Multilocus Sequence Typing of Urogenital *Chlamydia trachomatis* From Patients With Different Degrees of Clinical Symptoms

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Background: In the past, contradictory results have been obtained linking *Chlamydia trachomatis* serovars (*ompA* gene) to different clinical courses of infection.

Methods: A high resolution multilocus sequence typing (MLST) system was used to genotype 6 genetic regions, including *ompA,* in 70 Dutch urogenital *C. trachomatis* strains from patients with different degrees of defined clinical symptoms (asymptomatic, symptomatic, and lower abdominal pain), to determine if MLST genotypes correlated with clinical manifestations of infection.

Results and conclusions: We identified 46 MLST types, with only a small overlap to Swedish MLST types. This study found no correlation between MLST profiles and symptomatology. To understand the clinical course of infection, future studies should not only consider bacterial factors but also look on the immunogenetics of the host.

Urogenital infection with the intracellular bacterium *Chlamydia trachomatis* is the most common curable sexually transmitted bacterial infection in the United States and Europe.[1] About 50% of infected men and 70% of infected women remain asymptomatic.[2] If symptoms in females occur they are usually mild and atypical, and include mucopurulent vaginal discharge, contact bleeding, and slight abdominal discomfort or pain. In a minority of females, urogenital chlamydia infection causes pelvic inflammatory disease characterized by lower abdominal pain, fever, and malaise. Chronic infection can cause fibrosis and scarring of the fallopian tubes and severe sequelae such as ectopic pregnancy and infertility. The conventional view that the damage is caused by antigen-specific adaptive immune responses is not supported by unambiguous proof. It has instead been suggested that the tissue damage leading to severe sequelae is caused by innate host immune responses. Progressive disease is probably a combination of both host-specific innate immune responses and pathogen-specific antigens as well as other biologic properties of the pathogen.[3–6]

Traditional subtyping of *C. trachomatis* was performed using antibodies targeting the major outer membrane protein, encoded by the *ompA* gene, and later by using polymerase chain reaction (PCR) amplification of the gene directly and subsequent restriction length fragment polymorphism or DNA sequencing. A number of reports have been published on clinical manifestations and serotype, but the conclusions are contradictory.[7]

The multilocus sequence typing (MLST) system developed by Klint et al. for *C. trachomatis* is based on PCR amplification and DNA sequencing of 5 different target regions and offers a 3-fold higher resolution than *ompA* genotyping.[8] Two of these 5 target regions comprise partial sequences of known genes: *hctB* and *pbpB*. The *hctB* gene encodes a histone H1-like protein that functions as a global regulator of chromatin structure and gene expression, while the *pbpB* gene encodes a penicillin binding protein that is a putative outer membrane protein potentially involved in the interaction with the host cell.[8,9] The other 3 target regions contain hypothetical open reading frames, encoding putative membrane proteins, or unknown proteins.

In this study, we hypothesized that pathogen-specific factors contribute to the different clinical manifestations of urogenital chlamydia infections in females. We used the MLST system to genotype 70 well-defined urogenital *C. trachomatis* strains isolated from women with different degrees of clinical symptoms, to determine if the multilocus genotype correlated with clinical manifestations of infection.

MATERIALS AND METHODS

Clinical Isolates

The study was performed in accordance with the Helsinki declaration and approved by the Ethical Committee of the Academic Medical Centre, University of Amsterdam, Amsterdam. *C. trachomatis* strains isolated from consenting female white visitors of the Amsterdam Sexually Transmitted Diseases outpatient clinic between 2001 and 2005 were propagated in eukaryotic HeLa cell cultures, using standard techniques. The women were asked to fill out a questionnaire describing urogenital complaints (i.e., vaginal discharge, contact bleeding,

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abdominal pain, and dysuria). The strains were isolated as part of a larger study to investigate bacterial and host factors related to the course of *C. trachomatis* infection. The current study focused on bacterial components. A total of 70 strains representing the dominantly prevailing urogenital serovars were selected from cases in which evidence for all other sexually transmitted diseases (including HIV, *Trichomonas vaginalis*, and *Neisseria gonorrhoeae*) was absent, so that *C. trachomatis* was the presumed cause of any patient reported symptoms. Other diseases associated with vaginal discharge like vaginal candidiasis and bacterial vaginosis were excluded by routine wet mount light microscopic slide evaluation. Patient groups were formed based on clinical manifestation: asymptomatic (n = 30), symptomatic (vaginal complaints like discharge, discomfort, irregular and/or contact bleeding after manipulation of the cervix to obtain diagnostic swabs) without lower abdominal pain (LAP) associated with adnexal pathology (n = 23) and symptomatic with LAP (n = 17). LAP was confirmed with bimanual examination. The *C. trachomatis* positive women with lower abdominal pain were clinically treated as pelvic inflammatory disease cases and received standard treatment for this condition. The Dutch isolates were compared to specimens collected from heterosexuals in Örebro county in Sweden in 2006.

DNA Purification

DNA was purified from culture, using a MagAttract DNA Mini M48 kit (QIAGEN, Hilden, Germany) on a BioRobot M48 workstation (QIAGEN), according to the manufacturer's instructions.

PCR Amplification

PCR amplification of the *ompA* gene and the 5 target regions of the MLST system was performed with a high fidelity polymerase as previously described.[8,10]

Sequencing

Sequencing PCR using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), as well as subsequent purification, was carried out according to the manufacturer's instructions. Sequencing was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and the data were analyzed using BioEdit 7.0.9 (Ibis Therapeutics, Carlsbad, CA) and ContigExpress, a component of Vector NTI Advance 10.3.0 (Invitrogen). All novel mutations were reamplified and resequenced to assure their authenticity.

Statistics

The 3 clinical categories were investigated statistically for association with MLST genogroups, MLST profiles, individual variants in each of the 5 MLST regions, *ompA* genotypes and the B-, C-, and intermediate complexes of *ompA*. The 3 clinical categories were also simplified into 2 categories, asymptomatic and symptomatic (LAP included), and the statistical analysis was redone as described above. A χ^2 goodness of fit test or a 2-tailed Fisher exact test was used for statistical analysis and a P value less than 0.05 was considered statistically significant.

Phylogeny

Phylogenetic analysis was carried out using the neighbor-joining algorithm included in the Phylip 3.68 software package.

RESULTS

The 70 *C. trachomatis* isolates could be separated into 46 MLST genotypes whereas the conventional *ompA* genotyping system identified 18 genotypes (Table 1). Overall, the MLST system had a 2.5-fold higher resolution than conventional *ompA* genotyping. The MLST resolution was 7- and 6-fold higher within serovar K and E, respectively. MLST profile number 34 was found in both serovar D and H.

All MLST profiles represented by more than 1 isolate included isolates from different clinical categories. Certain MLST profiles differed from each other with only a single point mutation in 1 genetic region and therefore phylogenetic analyses were carried out and the MLST profiles were grouped into 8 different genogroups (Fig. 1). These genogroups did not reflect the serovar distribution, with exception of genogroup 2, which contained only 2 isolates that were serovar H, and genogroup 7, which contained 5 isolates that were serovar K. No statistically significant correlation could be established between the clinical manifestations of infection and the MLST genogroups, MLST profiles, individual genetic variants in each of the 5 MLST regions, the *ompA* genotypes, or the *ompA* B-, C-, or intermediate complexes.

The 46 MLST profiles of these 70 Dutch isolates were compared to 95 specimens collected from heterosexuals in Örebro county in Sweden in 2006.[11] Seven MLST profiles, comprising 25 of the 165 specimens (15%), were present in both specimen populations. MLST profile number 100 was found in serotype I among the Dutch isolates, but in serotype J in the Swedish specimens.

DISCUSSION

Convincing data identifying antigens that are associated with the pathogenesis of urogenital *C. trachomatis* infection have not yet been presented. There have been a number of studies based on the *ompA* gene or its coded protein, but the results have been contradictory, perhaps partly because of the limited numbers of specimens. However, the consensus appears to be that there is no clear correlation between *ompA* and clinical manifestations.[7] The chlamydial heat shock protein 60 is another candidate that has been extensively investigated, but with equivocal results.[3] The current study utilized a MLST system based on 5 highly variable genetic regions to investigate a potential correlation with the clinical symptoms of infection. No statistically significant correlation could be found however. Pathogen-specific factors that are involved in disease development might be found in other regions of the *C. trachomatis* genome that are not linked to the MLST genotypes, or disease development might be due to host-specific factors, having nothing or little to do with genetic variation in *C. trachomatis*.

A limitation in the current study is the high variability in the genetic regions investigated and the limited number of specimens, which might mask a complex correlation. The MLST system used here is not based on housekeeping genes, as is the case in 2 other MLST systems used for genotyping chlamydia.[12,13] These 2 systems have a low discriminatory capacity which gives them limited usefulness in *C. trachomatis* strain discrimination and outbreak investigations, but which might be advantageous when looking for virulence factors that perhaps have evolved slowly over time and in parallel with the housekeeping genes.

Another limitation is the selection of symptomatic patients. Although extensive efforts were performed to exclude patients with symptoms due to other causes than chlamydial infection, one can never be sure that other (yet unknown)

TABLE 1. Genetic Profiles of All 70 Isolates

					MLST Profile				
Isolates	Clinical Category	Serovar	*ompA*	MLST Profile Number	*hctB*	CT058	CT144	CT172	*pbpB*
n = 2	Symp/LAP	B	30*	74	8	8	1	7	18
n = 1	LAP	D	1†	80	5	4	7	1	4
n = 1	Symp	D	1†	79	5	19	7	1	37
n = 1	Symp	D	1†	78	7	27	18	2	34
n = 1	Asymp	D	1†	75	12	12	7	2	10
n = 2	Asymp/symp	D	31‡	77	5	19	7	2	37
n = 1	Asymp	D	31‡	76	5	26	7	2	40
n = 2	Symp/LAP	D	2§	35	10	8	1	4	23
n = 1	Asymp	D	2§	82	10	4	1	3	23
n = 1	Symp	D	2§	83	10	4	9	3	23
n = 1	LAP	D	2§	34	10	8	1	4	21
n = 1	Asymp	D	2§	81	34	4	1	4	23
n = 1	Asymp	D	32¶	21	10	4	1	4	23
n = 1	Asymp	D	33	84	5	28	7	2	37
n = 1	Symp	D	34	85	5	8	7	2	4
n = 2	Asymp/symp	E	6‖	87	35	2	6	2	2
n = 2	Asymp/LAP	E	6‖	86	1	2	6	14	2
n = 1	Symp	E	6‖	56	1	19	7	2	1
n = 1	Asymp	E	6‖	88	5	19	6	2	41
n = 1	LAP	E	6‖	89	5	19	7	3	1
n = 1	LAP	E	6‖	63	7	19	7	2	1
n = 5	Asymp/symp/LAP	F	24**	12	5	19	7	1	4
n = 1	LAP	F	24**	90	5	19	1	1	4
n = 1	LAP	F	24**	91	5	19	5	2	4
n = 1	Symp	G	11††	92	10	19	1	1	6
n = 4	Asymp/symp	G	9‡‡	27	10	6	10	1	6
n = 3	Asymp/symp/LAP	G	11‡‡	95	10	8	1	3	5
n = 1	Asymp	G	11‡‡	94	10	5	12	3	5
n = 1	Asymp	G	11‡‡	93	10	8	1	4	42
n = 1	Asymp	H	18§§	34	10	8	1	4	21
n = 3	Asymp/symp/LAP	H	35¶¶	97	12	12	11	9	21
n = 2	Asymp/symp	H	35¶¶	96	12	29	11	9	21
n = 2	Asymp/LAP	Ia	37‖‖	103	8	6	1	7	5
n = 1	LAP	Ia	37‖‖	102	10	30	1	7	5
n = 5	Asymp/symp	Ia	36***	100	10	5	12	7	18
n = 2	Asymp/symp	Ia	36***	101	38	5	12	7	18
n = 1	Symp	J	20†††	105	10	4	1	1	18
n = 1	Asymp	J	20†††	104	12	12	11	9	18
n = 1	LAP	Ja	38‡‡‡	99	36	15	7	1	43
n = 1	LAP	Ja	38‡‡‡	98	37	15	7	1	43
n = 1	Asymp	K	12§§§	106	10	4	1	7	44
n = 1	Symp	K	12§§§	32	10	7	9	4	8
n = 1	Symp	K	12§§§	30	10	7	9	3	8
n = 1	Symp	K	12§§§	133	10	7	9	1	8
n = 1	Asymp	K	12§§§	139	10	8	9	4	8
n = 1	LAP	K	12§§§	107	12	12	21	9	24
n = 1	Asymp	K	12§§§	140	33	7	20	4	8

The numbers are arbitrary designations from the *C. trachomatis* MLST database (available at: http://mlstdb.bmc.uu.se/) and correspond to specific DNA sequences. There were 46 MLST genotypes compared to 18 *ompA* genotypes.
*Identical to strain B/IU-1226 (AF063208.1).
†Identical to strain D/IC-CAL8 (DQ064285.1).
‡Identical to strain D/LSU-EP212 (AF279587.1).
§Identical to strain D/UW-3 (DQ064284.1).
¶Identical to strain DK-K35 (AM901184.1).
‖Identical to strain E/Bour (DQ064286.1).
**Identical to strain F/IC-CAL3 (DQ064287.1).
††Identical to strain G/IU-FW9155 (FJ261939.1).
‡‡Identical to strain G/11222 (CP001888.1).
§§Identical to strain UW-4 (AF304857.1).
¶¶Identical to strain CS-121/96 (DQ116395.1).
‖‖Identical to strain Ia/IU-TC0018ut (FJ261940.1).
***Identical to strain Ia/IU-4168 (AF063201.2).
†††Identical to strain J/UW-36 (DQ064292.1).
‡‡‡Identical to strain Ja/IU-FW4076 (FJ261932.1).
§§§Identical to strain DK-K7 (AM901164.1).
Asymp indicates asymptomatic; symp, symptomatic; LAP, lower abdominal pain.

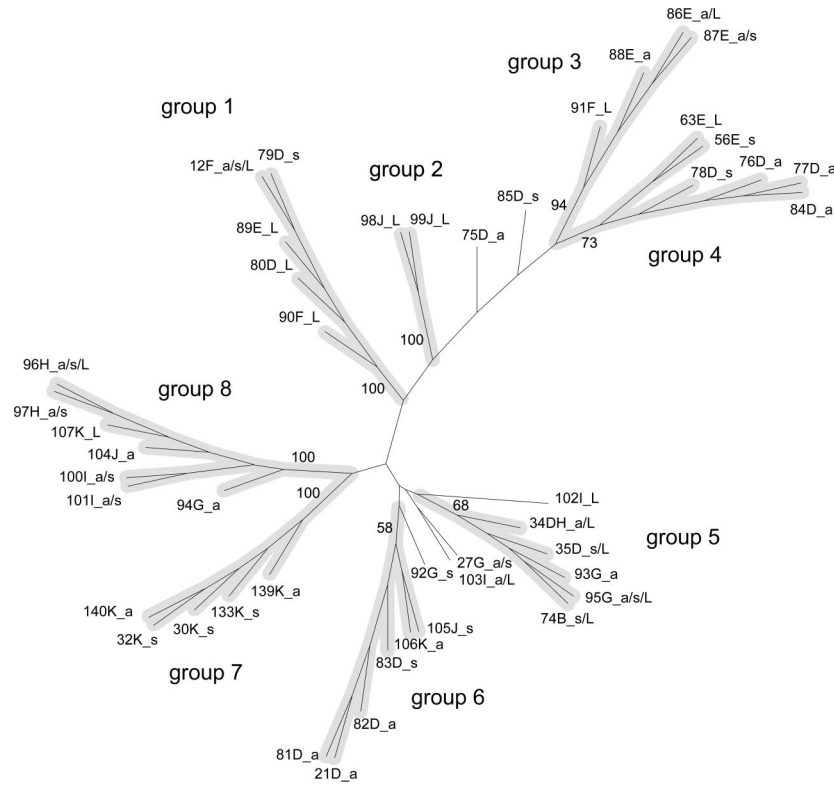

Figure 1. Unrooted cladogram, based on the neighbor joining algorithm, showing the genetic relationship between all 46 MLST profiles. The letter after each MLST profile number indicates serovar, based on the *ompA* sequence. Clinical category is indicated by the letter "a" for asymptomatic, "s" for symptomatic, and "L" for lower abdominal pain (LAP). The MLST profiles have been grouped into 8 genogroups, highlighted with a gray color. Bootstrap values for each genogroup are written in bold text and are shown as percentages of 1000 replicates.

causes have been overlooked. The diagnosis of LAP was based solely on the clinical picture (patient complaints plus a bimanual examination) and LAP can be associated with anaerobic bacterial infections which have not been excluded.

Comparison of the Dutch isolates to the Swedish specimens revealed a fairly small overlap in MLST genotypes, indicating that there is a limited exchange of *C. trachomatis* strains between the heterosexual populations in the 2 countries, as supported by the limited spread of the new variant *C. trachomatis* outside Sweden in recent years.[14] This highlights the usefulness of the MLST system in transmission studies and network analyses, where high resolution is needed to tell closely related strains apart.

Phylogenetic analysis of the MLST genotyping data revealed a genetic relationship dissimilar to that of the traditional serovar groupings and *ompA* genotyping. This is in accordance with previous conclusions that the *ompA* gene differs in phylogeny and rate of evolution from other regions of the genome, possibly due to recombination events.[15]

Recently, Bailey et al. showed using twin pairs that almost 40% of the differences in responses to *C. trachomatis* infection can be ascribed to host genetic factors.[16] The differences in the clinical course of infection are due to an interplay of both bacterial and host genetic factors and both should be taken into account in future studies,[17,18] though it appears that host factors contribute to a much higher degree. The European Union has funded the EpiGenChlamydia Consortium, which is led by Dr. Morré, and is in the process of creating large biobanks of patient-derived and bacterial specimens on which to perform studies to determine bacterial and host factors that play a role in the course of infection with chlamydia.[18]

In summary, MLST analysis of *C. trachomatis* isolates showed a high discriminatory capacity, but could not identify any multilocus genotypes that correlated with different defined clinical manifestations of female urogenital infection. This might in part reflect the genes chosen for MLST profiling in relation to the clinical course of infection, or, and consistent with the combined results of all studies to date, that bacterial factors if important need to be understood in the context of host factors. Thus, future studies should be directed at identifying host genetic factors that might play either a general role in the pathogenesis of chlamydial infection, or specifically in response to a particular bacterial factor or factors.

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