Title

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Journal

American journal of epidemiology, 187(9)

ISSN

0002-9262

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Publication Date

2018-09-01

DOI

10.1093/aje/kwy071

Peer reviewed

American Journal of Epidemiology

Vol. 187, No. 9
DOI: 10.1093/aje/kwy071
Advance Access publication: April 3, 2018

Original Contribution

Diversity of *Chlamydia trachomatis* in Trachoma-Hyperendemic Communities Treated With Azithromycin

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Initially submitted November 20, 2017; accepted for publication March 26, 2018.

Prior studies have theorized that low chlamydial genetic diversity following mass azithromycin treatments for trachoma may create a population bottleneck that prevents the return of infection, but little empirical evidence exists to support this hypothesis. In this study, a single mass azithromycin distribution was administered to 21 communities in the Gurage Zone of Ethiopia in 2003. All children aged 1–5 years had conjunctival swabs performed before treatment and 2 and 6 months after treatment. All swabs positive for *Chlamydia trachomatis* at 2 months underwent typing of the gene encoding the major outer membrane protein (*ompA*) of *C. trachomatis*, as did the same number of swabs per community from the pretreatment and 6-month visits. Diversity of *ompA* types, expressed as the reciprocal of Simpson's index, was calculated for each community. In total, 15 *ompA* types belonging to the A and B genovars were identified. The mean diversity was 2.11 (95% confidence interval: 1.79, 2.43) before treatment and 2.16 (95% confidence interval: 1.76, 2.55) 2 months after treatment ($P = 0.78$, paired *t* test). Diversity of *ompA* was not associated with the prevalence of ocular chlamydia ($P = 0.76$) and did not predict subsequent changes in the prevalence of ocular chlamydia ($P = 0.32$). This study found no evidence to support the theory that *ompA* diversity is associated with transmission of ocular chlamydia.

antibiotic; azithromycin; bacterial outer membrane proteins; *Chlamydia trachomatis*; genetic diversity; genotype; trachoma

Abbreviations: CI, confidence interval; *ompA*, gene encoding the major outer membrane protein.

Trachoma, the leading infectious cause of blindness, is caused by ocular infection with *Chlamydia trachomatis* serovars A, B, Ba, and C. Serovar classification is based on surface antigenic determinants of the major outer membrane protein gene (*ompA*) (1). The major outer membrane protein is thought to be an immunodominant antigen and may therefore play a role in chlamydia transmission as well as being a likely vaccine candidate. The diversity of *ompA* genotypes in a community may itself play an important role for chlamydia transmission. Because of poor cross-immunity to different *ompA* genovars, the collective immunity of the community to endemic chlamydial strains may be lower in settings with high *ompA* diversity, resulting in higher levels of transmission. Evidence for such a phenomenon was found in a previous cross-sectional study in Nepal, where *ompA* genotypic diversity was positively correlated with the prevalence of ocular chlamydia (2).

Based on the Nepal study, we speculated a priori that if *ompA* diversity were brought to low levels following a mass azithromycin distribution, the resulting population bottleneck of distinct immunogenic chlamydial strains would result in more widespread immunity to the reduced number of strains, and hence keep the prevalence of ocular chlamydia at a low level. Previous studies have been unable to test the hypothesis that lower genetic diversity is associated with lower rates of ocular chlamydia transmission either because they assessed *ompA* genotypes in the absence of mass azithromycin treatment or because they did not have *ompA* genotype data from a sufficient number of communities (2–5). Therefore, we designed a study using chlamydial swabs collected as part of a cluster-randomized trial of mass azithromycin for trachoma that we had completed in Ethiopia. This trial provided a population-based estimate of *ompA* diversity from a large number of communities both before and

after mass azithromycin treatments. We asked 3 main questions: 1) whether *ompA* diversity decreased after the mass azithromycin treatment, 2) whether diversity was associated with ocular chlamydia prevalence in a cross-sectional manner at each time point, and 3) whether posttreatment *ompA* diversity was associated with subsequent chlamydia prevalence.

METHODS

In a series of cluster-randomized clinical trials conducted in the Gurage zone of Ethiopia during 2003–2006, 40 randomly selected communities were treated with one of several different trachoma treatment strategies (6–8). The present report concerns a subset of these communities, each of which was treated identically for the first 6 months of the trial. After a baseline monitoring visit, all communities received a single mass azithromycin distribution (1 g for adults and 20 mg/kg for children), followed by monitoring visits at 2 months and 6 months (within 1 week) afterward. During monitoring visits, we collected Dacron swab (Puritan Medical Products, Guilford, Maine) samples from the right upper tarsal conjunctiva from all 1- to 5-year-old children in the community. We stored swabs on ice while in the field and then transported them to a −20° freezer within 8 hours; they were stored for several months at −20° before being transported to the processing point and then stored at −80° until processing.

For the underlying trial, swabs were processed for the presence of *C. trachomatis* using Roche COBAS AMPLICOR CT Test (Roche Diagnostics USA, Indianapolis, Indiana), with individual swabs tested at the pretreatment visit and then pools of 5 swabs tested for subsequent visits. Positive pools were not unpooled at the time they were originally processed; instead, maximum likelihood methods were used to estimate the prevalence of ocular chlamydia in each community (9). For the present study, we chose the 21 villages that had the highest number of positive pools at the 2-month visit, and unpooled all positive pools using AMPLICOR. We randomly selected an equal number of positive specimens from each village from the pretreatment and 6-month visits. The *ompA* gene region was evaluated as described previously (10). Briefly, DNA was extracted from each positive swab using the QIAmp DNA Micro Kit, the *ompA* region was polymerase-chain-reaction-amplified using the TopTaq DNA polymerase kit and published nested primers, and the polymerase-chain-reaction product was purified using the QIAquick PCR Purification Kit (all kits from Qiagen, Hilden, Germany; manufacturer's recommendations followed for each). The purified *ompA* polymerase-chain-reaction products were sequenced in both directions at Sequetech (Mountain View, California), edited with Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan), aligned to reference sequences (A/HAR-13; NC_007429 for A serovars and B/Tunis-864; DQ064280 for B serovars (GenBank; National Library of Medicine, Bethesda, Maryland)), and then assigned a genotype by considering a single-nucleotide base change from the reference sequence as a different strain. Genotypes were labeled with the genovar and an arbitrary number. DNA extraction and sequence analysis were performed by laboratory personnel masked to identifying information about the swab, including child, community, and visit. As a positive control, 12 randomly selected swabs were repeated in a masked fashion; all 12 pairs gave concordant sequences.

We expressed the diversity of *ompA* types in each community at each study visit as Jost's "effective number of species," which is equivalent to 1 ÷ Simpson's index (11). We repeated analyses with the unbiased Simpson's index as a sensitivity analysis. We assessed for changes in community-specific diversity over time using a paired *t* test. We assessed for a cross-sectional relationship between diversity and infection at the village level by including measurements from all study visits in a linear regression that modeled the prevalence of ocular chlamydia as a function of diversity and study visit; we used cluster-correlated variance estimates to account for nonindependence of communities and analytical weights to account for the different numbers of swabs taken in each community. We used linear regression to model the community prevalence of chlamydia at 6 months as a function of diversity and chlamydial prevalence at 2 months, using the number of swabs at month 2 as the analytical weight. All significance tests assumed a 2-sided α of 0.05. Analyses were performed with Stata, version 13 (StataCorp LLC, College Station, Texas).

Ethical approval for the study was obtained from the University of California, San Francisco, Committee for Human Research and the Ethiopian Science and Technology Commission. Due to the high rate of illiteracy in the study area, verbal informed consent was obtained from all caregivers prior to all study activities. The underlying trial was registered with ClinicalTrials.gov (NCT00221364).

RESULTS

A single dose of azithromycin was administered to all 21 study communities at month 0 (mean antibiotic coverage = 88.7%, 95% confidence interval (CI): 85.7, 91.8). The average prevalence of ocular chlamydia in the 21 villages decreased from 56.4% (interquartile range, 49.4–63.5) before treatment to 8.7% (95% CI: 6.9, 10.5) 2 months after the antibiotic distribution, and it then increased to 12.2% (95% CI: 9.1, 15.3) 6 months after the mass treatment. We identified 122 chlamydia-positive swabs from the 2-month visit. We randomly selected the same number of positive swabs from each community from the baseline monitoring visit ($n = 122$). We carried out the same sampling strategy for the 6-month visit; however, 4 of the communities had fewer positive swabs at the 6-month visit than at the 2-month visit, resulting in a total of 115 swabs from the 6-month visit. We extracted DNA and determined the *ompA* type from 122 (100%), 114 (93.4%), and 115 (100%) swabs from the pretreatment, 2-month, and 6-month visits, respectively. We identified 15 total *ompA* types from the 351 swabs, with all genotypes belonging to the A or B chlamydial serovars (Table 1). The most common *ompA* type (labeled A1 for this study) accounted for 177 (50.4%) swabs. In the case of a single swab, the sequencing results could not differentiate between A1 and A5; this swab was considered to have a mixed infection but we treated the swab as genotype A1 for the analysis.

Estimates of diversity for each community over the 3 study visits are shown in Table 2. We were unable to detect a significant change in this metric of diversity after a single mass azithromycin distribution; mean diversity was 2.11 (95% CI: 1.79, 2.43) before mass treatment compared with 2.16 (95% CI: 1.76, 2.55) 2 months afterward ($P = 0.78$, paired *t* test). We

Table 1. Description of Nucleotide Polymorphisms and Amino Acid Changes in the Major Outer Membrane Protein Gene of *Chlamydia trachomatis*, Gurage Zone of Ethiopia, 2003

ompA Type	Nucleotide Position and Reference Nucleotide (Amino Acid Position and Reference Amino Acid)[a]												No. at Each Study Visit		
	Type A									Type B					
	168C (56D)	281C (94A)	292A (98K)	304G (102A)	305C (102A)	355G (119E)	505G (169G)	506G (169G)	523A (175I)	269C (90A)	286A (96T)	577G (193A)	Month 0	Month 2	Month 6
A1[b]													64	57[c]	57
A2	T			A (Thr)					C (Leu)				14	13	9
A3					T (Val)								7	4	5
A4				A (Thr)					C (Leu)				5	2	3
A5							A (Ser)							5[c]	4
A6	T		G (Glu)	A (Thr)					C (Leu)					2	1
A7	T			A (Thr)				A (Asp)	C (Leu)					1	2
A8		T (Val)											1		
A9				A (Thr)										1	
A10	T			A (Thr)		A (Lys)			C (Leu)						1
A11								A (Asp)						1	
B1[d]													18	16	16
B2												T (Ser)	12	13	16
B3											G (Ala)		1		
B4										T (Val)		T (Ser)			1

Abbreviations: A, adenine; Ala, alanine; Asp, aspartic acid; C, cytosine; G, guanine; Glu, glutamic acid; Leu, leucine; Lys, lysine; *ompA*, gene encoding the major outer membrane protein; Ser, serine; Thr, threonine; T, thymine; Val, valine.

[a] Nucleotide polymorphism present in the respective *ompA* type relative to the reference strain, with any resultant amino acid changes in parentheses. Absence of an amino acid in parentheses indicates a synonymous mutation. All nucleotide bases, amino acid residues, and their positions are relative to the sequences found in GenBank (National Library of Medicine, Bethesda, Maryland); for sequence alignments, A/HAR-13 (accession number: NC_007429) was used as the A genovar reference strain and B/Tunis-864 (accession number: DQ064280) was used as the B genovar reference strain.

[b] Reference strain for A genovars; no difference between the A1 sequence and the A/HAR-13 reference sequence.

[c] Both A1 and A5 were detected in a single specimen.

[d] Reference strain for B genovars; no difference between the B1 sequence and the B/Tunis-864 reference sequence.

hypothesized that communities with a lower genetic diversity would have lower rates of chlamydial transmission and therefore a lower prevalence of ocular chlamydia infection. However, we were unable to detect a cross-sectional relationship between *ompA* diversity and the prevalence of ocular chlamydia ($P = 0.37$; Figure 1A). Furthermore, after controlling for the prevalence of ocular chlamydia, *ompA* diversity at month 2 was not predictive of ocular chlamydial infection 4 months later, with each 1-unit increase in diversity corresponding to a 1.9% increase in the prevalence of ocular chlamydia at month 6 (95% CI: −2.5, 6.2; $P = 0.38$; Figure 1B). Analyses using the unbiased Simpson's index were similar.

DISCUSSION

Contrary to our hypothesis, *ompA* diversity did not change significantly after a mass azithromycin distribution in an area hyperendemic for trachoma, was not associated with the prevalence of ocular chlamydia either before or after mass azithromycin treatments, and was not predictive of chlamydial prevalence 4 months later.

A previous cross-sectional study of 10 villages in Nepal found a positive association between community *ompA* diversity and chlamydial prevalence, suggesting that communities with more diverse populations of chlamydia might have higher rates of transmission (2). This finding could be explained by poor chlamydial cross-immunity: Individuals living in communities with a highly diverse set of chlamydial *ompA* types are likely to be exposed to many new chlamydial strains to which they have not yet developed immunity (12). The lack of cross-immunity makes it easier to be reinfected by these diverse strains, resulting in a higher prevalence of infection. The Nepal study speculated that a mass azithromycin treatment might decrease chlamydial diversity, which could in turn cause a population bottleneck and limit the ability of the chlamydial population to return to pretreatment levels. A subsequent study in 14 communities in the Gambia found supporting evidence for this theory by demonstrating a decrease in the number of distinct *ompA* types after a mass azithromycin distribution, although the ability to investigate the relationship between genotypic diversity and chlamydia transmission after mass antibiotic treatments was limited because infection was virtually eliminated in all but 2 of the communities (4).

The present study, which was unique in that it contained an estimate of *ompA* diversity from a large number of communities both before and after mass azithromycin treatments, was unable to confirm the results of the prior study from Nepal and did not provide strong evidence to support our underlying

Table 2. Community Diversity of the Major Outer Membrane Protein Gene of *Chlamydia trachomatis*[a], Gurage Zone of Ethiopia, 2003

Village	Before Treatment			2 Months After Treatment			6 Months After Treatment		
	Distinct	Total	Diversity	Distinct	Total	Diversity	Distinct	Total	Diversity
1	2	7	1.96	4	7	3.77	3	7	1.81
2	4	10	3.33	5	10	3.85	5	10	4.55
3	3	8	1.68	2	7	1.32	2	8	1.28
4	3	5	2.78	3	5	2.27	3	4	2.67
5	2	6	1.38	2	6	1.38	3	6	2.57
6	4	7	2.58	2	6	1.38	4	7	2.58
7	2	6	2.00	3	5	2.78	5	6	4.50
8	3	3	3.00	3	3	3.00	2	3	1.80
9	2	3	1.80	3	3	3.00	2	3	1.80
10	2	5	1.47	1	5	1.00	1	3	1.00
11	2	10	1.72	2	9	1.80	3	10	2.17
12	5	12	2.57	3	9	2.31	4	12	2.88
13	2	4	1.60	3	4	2.67	2	4	1.60
14	1	4	1.00	1	3	1.00	1	4	1.00
15	2	4	1.60	2	4	1.60	2	4	1.60
16	2	5	1.92	1	5	1.00	1	3	1.00
17	4	7	3.27	3	7	2.33	3	7	2.33
18	4	6	3.00	2	6	1.80	2	4	2.00
19	3	6	2.57	4	6	3.00	3	6	2.00
20	1	2	1.00	2	2	2.00	2	2	2.00
21	2	2	2.00	2	2	2.00	1	2	1.00

Abbreviation: *ompA*, gene encoding the major outer membrane protein.
[a] The number of distinct *ompA* genotypes and total number of successfully sequenced conjunctival swabs are shown for each village, along with the diversity of *ompA* types in that village, defined as (1 ÷ Simpson's Index).

hypothesis. While our study did not find an association between strain diversity and transmission, it is also possible that a relationship exists but was not detected. For example, the relationship between *ompA* diversity and chlamydial transmission may differ in areas with hypoendemic trachoma (e.g., Nepal and the Gambia) and hyperendemic trachoma (e.g., Ethiopia), or *ompA* diversity may be relevant only if ocular chlamydia is brought to low enough levels by mass azithromycin treatment. It is also possible that the level of diversity observed in this study was too low to meaningfully influence chlamydial transmission, although this does not easily explain the difference in results between the present study (median diversity, 1.96, interquartile range, 1.60–2.58) and the Nepal study (median diversity, 1.20, interquartile range, 1–1.64).

Mathematical models have suggested that genetic diversity plays a role in the collective immune status of communities, especially when immunity to an organism is short-lived or when lifelong immunity is present only for specific antigenic strains of the organism (13, 14). Ocular chlamydia infection, which causes trachoma, is thought to have both short-lived and strain-specific immunity, and therefore presents a model organism to test the role of genetic diversity for transmission of infection. Although we were unable to demonstrate a significant association between *ompA* diversity and ocular chlamydia prevalence in this study, the negative result is nonetheless notable because it empirically tests a well-accepted hypothesis generated from mathematical modeling. Such studies such are rarely done but serve as an important complement to modeling studies.

This study was population-based, included a relatively large number of communities, and had a relatively high yield of positive *ompA* genotype results. We chose to restrict our testing to the *ompA* gene instead of a more discriminative method, such as multilocus sequence typing (MLST) or whole-genome sequencing, because our hypothesis centered around antigenic diversity as it relates to the host immune system (15, 16). The major outer membrane protein, encoded by *ompA*, makes up the majority of the protein mass on the outer membrane of chlamydial bacteria and is thought to be a primary source of immune protection (17). Thus most of the antigen presented to the host immune system is likely coded for in the *ompA* gene, making it an ideal marker for antigenic diversity. More discriminatory methods could be useful for following evolutionary changes of chlamydia, but because these methods classify chlamydial strains based on proteins not present on the outer surface of the bacterium, they would be less useful in characterizing the importance of antigenic diversity for chlamydial transmission.

The study is limited by a relatively small number of positive chlamydia results per community, which may have limited the amount

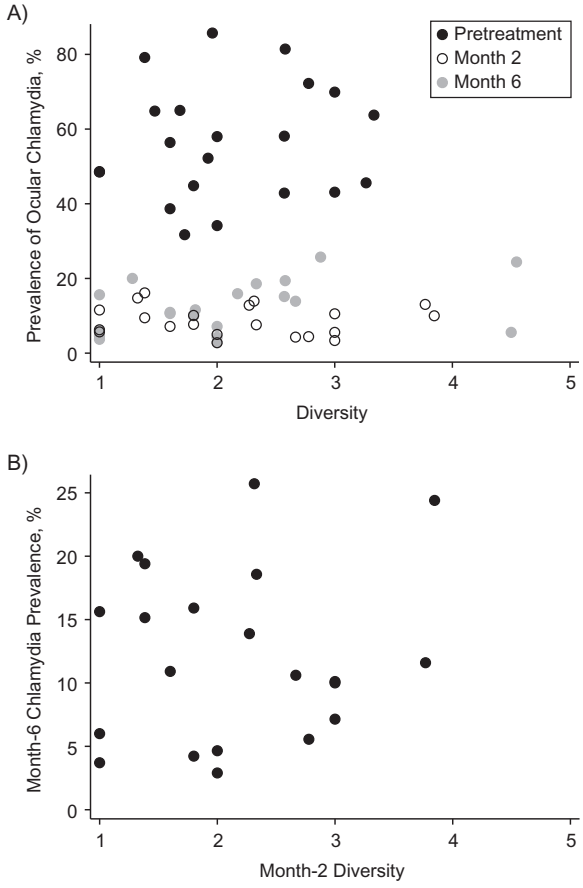

Figure 1. Community diversity of genotypes for the gene encoding the major outer membrane protein (*ompA*) of *Chlamydia trachomatis* was not associated with the prevalence of ocular chlamydia in the Gurage Zone of Ethiopia in 2003. Each point represents a community in the Gurage Zone of Ethiopia. A) Cross-sectional relationship between diversity and prevalence of ocular chlamydia infection at 3 different time points: before mass azithromycin treatment (black), 2 months after the mass treatment (white), and 6 months after the mass treatment (gray). B) Relationship between posttreatment diversity and the prevalence of ocular chlamydia 4 months later. Diversity is expressed as 1 ÷ Simpson's index, and prevalence was assessed in a random sample of children aged 1–5 years in each community.

of diversity that could be detected. We also did not have access to individual-level antibiotic treatment data and consequently could not explore the relationship between *ompA* type and azithromycin treatment—although we did know from community-level records that almost 90% of individuals in the study were treated.

In conclusion, we were unable to detect a relationship between *ompA* community diversity and the likelihood of current or subsequent ocular chlamydial infection. Studies in less endemic settings may be helpful to characterize a subtler role of chlamydial diversity on trachoma transmission.

ACKNOWLEDGMENTS

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This study was supported by the International Trachoma Initiative, the National Institutes of Health (grants U10EY016214 and R21AI55752), the Bernard Osher Foundation, That Man May See, the Harper Inglis Trust, the South Asia Research Fund, and Research to Prevent Blindness.

Conflict of interest: none declared.

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