Influence of non-polio enteroviruses and the bacterial gut microbiota on oral poliovirus vaccine response: a study from south India

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Brief summary: Non-polio enteroviruses have a significant impact on oral poliovirus vaccine sero-response irrespective of infecting serotype, while the bacterial gut microbiota does not affect OPV sero-response significantly.

Footnote page

Conflict of interest statement

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Abstract

Background: Oral poliovirus vaccine (OPV) is less immunogenic in LMIC countries. We tested whether bacterial and viral components of the intestinal microbiota are associated with this phenomenon.

Methods: We assessed prevalence of enteropathogens using TaqMan array cards 14 days before and at vaccination in 704 Indian infants (6-11 months) receiving monovalent type3 OPV (CTRI/2014/05/004588). Non-polio enterovirus (NPEV) serotypes were identified by VP1 sequencing. In 120 infants, pre-vaccination bacterial microbiota was characterised by 16S rRNA sequencing.

Results: We detected 56 NPEV serotypes on the day of vaccination. Concurrent NPEVs were associated with a reduction in OPV seroconversion, consistent across species (odds ratios and 95% CIs of 0·57[0·36–0·90], 0·61[0·43–0·86], and 0·69[0·41–1·16] for species A, B, and C, respectively). Recently acquired enterovirus infections,detected at vaccination, but not 14 days earlier had greater interfering effect on mOPV3 sero-response compared to persistent infections,with enterovirus detected at both time points (44/127[35%] vs 63/129[49%] seroconversion,p=0.021). Abundance of specific bacterial taxa did not differ significantly according to OPV response, although microbiota diversity was higher in non-responders at the time of vaccination.

Conclusion: Enteric viruses have greater impact on OPV response than the bacterial microbiota with recent enterovirus infections having greater inhibitory effect than persistent infections.

Key words: Non-polio enteroviruses, bacterial microbiota, 16S rRNA, OPV, next generation sequencing

Background

Oral vaccines have consistently proven to be less immunogenic in low- and middle-income countries (LMICs) than high-income countries[1-3]. Several mechanisms may contribute to this phenomenon, including maternal factors (e.g. transplacental antibodies), heritable factors (e.g. genes determining histo-blood group antigen structure), and environmental factors (e.g. enteric pathogen exposure) [4]. The presence of asymptomatic enteric viruses – particularly non-polio enteroviruses (NPEVs) – has consistently been linked with a reduction in the immunogenicity of oral poliovirus vaccine (OPV)[5], potentially reflecting competition between viruses at the cellular level[6], activation of innate antiviral immune pathways that inhibit OPV replication, or changes in lymphocyte responsiveness to poliovirus antigen. Notably, more than 100 enterovirus serotypes are known to infect humans. These can be separated into four distinct species (A–D) based on the sequence of the VP1 gene (one of the major determinants of antigenicity), of which polioviruses fall within species C[7;8]. However, the relative inhibitory effect of different NPEV species or serotypes has not been investigated in any detail.

Other components of the gut microbiota may also be pertinent to OPV outcome. Enteric viruses appear to exploit signals from the bacterial microbiota when colonising the intestinal mucosa[9]. Among infants in Bangladesh, *Bifidobacterium* abundance at the time of OPV administration correlated with poliovirus-specific IgG and CD4+ T-cell responses[10]. However, the potential influence of microbiota composition on OPV replication and neutralising antibody levels remains uncertain.

During a recent clinical trial in India, the presence of enteric viruses (of which the majority were NPEVs) was associated with reduced seroconversion to monovalent type 3 OPV (mOPV3)[11]. Here, we report on a follow-up study in which we tested whether OPV

response was associated with specific enterovirus serotypes or species, short-term changes in enteric virus burden, or bacterial microbiota composition.

Methods

Study design

Between 5 August 2014 and 21 March 2015, we carried out a randomised, placebo-controlled trial evaluating the effect of azithromycin on the immunogenicity of mOPV3 among 6–11-month-old Indian infants (CTRI/2014/05/004588). The protocol and primary outcomes of this study have been published[11]. Briefly, 754 infants lacking serum neutralising antibodies to type 3 poliovirus were randomised 1:1 to receive a 3-day course of oral azithromycin (administered once daily at a dose of 10 mg/kg) or placebo, starting 14 days before the administration of a single dose of mOPV3. The study was approved by the institutional review board of the Christian Medical College, Vellore, India, and good clinical practice guidelines were followed throughout.

Laboratory testing and bioinformatics

Enteropathogen detection. Stool samples were collected from all infants before treatment (day -14) and on the day of vaccination (day 0) and tested for the presence of 40 different enteric bacterial, viral, and eukaryotic targets using Taqman array cards (TACs), as previously described (figure 1A)[11]. A cycle threshold (Ct) value of 30 was used as a universal cut-off for pathogen detection. In all day 0 samples that were positive for enteroviruses in the TAC assay (targeting the 5' UTR), we carried out a semi-nested PCR assay targeting the VP1 gene[12]. Sanger sequencing of the amplified products was performed on a 3730XL DNA Analyzer and enterovirus serotypes were assigned using the RIVM Enterovirus Genotyping Tool (version 1.0)[13].

Characterisation of the bacterial microbiota. We assessed bacterial microbiota composition in 120 infants (60 per study arm) selected at random from the first 300 trial participants. Our laboratory and bioinformatic methods for microbiota assessment in this cohort have been published[14]. For each infant, we included samples collected before treatment (day -14) and before vaccination (day 0). We also assessed samples from 40 adults living with trial participants to provide community-specific mature microbiota profiles (used as a reference for calculating microbiota age). Following amplification and sequencing of the 16S rRNA gene V4 region via Illumina MiSeq, reads were assembled with FLASH, clustered *de novo* into operational taxonomic units (OTUs) using uclust (implemented in MacQIIME version 1.8.0), and taxonomically assigned using the RDP classifier.

OPV outcome. Serum samples collected 21 days after mOPV3 administration were tested for neutralising antibodies against type 3 poliovirus using a modified microneutralization assay with two-fold serial dilutions ranging from 1:4 to 1:512[15]. Seroconversion was defined as the detection of neutralising antibodies at a dilution of ≥1:8. In a subset of 300 infants, shedding of type 3 poliovirus was assessed in stool samples collected 7 days after vaccination using real-time PCR[16].

Statistical analysis

Enteropathogen burden. We included all per-protocol infants with available day 0 TAC data in the final analysis (n=704). The association between each enterovirus species and OPV response (seroconversion or shedding as categorical dependent variables) was assessed by logistic regression. Age and study arm were included as covariates to account for the potential confounding of these variables with viral infection status and OPV outcome. We fitted univariate models for each species in turn and multivariate models that included all three species. Separate models were fitted for individual serotypes present in at least 1% of infants. Fisher's exact test was used to compare the prevalence of each enterovirus species by

study arm. To examine whether mixed infections had an additive effect on OPV immunogenicity, we assessed the effect of enterovirus species count (as a categorical variable) on the odds of seroconversion. For each species in turn, we also compared the likelihood of heterospecific co-infection (≥1 enterovirus of another species) in infected versus uninfected infants using Fisher's exact test.

To test whether the association between infection and OPV response varied across enterovirus species, we used the likelihood ratio test (LRT) to compare the fit of logistic regression models that only included presence or absence of any enterovirus with models that included enterovirus species as a categorical variable. Where multiple species were detected, infections were classified as 'mixed', whilst untypeable infections were classified as 'unassigned'.

To examine whether changes in the burden of viral TAC targets (adenovirus, astrovirus, enterovirus, norovirus, rotavirus and sapovirus) in the 14 days preceding immunisation influenced OPV outcome, we classified viruses as absent (not detected on days -14 or 0), resolved (present on day -14, absent on day 0), recently acquired (absent on day -14, present on day 0), or persistent (present on days -14 and 0) – hereafter referred to as 'infection subclasses'. The impact of infection subclasses on OPV response was assessed by logistic regression, with age and study arm included as covariates. Infection subclasses were included if they contained at least ten infants. We directly compared OPV outcome between infants with recently acquired versus persistent infections using Fisher's exact test; if a significant discrepancy was observed, we assessed whether this was associated with a difference in pathogen abundance (based on TAC Ct value) using Wilcoxon's rank sum test. Analyses were implemented in the programming language R[17], and associations with a p value of <0·05 were considered statistically significant.

Microbiota composition. Among per-protocol infants in the microbiota subset, we compared baseline health and demographic characteristics by seroconversion status using Wilcoxon's rank sum test (for continuous variables) or Fisher's exact test (for categorical variables). We obtained a minimum of 7,708 sequences per sample after bioinformatic processing. To standardise sequencing depth, analyses were performed using 7,500 sequences per sample. Microbiota measures of interest included alpha diversity, beta diversity, microbiota stability, microbiota age, and relative taxon abundance. These were compared according to OPV outcome using Wilcoxon's rank sum test (for comparisons of taxon abundance), linear regression (for alpha diversity, microbiota stability, and microbiota age), or Adonis (for beta diversity), adjusting for infant age and study arm where possible. Adonis is a permutation-based analysis of variance. To account for multiple comparisons, p values for relative abundance comparisons were adjusted by Benjamini–Hochberg false discovery rate (FDR) correction[18], applied separately at each taxonomic rank. The association between alpha diversity and enterovirus infection subclass was assessed via linear regression. We also used the machine-learning algorithm Random Forests to predict OPV outcome based on OTU relative abundances. For each model, we carried out 20 cycles of 10-fold cross-validation using the R package *crossval*, with 5,000 trees per forest. Out-of-bag error rates and variable importance scores (based on Gini impurity) were determined across each of the 200 iterations of cross-validation. Sensitivity analyses of alpha and beta diversity were carried out in which azithromycin recipients were excluded.

Data availability. 16S rRNA sequences have been deposited in the European Nucleotide Archive (accession number PRJEB20773). An OTU table and relevant metadata have previously been published[14].

Role of the funding source

The funder had no role in the 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

NPEV burden

Enteroviruses were present at the time of vaccination (day 0) in 107/367 (29%) infants who seroconverted and 149/337 (44%) infants who failed to seroconvert (odds ratio [OR] 0·49, 95% CI 0·35–0·67). Based on partial sequencing of the VP1 gene, species-level assignments were made in 247 (96%) of the 256 enterovirus-positive samples and serotype-level assignments were made in 234 (91%). Overall, we detected 56 NPEV serotypes, of which 11 were present in at least 1% of the study population (figure 1B). Enteroviruses of species A, B, and C were detected in 90 (13%), 184 (26%), and 65 (9%) infants, respectively. Viruses in species D were absent. The receipt of azithromycin did not significantly impact the prevalence of any enterovirus species (figure S1). Infection with multiple enterovirus species was common – 76/704 (11%) infants harboured two species, while 8/704 (1%) were infected with three (figure S2). Moreover, the detection of any one enterovirus species was a significant risk factor for the presence of another (Table 1).

Association between NPEVs and OPV response

A negative association between NPEV detection and seroconversion to OPV3 was observed for each enterovirus species (ORs of 0·59 [0·37–0·93], 0·62 [0·44–0·87], and 0·67 [0·40–1·13] for species A, B, and C, respectively, after adjusting for age and study arm; figure 1C). Although this effect was significant only for species A and B, there was no evidence that the effect size differed significantly between species (LRT, p=0·192). ORs were generally consistent in a multivariate model that included all species (Table S1), and the presence of

multiple species did not have an additive inhibitory effect on seroconversion (ORs of 0·45 [0·31–0·65], 0·67 [0·41–1·09], and 0·43 [0·09–1·80] for infants infected with one, two, or three species, respectively). With the exception of echovirus 14, associations between individual serotypes and seroconversion were not significant (Table S2). Consistent with the data for seroconversion status, the association between concurrent NPEVs and post-vaccination shedding did not vary significantly by species (LRT, p=0·218; Table S1).

Recently acquired, persistent, and resolved viruses

To investigate the relationship between recent changes in viral infection status and OPV response, we classified viruses as recently acquired, persistent, and resolved (Table 2). "Recently acquired" enteroviruses were the only infection subclass to be significantly associated with OPV response (OR 0·38 [0·25–0·59], p<0·001). Notably, the seroconversion rate was significantly lower among infants with recently acquired as opposed to persistent enteroviruses (44/127 [35%] vs 63/129 [49%]; Fisher's test, p=0·023). The Ct values of persistent versus recently acquired enterovirus infections on day 0 did not differ significantly (27·0 ± 2·2 [mean ± standard deviation] vs 27·2 ± 2·1; Wilcoxon's rank sum test, p=0·475), suggesting that the difference in immunogenicity between these groups was not driven by viral copy number.

The odds of OPV shedding were reduced in infants with either recently acquired or persistent enteroviruses on day 0 (ORs of 0·55 [0·28–1·08] and 0·47 [0·25–0·91], respectively). In contrast to the immunogenicity data, shedding rates did not significantly differ between these groups (25/53 [47%] vs 25/58 [43%] for recently acquired vs persistent enteroviruses; Fisher's test, p=1·000). No other viral infection subclasses were significantly associated with vaccine virus shedding (Table S3).

Association between bacterial microbiota composition and OPV response

Of the 120 infants included in the microbiota subset, 114 (95%) completed the study per protocol. The effects of azithromycin on microbiota composition in these infants, including a reduction in the abundance of Proteobacteria and Verrucomicrobia, have previously been reported[14]. Baseline health and socio-demographic characteristics were generally comparable between OPV responders and non-responders in the microbiota subset (Table S4), although failure to seroconvert was associated with a lower height-for-age Z score at enrolment (day -14) – a discrepancy not apparent in the trial population as a whole[11].

We did not observe a strong correlation between composition of the bacterial microbiota at the time of vaccination (day 0) and OPV immunogenicity (Figure 2; Table 3). Unifrac distance from adults (an indicator of increased microbiota age) was lower in non-responders compared with responders (Table 3), while seroconversion status accounted for a significant but small proportion of variance among samples based on Unifrac distances (adonis, p=0·031, R^2=0·013; Table 3; Figure 2C). We did not observe significant differences in OTU count, Shannon index, microbiota stability, or relative taxon abundances according to seroconversion status (Table 3; Figure 2B). Random Forest models based on OTU abundance data failed to accurately distinguish infants according to OPV outcome (figure 2D).

Similar results were obtained when comparing individuals according to vaccine shedding status (Table 3). In contrast to the immunogenicity data, however, OTU count and Shannon index were significantly higher among non-shedders than shedders, and the class Clostridia was enriched in non-shedders (relative abundance, 11·6 ± 10·2% vs 5·6 ± 7·7%; Wilcoxon's rank sum test, FDR-corrected p=0·044; Table S5). Neither OTU count nor Shannon index varied significantly according to enterovirus infection subclass (LRT, p values >0·05; Figure S3), suggesting that the observed discrepancies in alpha diversity according to OPV outcome were not related to NPEV infection.

We observed no significant differences in microbiota composition according to OPV outcome in baseline (day -14) samples (Table S6). Moreover, the primary outcomes were largely unaffected when infants in the azithromycin arm were excluded (Table S6).

Discussion

The notion that concurrent enteroviruses may interfere with OPV immunogenicity dates back to the earliest trials of this vaccine[5]. Aside from a handful of relatively small association studies[19;20], however, the extent to which this effect varies among enterovirus species or serotypes has not been tested. In a large cohort of Indian infants who received a single dose of mOPV3, we observed a significant inhibitory effect of concurrent NPEVs on OPV response. Moreover, we found this effect to be consistent across enterovirus species and enhanced when infection with these viruses was recently acquired.

This study is among the first to characterise NPEV burden in asymptomatic infants living in a LMIC. Our findings highlight not only the high prevalence of NPEVs in this setting (35%), but their considerable diversity. We observed a total of 56 different NPEV serotypes, of which Coxsackievirus A6 – one of the major serotypes implicated in clinical conditions such as hand-foot-and-mouth disease[21] – was the most common. Two or more enterovirus species were detected in 11% of infants, and the presence of any one species was associated with an increased likelihood of hetero-specific co-infection, suggesting that shared risk factors (e.g. poor sanitation and hygiene) prompt multiple NPEV exposures. Several previous studies have employed cell culture to characterise the burden of NPEVs among children presenting with acute flaccid paralysis. For example, NPEVs were documented in 15% of cases in West Africa[22] and 19% in south-western India[23]. The higher prevalence of

NPEVs reported here among asymptomatic infants may reflect the enhanced sensitivity of PCR- versus culture-based detection methods[24].

Of the 56 NPEV serotypes observed in this population, 45 were present in <1% of infants, limiting our capacity to compare the effect of individual serotypes on OPV. The consistent species-level associations suggest that evolutionary (and antigenic) distance from poliovirus – a species C enterovirus – does not influence the outcomes of concurrent infection. Likewise, in cell culture, numerous NPEV serotypes have been shown to interfere with poliovirus replication[6]. Notably, enteroviruses exhibit long-term cycles in transmission that vary across serotypes – whereas some strains show regular annual peaks, others cause epidemics once every few years[25]. Our study was performed between August and March – a period typically associated with reduced NPEV transmission across India[26]. It is possible that distinct serotype-specific effects on OPV will only be apparent in particular seasons or years. Crucially, our findings are consistent with reports of seasonal variation in OPV immunogenicity[26] and suggest that planning OPV campaigns at times of year when enterovirus transmission is low may improve vaccine response.

By considering viral infection burden at multiple time points before vaccination, we observed a reduction in OPV immunogenicity in infants with newly acquired as opposed to persistent enterovirus infections. This finding should be interpreted with caution given its modest effect size and the fact that the discrepancy was absent when considering OPV shedding. Nonetheless, the possibility that recently acquired viruses may have an enhanced inhibitory effect on OPV response is plausible. Viral infections initiate a cascade of innate immune effectors that typically peak within several days of exposure[27]. Meanwhile, enteroviruses interfere with protein synthesis and secretion pathways to repress host interferon responses[28]. If the induction of innate antiviral immunity is responsible for the inhibitory effect of enteroviruses on OPV, the greater effect of recently acquired NPEVs compared to

persistent infections could be due to the waning and/or repression of innate antiviral responses over time.

Intestinal viruses other than enteroviruses may also interfere with OPV. During the primary analysis of this cohort, rotaviruses and adenoviruses were associated with a reduction in OPV immunogenicity[11]. The same trends were evident here; however, the statistical power of individual comparisons was reduced by the distinction between persistent and recently acquired infection subclasses. Despite the potential inhibitory effect of other viruses, the considerably higher prevalence of enteroviruses ensures that this genus is likely to account for a much greater fraction of OPV failure.

In addition to the effect of intestinal viruses, dysbiosis of the bacterial microbiota has been proposed as a possible cause of impaired oral vaccine performance in LMICs[29;30]. Although lacking a consistent definition, dysbiosis has typically been linked with a reduction in microbiota diversity and a detectable shift (or 'perturbation') in overall composition[31]. In this study, microbiota diversity was negatively correlated with OPV shedding. Given the modest effect size of the observed discrepancies and the fact that several previous studies have observed no such correlation[10;32;33], it appears unlikely that increased microbiota diversity represents a significant risk factor for vaccine failure, although it may be a marker for exposure to enteric infections. Other discrepancies in microbiota composition with respect to OPV response were modest, as exemplified by the inability of Random Forests to accurately distinguish responders from non-responders based on OTU abundance data. Thus, non-responders did not exhibit any clear manifestations of microbiota dysbiosis.

Several other studies have looked at the possible effect of the bacterial microbiota on oral vaccine outcome. Among infants in Bangladesh, OPV response was positively correlated with *Bifidobacterium* abundance[10]. In Ghana, oral rotavirus vaccine immunogenicity was associated with an increased abundance of *Streptococcus bovis* and decreased abundance of

the phylum Bacteroidetes[32], although no such discrepancies were apparent during a rotavirus vaccine study in Vellore, India[33]. Here, we did not identify any OTUs or genera that were significantly associated with OPV response. Although abundance of the class Clostridia was negatively correlated with vaccine shedding, given that older infants were less likely to respond to OPV[11], the observed discrepancy likely reflects the confounding of Clostridia abundance with age[14]. Differences in infant age, vaccine, laboratory methods, trial setting, and immunogenicity measure may all have contributed to the discrepancies in findings among studies reporting on the association between bacterial microbiota composition and oral vaccine response. For now, reproducible signatures of oral vaccine failure within the bacterial microbiota remain elusive.

Our study has several limitations. Biases in amplification efficiency may have undermined our characterisation of the bacterial microbiota[34], while a focus on relative rather than absolute taxon abundance (accounting for variation in total microbial load) may have obscured potentially relevant associations[14;35]. By applying a Ct cut-off of 30 for pathogen targets during the TAC assays, we may have failed to characterise the effect of enteric viruses present at low abundance. Finally, during our analysis of infection subclasses, it is possible that the replacement of one virus with another would be mistakenly categorised as a persistent infection as we did not determine enterovirus serotype in samples collected 14 days prior to the vaccination.

In conclusion, we did not observe any signs of bacterial microbiota dysbiosis among infants in India who failed to respond to mOPV3. The presence of NPEVs was associated with a lower response to OPV that was consistent across enterovirus species, and recently acquired enteroviruses appeared to inhibit OPV immunogenicity more than persistent infections. Although these findings do not preclude a role for more entrenched risk factors of OPV failure, such as the chronic inflammation associated with environmental enteropathy, they

suggest that the likelihood of responding to OPV may fluctuate from week to week and are consistent with seasonal trends in OPV immunogenicity that may reflect the abundance of NPEVs.

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Declaration of interests

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Table 1. Co-occurrence of enterovirus species.

Subset	Prevalence of ≥1 heterotypic EV
EV-A positive	59/90 (65·6)
EV-A negative	157/614 (25·6)
EV-B positive	65/184 (35·3)
EV-B negative	63/520 (12·1)
EV-C positive	52/65 (80·0)
EV-C negative	182/639 (28·5)

Data are n/N (%). P values for all comparisons <0·0001 (Fisher's exact test). EV, enterovirus.

Table 2. Association between viral infection subclasses and OPV3 seroconversion.

Pathogen	Infection status	N	Seropositive, n (%)	OR (95% CI)	p	p $^{Rec\ vs\ Pe}$
Adenovirus	Absent	507	269 (53·1)			
	Resolved	74	45 (60·8)	1·36 (0·83–2·27)	0·227	
	Recently acquired	88	40 (45·5)	0·73 (0·46–1·16)	0·187	
	Persistent	35	13 (37·1)	0·53 (0·26–1·07)	0·083	0·427
Astrovirus	Absent	684	355 (51·9)			
	Resolved	10	5 (50·0)	1·10 (0·30–4·05)	0·886	
	Recently acquired	10	7 (70·0)	2·30 (0·58–10·81)	0·235	
	Persistent	0	-	-	-	-
Enterovirus	Absent	313	178 (56·9)			
	Resolved	135	82 (60·7)	1·24 (0·82–1·90)	0·304	
	Recently acquired	127	44 (34·6)	0·38 (0·25–0·59)	<0·001	
	Persistent	129	63 (48·8)	0·70 (0·46–1·06)	0·091	0·023
Norovirus	Absent	604	318 (52·6)			
	Resolved	39	22 (56·4)	1·18 (0·61–2·32)	0·616	

	Recently acquired	46	20 (43·5)	0·70 (0·38–1·28)	0·246	
	Persistent	15	7 (46·7)	0·67 (0·23–1·89)	0·440	1·000
Rotavirus	Absent	685	362 (52·8)			
	Resolved	6	2 (33·3)	-	-	
	Recently acquired	12	3 (25·0)	0·34 (0·07–1·16)	0·109	
	Persistent	1	0 (0·0)	-	-	-
Sapovirus	Absent	671	348 (51·9)			
	Resolved	15	8 (53·3)	1·11 (0·39–3·23)	0·844	
	Recently acquired	16	9 (56·2)	1·30 (0·47–3·7)	0·615	
	Persistent	2	2 (100·0)	-	-	-

Age and study arm were included as covariates in all logistic regression models. CI, confidence interval; Rec, Recently acquired; OR, odds ratio; Pe, persistent.

Table 3. Association between bacterial microbiota comparison at the time of vaccination (day 0) and oral poliovirus vaccine response.

				Seroconversion			Shedding		
Comparison	Description	Test	Age/arm as covariates	Seropositive (n=62)[a]	Seronegative (n=52)	p	Shedders (n=42)	Non-shedders (n=33)	p
Alpha diversity	Number of OTU	LR	yes	68·8 ± 14·1	73·4 ± 15·3	0·198	65·4 ± 14·5	72·0 ± 16·4	0·034
	Shannon index	LR	yes	2·62 ± 0·47	2·82 ± 0·55	0·077	2·57 ± 0·50	2·84 ± 0·59	0·032
Beta diversity	Unifrac distances between samples	adonis	Yes	R^2=0·013		0·036	R^2=0·025		0·007
Microbiota age	Mean Unifrac distance from samples collected from non-cohabiting adults	LR	Yes	0·833 ± 0·042	0·811 ± 0·050	0·013	0·838 ± 0·046	0·806 ± 0·052	0·001
Microbiota stability	Unifrac distance between day 0 and 14	LR	yes	0·470 ± 0·071	0·460 ± 0·085	0·699	0·476 ± 0·078	0·455 ± 0·088	0·196
Taxon abundance	Phylum-, class-, genus-, and OTU-level relative abundances	WRS	no[c]	No discrepancies with FDR p<0·15			Clostridia enriched in non-shedders[b]		
Other	Cross-validation accuracy of Random Forests	-	no[c]	Median 54·5% (IQR 45·5–63·6%; baseline accuracy[d]: 54·5%)			Median 62·5% (IQR 50·0–75·0%; baseline accuracy[d]: 56·0%)		

Data are mean ± standard deviation unless otherwise stated. FDR, adjusted for false discovery rate; IQR, interquartile range; LR, linear regression; OTU, operational taxonomic unit; WRS, Wilcoxon rank sum.

[a] 120 infants were included in the microbiota subset, of which 114 completed the study per protocol and were included in the final analyses.

[b] See table S5 for full results.

[c] Whilst age and study arm could be included as covariates when applying linear regression or adonis, it was not possible to adjust for these variables when applying the Wilcoxon rank sum test (a non-parametric test) or Random Forests.

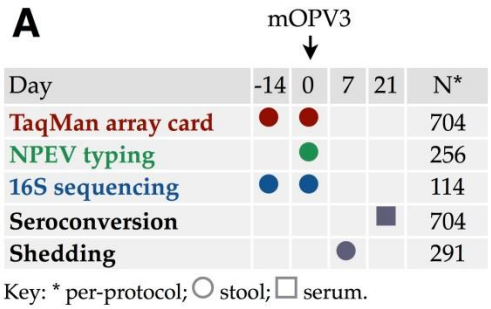
[d] Expected accuracy if all individuals are assigned to the majority class.

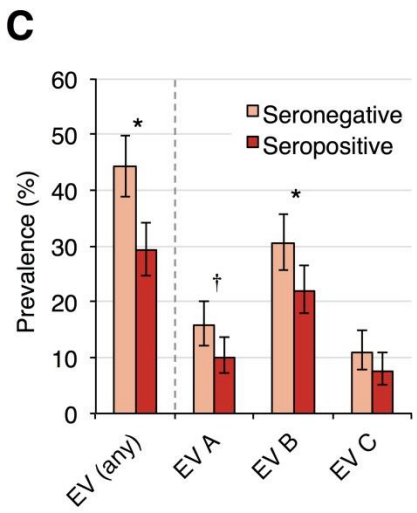
Figure legends

Figure 1. Association between non-polio enteroviruses and seroconversion. (A) Study design. **(B)** Enterovirus serotypes with a prevalence of at least 1%. The diameter of the circle at each branch tip is proportional to serotype prevalence. Phylogenetic relationships are as described by Oberste and colleagues.[7] Branch lengths are not proportional to phylogenetic distance. **(C)** Enterovirus prevalence by seroconversion status. Error bars indicate 95% CIs. CV, coxsackievirus; E, echovirus; EV, enterovirus; mOPV3, monovalent type 3 oral poliovirus vaccine; NPEV, non-polio enterovirus; * p<0·01; † p<0·05.

Figure 2. Association between microbiota composition and seroconversion. (A) OTU count and Shannon index (mean ± standard error). **(B)** Class-level composition of the bacterial microbiota. **(C)** Unweighted Unifrac distances between samples, visualised by principal coordinates analysis. Mean values for each principal coordinate are indicated by dotted lines. **(D)** Cross-validation accuracy of Random Forest models based on OTU abundance data (median and inter-quartile range). The baseline accuracy is the expected accuracy if all individuals are assigned to majority class. OTU, operational taxonomic unit; PC, principal coordinate; * p<0·05.

Figure 1





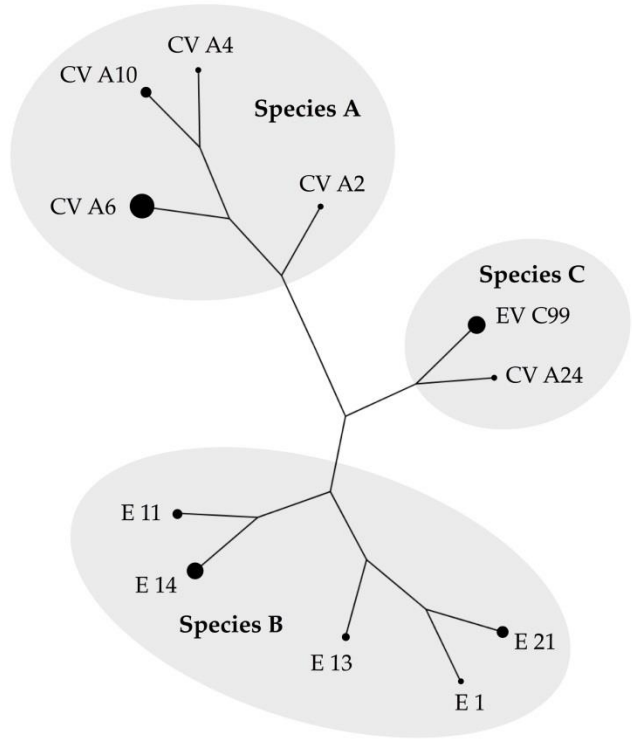


Figure 2