CHAPTER IV

RESULTS

1. Isolation of plasmid from *C. trachomatis* serotype L2 (pCHL2)

C. trachomatis was grown in cycloheximide treated McCoy cells. The plasmid was isolated by the method that described in chapter III and 10 ul of each isolated plasmid were analysed by electrophoresis on 0.7 % Tris-acetate agarose gel. The results showed more than one conformations of the plasmid which gave a single band of approximately 7.5 kb fragment when cleaved with BamHI restriction endonuclease. The concentration of the plasmid (pCHL2 No.2) was estimated from the agarose gel electrophoresis compared with 1.5 ul of λ/HindIII fragments (molecular size marker). The intensity of pCHL2 No.2 bands was corresponded to 1/20 times of the 4.3-kb λ/HindIII fragment intensity or 2 times of the 0.5-kb λ/HindIII fragment intensity. Accordingly, the concentration of the pCHL2 No.2 was approximately 2 ng per ul (Fig.5). The pCHL2 No.2 was diluted 1:100 with TE buffer and 1 ul of diluted pCHL2 was used as a template for setting up PCR reaction mixture for detection of *C. trachomatis* DNA.

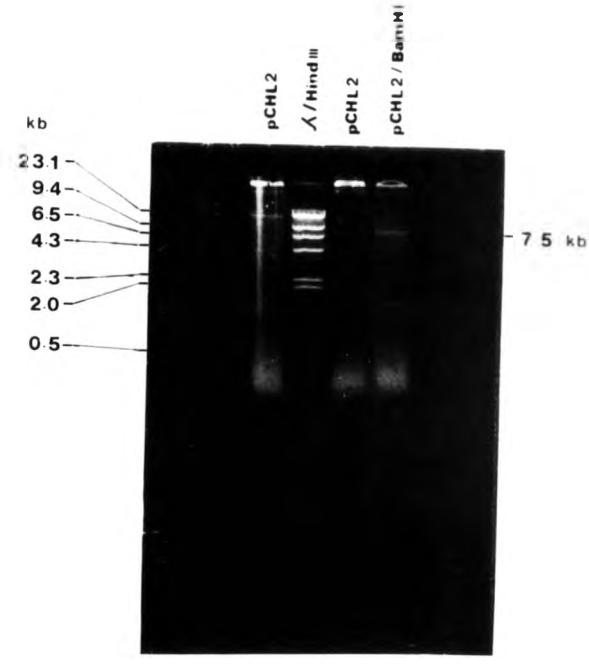


Fig.5 Analysis of pCHL2 isolated from *C. trachomatis* infected McCoy Cells by using agarose gel electrophoresis :

lane 1 ; pCHL2 No.1 lane 2 ; λ/HindIII fragments

lane 3 ; pCHL2 No.2 lane 4 ; pCHL2 (No.1+ No.2) cleaved with BamHI restriction endonuclease enzyme.

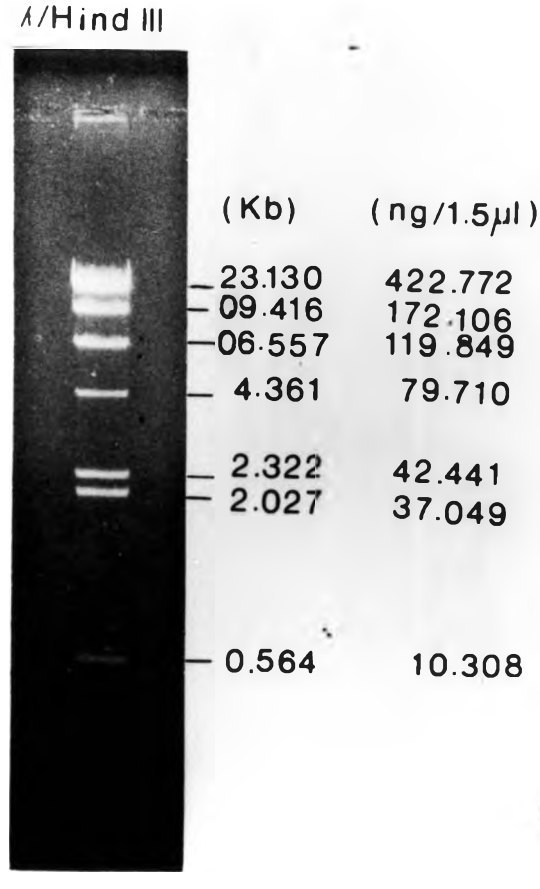


Fig.6 The concentration of λ/HindIII fragments (ng/1.5 ul)

2. Setting up the PCR reaction mixture

In the initial setting of the PCR reaction mixture for amplification with primer A and primer B , the optimal concentration of $MgCl_2$ was determined by varying the $MgCl_2$ in different concentration (1.0, 1.5, 2.0, 2.5, 3.0 mM) and using the standard PCR reaction as described in chapter III with 0.1 uM of each primer in 50 ul volume. The cycling of three- step reaction : 94°C for 1 min (denaturation), 52°C for 1 min (primer annealing), 72°C for 1 min (primer extension) was repeated until 30 cycles and then at 72°C for 10 min in order to complete the extension. The optimal concentration of $MgCl_2$ was 2.0 mM (Fig.7).

After the optimal $MgCl_2$ concentration was known, the optimal concentration of *Taq* polymerase was determined by varying the *Taq* polymerase in different concentration (1.0, 1.5, 2.0, 2.5, and 3.0 unit per 50 ul volume of the reaction mixture) and using the standard PCR reaction with 2.0 mM $MgCl_2$. The reaction was run as described above. The least concentration of the *Taq* polymerase which gave a satisfactory result was 2.0 unit per 50 ul volume of reaction mixture (Fig.8).

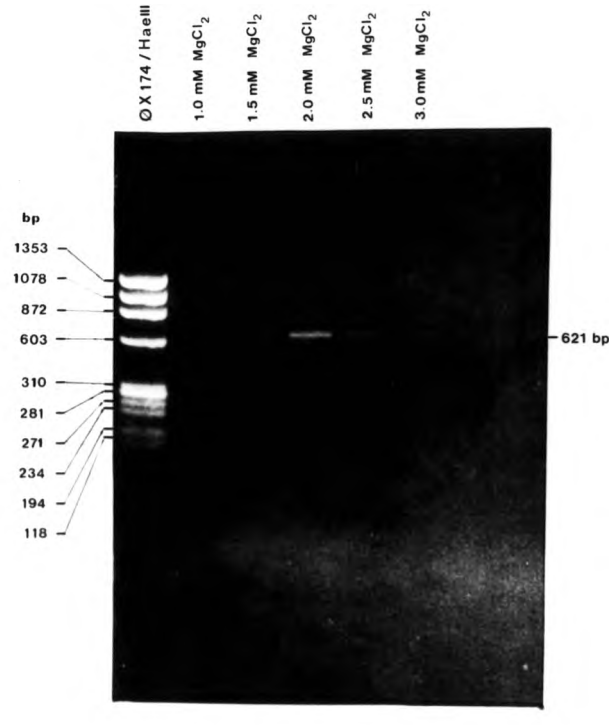


Fig.7 Setting up the PCR reaction mixture : Amplification with 0.1 uM of each primer (A,B) by varying the $MgCl_2$ concentration and using the standard PCR reaction : lane 1; ØX174 / HaeIII fragments, lane 2; 1.0 mM $MgCl_2$, lane 3; 1.5 mM $MgCl_2$, lane 4; 2.0 mM $MgCl_2$, lane 5; 2.5 mM $MgCl_2$, lane 6; 3.0 mM $MgCl_2$.

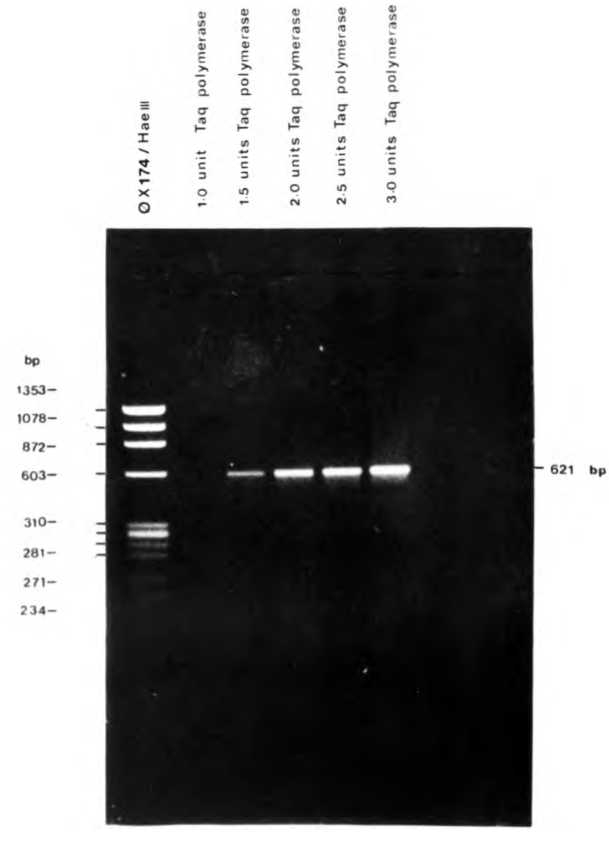


Fig.8 Amplification with 0.1 uM of each primer (A,B) by using the standard PCR reaction with 2.0 mM $MgCl_2$ and varying the *Taq* polymerase :

lane 1 ; ØX174/HaeIII fragments, lane 2 ; 1 unit of *Taq* polymerase, lane3 ; 1.5units of *Taq* polymerse, lane 4; 2.0 units of *Taq* polymerase, lane 5; 2.5 units of *Taq* polymerase, lane 6; 3.0 units of *Taq* polymerase. The reaction was done in 50 ul volume.

3. Determination of suitable number of PCR cycles

To determine the least number of PCR cycles that gave a satisfactory product, the cycling was repeated approximately 30 cycles , 35 cycles , 40 cycles , 45 cycles , and 50 cycles, respectively. The amplified products were analysed on 1.5 % Tris-acetate agarose gel electrophoresis. The suitable number of PCR cycles for this experiment was 30 cycles (Fig.9).

4. Optimization of dUTP concentration

In order to prevent amplicon carryover, the dUTP was used instead of dTTP and 0.5 units of the UDG was added in the reaction mixture to cleave the amplicons into small fragments prior to the PCR. The initial denaturation step in the first PCR cycle was extended to 10 min and the amplified products were soaked at 72°C. Because the amplification may be somewhat less efficient when incorporating dUTP in place of dTTP, this resulted in slightly decreased yield of products. Additional cycles would allow comparable yield of products to be obtained. The concentration of dUTP was optimized for satisfactory results by varying the dUTP concentration to 200 uM, 400 uM, 600 uM, 800 uM, 1.0 mM, 1.2 mM, 1.4 mM, 1.6 mM, 1.8 mM, and 2.0 mM, respectively. The PCR was run at 94°C for 10 min for 1 cycle, 94°C for 1 min, 52°C for 1 min and 72°C for 1 min for 32 cycles, and 72°C for 10 min for 1 cycle. The optimal concentration of dUTP used in the reaction mixture was 2.0 mM (Fig.10).

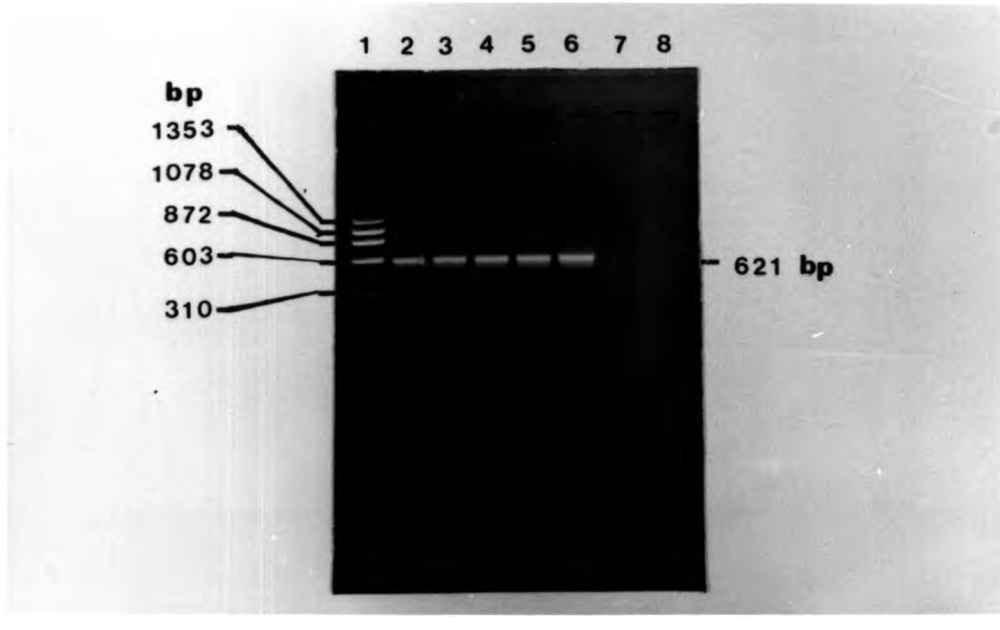


Fig.9 Amplification with 0.1 uM of each primer (A, B) by using the standard PCR reaction with 3.0 mM $MgCl_2$,200 mM of each dNTP (dATP,dCTP,dGTP), 2.0 mM of dUTP, 0.5 units of UDG, and 2.0 units of *Taq* polymerase per 50 ul volume and run at the different number of cycle:

lane 1 ; ØX174/HaeIII fragment. lane 2 ; 30 cycles.

lane 3 ; 35 cycles. lane 4 ; 40 cycles.

lane 5 ; 45 cycles. lane 6 ; 50 cycles.

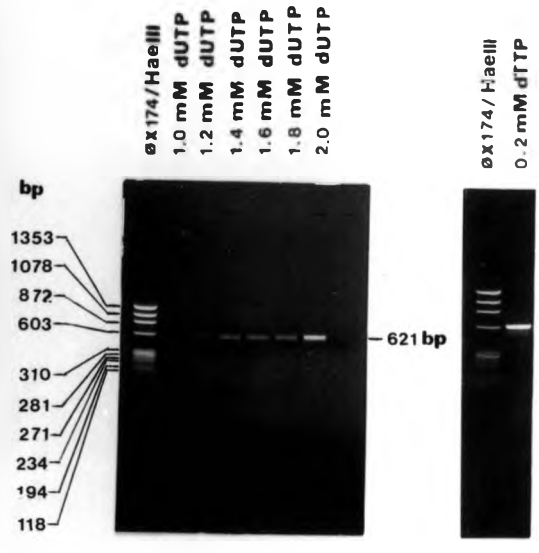


Fig.10 Amplification with 0.1 uM of each primer (A,B), 2.0 mM $MgCl_2$, 2.0 units of *Taq* Polymerase, 200 uM of each dNTP (dATP,dCTP,dGTP), 0.5 unit of UDG in 1x PCR buffer and varied the concentration of dUTP. The PCR was run as described above:

lane 1 ; ØX174/HaeIII fragments lane 2 ; 1.0 mM of dUTP

lane 3 ; 1.2 mM of dUTP lane 4 ; 1.4 mM of dUTP

lane 5 ; 1.6 mM of dUTP lane 6 ; 1.8 mM of dUTP

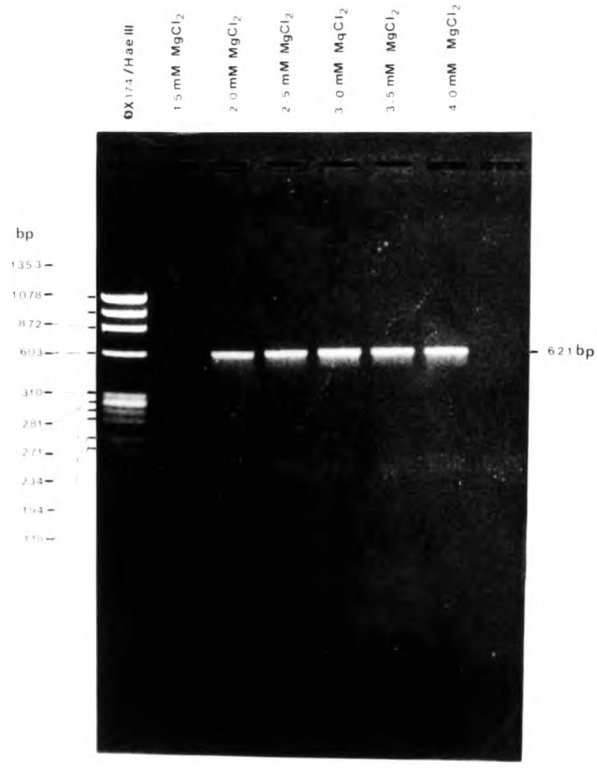
lane 7 ; 2.0 mM of dUTP

5. Adjustment of $MgCl_2$ concentration

Because high concentration of dUTP was used in the PCR, $MgCl_2$ concentration was adjusted in order to obtain the highest yield by varying $MgCl_2$ concentration to 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM , 3.5 mM , and 4.0 mM , respectively. The optimal concentration of $MgCl_2$ was 3.0 mM (Fig. 11)

6. Setting up the PCR for amplifying the 473-bp fragment situated in 621-bp fragment of *C. trachomatis* plasmid

Amplification of the 473-bp fragment was carried out by using the standard PCR reaction mixture with 0.1 uM of each primer (C,D) and varying the $MgCl_2$ concentration. The cycling was repeated approxymately 30 cycles of the three-step reaction: 94°C for 1 min, 45°C for 1 min,72°Cfor 1 min and extended the last extension step at 72°C for 10 min. The optimal concentration of $MgCl_2$ was 2.5 mM (Fig.12).



OPTIMIZATION OF THE PCR

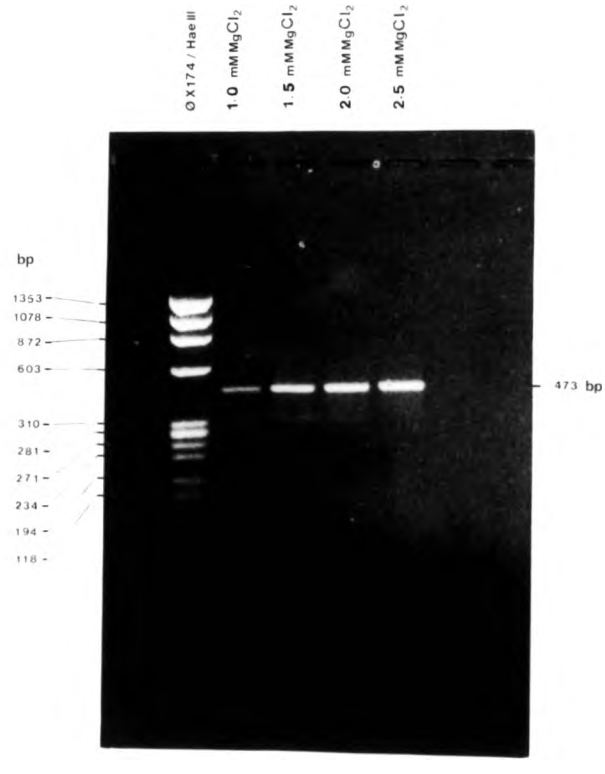
Fig. 11 Amplification with 0.1 uM of each primer (A,B) by using 2 units of *Taq* polymerase, 200 uM of each dNTP (dATP, dCTP, dGTP), 2.0 mM dUTP, 1x PCR buffer (Promega), 0.5 units of UDG and varied $MgCl_2$ concentration.

lane 1 ; ØX174/HaeIII fragments lane 2 ;1.5 mM $MgCl_2$

lane 3 ; 2.0 mM $MgCl_2$ lane 4 ;2.5 mM $MgCl_2$

lane 5 ; 3.0 mM $MgCl_2$ lane 6 ;3.5 mM $MgCl_2$

lane 7 ; 4.0 mM $MgCl_2$.



OPTIMIZATION OF THE PCR

Fig.12 Optimization of the amplification with o.1 uM of each primer C and D by using the standard PCR reaction and varying the $MgCl_2$ concentration.

lane 1 ; ØX174/HaeIII fragments lane 2 ; 1.0 mM $MgCl_2$

lane 3 ; 1.5 mM $MgCl_2$ lane 4 ; 2.0 mM $MgCl_2$

lane 5 ; 2.5 mM $MgCl_2$

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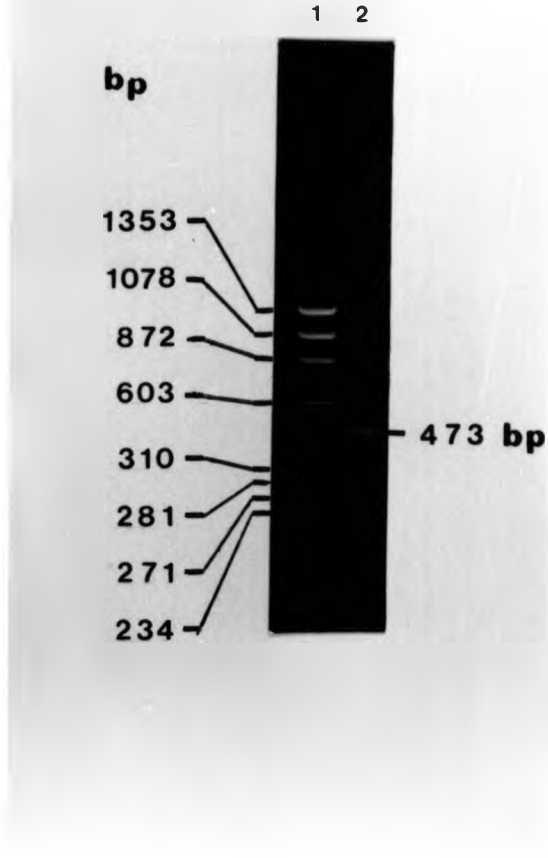

Fig.13 Analysis of the 473-bp biotinylated probe.

lane 1 ; 1.5 ul of ØX174/HaeIII fragments

lane 2 ; 10 ul of the recovered 473-bp biotinylated probe.

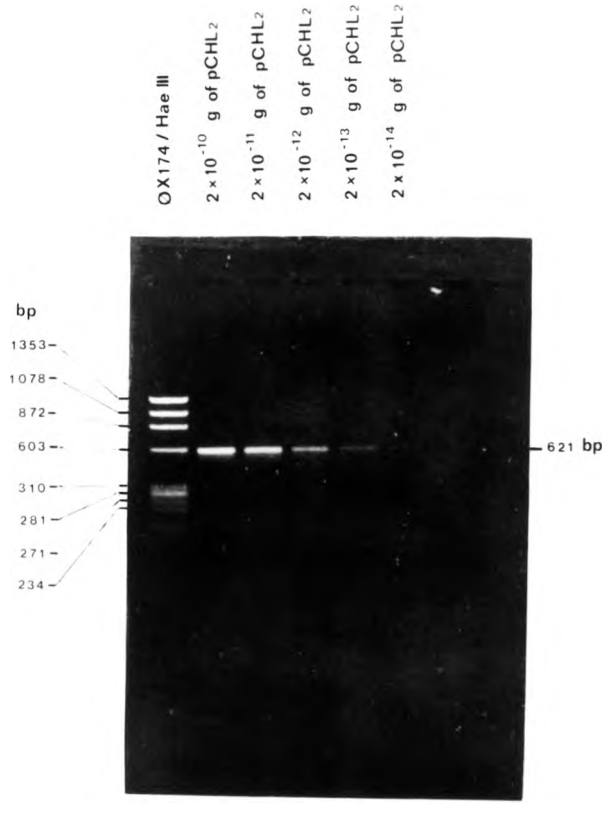

Fig.14 Evaluation of the PCR sensitivity : the amplification with primer A and B by using decreased amount of pCHL2. The amplified products were analysed by agarose gel electriphoresis : lane 1; ØX174/HaeIII fragments lane 2; 2 x 10^{-10} g of pCHL2 lane 3; 2 x 10^{-11} g of pCHL2 lane 4; 2 x 10^{-12} g of pCHL2 lane 5; 2 x 10^{-13} g of pCHL2 lane 6; 2 x 10^{-14} g of pCHL2 lane 7; 2 x 10^{-15} g of pCHL2 lane 8; 2 x 10^{-16} g of pCHL2

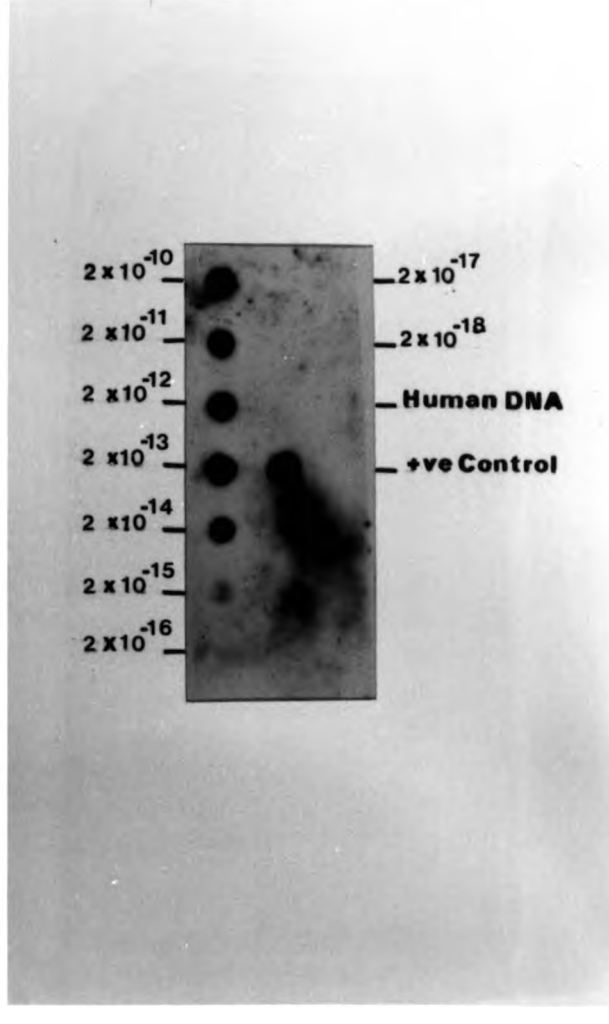

Fig.15 Determination of the PCR sensitivity : the amplification with primer A and B by using decreased amount of pCHL2. The amplified products were analysed by dot blot hybridization with 473-bp biotinylated probe.

9. Evaluation of PCR for detection of *C. trachomatis* from clinical specimens

The endocervical specimens from 100 women attending a clinic for sexually transmitted diseases were determined for *C. trachomatis* by PCR and cell culture as described above. The PCR products were analysed by electrophoresis in 1.5 % agarose gel and dot blot hybridization. Agarose gel electrophoresis results were shown in Fig.16 and dot blot hybridization results were shown in Fig. 17. There were 12 samples that PCR and cell culture test were positive and 84 samples that the PCR and cell culture test were negative. There are two samples that PCR results were positive but cell culture results were negative (Table 4). The sensitivity and the specificity of the PCR were 100% and 97.7 % ,respectively. The positive predictive value was 85.7 % and the negative predictive value was 100 % .

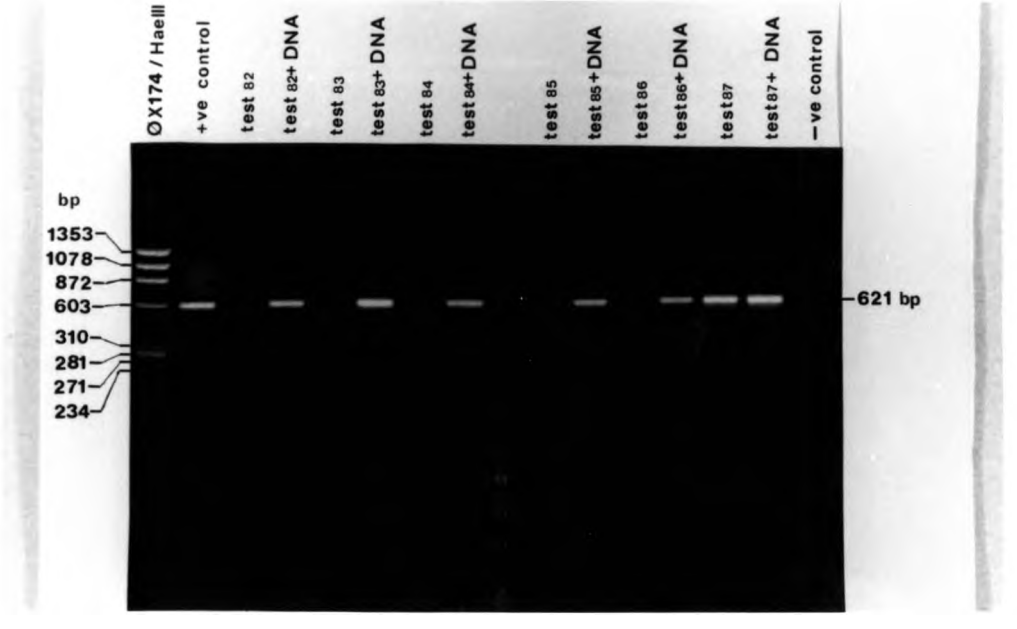

Fig.16 The results of *C. trachomatis* detection from endocervical samples by the PCR and analysed on agarose gel electrophoresis.

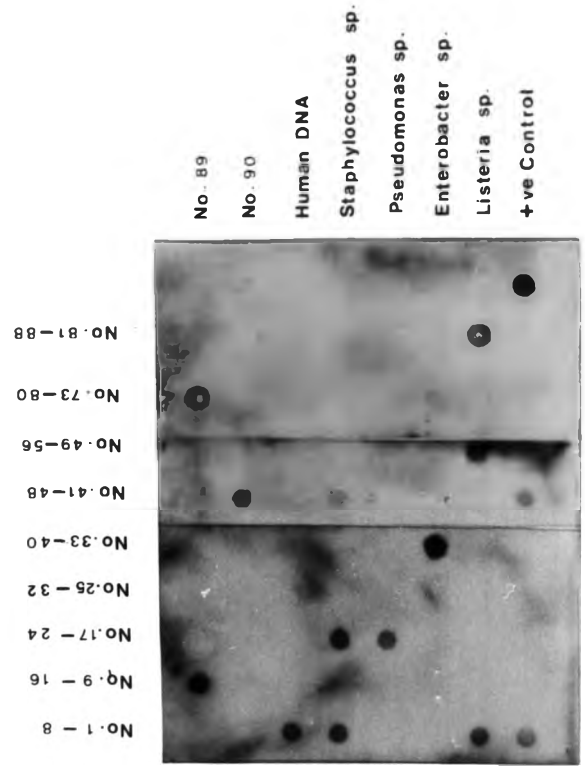

Fig.17 The results of *C. trachomatis* detection from endocervical samples by the PCR and analysed by dot blot hybridization with 473-bp biotinylated probe situated in the 621-bp fragments amplified products.

Table 4. Number of positive and negative specimens tested by culture and PCR

PCR result	No. of culture results		
	Positive	Negative	Total
PCR (GEL)[a]			
Positive	11	0	11
Negative	1	88	89
Total	12	88	100
PCR (DH)[b]			
Positive	12	2	14
Negative	0	86	86
Total	12	88	100

[a] PCR (GEL): PCR products were analysed by gel electrophoresis

[b] PCE (DH) : PCR products were analysed by dot blot hybridization