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Cessation of long-term cotrimoxazole prophylaxis in HIV-infected children does not alter the carriage of antimicrobial resistance genes

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FF analyzed the dataset and wrote the manuscript. EG assisted in analysis of dataset. TE worked on the sequencing data and mapped it to the CARD database. MG worked on the DNA extraction and library preparation for sequencing. CB, MD, AS, KN and DG assisted in data collection and curation. AP and AM guided the authors with the research question and analysis of the dataset. All authors contributed to writing and editing of the manuscript.

Competing Interests:

No competing interests to declare.

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Abstract

Background: Cotrimoxazole (CTX) is a broad-spectrum antimicrobial, combining trimethoprim and sulfamethoxazole. CTX prophylaxis reduces mortality and morbidity among people living with HIV in regions with high prevalence of bacterial infections and malaria. The Antiretroviral research for Watoto (ARROW) trial evaluated the effect of stopping versus continuing CTX prophylaxis in sub-Saharan Africa.

Methods: In this study, 72 HIV-infected Zimbabwean children, on antiretroviral therapy, provided fecal samples at 84- and 96-weeks after randomization to continue or stop CTX. DNA was extracted for whole metagenome shotgun sequencing, with sequencing reads mapped to the Comprehensive Antibiotic Resistance Database (CARD) to identify CTX and other antimicrobial resistance genes.

Results: There were minimal differences in the carriage of CTX resistance genes between groups. The *dfrA1* gene, conferring trimethoprim resistance, was significantly higher in the continue group (p=0.039) and the *tetA(P)* gene conferring resistance to tetracycline was significantly higher in the stop group (p=0.013). CTX prophylaxis has a role in shaping the resistome, however stopping prophylaxis does not decrease resistance gene abundance.

Conclusion: No differences were observed in resistance gene carriage between the stop and continue groups. The previously shown multi-faceted protective effects of CTX in ARROW Trial clinical outcomes are not outweighed by the risk of multi-drug resistance gene selection due to prophylaxis. These findings are reassuring, given current recommendations for long-term CTX prophylaxis among children living with HIV in sub-Saharan Africa to decrease mortality and morbidity.

Clinical Trial Number: ISRCTN24791884; **Trial duration:** October 02, 2006 to March 14, 2012

Keywords:

1. Drug Resistance, Microbial
2. Antibiotic Prophylaxis
3. Child Health
4. HIV infections
5. Trimethoprim, Sulfamethoxazole Drug Combination
6. Gastrointestinal microbiome

Introduction

World Health Organization (WHO) guidelines recommend long-term cotrimoxazole (CTX) prophylaxis for people living with HIV in areas where malaria or severe bacterial infections are highly prevalent [1]. This recommendation is based on randomized trials from sub-Saharan Africa in both adults and children which showed reduced morbidity and mortality with CTX use, regardless of HIV disease stage or antiretroviral therapy (ART) use [2–5]. However, global coverage of CTX among people living with HIV remains poor[6]. One concern with widespread and lifelong use of CTX is selection for and amplification of CTX-specific and overall antimicrobial resistance genes in the intestinal reservoir, leading to a potential increase in multidrug-resistant bacterial infections. CTX resistance genes are found in up to 80% of bacterial isolates from urinary tract, wound and blood infections in areas where prophylaxis is implemented [7]. However, the effectiveness of CTX prophylaxis in reducing morbidity and mortality still remains high despite the prevalence of resistance, possibly due to the anti-inflammatory, immunomodulatory, and taxon-specific antimicrobial effects of CTX [4,5,8–10]. As CTX coverage and duration is scaled up in sub-Saharan Africa, it is critical to understand whether this will drive further antimicrobial resistance in high HIV prevalence areas, which could have important downstream clinical impact.

In this study, we examined carriage of CTX and other resistance genes using metagenome sequencing among HIV-infected children in Zimbabwe who were randomized to continue or stop CTX prophylaxis in the Antiretroviral Research for Watoto (ARROW) trial.

Methods:

Study setup:

ARROW was an open-label parallel-group randomized trial evaluating several management strategies in HIV-infected children between 2006 and 2012, as previously reported [4,11,12]. Children over 3 years of age (median age, 7.9 years; interquartile range, 4.6 to 11.1), enrolled in ARROW and receiving ART with CTX prophylaxis for more than 96 weeks, who had not previously had *Pneumocystis jirovecii* pneumonia and were using insecticide-treated bed nets (in malaria-endemic areas), were randomized to stop versus continue daily CTX prophylaxis [4]. A sub-group of Zimbabwean children in the last 6 months of trial recruitment from stop (n=36 at week 84; n = 35 at week 96) and continue (n=36 at week 84; n=33 at week 96) groups had fecal samples collected. Caregivers gave written informed consent, and children gave assent depending on age, for the main trial and for this substudy, which were approved by ethics committees in the UK and Zimbabwe.

Stool specimens for the fecal metagenome study were identified from a gut epithelium sub-study investigating intestinal inflammation in the ARROW trial (Figure 1). The 72 children (40 female and 32 male) with a median age of 8.9 years (interquartile range, 5.7 to 11.1) at the time of randomization, did not substantially differ in baseline characteristics from the full ARROW trial population (*data not shown*). The samples were stored at -80^0 Celsius and shipped to British Columbia Centre for Disease Control (BCCDC), Vancouver, BC for processing. The objective of these analyses was to identify the impact of stopping CTX on the intestinal carriage of antibiotic resistance genes. The protocol for analysis was approved by the research ethics board at the University of British Columbia (protocol number: H14-00300).

DNA extraction and sequencing:

DNA extraction from 150mg of fecal samples was performed using Qiagen Power Soil DNA extraction kit with bead-beating. DNA was quantified using Quant-iT Pico Green dsDNA assay. DNA libraries were prepared using Illumina TruSeq Nano DNA Library Preparation kits. Libraries were quantified and normalized using qPCR and validated using the Agilent TapeStation system. Twenty-four libraries were pooled per sequencing lane. Whole metagenome sequencing was performed using the Illumina HiSeq 2500 platform at Canada's Michael Smith Genome Sciences Centre, Vancouver, Canada using dual-index, paired end sequencing.

Analysis:

The reads were trimmed, filtered and mapped to the Comprehensive Antibiotic Resistance Database (CARD) using the resistance gene identifier (RGI) tool. Genes with >80% coverage and 100% homology were defined as present. The 'vegan' analysis package in R was used to evaluate the alpha and beta diversity measures for the resistant genes. The alpha diversity of resistant genes is defined as the read count normalized Shannon diversity metric within a sample. The beta diversity of resistance genes was defined as the Bray-Curtis dissimilarity metric between the samples. Differences in alpha and beta diversity of resistance genes between the stop and continue groups was conducted using analysis of variance test. Logistic regression was performed to compare differences in the presence and absence of antimicrobial resistance genes between the stop and continue groups. Negative binomial regression was performed to compare normalized gene counts (with sequencing depth as the offset), including an adjustment for timing of sample collection between the randomized groups. Results are reported as percent of subjects

positive for a specific gene class and by total gene count for each gene class for all subjects in each group. Analyses were undertaken using R program version 3.5.1.

Results:

In total, 2900 resistance genes across all gene classes in continue group members and 3021 resistance genes in the stop group were identified through CARD alignment of metagenome data. There were no significant differences observed in the analysis of variance of the read-count normalized gene-level Shannon alpha diversity (P-value = 0.62) and Bray-Curtis dissimilarity-based beta diversity (P-value = 0.67) of the genes between the stop and continue groups. CTX resistance genes, including the s*ul* gene conferring sulfonamide resistance and *dfr* gene conferring trimethoprim resistance, were persistently carried by all subjects regardless of CTX discontinuation (Table 1). The *dfrA1* gene, encoding trimethoprim-resistant dihydrofolate reductase, was on average five-fold higher in the continue group (odds ratio (OR), 4.82; 95% confidence interval (CI), 1.24 to 31.62) than in the stop group, although it was present in only 6.3% of the total samples and represented only 0.3% of the observed resistance gene counts in the continue group. Sulfonamide resistance genes (*sul*1-3) were also present in all subjects but contributed to a small fraction of total resistance gene counts (2.5%) (Table 1).

Genes encoding beta-lactamases, and genes conferring resistance to aminoglycoside antibiotics and tetracycline, were very common in the stop and continue groups (Table 1). Together, *tet* (tetracyline resistance) and *cfx*A (beta-lactamase) genes represented almost one-third of the total resistant gene counts in both groups. Extended-spectrum beta-lactamase genes (*bla*TEM) were found in one stop and one continue sample at the 84-week time-point, and no carbapenem-resistance genes were identified. The tetracycline-resistance gene count for *tetA(P)* gene

encoding an inner membrane tetracycline efflux protein found in *Clostridium* species, was on average three-fold higher in the stop group (OR, 2.51; 95% CI, 1.25 to 5.45) than in the continue group. The increased occurrence of *tetA(P)* gene in the stop group might be attributed to the recovery of *tetA(P)*-carrying *Clostridium* species, such as *Clostridium bartlettii*, which is more abundant in the stop group at both time points [8]. The abundance of all other antimicrobial genes in both groups was similar, and there were few differences in the carriage of antimicrobial resistance genes between the 84-week (only APH(6)-Id) and 96-week (only *tet*44 and *tet*A(P)) sample time-points within subjects. There were also no differences in microbial diversity, enteric pathogen carriage or microbiota composition, aside from the presence of fewer viridans group streptococci in the continue group at both time-points, as previously reported [8].

Discussion:

The observed differences in resistance gene carriage profiles in ART-treated children randomized to stop or continue CTX were minimal; only the *dfr*A1 gene was higher, while the *tetA* gene was lower, in children continuing CTX. None of the children in the trial were CTX-naïve as they had all received prophylactic CTX for at least 96 weeks prior to randomization to either stop or continue CTX, which explains the universal carriage of *sul* and *dfr* genes. The Botswana Mpepu study showed up to 57% and 37% of the sample population was positive for CTX-resistant *Escherichia coli* and *Klebsiella* spp., respectively, prior to CTX exposure. CTX prophylaxis increased the proportion of participants with CTX resistance at 3 and 6 months post-exposure [13]. A culture-based study done at the Bugando Medical Centre in Tanzania reported that up to 77% of the healthy population defined as HIV negative with no antibiotic prophylaxis grew at least one organism resistant to CTX; and of the HIV positive population in the study 17% more CTX positive cultures were observed in those who continued CTX prophylaxis

relative to the no prophylaxis group[14]. This shows that CTX resistance inherently present in the population before CTX exposure, is amplified by CTX prophylaxis. Another longitudinal sub-study of a randomized controlled trial in South Africa evaluating the impact of CTX exposure in HIV-exposed but uninfected infants in the first year of life showed that CTX prophylaxis increased the abundance and alpha diversity of the CTX resistance genes in the gut relative to a CTX-naive group, but decreased the resistant gene beta diversity in children receiving CTX prophylaxis [15,16]. No significant differences in the occurrence of CTX resistance genes were observed in our study over the two sampling time points and minimal differences in the overall resistance gene counts were noted. This indicates that CTX use plays a role in shaping the resistome; however, removal of CTX selective pressure does not decrease the abundance nor diversity of the resistome established by CTX prophylaxis. Other studies have also shown minimal or no differences in the diversity of gut microbiota after long- and short-term CTX use [8,17]. The combination of these findings suggests that CTX prophylaxis shapes the development of the microbiome in childhood such that people with CTX exposure have lower taxonomic and resistance beta diversity, but high resistance alpha diversity. However, our results show that the resistance gene profiles remain largely unchanged for up to 22 months post-CTX discontinuation. Though the participants in our study were older, perinatal exposure to CTX could also alter the development of the intestinal microbiome. The effect of perinatal CTX exposure during gestation and through breastfeeding needs to be further characterized to evaluate the granular impact of oral CTX prophylaxis for infants in shaping their microbiome, as passage of CTX into breastmilk and the inheritance of antibiotic resistant genes from mother have been previously described [18–20].

Information regarding additional non-CTX antibiotic exposure for ARROW participants was not available. Metagenome identification of resistance genes does not confirm phenotypic expression of resistance and precludes accurate localization of genes to specific plasmids or genomes; therefore, only gene presence, absence and gene counts are reported. Higher tetracycline resistance gene counts could also be explained by higher overall microbial load due to the absence of CTX selection pressure in the gut of children who stopped CTX prophylaxis; adjustment for starting microbial load in processed fecal samples was not possible.

Conclusion:

The efficacy of CTX prophylaxis in reducing mortality and morbidity has been demonstrated in multiple trials among people living with HIV, even in areas with high baseline levels of CTX resistance [3,4,8]. This may partly be because CTX has properties beyond its broad-spectrum antimicrobial activity, including anti-inflammatory, immunomodulatory, and narrow microbiome modulating benefits [8]. This study shows that the vital population health benefits of long-term CTX prophylaxis in areas of high HIV prevalence, do not appear to be outweighed by a greater risk of antibiotic resistance gene carriage. However, our results, in combination with D'Souza et al [15], suggest that long-term CTX prophylaxis has a lasting effect on intestinal antibiotic resistance gene carriage and these effects persist even after many months without any CTX exposure. Our results provide reassurance to policymakers to continue scaling up long-term CTX prophylaxis in sub-Saharan Africa, since continuing CTX does not appear to drive accumulation of further antimicrobial resistance. To further reduce mortality in high HIV prevalence areas, efforts to scale-up widespread adoption of CTX prophylaxis in HIV-infected children remain a public health priority.

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Figure 1: Sample origin for the fecal metagenome study

Of the 760 participants who underwent initial randomization in ARROW, 293 were enrolled in an immunology sub-study. The 72 fecal samples analysed in our study originated from a sub-group of participants recruited for the gut epithelium sub-study in the final 6 months of the trial in Zimbabwe.

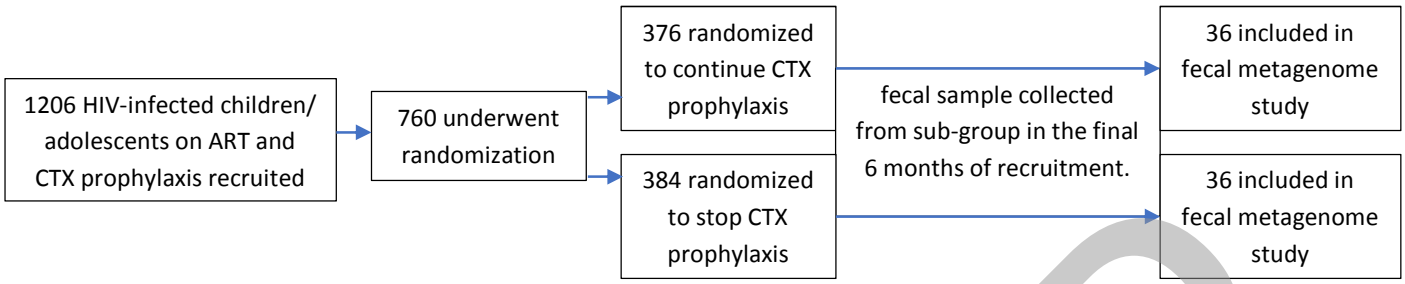
Table 1: Occurrence of all cotrimoxazole resistance and other clinically relevant resistance genes in the fecal metagenome of HIV-infected children randomized to continue or stop cotrimoxazole prophylaxis in the ARROW Trial.

Gene name	Resistance conferred	Gene class positive subjects[1]		Gene counts by class[2]	
		Continue n (%)	Stop n (%)	Continue Counts (%)	Stop Counts (%)
dfr(A1*,*A5,A7,A8,A12,A14,A15,A17, F)* (dihydrofolate reductase)	Trimethoprim	36 (100)	36 (100)	122 (4.2)	109 (3.6)
folP (dihydropteroate synthase)	Sulfonamide	18 (50)	20 (55.6)	24 (0.8)	27 (0.9)
sul(1,2,3) (dihydropteroate synthase)	Sulfonamide	36 (100)	36 (100)	79 (2.7)	70 (2.3)
Erm(B,F,G,Q,T) (ribosomal RNA methyltransferase)	Macrolides, Lincosamides, Streptogramins	34(94.4)	31 (86.1)	193 (6.7)	217 (7.2)
tet(A(P*)*-D,J-O,Q-S,W,X, 32,40,44)* (tetracycline-resistant ribosomal protein)	Tetracycline	36 (100)	36 (100)	500 (17.2)	533 (17.6)
CfxA(1-6) (beta-lactamase)	Cephamycin	36 (100)	36 (100)	311 (10.7)	341 (11.3)
APH (phosphorylation of streptomycin)	Streptomycin	31 (86.1)	25 (69.4)	147 (5.1)	123 (4.1)

[1]Number of subjects who are gene class positive in either or both week 84 and week 96 samples.

[2]The total count of resistant genes by gene class in the stop or continue groups. Proportion of gene class counts out of the total, 2900 and 3021 genes in the continue and stop groups, respectively.

*The asterisk defines genes that were significantly different between the stop and the continue groups using negative binomial regression, normalized for read depth. *dfrA1*: five-fold higher in the continue group; odds ratio (OR), 4.82; 95% confidence interval (CI), 1.24 to 31.62. *tetA(P):* three-fold higher in the stop group; OR, 2.51; 95% CI, 1.25 to 5.45.



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