II. LITERATURE REVIEWS

1. Common characteristics

Chlamydiaceae is a family of obligatory intracellular bacteria with a tropism for columnar epithelial cells lining the mucous membranes. They possess a cell wall similar to that of gram negative bacteria, contain DNA, RNA and ribosomes, and synthesis their own proteins, nucleic acids and lipids. They are susceptible to a wide range of antibodies (1).

The chlamydiae are non-motile, lacking in flagella and non-pilated. They do, however, possess unusual cylindrical surface projections, numbering 18 on average and arranged in a hexagonal array. During the developmental cycle, the chlamydiae has two morphologically distinct forms: the elementary body (EB) and the reticulate body (RB). Some characteristics of chlamydiae in comparison to those of bacteria, mycoplasmas and viruses are shown in table 1.

The EB is a small dense spherical body ranging from 0.2 to 0.4 μm in diameter. It is an infectious form of the organism, responsible for attaching to the target host cell and promoting its entry. The rigidity of the cell wall permits survival of the EB during its limited extracellular existence.

The RB is an intracellular, metabolically active form that divides by binary fission. It is larger than the EB, approximately 0.6 to 1.0 μm in diameter, and is osmotically fragile. The RBs synthesize their own DNA, RNA and proteins, but some of their metabolic capabilities are limited. For example, They cannot generate high-energy phosphate bonds. Thus, their adaptation for an intracellular habitat is due apparently to their total dependency on eucaryotic cells for energy.

Chlamydiae are sensitive to heat. Infectivity is lost completely within 10 minutes at 60°C and is drastically reduced within 48 hours at 37°C. Chlamydiae survive satisfactorily for about 24 hours. When freezing, an initial loss in infectivity occurs, but thereafter, it will remain stable at temperatures of -40°C to -70°C. Lympholization greatly reduces infectivity, but organisms that survive will remain infectious over a long period.

The stability of chlamydia is enhanced by adding protein, lipids, or certain ions to the diluent, such as 10% skim milk (SM), sucrose in a sodium phosphate buffer (SP) and sucrose in potassium phosphate with glutamate (SPG).

Chlamydiae are naturally sensitive to many antibiotics. All penicillin and cycloserine inhibit their replication through the cell wall synthesis. Tetracyclin, erythromycin and chloramphenicol inhibit the protein synthesis, while sulfonamide exhibit the inhibition of bacterial folate metabolism. These antibiotics can also inhibit chlamydial growth in cell cultures and sulfonamide is commonly the most used for clinical treatment (3).

Chlamydiae is classified on the basis of antigenic composition, intracellular inclusions, sulfonamide susceptibility and disease production. The family contains one genus: chlamydia and four species; *C. trachomatis*, which is inhibited by sulphonamides and produces iodine staining cytoplasmic inclusions, and *C. psittaci* and *C. pneumoniae* that are not inhibited by sulphonamides and do not produce iodine staining cytoplasmic inclusions. The last species is *C. pecorum*, which is found mainly in sheep and cattle, while the first three species cause diseases in humans (4).

2. Developmental cycle

The development of *C. trachomatis* in its host cells is about 48-72 hours. It consists essentially of four major steps.

Table 1. Characteristics of chlamydiae, bacteria, mycoplasma, and virus (2).

Characteristics	Chlamydia	Bacteria	Mycoplasma	Virus
Size (<500nm)	+	-	+	+
Obligatory intracellular	+	-	-	+
DNA/RNA	+	+	+	-
Cell wall	+	+	-	-
Ribosomes of prokaryotic type	+	+	+	-
Metabolism of carbohydrate	+	+	+	-
Incorporation with host nucleic acid	-	-	-	+
Binary fission	+	+	+	-

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2.1 Attachment and penetration

During the first six to eight hours, the infected EBs become attached to the microvilli of a susceptible columnar epithelial cell. They travel down the microvilli and localize in the indentations of the plasma membrane resembling the coated pits. EBs containing endosomes begin to fuse with one another and, perhaps, lysosomes. The chlamydiae remain bound and protected by the endosome membrane throughout their intracellular development.

2.2 Transition and development of RBs

The EBs undergo a transition to the RB's form and they lose their infectivity. The cell wall loses its rigidity and becomes a spheroplastlike. At this time, DNA, RNA and proteins are synthesized. The RBs increase in number and size. They are divided by binary fission. Only the RBs are seen in the vacuole after 18-24 hours of infection.

2.3 Maturation of RBs

During 24-48 hours of infection, the immature RBs condense to become infectious EBs. The RNA production decreases, while DNA and Glycogen increase.

2.4 Release of EBs

About 48-72 hours after infection, infectivity reaches its maximum. Glycogen degrades and eventually becomes undetectable. The inclusion becomes filled with mature EBs, which are then released by the destruction of the host cell.

3. *Chlamydia trachomatis* infections

Human infections caused by *C. trachomatis* primarily involve the eyes and genital tract. The organism is responsible for significant diseases, including blinding trachoma, severe genitourinary pathology, and neonatal pneumonia. Chlamydial diseases are distributed worldwide. However, the incidence of a specific disease may be more prevalent within certain populations, as determined by geographic location and/or socio-economic conditions.

3.1 Ocular infections

Trachoma is a chronic keratoconjunctivitis caused by *C. trachomatis* serotype A, B, Ba, and C, and is transmitted from eye to eye in endemic areas (2). Children act as the main reservoir for transmission of the disease. Serotypes D through K have been isolated from the eyes of trachoma patients in non-endemic areas and here the disease depends on genital tract to eye transmission. The *C. trachomatis* replicates on mucosal surfaces within columnar or transitional epithelial cells and stimulates a brisk infiltration of PMN cells. Submucosal lymphocytic infiltration is also impressive, leading to lymphoid follicle formation and fibrotic changes. As a result of the necrosis of the follicles, scars may develop at the conjunctiva and cornea. They may become damaged, due to both mechanical and secondary infection, resulting in blindness.

Inclusion conjunctivitis is a disease of the new born that derives from the passage through the infected maternal birth canal. It is caused by *C. trachomatis* serotypes D through K. The disease usually becomes clinically apparent 5 to 12 days after birth. It is characterized by a sticky exudate and conjunctivitis.

3.2 Genital tract infections

Chlamydia trachomatis infection of the genital tract is divided into two types:(1) that caused by the oculogenital serotypes D through K and (2) LGV caused by serotypes L1-L3. Chlamydial urogenital infections are caused mostly by serotypes D through K. *C. trachomatis* infection in men is the most common cause identified of nongonococcal urethritis (NGU) (5,6). Although urethritis usually results in a scant mucoid discharge, the disease cannot be distinguished clearly from gonococcal urethritis on clinical grounds: a spectrum from absence of discharge to a frankly purulent discharge from approximately 35-50% of men with NGU (7). The infection in adult women includes chronic cervicitis, urethritis, and salpingitis and pelvic inflammatory disease. Postpartum fever in infected mothers is also common. In addition, maternal cervical infection is associated with an increased rate of premature deliveries and perinatal morbidity. Ectopic pregnancies, may apparently be secondary to chlamydial salpingitis. This has increased in incidence over the past decade (8).

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by *C. trachomatis* serotypes L1, L2 and L3. These serotypes differ from oculo-genital groups, not only antigenically, but also biologically. LGV serotypes are more invasive than the other serotypes of *C. trachomatis*. The preferrential site for the multiplication of LGV is the regional lymph nodes, whereas serotypes of the trachoma group prefer squamocolumnar epithelial cells. The usual incubation period of LGV is 1 to 4 weeks. Early constitutional symptoms such as fever, headache, and myagia are common. The primary lesions are painless, small, inconspicuous, and vesicle, and they often escape notice. Characteristically, the complaint presented concerns enlarged matted inguinal and femoral lymphnodes, which are moderately painful and firm, and may fluctuate. In the United States, especially male homosexuals are likely to experience LGV infection and they constitute a major reservoir of the disease.

3.3 Respiratory tract infection

Neonatal Pneumonia is caused by *C. trachomatis* serotype D through K. The infection usually occurs at the age of 4 to 16 weeks. The disease has prominent respiratory symptoms such as wheezing, coughing, and a lack of systemic findings of fever or toxicity. They may be eosinophilic and have elevated serum IgG and IgM, with a very pronounced titer to the infecting serotype. Chlamydial neonatal conjunctivitis often precedes the onset of pneumonia.

4. Antigens and immunotypes

At least three major groups of *C. trachomatis* antigen have been identified: genus-specific antigens, species-specific antigens, and serotype-specific antigens (1).

4.1 Genus-specific antigens

All members of the genus share a common heat stable (100 °C for 30 min) complement-fixing antigen, referred to as the "Chlamydial group antigen". This group antigen is extracted readily with ethyl ether, sodium lauryl sulfate, or sodium deoxycholate. The chemical nature of the group antigen is a lipocarbohydrate. The ability to fix the complement is lost after the removal of lipid or periodate oxidation. Dhir *et al.*(9) isolated a large water-soluble polysaccharide ($0.2\text{-}2.0\times10^6$ dalton) after the alkaline hydrolysis of partially purified ethyl ether-extracted antigen. The isolated polysaccharide failed to fix the complement, but was capable of doing so after using crude antigen. It was identified as an acidic glycosidically polysaccharide, bound to serologically inactive neutral carbohydrates. The immunodominant antigen was similar, but not identical to the 2-keto-3 deoxyoctanoic acid of salmonella lipopolysaccharide.

4.2 Species-specific antigens

This antigen was shown to be common in all 18 serotypes of *C. trachomatis*, but it was not shared by the mouse pneumonitis strain or *C. psittaci*. The *C. trachomatis* species-specific antigens were purified to homogeneity by immunoadsorbtion, using a monospecific antibody (10). The antigen was shown to be a protein with a subunit molecular weight of 1.55×10^5 dalton, whose antigenicity was destroyed by heating at 56 °C for 30 minutes or by treatment with pronase. This antigen appeared to be highly immunogenic and was common to all *C. trachomatis*, which made it an attractive candidate for a serologic test antigen. Similarly, purified reticulate bodies appeared to be species specific by immunofluorescence, and could be useful in serology (11).

4.3. Serotype-specific antigens

Serotype-specific antigens are the determinants common only to certain chlamydial isolates within a species and have been referred to as subspecies antigens. They have been used for serologically classified chlamydial isolates. Bell *et al.*(12) reported that the mouse toxicity prevention test (MTPT) was utilized to serologically classify the ocular trachoma strains of *C. trachomatis*. Wang *et al.*(13) developed an indirect microimmunofluorecence (MICRO-IF) test for simultaneous identification of the *C. trachomatis* strain, using terminal dilutions of antisera. To date, 18 distinct antigenic serotypes of *C. trachomatis* have been identified (14).

The chlamydial antigen that confers type, group and species specificities of the organism, is the major outer membrane protein (MOMP)(15,16,17). A comparative analysis of their amino acid sequences showed that the MOMP genes encoded a highly conserved protein structure that contained four variable domains (VD1-VD4), whose sequences varied among the different serotypes. The locations of these VDs in MOMP were VD1, amino acid residues 64 to

83; VD2, residues 139 to 160; VD3, residues 224 to 237 and VD4, residues 288 to 317.

The serotypes and serogroup specific determinants were mapped to three domains: VD1, VD2 and VD4. The VD1 and VD2, the greatest sequence variations, were the locations of serotype-specific determinants. VD4, the largest domain located near the C-terminus of the protein, was the location of the subspecies, serogroup and species-specific antigenic determinant. No antigenic determinants were mapped to VD3-the smallest domain of the MOMP gene. VD1, VD2 and VD4 protruded toward the external environment of the chlamydial surface, as shown by their accessibility to antibody binding. In addition, tryptic cleavage in both VD1 and VD4 decreased chlamydial binding to the cell culture. This suggested that these domains could function as a chlamydia ligand, responsible for both pathogenesis and protective immunity.

5. Laboratory diagnosis

5.1 Direct cytopathologic examination

Chlamydia trachomatis infected cells develop compact, clearly defined, glycogen-containing, intracytoplasmic microcolonies or inclusions. These are usually found in infected yolk sac preparations, infected animal tissue, inoculated tissue culture cells and conjunctival scrapings of persons with active trachoma or inclusion conjunctivitis. The impression smears may be stained by Giemsa or iodine. The Giemsa's stain is simple to perform, inexpensive and it provides a permanent preparation. The iodine staining for glycogen-containing inclusion of *C. trachomatis* is also simple and rapid for screening many sides in a relatively short period of time. However, the sensitivity of this method is poor, and it is unreliable since glycogen is present only during certain periods in the developmental cycle.

5.2 Rapid antigen detection methods

Direct fluorescent antibody (DFA) staining and enzyme immunoassay (EIA) methods are for the direct detection of *C. trachomatis* from genital, conjunctival and urine samples. These techniques are more sensitive than conventional direct-staining methods. They have the advantage in detecting small amounts of specific antigens or intact organisms (18). DFA staining requires considerable experience and thoughtful interpretation. It is not well suited for processing a large number of specimens. EIA is automated and can be applied to process large numbers of samples. It requires minimal training and equipment, and provides quantitative results. The recent development of rapid monoclonal antibody-based membrane culture EIA devices may offer further advantages in sensitivity and ease to perform the detection of the chlamydia antigen.

5.3 Isolation of *C. trachomatis*

The isolation of *C. trachomatis* was accomplished by the inoculation of infected material into embryonated eggs, experimental animals or selected cell lines (1). The isolation in embryonated eggs was an impractical method for clinical laboratories since it was tedious and time-consuming. Some strains of chlamydia could infect mice, which are dependent on the route of inoculation. This made it possible to partially characterize a strain of chlamydia. LGV strains usually infected mice inoculated intracerebrally, but other *C. trachomatis* did not infect them by any route of infection. Moreover, they caused a rapid toxic death in mice when injected intravenously. The isolation of *C. trachomatis* in mice was less sensitive than inoculation in either cell cultures or embryonated eggs. However, the inoculation in all cultures was the most sensitive and specific method for the isolation and identification of chlamydia. The cell lines, which were commonly used to isolate chlamydia, were McCoy, Hela 229 and BHK (Baby Hamster Kidney) 21. After

incubating the cultures for 48 to 72 hours, chlamydial inclusions were visualised from using Giemsa, iodine or FA staining. Of these three methods, FA staining was the most sensitive for identifying the inclusion bodies after primary culture (19).

5.4 Serologic diagnosis

The microimmunofluorescence (MICRO-IF) and complement fixation (CF) tests are for the direct detection of antibodies in clinical samples such as serum, tears and genital secretions. The CF test, using a genus- specific antigen, is applied for the diagnosis of infections caused by *C. psittaci, C. pneumoniae* and the LGV strains of *C. trachomatis*. The MICRO-IF test has been used widely in the diagnosis of ocular and genital infections caused by *C. trachomatis*. This method is more sensitive than the CF test (13). In primary infection, the early antibody formation is the IgM class, which persists for approximately 1 month before being replaced by the IgG. For reinfection with the same serotypes, results in increased IgM titers to the new serotypes and the level of IgG to the previous immunotype are also elevated.

5.5 Direct nucleic acid detection

Nucleic acid hybridization is the molecular technique that couples the target nucleotides (DNA or RNA) and the detecting nucleotide probe. The detecting nucleotide probe is a short chain, containing 20-30 nucleotides that can be DNA or RNA. The hybridization occurs when the target and probing DNA are complementary. The DNA probe is labelled with radioisotopic or non-radioisotopic material. This technique can detect the Chlamydial from the specimen directly, but it still has a limitation if the specimen contains rare chlamydial organisms (19,20).

5.6 Polymerase chain reaction (PCR)

Polymerase chain reaction is an ingenious new tool for molecular biology. It is sensitive in that a single DNA molecule can be amplified and visualized as distinct bands (8,21). PCR is an *in vitro* method of nucleic acid synthesis, by which a particular segment of DNA can be specifically amplified. It involves two oligonucleotide primers that frank the DNA fragment, then amplify in repeating cycles. Each cycle consists of heat denaturation of the DNA, annealing the primers to their complementary sequence target at the 3-end, and finally, extending the annealed primers with *Tag* DNA polymerase. These primers anneal to opposite strands of the target sequence and are oriented so that the DNA synthesis, by polymerase, proceeds across the region between them. Since the extension products themselves are also complementary and capable of binding to the primers, successive cycles of amplification essentially double the amount of the target DNA synthesis in the previous cycle. The result is an exponential accumulation of the specific target fragment, of approximately 2^n, where "n" is the number in cycles of the amplification performed (22,23).

It has been found that the PCR technique is highly specific in the detection of *C. trachomatis* in clinical samples (24,25), and about 100 fold more sensitive than the commonly used cell culture method. Because of the simplicity and sensitivity of PCR, it may be useful and also applicable for the routine diagnosis of *C. trachomatis* infection, especially in studying the prevalence of asymptomatic *C. trachomatis* infection. This infection has been often associated with low copy numbers of *C. trachomatis*. Lan *et al.* reported that the prevalence rates of *C. trachomatis* in asymptomatic and symptomatic women younger than 30 years in the Netherlands were 9.2 % and 11.8 %, respectively (26). In addition, the prevalence rates of *C. trachomatis* infection in Chiang Mai, Thailand were found by PCR to be 12.4 % and 4 % in a high and low risk group, respectively (27). Whereas, a previous

report showed the prevalence rate as 14.39% in a high risk group by using the nucleic acid hybridization method (28).

6. Genotyping of *C. trachomatis*

The serotype classification of *C. trachomatis* is based on the serologic recognition of antigenic epitopes on the MOMP. The first method for typing *C. trachomatis* was the microimmunofluorescence (MICRO-IF) test (29,30). For this test, an early immune serum was obtained from mice that had been immunized intravenously with the *C. trachomatis* isolate. The elementary body (EB) was used as a test antigen that reacted with immune sera to different strains of the organisms. The MICRO-IF test used polyvalent antisera that could identify 15 serotypes of *C. trachomatis*. These 15 serotypes were placed into three groups : C (C, J, A, H, I), B (B, Ba, D, E, L1, L2) and intermediate (G, F, K, L3) (31,32). Then the MOMP-specific monoclonal antibodies became available and the identification of three new subtypes or serotypes: Da, Ia and L2a, was presented (14). However, this technique still has some limitations in propagation of the organisms, since some strains cannot be cultured or they grow poorly in the cell culture. Also, a large number of infected cells are needed for typing. In addition, this technique requires a panel of monoclonal antibodies, which are expensive and not commercially available.

The genotyping of different *C. trachomatis* serotypes using the restriction fragment length polymorphism (RFLP) technique has been studied by many investigators (15,33,34). This method is based on the nucleotide variation in the MOMP gene of different serotypes of *C. trachomatis*. The epitopes that distinguish the serotypes of *C. trachomatis* reside principally on the MOMP, which is responsible for the antigenic variation of the protein. The MOMP consists of five constant domains (CDs) separated by four variable domains (VDs). The polymerase chain

reaction (PCR) technique was used to amplify a part of DNA including the four VDs (1,200 bp) for further use in RFLP and sequencing.

The RFLP genotyping of *C. trachomatis* is based upon the differential restriction endonuclease digestion of PCR-amplified MOMP gene sequences. Frost *et al.*(33) and Rodriguez *et al.*(35) also amplified the MOMP gene of *C. trachomatis* reference strains and performed RFLP by using one to four restriction endonuclease (RE). This capably distinguished at least 13 different serotypes of *C. trachomatis*. The overall distribution of *C. trachomatis* serotypes in worldwide clinical specimens tested by the RFLP showed that serotypes E (22.9-52.0%), D (12.5-31.4%), and F (8.8-31.0%) were the most prevalent. However, serotypes E, D and F together accounted for 60-70% of *C. trachomatis* infection (15,33,34,35,36). Frost *et. al.* reported that the distribution of *C. trachomatis* serotypes did not differ significantly between men and woman (37). The serotypes of *C. trachomatis* were observed from 28 PCR positive samples in Chiang Mai, Thailand by using RFLP, and the *C. trachomatis* serotypes F and H/J were found to predominate among the clinically isolated (27).

A problem often occured when the number of amplified DNA fragments was greater than 1,000 base pair including 5 CDs and 4 VDs. The PCR product was very low, and not enough for subsequent digestion by the restriction enzyme. In addition, the nucleotide sequence of the MOMP gene differed not only in the VDs, but also, and to a lesser extent, in the CDs that make a more complex banding pattern after RFLP. This made identification difficult . In contrast to earlier efforts, only the largest variable domain, VD4 of the MOMP gene, was chosen for amplification and subsequent typing to overcome the problem. A small (350 bp) amplified product gave a fewer number of less complex DNA banding patterns after RFLP, thus enabling differentiation from each other.

Leechanachai *et al.*(38) showed the restriction pattern of the VD4-MOMP gene in 18 serotypes of *C. trachomatis*, with the reference strain analysed by the computer programme, DNASIS version 2.1, as shown in Table 2. The nucleotide sequences of *C. trachomatis* reference strains were download from the GenBank (National Center for Biotechnology Information, NCBI). The Nest 2 and Nest 4 primers derived from the conserved sequence with in the MOMP gene and covered only the VD4. The sequence bracketed by these primers was simulated. The Nest 2 - Nest 4 fragment of each serotype was analysed for the restriction site of all different restriction endonuclease by using the above mentioned computer programme. The number and sizes of the restricted fragments, generated by each enzyme, were determined in order to find an appropriate restriction treatment of the fragment which could, in turn, give a different electrophoresis profile for each serotype. According to the results of the computer analysis, the Nest 2-Nest 4 amplified DNA were separated by digesting with 4 restriction endonucleases: *Alu*I, *Hind*III, *Dde*I and *EcoR*II. The projected digested fragment size of all *C. trachomatis* serotypes was tabulated (Table 2.). At least 10 out of 18 serotypes were identified as A/I/L3, H/J/Ia, C, E, L2/L2a, D/Da/L1, B/Ba, F, G, and K. If necessary, serotypes A/I/L3 could be separated further by using *Hpa*II and *Mae*III, while serotypes D/Da and L1 were divided by *BsmA*I. The VD4-RFLP successfully separated most of the predominate genital serotypes of *C. trachomatis* presented.

Table 2. The fragment sizes of PCR-RFLP in the VD4-MOMP gene of 18 *C. trachomatis* serotypes, generated from the GenBank database and analysed by computer programme DNASIS version 2.1.

Group	Serotype of *C. trachomatis*	*Alu*I	*Hind*III	*Dde*I	*EcoR*II
B-complex	B	142	267	238	348
	Ba	142	267	238	348
	D	145	267	348	348
	Da	145	267	348	348
	L1	142	267	348	348
	E	142	348	348	348
	L2	142	348	237	348
	L2a	142	348	237	348
Intermediate	F	160	270	351	351
	G	160	350	350	350
C-complex	K	173	350	242	350
	C	330	350	241	348
	A	348	348	237	348
	I	350	350	240	350
	L3	351	351	241	351
	H	351	351	240	146
	Ia	351	351	240	146
	J	350	350	240	146

7. Genetic polymorphism of the MOMP gene

The sequencing of the PCR-amplified MOMP DNA fragment was applied to the molecular epidemiology of *C. trachomatis.* At least 18 serotypes were found among clinical isolates of *C. trachomatis* (14,39). The MOMP gene sequence comparisons among serotypes were revealed in all VDs.

From the VDs of *C. trachomatis* (ATCC), 14 serotypes were sequenced by Yaun *et al.*(15) and Yang *et al.*(12). These serotypes were A/Har-13 (VR 571B), Ba/AP-2 (VR 347), C/TW-3 (VR 578), D/UW-3 (VR885), E/Bour (VR 348B), F/IC-Cal-3 (VR 346), G/UW-57 (VR 878), H/UW-4 (VR 879), I/UW-12 (VR 880), J/UW-36 (VR 886), K/UW-31 (VR 887), L1-440 (VR 901B), L2-434 (VR 902B) and L3-404 (VR 903B). In addition, the clinically isolated strains, Da/TW-448, Ia/UW-202 and L2a/TW-396, were sequenced by Dean *et al.*(21), while serotype B/TW-5 was sequenced by Yaun *et al.*(16). All serotypes were already immunotyped by the MICRO-IF test and their nucleotide sequence was generated from the GenBank database.

The comparative analysis of nucleotide sequence homologies of all 4 VDs of 18 serotypes were separated and placed into three groups: B complex group, serotypes B, Ba, D, Da, E, L1, L2,and L2a; intermediate group, serotypes G and F; and C complex group, serotypes A, C, H, I, Ia, J, K, and L3. On the basis of MOMP gene diversity among *C. trachomatis* serotypes, the clustered nucleotide substitution was found to be closely related to the serotypes, while the insertions or deletions were observed among distantly related serotypes. These diversities were found mostly in VD1, VD2 and VD4, while the VD3 sequences of most serotypes were rather conserved. Three new chlamydial serotypes were compared to their prototype serotypes. Dean *et al.*(21) reported that the serotype Da differed with only one nucleotide substitution and one amino acid transition in VD4. As the serotype L2a varied from the L2 serotype in VD2 with one nucleotide substitution and one amino

acid transition. Meanwhile, the Ia differed from the I serotype in VD1 and VD2 with one nucleotide substitution and one amino acid transition, in the VD4, 4 nucleotide and amino acid substitution.

The VD4 was the largest domain, with the position of nucleotide sequence located from 928 to 1017, which resulted in amino acid from 288 to 317 on the MOMP gene. The nucleotide sequences of the VD4-MOMP gene of the 18 *C. trachomatis* reference serotypes were available in the GenBank database, and they were then used as a prototype sequence for the comparative analysis of the VD4-MOMP gene of serotypes from clinical samples. These nucleotide sequences are shown in Figure 1.

The serotypes B, Ba, D, Da, E, L1, L2, L2a, A and I, contained 90 nucleotides, encoded 30 amino acids, and 93 nucleotides encoded 31 amino acids for the remaining serotypes C, F, G, H, Ia, J, K and L3in the VD4-MOMP gene. The serotypes B and Ba had identical nucleotide sequences, as did serotypes L2 and L2a. Serotypes Ia could be differentiated from serotype I, but serotypes H had identical nucleotide sequences.

This nucleotide sequencing method will be useful in future for the identification of chlamydial genotypes and the evolution of variants or new types. Yang *et al.* reported that the nucleotide sequences of VD1, VD2 and VD4-MOMP genes of *C. trachomatis* were determined in 48 positive clinical samples. Thirty samples (61%) had prototype VD sequences and 15 (31%) had those that varied from prototype sequences. Eleven of 15 (73%) nucleotide sequence variations had two or fewer nucleotide substitutions in one or two VDs, which represented point mutation, while the other 4 (27%) had a large number of nucleotide changes that represented genetic recombination. This exhibited a nucleotide sequence of some VDs that were mostly close to one genotypes, while the other VDs resembled other genotypes (34).

Serotype	Stain	928 ↓ Nucleotide sequence of VD4-MOMP gene of *C. trachomatis* 1017 ↓
B	B/TW5/OTS	TCAGCCGAGACTATCTTTGATGTTACCACTCTGAACCCAACTATTGCTGGAGCTGGCGATGTGAAAACTAGC GCA- - - GAGGGTCAGCTCGGA
Ba	Ba/AP-2	. . . . - - - . . . .
D	D/UW-3	. . . . . TACAG . . . . T . . . . . . AC . . . . . .G . .T. . . . . .G . . . . . - - - . . . .
Da	Da/TW-448	. . . . . TACAG . . . . AC . . . . . .G . . T. . . . . .G . . . . . - - - . . . .
E	E/Bour	. . . . . TACAG . . . . AC . . . . . .G . . T. . . . . .G. . . . . . . - - - . . . .
L1	L1-440	. T . . . TACAG . . . . AC . . . . . .G . . T. . . . . .G . . . . . .G . . . A. . . . . - - - . . . .
L2	L2-434	. . . . . TACA . . . G . . . . AC . . . . . .G . . T. . . . . .G . . . . - - - . . . .
L2a	L2a/TW-396	. . . . . TACA . . . G . . . . AC . . . . . .G . . T. . . . . .G . . . . - - - . . . .
F	F/IC-Cal-3	TTGGTAACAC CTGTTGTAGATATTACAACCCTTAAC CCAACTATTGCAGGATGCGGCAGTGTAGCTGGAGCTAACACGGAAGGACAGATATCT
G	G/UW-57	. . . . C . . A . . . . TC . C . . . . T. . . . .
C	C/TW-3/OT	TTGGCTGAAGCAATCTTGGATGTCACTACTCTGAACCCGACTATCGCTGGTAAAGGAAGTGTGGTCTCTGCCGGAACCGATAACGAACTGGCT
A	A/Har-13	. . . . . . A . . C . . G. . . . . AC . . . . A . . . . C . . . . .C . . . . T . . .C . - - - . .A . . . .
H	H/UW-4/Cx	. . . . A . . . . C . . . . .C . . . . G . . T . . . . . G . . . . C. . . . . .
I	I/UW-12/Ur	. . . . A . . . . C . . . . C . . . . T . . . . . - - - . . . .
Ia	Ia/UW-202	. . . . A . . . . C . . . . .C . . . . . . .G . . T . . . . . G . . . . C. . . . .
J	J/UW-36	. . . . A . . . . C . . . . .C . . . . . . .G . . T . . . . . G . . . A. . . . . C. . . . . .
K	K/UW-31	. . . . A . . . . C . . . A . . . . GC . . . . T . . . . . G . . . .
L3	L3-404	. . . . G . . . . A . . . . C . . . . .G . . T . . . . C. G . . . A . . . .

Figure 1. Nucleotide sequences of the VD4-MOMP gene of the 18 *C. trachomatis* reference serotypes, generated from the GenBank database.

Brunham *et al.*(40) studied the nucleotide sequence variation of the MOMP gene of *C. trachomatis* that was isolated from patients with sexually transmitted diseases (STD), who were high risk and exposed repeatedly to a STD pathogen. They found that 63% had a variant VD sequence, while 32% possessed the prototype. Among the variant type, 48% had a point mutation and 52% displayed a genetic recombination. This recombination could have resulted from co-infection with multiple *C. trachomatis* genotypes. Furthermore, Lampe *et al.*(41) also reported that genetic recombination was a potential genetic mechanism for the generation of MOMP sequence polymorphism. However, in both the genetic recombination of VDs between two distinct genotypes and accumulated point mutation with nucleotide substitution, deletion or insertion converging on prototype sequence could be the genetic mechanism for generating MOMP sequence polymorphism. This polymorphism could have aided the organisms in establishing infection or persistence in persons who had neutralizing antibodies to non-variant MOMP epitopes.

In this study, the nucleotide sequences of the *C. trachomatis* VD4-MOMP gene was determined by using the cycle sequencing technique with the ABI PRISM^{TM} dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, USA). This method was based on the dideoxynucleotide chain termination technique of Sanger *et al.*(42). Since the 2', 3' dideoxyribonucleoside triphosphates (ddNTPs) lacked a hydroxyl residue at the 3' position of deoxyribose, the formation of a phosphodiester bond with the succeeding dNTP was prevented. The extension of the growing DNA chain was therefore terminated.

Interestingly, with the dye terminators labelled in this kit, each of the four ddNTPs was tagged with four different fluorescent dyes: A-dye T, C-dye T, G-dye T and T-dye T. They were able to be incorporated into a DNA extension product by Ampli *Tag* DNA polymerase, FS enzyme. Thus, the growing chain was terminated simultaneously and labelled with the dye that corresponded to that base. While Ampli

Tag DNA polymerase, FS enzyme was a member of the *Tag* F667Y family, in which phenylalanine was substituted by a tyrosine at position 667, it could incorporate chain termination ddNTPs more efficiently than the *Tag* DNA polymerase. This enzyme provided rapid nucleotide incorporation and had no 3' to 5' exonuclease activity. Moreover, this kit was suitable for performing cycle sequencing reaction on a single- or double-stranded DNA template or PCR product (43).

The ABI 310 automated DNA sequencer detected fluorescence from four difference dyes that were used to identify the A, C, G and T extension reactions. Each dye emitted light at a different wavelength when excited by a laser. Thus, all four colours in the single reaction could be detected and distinguished in capillary electrophoresis. The direct on-line monitoring of the sequencing gel capillary electrophoresis and direct data entry were controlled by computer programme ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2 (Perkin-Elmer, Applied Biosystems, USA).