

The effect of azithromycin on the immunogenicity of oral poliovirus vaccine: a double-blind randomised placebo-controlled trial in seronegative Indian infants



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Summary

Background Oral poliovirus vaccine is less immunogenic and effective in low-income countries than in high-income countries, similarly to other oral vaccines. The high prevalence of intestinal pathogens and associated environmental enteropathy has been proposed to explain this problem. Because administration of an antibiotic has the potential to resolve environmental enteropathy and clear bacterial pathogens, we aimed to assess whether antibiotics would improve oral poliovirus vaccine immunogenicity.

Methods We did a double-blind, randomised, placebo-controlled trial of the effect of azithromycin on the immunogenicity of serotype-3 monovalent oral poliovirus vaccine given to healthy infants living in 14 blocks of Vellore district, India. Infants were eligible to participate if they were 6–11 months old, available for the study duration, and lacked serum neutralising antibodies to serotype-3 poliovirus. Infants were randomly assigned (1:1) at enrolment to receive oral 10 mg/kg azithromycin or placebo once daily for 3 days, followed by serotype-3 monovalent oral poliovirus vaccine on day 14. The primary outcome was detection of serum neutralising antibodies to serotype-3 poliovirus at a dilution of one in eight or more on day 35 and was assessed in the per-protocol population (ie, all those who received azithromycin or placebo, oral poliovirus vaccine, and provided a blood sample according to the study protocol). Safety outcomes were assessed in all infants enrolled in the study. The trial is registered with the Clinical Trials Registry India, number CTRI/2014/05/004588.

Findings Between Aug 5, 2014, and March 21, 2015, 754 infants were randomly assigned: 376 to receive azithromycin and 378 to placebo. Of these, 348 (93%) of 376 in the azithromycin group and 357 (94%) of 378 infants in the placebo group completed the study per protocol. In the azithromycin group, 175 (50%) seroconverted to serotype-3 poliovirus compared with 192 (54%) in the placebo group (risk ratio 0·94, 95% CI 0·81–1·08; $p=0\cdot366$). Azithromycin reduced faecal biomarkers of environmental enteropathy (calprotectin, myeloperoxidase, α 1-antitrypsin) and the prevalence of bacterial but not viral or eukaryotic pathogens. Viral pathogens were associated with lower seroconversion. Three serious adverse events were reported (two in the azithromycin group and one in the placebo group), but none was considered related to the study interventions.

Interpretation Azithromycin did not improve the immunogenicity of oral poliovirus vaccine despite reducing biomarkers of environmental enteropathy and the prevalence of pathogenic intestinal bacteria. Viral interference and innate antiviral immune mechanisms might 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 finding 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, 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 might be common to several oral vaccines, such as interference with the immune response in infants by high concentrations of homologous maternal antibodies transferred via the placenta or breast feeding.^{5,6} Others might be specific to particular vaccines. For example, infection with enteroviruses at the time of vaccine administration is associated with reduced immunogenicity of oral poliovirus vaccine, perhaps as a result of cross-neutralising antibodies or direct interference at the cellular level.⁷ However, definitive

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Research in context

Evidence before this study

We previously did a systematic review of the association of intestinal infections and environmental enteropathy with the immune response to oral poliovirus vaccine published in 2014. In that review, we searched PubMed and ISI Web of Knowledge in October, 2012, without language or date restrictions. Meta-analysis of included studies identified a significant reduction in the immunogenicity of oral poliovirus vaccine in children infected with non-polio enteroviruses or who had diarrhoea at the time of vaccination. There was also evidence for an association between oral poliovirus vaccine 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 environmental enteropathy met the inclusion criteria.

In an update of the review of environmental enteropathy on Jan 26, 2016, using the same databases and criteria, we identified a single study published in 2015 of environmental enteropathy and the immunogenicity of oral poliovirus vaccine given on a routine schedule. Several associations between environmental enteropathy biomarkers and the immunogenicity of oral poliovirus vaccine and oral rotavirus vaccine (Rotarix) were reported, although the directions of these associations were variable and not consistent with a clear role of environmental enteropathy 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 human beings were identified.

Added value of this study

To our knowledge, this is the first randomised clinical trial of the effect of antibiotics on the immunogenicity of oral poliovirus vaccine (or any oral vaccine). Additionally, we report on the effect of antibiotic treatment on intestinal pathogens, biomarkers of environmental enteropathy, and their association with oral poliovirus vaccine 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 environmental enteropathy. However, these and other biomarkers of environmental enteropathy were not associated with seroconversion after a subsequent dose of oral poliovirus vaccine, which was no different for infants in the azithromycin or placebo groups. These results suggest that environmental enteropathy is not a major determinant of oral poliovirus vaccine immunogenicity. Instead, viral pathogens were more prevalent and abundant in infants who did not seroconvert after oral poliovirus vaccine.

Implications of all the available evidence

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

mechanisms that can explain the substantial differences in immunogenicity of oral vaccines between populations have not been identified.^{1,2} Identification of these mechanisms 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 oral poliovirus vaccine.^{7,9,10} Repeated exposure to bacterial and other pathogens is thought to underlie environmental (or tropical) enteropathy, a subclinical 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 environmental enteropathy have been used to establish its extent and role in growth faltering and malnutrition.¹⁵ Some of these biomarkers are associated with the outcome of immunisation with oral rotavirus and poliovirus vaccines.¹⁶

We investigated the association between infection with intestinal pathogens, environmental enteropathy, and the immune response to oral vaccination by assessing the effect of a 3-day course of oral azithromycin on the immunogenicity of a subsequent dose of serotype-3 monovalent oral poliovirus vaccine given to Indian infants who did not have 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 immunomodulator in the treatment of several conditions, including cystic fibrosis, but its effectiveness as treatment for environmental enteropathy has not been examined.¹⁸ The effect of antibiotics on the response to oral vaccination has also not previously been examined.¹⁹ We measured the effect of azithromycin on the development of serum neutralising antibodies and poliovirus shedding after oral poliovirus vaccination, in addition to faecal and plasma biomarkers of environmental enteropathy and intestinal pathogens in stool. We were therefore able to assess the effect of antibiotic treatment on environmental enteropathy and intestinal pathogens, and whether this improved the immune response to oral poliovirus vaccination.

Methods

Study design and participants

We did a double-blind, randomised, placebo-controlled trial among infants living in 14 blocks of Vellore district in south India. Infants were identified in the community based on records of births held at 210 health subcentres and screened for serum neutralising antibodies to serotype-3 poliovirus by taking a single 3 mL blood draw. A questionnaire recording basic demographic and vaccination history data was administered. Infants were eligible for screening if they lived in the area, had no reported receipt of inactivated poliovirus vaccine, and would be 6–11 months old at study enrolment and available for the duration of the study. After screening, infants who did not have detectable neutralising antibodies at a dilution of one in eight, 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. Infants were excluded if they had received oral poliovirus vaccine since the screening visit, had a history of allergic reaction after oral poliovirus vaccine, had 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 hospital admission were temporarily excluded for up to 2 weeks until they were either able to participate or met a permanent exclusion criterion. All infants who were seronegative but ineligible after screening or at completion of the clinical trial were offered inactivated poliovirus vaccine. Separate written informed consent for screening and for enrolment in the trial were sought from a parent or caregiver. The trial was done in accordance with the good clinical practice and ethical principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Christian Medical College and the Drugs Controller General of India.

Randomisation and masking

Infants were randomly assigned (1:1) 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 oral poliovirus vaccine. The randomisation sequence was computer generated with a block 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 participant 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 were blinded to group assignment.

Procedures

Infants were given azithromycin at a dose of 10 mg/kg in a syrup (Zithrox, MacLeods Pharmaceuticals Ltd, Mumbai, India) or a placebo syrup matched in colour and taste (Christian Medical College pharmacy, Vellore, India) once a day for the first 3 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 oral poliovirus vaccine 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, Rixensart, Belgium).

Serum was tested for serotype-3 poliovirus-specific neutralising antibodies with a modified micro-neutralisation assay at one in four and one in eight dilutions for eligibility screening and in two-fold serial dilutions from one in four to one in 512 for infants enrolled in the trial on day 35.²⁰ 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.²¹ A complete list of pathogen sequence targets is provided in the appendix. Faecal biomarkers of intestinal inflammation, protein-losing enteropathy, and immune activation (myeloperoxidase, calprotectin, α1-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 with enzyme-linked immunosorbent assays.^{14,15} Shedding of poliovirus in stool samples was assessed with quantitative real-time PCR (appendix). Infants randomly assigned to azithromycin or placebo were enrolled in the study for 35 days.

Outcomes

The primary outcome of the study was seroconversion, defined as the detection of serotype-3 poliovirus-specific serum neutralising antibodies at a dilution of one in

See Online for appendix

eight or higher in blood taken 21 days after vaccination (day 35). Secondary outcomes included the prevalence and abundance of intestinal pathogens, biomarkers of environmental enteropathy, and shedding of serotype-3 poliovirus. Stool samples were collected at enrolment (day 0, before treatment) and on the day of oral poliovirus vaccine administration (day 14, before vaccination) and tested for the presence of intestinal pathogens. In a subset of infants, biomarkers of environmental enteropathy 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

oral poliovirus vaccine (day 21). Additionally, poliovirus-specific faecal IgA and T-cell assays were planned as secondary and exploratory objectives and will be reported elsewhere. Adverse events were solicited through daily visits on the days study drugs were administered, followed by biweekly visits for the next fortnight and weekly visits until the child completed the study. All serious adverse events were reported to the data and safety monitoring board and the Christian Medical College institutional review board. The safety analysis was performed on all participants who received a study intervention.

Statistical analysis

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 oral poliovirus vaccine on the basis of an estimated seroconversion in the placebo group of 60%, prevalence of treatable intestinal pathogens of 40%, reduction in oral poliovirus vaccine seroconversion in infants infected with these pathogens of 66%, treatment efficacy of 75% and reinfection in about 10% between treatment and vaccination (resulting in 72% seroconversion expected in the azithromycin group), 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 environmental enteropathy and poliovirus shedding.

Infants who received azithromycin or placebo daily on study days 0–2 (or up to 1 day late), oral poliovirus vaccination on day 14 (plus or minus 1 day), and provided a blood sample on day 35 (minus 1 or plus up to 7 days) were considered to have completed the study per protocol and were included in the primary analysis. Supportive analyses were done in all infants who provided a blood sample at the final visit.

The proportion of infants who seroconverted or shed poliovirus in each study group was compared with Fisher's exact test and the risk ratio (RR) was calculated with 95% CIs based on the delta method. Geometric mean titres 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 groups with Wilcoxon's rank sum test. Baseline characteristics of infants were compared between study groups with Fisher's exact test for binary data and Wilcoxon's rank sum 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 a cross-sectional analysis comparing the proportion of infants with each pathogen between study groups on day 14 with Fisher's exact test, and a longitudinal analysis of the change in this proportion between study day 0 and 14 in each group with McNemar's exact test for paired data. All proportions are presented with Clopper-Pearson 95% CIs. Equivalent

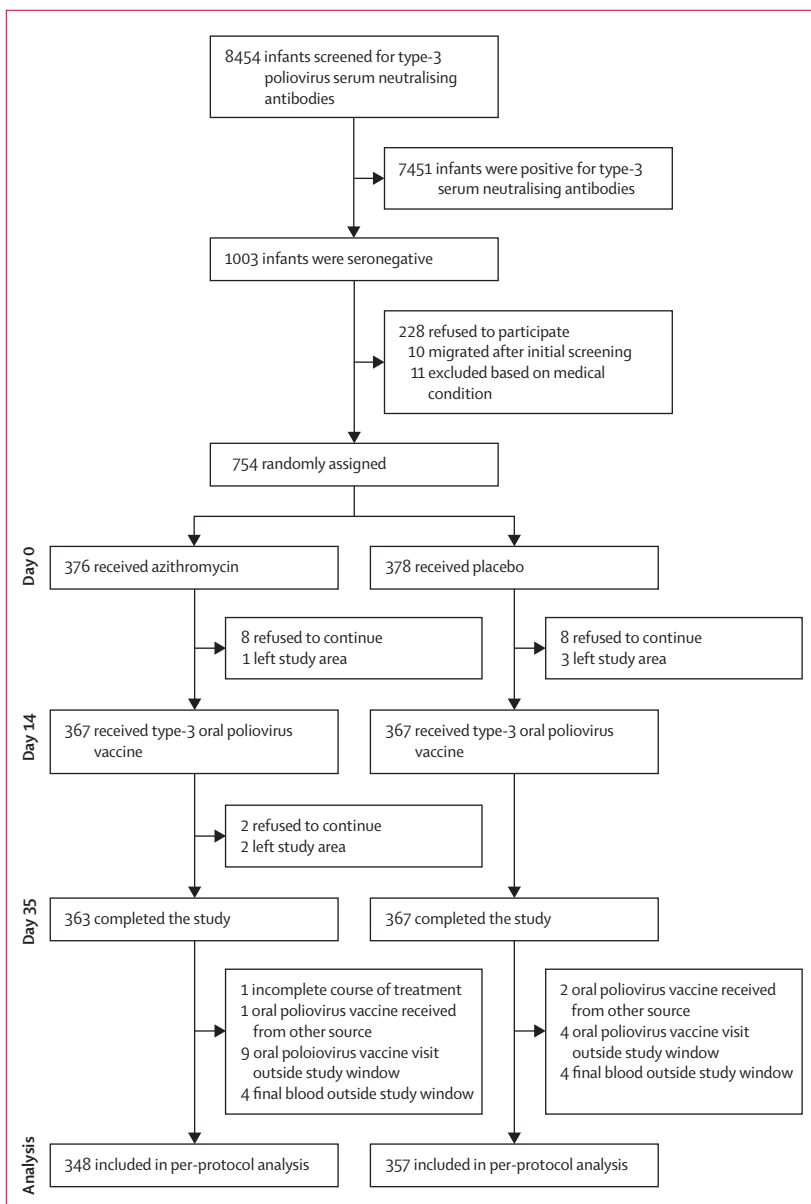


Figure 1: Trial profile

analyses of the abundance of intestinal pathogens based on the quantitative PCR cycle threshold value were done with Wilcoxon's rank sum test and signed rank test (for paired data). Change in the number of pathogens detected in each infant was also examined in each study group with Wilcoxon's rank sum test.

The effect of azithromycin on faecal biomarkers of environmental enteropathy was assessed in analogous cross-sectional and longitudinal analyses with Wilcoxon's rank sum and signed rank tests. Plasma biomarkers of environmental enteropathy were available for study day 14 only and compared with Wilcoxon's rank sum test. Additionally environmental enteropathy was examined when defined by a binary variable equal to one if one or more biomarkers were in the top quartile for measurements of stool or plasma and zero otherwise, and using an environmental enteropathy score based on faecal biomarkers as defined by Kosek and colleagues.¹⁵ The association between biomarkers of environmental enteropathy and the number of intestinal pathogens by group (bacteria, viruses, eukaryotes) was assessed with log-linear regression.

The same statistical methods were used in cross-sectional analyses comparing biomarkers of environmental enteropathy and presence of pathogens between infants according to whether they seroconverted or shed poliovirus after oral poliovirus vaccination. Additionally, the association between baseline characteristics of infants, the prevalence and abundance of intestinal pathogens, and seroconversion was assessed with logistic regression. Oversight of the study was provided by an independent data safety and monitoring board. All analyses were conducted using the statistical programming language R. The trial is registered with the Clinical Trials Registry India, number CTRI/2014/05/004588.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding and senior authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Infants participated in the clinical trial during the study period Aug 5, 2014, to March 21, 2015. 754 participants were randomly assigned to receive azithromycin (n=376) or placebo (n=378) following screening of 8454 infants for serum neutralising antibodies 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. Trivalent oral poliovirus vaccine offered through national immunisation days (Jan 18, 2015, and Feb 22, 2015) was withheld from infants who were enrolled in the trial at that time. Demographic characteristics, number of oral poliovirus vaccine doses, and health status did not differ between study groups (table 1).

In infants in the per-protocol analysis, 175 (50%) of 348 participants in the azithromycin group and 192 (54%) of 357 participants in the placebo group seroconverted after oral poliovirus vaccine (RR=0.94, 95% CI 0.81–1.08; Fisher's p=0.366). Similar results were obtained for the group consisting of all infants that completed the study (183 [50%] of 363 vs 196 [53%] of 367; RR=0.94, 95% CI 0.82–1.09; Fisher's p=0.459). For infants who completed the study per protocol, the geometric mean titres of serum neutralising antibodies on day 35 did not differ by study group (75.2 in the azithromycin group and 81.9 in the placebo group; Wilcoxon's p=0.281; appendix p 14).

In the subset of infants tested for serotype-3 poliovirus shedding on day 21 and who completed the study per protocol, 74 (51%) of 144 in the azithromycin group and 83 (56%) of 148 in the placebo group had detectable serotype-3 poliovirus in their stool 7 days after vaccination (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<0.0001; appendix p 4).

Older infants were less likely to seroconvert than younger infants (140 [47%] of 297 for 8–11 month olds compared with 227 [56%] of 408 for ages 6–7 month olds; Fisher's p=0.027). Infants who seroconverted reported slightly fewer previous doses of oral poliovirus vaccine 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 p 5). Infants with detectable

	Azithromycin (n=376)	Placebo (n=378)
Age (months)	7.46 (0.08)	7.49 (0.08)
Sex		
Male	180 (48%)	175 (46%)
Female	196 (52%)	203 (54%)
Mother's education		
Illiterate	10 (3%)	29 (8%)
Primary (1–5 years)	39 (10%)	34 (9%)
Middle (6–8 years)	99 (26%)	94 (25%)
Secondary (9–12 years)	193 (51%)	192 (51%)
University graduate (13+ years)	35 (9%)	29 (8%)
House roof type		
Concrete	189 (50%)	183 (48%)
Tiled	59 (16%)	67 (18%)
Thatched	128 (34%)	128 (34%)
Trivalent oral poliovirus vaccine doses	4.02 (0.05)	4.11 (0.05)
Health status		
Diarrhoea at enrolment	8 (2%)	6 (2%)
Breastfed	339 (90%)	339 (90%)
Mean height (cm)	66.9 (0.17)	66.9 (0.16)
Mean weight (kg)	7.3 (0.05)	7.3 (0.05)

Data are mean (SE) or n (%).

Table 1: Baseline characteristics

neutralising antibodies at enrolment did not differ significantly in their probability of seroconversion from those that did not have neutralising antibodies at enrolment (48 [61%] of 79 in those with neutralising antibodies at a one in four dilution vs 319 [51%] of 626 for those with undetectable neutralising antibodies; $p=0.120$). No other baseline characteristics were associated with seroconversion (appendix p 5).

Among infants who completed the study per protocol, TaqMan array card data were available for 705 (100%) of 705 on study day 0 and 704 (99.9%) of 705 infants on study day 14. Comparison of the prevalence of intestinal pathogens between study groups 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* and

Campylobacter were significantly less prevalent in the azithromycin group compared with the placebo group. This difference was also apparent when comparing the abundance of each bacterial pathogen on the basis of the quantitative PCR, and even in those bacteria without a significant reduction in prevalence, abundance was often reduced (appendix p 6). Overall, the number of bacterial pathogens detected at the time of vaccination (day 14) in infants who had received azithromycin was lower compared with infants who received placebo (mean 0.98 [SE 0.05] vs 1.78 [0.06]; Wilcoxon's $p<0.0001$). This difference was not apparent for viruses (0.72 [0.04] vs 0.65 [0.04]; Wilcoxon's $p=0.183$) or eukaryotic pathogens (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 groups 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* spp, *Bacteroides fragilis*, and *Salmonella* spp in stool collected on day 14 compared with day 0 in infants who received azithromycin but not in those who received placebo (appendix p 7, 15). 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 p 8).

Infants who seroconverted after oral poliovirus vaccine 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 p 9). 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 p 9). Overall, infants who seroconverted had fewer viral pathogens at the time of vaccination compared with those who did not (mean 0.57 [SE 0.04] vs 0.81 [0.04]; Wilcoxon's $p<0.0001$), and this remained significant after adjusting for age in a logistic regression ($p<0.0001$) 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 pathogens in infants who seroconverted was 1.37 [SE 0.06] compared with 1.41 [0.06] in those who

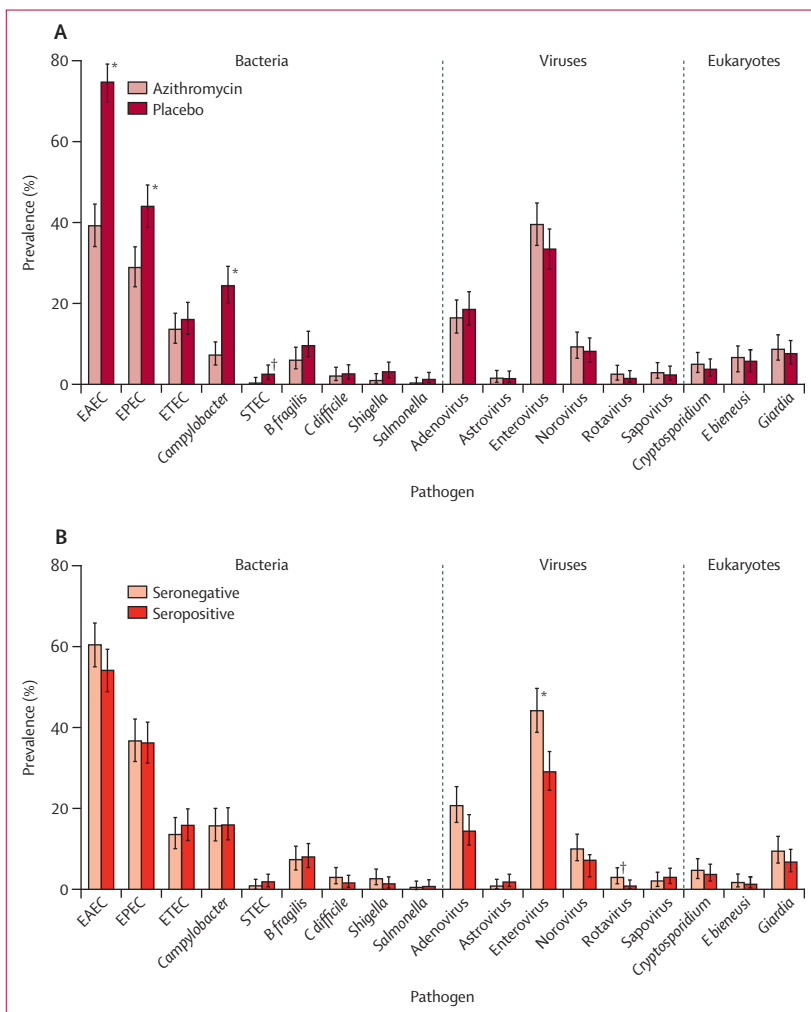


Figure 2: Prevalence of intestinal pathogens at the time of vaccination with oral poliovirus vaccine by study group (A) and by subsequent seroconversion status (B)
 Only pathogens with a prevalence of at least 1% across all samples are shown. Error bars indicate 95% CIs. Exact p values are given in the appendix (p 6,9). 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 bienesi*=*Enterocytozoon bienesi*. * $p<0.01$. † $p<0.05$.

did not seroconvert; Wilcoxon's $p=0.467$, and mean number of eukaryotic pathogens in infants who seroconverted was $0.13 [0.02]$ compared with $0.16 [0.02]$ in those who did not seroconvert; Wilcoxon's $p=0.153$; appendix p 9). 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 p 10, 16).

In stool samples collected on day 14 with enterovirus detected at a PCR cycle threshold value of less than 35 with the TaqMan array card data, only four (1%) of 300 tested were positive for any of the Sabin polioviruses with multiplex real-time PCR, indicating little secondary exposure to oral poliovirus vaccine in our study.

Faecal biomarkers were measured on study day 0 in 292 (100%) and on study day 14 in 291 (99.7%) of 292 infants included in the subset identified for biomarker assessment and who completed the study per protocol. Plasma biomarkers were measured for the same number of participants on study day 14 only. Faecal biomarkers of intestinal inflammation and permeability measured on the day of vaccination (day 14) were significantly lower in 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 groups (day 14). In the longitudinal analysis of faecal biomarkers there was a significant decrease between day 0 and 14 in myeloperoxidase and calprotectin in infants who received azithromycin but not in those who received placebo (appendix p 11). Neopterin showed a significant increase in the azithromycin but not placebo group ($p=0.030$), although this was not reflected in the comparison of this biomarker between study groups on day 14.

Faecal and plasma biomarkers of environmental enteropathy 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 concentrations were lower in infants who seroconverted for all biomarkers with the exception of faecal neopterin. Infants who shed poliovirus 7 days after oral poliovirus vaccine administration also had lower concentrations of faecal and plasma biomarkers of environmental enteropathy and this difference was significant for faecal calprotectin ($p=0.021$) and $\alpha 1$ -antitrypsin (0.044 ; 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 (84 [52%] of 163 vs 63 [49%] of 128; $p=0.724$ for faecal biomarkers and 78 (46%) of 171 vs 69 (58%) of 120; $p=0.057$ for plasma biomarkers). Similarly, seroconversion was not significantly correlated with the faecal biomarker environmental enteropathy score defined by Kosek and colleagues in 2013¹⁵ (odds ratio of seroconversion for each unit increase in environmental enteropathy score $0.93 [95\% \text{ CI } 0.85-1.02; p=0.145]$).

In multivariable log-linear regression analyses, myeloperoxidase ($p=0.005$) and calprotectin ($p=0.001$) measured in stool collected at enrolment were positively correlated with the number of bacteria detected in that stool (appendix p 12). Faecal neopterin was negatively correlated with the number of eukaryotic pathogens (mainly *Giardia* spp and *Cryptosporidium* spp, $p<0.001$). 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. Generally, plasma biomarkers were not correlated with faecal biomarkers (appendix p 13). Faecal biomarkers were positively correlated with one another, although this correlation was abrogated to some extent in the azithromycin group.

Solicited adverse events including cough or cold, diarrhoea, and fever were recorded in 216 (59%) of 367 infants receiving azithromycin and 227 (62%) of 367 receiving placebo between enrolment and vaccination, and in 160 (44%) of 363 and 163 (44%) of

	Study group		p value	Seroconversion			Poliovirus shedding		
	Azithromycin	Placebo		Seroconversion	No seroconversion	p value	Poliovirus shedding	No poliovirus shedding	p value
Stool									
Myeloperoxidase (ng/mL)	15780 (1277)	21251 (1407)	0.001	17425 (1276)	19724 (1445)	0.297	17318 (1216)	20001 (1525)	0.302
Calprotectin ($\mu\text{g/g}$)	862 (65)	1094 (61)	0.001	918 (61)	1043 (67)	0.191	892 (60)	1082 (68)	0.021
Neopterin (nmol/L)	6644 (396)	6934 (423)	0.612	6871 (444)	6710 (372)	0.757	6779 (392)	6806 (431)	0.779
$\alpha 1$ -antitrypsin (mg/g)	1.028 (0.08)	1.269 (0.108)	0.015	1.025 (0.071)	1.279 (0.115)	0.126	1.047 (0.09)	1.27 (0.102)	0.044
Plasma									
Soluble CD14 (ng/mL)	2623 (133)	2750 (142)	0.419	2589 (132)	2788 (143)	0.291	2662 (135)	2716 (141)	0.735
I-FABP (pg/mL)	823 (41)	810 (40)	0.520	770 (35)	863 (45)	0.138	768 (34)	873 (47)	0.091
EndoCAB IgG (MU/mL)	77 (14)	56 (8)	0.832	58 (10)	75 (13)	0.074	57 (9)	77 (14)	0.098

Data are mean (SE). Differences between groups were assessed using Wilcoxon rank sum test.

Table 2: Faecal and plasma biomarkers of environmental enteropathy at the time of vaccination with serotype-3 monovalent oral poliovirus vaccine (day 14) according to study group, seroconversion (day 35), and detection of poliovirus shedding (day 21)

367 for these same groups between vaccination and completing the study. Three serious adverse events were reported (two in the azithromycin group and one in the placebo group), but none was considered related to the study interventions.

Discussion

Treatment with azithromycin reduced the prevalence and abundance of bacterial intestinal pathogens and faecal markers of environmental enteropathy but did not improve seroconversion after a subsequent dose of serotype-3 monovalent oral poliovirus vaccine. Seroconversion 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. Faecal and plasma biomarkers of environmental enteropathy were typically lower in children who seroconverted or shed poliovirus after oral poliovirus vaccine. However, this association was not statistically significant for individual biomarkers or for environmental enteropathy scores based on aggregated biomarker data, with the exception of faecal calprotectin and α 1-antitrypsin that were significantly lower in infants who shed poliovirus (but not in those who seroconverted). Therefore, insofar as we can measure environmental enteropathy, it does not appear to be a major determinant of oral poliovirus vaccine immunogenicity in Indian infants, although it is more common in populations where the immunogenicity of oral vaccines is lower. In fact, environmental enteropathy is thought to develop after repeated exposure to bacterial and viral pathogens, and it may only be exposure to viral pathogens that is mechanistically linked to poor oral poliovirus vaccine immunogenicity.

The number of viral pathogens was significantly lower in infants who seroconverted or shed poliovirus after oral poliovirus vaccine, and this association was especially marked for enteroviruses (figure 2). Infants did not receive oral poliovirus vaccines except through participation in this trial and most (>98%) enteroviruses we detected were non-polio enteroviruses. The association between non-polio enterovirus infection and an inability to seroconvert after oral poliovirus vaccination has been observed in several studies dating back to the earliest human trials of live-attenuated polioviruses and was confirmed in a systematic review and meta-analysis.⁷ We also identified 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 other viral infections and seroconversion to oral poliovirus vaccine has not previously been reported. Immunisation with live-attenuated oral rotavirus vaccine does not appear to significantly lower seroconversion to co-administered

oral poliovirus vaccine, but pre-existing wild-type rotavirus infection might be expected to have greater significance for oral poliovirus vaccine replication.²³

The association between seroconversion and the presence of viral pathogens was apparent for stool collected on the day of vaccination (day 14) but not for stool collected at enrolment (day 0). This finding suggests an effect of concurrent infection on oral poliovirus vaccine immunogenicity that is not sustained after infection is cleared, which would be consistent with an innate antiviral immune response induced by these pathogens that is effective in suppressing oral poliovirus vaccine replication or direct viral interference within infected cells, or both.²⁴ Further studies of innate immune activation in the intestine in human and animal models 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 in infants at enrolment to our study, similarly to concentrations 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 finding might reflect an inherent limitation in the effectiveness of azithromycin against environmental enteropathy or the short course of treatment (3 days). Sustained suppression of inflammation through a longer course of treatment might have allowed epithelial healing and a reduction in microbial translocation and immune activation, the hallmarks of environmental enteropathy.^{13,14}

We are aware of only one other randomised trial of antibiotics on environmental enteropathy, which found no effect of a 7-day course of rifaximin given to Malawian children on intestinal permeability measured with a sugar absorption test (lactulose-to-mannitol ratio in urine).²⁸ This non-absorbed antibiotic has a different antimicrobial spectrum to azithromycin (eg, *Campylobacter* spp are 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 effect of a longer course of azithromycin on environmental enteropathy might 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 susceptible 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 environmental enteropathy might have an important role.

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

We administered oral poliovirus vaccine on day 14 of the study to allow for the resolution of intestinal inflammation following treatment of bacterial pathogens. Reinfection after clearance of azithromycin from mucosal tissues (half-life 2–4 days) might have occurred, diminishing the effect of the 3-day course of treatment on the prevalence and abundance of bacterial pathogens at this time. Nonetheless, the significant effect that we observed and the absence of association between these pathogens and oral poliovirus vaccine 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 have 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 than traditional methods. The biological relevance of a pathogen detected at a high cycle threshold value (low target copy number) might therefore be questionable, and association with a clinically measurable outcome such as gastroenteritis is more common for lower cycle threshold values.²¹ For this reason, we did a quantitative statistical analysis of cycle threshold values in addition to analysis of pathogen prevalence and we found consistent results with both approaches. We were also reliant on molecular biomarkers of environmental enteropathy in stool or plasma and did not do intestinal biopsies in this asymptomatic population to confirm enteropathy. These biomarkers are widely used in studies of environmental enteropathy, capture many related pathological processes, and are used to support diagnosis of enteropathies (eg, calprotectin for inflammatory bowel disease). Nonetheless, tissue samples from the small intestine would have probably provided additional information

about the processes affecting oral poliovirus vaccine 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 oral poliovirus vaccine in low-income countries. Although a 3-day course of azithromycin was effective in achieving these aims, it had no effect on oral poliovirus immunogenicity. Instead, we found that immunogenicity was reduced in the presence of viral pathogens, potentially implicating viral interference or innate antiviral immune mechanisms, or both. An understanding of these mechanisms and the factors that determine viral infection in the intestine in early infancy might help in the design of new oral vaccines and vaccine schedules that are more effective in low-income countries.

Contributors

NCG, SBah, JJ, 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

HHU declares unrelated collaborations with Eli Lilly, UCB Pharma, and Vertex Pharmaceuticals and travel support from Actelion and Merck Sharp & Dohme. MI-G received support from an Institutional Strategic Support Fund Wellcome Trust grant awarded to the University of Liverpool and the Health Protection Research Unit in Gastrointestinal Infections, National Institute for Health Research at the University of Liverpool (grant number NIHR HPRU 2012-10038). All other authors declare no competing interests. The views expressed are those of the author(s) and do not necessarily represent those of the National Health Service, the National Institute for Health Research, the Department of Health, or Public Health England. SBah is a staff member of the World Health Organization and he alone is responsible for the views expressed in this publication that do not necessarily represent the decisions or policies of the World Health Organization.

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