GENOTYPE DISTRIBUTION OF GENITAL *CHLAMYDIA TRACHOMATIS* IN CHIANG MAI, THAILAND

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Abstract. Urogenital isolates (N=84) of *Chlamydia trachomatis* collected from high-risk STD subjects in Chiang Mai and the surrounding areas were investigated for genotype distribution. *C. trachomatis* genotypes were determined by the PCR-based RFLP technique and confirmed by nucleotide sequencing. By this method, the VD4 DNA of the MOMP gene was amplified and digested separately with 4 restriction endonucleases, *Alu*I, *Hind*III, *Dde*I, and *Eco*RII. The nucleotide sequence was determined by dye terminator cycle sequencing. Eight different *C. trachomatis* genotypes were identified: genotype D (34.5%), F (21.4%), K (13.1%), H/Ia (8.3%), E (7.1%), B/Ba (7.1%), G (6.0%), and J (2.4%). Genotype D and F were the commonest, accounting for 56% of the *C. trachomatis* infections. When nucleotides of the VD4-MOMP gene were anlyzed, 43 samples (51.2%) had nucleotide sequences that differed from the prototypes, while 41 (48.8%) were identical. Nucleotide substitution mutation was the major mechanism in these variants; changes in nucleotide sequences usually resulted in amino acid substitution, which could lead to a modification of antigenic determinants and the consequent evasion of immune responses.

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium that causes a wide spectrum of human diseases; it affects millions of people worldwide. The most notable diseases are blinding trachoma in developing countries, caused by *C. trachomatis* serotypes A, B, Ba and C, and genitourinary tract infections in developed countries, caused by serotypes D through K (Schachter, 1999). *C. trachomatis* genital infection in women is usually subclinical, although more complicated forms, such as pelvic inflammatory disease (PID), tubal infertility, and ectopic pregnancy, are not rare. In men, urogenital *C. trachomatis* infection causes urethritis. The severe genital infection, lymphogranuloma venereum, results from infection with L1, L2 or L3 (Stamm, 1999). *C. trachomatis*, has been serologically differentiated into 19 serotypes by both polyclonal and monoclonal antiboides directed against epitopes of the major outer membrane protein (MOMP). The MOMP gene contains four hypervariable domains (VD1-4) that are separated and flanked by five constant domains. Epitope mapping revealed that proteins encoded from the VD1, 2, and 4 regions are surface-exposed and contain serotype-specific determinants, which play a role in protective immunity. While the protein encoded from VD3 does not protrude from the membrane, it can elicit a T -cell helper function (Baehr *et al*, 1988; Stephen *et al*, 1988; Allen *et al*, 1991).

Recently, a number of sequence variations have been observed in the VD regions of the MOMP gene among *C. trachomatis* trachoma and genital serotypes isolated in different geographical areas (Dean *et al*, 1992; Lampe *et al*, 1993; Morre *et al*, 1998). These variations may occur by random mutation or immune selection. However, alteration in the surface antigenic molecules of microorganisms may result in virulence or the evasion of host immune surveillance (Bruham *et al*, 1994). Differentiation of *C. trachomatis* serotypes and sequence variation of the VD regions have been necessary in epidemiological studies in order to establish the community serotype prevalence of organisms and promote vaccine preparation. However, the serotyping of this organism is limited, due to the lack of commercially available antisera and the need for time consuming culture. Moreover, the monoclonal antibodies react only with known serotype - specific MOMP antigens. New variant serotypes connot be detected. Genotyping of *C. trachomatis* using molecular techniques has been developed in order to solve these problems. Restriction fragment length polymorphism (RFLP) and nucleotide sequencing of the MOMP gene are now widely used (Frost *et al*, 1991; Lan *et al*, 1993; Yang *et al*, 1993; Brunham *et al*, 1994). The PCR-RFLP of the whole MOMP gene often shows the complex banding patterns of the digested fragments, which make it difficult to interpret the result. The PCR- RFLP of a short sequence, especially the VD4 region, gives less complex patterns and is also useful in differentiating genital *C. trachomatis*

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genotypes (Leechanachai, 1996). Nevertheless, in order to detect the type of variant, nucleotide sequencing is needed because only the nucleotides that change in the recognition sequence can be detected as an altered RFLP pattern.

At present, there is no data available regarding *C. trachomatis* genotype infection among the Thai population. In this study, the genotypes of *C. trachomatis* isolated from clinical samples collected in Chiang Mai and the surrounding areas were determined by PCR-RFLP of the VD4-MOMP gene and confirmed by nucleotide sequencing.

MATERIALS AND METHODS

Clinical samples

The endocervical swabs were obtained from female sex workers who attended the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai Province, between January 1997 and August 1999. The patients came routinely for physical examination every two weeks and had no clinical symptoms of *C. trachomatis* infection at the time of investigation. The swabs were placed into a Gen-Probe lysis buffer and tested for the presence of *C. trachomatis* DNA by the nucleic acid hybridization test (Gen-Probe PACE 2 System, USA) at the VD and AIDS Control Center. Eighty-four positive samples were sent for genotyping and nucleotide sequence analysis at the Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University.

DNA extraction

DNA was extracted in a phenol/chloroform solution according to the protocol recommended by Sambrook *et al* (1989). Two hundred microliters of DNA lysate were transferred to a 1.5ml microcentrifuge tube. An equal volume of phenol/chloroform solution was added and centrifuged at 10,000rpm for 10 minutes. After collecting the supernatant from an aqueous phase, DNA was precipitated with a 0.5 volume of 7.5M sodium acetate and a 2.0 volume of absolute ethanol. The mixture was stored at -70˚C for 10 minutes and centrifuged at 10,000rpm for 30 minutes before the supernatant was discarded. The pellet was rinsed once with 70% ethanol and then air-dried. The pellet was resuspended in 50µl of sterile distilled water and kept at -70˚C for PCR amplification.

PCR amplification

The VD4 sequence of the MOMP gene was amplified by N2 and N4 primers, which generated the product to approximately 350bp. The oligonucleotide primers were synthesized according to the sequences shown at the WHO Chlamydial PCR Workshop from June 2 to 5, 1992 (England). The nucleotide sequence and location of these primers are shown in Fig 1. PCR was performed with a total volume of 50µl. The final reaction mixture contained 0.2mM of each of the following: deoxynucleotide triphosphate (dATP, dTTP, dGTP, and dCTP); PCR buffer (50mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 8.8); 50pmol of both the N2 and N4 primer; 1.25U of *Taq* DNA polymerase. The initial PCR reaction contained 10µl of DNA template. The reaction was amplified for 30 cycles in a thermocycler (GeneAmp 2400, Perkin-Elmer, Cetus, USA). Each cycle consisted of denaturation at 94˚C, annealing at 60˚C, and extension at 72˚C for 1 minute.

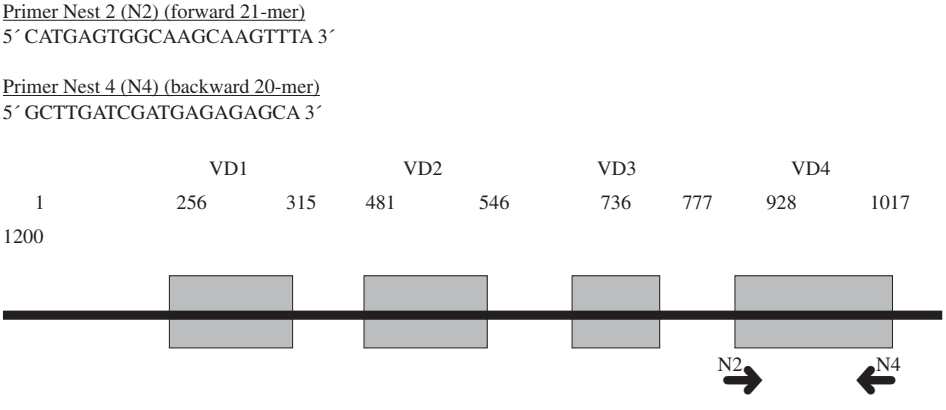

Fig 1- The sequences of the N2 and N4 oligonucleotide primers and their positions on the MOMP gene of *C. trachomatis*.

The VD4-MOMP amplified products were examined by agarose gel electrophoresis. The products were subsequently used for RFLP and nucleotide sequence analysis.

Genotyping by RFLP analysis

The RFLP analysis was performed with four restriction endonucleases (RE): *Alu*I, *Hind*III, *Dde*I, and *Eco*RII (GIBCO-BRL, Gaintherburg, USA). Ten microliters of the VD4-MOMP amplified product were digested separately with 5U of each RE in 20µl of reaction volume and incubated at 37˚C for 4 hours. The digested products were analyzed on 6% polyacrylamide gel electrophoresis. The DNA fragments were visualized under long wavelength ultraviolet light using an ultraviolet transilluminator, and measured for fragment size by reference to a 50bp DNA ladder marker (GIBCO-BRL, Gaintherburg, USA). The genotypes were identified by comparison with the RFLP pattern of the known *C. trachomatis* prototype generated with the aid of computer software (DNASIS 2.1) (Table 1).

Nucleotide sequencing

The amplified product of the VD4-MOMP gene was purified and used as the template for sequence analysis by ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Applied Biosystems, USA). The DNA template was sequenced in both directions using the N2 and N4 primers. The N2 primer was used to sequence forward through the VD4-region, while the N4 was a backward primer. The sequencing products were analyzed by the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA). The nucleotide sequence of the VD4 from each sample was analysed by comparison with the reference prototype strains via the computer program, Autoassembler™ 1.4.0 (Perkin-Elmer, Applied Biosystems, USA).

RESULTS

Genotyping of *C. trachomatis*

Among the 84 endocervical samples, 8 genotypes of *C. trachomatis* were identified. Twenty-nine were typed as genotype D/Da/L1, 18 were typed as genotype F, 11 were typed as genotype K, 9 were typed as genotype H/Ia/J, 6 were typed as both E and B/Ba, and 5 were typed as G. The data are shown in Table 2.

Since the nucleotide sequences in the VD4 region of genotype D, Da, and L1 were different in only a few bases, and as they were not in the recognition site of the restriction endonuclease used in the analysis, it was impossible to distinguish them into a single genotype by RFLP in this study. A group of genotype B/Ba and H/Ia/J exhibited the same problem.

Table 1
The fragment size of PCR-RFLP in the VD4-MOMP gene of 18 *C. trachomatis* genotypes, generated from the GenBank database and analyzed by computer program (DNASIS, version 2.1).

Genotype of *C. trachomatis*	*Alu*I	*Hind*III	*Dde*I	*Eco*RII
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
F	160	270	351	351
G	160	260	350	350
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

Nucleotide sequence of the VD4 region of the MOMP gene

In order to ensure that the genotypes were classified correctly from the RFLP pattern, nucleotide sequences of the VD4 region were determined in all 84 samples. The results of *C. trachomatis* genotyping by RFLP analysis corresponded to the results obtained by nucleotide sequencing in all samples. The data are shown in Table 3. In addition, the nucleotide sequence analysis differentiated a group of genotypes identified by RFLP as a single genotype (D/Da/L1 and H/Ia/J). Twenty-nine samples identified previously as a group of genotype D/Da/L1 were all genotype D, and 2 of genotype J were separated from 9 of genotype H/Ia/J. Furthermore, in a number of the type variants, 43 (51.2%) samples were identified in this study. They were 29 (34.5%) of genotype D, 2 (2.4%) of genotype G, 1 (1.2%) of genotype H/Ia, and 11 (13.1%) of genotype K. The remaining 41 samples (48.8%) had nucleotide sequences of the VD4 region identical to those of the reference prototypes (Table 4).

Table 2
Genotype distribution of *C. trachomatis* determined by VD4 PCR-RFLP.

RFLP genotype	n=84	%
B/Ba	6	7.1
D/Da/L1	29	34.5
E	6	7.1
F	18	21.4
G	5	6.0
H/Ia/J	9	10.7
K	11	13.1

DISCUSSION

By RFLP analysis of the VD4 region, 4 single (E, F, G, K) and 3 group (B/Ba, D/Da/L1, H/Ia/J) genotypes of *C. trachomatis* were identified. It was found that the most prevalent genotype was D/Da/L1 (34.5%), followed by genotype F (21.4%), K (13%), and H/Ia/J (10.7%). Genotype E, the commonest genotype in North America and many European countries (Rodriguez *et al*, 1993; Borrego *et al*, 1997; Van Duynhoven *et al*, 1998; Morre *et al*, 2000; Sturm Ramirez *et al*, 2000), was found in only 7%. Our findings corresponded with those reported from other Asian countries, such as Japan and India, where genotype D is predominant (NIH Japan, 1998; Mittal 1998). This might suggest a genotype-specific geographical dependence or a transmission advantage of genotype D over the other genotypes in this area. However, RFLP could not generally differentiate between some closely related *C. trachomatis* genotypes that had identical nucleotide sequences in the recognition sites of the appropriate restriction endonucleases. Therefore, genotypes B/Ba, D/Da/L1, and H/Ia/J could not be differentiated by this technique. After the nucleotide sequences of the VD4-MOMP gene were analyzed, all 29 samples originally identified as genotype D/Da/L1 by RFLP were classified as genotype D. However, their sequences were different from the previously reported prototype (Yuan *et al*, 1989), but similar to the D variant observed by both Pool and Lamont (1992) and Sayada *et al* (1995). The D variant genotypes are now more often found, along with the prototype; 73% of the D genotype reported in France were the sequence variant (Sayada *et al*, 1995).

Among the genotype H/Ia/J identified in this study, two were later classified as genotype J after nucleotide sequencing and the other seven were genotype H or

Table 3
Comparison of *C. trachomatis* genotypes determined by VD4 PCR-RFLP and nucleotide sequencing of the VD4-MOMP gene.

RFLP of the VD4-MOMP gene		Nucleotide sequencing of the MOMP gene	
Genotype	No. of samples n=84	Genotype	No. of samples n=84
B/Ba	6	B/Ba	6
D/Da/L1	29	D	29
E	6	E	6
F	18	F	18
G	5	G	5
H/Ia/J	9	H/Ia	7
		J	2
K	11	K	11

Table 4
Nucleotide sequence variation in the MOMP gene of *C. trachomatis*.

Nucleotide sequence variation of the MOMP gene	Genotype	N=84 (%)
Variant	D	29 (34.5)
	G	2 (2.4)
	H/Ia	1 (1.2)
	K	11 (13.1)
		43 (51.2)
Prototype	B/Ba	6 (7.1)
	E	6 (7.1)
	F	18 (21.4)
	G	3 (3.6)
	H/Ia	6 (7.1)
	J	2 (2.4)
		41 48.8

Ia. Since the VD4 sequence of genotype H and Ia and genotype B and Ba are identical, they could not be differentiated by this technique except that the VD1 or VD2 were included. The genotype K found in this study was different from that of the prototype strain K/UW-31/CX in the VD4 sequence. However, when that prototype strain was resequenced by Pool and Lamont (1992) and Stothard *et al* (1998), it was different from those originally reported by Yuan *et al* (1989) but identical to the K sequence reported later by several investigators, including those conducting this study. It has been argued controversially, that the K strain sequence reported earlier by Yuan *et al* (1989) might actually be a variant. If so, the K variants found in this study, as well as in others, might be non-variants.

The nucleotide sequence variations in the MOMP gene are commonly found in most countries, including Thailand. The high prevalence of the variant type found in these populations was correlated to the risk of STD (Brunham *et al*, 1994). Individuals in a high-risk group had probably had repeated infections, which might have boosted mucosal antibody titers and led, ultimately, to the selection a variant, especially in genes coding the surface proteins (Lampe *et al*, 1997; Stephen *et al*, 1987). The genetic mechanisms for the diversification of the MOMP gene are still unclear. However, most sequence variation usually results in amino acid transition, which might confer some antigenic changes. It has been reported that MOMP variants can escape neutralization by both serotype-specific monoclonal antibodies and human immune sera (Lampe *et al*, 1997). It is likely that the immune selection mechanism plays an important role in the adaptability of organisms and the evasion of host immune surveillance. Study of genotype distribution and sequence variation of *C. trachomatis* may benefit the control of chlamydial disease, especially by vaccination.

ACKNOWLEDGEMENTS

This work was supported in part by the National Research Council of Thailand (1998). The authors would like to thank the STD Clinic of the Venereal Disease and AIDS Control Center, Region 10, Chiang Mai, and the Research Institute for Health Sciences, Chiang Mai University, for providing samples.

REFERENCES

Allen JE, Locksley RM, Stephen RS. A singer peptide from the major outer membrance protein of *C. trachomatis* elicit T helper cell for the production of antibodies to protective determinants. *J Immunol* 1991;147:674-9.

Baehr W, Zhang XI, Joseph T, *et al*. Mapping antigenic domains expressed by *C. trachomatis* major outer membrane protein genes. *Proc Nat Acad Sci USA* 1988;85:4000-4.

Borrego MJ, Gomes JP, Lefebvre JF, Eb F, Orfila J, Catry MA. Genotyping of Portuguese *C. trachomatis* urogenital isolates. *Genitourin Med* 1997;73:561-3.

Brunham R, Chunlin Y, Maclean I, Josshua K, Maitha G, Plummer F. *C. trachomatis* from invidiuals in a sexually transmitted disease core group exhibit frequent sequence variation in major outer membrane protein (omp1) gene. *Clin Invest* 1994;94:458-63.

Dean D, Schachter J, Dawson CR, *et al*. Comparison of the major outer membrane protein variant sequence regions of B/Ba isolates: a molecular epidemiologic approach to *C. trachomatis* infection. *J Infect Dis* 1992;166:383-92.

Frost EH, Reslandes S, Velleux S, Ramoisy DB. Typing *C. trachomatis* by detection of restriction fragment length polymorphism in the gene encoding the major outer membrane protein. *J Infect Dis* 1991;163:1103-7.

Lampe MF, Wong KG, Kuehl LM, Stamm WE. *C. trachomatis* major outer membrane protein variants escape neutralization by both monoclonal antibodies and human immune sera. *Infect Immun*

1997;65:317-9.

Lampe MF, Suchland RJ, Stamm WE. Nucleotide sequence of a variable domains with major outer membrane protein gene from serovariants of *C. trachomatis. Infect Inumum* 1993;61:213-9.

Lan J, Walboomers JM, Roosendaal R, *et al*. Direct detection and genotyping of *C. trachomatis* in cervical scrapes by using PCR and restriction fragment length polymorphism analysis. *J Clin Microbiol* 1993;31:1060-5.

Leechanachai P. Restriction fragment length polymorphism. In: Chantraratit W, Leechanachai P, Sirirungsri W, eds. Modern techiques in chromosomes and gene analysis. 1st ed. Bangkok: Pongsawad Karnpim, 1996: 17-1; 17-5 (In Thai).

Mittal A. Serovar distribution of *C. trachomatis* isolates collected from the cervix: use of the polymerase chain reaction and restriction endonuclease digestion. *Br J Biomed Sci* 1998;55:179-83.

Morre SA, Ossewaarde JM, Lan J, *et al*. Serotyping and genotyping of genital *C. trachomatis* isolates reveal variants of serovars Ba, G and J as confirmed by *omp* 1 nucleotide sequence analysis. *J Clin Microbiol* 1998;36:345-51.

Morre SA, Rozendaal L, Van Valkengoed IG, *et al*. Urogenital *C. trachomatis* serovars in men and women with a symptomatic or asymptomatic infection: an association with clinical manifestations. *J Clin Microbiol* 2000;38:2292-6.

National Institute of Health, Japan. Serotypes of *C. trachomatis* isolated from the cervixes and clinical symptoms of the patients in Japan. *Infect Agents Surv Rep* 1998;19.

Poole E, Lamont I. *C. trachomatis* serovar differentiation by direct sequence analysis of the variable segment 4 region of outer membrane protein gene. *Infect Immun* 1992;60:1089-94.

Rodriguez P, Persson B, Dutilt K, *et al*. Evaluation of molecular typing for epidemiological study of *C. trachomatis* genital infection. *J Clin Microbiol* 1993;31:2238-40.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, 1989:E3-4.

Sayada C, Denamure E, Grandchamp B, *et al*. Denaturing gradient gel electrophoresis analysis for the detection of point mutation in the *C. trachomatis* major outer membrane protein gene. *J Med Microbiol* 1995;43:14-25.

Schachter J. Biology of *C. trachomatis*: Bacteria. In: Holmes KK, Mardh P, Sparling PF, eds. Sexually transmitted diseases. 3rd ed. New York: McGraw-Hill, 1999:391-450.

Stamm WE. *Chlamydia trachomatis* infections of the adult: Bacteria. In: Holmes KK, Mardh P, Sparling, PF, eds. Sexually transmitted diseases. 3rd ed. New York: McGraw-Hill, 1999:407-32.

Stephen RS, Wager EA, Schoolnik GK. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *C. trachomatis*. *J Exp Med* 1988;167:817-31.

Stephen RS, Sanchez-Pescador R, Wagar EA, *et al*. Diversity of *C. trachomatis* major outer membrane protein genes. *J Bacteriol* 1987;167:3879-85.

Stothard DR, Bogusslawsk G, Jones RB. Phylogenetic analysis of the *C. trachomatis* major outer membrane protein (MOMP) and examination of potential pathogenic determinants. *Infect Immun* 1998;66:3618-25.

Sturm-Ramirez K, Brumblay H, Diop K, *et al*. Molecular epidemiology of genital *C. trachomatis* infection in high risk women in Senegal, West Africa. *J Clin Microbiol* 2000;38:138-45.

Van Duynhoven YTHP, Ossewaarde JM, Derksen-Nawrocki RP, *et al*. *C. trachomatis* genotypes: correlation with clinical manifestations of infection and patient's characteristics. *Clin Infect Dis* 1998;26:314-22.

Yang CL, Maclean I, Brunham RC. DNA sequence polymorphism of the *C. trachomatis* omp1 gene. *J Infect Dis* 1993;168:1225-30.

Yuan Y, Zhang YX, Watkins NG, Coldwell HD. Nucleotide and deduced amino acid sequence for the four variable domains of the major outer membrane protein of 15 *C. trachomatis* serovars. *Infect Immun* 1989;57:1040-9.