The Development a Polymerase Chain Reaction (PCR) to detect C.Pneumoniae and C.Psittaci

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ABSTRACT

C.pneumoniae and *C.psittaci* both cause respiratory infections in human. detection of these organisms in tissue culture is difficult and serological testing is unreliable. There are no sensitive and reliable tests for the detection of these organisms. The polymerase chain reaction (PCR) has provided an alternative diagnostic method for the detection of these fastidious organisms. The aim of this study was to develop a PCR to detect *C.pneumoniae* and *C.psittaci* from clinical samples. The PCR was optimized in a series of experiments. To determine if the optimized PCR could be applied to clinical samples, mock positive specimen were produced by adding *chlamydiae* to throat swab from healthy adults. The DNA was extracted by phenol/chloroform. When tested by PCR all the throat swabs were negative. However, when diluted and retested, many of the swabs were positive and 10 IFU of *C.psittaci* and 100 IFU of *C.pneumoniae* could be detected. This experiment indicates that inhibitor to PCR are found in throat swab and further work is needed on specimen preparation. The PCR was optimized in a series of experiments. The optimal conditions were to use a two segment PCR with 68°c annealing and polymerization temperature, 2.0mM Mgcl2, 0.2μM primers and 40 cycles. The PCR was highly sensitive and could detect one inclusion forming unit with both *C.pneumoniae* and *C.psittaci* strains. Two human strains and one nonhuman strain of *C.pneumoniae* and two avian strains and three mammalian strains of C.psittaci were used to determine the specificity of PCR. The PCR detected all these different strains. *C.trachomatis* strains were not detected. Various bacterial strains, fungi DNA, and human DNA were negative in PCR and no amplification DNA was found in negative controls.

Keywords: PCR; *Chlamydia;* IFU; *Pneumoniae; Psittaci*

INTRODUCTION

Chlamydiae are a group of obligate intracellular parasites. They are gram-negative bacteria [1] with a unique developmental cycle. These microorganisms cause variety of diseases in human and other animals. They are divided into four species, C.psittaci, C.trachomatis, C.pneumoniae and C.pecorum [2-4]. *Chlamydia* are obligate intracellular parasites unable to produce their own ATP and so are "energy parasites" of their host eukaryotic cell. The genome of *chlamydiae* is about one million base pair [5]. They have two distinct forms, the elementary body (EB) which is infectious and the reticulate body (RB) [6,7]. The cell wall in *Chlamydia* resembles that of gram-negative bacteria, but it has no peptidoglycan [8]. Rigidity in the chlamydiae is thought to be due to disulfide bonds cross linking the major outer membrane protein (MOMP) and other cystine rich proteins [9]. The MOMP DNA has been sequenced and has become one of the most useful targets for chlamydial polymerase chain reaction tests [10-13]. *C.pneumoniae* strain TWAR is a human pathogen that causes respiratory infections including pneumonia. It is transmitted from human to human [14], probably by inhalation. The serological testes are not reliable and ELISA produces many false positive results. PCR is potentially a specific and sensitive method for detection of organisims from clinical specimens. The aim of this project is to develop a PCR which can detect C.pneumoniae and C.psittaci from clinical specimens.

MATERIALS AND METHODS

In this study Ten *Chlamydia* strains were used, *C.pneumoniae* (IOL-207Human, VR-1310Human, N16Horse), *C.psittaci* 6BCAvian, N352Avian, Fpn Pring cat, Gpic

Guinea pig, EAE Lx sheep, *C.trachomatis* E T181Human, L2Human. DNA was extracted from clinical samples using proteinase K digestion followed by phenol-chloroform extraction. The samples were digested with proteinase K (250µg/ml) and 0.5% SDS (sodium dodesilsulfate) then incubated in 56°for 3 hours. An equal volume of phenol/chloroform was added, mixed and centrifuged for three minutes at 11000g.The aqueous layer was removed and then extracted once with a volume of chloroform. The aqueous layer was precipitated with 1/10 volume of sodium acetate(3M pH 5.2)and 2 volumes of 100% ethanol, mixed and left to precipitate at -20°C for 10 minutes. Then, it was centrifuged at 4°C at 52000g for 15 minutes. The pellet was washed in 70% ethanol and re-centrifuged at 52000g for 15 minutes. The precipitated DNA was dried, then re-suspended in 100µl of TE (10mM-Tris/HCL pH 8.0, 1mM EDTA) and stored at 4°C. Because of its exquisite sensitive one needs adequate care in procedures not introduce contaminating DNA in the different steps in setting up a PCR. In DNA free room, the reaction mixture was prepared which contained different components. In the following table the concentration of each component is demonstrated.

Table 1 shows concentration of each component in the form of stock, 10 reaction mixture or final concentration.

Table 1. PCR protocol

	Stock concentration	10 Reaction Mixture	Final concentration
Primer cpnA	130 µg/ml	19µl	0.2µM
Primer cpnB	120 µg/ml	15µl	0.2µM
Dinucleotide triphosphate[1](dNTP)	20 mM	44µl	800µM
$MgCl_2$[2]	100mM	5.0µl	0.5mM
Taq polymerase	5µ/µl	2.0µl	1.0u/100µl
10x buffer[2]	Tris-HCL100mM,$MgCL_2$ 15mM,KCL500mM,Gelatin1 mg/ml pH 8.3	105µl	Tris-HCL10mM, $MgCL_2$ 1.5mM,KCL50mM,Gelatin0.1m g/ml
Double distilled water	DDW	760µl	

[1]Stock solution of dNTP(contained 5mM dATP,dCTP,dGTP,dTTP). Reaction mixture contained 200uM of each.
[2]0.5mM $MgCL_2$ per reaction from 100mM stock solution and 1.5mM magnesium chloride per reaction from buffer.

All the PCR reagents were stored in -20°C.The ten-reaction mixture was prepared and mixed well, divided into ten eppendorf tube, then two drops paraffin oil were added to prevent evaporation. The 10ul DNA per one reaction was added to each tube then was put in the (PHC_2) Techno Ltd machine. Two tubes as negative and positive controls were utilized in our method containing 10µl DDW in negative control and in positive control 10µl of *Chlamydia* DNA.

Table 2. The Initially PCR in 30 cycles

	Temp	Time	Annealing	Time	Extension	Time
First cycle	94°C	7min	55°C	1min	72°C	0.5min
2-29 cycles	92°C	2min	55°C	1min	72°C	0.5min
30 cycle	92°C	2min	55°C	1min	72°C	5min

After optimization, the number of cycle and annealing temperature were changed to 40 cycle and 68°C for annealing. The PCR with three segments was altered to two segments with combining the annealing and extension of the primers.

Table 3. PCR after optimazation

	Temp	Time	Annealing and Extension	Time	Time
First cycle	94℃	7min	68℃	1min	1min
2-39 cycles	92℃	2min	68℃	1min	1min
40 cycle	92℃	2min	68℃	1min	5min

The PCR with three segments was altered to two segments with combining the annealing and extension of the primers.

ELECTROPHORESIS

PCR products were analyzed by agarose gel electrophoresis. The 1.2% agarose gels were made up in Tris –phosphate EDTA buffer (0.09M Tris-phosphates 0.002M EDTA pH 8.0). The samples were mixed with 5µl of loading buffer (bromophenol blue 0.25% and 40% sucrose) and were then loaded onto gel. The one Kbp ladder (BRL) was used as a marker. Then, gel was stained in ethidium bromide (1µg/ml of distilled water) for 30 minutes and de-stained in distilled water. The gel was examined under UV light and photographed with Polaroid 667 film.

RESULTS

Design of primers for Chlamydia PCR

A PCR was designed in this study to detect both *C.pneumoniae* and *C.psittaci*. The MOMP gene was chosen as the target, as more is known about this gene than any other chlamydial gene. The MOMP gene in *Chlamydia* is composed of 4 variable domains flanked by conserved regions. The primers were chosen from the regions outside of variable domain I where there is minimal nucleotide variation. This region would be amplified as follows:

I……I…VDI…I…...…I…VDII…I……I…VDIII……I……I…VDIV….I…I

Analysis of the MOMP nucleotide sequences predicted that the chosen primers CPnA, CPnB would bind to all different strains. After amplification with the above primers, a fragment with a predicted length of 336bp for C.pneumoniae and 351bp fragment for C.psittaci would be produced. The PCR products could be differentiated by using a hybridization probe or by using restriction endonuclease analysis. The restriction endonuclease enzymes, by cleaving the DNA in specific restriction sites, would give different sizes of DNA fragment. Two enzymes, Pst and Dra III, could be used to cut the PCR product. Pst I cleaves C.pneumoniae to give one 78 bp fragment and one 258bp fragment. Dra III cleaves C.psittaci which produces one 62bp fragment and 289bp fragment. Therefore, C.pneumoniae could be differentiated from C.psittaci by the size of restriction fragments.

Table 4. Primer position

Primer	sequence	position
CpnA	5' CTCCTTACAAGCCTTGCCTGTAGGG 3'	57-81
CpnB	5' GCGATCCCAAATGTTTAAGGC 3'	393-373

Numbered from the A of the ATG start codon

The optimization of the PCR

Experiments were conducted to find the optimal concentration of each component in the PCR. The different variables, primer concentration, MgCL2 concentration, cycle number and annealing temperature were changed to determine the optimal conditions. C.pneumoniae TWAR strain was used in these experiments. The PCR product was analyzed by agarose gel electrophoresis, and the results were obtained by the intensity of the products after staining with ethidium bromide. The primer concentration of 0.2µM had a good

yield and further increase in primer concentration did not produce more products. Figure 1 shows the titration of primers. The MgCL2 concentration with good yield was 2.0mM. Figure2 shows the titration ofMgCL2 concentration. The dNTP concentration in each reaction was 800uM(200uM for each nucleotide).The highest temperature that provided strong band was 68°C.Figure 3 shows the sensitivity of the PCRat 3annealing tempreture. To anneal and the extend of primers at the same time to simplify the procedure. Therefore, the three steps of a PCR can be changed to two steps by combining the annealing temperature and extension of the primers. The yield of PCR product was improved when 40 cycles were used. Figure 4 shows the effect of altering cycle number on the yield of PCR product. Also, Figure 5 shows the optimized PCR with 2 segments and 40 cycles, 68°C annealing temperature and DNAfrom c.pneumoniae.

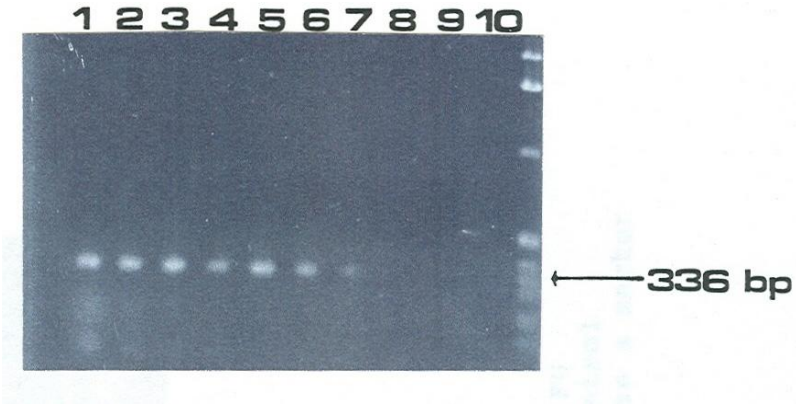

Figure1. Titration of primers CpnA and CpnB

Lane1 3.6uM primer	Lane6 0.1uM primer
Lane2 1.6uM primer	Lane7 0.05uM primer
Lane3 0.8uM primer	Lane 8 0.025uM primer
Lane4 0.4uM primer	lane 9 0.0125uM primer
Lane5 0.2uM primer	Lane 10 negative control
TWAR DNA with 2x 106 IIFU	Lane 11 1Kb ladder as a marker

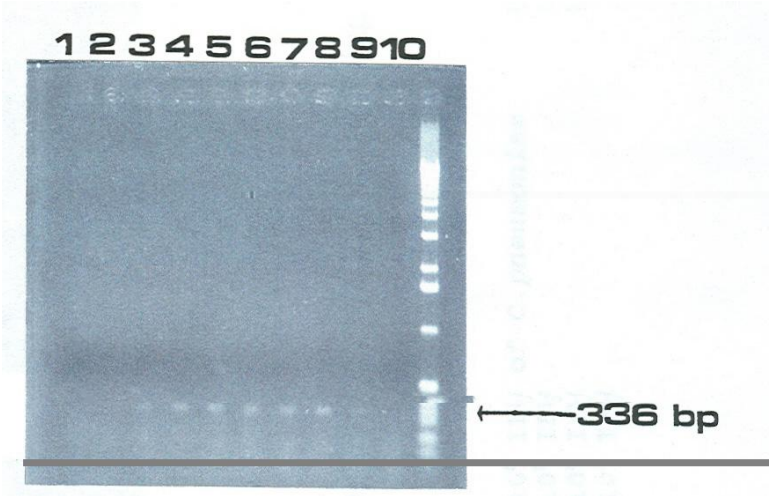

Figure 2. Titration of MgCL2 Concentration

Lane 1 without MgCL2	Lane 6 2.5 mM ″
Lane 2 0.5 mM ″	Lane 7 3.0 mM ″
Lane 3 1.0 mM ″	Lane 8 3.5 mM ″
Lane 4 1.5 mM ″	Lane 9 4.0 mM ″
Lane 5 2.0 mM ″	Lane 10 negative control
Lane 11 1Kb ladder as a marker	
TWAR DNA was used with 2x 106 IFU	

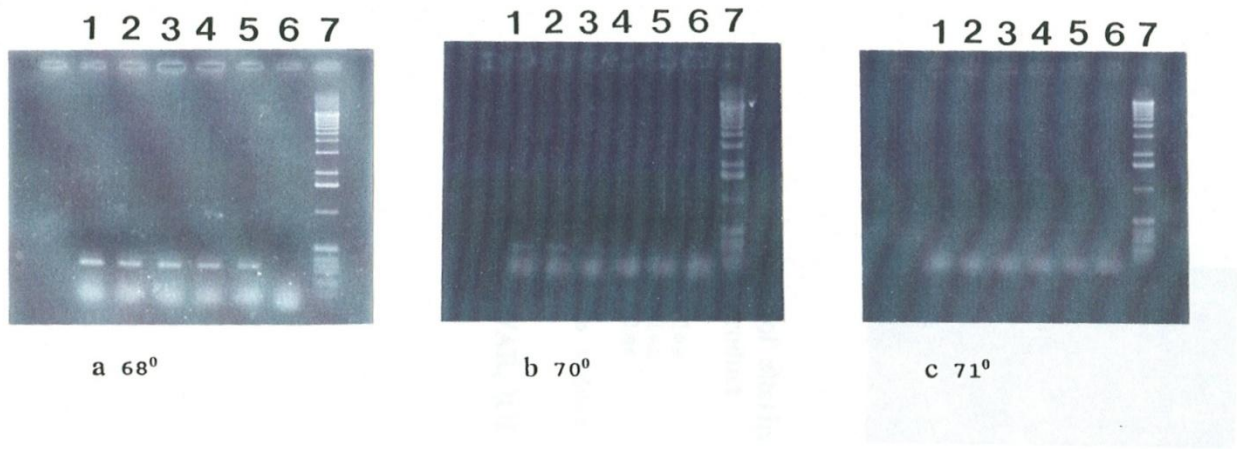

Figure 3. The sensitivity of the PCR at 3 annealing temperature a)68°C b)70°C c)71°C

Lane 1. 100 x 106 IFU of *C.Pneumoniae*
Lane 2. 50 x 106 IFU
Lane 3. 25 x 106 IFU
Lane 4. 12.5 x 106 IFU
Lane 5. 6.25 x 106 IFU
Lane 6. Negative control
Lane 7. 1Kb ladder as a marker

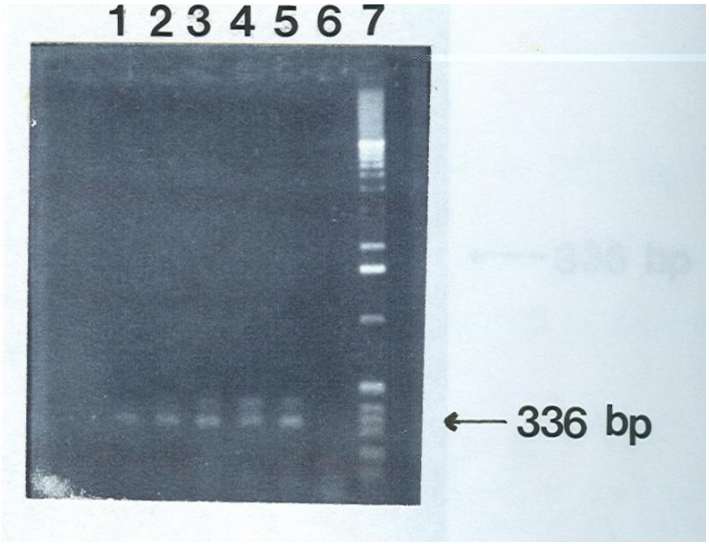

Figure 4. Effect of altering cycle number on the yield of PCR product

Lane 1 30 cycles
Lane 2 35 cycles
Lane 3 40 cycles
Lane 4 45 cycles
Lane 5 50 cycles
Lane 6 negative control
Lane 7 1Kb ladder as a marker
C.Pneumoniae DNA with 7.5 x 104 IFU

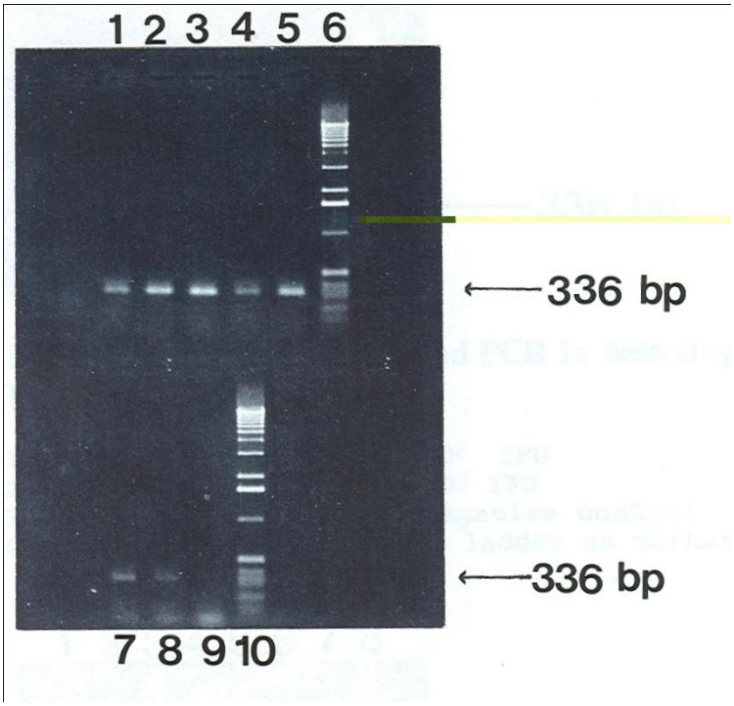

Figure 5. Optimized PCR with 2 segments and 40 cycles, 68°C annealing temperature and DNA from *C.Pneumoniae* (IOL-207)

Lane 1	7.5 x 106 IFU	Lane 6	1Kb ladder as a marker
Lane 2	7.5 x 105 IFU	Lane 7	1.5 x 102 IFU
Lane 3	7.5 x 104 IFU	Lane 8	1.5 x 10 IFU
Lane 4	1.5 x 104 IFU	Lane 9	negative control
Lane 5	7.5 x 103 IFU	Lane 10	1Kb ladder as a marker

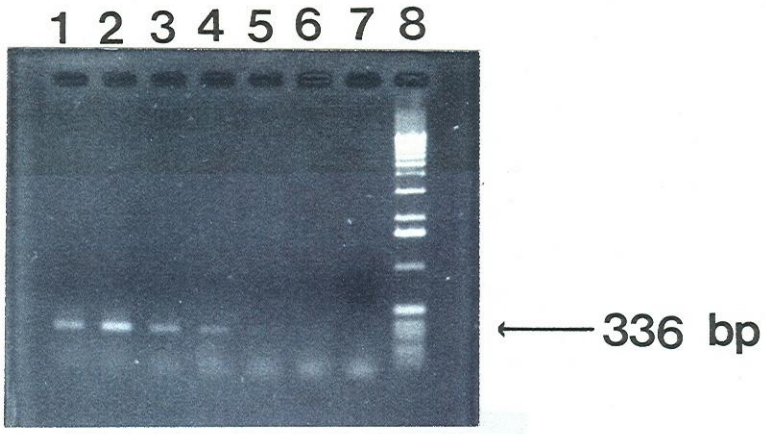

Figure 6. The sensitivity of the optimized PCR in detecting TWAR DNA (IOL-207)

Lane 1	104 IFU	Lane 5	100 IFU
Lane 2	103IFU	Lane 6	10-1 IFU
Lane 3	102 IFU	Lane 7	negative control
Lane 4	101 IFU	Lane 8	1Kb ladder as marker

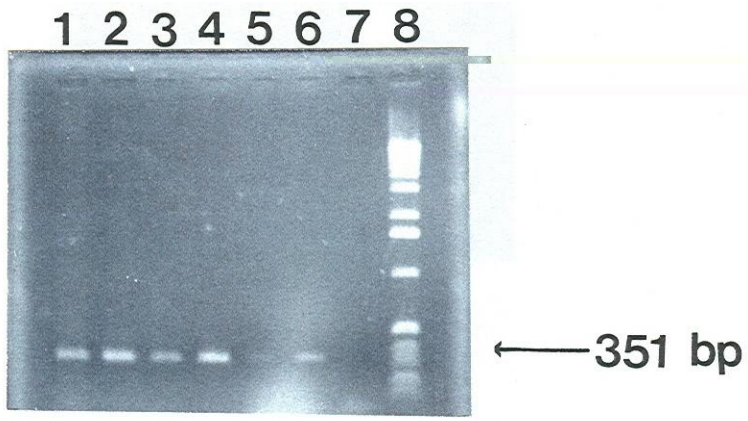

Figure 7. The sensitivity of the optimized PCR in detecting *C.Psittaci* (6BC)

Lane 1	105 IFU	Lane 5	101 IFU
Lane 2	104 IFU	Lane 6	1 IFU
Lane 3	103 IFU	Lane 7	negative control
Lane 4	102 IFU	Lane 8	1Kb ladder as a marker

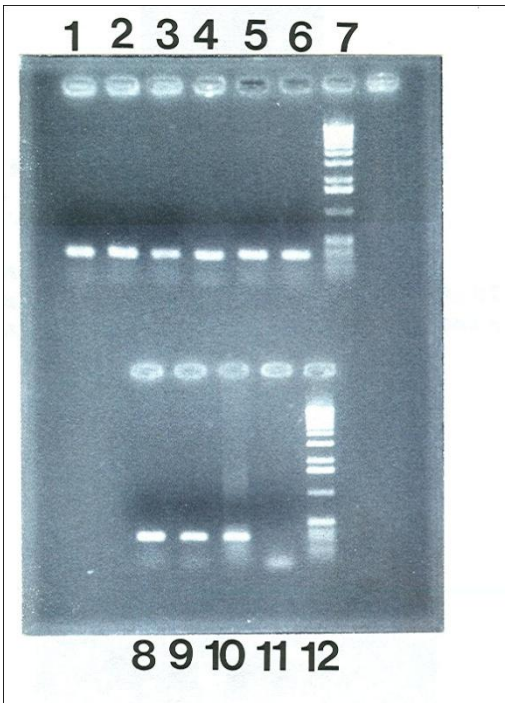

Figure 8. Detection of *C.Pneumoniae* and *C.psittaci* strains using the optimized PCR

Lane 1	6BC DNA	Lane 7	1Kb ladder as a marker
Lane 2	N352 DNA	Lane 8	IOL-207
Lane 3	EAE DNA	Lane 9	N16 DNA
Lane 4	GPIC DNA	Lane 10	VR 1310DNA
Lane 5	Fpn DNA	Lane 11	negative control
Lane 6	IOL-207	Lane 12	1Kb ladder

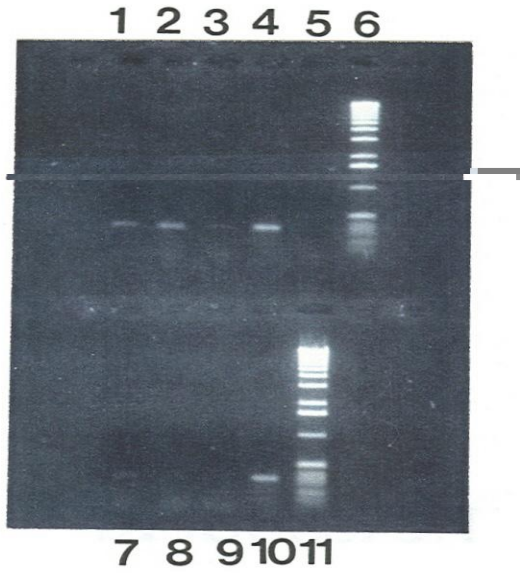

Figure 9. Detection of *C.Pneumoniae*(IOL-207) ind *C.Psittaci* (6BC)

Lane 1 103 IFU From *C.Pneumoniae*
Lane 2 102 IFU From *C.Psittaci*
Lane 3 10 IFU From *C.Psittaci*
Lane 4 103
Lane 5 1 IFU From *C.Psittaci*
Lane 6 11 1Kb ladder
Lane 7 102 IFU From *C.Pneumoniae*
L*ane* 8 10 IFU
Lane 9 1 IFU ″
Lane 10 positive control

Determination of sensitivity in PCR

The PCR sensitivity was evaluated by comparing the PCR result with infectivity. The DNA was diluted serially in distilled water and sensitivity of the PCR was one IFU as shown in Figures 6 and 7.

Determining the specificity of Chlamydia PCR

The specificity of the PCR was determined by testing three strains of C.pneumoniae, five strains of C.psittaci and two strains of *C.trachomatis*. All the strains were positive and produced a band of the expected size (336bp). All the *C.psittaci* strains were also positive with 351bp. Figure 8 shows different strains of Chlamydia.

The specificity of the PCR was determined by testing throat swabs as shown in Figure 9.

DISCUSSION

In this study polymerase chain reaction was developed to detect Chlamydia in throat swabs. The MOMP gene in different species was sequenced, and it showed there are regions with high degree of homology between the different species. We selected the primers from highly conserved regions of the MOMP gene which flanked variable domain I(VDI) which would be able to detect *C.pneumoniae* and *C.psittaci*. Tong and Sillis in 1993[15] used primers from same region of the MOMP gene in nested PCR with four primers. The optimization process showed a two-step PCR is a suitable method. The sensitivity was measured in term of infectivity (IFU), as it was assumed that in *chlamydial* one IFU represents one organism. A sensitiviry of one IFU was the aim, as other studies suggest that this level of sensitivity is required to be useful in a diagnostic situation. Tjhie et al. (1993) used PCR in detection of *Chlamydia* species using the MOMP gene as the target and also reported a sensitivity of one IFU after hybridization with a probe. The sensitivity of PCR in this study was equivalent to Tjhie's studies but without hybridization. It also reported the same sensitivity of one IFU after hybridization with a probe [13]. In this study, the sensitivity of PCR was equivalent to Tjhie's studies but without hybridization. The results from clinical samples indicated the presence of inhibitors which inhibits PCR. To overcome this, the samples were diluted ½ in

DDW, and were then retested by PCR. The sensitivity of PCR after dilution was 10 IFU for *C.psittaci* and 100IFU for *C.pneumonia*[16]. More recent studies have shown there is some variability in C.pneumoniae strains [10,17,18] and have reported variation in DNA sequences [19], antigenic variability and [20] morphology variation. The method for preparation of clinical samples is very important as others have found. In most of these studies proteinase K digestion was used to extract DNA, but in our study phenol/chloroform in addition to PK digestionwas used. The protainase K digestion and phenol/chloroform for DNA extraction were used.

Tjhie [13] used one freeze thaw step and boiling for 10 minutes, and then added DNA to PCR mixture. In most of these studies protainase K digestion was used to extract DNA, but in our study phenol/chloroform in addition to PK digestion was used.

It was thought that this would produce a clean DNA sample and have less problems with inhibitors. It is possible that it was residual phenol/chloroform in the DNA sample that affected the PCR and not inhibitors in the samples. Therefore, sample preparation needs more investigation to find the best method. PCR is an extremely sensitive and specific test which with further investigation of the specimen preparation could be used to test clinical samples. This method has the potential to be used as a rapid diagnostic test that would give a result in time to be helpful in choosing the antibiotic treatment for a patient with a respiratory illness. The PCR which is described here could be further developed into a multiplex PCR to simultaneously detect other microorganism such as *Mycoplasma Pneumoniae,* as well as *Chlamydia* in a single test. This type of multiplex test has great potential in the future as a screen for patients with atypical pneumonia.

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