

The impact of antibiotic treatment on the immunogenicity of oral poliovirus vaccine: a randomised placebo-controlled trial among seronegative Indian infants

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Summary

Background

Oral poliovirus vaccine (OPV) is less immunogenic and effective in low-income countries, in common with other oral vaccines. The high prevalence of intestinal pathogens and associated environmental enteropathy (EE) have been proposed to explain this phenomenon. Administration of an antibiotic has the potential to resolve EE and clear bacterial pathogens, thereby improving OPV immunogenicity.

Methods

We did a randomised, double-blind placebo-controlled trial of the effect of azithromycin on the immunogenicity of serotype-3 monovalent OPV (mOPV3) given to healthy infants aged 6-11 months in Vellore, India, who lacked antibodies to this serotype. Infants were randomised (1:1) at enrolment to receive oral azithromycin at 10mg/kg or placebo once daily for three days, followed by mOPV3 on day 14. The primary outcome was detection of serum neutralising antibodies to serotype-3 poliovirus at $\geq 1/8$ dilution on day 35. Statistical analysis was planned for all infants who completed the study per-protocol. The trial was registered with Clinical Trials Registry of India (CTRI/2014/05/004588).

Findings

754 infants were randomised into study groups during the study period August 5, 2014 to March 21, 2015. 92.6% (348/376) and 94.4% (357/378) of infants in the azithromycin and placebo groups respectively completed the study per-protocol. In the azithromycin group 175 (50.3%) seroconverted to serotype-3 poliovirus compared with 192 (53.8%) in the placebo group (risk ratio 0.94, 95% CI 0.81-1.08, $p=0.366$). Azithromycin reduced faecal biomarkers of EE (calprotectin, myeloperoxidase, $\alpha 1$ -antitrypsin) and the prevalence of bacterial but not viral or eukaryotic pathogens. Viral pathogens (~~enterovirus, rotavirus~~) were associated with lower seroconversion. No adverse events were related to the study interventions.

Interpretation

Azithromycin did not improve the immunogenicity of OPV despite ~~significantly~~ reducing biomarkers of EE and the prevalence of pathogenic intestinal bacteria. Viral interference and/or innate antiviral immunity may be more important determinants of the immunogenicity of live-virus oral vaccines.

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Introduction

The immunogenicity and efficacy of oral vaccines are impaired when given to infants in low-income countries compared with the same vaccines given in high-income countries.^{1,2} This phenomenon has been observed for live-attenuated and killed oral vaccines against bacterial and viral pathogens – including licensed vaccines against poliovirus, rotavirus and cholera – substantially reducing their public health benefit. In the case of oral poliovirus vaccine (OPV), impaired efficacy of the vaccine has stalled eradication and required the use of frequent, often monthly mass vaccination campaigns in endemic regions.^{3,4}

The biological mechanisms underlying poor oral vaccine performance in low-income countries have not been elucidated, although several candidates have been proposed. Some may be common to a number of oral vaccines, such as interference with the immune response in infants by high-levels of homologous maternal antibodies transferred via the placenta or breast-feeding.^{5,6} Others may be specific to particular vaccines. For example, infection with enteroviruses at the time of vaccine administration is associated with reduced immunogenicity of OPV, perhaps as a result of cross-neutralising antibodies or direct interference at the cellular level.⁷ However, definitive mechanisms that can explain the substantial differences in immunogenicity of oral vaccines between populations have not been identified.^{1,2} This is a priority for efforts to design better oral vaccines and adjuvants, and to identify interventions to improve the efficacy of existing vaccines.

Children in low-income countries are exposed to a high burden of intestinal pathogens.⁸ These pathogens induce innate and adaptive immune responses in the intestine, changes to the microbiota, and have been associated with diminished immunogenicity of OPV.^{7,9,10} Repeated exposure to bacterial and other pathogens is thought to underlie environmental (or 'tropical') enteropathy (EE), a sub-clinical condition commonly found in children and adults in low-income countries, which affects the small intestine and is characterised by inflammation, increased permeability and malabsorption.¹¹⁻¹⁴ Faecal and blood biomarkers of EE have been used to determine its extent and role in growth faltering and malnutrition.¹⁵ Some of these biomarkers have also been found to correlate with the outcome of immunisation with oral rotavirus and poliovirus vaccines.¹⁶

We investigated the association between infection with intestinal pathogens, EE and the immune response to oral vaccination in a randomised placebo-controlled trial of the effect of a 3-day course of oral azithromycin on the immunogenicity of a subsequent dose of serotype-3 monovalent OPV given to Indian infants who lacked immunity to this serotype. Azithromycin is a broad spectrum, bacteriostatic macrolide antibiotic with a long half-life that has been shown to be effective against a range of intestinal pathogens and has been safely used in many countries in mass treatment campaigns to prevent trachoma.¹⁷ It also has a direct anti-inflammatory effect and is used as an immunomodulatory agent in the treatment of a number of conditions including cystic fibrosis, but its effectiveness as treatment for EE has not been examined.¹⁸ The impact of antibiotics on the response to oral vaccination has also not previously been examined.¹⁹ We measured the impact of azithromycin on the development of serum neutralising antibodies and poliovirus shedding after OPV, in addition to faecal and plasma biomarkers of EE and intestinal pathogens in stool. We were therefore able to determine the impact of antibiotic treatment on EE and intestinal pathogens, and whether this improved the immune response to OPV.

Methods

Study design and participants

Infants living in 14 blocks of Vellore District in south India were identified in the community based on records of births held at 210 health sub-centres and screened for serum neutralising antibodies (NAb) to serotype 3 poliovirus by taking a single 3 ml blood draw. Written informed consent for screening was sought from a parent or caregiver and a questionnaire recording basic demographic and vaccination history data administered. Infants were eligible for screening if they lived in the area, had no reported receipt of inactivated poliovirus vaccine, would be 6-11 months old at study enrolment and available for the duration of the study. Following screening, infants who lacked detectable NAb at a dilution of 1 in 8, whose results were available within 14 days and who were determined by study doctors to be medically fit were invited to participate in the clinical trial. Informed consent for participation in the clinical trial was obtained from a parent or caregiver. Infants were excluded if they had received OPV since the screening visit, a history of allergic reaction following OPV, chronic diarrhoea (>14 days), were receiving immunosuppressant medication, or they or their mother had syndromic or documented evidence of being immunocompromised. Eligible infants who were febrile (>38°C) at the time of the enrolment visit or required hospitalisation were temporarily excluded for up to 2 weeks until they were either able to participate or met a permanent exclusion criterion. All infants found to be seronegative but ineligible after screening or at completion of the clinical trial were offered inactivated poliovirus vaccine.

Infants were randomised at enrolment in a parallel group design to receive either a 3-day course of azithromycin or placebo (study day 0). On study day 14 all infants were given a single dose of serotype-3 monovalent OPV. The primary outcome of the study was seroconversion, defined as the detection of serotype-3 poliovirus-specific serum NAb at a dilution of 1 in 8 or higher in blood taken 21 days after vaccination (day 35). Secondary outcomes included the prevalence and abundance of intestinal pathogens, biomarkers of EE and shedding of serotype-3 poliovirus. Stool samples were collected at enrolment (day 0, before treatment) and on the day of OPV administration (day 14, before ~~treatment or~~ vaccination ~~respectively~~) and tested for the presence of intestinal pathogens. In a subset of

infants, biomarkers of EE were measured in stool collected at enrolment (day 0) and in blood and stool samples collected on the day of vaccination (day 14). In the same infants, shedding of serotype-3 poliovirus was measured in stool samples collected 7 days after administration of OPV (day 21). **Additionally, poliovirus-specific faecal immunoglobulin-A and T-cell assays were planned as secondary and exploratory objectives and will be reported in another paper.**

The trial was conducted in accordance with the principles of good clinical practice and the ethical principles in the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Christian Medical College (CMC) and the Drugs Controller General of India. Oversight of the study was provided by an independent data safety and monitoring board. The trial was registered prospectively with the Clinical Trials Registry India (CTRI/2014/05/004588).

Randomization and masking

Children were randomized in a 1:1 ratio to receive azithromycin or placebo at enrolment in a double-blind controlled trial. The randomisation sequence was computer generated using a blocked randomisation procedure with variable block sizes of 6, 12 and 18 by an independent statistician. **An independent pharmacist provided azithromycin or placebo in identical bottles labelled with an allocation code A to F.** The allocation code for each subject was concealed in sequentially numbered opaque covers that were opened at the time of enrolment by study staff. All biological samples were given a unique ID linked to the study participant ID, such that laboratory staff conducted blinded assessments.

Interventions

Infants were given azithromycin at a dose of 10mg/kg in a syrup (Zithrox, MacLeods Pharmaceuticals Ltd.) or a placebo syrup matched in colour and taste (CMC pharmacy) once a day for the first three days of the study. The first dose was administered in the study clinic and subsequent two doses at home under observation by a member of the study team. Vaccination was with monovalent OPV containing at least $10^{5.8}$ CCID₅₀ (median cell culture infective doses) of serotype-3 poliovirus, Leon-12a,b strain produced in Vero cells (GlaxoSmithKline Biologicals).

Sample size

We estimated that enrolment of approximately 750 infants would provide 90% power with $\alpha=0.05$ to detect an effect of treatment on the immunogenicity of OPV based on estimated seroconversion in the placebo arm of 60%; prevalence of treatable intestinal pathogens of 40%, a 66% reduction in OPV seroconversion among infants infected with these pathogens, treatment efficacy of 75% and reinfection in about 10% between treatment and vaccination (resulting in 72% seroconversion expected in the azithromycin arm); and loss to follow-up of 10%. The first 300 infants enrolled in the trial with sufficient sample volumes were included in a subset assessed for biomarkers of EE and poliovirus shedding.

Laboratory methods

Serum was tested for serotype 3 poliovirus-specific NAb using a modified micro-neutralisation assay at 1/4 and 1/8 dilution for eligibility screening and in 2-fold serial dilutions from 1/4 to 1/512 for infants enrolled in the trial.²⁰ The presence of bacterial, viral and eukaryotic pathogens in stool samples was assessed through quantitative PCR of extracted DNA and RNA using TaqMan® array cards (TACs).²¹ A complete list of pathogen sequence targets is provided in the appendix ~~table 1~~. Faecal biomarkers of intestinal inflammation, protein-losing enteropathy and immune activation (myeloperoxidase, calprotectin, α 1-antitrypsin, neopterin) and plasma biomarkers of microbial translocation (soluble CD14 and endotoxin-core IgG (EndoCAb)) and epithelial damage (intestinal fatty acid binding protein (I-FABP)) were measured using enzyme-linked immunosorbent assays.^{14,15} Shedding of poliovirus in stool samples was assessed using quantitative real-time PCR. Further details are provided in the appendix.

Statistical methods

Infants who received azithromycin or placebo daily on study days 0-2 (or up to 1 day late), OPV on day 14 (\pm 1 day) and provided a blood sample on day 35 (-1, +7 days) were considered to have completed the study per protocol and were used for the primary analysis. Supportive analyses were conducted using an intention to treat (ITT) group consisting of all infants who provided a blood sample at the final visit.

The proportion of infants who seroconverted or shed poliovirus in each study arm was compared using Fisher's exact test and the risk ratio (RR) calculated with 95% confidence intervals based on the delta method.- Geometric mean titres (GMTs) of serum neutralising antibodies were calculated by assigning a value of 1:3 and 1:728 for the censored values below and above the limits of the dilution series and compared between study arms using Wilcoxon's rank sum test. Baseline characteristics of infants were compared between study arms using Fisher's exact test for binary data and Wilcoxon's rank sum (~~WRS~~) test for continuous data. A significance level of 0.05 was used for all statistical tests.

The effect of azithromycin on the prevalence of intestinal pathogens in stool was assessed in i) a cross-sectional analysis comparing the proportion of infants with each pathogen between study arms on day 14 using Fisher's exact test, and ii) a longitudinal analysis of the change in this proportion between study day 0 and 14 in each arm using McNemar's exact test for paired data. All proportions are presented with Clopper-Pearson 95% confidence intervals. Equivalent analyses of the abundance of intestinal pathogens based on the quantitative PCR cycle threshold (CT) value were performed using Wilcoxon's rank sum test and signed rank test (for paired data) respectively. Change in the number of pathogens detected in each infant was also examined in each study arm using Wilcoxon's rank sum test.

The effect of azithromycin on faecal biomarkers of EE was assessed in analogous cross-sectional and longitudinal analyses using Wilcoxon's rank sum and signed rank tests respectively. Plasma biomarkers of EE were available for study day 14 only and compared using Wilcoxon's rank sum test. Additionally EE was examined when defined by a) a binary variable equal to one if ≥ 1 biomarker was in the top quartile for measurements on stool or plasma and zero otherwise, and b) an EE score based on faecal biomarkers as defined by Kosek et al. 2013.¹⁵ The association between biomarkers of EE and the number of intestinal pathogens by group (bacteria, viruses, eukaryotes) was assessed using log-linear regression.

The same statistical methods were used in cross-sectional analyses comparing biomarkers of EE and presence of pathogens between infants according to whether they seroconverted or shed poliovirus after OPV. Additionally, the association between baseline characteristics of

infants, the prevalence and abundance of intestinal pathogens and seroconversion was assessed using logistic regression.

Role of the funding source

This is an investigator initiated study. The funders had no role in the design of the study; collection, analysis and interpretation of data; writing of the report; or the decision to submit the paper for publication. The corresponding and senior authors had full access to all the data from the study and had final responsibility for the decision to submit for publication.

Results

Participants

Infants participated in the clinical trial during the study period August 5, 2014 to March 21, 2015, with each infant enrolled for 35 days. Trivalent OPV offered through national immunisation days (January 18 and February 22, 2015) was withheld from infants who were enrolled in the trial at that time. In total 754 infants were randomised following screening of 8454 infants for serum NAb to serotype-3 poliovirus (Figure 1). 730 provided a blood sample at the final visit and 705 were considered to have completed the study per protocol. Infants in each study arm did not differ in their demographic characteristics, vaccination history or health status (Table 1).

OPV immunogenicity

The proportion seroconverting after OPV was 50.3% (175/348) in the azithromycin arm and 53.8% (192/357) in the placebo arm among infants who completed the study per protocol (RR=0.94, 95% confidence interval (CI): 0.81–1.08; Fisher's $p=0.366$). Similar results were obtained for the ITT group (50.4% (183/363) vs. 53.4% (196/367); RR=0.94, 95% CI: 0.82–1.09; Fisher's $p=0.459$). **The GMT of serum neutralising antibodies on day 35 did not differ by study arm (75.2 and 81.9 for azithromycin and placebo arm respectively, for infants who completed per protocol, Wilcoxon's $p=0.281$; appendix p14).**

In the subset of infants tested for serotype-3 poliovirus shedding and who completed the study per protocol, 51.4% (74/144) in the azithromycin arm and 56.1% (83/148) in the placebo arm had detectable serotype-3 poliovirus in stool 7 days after vaccination (day 21) (RR=0.92, 95% CI: 0.74–1.13; Fisher's $p=0.481$). Shedding of poliovirus and seroconversion were strongly correlated (Fisher's $p<0.001$; appendix [table 2p4](#)).

Older infants were less likely to seroconvert than younger infants (47.1% (140/297) for ages 8-11 months compared with 55.6% (227/408) for ages 6-7 months, Fisher's $p=0.027$). Infants who seroconverted reported slightly fewer previous doses of OPV than those who did not (3.99 vs. 4.18, $p=0.021$) but the number of doses was confounded with age and this association was no longer significant in a multivariable logistic regression (appendix [table](#)

p53). Infants with detectable NAb at enrolment did not differ significantly in their probability of seroconversion (60.8% (48/79) among those with NAb at 1/4 dilution vs. 51.0% (319/626) for those with undetectable NAb, $p=0.120$). No other baseline characteristics were associated with seroconversion (appendix table 3p5).

Intestinal pathogens

Among infants who completed the study per protocol, TAC data were available for 100% (705/705) and 99.9% (704/705) of infants on study day 0 and 14 respectively. Comparison of the prevalence of intestinal pathogens between study arms on the day of vaccination (day 14) revealed an effect of azithromycin on bacterial pathogens (figure 2). Enteroaggregative, enteropathogenic and shiga toxin-producing *Escherichia coli* (*E. coli*) and *Campylobacter* were significantly less prevalent in the azithromycin compared with placebo arm. This difference was also apparent when comparing the abundance of each bacterial pathogen based on the quantitative PCR, and even among those bacteria without a significant reduction in prevalence, abundance was often reduced (appendix table 4p6). Overall, the number of bacterial pathogens detected at the time of vaccination (day 14) among infants who had received azithromycin was lower compared with infants who received placebo (mean 0.98 (standard error: 0.05) vs. 1.78 (0.06), Wilcoxon's $p<0.001$). This difference was not apparent for viruses (mean 0.72 (0.04) vs. 0.65 (0.04), Wilcoxon's $p=0.183$) or eukaryotic pathogens (mean 0.16 (0.02) vs. 0.13 (0.02), Wilcoxon's $p=0.187$), and there were no significant differences in the prevalence of any of these individual pathogens between study arms on day 14 (figure 2).

The effect of azithromycin on bacterial pathogens was also apparent in the longitudinal analysis with a significant reduction in the prevalence of *E. coli* (enteroaggregative, enteropathogenic, enterotoxigenic and shiga-toxin producing), *Campylobacter*, *Bacteroides fragilis* and *Salmonella* in stool collected on day 14 compared with day 0 among infants who received azithromycin but not among those who received placebo (appendix figure 1p7, 15; appendix table 5). There were no statistically significant changes in the prevalence of viruses or eukaryotic pathogens after treatment with azithromycin or placebo. Significant reductions in the abundance of bacterial pathogens were also observed following treatment with azithromycin but not placebo (appendix table 6p8).

Infants who seroconverted after OPV had a significantly lower prevalence and abundance of enterovirus and rotavirus in stool at the time of vaccination (day 14) compared with those who did not (figure 2; appendix ~~table 7~~p9). The prevalence and abundance of other viral pathogens did not show significant differences by seroconversion status. **Adjusting for age in a logistic regression gave very similar results, although the association with rotavirus was no longer statistically significant (p=0.073) and that with adenovirus became significant (p=0.035; appendix p9).** Overall, infants who seroconverted had fewer viral pathogens at the time of vaccination compared with those who did not (mean 0.57 (0.04) vs. 0.81 (0.04), Wilcoxon's $p < 0.001$), **and this remained significant after adjusting for age in a logistic regression (p<0.001) and if enteroviruses were excluded (mean 0.28 (0.03) vs. 0.37 (0.03), p=0.038).** This difference was not apparent when comparing stool samples collected on day 0 (0.68 (0.04) vs. 0.63 (0.04), Wilcoxon's $p = 0.403$). There were no significant differences in the prevalence, abundance or overall number of bacterial or eukaryotic pathogens detected in stool at the time of vaccination (day 14) according to seroconversion status (mean number of bacterial and eukaryotic pathogens among infants who seroconverted was 1.37 (0.06) and 0.13 (0.02) compared with 1.41 (0.06) and 0.16 (0.02) respectively, Wilcoxon's $p = 0.467$ and 0.153; appendix ~~table 7~~p9). Similar results were obtained for the subset of infants assessed for poliovirus shedding, when comparing the prevalence of pathogens in stool samples collected on day 14 in infants who shed poliovirus on day 21 with those who did not shed (appendix p10, ~~figure 2; appendix table 8~~).

Among stool samples collected on day 14 with enterovirus detected at a CT value < 35 using the TAC, just 4 (1.3%) of 300 tested were positive for any of the Sabin polioviruses using a multiplex real-time PCR, **indicating minimal secondary exposure to OPV in our study.**

Biomarkers of EE

Faecal biomarkers on study days 0 and 14 were measured in 100.0% (292/292) and 99.7% (291/292) respectively of infants included in the subset identified for biomarker assessment and who completed the study per protocol. Plasma biomarkers were measured for the same number on study day 14 only. Faecal biomarkers of intestinal inflammation and permeability

measured on the day of vaccination (day 14) were significantly lower among infants who had received azithromycin compared with placebo for three of the four biomarkers measured (table 2). Plasma biomarkers of microbial translocation and epithelial damage did not differ between the study arms (day 14). In the longitudinal analysis of faecal biomarkers there was a significant decline between day 0 and 14 in myeloperoxidase and calprotectin among infants who received azithromycin but not among those who received placebo (appendix [table 9p11](#)). Neopterin showed a significant increase in the azithromycin but not placebo arm ($p=0.030$), although this was not reflected in the comparison of this biomarker between study arms on day 14.

Faecal and plasma biomarkers of EE measured at the time of vaccination (day 14) did not differ significantly between infants according to whether they subsequently seroconverted or not (table 2). However, mean levels were lower among infants who seroconverted for all biomarkers with the exception of faecal neopterin. Infants who shed poliovirus 7 days after OPV administration also had lower levels of faecal and plasma biomarkers of EE and this difference was significant for faecal calprotectin and $\alpha 1$ -antitrypsin ($p=0.021$ and 0.044 respectively; table 2). Infants with one or more biomarker in the top quartile did not differ significantly in their probability of seroconversion compared with other infants (51.5% (84/163) vs 49.2% (63/128)), $p=0.724$ and 45.6% (78/171) vs. 57.5% (69/120), $p=0.057$ for faecal and plasma biomarkers respectively). Similarly, seroconversion was not significantly correlated with the faecal biomarker EE score defined by Kosek et al. 2013¹⁵ (relative odds of seroconversion for each unit increase in EE score was 0.93 (95% CI: 0.85-1.02), $p=0.145$).

In multivariable log-linear regression analyses, myeloperoxidase and calprotectin measured in stool collected at enrolment were found to be positively correlated with the number of bacteria detected in that stool ($p=0.005$ and 0.001 respectively; appendix [table 10p12](#)). Faecal neopterin was negatively correlated with the number of eukaryotic pathogens (mainly *Giardia* and *Cryptosporidium*, $p<0.001$). Plasma biomarkers measured at the time of vaccination in infants receiving placebo did not show any correlation with the number of intestinal pathogens in stool collected at the same time. In general, plasma biomarkers were not correlated with faecal biomarkers (appendix [table 11p13](#)). The latter were positively

correlated with one another, although this correlation was abrogated to some extent in the azithromycin arm.

Adverse events

Solicited adverse events including cough/cold, diarrhoea and fever were recorded among 58.9% (216/367) of infants receiving azithromycin and 61.9% (227/367) receiving placebo between enrolment and vaccination, and among 44.1% (160/363) and 44.4% (163/367) for these same groups between vaccination and completing the study. Three serious adverse events were reported (2 in the azithromycin group and 1 in the placebo group), which were not considered related to the study interventions.

Discussion

Treatment with azithromycin reduced the prevalence and abundance of bacterial intestinal pathogens and faecal markers of EE, but did not improve seroconversion after a subsequent dose of serotype-3 monovalent OPV. This remained low at approximately 50%, compared with an average of 94% in temperate countries.²² Seroconversion did, however, correlate (negatively) with the presence of viral pathogens detected in stool at the time of vaccination, which were unaffected by azithromycin. ~~The number of viral pathogens was significantly lower among infants who seroconverted or shed poliovirus after OPV, and this association was individually significant for enteroviruses and rotavirus (figure 2).~~

Faecal and plasma biomarkers of EE were typically lower among children who seroconverted or shed poliovirus after OPV. However, this association was not statistically significant for individual biomarkers or for EE scores based on aggregated biomarker data, with the exception of faecal calprotectin and α 1-antitrypsin that were significantly lower among infants who shed poliovirus (but not among those who seroconverted). Therefore, insofar as we can measure EE, it does not appear to be a major determinant of OPV immunogenicity in Indian infants, although it is more common in populations where the immunogenicity of oral vaccines is lower. In fact, EE is thought to develop following repeated exposure to bacterial and viral pathogens, and it may only be the latter that are mechanistically linked to poor OPV immunogenicity.

~~The number of viral pathogens was significantly lower among infants who seroconverted or shed poliovirus after OPV, and this association was especially marked for enteroviruses (figure 2).~~ Infants did not receive OPV except through the clinical trial and so the majority (>98%) of enteroviruses we detected were non-polio enteroviruses (NPEV). The association between NPEV infection and a failure to seroconvert after OPV has been observed in a number of studies dating back to the earliest human trials of live-attenuated polioviruses and was recently confirmed in a systematic review and meta-analysis.⁷ ~~We also found an association between the number of other viral pathogens and seroconversion, although this was only of borderline significance when individual pathogens were compared (rotavirus, adenovirus).~~ An association between ~~rotavirus-other viral infections~~ and seroconversion to OPV has not previously been reported. Immunisation with live-attenuated oral rotavirus

vaccine does not appear to significantly lower seroconversion to co-administered OPV, but pre-existing wildtype rotavirus infection might be expected to have greater significance for OPV replication.²³ ~~Other viral pathogens (adenovirus, norovirus) were also more common among infants who did not seroconvert after OPV, although these associations were not statistically significant.~~

The association between seroconversion and the presence of viral pathogens ~~that we observed~~ was apparent for stool collected on the day of vaccination (day 14) but not for stool collected at enrolment (day 0). This suggests an effect of concurrent infection on OPV immunogenicity that is not sustained after infection is cleared. This would be consistent with an innate antiviral immune response induced by these pathogens that is effective in suppressing OPV replication and/or direct viral interference within infected cells.²⁴ Further studies of innate immune activation in the intestine in human and animal studies of viral infection and oral vaccine response would **provide more robust data on the significance of viral pathogens for the immune response to vaccination and** allow these mechanistic hypotheses to be tested.

Azithromycin significantly reduced faecal biomarkers of neutrophil activity (myeloperoxidase and calprotectin) and protein-losing enteropathy (α 1-antitrypsin), which were high among infants at enrolment to our study, comparable to levels found in inflammatory bowel disease.^{15,25} However, faecal neopterin – a marker of activated cell-mediated immunity – was unaffected and remained high relative to normal values observed in healthy children in high-income countries.¹⁵ Plasma biomarkers of microbial translocation from the intestine to systemic sites (EndoCAb, sCD14) and of damage to the intestinal epithelium (I-FABP) were also unaffected and remained high relative to values observed in high-income countries.^{26,27} This may reflect an inherent limitation in the effectiveness of azithromycin against EE or the relatively short course of treatment (3 days). It is possible that sustained suppression of inflammation through a longer course would have allowed epithelial healing and a reduction in microbial translocation and immune activation, the hallmarks of EE.^{13,14} We are aware of only one other randomised trial of antibiotics on EE, which found no effect of a 7-day course of rifaximin given to Malawian children on intestinal permeability measured using a sugar absorption test (lactulose-to-mannitol ratio in urine).²⁸ This non-absorbed antibiotic has a

different anti-microbial spectrum to azithromycin (e.g. *Campylobacter* is unaffected) and unfortunately, biomarkers of inflammation and microbial translocation were not assessed. Given the reduction in intestinal inflammation and protein-losing enteropathy that we observed, future studies of the impact of a longer course of azithromycin on EE may therefore be warranted. This would be most relevant in the context of nutritional interventions that aim to prevent growth faltering and severe acute malnutrition in the most vulnerable children. Indeed, antibiotics are known to promote growth in malnourished children.²⁹ The mechanisms of action are not known, although reduction in intestinal inflammation and resolution of EE may play an important role.

The number of bacterial pathogens detected in stool significantly correlated with faecal biomarkers of intestinal inflammation among infants at enrolment, and the reduction in these biomarkers following treatment with azithromycin was associated with a reduction in the prevalence and abundance of bacterial pathogens. It is therefore plausible that the effect of azithromycin on intestinal inflammation was mediated through a reduction in the abundance of these pathogens. However, azithromycin is also known to have direct anti-inflammatory properties, potentially modulating a number of immune pathways.¹⁸ Unfortunately, the high prevalence of bacterial pathogens in this population precluded examination of a direct effect of azithromycin on EE biomarkers in the absence of detectable infection.

We administered OPV on day 14 of the study to allow for the resolution of intestinal inflammation following treatment of bacterial pathogens. It is likely that reinfection following clearance of azithromycin from mucosal tissues (half-life 2-4 days) occurred, diminishing the impact of the 3-day course of treatment on the prevalence and abundance of bacterial pathogens at this time. Nonetheless, the significant impact that we observed and the absence of association between these pathogens and OPV immunogenicity suggest earlier vaccination would not have led to a different study outcome.

A potential limitation of our study was the reliance on molecular detection methods for intestinal pathogens. These methods show good sensitivity (85%) when compared with conventional methods based on immunoassay, culture or microscopy.³⁰ However, they have

a lower limit of detection and typically detect more pathogens in each sample compared with traditional methods. The biological relevance of a pathogen detected at a high CT value (low target copy number) may therefore be questionable, and association with a clinically measurable outcome such as gastroenteritis is more common for lower CT values.²¹ For this reason we performed quantitative statistical analysis of CT values in addition to analysis of pathogen prevalence and we found consistent results with both approaches. We were also reliant on molecular biomarkers of EE in stool/plasma and did not perform intestinal biopsies in this asymptomatic population to confirm enteropathy. These biomarkers are widely used in studies of EE, capture a number of related pathological processes and are used to support diagnosis of enteropathies (e.g. calprotectin for inflammatory bowel disease). Nonetheless, tissue samples from the small intestine would likely provided additional information about the processes affecting OPV replication and immune response.

In this study we aimed to reduce the prevalence of intestinal bacterial pathogens and inflammation, which we hypothesised were important determinants of the poor immunogenicity of OPV in low income countries. Although a 3-day course of azithromycin was effective in achieving these aims, it had no impact on OPV immunogenicity. Instead, we found that immunogenicity was reduced in the presence of viral pathogens, potentially implicating viral interference and/or innate antiviral immune mechanisms. An understanding of these mechanisms and the factors that determine viral infection in the intestine in early infancy may help in the design of new oral vaccines and vaccine schedules that are more effective in low income countries.

Research in context

Evidence before this study

We previously did a systematic review of the association of intestinal infections and environmental enteropathy (EE) with the immune response to oral poliovirus vaccine (OPV), published in 2014. Meta-analysis of included studies identified a significant reduction in the immunogenicity of OPV in children infected with non-polio enteroviruses or who had diarrhoea at the time of vaccination. There was also evidence for an association between OPV immunogenicity and the presence of pathogenic bacteria and parasites in stool at the time of vaccination, although the number of published studies was small and there was risk of publication bias. No studies of EE met the inclusion criteria. In an update of the review of EE in January 2016 we identified a single study published in 2015 of EE and the immunogenicity of OPV given on a routine schedule. A number of associations between EE biomarkers and the immunogenicity of OPV and oral rotavirus vaccine (Rotarix) were reported, although the directions of these associations were variable and not consistent with a clear role of EE in oral vaccine failure. In 2015 we published a review of the effect of antibiotics and probiotics on the response to oral vaccines. A study of rotavirus infection in mice as a model for vaccination found that sustained treatment with ampicillin and neomycin before and after inoculation led to more durable rotavirus-specific antibody production in serum and stool. A study of anthelmintics given to children infected with *Ascaris lumbricoides* found a modest improvement in the immunogenicity of a subsequent dose of live-attenuated oral cholera vaccine compared with children given placebo. No studies reporting the effect of antibiotics on oral vaccine immunogenicity in humans were identified.

Added value of this study

This is the first randomised clinical trial of the effect of antibiotics on the immunogenicity of OPV (or any oral vaccine). In addition, we report on the impact of antibiotic treatment on intestinal pathogens, biomarkers of EE and their association with OPV immunogenicity. We found that a 3-day course of azithromycin was effective in reducing the prevalence and abundance of pathogenic intestinal bacteria and biomarkers of intestinal inflammation and permeability associated with EE. However, these and other biomarkers of EE were not associated with seroconversion after a subsequent dose of OPV, which was no different for infants in the azithromycin or placebo groups. These results suggest that EE is not a major determinant of OPV immunogenicity. Instead, viral pathogens (~~enterovirus, rotavirus~~) were found to be more prevalent and abundant among infants who did not seroconvert after OPV.

Implications of all the available evidence

Although azithromycin appears to offer effective treatment of some aspects of EE, it does not offer a solution to the poor immunogenicity and efficacy of OPV in low-income settings. Intestinal viral infections may be more important determinants of the immunogenicity of OPV and other live oral viral vaccines. Strategies to alter exposure to enteric viruses in early infancy (~~e.g.- maternal immunisation or water, sanitation and hygiene interventions~~) or changes in the immunisation schedule (~~e.g. neonatal doses~~) may be needed to improve the effectiveness of these vaccines.

References

1. Qadri F, Bhuiyan TR, Sack DA, Svennerholm AM. Immune responses and protection in children in developing countries induced by oral vaccines. *Vaccine* 2013; **31**(3): 452-60.
2. Levine MM. Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. *BMC Biol* 2010; **8**.
3. Grassly NC, Fraser C, Wenger J, et al. New strategies for the elimination of polio from India. *Science* 2006; **314**(5802): 1150 - 3.
4. O'Reilly KM, Durry E, ul Islam O, et al. The effect of mass immunisation campaigns and new oral poliovirus vaccines on the incidence of poliomyelitis in Pakistan and Afghanistan, 2001-11: a retrospective analysis. *Lancet* 2012; **380**: 491-8.
5. Sabin AB, Michaels RH, Krugman S, Eiger ME, Berman PH, Warren J. Effect of oral poliovirus vaccine in newborn children: I. Excretion of virus after ingestion of large doses of type I or of mixture of all three types, in relation to level of placentally transmitted antibody. *Pediatrics* 1963; **31**(4): 623-40.
6. Appaiahgari MB, Glass R, Singh S, et al. Transplacental rotavirus IgG interferes with immune response to live oral rotavirus vaccine ORV-116E in Indian infants. *Vaccine* 2014; **32**(6): 651-6.
7. Parker EPK, Kampmann B, Kang G, Grassly NC. Influence of enteric infections on response to oral poliovirus vaccine: a systematic review and meta-analysis. *J Infect Dis* 2014; **210**: 853-64.
8. Kotloff KL, Nataro JP, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* 2013; **382**: 209-22.
9. World Health Organization Collaborative Study Group on Oral Poliovirus Vaccine. Factors affecting the immunogenicity of oral poliovirus vaccine - a prospective evaluation in Brazil and the Gambia. *J Infect Dis* 1995; **171**(5): 1097-106.
10. Maldonado YA, PenaCruz V, Sanchez MD, et al. Host and viral factors affecting the decreased immunogenicity of Sabin type 3 vaccine after administration of trivalent oral polio vaccine to rural Mayan children. *J Infect Dis* 1997; **175**(3): 545-53.

11. Kau AL, Planer JD, Liu J, et al. Functional characterization of IgA-targeted bacterial taxa from undernourished Malawian children that produce diet-dependent enteropathy. *Sci Transl Med* 2015; **7**(276): 276ra24.
12. Kelly P, Menzies I, Crane R, et al. Responses of small intestinal architecture and function over time to environmental factors in a tropical population. *Am J Trop Med Hyg* 2004; **70**(4): 412-9.
13. Korpe PS, Petri WA. Environmental enteropathy: critical implications of a poorly understood condition. *Trends Mol Med* 2012; **18**(6): 328-36.
14. Keusch GT, Denno DM, Black RE, et al. Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences. *Clin Infect Dis* 2014; **59**(suppl 4): S207-S12.
15. Kosek M, Hague R, Lima A, et al. Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants. *Am J Trop Med Hyg* 2013; **88**(2): 390-6.
16. Naylor C, Lu M, Haque R, et al. Environmental enteropathy, oral vaccine failure and growth faltering in infants in Bangladesh. *EBioMedicine* 2015: [dx.doi.org/10.1016/j.ebiom.2015.09.036](https://doi.org/10.1016/j.ebiom.2015.09.036).
17. Lode H, Borner K, Koeppe P, Schaberg T. Azithromycin - Review of key chemical, pharmacokinetic and microbiological features. *J Antimicrob Chemother* 1996; **37**: 1-8.
18. Shinkai M, Henke MO, Rubin BK. Macrolide antibiotics as immunomodulatory medications: Proposed mechanisms of action. *Pharmacol Ther* 2008; **117**(3): 393-405.
19. Praharaj I, John SM, Bandyopadhyay R, Kang G. Probiotics, antibiotics and the immune responses to vaccines. *Philos Trans R Soc B-Biol Sci* 2015; **370**(1671): 12.
20. World Health Organisation. Manual of laboratory methods for testing of vaccines used in the WHO Expanded Programme on Immunization. WHO/VSQ/97.04. 1997.
21. Liu J, Kabir F, Manneh J, et al. Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. *Lancet Infect Dis* 2014; **14**(8): 716-24.
22. Caceres VM, Sutter RW. Sabin monovalent oral polio vaccines: Review of past experiences and their potential use after polio eradication. *Clin Infect Dis* 2001; **33**(4): 531-41.
23. Patel M, Steele AD, Parashar UD. Influence of oral polio vaccines on performance of the monovalent and pentavalent rotavirus vaccines. *Vaccine* 2012; **30**: A30-A5.

24. Wang HP, Moon S, Wang YH, Jiang BM. Multiple virus infection alters rotavirus replication and expression of cytokines and Toll-like receptors in intestinal epithelial cells. *Virus Res* 2012; **167**(1): 48-55.
25. Lin JF, Chen JM, Zuo JH, et al. Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity. *Inflamm Bowel Dis* 2014; **20**(8): 1407-15.
26. Lundell AC, Adlerberth I, Lindberg E, et al. Increased levels of circulating soluble CD14 but not CD83 in infants are associated with early intestinal colonization with *Staphylococcus aureus*. *Clin Exp Allergy* 2007; **37**: 62-71.
27. Prendergast AJ, Rukobo S, Chasekwa B, et al. Stunting is characterized by chronic inflammation in Zimbabwean infants. *Plos ONE* 2014; **9**: doi: 10.1371/journal.pone.0086928.
28. Trehan I, Shulman RJ, Ou CN, Maleta K, Manary MJ. A randomized, double-blind, placebo-controlled trial of rifaximin, a nonabsorbable antibiotic, in the treatment of tropical enteropathy. *Am J Gastroenterol* 2009; **104**(9): 2326-33.
29. Gough EK, Moodie EEM, Prendergast AJ, et al. The impact of antibiotics on growth in children in low and middle income countries: systematic review and meta-analysis of randomised controlled trials. *Br Med J* 2014; **348**: g2267.
30. Liu J, Gratz J, Amour C, et al. A laboratory developed TaqMan array card for simultaneous detection of nineteen enteropathogens. *J Clin Microbiol* 2012; **51**(2): 472-80.

Contributors

NCG, JJ, SBah and GK conceived the study, wrote the protocol and obtained all approvals. SPK, SV, SD, UR and JJ managed infant screening and the clinical trial. JVP oversaw preparation of the test articles. MP, SJ, SBal, JR and RS implemented study procedures. IP, SBab, SG, EPKP, AA, JL, MI-G, HHU, ERH and GK led the laboratory work. NCG, JM and JJ led the statistical analysis. All authors contributed to the interpretation of the data, writing of the report, and approved the final manuscript.

Declaration of interests

The authors declare no conflict of interests. HHU declares unrelated collaborations with Eli Lilly, UCB Pharma and Vertex Pharmaceuticals, and travel support from Actelion and Merck Sharp & Dohme.

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Table 1: Characteristics of the study population at enrolment (day 0).

Characteristic	Study arm	
	Azithromycin	Placebo
<i>Demography</i>		
Age in months	7.46 (0.08)	7.49 (0.08)
WomenFemale	196 (52.1)	203 (53.7)
Mother's education (primary or below)	49 (13.0)	63 (16.7)
illiterate	10 (2.7)	29 (7.7)
primary	39 (10.4)	34 (9.0)
middle	99 (26.3)	94 (24.9)
secondary	193 (51.3)	192 (50.8)
graduate	35 (9.3)	29 (7.7)
House roof type (concrete floor or better)	268 (71.3)	265 (70.1)
concrete	189 (50.3)	183 (48.4)
tiled	59 (15.7)	67 (17.7)
thatched	128 (34.0)	128 (33.9)
<i>Vaccination history</i>		
Trivalent OPV doses	4.02 (0.05)	4.11 (0.05)
<i>Health status</i>		
Diarrhoea at enrolment	8 (2.1)	6 (1.6)
Currently breastfed	339 (90.2)	339 (89.7)
Length (cm)	66.9 (0.17)	66.9 (0.16)
Weight (kg)	7.32 (0.05)	7.3 (0.05)

Data are mean (SE) or n (%)

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Table 2 Faecal and plasma biomarkers of EE at the time of vaccination with serotype-3 monovalent OPV (day 14) according to study arm, seroconversion (day 35) and detection of poliovirus shedding (day 21).

Biomarker	Study arm		p-value	Seroconversion		p-value	Poliovirus shedding		p-value
	Azithromycin	Placebo		No	Yes		No	Yes	
<i>Stool</i>									
myeloperoxidase (ng/mL)	15780 (1277)	21251 (1407)	0.001	19724 (1445)	17425 (1276)	0.297	20001 (1525)	17318 (1216)	0.302
calprotectin (µg/g)	862 (65)	1094 (61)	0.001	1043 (67)	918 (61)	0.191	1082 (68)	892 (60)	0.021
neopterin (nmol/L)	6644 (396)	6934 (423)	0.612	6710 (372)	6871 (444)	0.757	6806 (431)	6779 (392)	0.779
α1-antitrypsin (mg/g)	1.028 (0.08)	1.269 (0.108)	0.015	1.279 (0.115)	1.025 (0.071)	0.126	1.27 (0.102)	1.047 (0.09)	0.044
<i>Plasma</i>									
soluble CD14 (ng/mL)	2623 (133)	2750 (142)	0.419	2788 (143)	2589 (132)	0.291	2716 (141)	2662 (135)	0.735
I-FABP (pg/mL)	823 (41)	810 (40)	0.520	863 (45)	770 (35)	0.138	873 (47)	768 (34)	0.091
EndoCAb IgG (MU/mL)	77 (14)	56 (8)	0.832	75 (13)	58 (10)	0.074	77 (14)	57 (9)	0.098

data are mean (SE); differences between groups assessed using Wilcoxon rank sum test.

Figure legends

Figure 1 Trial profile. Children randomized to azithromycin or placebo were enrolled in the study for a total of 35 days.

Figure 2 Prevalence of intestinal pathogens at the time of vaccination with OPV. Shown in (A) by study arm and in (B) by subsequent seroconversion status. Only pathogens with a prevalence of at least 1% across all samples are shown. Error bars indicate 95% confidence intervals. * p-value < 0.05; ** p-value < 0.01. All p-values are given in appendix [table 4p6](#). Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, shiga toxin-producing *E. coli*; *B. fragilis*, *Bacteroides fragilis*; *C. difficile*, *Clostridium difficile*; *E. bieneusi*, *Enterocytozoon bieneusi*.

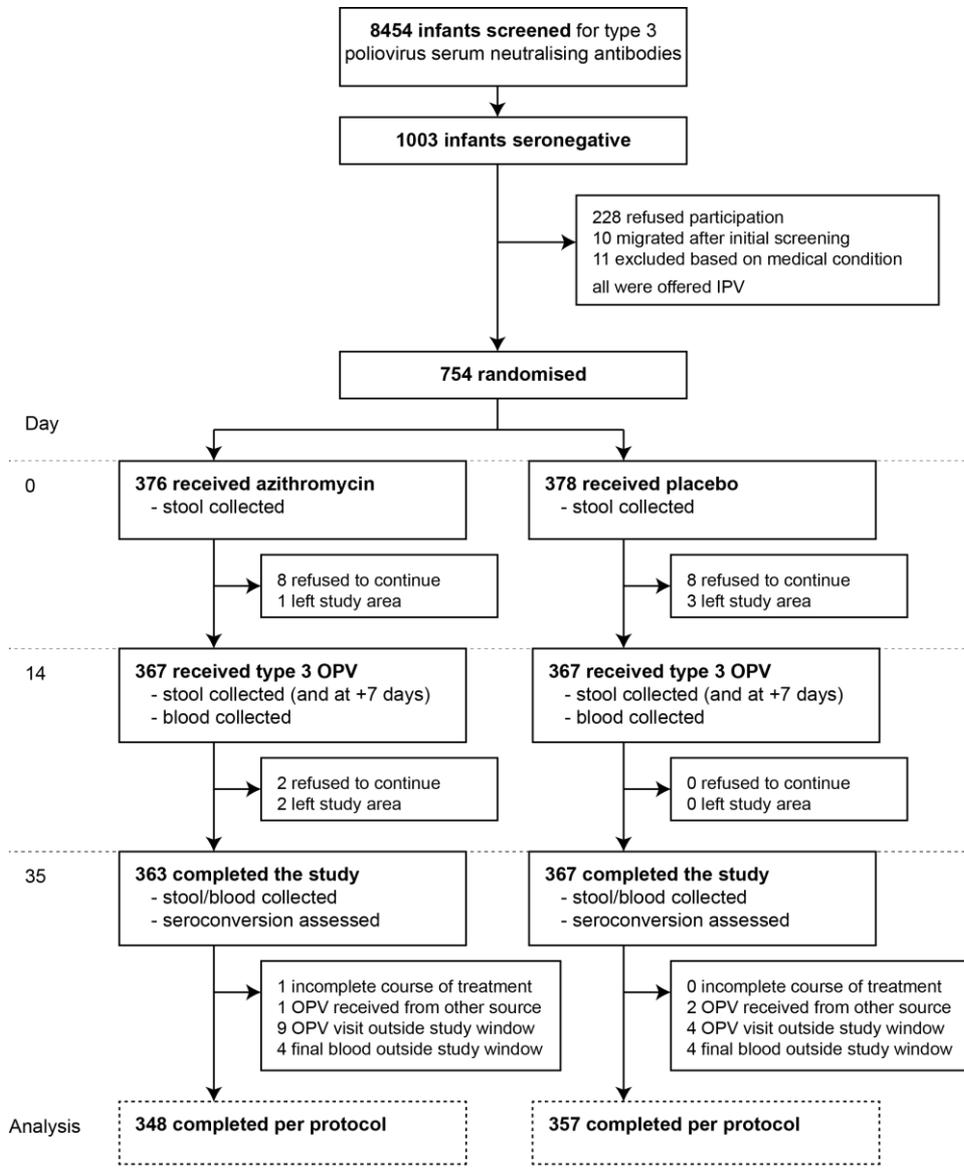
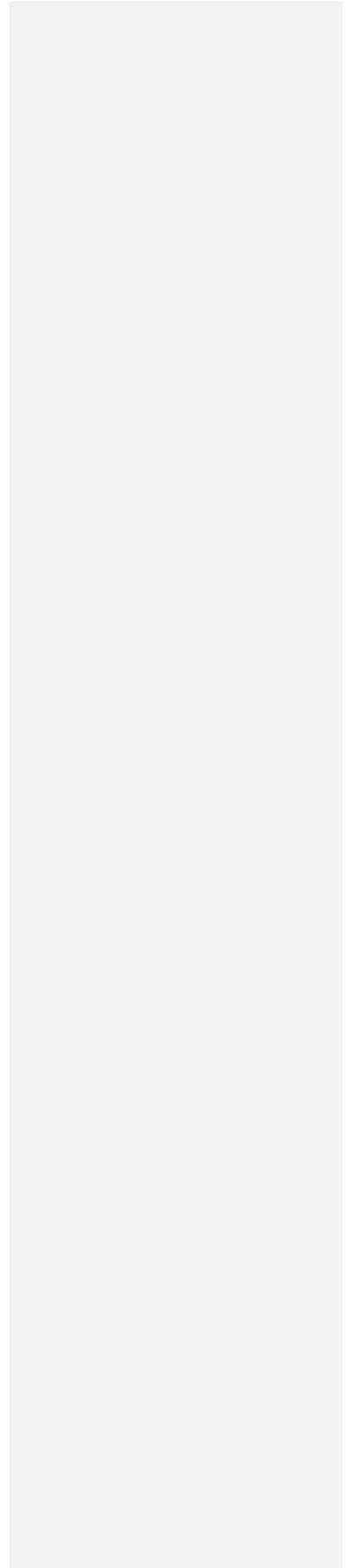
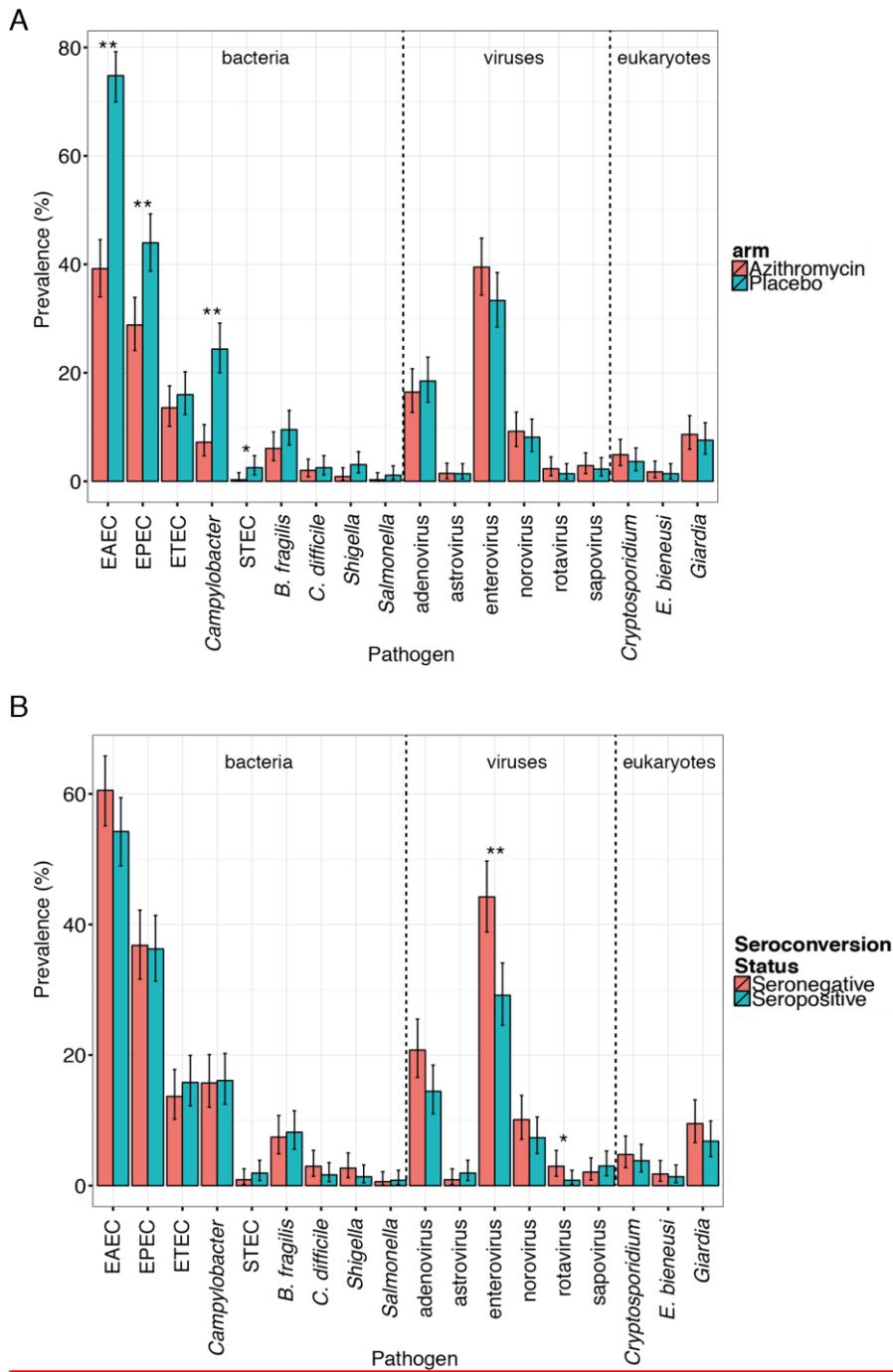


Figure 1

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Figure 2