) and 5 μl for dot blot hybridization, the sensitivity was increased to 0.1 IFU (Fig 9).

2. Determination of the sensitivity of 16S rDNA-based PCR

The sensitivity of PCR with primers CpnA and CpnB was also tested by using serial dilutions of DNA extracted from elementary bodies of *C. pneumoniae*. At least 1 IFU of elementary bodies of *C. pneumoniae* was detected (Fig 6), when the products were analyzed by gel electophoresis and the PCR sensitivity was increased to 0.1 IFU when 1 μl of the first step products were amplified with the nested PCR (Fig 8) and 5 μl used for detection with dot blot hybridization (Fig 10).

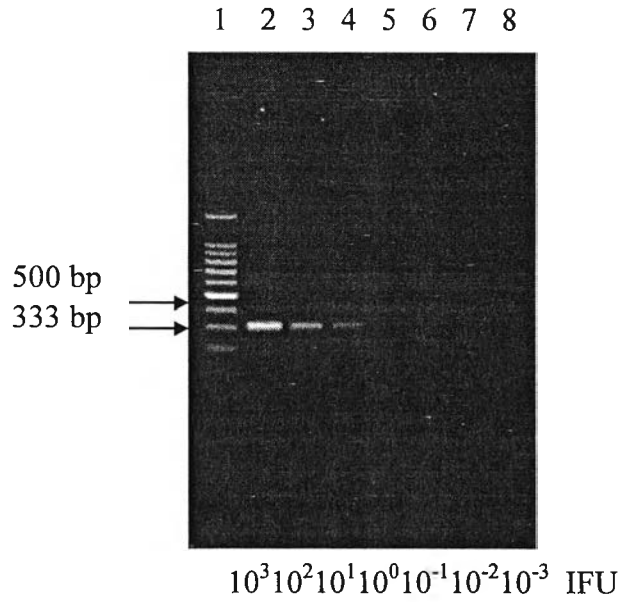

Fig 5 : Determination of the *omp1*-based PCR sensitivity; the first amplification with primer CP1 and CP2 by using decreased amount of DNA extracted from *C. pneumoniae* strain TWAR elementary body. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of *C. pneumoniae* DNA from 10^3, 10^2, 10^1, 1^0, 1^{-1}, 1^{-2}, 1^{-3} IFU/reaction

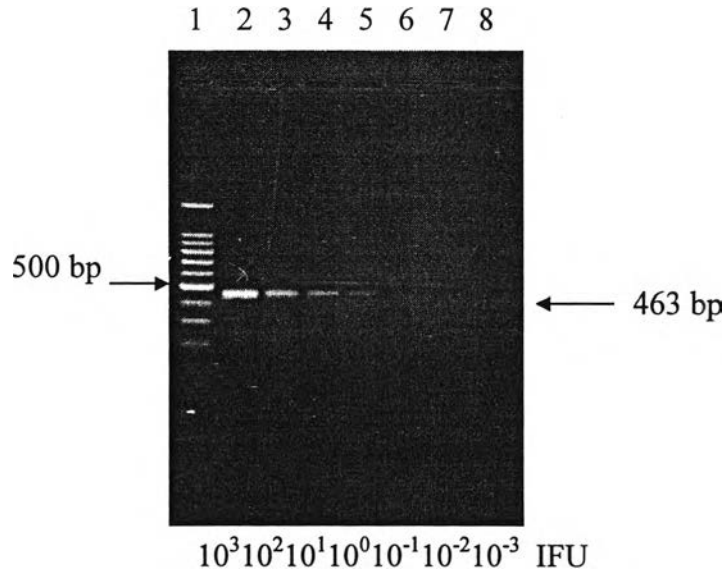

Fig 6 : Determination of the 16S rDNA-based PCR sensitivity; the first amplification with primer CpnA and CpnB by using decreased amount of DNA extracted from *C. pneumoniae* strain TWAR elementary body. The amplified products were analyzed by gel electrophpresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of *C. pneumoniae* DNA from 10^3, 10^2, 10^1, 1^0, 1^{-1}, 1^{-2}, 1^{-3} IFU/reaction

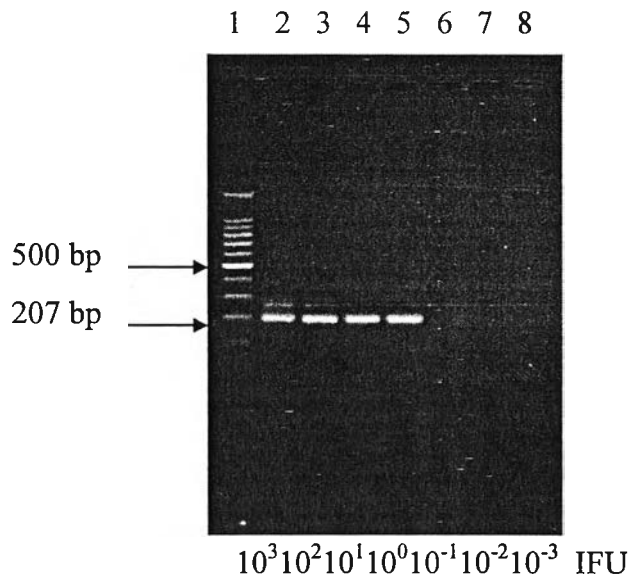

Fig 7 : Determination of the *omp1*-nested PCR sensitivity; the amplification with primer CPCand CPD by using 1 μl of 1:10 diluted first round PCR products as a template. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of *C. pneumoniae* DNA from 10^3, 10^2, 10^1, 1^0, 1^{-1}, 1^{-2}, 1^{-3} IFU/reaction

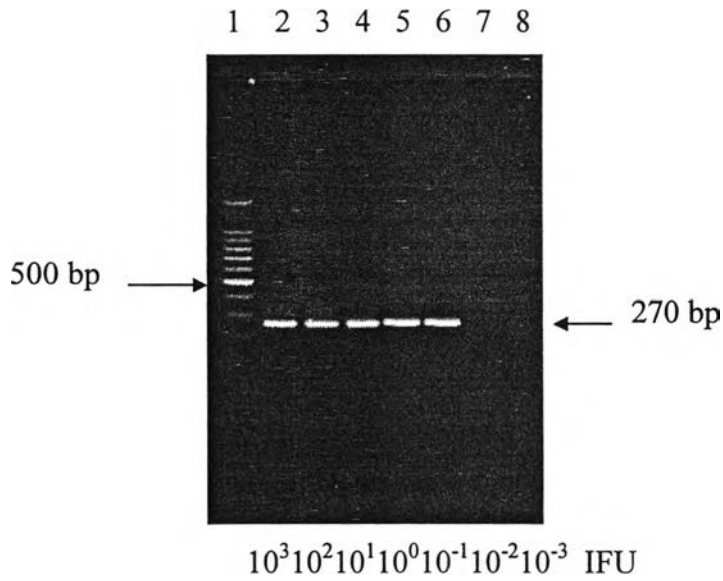

Fig 8 : Determination of the 16S rDNA nested PCR sensitivity; the amplification with primer pTW50 and pTW51 by using 1 μl of first round PCR products as a template. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of *C. pneumoniae* DNA from 10^3, 10^2, 10^1, 1^0, 1^{-1}, 1^{-2}, 1^{-3} IFU/reaction

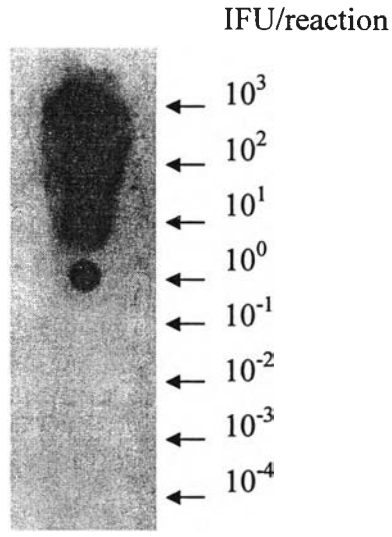

Fig 9 : Determination of the *omp1*-based PCR sensitivity by dot blot hybridization. The decreased amount of DNA extracted from *C. pneumoniae* strain TWAR elementary body were amplified by using primers CP1 and CP2. Five microliters of amplified products from each dilution were analyzed by dot blot hybridization with the 207-bp probe.

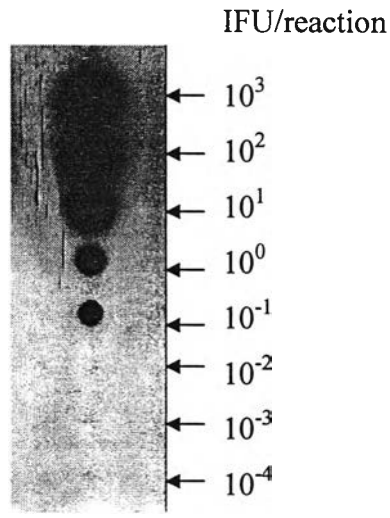

Fig 10 : Determination of the 16 S rDNA – based PCR sensitivity by dot blot hybridization. The decreased amount of DNA extracted from *C. pneumoniae* strain TWAR elementary body were amplified by using primers CpnA and CpnB. Five microliters of amplified products from each dilution were analyzed by dot blot hybridization with the 270-bp probe.

3. Determination of the amount of plasmid control used in PCR

The amount of plasmid control used for spiking in the sample was determined with serial ten-fold dilution of plasmid control pKW2 and pKW4. The concentration of 10^3 copies per reaction of plasmid control pKW2 and pKW4 that showed a single band of the 254 bp fragments for *ompl*-based PCR and the 400 bp fragments for 16S rDNA-based PCR was used for spiking in the samples as a positive control. The results were shown in Figure 11 - 14.

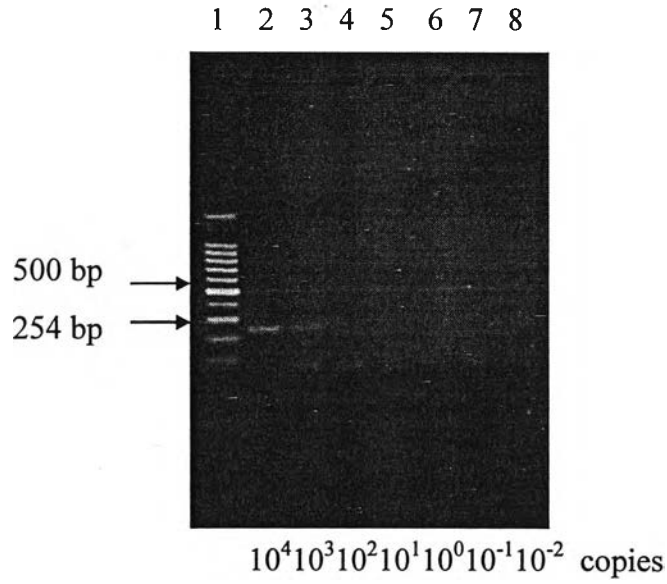

Fig 11 : Determination of the amount of *omp1*-based PCR plasmid control ; the decreased amount of plasmid control pKW2 were amplified with primer CP1 and CP2. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of plasmid control pKW2 $10^4 10^3 10^2 10^1 10^0 10^{-1} 10^{-2}$ copies/reaction

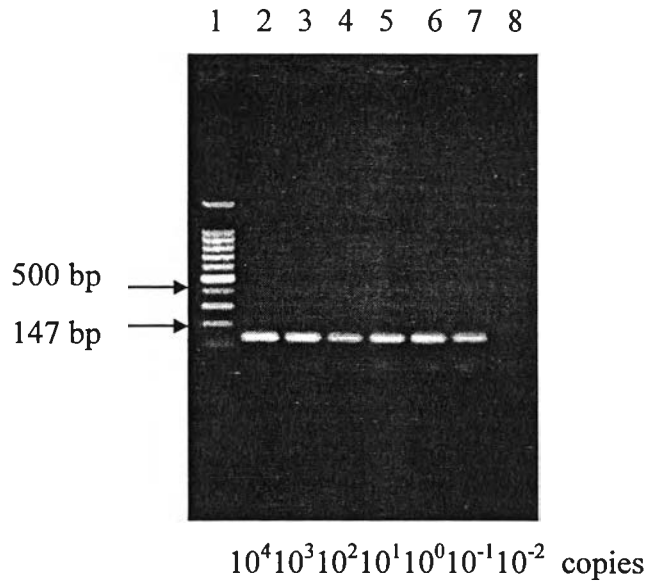

Fig 12 : Determination of the amount of plasmid control by *ompl* nested PCR; the amplification with primers CPC and CPD by using 1 μl of 1:10 diluted first round amplified products from each dilution of plasmid control pKW2. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of plasmid control pKW2 from 10^4,10^3, 10^2, 10^1,10^0, 10^{-1}, 10^{-2} copies/reaction

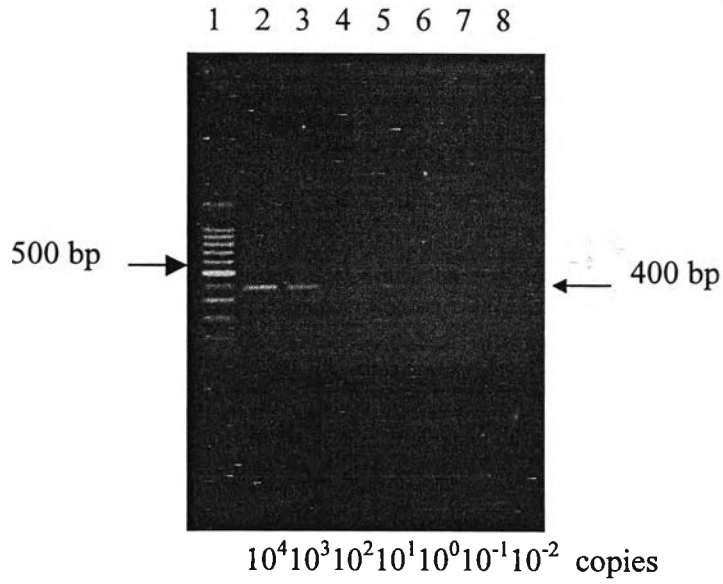

Fig 13 : Determination of the amount of 16 S rDNA – based plasmid control; the amplification with primer CpnA and CpnB by using decreased amount of plasmid control pKW4. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of plasmid control pKW4 from 10^4,10^3, 10^2, 10^1,10^0, 10^{-1}, 10^{-2} copies/reaction

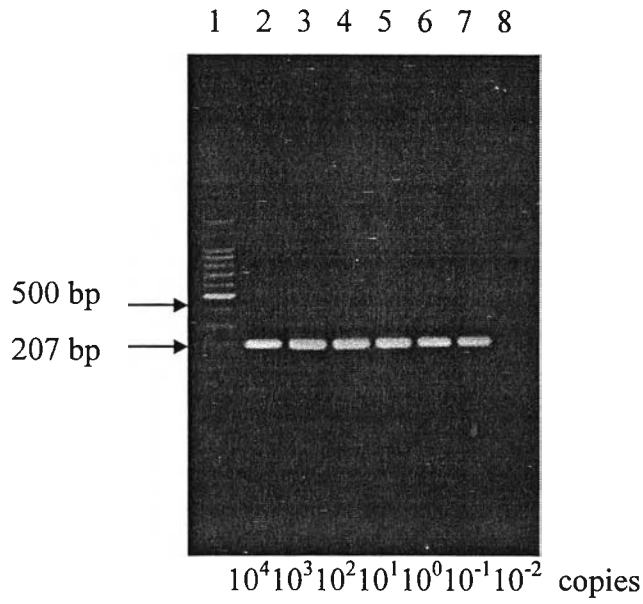

Fig 14 : Determination of the amount of plasmid control by 16S rDNA nested PCR; the amplification with primers pTW50 and pTW51 by using 1 μl of first round amplified products from each dilution of plasmid control pKW4. The amplified products were analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2 – 8 : Serial ten-fold dilution of plasmid control pKW4 from 10^4,10^3, 10^2, 10^1,10^0, 10^{-1}, 10^{-2} copies/reaction

4. Evaluation of PCR for detection of *C. pneumoniae* from clinical samples

One hundred and fifteen throat swab samples from 114 patients with clinically suspected of community-acquired pneumonia were determined by 2 PCR-based protocols. Fifteen throat swab samples from 14 patients were positive by PCR. Of 15 samples, 10 were positive by both *omp1*-based PCR and 16S rDNA-based PCR when the amplified products were detected by dot blot hybridization. Five samples were positive only after analyzed by 16S rDNA nested PCR. All of these 15 samples were negative by using *omp1* nested PCR. Inhibition of the amplification was found in 4 (3.5 %) samples. After 1:10 dilution of these samples, the inhibitors were eliminated and non had positive result. The results of *omp1*-based PCR and 16s rDNA-based PCR were shown in Figure 15 – 19.

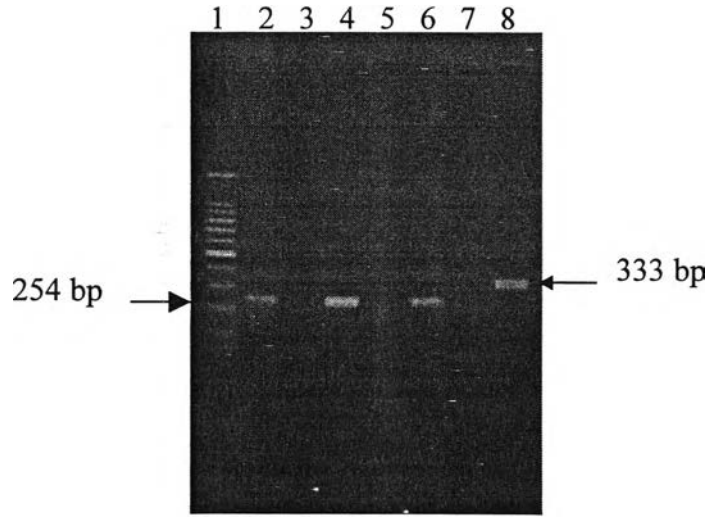

Fig 15 : The results of the first round amplification of *ompl* - based PCR for detection of *C. pneumoniae* in throat swab samples and analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2, 4, 6 : positive control of sample No. 3, 4 and 5 spiked with 10^3 copies of plasmid control pKW2

Lanes 3, 5, 7 : *ompl* first round PCR products from throat swab samples No. 3, 4, 5

Lanes 8 : *ompl* first round PCR products from *C. pneumoniae* strain TWAR elementary bodies

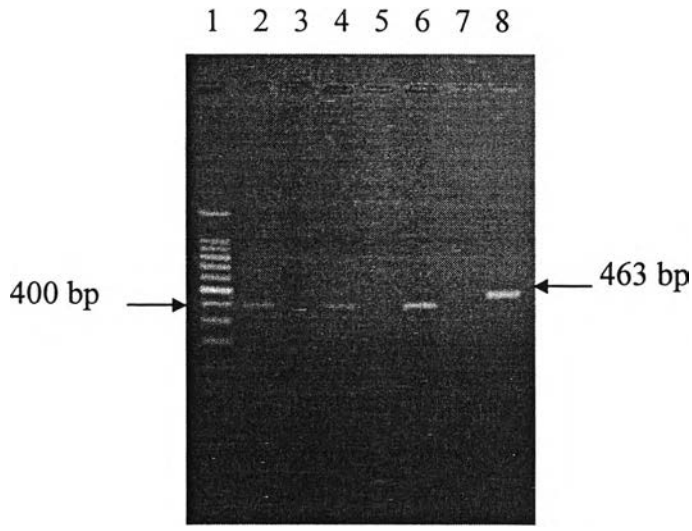

Fig 16 : The results of the first round amplification of 16S rDNA-based PCR for detection of *C. pneumoniae* in throat swab samples and analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lanes 2, 4, 6 : positive control of sample No. 3, 4 and 5 spiked with 10^3 copies of plasmid control pKW4

Lanes 3, 5, 7 : 16S rDNA first round PCR products from throat swab samples No. 3, 4, 5

Lanes 8 : 16S rDNA first round PCR products from *C. pneumoniae* strain TWAR elementary bodies

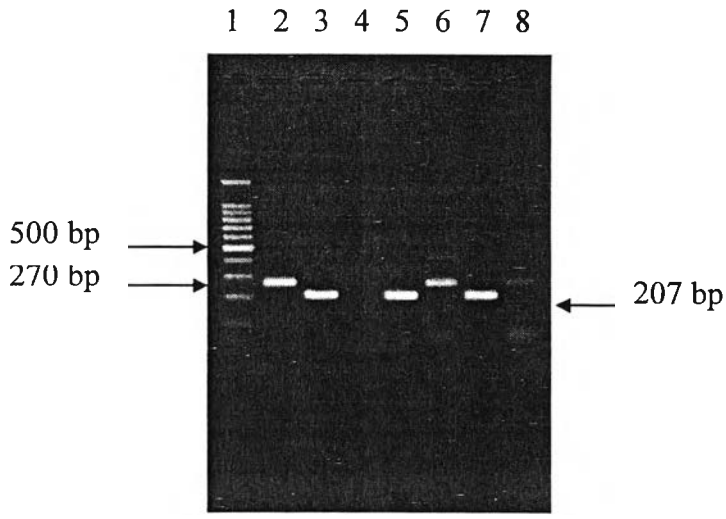

Fig 17 : The results of 16S rDNA nested PCR for detection of *C. pneumoniae* in throat swab samples and analyzed by agarose gel electrophoresis.

Lane 1 : 100-bp molecular size marker

Lane 2 : products of plasmid control pKW3 as marker for positive results

Lane 3 : products of plasmid contol pKW4

Lane 4 : negative control of 16S rDNA nested PCR

Lanes 5, 7 : PCR products of sample No. 5 and 63 spiked with 10^3 copies of plasmid control pKW4

Lanes 6, 8 : 16S rDNA nested PCR products from throat swab samples No. 5 and 63

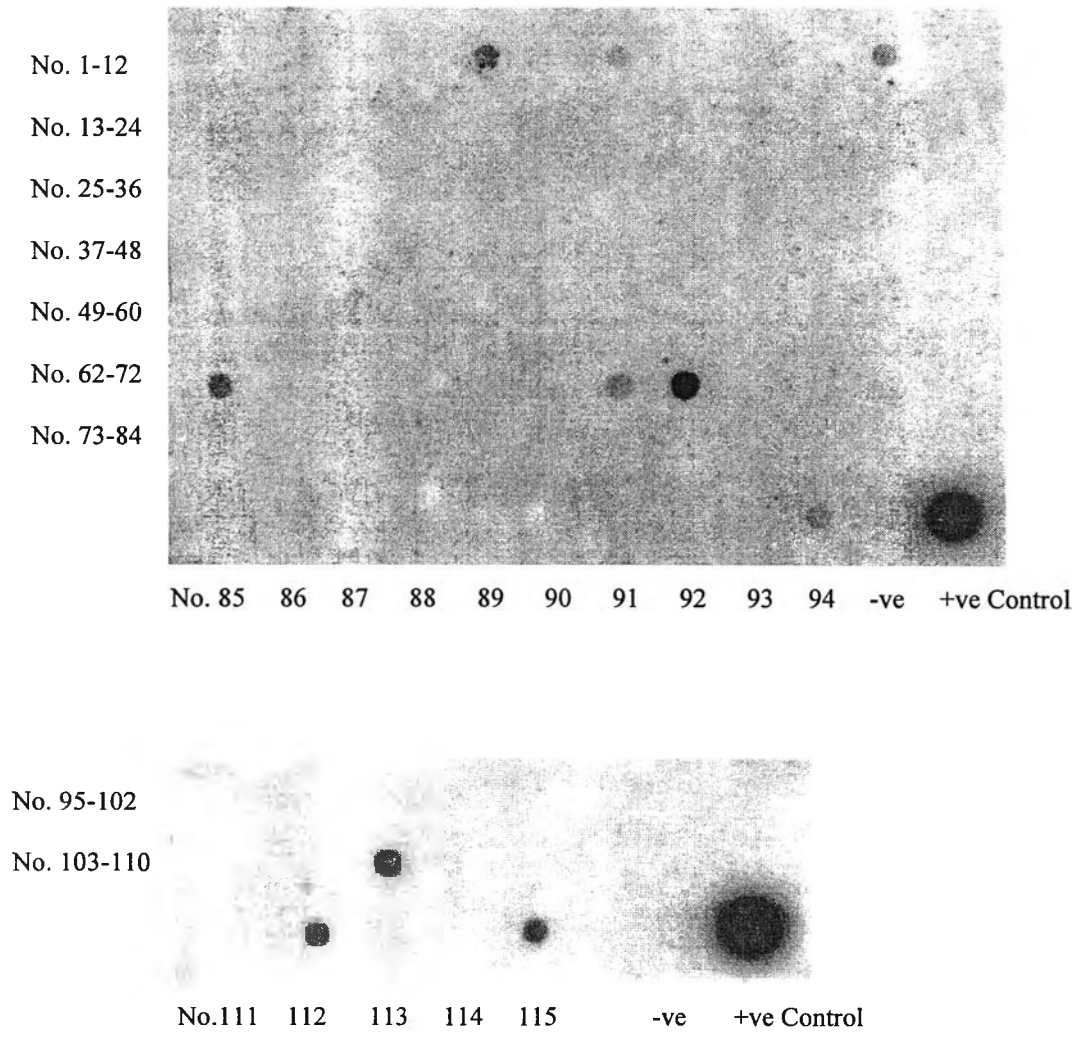

Fig 18 : The results of *omp1*-based PCR for detection of *C. pneumoniae* in throat swab samples and analyzed by dot blot hybridization with 207-bp probe situated in the 333-bp amplified products.

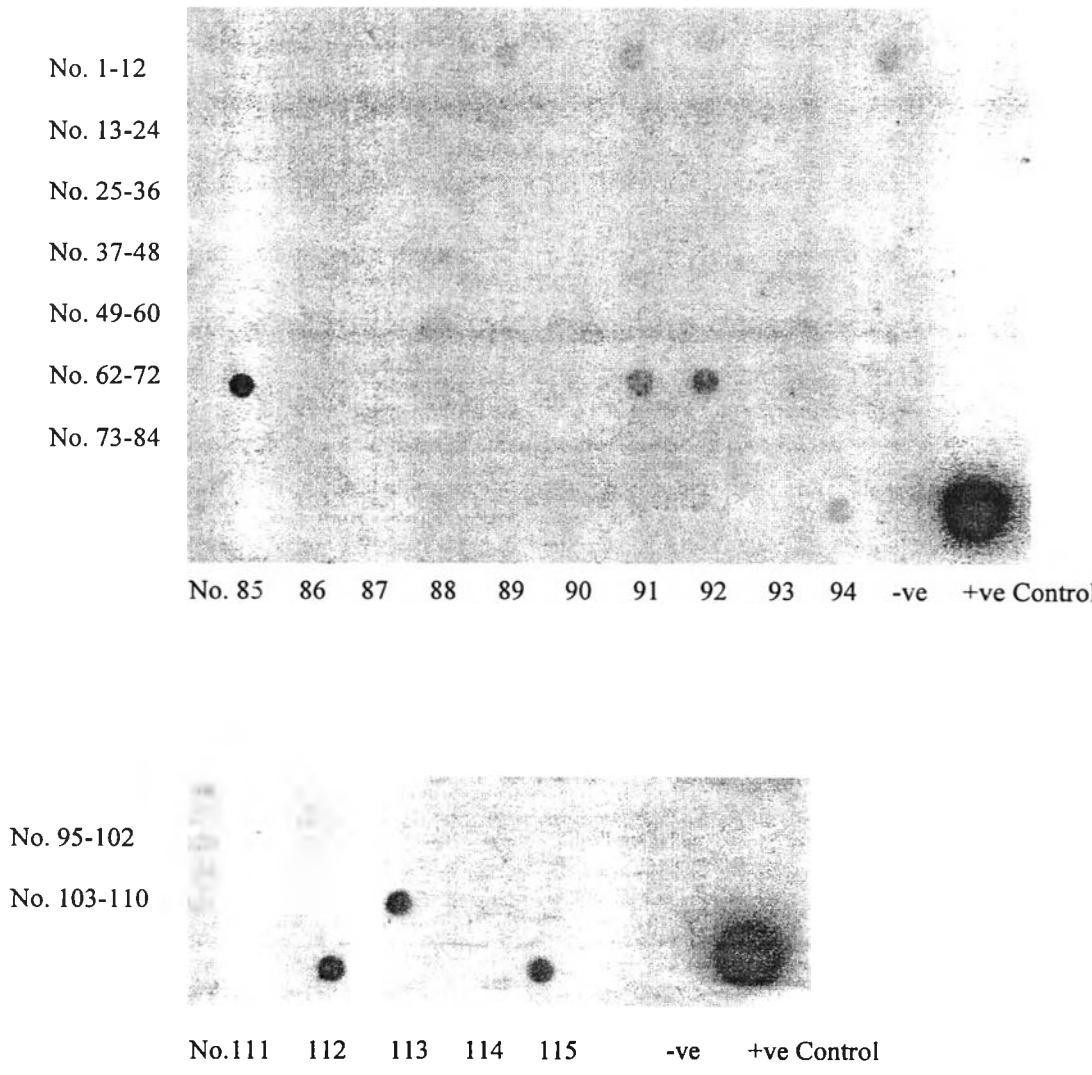

Fig 19 : The results of 16S rDNA-based PCR for detection of *C. pneumoniae* in throat swab samples and analyzed by dot blot hybridization with 270-bp probe situated in the 463-bp amplified products.

One hundred and fifteen throat swab specimens were examined for the presence of *C. pneumoniae* by *omp1*-based and 16S rDNA-based PCR. *C. pneumoniae* sequences amplified from *omp1* and 16S rRNA genes were analysed by nested PCR before ethidium bromide staining of agarose gels and dot blot hybridization. Comparison of the results of these two PCR protocol was shown in Table 3.

Table 3. Results of PCR methods in the detection of *C. pneumoniae* DNA from 115 clinical specimens

Method	No. (%) of clinical specimens with result	
	Positive	Negative
omp1-based PCR		
Nested	0 (0%)	115 (100%)
Dot blot hybridization	10 (8.7%)	105 (91.3%)
16S rDNA-based PCR		
Nested	15 (13.0%)	100 (87.0%)
Dot blot hybridization	10 (8.7%)	105 (91.3%)

Of 15 throat swab samples that were PCR positive, 10 samples were positive by both *omp1*-based and 16S rDNA-based PCR after detection with dot blot hybridization. Five samples had only 16S rDNA nested PCR positive, but they were negative after detection with dot blot hybridization. Of these 15 samples that 16S rDNA-based PCR were positive, none of them had positive result when using *omp1*-nested PCR.

On the basis of 115 serum specimens tested by MIF from 114 patients with CAP, 42 patients had only acute serum available. Fifty eight (50.9 %) patients had MIF results indicating acute *C. pneumoniae* infection. Twelve of these subjects (20.7 %) were *C. pneumoniae* PCR positive. Three patients (5.3 %) had positive PCR results without serological evidence of acute *C. pneumoniae* infection. MIF titers of serum samples from patients with PCR positive results were shown in Table 4. Among the eight patients who had only single serum sample, 6 (75.0 %) had titers considered to be diagnostic of acute *C. pneumoniae* infection. Of 7 patients with positive PCR whose paired serum were available, 6 of them (85.7%) had titers considered to be acute infection included 1 (14.3 %) had four fold rise in IgG titer and IgM antibody levels of ≥ 1:16, 2 (28.6 %) demonstrated only fourfold rise in IgG, 1 (14.3%) had only IgM level of ≥1:16, 2 (28.6%) had IgG ≥ 1:512. There was only 1 patient (14.3 %) who had stable IgG level of 1:256 and was defined as past infection.

Table 4. Results of interpretation of MIF test in patients with PCR positive

Specimen No.	MIF titer of				Final interpretation of MIF
	acute serum		convalescent serum		
	IgG	IgM	IgG	IgM	
4	1:256	-	1:512	-	+
5*	1:512	1:16	1:2048	1:16	+
7	1:2048	-	NA	NA	+
11	1:128	-	NA	NA	-
31	1:1024	-	1:512	-	+
32	1:256	-	1:1024	-	+
62	1:512	-	NA	NA	+
68	1:64	-	NA	NA	-
69	1:512	-	NA	NA	+
84	1:128	-	1:1024	-	+
94	1:16	1:16	1:16	1:16	+
105	1:256	-	1:256	-	-
112*	1:512	1:64	NA	NA	+
114	1:2048	1:32	NA	NA	+
115	1:512	-	NA	NA	+

* samples obtained 15 months apart from the same patient.

(-) Negative result indicating a titer of < 1:16.

NA : No serum sample available.

In our study, specimens were considered as positive if there was an agreement of the results of at least two different methods for detection, i.e. MIF test and PCR for one or both primer pairs or IFA and PCR for one or both primer pairs or MIF and IFA. Of 115 throat swab samples, none had IFA positive result. Comparison of the results from throat swab samples that had PCR positive and MIF test was shown in Table 5.

Table 5. Results from MIF test of the clinical specimens positive by PCR and final interpretation

Specimen No.	PCR results				MIF test results	Interpretation
	ompl-based		16S rDNA-based			
	Nested	Dot blot	Nested	Dot blot		
4	-	-	+	-	+	+
5*	-	+	+	+	+	+
7	-	+	+	+	+	+
11	-	+	+	+	-	-
31	-	-	+	-	+	+
32	-	-	+	-	+	+
62	-	+	+	+	+	+
68	-	+	+	+	-	-
69	-	+	+	+	+	+
84	-	-	+	-	+	+
94	-	+	+	+	+	+
105	-	+	+	+	-	-
112*	-	+	+	+	+	+
114	-	-	+	-	+	+
115	-	+	+	+	+	+

* samples obtained 15 months apart from the same patient.

Table 6. Comparison of PCR methods for detection of *C. pneumoniae* in throat swab samples that had final interpretation

Test and Result	Number of throat swab samples with result [a]	
	Positive	Negative
ompl-based PCR (Nested)		
Positive	0	0
Negative	12	103
ompl-based PCR (Dot blot)		
Positive	7	3
Negative	5	100
16S rDNA-based PCR (Nested)		
Positive	12	3
Negative	0	100
16S rDNA-based PCR (Dot blot)		
Positive	7	3
Negative	5	100

[a] result after final interpretation based on the agreement of results from two different methods.

Of 115 throat swab samples, 12 samples were accepted as true positive. The sensitivity of the *ompl*-based PCR when analyzed by dot blot hybridization was 58.3 % and the specificity was 97.1 %. In contrast to *ompl*-based PCR, 16S rDNA had sensitivity of 58.3 % and specificity of 97.1% when analyzed by dot blot hybridization. The sensitivity of 16S rDNA nested PCR was 100 % and the specificity was 97.1 %.