View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by LSHTM Research Online

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

LSHTM Research Online

Last, AR; Roberts, CH; Cassama, E; Nabicassa, M; Molina-Gonzalez, S; Burr, SE; Mabey, DC; Bailey, RL; Holland, MJ; (2013) Plasmid copy number and disease severity in naturally occurring ocular Chlamydia trachomatis infection. Journal of clinical microbiology. ISSN 0095-1137 DOI: https://doi.org/10.1128/JCM.02618-13

Downloaded from: http://researchonline.lshtm.ac.uk/1320569/

DOI: https://doi.org/10.1128/JCM.02618-13

Usage Guidelines:

Please refer to usage guidelines at https://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/

https://researchonline.lshtm.ac.uk

JCM
Journals.ASM.org

Plasmid Copy Number and Disease Severity in Naturally Occurring Ocular *Chlamydia trachomatis* Infection

Anna R. Last,[a] Chrissy h. Roberts,[a] Eunice Cassama,[b] Meno Nabicassa,[b] Sandra Molina-Gonzalez,[a] Sarah E. Burr,[a,c] David C. W. Mabey,[a] Robin L. Bailey,[a] Martin J. Holland[a]

Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom[a]; Programa Nacional de Saúde de Visão, Ministério de Saúde Publica, Bissau, Guinea-Bissau[b]; Disease Control and Elimination Theme, Medical Research Council Unit The Gambia, Fajara, The Gambia[c]

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.

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-borne *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 treatment-naive population on the Bijagós Archipelago of Guinea Bissau where trachoma is hyperendemic.

This study was conducted in accordance with the declaration of Helsinki. Ethical approval was obtained from the Comitê Nacional de Ética e Saúde (Guinea Bissau), the LSHTM Ethics Committee (United Kingdom), and 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.

Individuals from 300 randomly selected households from 38 villages on four islands were examined by a single trained examiner using the simplified WHO and modified FPC grading systems (10, 11). In the FPC system, follicles (F), papillae (P), and conjunctival scarring (C) are separately scored on a scale of 0 to 3. Active disease (TF [follicular trachoma] or TI [inflammatory trachoma] according to the simplified WHO 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 h 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 master mix preparation, droplet generation, thermal cycling conditions, droplet reading, target DNA concentration calculation, and retesting 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, TX) 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 was 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 to 88 years), and 57% were female. Most participants were of the Bijagós ethnic group. The prevalence of active trachoma (TF/TI) in 1- to 9-year-

Received 21 September 2013 Returned for modification 24 October 2013
Accepted 25 October 2013
Published ahead of print 6 November 2013
Editor: E. Munson
Address correspondence to Anna R. Last, anna.last@lshtm.ac.uk.
Copyright © 2014 Last et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.
doi:10.1128/JCM.02618-13

TABLE 1 Primer and probe sequences for control and *C. trachomatis* targets using the ddPCR system[a]

Molecular target and primer or probe	Nucleotide sequence and modifications
Homo sapiens RNase P/MRP 30-kDa subunit (RPP30) (internal control)	
Forward primer (RPP30-F)	5′ AGA TTT GGA CCT GCG AGC G 3′
Reverse primer (RPP30-R)	5′ GAG CGG CTG TCT CCA CAA GT 3′
Probe (RPP30_HEX_BHQ1)	5′ HEX-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1 3′
C. trachomatis cryptic plasmid pLGV440 (circular; genomic DNA; 7,500 bp)	
Forward primer (Ct-plasmid-F)	5′ CAG CTT GTA GTC CTG CTT GAG AGA 3′
Reverse primer (Ct-plasmid-R)	5′ CAA GAG TAC ATC GTT CAA CGA AGA 3′
Probe (Ct-plasmid_FAM_BHQ1)[b]	5′ 6FAM-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3′
Probe (Ct-plasmid_HEX_BHQ1)[c]	5′ HEX-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3′
C. trachomatis (serovar A) *omcB* gene	
Forward primer (Ct-*omcB*-F)	5′ GAC ACC AAA CGC AAA GAC AAC AC 3′
Reverse primer (Ct-*omcB*-R)	5′ ACT CAT GAA CCG GAG CAA CCT 3′
Probe (Ct-*omcB*-FAM-BHQ1)	5′ 6FAM-CCA CAG CAA AGA GAC TCC CGT AGA CCG-BHQ1 3′

[a] MRP, mitochondrial RNA processing endoribonuclease; 6FAM, 6-carboxyfluorescein reporter; BHQ1, black hole quencher 1; HEX, hexachlorofluorescein reporter.
[b] *C. trachomatis* plasmid probe used in screening (first) assay.
[c] *C. trachomatis* probe used in quantitative (second) assay.

TABLE 2 Relationship between plasmid copy number and *C. trachomatis* load[a]

No. of *omcB* copies/swab	No. of samples	Plasmid copy no.			
		Variance	Minimum	Median	Maximum
<100	41	19.8139	1	4.1514	18.0291
100–10,000	82	2.7136	1	5.3421	9.2819
>10,000	62	1.0814	3.6164	5.4261	8.3947

[a] Kruskall Wallis H (χ^2) = 4.58; df = 2; P = 0.10.

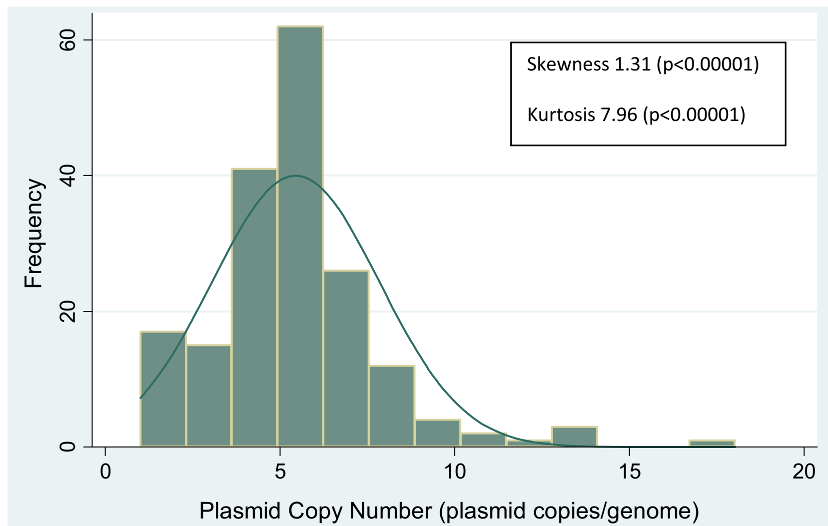
olds was 21% (136/660) (95% confidence interval [CI], 17.89 to 24.11%). Overall, 11% had clinically active trachoma (164/1,508) (95% CI, 9.42 to 12.58%). *C. trachomatis* plasmid DNA was detected in 16% overall (233/1,507) (26% of 1- to 9-year-olds). All samples were adequate according to criteria described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

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 number of *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 [χ^2] = 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 to 1.12; P = 0.960), severity of inflammatory (OR, 1.04; 95% CI, 0.927 to 1.162; P = 0.515) or follicular (OR, 1.03; 95% CI, 0.922 to 1.159; P = 0.572) disease, or *C. trachomatis* load (Table 2). At lower loads, the variance was highly heterogeneous (Levene's W_0 = 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 *omcB* copy numbers within the same reaction. We have discussed the precision and accuracy of our diagnostic ddPCR assay elsewhere (14).



n	Min	Max	Mean	se(mean)	Median	25%	75%
184	1	18.03	5.45	0.177	5.34	4.39	6.46

FIG 1 Distribution of plasmid copy number variation in naturally occurring ocular infections with *C. trachomatis* within the study population. se, standard error.

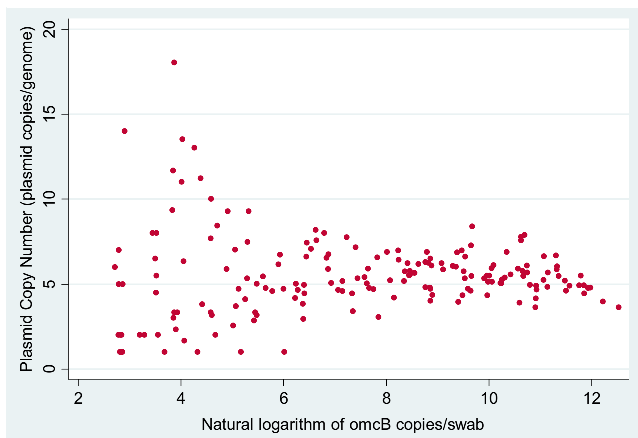


FIG 2 *C. trachomatis* load and plasmid copy number variation.

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 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 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.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust (grant 097330/Z/11/Z).

All reagents for the study were purchased at full list price, but we are grateful to Bio-Rad Laboratories Inc. for the loan of the QX-100 platform free of charge for use in this study. We extend thanks to the study participants and field research team in Guinea Bissau.

We have no conflicts of interest to declare.

REFERENCES

1. **Kari L, Whitmire WM, Olivares-Zavaleta N, Goheen MM, Taylor LD, Carlson JH, Sturdevant GL, Lu C, Bakios LE, Randall LB, Parnell MJ, Zhong G, Caldwell HD.** 2011. A live-attenuated chlamydial vaccine prevents against trachoma in non-human primates. J. Exp. Med. **208:**2217–2223. http://dx.doi.org/10.1084/jem.20111266.
2. **Frazer LC, Darville T, Chandra-Kuntal K, Andrews CW, Jr, Zurenski M, Mintus M, AbdelRahmen YM, Belland RJ, Ingalls RR, O'Connell CM.** 2012. Plasmid-cured *Chlamydia caviae* activates TLR2-dependent signaling and retains virulence in the guinea pig model of genital tract infection. PLoS One **7:**e30747. http://dx.doi.org/10.1371/journal.pone.0030747.
3. **O'Connell CM, AbdelRahman YM, Green E, Darville HK, Saira K, Smith B, Darville T, Scurlock AM, Meyer CR, Belland RJ.** 2011. Toll-like receptor 2 activation by *Chlamydia trachomatis* is plasmid dependent, and plasmid-responsive chromosomal loci are coordinately regulated in response to glucose limitation by *C. trachomatis* but not by *C. muridarum*. Infect. Immun. **79:**1044–1056. http://dx.doi.org/10.1128/IAI.01118-10.
4. **Russell M, Darville T, Chandra-Kuntal K, Smith B, Andrews CW, Jr, O'Connell CM.** 2011. Infectivity acts as *in vivo* selection for maintenance of the chlamydial cryptic plasmid. Infect. Immun. **79:**98–107. http://dx.doi.org/10.1128/IAI.01105-10.
5. **Song L, Carlson JH, Whitmire WM, Kari L, Virtaneva K, Sturdevant DE, Watkins H, Zhou B, Sturdevant GL, Porcella SF, McClarty G, Caldwell HD.** 2013. *Chlamydia trachomatis* plasmid-encoded *pgp4* is a transcriptional regulator of virulence associated genes. Infect. Immun. **81:**636. http://dx.doi.org/10.1128/IAI.01305-12.
6. **Carlson JH, Whitmire WM, Crane DD, Wicke L, Virtaneva K, Sturdevant DE, Kupko JJ, III, Porcella SF, Martinez-Orengo N, Heinzen RA, Kari L, Caldwell HD.** 2008. The *Chlamydia trachomatis* plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. Infect. Immun. **76:**2273–2283. http://dx.doi.org/10.1128/IAI.00102-08.
7. **Pickett MA, Everson JS, Pead PJ, Clarke IN.** 2005. The plasmids of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. Microbiology **151:**893–903. http://dx.doi.org/10.1099/mic.0.27625-0.
8. **Seth-Smith HM, Harris SR, Persson K, Marsh P, Barron A, Bignall A, Bjartling C, Clarke L, Cutcliffe LT, Lambden PR, Lennard N, Lockey SJ, Quail MA, Salim O, Skilton RJ, Wang Y, Holland MJ, Parkhill J, Thomson NR, Clarke IN.** 2009. Co-evolution of genomes and plasmids within *Chlamydia trachomatis* and the emergence in Sweden of a new variant strain. BMC Genomics **10:**239–249. http://dx.doi.org/10.1186/1471-2164-10-239.
9. **Ferreira R, Borges V, Nunes A, Borrego MJ, Gomes JP.** 2013. Assessment of the load and transcriptional dynamics of *Chlamydia trachomatis* plasmid according to strains' tissue tropism. Microbiol. Res. **168:**333–339. http://dx.doi.org/10.1016/j.micres.2013.02.001.
10. **Dawson CR, Jones BR, Tarizzo ML.** 1981. Guide to trachoma control in programs for the prevention of blindness. World Health Organization, Geneva, Switzerland.
11. **Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR.** 1987. A simple system for the assessment of trachoma and its complications. Bull. World Health Organ. **65:**477–483.
12. **Keenan JD, Lakew T, Alemayehu W, Melese M, Porco TC, Yi E, House JI, Zhou Z, Ray KJ, Acharya NR, Whitcher JP, Gaynor BD, Lietman TM.** 2010. Clinical activity and polymerase chain reaction evidence of chlamydial infection after repeated mass antibiotic treatments for trachoma. Am. J. Trop. Med. Hyg. **82:**482–487. http://dx.doi.org/10.4269/ajtmh.2010.09-0315.
13. **Stare D, Harding-Esch E, Muñoz B, Bailey R, Mabey D, Holland M, Gaydos C, West S.** 2011. Design and baseline data of a randomised trial to evaluate coverage and frequency of mass treatment with azithromycin: The Partnership for the Rapid Elimination of Trachoma (PRET) in Tanzania and The Gambia. Ophthalmic Epidemiol. **18:**20–29. http://dx.doi.org/10.3109/09286586.2010.545500.
14. **Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabi-**

cassa M, McCarthy E, Burr SE, Mabey DC, Bailey RL, Holland MJ. 2013. Development and evaluation of a next generation digital PCR diagnostic assay for ocular *Chlamydia trachomatis* infections. J. Clin. Microbiol. **51:**2195–2203. http://dx.doi.org/10.1128/JCM.00622-13.

15. **Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M.** 2013. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat. Methods **10:**1003–1005. http://dx.doi.org/10.1038/nmeth.2633.
16. **Palmer L, Falkow S.** 1986. A common plasmid of *Chlamydia trachomatis*. Plasmid **16:**52–62. http://dx.doi.org/10.1016/0147-619X(86)90079-X.
17. **Tam JE, Davis CH, Thresher RJ, Wyrick P.** 1992. Location of the origin of replication for the 7.5-kb *Chlamydia trachomatis* plasmid. Plasmid **27:** 231–236. http://dx.doi.org/10.1016/0147-619X(92)90025-6.
18. **Twigg AJ, Sheratt D.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature **283:**216–218. http://dx.doi.org/10.1038/283216a0.
19. **Thomas NS, Lusher M, Storey CC, Clarke IN.** 1997. Plasmid diversity in chlamydia. Microbiology **143:**1847–1854. http://dx.doi.org/10.1099/00221287-143-6-1847.
20. **An Q, Radcliffe G, Vassallo R, Buxton G, O'Brien WJ, Pelletier DA, Weisberg WG, Klinger JD, Olive DM.** 1992. Infection with a plasmid-free variant Chlamydia related to *Chlamydia trachomatis* identified by using multiple assays for nucleic acid detection. J. Clin. Microbiol. **30:** 2814–2821.
21. **Peterson EM, Markoff BA, Schacter J, de la Maza LM.** 1990. The 7.5kb plasmid present in *Chlamydia trachomatis* is essential for the growth of the micro-organism. Plasmid **23:**144–148. http://dx.doi.org/10.1016/0147-619X(90)90033-9.
22. **Nunes A, Borrego MJ, Gomes JP.** 2013. Genomic features beyond *C. trachomatis* phenotypes: what do we think we know? Infect. Genet. Evol. **16:**392–400. http://dx.doi.org/10.1016/j.meegid.2013.03.018.
23. **O'Connell CM, Ingalls RR, Andrews CW, Jr, Scurlock AM, Darville T.** 2007. Plasmid-deficient *Chlamydia muridarum* fail to induce immune pathology and protect against oviduct disease. J. Immunol. **179:**4027–4034.
24. **Comanducci M, Manetti L, Bini A, Santucci A, Pallini V, Cevenini R, Sueur JM, Orfila J, Ratti G.** 1994. Humoral immune response to plasmid protein *pgp-3* in patients with *Chlamydia trachomatis* infection. Infect. Immun. **62:**5491–5497.
25. **Kari L, Whitmire WM, Carlson JH, Crane DD, Reveneau N, Nelson DE, Mabey DCW, Bailey RL, Holland MJ, McClarty G, Caldwell HD.** 2008. Pathogenic diversity among *Chlamydia trachomatis* ocular strains in nonhuman primates is affected by subtle genomic variations. J. Infect. Dis. **197:**449–456. http://dx.doi.org/10.1086/525285.
26. **Miyairi I, Laxton JD, Wang X, Obert CA, Arva Tatireddigari VR, van Rooijen N, Hatch TP, Byrne GI.** 2011. *Chlamydia psittaci* genetic variants differ in virulence by modulation of host immunity. J. Infect. Dis. **204:** 654–663. http://dx.doi.org/10.1093/infdis/jir333.