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Influence of the intestinal microbiota on the immunogenicity of oral rotavirus vaccine given to infants in south India

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ABSTRACT

Oral rotavirus vaccines have consistently proven to be less immunogenic among infants in developing countries. Discrepancies in the intestinal microbiota, including a greater burden of enteropathogens and an altered commensal community composition, may contribute to this trend by inhibiting the replication of vaccine viruses. To test this possibility, we performed a nested case–control study in Vellore, India, in which we compared the intestinal microbiota of infants who responded serologically or not after two doses of Rotarix delivered at 6 and 10 weeks of age as part of a clinical trial (CTRI/2012/05/002677). The prevalence of 40 bacterial, viral, and eukaryotic pathogen targets was assessed in pre-vaccination stool samples from 325 infants using singleplex real-time PCR on a Taqman array card (TAC). In a subset of 170 infants, we assessed bacterial microbiota composition by sequencing the 16S rRNA gene V4 region. Contrary to expectations, responders were more likely than non-responders to harbor ≥1 bacterial enteropathogen at dose 1 (26% [40/156] vs 13% [21/157] of infants with TAC results who completed the study per protocol; χ^2, $P = .006$), although this was not apparent at dose 2 (24% [38/158] vs 23% [36/158]; $P = .790$). Rotavirus shedding after dose 1 was negatively correlated with the replication of co-administered oral poliovirus vaccine (OPV). We observed no consistent differences in composition or diversity of the 16S bacterial microbiota according to serological response, although rotavirus shedding was associated with slightly more bacterial taxa pre-vaccination. Overall, our findings demonstrate an inhibitory effect of co-administered OPV on the first dose of Rotarix, consistent with previous studies, but in the context of OPV co-administration we did not find a strong association between other components of the intestinal microbiota at the time of vaccination and Rotarix immunogenicity.

1. Introduction

Each year, an estimated 215,000 children die of severe gastroenteritis associated with rotavirus infection, including between 47,000 and 79,000 in India [1,2]. Although two internationally-licensed oral rotavirus vaccines, Rotarix (RV1) and RotaTeq, are currently available, their efficacy is impaired in low-income countries [3]. Mechanisms responsible for this phenomenon remain uncertain, but may include maternal antibodies, histo blood group antigen phenotype, malnutrition, environmental enteropathy, and interference by enteric infections [4–7]. In a systematic review of oral poliovirus vaccine (OPV) trials, we observed a reduction in the odds of seroconversion and vaccine virus shedding among individuals infected with non-polio enteroviruses (NPEVs) [8]. Similarly, during a recent study in Bangladesh, enterovirus quantity at the time of immunization was negatively correlated with the immunogenicity of both OPV and RV1 [9].

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The composition of the bacterial microbiota may also shape response to oral vaccines. Viruses exploit microbiota-derived compounds to replicate efficiently in the intestinal mucosa, as evidenced by the reduced pathogenicity of poliovirus and rotavirus in antibiotic-treated mice [10,11]. Significant geographic variation occurs in the composition of the infant microbiota [12,13], which may in turn contribute to discrepancies in vaccine performance.

We carried out a nested case–control study among infants enrolled in a clinical trial of RV1 immunogenicity in India [14]. Herein, we tested the hypothesis that failure to seroconvert would be associated with an elevated pathogen burden and an altered bacterial microbiota composition.

2. Materials and Methods

2.1. Study population

Full details of the study design, laboratory procedures, and statistical analyses are provided in the Supplementary Methods. Samples were obtained from a randomized, placebo-controlled trial assessing the impact of daily supplements of zinc and/or probiotics (*Lactobacillus rhamnosus* GG) on the immunogenicity of RV1 and OPV doses co-administered at 6 and 10 weeks of age [14]. The trial was performed in Chinnallapuram, a densely populated urban area in Vellore, India [15]. Infants were considered eligible for enrollment if they were between 35 and 41 days of age, weighed at least 3.2 kg, were available for the duration of the follow-up period, and had no medical conditions that precluded involvement. Written informed consent was obtained from parents or guardians prior to recruitment. Infants received routine vaccines according to the national schedule in India, including OPV at birth, but were excluded if they had received any other doses of OPV or rotavirus vaccine.

Serum anti-rotavirus VP6 IgA antibodies were measured at 6 and 14 weeks of age using an antibody-sandwich enzyme immunoassay [16]. Rotavirus seroconversion was defined as a four-fold increase in anti-VP6 IgA concentration or detection of antibodies at $\geq$20 U/ml in previously seronegative infants. Hereafter, we refer to infants who seroconverted to rotavirus as responders and infants who failed to seroconvert as non-responders.

Following completion of the trial, we conducted a nested case–control study to assess the association between enteropathogens and RV1 response. Infants were considered eligible for the study if they received supplements or placebo, received scheduled doses of OPV and RV1, and provided paired serum samples. To meet sample size requirements (Supplementary Methods), we analyzed stool samples from all responders, subject to constraints in sample availability (n = 162). We randomly selected an approximately equal number of non-responders from each study arm (n = 163) to account for the potential confounding of treatment group with enteropathogen burden. Baseline characteristics were comparable between responders and non-responders (Table 1).

In a subset of 170 infants that had been assessed for enteropathogen burden (including 85 responders), we sequenced the 16S rRNA gene V4 region in stool samples collected before each RV1 dose to assess the intestinal bacterial microbiota. For this microbiota subset we preferentially sampled recipients of placebo-only and probiotics-only, enabling us to assess the effect of probiotics on microbiota composition as a secondary objective.

2.2. Enteropathogen testing by TaqMan array card

Stool samples were obtained on the day of or preceding each vaccine dose. These were kept at room temperature until collection (which typically occurred within 4 h), transported in cold boxes to the laboratory, then stored at −70 °C until testing, with up to two intervening freeze–thaw cycles for aliquoting. We extracted DNA and RNA from 200 mg of the 6- and 10-week pre-vaccination stools from each infant and assessed the presence of 40 enteropathogen targets via real-time reverse transcription PCR (RT-PCR) using TaqMan array cards (TACs) [17,18]. A threshold cycle (Ct) value of 35 was used as a cut-off for pathogen detection [17]. Enterovirus-positive samples were assessed for the presence of Sabin poliovirus using multiplex RT-PCR [19]. To assess RV1 replication (or 'take'), we quantified rotavirus shedding in samples collected pre-vaccination (indicative of natural rotavirus exposure) and 4 and 7 days after the 6-week dose using a VP6-specific real-time RT-PCR assay [20,21].

2.3. Characterization of the intestinal microbiota by 16S rRNA gene sequencing

Our laboratory and bioinformatic pipelines for assessment of the bacterial microbiota have previously been described [22]. We amplified the 16S rRNA gene V4 region using primers 515F (5′-GTGCCAGCAGCCGCGGTAA-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′) in DNA extracted from stool samples collected at 6 and 10 weeks of age in each infant. Purified PCR products were sequenced in two Illumina MiSeq runs (2 × 151 bp) [23]. Reads were assembled using FLASH [24] and analyzed using QIIME (MacQIIME version 1.8.0) [25]. After quality filtering [26] and chimera removal, sequences were clustered *de novo* into operational taxonomic units (OTUs) with $\geq$97% nucleotide identity using uclust and taxonomically assigned using the RDP classifier [27].

2.4. Statistical analysis

2.4.1. Enteropathogen burden

Analyses were performed on infants who completed the study per protocol (as defined by Lazarus et al. [14]). Our primary outcome was the association between rotavirus seroconversion and the presence of $\geq$1 enteropathogen at 6 or 10 weeks of age, as determined via logistic regression. We excluded enteroaggregative *Escherichia coli* (EAEC) from the primary outcome analysis based on the high prevalence of this target in an interim analysis and its limited association with diarrhea during previous studies in resource-poor settings using TACs [28], and enteroviruses given that they may reflect replication of OPV rather than natural enteropathogen exposure.

As secondary outcomes, we compared the prevalence of individual pathogens, pathogen groups (bacterial, viral, eukaryotic, or any), mixed infections (defined as >1 enteropathogen), Sabin viruses, and concurrent diarrhea (defined as $\geq$3 loose stools in a 24-h period within the 7 days preceding vaccination) according to RV1 outcome (seroconversion/shedding) at each dose using the χ^2 test or Fisher's exact test (the latter applied if there were <5 infected or uninfected individuals in a given comparison). The presence of an enterovirus in the absence of any Sabin viruses was defined as an NPEV, though notably our assays did not allow distinction of samples positive for both Sabin viruses and NPEVs. For prevalence estimates, 95% confidence intervals (CIs) were calculated using the Clopper–Pearson exact method [29]. Wilcoxon's rank sum test was used to compare total pathogen count and Ct values at each dose according to RV1 outcome; lower Ct values correspond to higher target copy numbers and were used as an indicator of increased pathogen abundance. Rotaviruses were excluded from analyses of mixed infections, pathogen groups, and pathogen count given that, in contrast to the hypothesized inhibitory effect of enteropathogens, one would expect natural rotavirus exposure or RV1 shedding to be positively correlated with rotavirus seroconversion. Across the 6- and 10-week doses,