The Chlamydia trachomatis plasmid is a virulence factor. Plasmid copy number, C. trachomatis load and disease severity were assessed in a treatment-naive population where trachoma is hyperendemic. By using droplet digital PCR, plasmid copy number was found to be stable (median, 5.34 [range, 1 to 18]) and there were no associations with C. trachomatis load or disease severity.
TABLE 1 Primer and probe sequences for control and \textit{C. trachomatis} targets using the ddPCR system$^a$

<table>
<thead>
<tr>
<th>Molecular target and primer or probe</th>
<th>Nucleotide sequence and modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Homo sapiens} RNase P/MPR 30-kDa subunit (RPP30) (internal control)</td>
<td></td>
</tr>
<tr>
<td>Forward primer (RPP30-F)</td>
<td>5\textsuperscript{'}G AGA TTT GGA CCT GCG AGC G 3\textsuperscript{'})</td>
</tr>
<tr>
<td>Reverse primer (RPP30-R)</td>
<td>5\textsuperscript{'}C AAG TAC ATC TTT GCA CTA CAA GT 3\textsuperscript{'})</td>
</tr>
<tr>
<td>Probe (RPP30_HEX_BHQ1)</td>
<td>5\textsuperscript{'}G AGA TTT GGA CCT GCG AGC G 3\textsuperscript{'})</td>
</tr>
</tbody>
</table>

\textit{C. trachomatis} cryptic plasmid

pLGV440 (circular; genomic DNA; 7,500 bp)

Forward primer (Ct-plasmid-F) | 5\textsuperscript{'}G CAG TTT GTA GTC CTG CTT GAG AGA 3\textsuperscript{'}) |

Reverse primer (Ct-plasmid-R) | 5\textsuperscript{'}G CAAG TAC ATC TTT GCA CTA CAA GT 3\textsuperscript{'}) |

Probe (Ct-plasmid_FAM_BHQ1)$^b$ | 5\textsuperscript{'}G 6FAM-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3\textsuperscript{'}) |

Probe (Ct-plasmid_HEX_BHQ1)$^c$ | 5\textsuperscript{'}G HEX-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3\textsuperscript{'}) |

\textit{C. trachomatis} (serovar A) \textit{omcB} gene

Forward primer (Ct-omcB-F) | 5\textsuperscript{'}G GAC ACC AAA CGC AAA GAC AAC AC 3\textsuperscript{'}) |

Reverse primer (Ct-omcB-R) | 5\textsuperscript{'}G ACT CAT GAA CCG GAG CAA CTT 3\textsuperscript{'}) |

Probe (Ct-omcB-FAM_BHQ1)$^c$ | 5\textsuperscript{'}G 6FAM-CCA CAG CAA AGA GAG TCC CGT AGA CCG-BHQ1 3\textsuperscript{'}) |

$^a$ MRP, mitochondrial RNA processing endoribonuclease; 6FAM, 6-carboxyfluorescein reporter; BHQ1, black hole quencher 1; HEX, hexachlorofluorescein reporter.

$^b$ \textit{C. trachomatis} plasmid probe used in screening (first) assay.

$^c$ \textit{C. trachomatis} probe used in quantitative (second) assay.

mid-positive samples. In 21% of samples where plasmid load was very low, \textit{omcB} was below the level of detection.

The geometric mean estimated number of \textit{omcB} copies/swab varied by clinical phenotype: 294 copies/swab (95% CI, 165 to 524) in 73 subjects with normal conjunctivae, 8,562 copies/swab (95% CI, 5,412 to 13,546) in 92 with active trachoma, and 928 copies/swab (95% CI, 280 to 2,074) in 19 with scarring.

The median plasmid copy number was 5.34 (1 to 18.03) (Fig. 1). Plasmid copy number was stable in infections across the four study islands (Kruskal-Wallis $H = 4.58; df = 2; P = 0.10$). Plasmid copy number was not associated with the presence of active trachoma (OR, 1.00; 95% CI, 0.88 to 1.12; $P = 0.96$), severity of inflammatory (OR, 1.04; 95% CI, 0.927 to 1.16; $P = 0.51$) or follicular (OR, 1.03; 95% CI, 0.922 to 1.15; $P = 0.57$) disease, or \textit{C. trachomatis} load (Table 2).

At lower loads, the variance was highly heterogeneous (Levene’s $W = 55.3; df = 2; P < 0.000000001$) (Fig. 2).

The theoretical advantages of ddPCR are presented by Hindson et al. (15). These include nanoliter-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 \textit{omcB} copy numbers within the same reaction. We have discussed the precision and accuracy of our diagnostic ddPCR assay elsewhere (14).

old's was 21% (136/660) (95% confidence interval [CI], 17.89 to 24.11%). Overall, 11% had clinically active trachoma (164/1,508) (95% CI, 9.42 to 12.58%). \textit{C. trachomatis} plasmid DNA was detected in 16% overall (233/1,507) (26% of 1- to 9-year-olds). All samples were adequate according to criteria described previously (14).

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

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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 in 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 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 numbers carries an 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 or 6 plasmids per genome may maximize infectivity or intracellular survival while 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.

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We have no conflicts of interest to declare.

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