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

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Cover letter

Multilocus Sequence Typing of Urogenital *Chlamydia trachomatis* from Patients with Different Degrees of Clinical Symptoms

L. Christerson, H. J.C. de Vries, M. Klint, B. Herrmann and S. A. Morré

Dear Editor,

Please find enclosed the above-mentioned manuscript, which we submit for publication as an Original Article in the journal of *Sexually Transmitted Diseases*.

In the past contradictory results have been obtained by linking *Chlamydia trachomatis* serovars (*ompA* gene) to the different clinical courses of infection. In this study we used a high resolution multilocus sequence typing (MLST) system to genotype six genes including *ompA* in 70 Dutch urogenital *C. trachomatis* strains from patients with different degrees of well-defined clinical symptoms to see if the genotyping results could be correlated with the clinical manifestations of infection. We identified 46 MLST types indicating a high discriminating capacity, but the study could not show any correlation between MLST profiles and symptomatology.

The manuscript has not been published in any other journal and is not being considered for publication elsewhere. However, a summary of the results is accepted for the 12th International Symposium on Human Chlamydial Infections, which you are familiar to.

I hope you find our work interesting for publication in *STD*.

Best regards,

Björn Herrmann

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Dear Editor,

We have now considered the reviewers' comments and below are point by point answers to their criticism. We thank you for taking time to reconsider our revised manuscript.

Best regards,
Björn Herrmann

----- Weitergeleitete Nachricht von std@ucsf.edu -----
Datum: 24 Jun 2010 01:06:47 -0400
Von: Sexually Transmitted Diseases <std@ucsf.edu>
Antwort an: Sexually Transmitted Diseases <std@ucsf.edu>
Betreff: Your Submission
An: Björn Herrmann <bjorn.herrmann@medsci.uu.se>

Ref.: Ms. No. STD10-71
Multilocus Sequence Typing of Urogenital Chlamydia trachomatis from Patients with Different Degrees of Clinical Symptoms
Sexually Transmitted Diseases

Dear Mr. Herrmann,

Your manuscript has been read and reviewed by members of the Editorial Board. The reviewers had reservations about the manuscript (see comments appended below). Thus, we are unable to accept the manuscript for publication in its present form.

We would be receptive to a resubmission that deals directly with the reviewer's comments. If you are prepared to undertake the work required, then we would reconsider our decision. The manuscript would again go through our review process before a decision can be made as to its acceptability for publication.

If you decide to revise your manuscript, then please include a list of changes or rebuttals against each point which is being raised when you submit your revision. Your revised manuscript must be returned within 6 weeks of this e-mail message.

To submit a revision, go to http://std.edmgr.com/ and log in as an Author. You will find your manuscript in the menu item call Submission Needing Revision. Instructions on how to submit a revised manuscript can be found on the STD web site under Files & Resources, Revision Guidelines.

Yours sincerely,

Julius Schachter, PhD
Editor
Sexually Transmitted Diseases

Reviewer #1:
In this study a high resolution multilocus sequence typing (MLST) system was used to genotype six genes including ompA in 70 Dutch urogenital C. trachomatis strains obtained from patients categorized clinically as being asymptomatic, symptomatic or having lower abdominal pain, to determine if the genotyping results could be correlated with clinical manifestations of infection. In general the paper is in need of editing to clarify sentence structure and misuse of certain words.

Answer: We have asked a native American Chlamydia researcher to read and correct the manuscript. His name is Joseph Lyons and we have mentioned him in the acknowledgements.

More specific comments follow.

1. The MLST system is based on 5 variable genes and the details of the methods for amplification, sequencing, etc have been published elsewhere. Two methods are used to perform ompA typing: one is based on RFLP patterns and the other is based on direct sequencing of a nested PCR product. In Table 1 the column labeled ompA contains numbers which I am guessing represent ompA sequence based genotype assignments. This is confusing as neither the methods section nor the Table footnote provides any explanation of what these numbers mean.

Answer: The Table footnote states: "The numbers are arbitrary designations from the C. trachomatis MLST database (http://mlstdb.bmc.uu.se/)"

To further clarify, the above sentence has now been changed to: "The numbers are arbitrary designations from the C. trachomatis MLST database (http://mlstdb.bmc.uu.se/) and correspond to specific DNA sequences." (line 274-276)

Additionally, strain name and accession numbers to all *ompA* variants found in GenBank has been added as footnotes. (lines 279-294)

Furthermore, the relationship between the 2 ompA genotyping systems is not clear. For example in the results we are told that the "conventional" ompA typing system resulted in 18 genotypes. Depending on your point of view, either of the 2 genotyping systems could the "conventional" system. The authors need to clarify why they present the results of 2 ompA genotyping systems and how they are using the data in the analysis. If the data from one of the ompA genotyping systems is not being used in this analysis then that data should be removed from Table 1 for the sake of clarity.

Answer: We agree and have therefore removed the RFLP results and RFLP methodology description from the manuscript. The serovar designations are now based on the *ompA* sequence instead.

2. Line 151 -"No MLST profile represented by more than one isolate was

found within a single clinical category only." This sentence does not make sense.

Answer: The sentence has been changed to: "All MLST profiles represented by more than one isolate included isolates from different clinical categories." (line 150-151)

It would be a good idea to ask someone outside of the research group to edit the paper. Such an individual without prior knowledge of the study results and methods would be able to quickly identify sentences that are unclear and need re writing.

Answer: We have asked a native American Chlamydia researcher to read and correct the manuscript. His name is Joseph Lyons and we have mentioned him in the acknowledgements.

3. A weakness of this study is that the definitions of clinical categories used are based on patient histories only. Symptoms alone are relatively non specific for chlamydial infection. Was the Dutch study carried out specifically to collect chlamydia strains for genotyping or was this study done originally for other reasons? Since physical examination findings that could have more precisely categorized the patients were not used, I suspect that the specimens were collected for another purpose and that the study reported here was designed to make use of the available chlamydia isolates and whatever clinical data had been previously collected.

Answer: This study was collected specifically for having a culturable set of clinically well defined isolates. These isolates were obtained from a larger study in which NO culture was obtained from the other isolates, and this study aimed to study both host and bacterial factors in relation to the course of CT infections, thus the samples were not "just picked from another study" bacterial typing was already initially part of the aim. The way samples are collected based on the largest STD clinic in The Netherlands is that samples are collected initially based on the patient reportance on symptoms or reasons (Asymptomatic but wanting to check based on a new relation. This was also the way the METC allowed us to collect the samples.

This should be made clear in the methods section.
Answer: We have added additional information in the Methods section on the origin on the samples as suggested by the reviewer.(lines 95-109)

Then there should be a short "limitations" paragraph in the Discussion section. Here the authors should indicate that the negative findings in this study do not disprove that some stains of chlamydia are more virulent than others and could suggest that future studies should look at mucopurulent cervicitis and pelvic inflammatory disease using clearly defined examination findings to form clinical definitions.

Answer: We have not stated that the results disprove that there might be "some stains of chlamydia that are more virulent than others". Quite contrary, the discussion already contained the following sentence:
"The immune responses leading to symptoms and sequelae might be initiated by antigens encoded by other regions of the *C. trachomatis* genome..."

To meet the criticism we have nevertheless clarified and expanded on the limitations of the study in the discussion (lines 178-184).

4. Lines 161-65: Only 7 Dutch MLST types were also seen in Sweden. Are the other 39 Dutch MLST types new or have they been seen in other countries? Please address this issue.

Answer: The other 39 Dutch MLST types were new. The C.trachomatis MLST database (http://mlstdb.bmc.uu.se/) do not contain any large specimen collections from heterosexual populations outside Sweden, hence a good comparison to other countries is not possible.

5. Line 177: "The immune responses leading to symptoms and sequelae might be initiated by antigens encoded by other regions of the C. trachomatis genome?" What is meant by this phrase?

Are the authors referring to antigens encoded by genes other than those used for their MLST system?

Answer: Yes. We consider the sentence to be quite clear, but since both reviewers ask questions about the same sentence it has now been rewritten in the manuscript (lines 178-181)

Do MLST genes in their system code for proteins that are likely to be recognized by the host immune system? If so that should be stated.

Answer: It is already stated. In the introduction it says (lines 75-78): "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."

Generally it is assumed that a genotyping system is a surrogate for associated genes within a given strain variant that have virulence potential. Please clarify the intent of this phrase. Might want to just drop it.

Answer: A correlation between MLST genotypes and disease could mean two things:
1. That there are important mutations in the MLST target regions themselves influencing the pathogenesis.
2. That there are mutations in the MLST target regions that are linked to mutations in other regions influencing the pathogenesis.

The first step is to find a correlation. The second step is to investigate exactly which changes in the genome correspond to the correlation. We did however not find a correlation, and have therefore not continued with the second step.

6. Lines 186-190. This could be dropped. Discrimination index change from 3 to 2.5 is insignificant. This distracts from main points of the study

Answer: We agree. This has now been excluded from the manuscript.

7. The issue of utility of housekeeping genes vs. those chosen by these investigators for an MLST system is complex. Some would argue that the lower discriminatory index would be an advantage when searching for virulence factors as both would evolve slowly over time and perhaps more likely in parallel than more rapidly evolving genes. It is not clear to me which is better, but expanded discussion of this issue would be of interest to readers. It would also help explain to those not familiar with the issues surrounding developing MLST systems why the authors have brought the issue up in the first place.

Answer: We agree and have added this to the manuscript (lines 184-190).

8. Lines194-96: This sentence could be part of your discussion of your MLST system vs. the housekeeping gene based systems.

Answer: Yes, it could. But we feel it also fits nicely where it is currently written.

Reviewer #2:
This is an interesting paper which has clearly involved a considerable amount of work. However I am unconvinced by the rational behind it and given its size I am not surprised no association was found. It does however highlight the difficulties in undertaking genotyping studies with C. trachomatis in humans.

I have the following comments:

1) The central hypothesis in this study seems to be that this type of study is able to provide information on how the immune response to the antigens studied may influence disease

Answer: No. The central hypothesis in this study is that differences in the genetic composition of C. trachomatis strains can influence the development of urogenital disease. Pathogen specific genetic factors that unambiguously explain the pathogenesis of C. trachomatis have not yet been clearly identified. Therefore we decided to try previously untested genetic regions, i.e. the MLST target regions, in order investigate this hypothesis, by looking for a correlation between the MLST results and the clinical symptoms of disease. The last paragraph in the introduction has been slightly rephrased (lines 82-86) to avoid future misunderstandings.

- "the immune responses
leading to symptoms and sequelae might be initiated by antigens encoded by other regions of the C. trachomatis genome, or it might be due to host specific innate immune responses, having nothing or little to do with strain specific antigens" (lines 177-9). I do not believe this is a reasonable assumption.

Answer: The text quoted from our article is a view which has been expressed in several previous publications by various authors:
1. Stephens RS. The cellular paradigm of Chlamydial pathogenesis. Trends Microbiol. 2003; 11: 44-51. Review.
2**. Brunham RC, Rekart ML. Considerations on Chlamydia trachomatis disease expression. FEMS Immunol Med Microbiol. 2009; 55: 162-166. Review.**
3. Darville T, Hiltke TJ. Pathogenesis of genital tract disease due to Chlamydia trachomatis. J Infect Dis. 2010 Jun 15;201 Suppl 2:S114-25. Review.

But to further clarify the sentence has now been rewritten (lines 178-181).

For three reasons:
a. The association of disease is may be due to genetic linkage. For example genotyping C. trachomatis using MOMP reveals 3 distinct groups characterised by different disease patterns. The trachoma serovars are genetically linked to a defective tryptophan dehydrogenase gene which is likely to be important in pathogenesis[1, 2]. LGV is a much more aggressive infection capable of infecting a much wider range of cells than serovars A-J and is associated with invasive disease. This is unlikely to be due to differences in the immune response to MOMP, it most likely is a consequence of genetic linkage to other genes which control replication dynamics and attachment although it may directly influence cell tropism as there is some evidence MOMP may be involved in cell attachment and entry.[3] Thus pathogenesis of disease will be related to the immunobiology of the host pathogen interaction not just the immune response.

Answer: We agree. As previously stated, the last paragraph in the introduction has been slightly rephrased (lines 82-86) to avoid future misunderstandings.

Given the number of genes present in C. trachomatis, MLST studies may identify association with disease directly related to those genes selected or as a consequence of genetic linkage.

Answer: We agree and this has not been contradicted anywhere in the article.

b. The MLST profiles are likely to only be important in the immune response if they involve (or are linked) to critical B or T cell epitopes. Thus the failure to demonstrate an association with disease does not necessarily exclude these antigens as being important in the immune response. This is consistent with what we know about the immune response and MOMP serovar.

Answer: It is unclear what the reviewer means, perhaps because of misunderstanding the central hypothesis of the article. The known biological functions of the MLST targets *hctB* and *pbpB* are described in the introduction (lines 75-78).

c. It is possible that these base pair differences could effect individual gene function and as a result change the biological characteristics of the isolate and thus its pathogenicity.

Answer: We agree that genetic variation could affect individual gene function and pathogenicity. That is the central hypothesis in this study and that is why the study has been performed.

Thus association of disease with distinct MLST patterns may be due to differences in the immune response but may also result from differences in cellular biology in vivo which may or may not be as a direct consequence of the gene being studied.

Answer: We agree and to avoid misunderstandings lines 62-64 in the introduction and, as previously mentioned, lines 178-181 in the discussion has been rewritten.

2. Nevertheless important differences in clinical presentation may be related to C. trachomatis genotypes within serovars D-K. Although as stated the evidence is inconclusive, Geisler and colleagues have published on the potential interaction between serovar J/Ja and the immune system - being associated with early clearance.[4] This may reflect slower replication rate.[5] Geisler has demonstrated a significant association of serovar F with abdominal pain in women, although no association was found between serovars and disease.[6]

Answer: The aim of our study was not to investigate ompA/MOMP. This has already been done with varying, often contradictory results in many studies over the last three decades. Figure 1 has nevertheless been updated with serovar information (based on the ompA sequences) for those who are still interested in ompA.

I. With 46 MLST profiles it is my understanding that a very large number of clinical samples would be needed in order to identify a significant association with disease (which is not due to chance). Have you sought statistical advice on this- if so this should be explicit. It would be informative for the reader to know that as the discriminatory power of typing techniques increases the larger the number of characterised clinical samples required in order to reliably demonstrate a significant association.

Answer: We agree that the number of analysed cases is limited which affects statistical analysis. To overcome this limitation the complexity of the results where reduced in several ways. The 46 MLST profiles were for example grouped into 8 genogroups, and the clinical categories where simplified to only asymptomatic and symptomatic (including lower abdominal pain), the regions were analysed individually and so on. Furthermore the discussion of limitations in the current study has been expanded (lines 183-190)

a. Have you grouped the OmpA serovars according to B, C and intermediate complex in order to reduce the number of groups for comparison?[6]

Answer: This has now been included into the manuscript (lines 132 and 158-159).

b. No evidence is presented to suggest that the 5 genes selected for MLST analysis are likely to be important in the immunobiology of infection.
My understanding is that this typing system is primarily of value "in transmission studies and network analyses, where high resolution is needed to tell closely related strains apart." (discussion lines 194-6). Surely for the purposes of this study as stated, it would be sensible to use genes which are known (or believed to be) important in the immunobiology of disease based on in vitro and animal studies- (I acknowledge that this is an area which remains poorly understood but it should be discussed)

Answer: Pathogen specific antigens unambigously explaining the pathogenesis of C. trachomatis are still poorly identified. Therefore, instead of trying targets that have already been investigated, we decided to try novel targets, i.e. the MLST target regions, to see if we could find a correlation to clinical symptoms. In the introduction we explain that: "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." (lines 75-78) It might have been a long shot to try and correlate the MLST target regions to symptoms, but in our opinion, still worth a try.

II. Relating symptoms to disease is complex in C. trachomatis infection. Vaginal discharge is non-specific and often due to other aetiologies such as bacterial vaginosis. Abdominal pain is also non-specifc, as you have acknowledged in previous communications[7]. Where the women recruited examined? Details are only provided of clinical symptoms. It would be helpful to know if those with abdominal pain had clinical PID. [8] This needs to be discussed.

Answer: The samples selected were all samples in which all others STDs were excluded so only CT was present, specifically to circumvent the issue raised by the reviewer. We have now stated this in the Methods section.(lines 100-103) In addition, the women with CT and lower abdominal pain were treated as PID cases, we have also made this clear in the methods section "clinical isolates" now as requested by the reviewer.(lines 106-109) We want to thank the reviewer for this suggestions, this indeed makes the cohort description better.

III. Given that culture is only 60-80% sensitive it is possible that the isolates obtained by culture were biased and may reflect growth characteristics associated with easier propogation in culture. Do you have any details of those women sampled who were culture negative? Where women tested by a NAAT and is any specimen available from those

NAAT-positive culture negative? Did the proportion vary by symptom group? I would expect asymptomatic women to have lower chlamydial loads to be less likely to be culture positive[9, 10].

Answer: The culture efficiency was not different between symptomatic and asymptomatic cases and very high both to very stringent sample collection procedures having the samples directly frozen at -80C as well as the presence of 2 different samples to use for culture. There can be a slight though non significant selection since we selected such that we represented all urogenital serovars in the selection of strains. In addition we feel that the course of infection is not only a bacterial load issue but also a host issue based on how you combat infection based on your genetic make-up.

In conclusion although I agree with your conclusion that to "better understand the clinical course of infection future studies should not only consider bacterial factors but also look more on the immunogenetics of the host." I do not believe that the data as currently presented supports such a statement. Essentially we need very large studies, ideally using isolates from patients with well characterised clinical presentations which also includes human genotyping. Given the likely size this will need to be multi-centre, multidisciplinary and almost certainly international.

Answer: We completely agree with the reviewer that that question can only we answered by collecting very large cohorts collected in a multi centre approach. This is what is exactly done by the European Union funded EpiGenChlamydia Consortium which is cited in the Sources of support, but we have now also stated this in the discussion as suggested by the reviewer.

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(3) Su H, Raymond L, Rockey DD, Fischer E, Hackstadt T, Caldwell HD.
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*Manuscript with tracked changes

1 # Multilocus Sequence Typing of Urogenital *Chlamydia trachomatis* from
2 # Patients with Different Degrees of Clinical Symptoms

3

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18

19 Running title: MLST analysis of urogential *C. trachomatis* strains

20

21 Word count: Summary 28 words, Abstract ~~132~~ 121 words, Text ~~1637~~ 1732 words.

22

23 Number of figures: 1
24 Number of tables: 1

25

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29 Sixth Framework Programme through contract no. LSHG-CT-2007-037637. See
30 www.EpiGenChlamydia.eu for more details about this Consortium. The study was
31 supported by local funds at Uppsala University Hospital. All authors declare that they have
32 no conflicts of interest.

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33

34 Short summary: Multilocus sequence typing of Dutch urogenital *Chlamydia trachomatis*
35 isolates showed a high discriminatory capacity and a ~~fairly~~ small overlap to Swedish
36 genotypes, but could not be correlated with the symptomatology.

37

ABSTRACT

In the past, contradictory results have been obtained ~~by~~ linking *Chlamydia trachomatis* serovars (*ompA* gene) to ~~the~~ different clinical courses of infection. In this study we used a high resolution multilocus sequence typing (MLST) system to genotype six genetic regions, including *ompA*, in 70 Dutch urogenital *C. trachomatis* strains from patients with different degrees of well-defined clinical symptoms (asymptomatic, symptomatic and lower abdominal pain), to ~~see~~ determine if ~~the genotyping results could be~~ MLST genotypes correlated with ~~the~~ clinical manifestations of infection. We identified 46 MLST types, with only a ~~fairly~~ small overlap to Swedish MLST types. This study ~~showed that~~found no correlation between MLST profiles and symptomatology~~ could be made~~. To ~~better~~ understand the clinical course of infection, future studies should not only consider bacterial factors but also look ~~more~~ on the immunogenetics of the host.

Keywords: clinical symptoms; *Chlamydia trachomatis*; multilocus sequence typing (MLST); *ompA*

INTRODUCTION

Urogenital ~~chlamydia~~ infection ~~is caused by~~with the intracellular bacterium *Chlamydia trachomatis* ~~and~~ 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, ~~like~~ and include mucopurulent vaginal discharge, contact bleeding, and slight~~faint~~ abdominal discomfort or pain. In a minority of females urogenital chlamydia infection causes pelvic inflammatory disease (PID) characterized by lower abdominal pain, fever and malaise. Chronic infection can cause fibrosis and scarring of the fallopian tubes and ~~lead to~~ 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.~~3,4~~ It has instead been suggested that the tissue damage leading to severe sequelae ~~are~~ 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 biological properties of the pathogen.~~Probably it is a combination of both host specific innate immune responses and pathogen specific antigens that lead to symptoms and sequelae.~~[3,4,5,6]

Traditional subtyping of *C. trachomatis* ~~has been~~was performed ~~by~~ using antibodies targeting the major outer membrane protein, encoded by the *ompA* gene, and later by using 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.[~~6~~7]

The multilocus sequence typing (MLST) system developed by Klint *et al.* for *C. trachomatis* is based on PCR amplification and DNA sequencing of five different ~~genetic~~ target regions and offers a threefold higher resolution than *ompA* genotyping.[~~7~~8] Two of these five target regions comprise partial sequences of known genes: *hctB* and *pbpB*~~, respectively~~. 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.[~~7,8~~8,9] The other three target regions contain ~~complete or partial~~ hypothetical open reading frames, encoding putative membrane proteins or unknown proteins.

In this study we hypothesized that pathogen specific ~~antigens~~ factors contribute to the clinical manifestations of urogenital ~~C~~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 ~~see~~ determine if the ~~genetic composition~~multilocus genotype ~~of the strains could be~~ correlated ~~to symptoms~~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,

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Amsterdam. *C. trachomatis* strains isolated from consenting female Caucasian visitors of the Amsterdam STD 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~~,~~ ~~regarding~~ describing urogenital complaints (i.e. vaginal discharge, contact bleeding, 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 ~~selection~~ 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*) was absent, so that *C. trachomatis* was the presumed cause of any patient reported symptoms. ~~was made.~~ Patient groups were formed based on clinical manifestation: asymptomatic (n = 30), symptomatic (vaginal complaints like discharge, discomfort, irregular and/or contact bleeding) without lower abdominal pain (LAP) (n = 23) and symptomatic with LAP (n = 17). 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.

~~Serovar determination~~

~~*C. trachomatis* typing was performed by amplification of the *ompA* gene (1.1 kb) in a nested PCR using primers NLO and NRO and primers sero1A and sero2A as described previously for cervical and urethral swabs,9,10,11 and urine specimens.12 The PCR product was checked on an agarose gel for length. Subsequently, 10 µl of the PCR product was digested using different restriction enzymes. Serovars and variants were identified by their restriction fragment length polymorphism (RFLP) patterns after polyacrylamide gel electrophoresis.11~~

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 five target regions of the MLST system was performed with a high fidelity polymerase as previously described.[~~7~~8,1~~3~~0]

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 VectorNTIAdvance 10.3.0 (Invitrogen). All novel mutations were reamplified and resequenced to assure their authenticity.

Statistics

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The three clinical categories were investigated statistically for association with MLST genogroups, MLST profiles, individual variants in each of the five MLST regions, ~~and~~ *ompA* genotypes and the *ompA* B-, C- and intermediate complexes. The three categories were also simplified into two categories, asymptomatic and symptomatic (LAP included), and the statistical analysis was redone as described above. A chi-square goodness of fit test or a two-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 seven and six fold higher within serovar K and E respectively. MLST profile number 34 was found in both serovar D and H.

~~No~~ All MLST profiles represented by more than one isolate ~~was~~ included isolates from different ~~found within a single~~ clinical categoriesy ~~only~~. Certain MLST profiles differed from each other with only a single point mutation in one genetic region and therefore phylogenetic ~~analysis~~ analyses were carried out and the MLST profiles were grouped into eight different genogroups (Figure 1). These genogroups did not reflect the serovar distribution, with exception of genogroup 2, which containinged only two isolates, ~~–~~ that ~~which~~ were serovar H, and genogroup 7, which containinged five isolates ~~, which~~that were serovar K. No statistically significant correlation could be established between the clinical manifestations of ~~the *C. trachomatis*~~ infections and the MLST genogroups, MLST profiles, individual genetic variants in each of the five MLST regions, ~~or~~ 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.[~~14~~–11] Seven MLST profiles, comprising ~~in total~~ 25 ~~out~~ 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 ~~can explain~~ are associated with the pathogenesis of urogenital *C. trachomatis* infection has not yet been presented. There have been a number of studies based on the *ompA* gene or ~~the~~ its ~~coding~~ coded protein, but the ~~conclusions~~ results have been contradictory, perhaps partly because of the limited numbers of specimens. ~~The total picture h~~However, the consensus appears to be that there is no clear correlation ~~to be found~~ between *ompA* and clinical manifestations.[6–7] The chlamydial heat

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194 shock protein 60 is another candidate that has been ~~intensely~~ extensively investigated, but
195 with equivocal results~~undisputed data is still lacking~~.[3] The current study utilized a MLST
196 system based on five highly variable genetic regions to investigate a potential correlation
197 with the clinical symptoms of infection. No statistically significant correlation could be
198 found however. Pathogen specific factors that are involved in disease development might
199 be found in other regions of the *C. trachomatis* genome that are not linked to the MLST
200 genotypes, or disease development might be due to host specific factors, having nothing or
201 little to do with genetic variation in *C. trachomatis*.
202 ~~The immune responses leading to symptoms and sequelae might be initiated by antigens~~
203 ~~encoded by other regions of the *C. trachomatis* genome, or it might be due to host specific~~
204 ~~innate immune responses, having nothing or little to do with strain specific antigens.~~
205
206 A limitation in the present study is the high variability in the genetic regions investigated
207 and the limited number of specimens, which might mask a complex correlation. The MLST
208 system used here is not based on housekeeping genes, as is the case in two other MLST
209 systems used for genotyping ~~C~~chlamydia ~~bacteria~~.[~~15~~12,1~~6~~3] These two systems have a low
210 discriminatory capacity which gives them limited usefulness in *C. trachomatis* strain
211 discrimination and outbreak investigations, but which might be advantageous when looking
212 for virulence factors that perhaps have evolved slowly over time and in parallel with the
213 housekeeping genes.
214 ~~and could likely not link certain MLST profiles with clinical manifestations either.~~
215
216 ~~The MLST system had a 2.5 fold higher resolution than *ompA* genotyping in this study,~~
217 ~~which is lower than the 3.0 fold higher resolution shown in previous studies. This is due to~~
218 ~~the low number of serotype E strains included in this study. Serotype E is usually the most~~
219 ~~prevalent serotype in genital tract infections and it is where the MLST system previously~~
220 ~~has been the most discriminatory compared to *ompA* genotyping.~~ Comparison of the Dutch
221 isolates to the Swedish specimens revealed a fairly small overlap in MLST genotypes,
222 indicating that there is a limited exchange of *C. trachomatis* strains between the
223 heterosexual populations in the two countries, as supported by the limited spread of the new
224 variant *C. trachomatis* outside Sweden in recent years.[~~17~~14] This highlights the usefulness of
225 the MLST system in transmission studies and network analyses, where high resolution is
226 needed to tell closely related strains apart.
227
228 Phylogenetic analysis of the MLST genotyping data revealed a genetic relationship
229 dissimilar to that of the traditional serovar groupings and *ompA* genotyping. This is in
230 accordance with previous conclusions that the *ompA* gene differs in phylogeny and rate of
231 evolution from other regions of the genome, possibly due to recombination events.[~~18~~15]
232
233 Recently, Bailey *et al*. showed using twin pairs that almost 40% of the differences in
234 responses to *C. trachomatis* infection can be assessed to host genetic factors.[~~19~~16] The
235 differences in the clinical course of infection are due to an interplay of both bacterial and
236 host genetic factors and both should be taken into account in future studies~~,~~[~~20~~17,~~21~~18], though
237 it appears that host factors contribute to a much higher degree. The European Union has
238 funded the EpiGenChlamydia Consortium, which is led by Dr. Morré, and is in the process
239 of creating large biobanks of patient derived and bacterial specimens on which to perform

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240 studies to determine bacterial and host factors that play a role in the course of infection with
241 chlamydia.[18]
242
243
244 In summary, MLST analysis of *C. trachomatis* isolates showed a high discriminatory
245 capacity but could not identify any multilocus genotypes that correlated with different well-
246 defined clinical manifestations of female urogenital infectioncorrelate genotypes with
247 different degrees of clinical manifestations. This might in part reflect the genes chosen for
248 MLST profiling in relation to the clinical course of infection, or, and consistent with the
249 combined results of all studies to date, that bacterial factors if important need to be
250 understood in the context of host factors. Thus, future studies should be directed at
251 identifying host genetic factors that might play either a general role in the pathogenesis of
252 chlamydial infection, or specifically in response to a particular bacterial factor or factors.
253 and indicates that in future studies besides bacterial factors also host genetic factors should
254 be taken into account to better understand the clinical course of infection.
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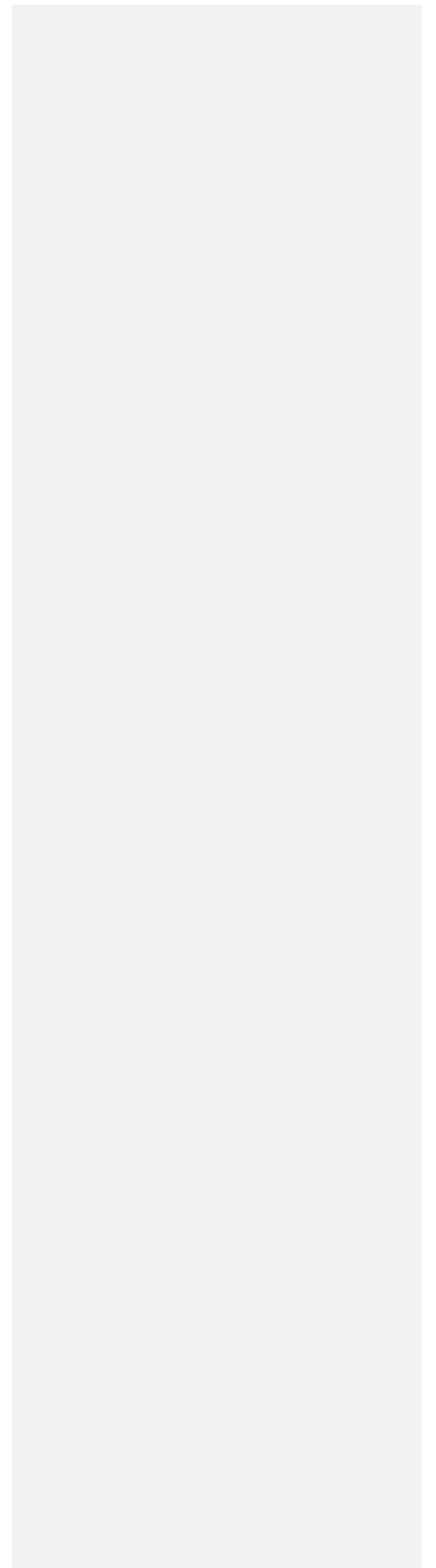


Table 1. Genetic profiles of all 70 isolates

Isolates	Clinical category	RFLP serovar	*ompA*	MLST profile number	MLST profile				
					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	Da	33	84	5	28	7	2	37
n = 1	symp	Da	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/Ga	9	27	10	6	10	1	6
n = 3	asymp/symp/LAP	Ga	11	95	10	8	1	3	5
n = 1	asymp	Ga	11	94	10	5	12	3	5
n = 1	asymp	Ga	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 = 1	LAP	H	38	99	36	15	7	1	43
n = 1	LAP	H	38	98	37	15	7	1	43
n = 2	asymp/LAP	I-	37	103	8	6	1	7	5
n = 5	asymp/symp	I-/Ia	36	100	10	5	12	7	18
n = 2	asymp/symp	Ia	36	101	38	5	12	7	18
n = 1	LAP	Ia	37	102	10	30	1	7	5
n = 1	symp	J	20	105	10	4	1	1	18
n = 1	asymp	J	20	104	12	12	11	9	18
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

Table 1. Genetic profiles of all 70 isolates

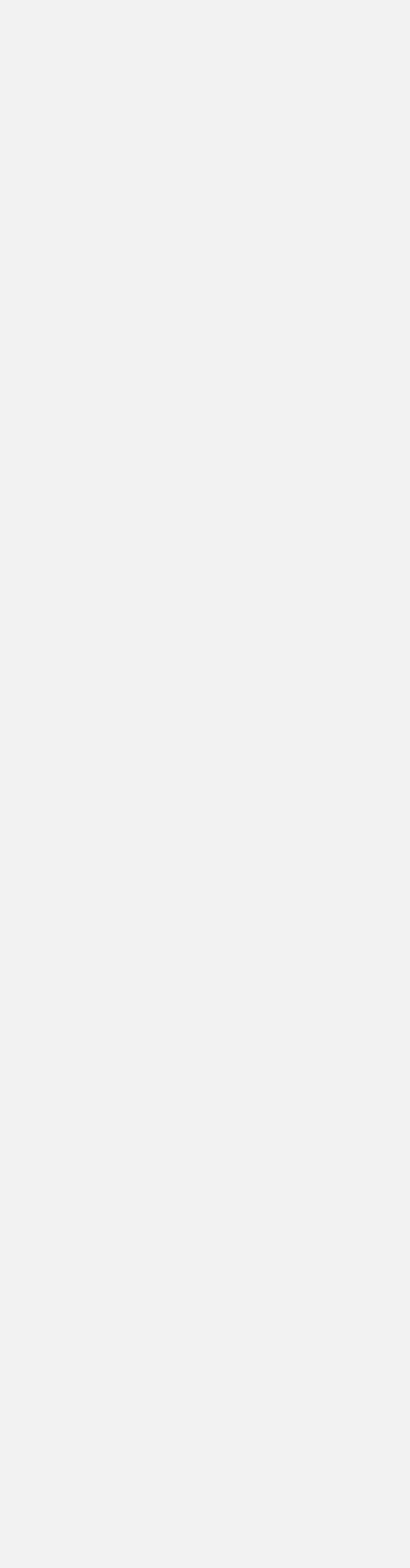
Isolates	Clinical category	Serovar	*ompA*	MLST profile number	MLST profile				
					hctB	CT058	CT144	CT172	*pbpB*
n = 2	symp/LAP	B	30[*]	74	8	8	1	7	18
n = 1	LAP	D	1[†]	80	5	4	7	1	4
n = 1	symp	D	1[†]	79	5	19	7	1	37
n = 1	symp	D	1[†]	78	7	27	18	2	34
n = 1	asymp	D	1[†]	75	12	12	7	2	10
n = 2	asymp/symp	D	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

323 The clinical categories have been abbreviated "asymp" for asymptomatic, "symp" for
324 symptomatic and LAP for lower abdominal pain. ~~RFLP is an abbreviation of restriction~~
325 ~~fragment length polymorphism.~~ The numbers are arbitrary designations from the *C.*
326 *trachomatis* MLST database (http://mlstdb.bmc.uu.se/) and correspond to specific DNA
327 sequences. There were 46 MLST genotypes compared to 18 *ompA* genotypes.
328
329 *Identical to strain B/IU-1226 (AF063208.1)
330 † Identical to strain D/IC-CAL8 (DQ064285.1)
331 ‡ Identical to strain D/LSU-EP212 (AF279587.1)
332 § Identical to strain D/UW-3 (DQ064284.1)
333 ¶ Identical to strain DK-K35 (AM901184.1)
334 # Identical to strain E/Bour (DQ064286.1)
335 ** Identical to strain F/IC-CAL3 (DQ064287.1)
336 †† Identical to strain G/IU-FW9155 (FJ261939.1)
337 ‡‡ Identical to strain G/11222 (CP001888.1)
338 §§ Identical to strain UW-4 (AF304857.1)
339 ¶¶ Identical to strain CS-121/96 (DQ116395.1)
340 ## Identical to strain Ia/IU-TC0018ut (FJ261940.1)
341 *** Identical to strain Ia/IU-4168 (AF063201.2)
342 ††† Identical to strain J/UW-36 (DQ064292.1)
343 ‡‡‡ Identical to strain Ja/IU-FW4076 (FJ261932.1)
344 §§§ Identical to strain DK-K7 (AM901164.1)
345
346
347

348 **FIGURE LEGEND**

349

350 Figure 1. Unrooted cladogram, based on the neighbor joining algorithm, showing the
351 genetic relationship between all 46 MLST profiles. The letter after each MLST profile
352 number indicates serovar, based on the *ompA* sequence. Clinical category is indicated by
353 the letter "a" for asymptomatic, "s" for symptomatic and "L" for lower abdominal pain
354 (LAP). The MLST profiles have been grouped into eight genogroups, highlighted with a
355 gray color. Bootstrap values for each genogroup are written in bold text and are shown as
356 percentages of 1,000 replicates.



*Manuscript

1 **Multilocus Sequence Typing of Urogenital *Chlamydia trachomatis* from**
2 **Patients with Different Degrees of Clinical Symptoms**

3

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19 Running title: MLST analysis of urogential *C. trachomatis* strains

20

21 Word count: Summary 28 words, Abstract 121 words, Text 1732 words.

22

23 Number of figures: 1

24 Number of tables: 1

25

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29 contract no. LSHG-CT-2007-037637. See www.EpiGenChlamydia.eu for more details
30 about this Consortium. The study was supported by local funds at Uppsala University
31 Hospital. All authors declare that they have no conflicts of interest.

32

33 Short summary: Multilocus sequence typing of Dutch urogenital *Chlamydia trachomatis*
34 isolates showed a high discriminatory capacity and a small overlap to Swedish genotypes,
35 but could not be correlated with symptomatology.

36

37 **ABSTRACT**

38 In the past, contradictory results have been obtained linking *Chlamydia trachomatis*
39 serovars (*ompA* gene) to different clinical courses of infection. In this study we used a high
40 resolution multilocus sequence typing (MLST) system to genotype six genetic regions,
41 including *ompA,* in 70 Dutch urogenital *C. trachomatis* strains from patients with different
42 degrees of well-defined clinical symptoms (asymptomatic, symptomatic and lower
43 abdominal pain), to determine if MLST genotypes correlated with clinical manifestations of
44 infection. We identified 46 MLST types, with only a small overlap to Swedish MLST
45 types. This study found no correlation between MLST profiles and symptomatology. To
46 understand the clinical course of infection, future studies should not only consider bacterial
47 factors but also look on the immunogenetics of the host.

48

49 **Keywords:** clinical symptoms; *Chlamydia trachomatis*; multilocus sequence typing
50 (MLST); *ompA*

51

52 **INTRODUCTION**

53 Urogenital infection with the intracellular bacterium *Chlamydia trachomatis* is the most
54 common curable sexually transmitted bacterial infection in the United States and Europe.[1]
55 About 50 % of infected men and 70 % of infected women remain asymptomatic.[2] If
56 symptoms in females occur they are usually mild and atypical, and include mucopurulent
57 vaginal discharge, contact bleeding, and slight abdominal discomfort or pain. In a minority
58 of females urogenital chlamydia infection causes pelvic inflammatory disease (PID)
59 characterized by lower abdominal pain, fever and malaise. Chronic infection can cause
60 fibrosis and scarring of the fallopian tubes and severe sequelae such as ectopic pregnancy
61 and infertility. The conventional view that the damage is caused by antigen-specific
62 adaptive immune responses is not supported by unambiguous proof. It has instead been
63 suggested that the tissue damage leading to severe sequelae is caused by innate host
64 immune responses. Progressive disease is probably a combination of both host specific
65 innate immune responses and pathogen specific antigens as well as other biological
66 properties of the pathogen.[3,4,5,6]

67

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

73

74 The multilocus sequence typing (MLST) system developed by Klint *et al.* for
75 *C. trachomatis* is based on PCR amplification and DNA sequencing of five different target

76 regions and offers a threefold higher resolution than *ompA* genotyping.[8] Two of these five
77 target regions comprise partial sequences of known genes: *hctB* and *pbpB*. The *hctB* gene
78 encodes a histone H1-like protein that functions as a global regulator of chromatin structure
79 and gene expression, while the *pbpB* gene encodes a penicillin binding protein that is a
80 putative outer membrane protein potentially involved in the interaction with the host cell.[8,9]
81 The other three target regions contain hypothetical open reading frames, encoding putative
82 membrane proteins or unknown proteins.
83
84 In this study we hypothesized that pathogen specific factors contribute to the different
85 clinical manifestations of urogenital chlamydia infections in females. We used the MLST
86 system to genotype 70 well-defined urogenital *C. trachomatis* strains isolated from women
87 with different degrees of clinical symptoms, to determine if the multilocus genotype
88 correlated with clinical manifestations of infection.
89
90

91 **MATERIALS AND METHODS**

92 Clinical isolates

93 The study was performed in accordance with the Helsinki declaration and approved by the
94 Ethical Committee of the Academic Medical Centre, University of Amsterdam,
95 Amsterdam. *C. trachomatis* strains isolated from consenting female Caucasian visitors of
96 the Amsterdam STD outpatient clinic between 2001 and 2005 were propagated in
97 eukaryotic HeLa cell cultures using standard techniques. The women were asked to fill out
98 a questionnaire describing urogenital complaints (i.e. vaginal discharge, contact bleeding,
99 abdominal pain and dysuria). The strains were isolated as part of a larger study to

100 investigate bacterial and host factors related to the course of *C. trachomatis* infection. The
101 current study focused on bacterial components. A total of 70 strains representing the
102 dominantly prevailing urogenital serovars were selected from cases in which evidence for
103 all other sexually transmitted diseases (including HIV, *Trichomonas vaginalis*, and
104 *Neisseria*) was absent, so that *C. trachomatis* was the presumed cause of any patient
105 reported symptoms. Patient groups were formed based on clinical manifestation:
106 asymptomatic (n = 30), symptomatic (vaginal complaints like discharge, discomfort,
107 irregular and/or contact bleeding) without lower abdominal pain (LAP) (n = 23) and
108 symptomatic with LAP (n = 17). The *C. trachomatis* positive women with lower abdominal
109 pain were clinically treated as pelvic inflammatory disease cases and received standard
110 treatment for this condition. The Dutch isolates were compared to specimens collected from
111 heterosexuals in Örebro county in Sweden in 2006.
112
113 DNA purification
114 DNA was purified from culture using a MagAttract DNA Mini M48 kit (QIAGEN, Hilden,
115 Germany) on a BioRobot M48 workstation (QIAGEN), according to the manufacturer's
116 instructions.
117
118 PCR amplification
119 PCR amplification of the *ompA* gene and the five target regions of the MLST system was
120 performed with a high fidelity polymerase as previously described.[8,10]
121
122 Sequencing

123 Sequencing PCR using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied
124 Biosystems, Foster City, CA), as well as subsequent purification, was carried out according
125 to the manufacturer's instructions. Sequencing was performed on an ABI PRISM 3130
126 Genetic Analyzer (Applied Biosystems) and the data were analyzed using BioEdit 7.0.9
127 (Ibis Therapeutics, Carlsbad, CA) and ContigExpress, a component of VectorNTIAdvance
128 10.3.0 (Invitrogen). All novel mutations were reamplified and resequenced to assure their
129 authenticity.
130
131 Statistics
132 The three clinical categories were investigated statistically for association with MLST
133 genogroups, MLST profiles, individual variants in each of the five MLST regions, *ompA*
134 genotypes and the *ompA* B-, C- and intermediate complexes. The three categories were also
135 simplified into two categories, asymptomatic and symptomatic (LAP included), and the
136 statistical analysis was redone as described above. A chi-square goodness of fit test or a
137 two-tailed Fisher exact test was used for statistical analysis and a P value less than 0.05 was
138 considered statistically significant.
139
140 Phylogeny
141 Phylogenetic analysis was carried out using the neighbor-joining algorithm included in the
142 Phylip 3.68 software package.
143
144
145 **RESULTS**

146 The 70 *C. trachomatis* isolates could be separated into 46 MLST genotypes whereas the
147 conventional *ompA* genotyping system identified 18 genotypes (Table 1). Overall, the
148 MLST system had a 2.5 fold higher resolution than conventional *ompA* genotyping. The
149 MLST resolution was seven and six fold higher within serovar K and E respectively. MLST
150 profile number 34 was found in both serovar D and H.
151
152 All MLST profiles represented by more than one isolate included isolates from different
153 clinical categories. Certain MLST profiles differed from each other with only a single point
154 mutation in one genetic region and therefore phylogenetic analyses were carried out and the
155 MLST profiles were grouped into eight different genogroups (Figure 1). These genogroups
156 did not reflect the serovar distribution, with exception of genogroup 2, which contained
157 only two isolates that were serovar H, and genogroup 7, which contained five isolates that
158 were serovar K. No statistically significant correlation could be established between the
159 clinical manifestations of infection and the MLST genogroups, MLST profiles, individual
160 genetic variants in each of the five MLST regions, the *ompA* genotypes or the *ompA* B-, C-
161 or intermediate complexes.
162
163 The 46 MLST profiles of these 70 Dutch isolates were compared to 95 specimens collected
164 from heterosexuals in Örebro county in Sweden in 2006.[11] Seven MLST profiles,
165 comprising 25 of the 165 specimens (15 %), were present in both specimen populations.
166 MLST profile number 100 was found in serotype I among the Dutch isolates, but in
167 serotype J in the Swedish specimens.
168
169

170 **DISCUSSION**

171 Convincing data identifying antigens that are associated with the pathogenesis of urogenital
172 *C. trachomatis* infection has not yet been presented. There have been a number of studies
173 based on the *ompA* gene or its coded protein, but the results have been contradictory,
174 perhaps partly because of the limited numbers of specimens. However, the consensus
175 appears to be that there is no clear correlation between *ompA* and clinical manifestations.[7]
176 The chlamydial heat shock protein 60 is another candidate that has been extensively
177 investigated, but with equivocal results.[3] The current study utilized a MLST system based
178 on five highly variable genetic regions to investigate a potential correlation with the clinical
179 symptoms of infection. No statistically significant correlation could be found however.
180 Pathogen specific factors that are involved in disease development might be found in other
181 regions of the *C. trachomatis* genome that are not linked to the MLST genotypes, or disease
182 development might be due to host specific factors, having nothing or little to do with
183 genetic variation in *C. trachomatis*.
184
185 A limitation in the present study is the high variability in the genetic regions investigated
186 and the limited number of specimens, which might mask a complex correlation. The MLST
187 system used here is not based on housekeeping genes, as is the case in two other MLST
188 systems used for genotyping chlamydia.[12,13] These two systems have a low discriminatory
189 capacity which gives them limited usefulness in *C. trachomatis* strain discrimination and
190 outbreak investigations, but which might be advantageous when looking for virulence
191 factors that perhaps have evolved slowly over time and in parallel with the housekeeping
192 genes.
193

194 Comparison of the Dutch isolates to the Swedish specimens revealed a fairly small overlap
195 in MLST genotypes, indicating that there is a limited exchange of *C. trachomatis* strains
196 between the heterosexual populations in the two countries, as supported by the limited
197 spread of the new variant *C. trachomatis* outside Sweden in recent years.[14] This highlights
198 the usefulness of the MLST system in transmission studies and network analyses, where
199 high resolution is needed to tell closely related strains apart.
200

201 Phylogenetic analysis of the MLST genotyping data revealed a genetic relationship
202 dissimilar to that of the traditional serovar groupings and *ompA* genotyping. This is in
203 accordance with previous conclusions that the *ompA* gene differs in phylogeny and rate of
204 evolution from other regions of the genome, possibly due to recombination events.[15]
205

206 Recently, Bailey *et al*. showed using twin pairs that almost 40% of the differences in
207 responses to *C. trachomatis* infection can be ascribed to host genetic factors.[16] The
208 differences in the clinical course of infection are due to an interplay of both bacterial and
209 host genetic factors and both should be taken into account in future studies[17,18], though it
210 appears that host factors contribute to a much higher degree. The European Union has
211 funded the EpiGenChlamydia Consortium, which is led by Dr. Morré, and is in the process
212 of creating large biobanks of patient derived and bacterial specimens on which to perform
213 studies to determine bacterial and host factors that play a role in the course of infection with
214 chlamydia.[18]
215

216 In summary, MLST analysis of *C. trachomatis* isolates showed a high discriminatory
217 capacity but could not identify any multilocus genotypes that correlated with different well-

218 defined clinical manifestations of female urogenital infection. This might in part reflect the
219 genes chosen for MLST profiling in relation to the clinical course of infection, or, and
220 consistent with the combined results of all studies to date, that bacterial factors if important
221 need to be understood in the context of host factors. Thus, future studies should be directed
222 at identifying host genetic factors that might play either a general role in the pathogenesis
223 of chlamydial infection, or specifically in response to a particular bacterial factor or factors.
224

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275

Table 1. Genetic profiles of all 70 isolates

Isolates	Clinical category	Serovar	*ompA*	MLST profile number	MLST profile				
					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

276 The clinical categories have been abbreviated "asymp" for asymptomatic, "symp" for
277 symptomatic and LAP for lower abdominal pain. The numbers are arbitrary designations
278 from the *C. trachomatis* MLST database (http://mlstdb.bmc.uu.se/) and correspond to
279 specific DNA sequences. There were 46 MLST genotypes compared to 18 *ompA*
280 genotypes.

281

282 *Identical to strain B/IU-1226 (AF063208.1)

283 † Identical to strain D/IC-CAL8 (DQ064285.1)

284 ‡ Identical to strain D/LSU-EP212 (AF279587.1)

285 § Identical to strain D/UW-3 (DQ064284.1)

286 ¶ Identical to strain DK-K35 (AM901184.1)

287 # Identical to strain E/Bour (DQ064286.1)

288 ** Identical to strain F/IC-CAL3 (DQ064287.1)

289 †† Identical to strain G/IU-FW9155 (FJ261939.1)

290 ‡‡ Identical to strain G/11222 (CP001888.1)

291 §§ Identical to strain UW-4 (AF304857.1)

292 ¶¶ Identical to strain CS-121/96 (DQ116395.1)

293 ## Identical to strain Ia/IU-TC0018ut (FJ261940.1)

294 *** Identical to strain Ia/IU-4168 (AF063201.2)

295 ††† Identical to strain J/UW-36 (DQ064292.1)

296 ‡‡‡ Identical to strain Ja/IU-FW4076 (FJ261932.1)

297 §§§ Identical to strain DK-K7 (AM901164.1)

298

299

300

301 **FIGURE LEGEND**

302

303 Figure 1. Unrooted cladogram, based on the neighbor joining algorithm, showing the
304 genetic relationship between all 46 MLST profiles. The letter after each MLST profile
305 number indicates serovar, based on the *ompA* sequence. Clinical category is indicated by
306 the letter "a" for asymptomatic, "s" for symptomatic and "L" for lower abdominal pain
307 (LAP). The MLST profiles have been grouped into eight genogroups, highlighted with a
308 gray color. Bootstrap values for each genogroup are written in bold text and are shown as
309 percentages of 1,000 replicates.

Figure
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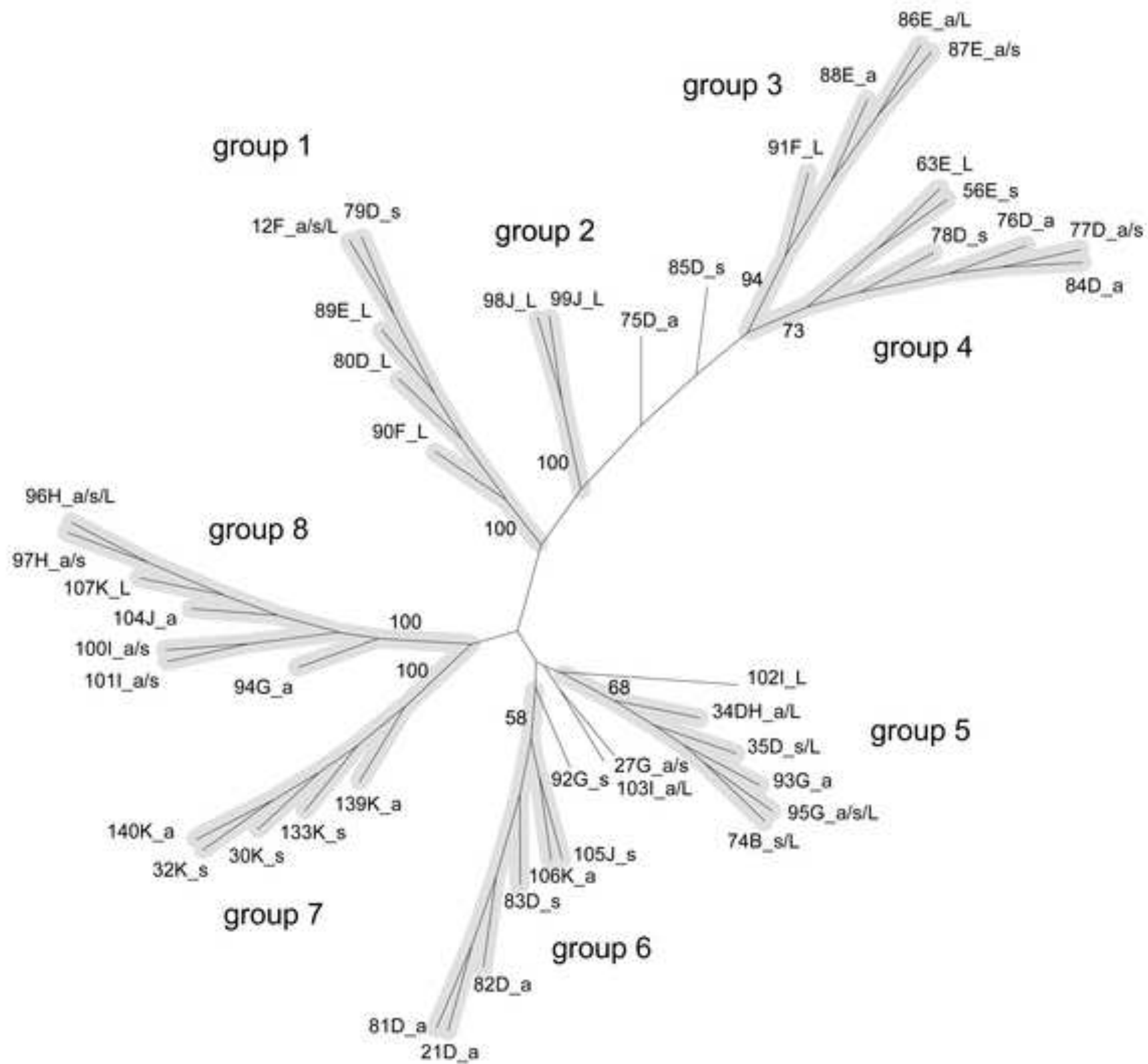

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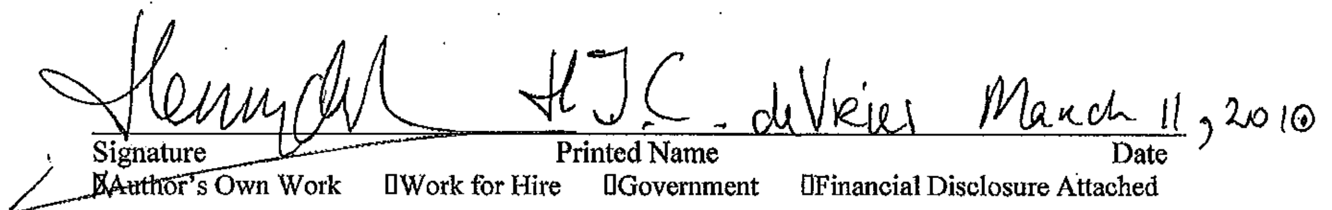

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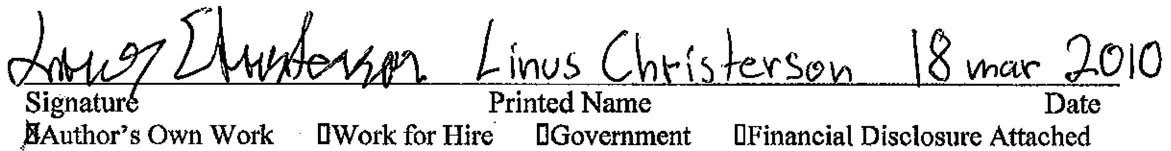

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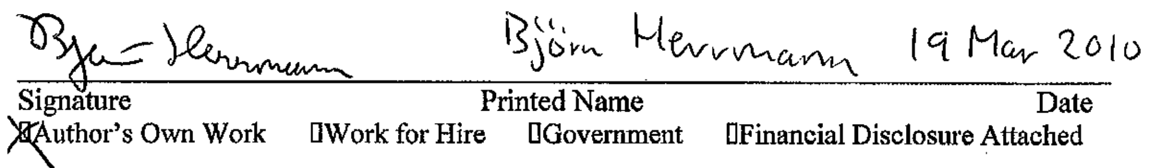

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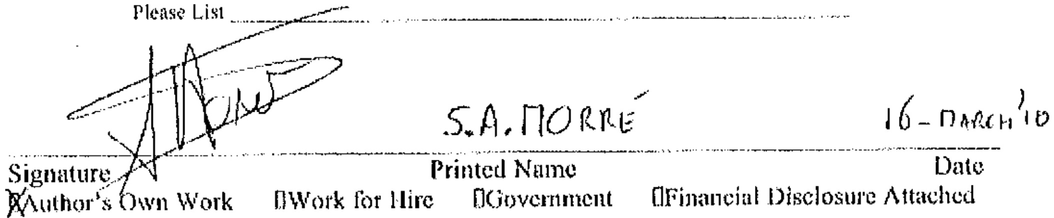

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