Plasmid copy number and disease severity in naturally occurring ocular *Chlamydia trachomatis* infection

**RUNNING TITLE**

Ocular *C. trachomatis* infection and plasmid copy number by ddPCR

Anna R Last¹#, Chrissy h Roberts¹, Eunice Cassama², Meno Nabicassa², Sandra Molina-Gonzalez¹, Sarah E Burr¹,³, David CW Mabey¹, Robin L Bailey¹, Martin J Holland¹

¹Clinical Research Department, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom

²Programa Nacional de Saúde de Visão, Ministério de Saúde Publica, P.O. Box 50, Avenida de Unidade Africana, Bissau, Guiné Bissau

³Disease Control and Elimination Theme, Medical Research Council Unit The Gambia, P.O. Box 273 Banjul, Atlantic Boulevard, Fajara, The Gambia

#CORRESPONDING AUTHOR  Mailing Address: Clinical Research Department, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom

Telephone: +44 (0)207 927 2566  Email: anna.last@lshtm.ac.uk
Abstract

The *Chlamydia trachomatis* (*Ct*) plasmid is a virulence factor. Plasmid copy number, *Ct* load and disease severity were assessed in a trachoma-hyperendemic treatment-naïve population. Using droplet digital PCR plasmid copy number was found to be stable (median 5.34 (1-18)) and there were no associations with *Ct* load or disease severity.

KEYWORDS *Chlamydia trachomatis*; trachoma; disease severity; plasmid copy number; ddPCR
Trachoma is caused by infection with ocular strains of *Chlamydia trachomatis*. The 7.5-kb *C. trachomatis* plasmid has been shown to function as a virulence factor in animal models (1,2). Phenotypic differences exist between plasmid-cured and wild-type *C. trachomatis* strains with respect to infectivity, glycogen accumulation, induction of inflammation and activation of toll-like receptor pathways (3,4). Plasmid deletion mutagenesis studies showed that deletion of the plasmid-encoded *pgp4* gene results in an *in vitro* phenotype identical to that of a plasmid-free strain (5). This supports bacterial transcriptome analysis showing a decrease in transcript levels of a subset of chromosomal genes in a naturally occurring plasmid-free strain of *C. trachomatis*, demonstrating that the plasmid is a transcriptional regulator of virulence-associated genes (6).

There is little information in the literature relating plasmid copy number (per genome) to virulence (7-9). The mechanisms of plasmid virulence are not clearly defined, particularly in naturally occurring infections. We assessed plasmid copy number variation and its association with disease severity in ocular *C. trachomatis* infection from a trachoma-hyperendemic treatment-naïve population on the Bijagós Archipelago of Guinea Bissau.

Individuals from 300 randomly selected households from 38 villages on four islands were examined by a single trained examiner using the WHO simplified and modified FPC grading systems (10,11). In the FPC system, follicles (F), papillae (P) and conjunctival scarring (C) are separately scored 0-3. Active disease (TF (follicular trachoma) or TI (inflammatory trachoma) by the WHO simplified system) equates to F2/3 and P3 respectively. C2/3 (and in some cases C1) is equivalent to TS (trachomatous scarring). Both systems were used to provide detailed phenotypic information and comparability with other studies. Individuals’ age, sex and ethnicity were recorded.
Swabs were taken from the left upper tarsal conjunctiva of each participant using a validated procedure (12,13). Swabs were collected dry into microcentrifuge tubes (Simport, Canada), kept on ice in the field and frozen to -80°C within 8 hours of collection. Measures were taken to avoid cross-contamination in the field and in the laboratory (13).

DNA extraction and droplet digital PCR (ddPCR) for detection of C. trachomatis plasmid were conducted as described previously (14). A second duplex assay was used to estimate plasmid and chromosome (omcB) target concentrations within the same reaction in plasmid positive samples. We used published primer-probe target sequences appropriate for quantitation of all genovars of C. trachomatis (7,14). We used a modified omcB probe to improve quenching efficiency and reduce background fluorescence (Table 1). Methods for mastermix preparation, droplet generation, thermal cycling conditions, droplet reading, target DNA concentration calculation and re-testing of saturated samples are described elsewhere (14). Estimated quantities of omcB and plasmid are expressed as copies/swab. C. trachomatis load refers to omcB copies/swab.

Plasmid copy number (per genome) was calculated using the plasmid:genome ratio.

Raw quantitation data were processed as previously described (14). Geometric mean omcB load and linear and logistic regression analyses (with odds ratios (OR)) were conducted in STATA 12 (Stata Corporation, College Station, Texas USA) to examine associations between plasmid copy number, load and detailed clinical phenotype. C. trachomatis load and plasmid copy number data were log-(e) transformed and robust standard error used where indicated.

Of 1,511 individuals enrolled, 1,508 individuals consented to ocular assessment and 1,507 conjunctival swabs were obtained. The median age of participants was 13 years (1 month-88 years) and 57% were female. Most participants were of the Bijagós ethnic group. The prevalence
of active trachoma (TF/TI) in 1-9 year olds was 21% (136/660) (95% CI 17.89-24.11%). Overall, 11% had clinically active trachoma (164/1508) (95% CI 9.42-12.58%). *C. trachomatis* plasmid DNA was detected in 16% overall (233/1507) (26% of 1-9 year olds). All samples were adequate by criteria described previously (14).

*C. trachomatis* load was estimated in 79% (184/233) of plasmid positive samples. In 21% of samples where plasmid load was very low, *omcB* was below the level of detection.

The geometric mean estimated *omcB* copies/swab varied by clinical phenotype (294 copies/swab (95% C.I. 165-524) in 73 with normal conjunctivae, 8562 copies/swab (95% C.I. 5412-13546) in 92 with active trachoma and 928 copies/swab (95% C.I. 280-2074) in 19 with scarring.

The median plasmid copy number was 5.34 (1-18.03) (Figure 1). Plasmid copy number was stable in infections across the four study islands (Kruskall Wallis H (Chi2)=4.5001(df=3,p=0.2123)). Plasmid copy number was not associated with the presence of active trachoma (OR 1.00, 95% CI 0.88-1.12, p=0.960), severity of inflammatory (OR 1.04, 95% CI 0.927-1.162, p=0.515) or follicular (OR 1.03, 95% CI 0.922-1.159, p=0.572) disease or *C. trachomatis* load (Table 2). At lower load the variance was highly heterogeneous (Levene’s W0=55.3, df=2, p<0.00000001) (Figure 2).

The theoretical advantages of ddPCR are presented by Hindson et al. (15). These include nanolitre-sized droplet partitioning of the reaction, which promotes optimal primer-template interaction conditions robust to variation in PCR efficiency, thus enabling accurate estimation of both plasmid and *omcB* copy numbers within the same reaction. We have discussed the precision and accuracy of our diagnostic ddPCR assay elsewhere (14).
There are a few published studies examining plasmid copy number in reference strains of *C. trachomatis* (7-9, 16-17). Pickett *et al.* showed that across 12 *C. trachomatis* serovars the plasmid copy number was not significantly different but there were variations depending on growth phase and condition during *in vitro* culture (7). Seth-Smith *et al.* showed an increased plasmid copy number in ocular relative to urogenital strains (8). We demonstrate a stable plasmid copy number distribution in naturally occurring ocular *C. trachomatis* infection that does not vary with geographic location, clinical phenotype or *C. trachomatis* load. Our data show that ddPCR may have limitations in measuring plasmid copy number at very low load infections (<200 *omcB* copies/swab) where plasmid copy number variance is greatest. This observation may reflect a breakdown in the assumptions required to apply the Poisson distribution to accurately estimate load with ddPCR. Despite the caveats, our data suggest plasmid copy number stability in naturally occurring ocular *C. trachomatis* infection.

Maintenance of the plasmid at low copy number carries inherent risk during cell partition (18) but naturally occurring plasmid-free strains are rare (19-21). A lower risk higher copy number system is metabolically expensive but may confer a ‘fitness’ advantage. Thus, the maintenance of 5-6 plasmids per genome may maximise infectivity or intracellular survival whilst provoking minimal host immune response.

Though there is convincing evidence that the chlamydial plasmid is a virulence factor (3, 4, 6, 22-24) our data suggest that plasmid copy number is not associated with disease severity and that additive gene dosage effects do not appear to correlate with pathogen virulence *in vivo*. This supports *in vitro* work showing no association between plasmid copy number and tissue tropism (9). Previous work *in vitro* and in animal models suggests that subtle genomic differences between chlamydial isolates are associated with differences in growth kinetics, immune
responses and pathology (25,26). Further epidemiological and *in vitro* studies using comparative pathogen genomics to examine these associations are required to fully understand the relationship between disease severity and chlamydial virulence.

Acknowledgements

This study was conducted in accordance with the declaration of Helsinki. Ethical approval was obtained from the Comité Nacional de Ética e Saúde (Guinea Bissau), the LSHTM Ethics Committee (UK) and the The Gambia Government/MRC Joint Ethics Committee (The Gambia). Written (thumbprint or signature) informed consent was obtained from all study participants or their guardians as appropriate. Following the survey all communities on the study islands were treated with azithromycin in line with WHO and national protocols.

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All authors declare no conflict of interests.


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Tables and Figures

Table 1. Primer and probe sequences for control and *C. trachomatis* targets using the ddPCR system

Table 2. The relationship between plasmid copy number and *C. trachomatis* load (*omcB* copies/swab)

Figure 1. Distribution of plasmid copy number variation in naturally occurring ocular infection with *C. trachomatis* within the study population

Figure 2. *C. trachomatis* load and plasmid copy number variation
Table 1. Primer and probe sequences for control and *C. trachomatis* targets using the ddPCR system

<table>
<thead>
<tr>
<th>Molecular Target</th>
<th>Nucleotide Sequence and Modifications</th>
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<tbody>
<tr>
<td>Internal Control</td>
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<tr>
<td><em>Homo sapiens</em> ribonuclease P/MRP 30kDa subunit (RPP30)</td>
<td></td>
</tr>
<tr>
<td>Forward (RPP30-F)</td>
<td>5’ AGA TTT GGA CCT GCG AGC G 3’</td>
</tr>
<tr>
<td>Reverse (RPP30-R)</td>
<td>5’ GAG CGG CTG TCT CCA CAA GT 3’</td>
</tr>
<tr>
<td>Probe (RPP30_HEX_BHQ1)</td>
<td>5’ HEX-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3’</td>
</tr>
<tr>
<td><strong>Target One</strong></td>
<td></td>
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<tr>
<td><em>C. trachomatis</em> cryptic plasmid pLGV440 (circular; genomic DNA; 7500bp)</td>
<td></td>
</tr>
<tr>
<td>Forward (Ct-plasmid-F)</td>
<td>5’ CAG CTT GTA GTC CTG CTT GAG AGA 3’</td>
</tr>
<tr>
<td>Reverse (Ct-plasmid-R)</td>
<td>5’ CAA GAG TAC ATC GTT CAA CGA AGA 3’</td>
</tr>
<tr>
<td>Probe (Ct-plasmid_FAM_BHQ1)</td>
<td>5’ 6FAM-CCC CAC CAT TTT TCC GGA GCG A-BHQ1-3’</td>
</tr>
<tr>
<td>Probe (Ct-plasmid_HEX_BHQ1)</td>
<td>5’ HEX-CCC CAC CAT TTT TCC GGA GCG A-BHQ1-3’</td>
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<td><strong>Target Two</strong></td>
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<tr>
<td><em>C. trachomatis</em> (Serovar A) <em>omcB</em> gene</td>
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<tr>
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<td>5’ GAC ACC AAA CGC AAA GAC AAC AC 3’</td>
</tr>
<tr>
<td>Reverse (Ct-omcB-R)</td>
<td>5’ ACT CAT GAA CCG GAG CAA CCT 3’</td>
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<tr>
<td>Probe (Ct-omcB-FAM-BHQ1)</td>
<td>5’ 6FAM-CCA CAG CAA AGA GAC TCC GTG AGA CCG-BHQ1-3’</td>
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*C. trachomatis* plasmid probe used in screening (first) assay  
*C. trachomatis* probe used in quantitative (second) assay
Table 2. The relationship between plasmid copy number and *C. trachomatis* load (omcB copies/swab)

<table>
<thead>
<tr>
<th>OmcB (copies/swab)</th>
<th>n</th>
<th>Variance</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
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<tr>
<td>&lt;100</td>
<td>41</td>
<td>19.8139</td>
<td>1</td>
<td>4.1514</td>
<td>18.0291</td>
</tr>
<tr>
<td>100-10000</td>
<td>82</td>
<td>2.7136</td>
<td>1</td>
<td>5.3421</td>
<td>9.2819</td>
</tr>
<tr>
<td>&gt;10000</td>
<td>62</td>
<td>1.0814</td>
<td>3.6164</td>
<td>5.4261</td>
<td>8.3947</td>
</tr>
</tbody>
</table>

Kruskall Wallis H (Chi2) = 4.58, df=2, p=0.10.
Figure 1. Distribution of plasmid copy number variation in naturally occurring ocular infection with *C. trachomatis* within the study population.

<table>
<thead>
<tr>
<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>se(mean)</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
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<tbody>
<tr>
<td>184</td>
<td>1</td>
<td>18.03</td>
<td>5.45</td>
<td>0.177</td>
<td>5.34</td>
<td>4.39</td>
<td>6.46</td>
</tr>
</tbody>
</table>

Skewness 1.31 (p<0.00001)

Kurtosis 7.96 (p<0.00001)
Figure 2. *C. trachomatis* load and plasmid copy number variation