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Journal

Cornea, 41(10)

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Publication Date

2022-10-01

DOI

10.1097/ICO.0000000000002863

Peer reviewed

HHS Public Access

Author manuscript

Cornea. Author manuscript; available in PMC 2023 October 01.

Effect of Azithromycin on the Ocular Surface Microbiome of Children in a High Prevalence Trachoma Area

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Abstract

Purpose: To evaluate the effect of 4 times per year mass azithromycin distributions on the ocular surface microbiome of children in a trachoma endemic area.

Methods: In this cluster-randomized controlled trial, children aged 1–10 years in rural communities in the Goncha Seso Enesie district of Ethiopia were randomized to either no treatment or treatment with a single dose of oral azithromycin (height-based dosing to approximate 20 mg/kg) every 3 months for 1 year. Post-hoc analysis of ocular surface *Chlamydia trachomatis* load, microbial community diversity, and macrolide resistance determinants was performed to evaluate differences between treatment arms.

Results: 1,255 children from 24 communities were included in the study. The mean azithromycin coverage in the treated communities was 95% (95% CI: 92% to 98%). The average age was 5 years ((95% CI: 4 to 5). Ocular surface *C. trachomatis* load was reduced in children treated with 4 times per year azithromycin (P=0.0003). *Neisseria gonorrhoeae*, *Neisseria lactamica*, and *Neisseria meningitidis* were more abundant in the no treatment arm compared to the treated arm. The macrolide resistance gene *ermB* was not different between arms (P=0.63), but mefA/E was increased (P=0.04) in the azithromycin treated arm.

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Declaration of competing interest

None of the authors have conflicts of interest to declare.

Trial Registration. Clinicaltrials.gov NCT00322972

Conclusions: We found a reduction in the load of *Chlamydia trachomatis* and 3 *Neisseria* species in communities treated with azithromycin. These benefits came at the cost of selection for macrolide resistance.

Keywords

ocular surface microbiome; children; trachoma; metagenomic sequencing; antibiotic resistance

INTRODUCTION

Intensive oral azithromycin treatment of children aged 1–10 years in trachoma-endemic areas significantly decreased the prevalence of ocular chlamydial infection.[1] The broad antimicrobial activity of azithromycin is expected to affect other ocular pathogens as well. While microbiological experiments showed an acute reduction in the prevalence of some ocular pathogens, the longer term effects are unclear.[2] In addition, little is known about the ocular surface microbiome of children in communities with high prevalence of trachoma. In this cluster-randomized controlled trial, we used metagenomic DNA deep sequencing to determine the long term effects on the ocular surface microbiome of children in communities treated with single-dose oral azithromycin 4 times in a year in the Amhara region of Ethiopia.

METHODS

Trial Oversight:

Approval for the study was obtained from the Committee for Human Research of the University of California, San Francisco (UCSF) and the Ethiopian Science and Technology Commission. The study was undertaken in accordance with the Declaration of Helsinki. Oral consents were obtained from adults and from guardians of children. No incentives were given for participation in the trial. The study started on May 2006 and concluded on May 2007.

Eligibility:

Rural communities in the Goncha Seso Enesie district of the Amhara Region of northern Ethiopia were eligible for participation.

Intervention:

72 communities were randomly assigned to one of six treatment groups of 12 communities each. This study only included comparison between communities randomized to the delayed treatment arm and the communities randomized to having children aged 1–10 years treated with a single dose of oral azithromycin (height-based dosing to approximate 20 mg/kg) every 3 months for 1 year. Medication intake was directly observed by health-care personnel.

Sample Collection:

60 children aged 1–10 years from each community were randomly selected for conjunctival swabbing at the 12-month time point (3 months after the 4th treatment) per standardized protocol. The protocol was as followed: (1) wearing latex gloves, the examiner used his/her fingertips to grasp the central portion of the child's right upper lid eyelashes, (2) the right upper lid was then everted, using a finger of the examiner's other hand (or the end of the sterile swab) as a fulcrum, positioned superior to the tarsal plate, (3) the everted lid was then held in place by the examiner's non-dominant hand, holding the eyelashes against the orbital rim, thus keeping the examiner's dominant hand free for swabbing the tarsal conjunctiva. If the right eye was difficult to evert, the examiner was allowed to evert the left eye instead, (4) a sterile dacron swab was used to firmly run on the upper tarsal conjunctiva in one direction, rotate the swab 120° along its axis and rub firmly again, and rotate and repeat a 3rd time. The samples were kept at 4°C in the field and stored at −20°C in Ethiopia until shipment to UCSF for long term storage at −80°C.

Laboratory Methods:

Samples were de-identified and communities were placed in a random order. Researchers processing and analyzing the samples were masked. All conjunctival samples collected were pooled at the community level for metagenomic DNA sequencing to evaluate for ocular surface microbiome. Nucleic acid extraction and sequencing libraries were prepared and sequenced as previously described.[3] In brief, DNA was extracted from the pooled conjunctival samples using the QIAGEN Allprep DNA/RNA Micro Kit per manufacturer's instructions. The extracted pooled DNA was used to prepare DNA sequencing libraries using the New England Biolabs' (NEB) NEBNext Ultra II DNA Library Prep Kit and then amplified with 16 PCR cycles and sequenced on the NovaSeq 6000 instrument using 125-base paired-end sequencing. Sequencing data were processed as previously described.[4,5] Macrolide resistance determinants were evaluated with *ermB* and *mefA/E* directed PCRs using previously described primers.[6,7]

Data Analysis:

The study was powered for the primary outcome, to detect a 6% difference in the prevalence of infection in individuals older than 11 years at 12 months.[1] The analyses done in this study, therefore, should be considered as exploratory as they were not prespecified. All analyses were done at the community level. Non-parametric Mann-Whitney test was used to compare the normalized *Chlamydia trachomatis* load and the presence of *ermB/mefA/E* genes between treatment arms. Diversity was compared by one-tailed permutation test of the absolute difference in community-diversity of the treated and the controlled groups. DESeq2 was used to perform differential abundance analysis on the bacteria at the species level and Topconfects algorithm was used to determine confident effect sizes.[8,9] Analyses were conducted in R v.3.5 for Mac (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Figure 1 shows the trial profile. In the 4 times per year azithromycin-treated arm, the mean coverage was 95% (95% confidence interval, CI, was 92% to 98%). The control arm

received no treatment until after the 12-month collections (Table 1). Conjunctival samples were obtained from 618 children from communities with delayed treatment and from 637 children from the azithromycin treated arm. The average age was 5 years (95% CI: 4 to 5) for both arms and gender was similar between arms (Table 1).

The 15 most abundant bacterial genera on the conjunctiva are shown in Figure 2A. *Chlamydia* reads were more abundant in the control group compared to the treatment group at the genus level (Fig. 2A). At the species level, *C. trachomatis* was significantly reduced with intensive azithromycin treatment (P=0.0003, Fig. 2B). The reduction of *C. trachomatis* in the treatment arm was associated with an increase in community-level bacterial diversity (P=0.03, Fig. 2C) compared to the control group. Differential analysis of the bacterial species revealed an increase in relative abundance of multiple *Acinetobacter*, *Enterobacter*, and *Pseudomonas* species on the conjunctiva of children from communities treated with azithromcyin. Notable was the relative reduction of *Neisseria gonorrhoeae*, *Neisseria lactamica*, and *Neisseria meningitidis* in those same children compared to the control group 3 months after the 4th azithromycin distribution (Fig. 2D).

We next examined the ocular surface for genotypic macrolide resistance. While there was no difference in *ermB* (P=0.63), *mefA/E* was more abundant in the intensively treated arm (P=0.04, Fig. 2E).

All children who had their conjunctiva swabbed also underwent a clinical examination to evaluate for clinical signs of trachoma in the form of trachomatous inflammation-follicular (TF) or trachomatous inflammation-intense (TI). Testing for infection was performed with the Amplicor PCR assay in Ethiopia. Here, we evaluated whether pooled DNA-seq at the community level correlated with the prevalence of clinical findings or microbiologic detection. Spearman correlation analysis showed that the normalized load of *C. trachomatis* with DNA-seq was positively correlated with the prevalence of TF, TI, and *C. trachomatis* infection by PCR, although we were unable to show these correlations within study arms. (Fig. 2F).

DISCUSSION

In this cluster-randomized trial of mass azithromycin distribution in a trachoma-endemic area, we detected a change in the ocular surface microbiome with associated reductions in ocular, respiratory, and sexually transmitted-related pathogens on the conjunctiva of children who received intensive azithromycin treatments for 1 year.

Previous work in a trachoma-endemic area of Nepal found a significant change in the ocular pathogens of children 14 days after a single dose of oral azithromycin compared to untreated children.[2] However, the authors were unable to detect significant changes in the distributions of any cultured ocular pathogens between treatment arms. In contrast, the results of this study showed a reduction in *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Neisseria lactamica*, and *Neisseria meningitidis* for up to 3 months after the 4th distribution. Ethiopia is located in the “meningitis belt” of sub-Saharan Africa and both *Neisseria lactamica* and *Neisseria meningitidis* are pathogens known to cause meningitis in children.[10,11] The

relative reduction of these pathogens with azithromycin treatment seen here is consistent with the verbal autopsy results of the MORDOR (*Macrolides Oraux pour Réduire les Décès avec un Oeil sur la Résistance)* study that assessed childhood mortality with mass azithromycin distribution.[12] The carriage of *N. gonorrhoeae* on the ocular surface of children in the control communities is presumably a reflection of the maternal carriage in the community and of the transmission from mother to child. Thus, while the goal of mass drug distribution is for the elimination of trachoma, it may also be effective in treating pediatric infections acquired during the perinatal period.

The load reduction of various pathogens seen in this study is tempered by the increase in macrolide resistance determinants on the ocular surface of children. This finding, however, is consistent with prior phenotypic data of isolates from the ocular surface and genotypic and phenotypic data from the nasopharynx and the gut of children who received multiple rounds of mass azithromycin distributions.[3,13,14]

Finally, we found that a single mean load of *C. trachomatis* for a community is as informative as the prevalence of individuals in the community. The community mean load is also highly correlated with the clinical findings at the individual level, suggesting that perhaps metagenomic deep sequencing at the community level may be another viable tool for trachoma surveillance.[15]

Limitations of this cluster-randomized trial included the absence of clinical data beyond ocular findings at the individual level and the non-prespecified nature of the analyses. Interpretation of the results beyond a high prevalence trachoma area should be done cautiously.

CONCLUSION

In summary, the ocular surface of Ethiopian children from communities treated with 4 single-dose oral azithromycin every 3 months had an associated reduction in the relative load of *Chlamydia* and other pathogens. Metagenomic deep sequencing of pooled samples has the potential to complement and improve upon the current surveillance approaches for trachoma.

Funding

This work was funded by the National Eye Institute of the National Institutes of Health under Award Numbers U10 EY016214 and K08EY026986, Research to Prevent Blindness Career Development Award, and an unrestricted grant from Research to Prevent Blindness (RPB, New York, NY). Pfizer donated all azithromycin used in this study, but did not participate in the design or analysis.

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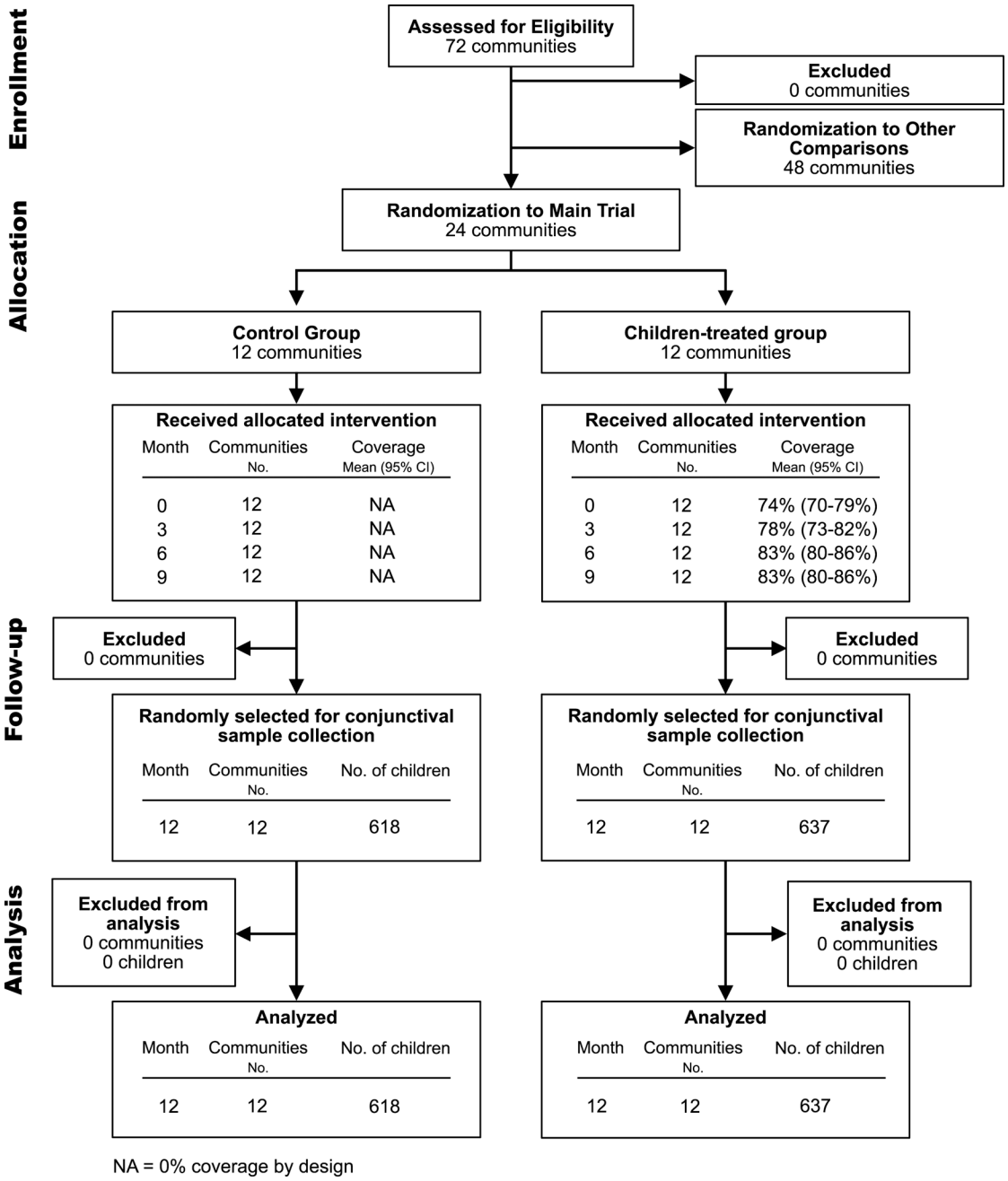

Figure 1:
Trial Profile

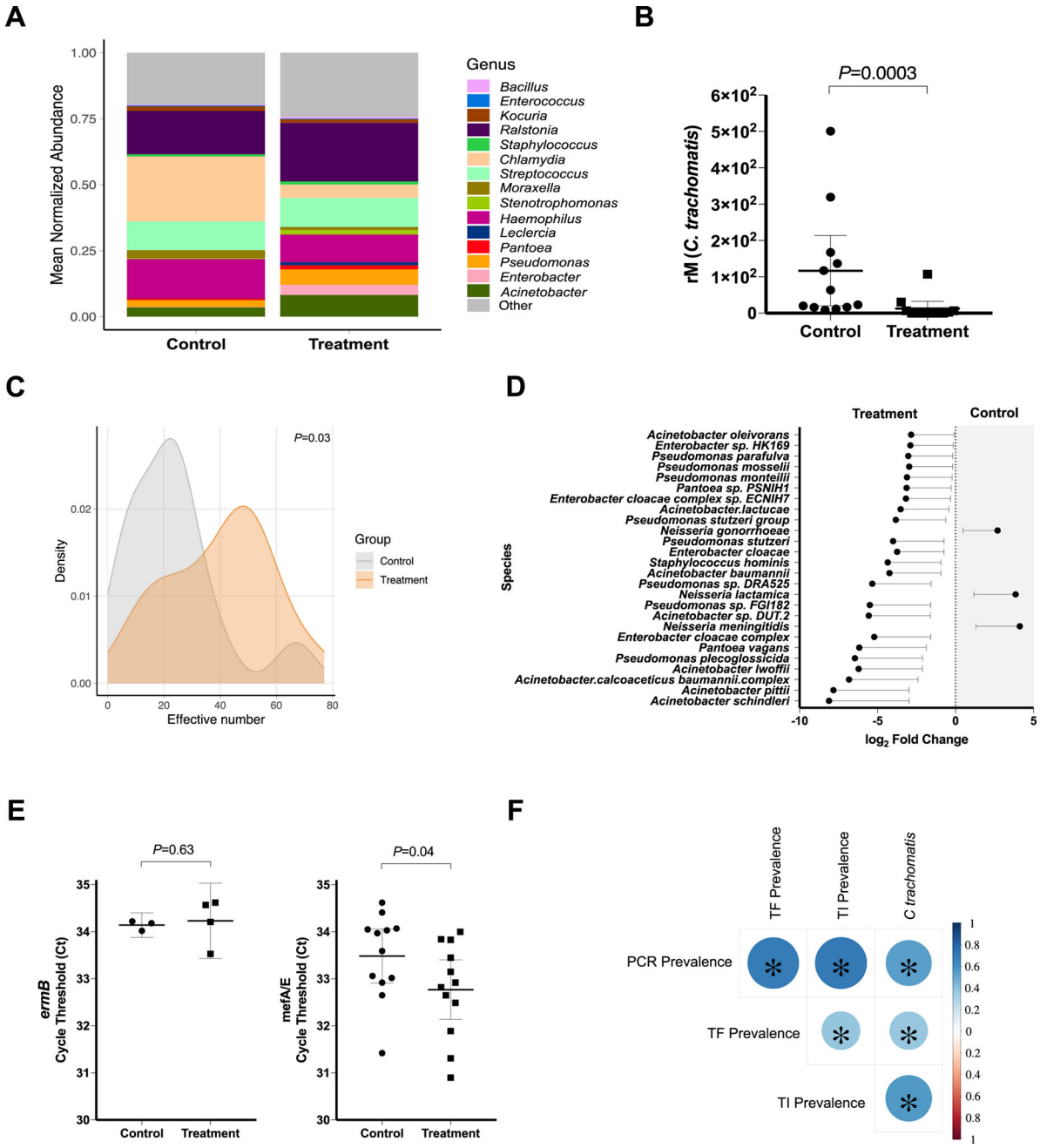

Figure 2:

Ocular surface bacterial composition and macrolide resistance of children 3 months after 4th azithromycin treatment (12-month time point) compared to untreated children. A, Mean relative abundance of the 15 most abundant genera at the 12-month time point for 1255 samples from 24 villages between arms. B, Normalized *Chlamydia trachomatis* sequencing reads at 12 months. Bars indicate the mean and 95% confidence intervals. Each point represents a village. C, Density plot for inverse Shannon's diversity index at 12 months (*P*=0.03, permuted with 10,000 simulations). D, Top bacterial species at 1% false discovery rate. For each species, the dot shows the $\log_2$ fold change with confidence

bound. Values in the shaded area represent more relative abundance in the controlled arm (no treatment), whereas values in the non-shaded area represent more relative abundance in the azithromycin-treated arm. E, Scatter plots of *ermB* and *mefA/E* genes for each arm. Non-parametric Mann-Whitney test to compare between arms. Error bars represent mean and 95% confidence intervals. F, Spearman correlation coefficients of ocular clinical findings and *Chlamydia trachomatis* prevalence as determined with Amplicor testing with *C. trachomatis* community load as determined with DNA-seq. Asterisk indicates $P<0.05$ after Benjamini-Hochberg corrections. Abbreviations: rM, matched read pairs per million read pairs; TF, Trachomatous Inflammation – Follicular; TI, Trachomatous Inflammation – Intense.

Table 1:

Demographics of Analyzed Participants

	12 Months *Conjunctival Swabs*	
	Control	**Treatment**
Number of villages	12	12
Treatment coverage	NA	95% (92% to 98%)
Number of children	618	637
Mean children per village (±SD)	52 (± 1)	53 (± 2)
Mean age, years (95% CI)	5 (4 to 5)	5 (4 to 5)
Female, % (95% CI)	52 % (47 to 56)	48% (43 to 52)