SUPPLEMENT ARTICLE

Evaluating Associations Between Vaccine Response and Malnutrition, Gut Function, and Enteric Infections in the MAL-ED Cohort Study: Methods and Challenges

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Most vaccine assessments have occurred in well-nourished populations of higher socioeconomic status. However, vaccines are often used in populations with high incidences of malnutrition and infections, in whom the effectiveness of some vaccines is inferior for unknown reasons. The degree and extent of vaccine underperformance have not been systematically studied for most vaccines across differing epidemiologic settings. This paper outlines the methods used and challenges associated with measuring immunological responses to oral vaccines against poliovirus and rotavirus, and parenteral vaccines against pertussis, tetanus, and measles in an observational study that monitored daily illness, monthly growth, intestinal inflammation and permeability, pathogen burden, dietary intake, and micronutrient status in children in 8 countries. This evaluation of vaccine response in the context of low- and middle-income countries is intended to address the gaps in knowledge of the heterogeneity in vaccine response in diverse epidemiological settings and the interplay between infections, nutrition, and immune response.

Keywords. vaccines; malnutrition; enteric infections; gut function; MAL-ED.

Multiple factors are associated with immune response to vaccines administered during childhood as measured by antibody titers. Among the most important is the timing of antigen exposure(s), including age of first presentation and the interval between vaccine doses. Other factors may include infections (recent and/or frequent diarrhea, respiratory illness, other concurrent infections) [1, 2], malnutrition, particularly micronutrient deficiencies (vitamin A, iron, and zinc deficiency), stunting and wasting [3–5], intestinal dysfunction (malabsorption, inflammation, overgrowth) [6], maternal exposures (maternal antibody level, breastfeeding) [7], and early exposures to environmental antigens influenced by high population density, sanitation, or siblings [8].

The amount of antigen presented to the immune system is also affected by the route of delivery. The quantity of antigen presented is likely to be less variable for parenteral vaccines, where a fixed dosage of antigen is injected, as compared to orally administered vaccines where the quantity of antigen presented to the immune system may be influenced by breastfeeding, diet, and enteric infection [9, 10]. Orally administered vaccines against polio (OPV), cholera, and rotavirus elicit poorer immune responses in lower socioeconomic settings [11–16]; however, the specific reasons for this lack of an adequate immune response are poorly understood. With the continued administration of OPV, and the

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recent introductions of oral rotavirus vaccines into vaccination programs in low- and middle-income countries, it is critical to understand the extent to which gut function and nutritional, infectious, and environmental exposures impact immune response to both oral and parenteral vaccines in early childhood.

The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) cohort study was designed to measure associations between antibody titer levels to vaccines commonly administered during the first 2 years of life (primarily based on the World Health Organization's [WHO] Expanded Programme on Immunization [EPI] schedule) and micro- and macronutritional status, stool pathogen carriage, diarrheal illness, and assessments of intestinal permeability and inflammation [17]. One of the specific goal was to examine a potentially diminished immune response in the context of gut dysfunction. This measure is crucial when evaluating suspected factors influencing the vaccine response in low- and middle-income countries [18]. The study followed children from birth to 2 years of age in 8 diverse sites (>200 children per site): Dhaka, Bangladesh (BGD); Fortaleza, Brazil (BRF); Vellore, India (INV); Bhaktapur, Nepal (NEB); Loreto, Peru (PEL); Naushahro Feroze, Pakistan (PKN); Venda, South Africa (SAV); and Haydom, Tanzania (TZH) [19–26]. This paper outlines the rationale, methodology, and definitions used for the assessment of vaccine response in the MAL-ED cohort study.

METHODS

Vaccine Schedules

The MAL-ED cohort study was observational and vaccines were not supplied or administered by study staff at the 8 study sites. Table 1 lists the national vaccine schedules followed for each of the study sites. Bacillus Calmette-Guerin (BCG), diphtheria-pertussis-tetanus (DPT), hepatitis B, oral polio vaccine (OPV), *Haemophilus influenzae* type B (Hib), and measles vaccine were administered at all sites; whereas, rotavirus, pneumococcal conjugate (PCV), and yellow fever (YF) vaccines were administered at 2 or more sites. Hib was administered at all sites throughout the study period except INV, where it was introduced in December 2011. In South Africa, inactivated polio vaccine (IPV) was administered as part of the EPI schedule along with OPV. In India and Pakistan, national vaccination campaigns using OPV occurred frequently, permitting measurement of response to a variable number of OPV doses. Vitamin A supplementation was coadministered according to the national vaccination schedule in 7 of 8 sites (Table 1).

Vaccine Coverage Data Collection

Although national vaccine schedules specified the ages for the administration of each included vaccine, the actual ages of receipt through routine immunization programs varied. Data on the frequency and timing of vaccine administration were actively collected by MAL-ED field-workers during monthly household visits, ideally within a 2-day window of the monthly birth anniversary [27]. During the monthly household visit, MAL-ED field-workers recorded the vaccines administered and dates of administration on the Monthly Form A/B (MOA/MOB). The information was ideally obtained from the vaccine card; however, when vaccine cards were not available, clinical records were utilized where possible. If neither vaccine nor clinical records were available, field-workers asked the mother or caregiver if vaccines were administered since the previous monthly visit, and inquired about the type and the date of vaccine administration. Additionally, a quarterly assessment of vaccines administered and dates of administration using the Vaccine Information Form (VIF) functioned as a validation tool for the data collected on the monthly forms. The source of vaccine data was also collected on the VIF (ie, vaccine record, clinical record, mother or caregiver's report) for all vaccines administered.

Natural Exposure

Natural exposure to a vaccine-preventable disease (VPD) could influence antibody titers. The MAL-ED study design included a twice-weekly visit and monitored for any reported or referred illness [17]. Although no environmental sampling was conducted, this active surveillance allowed for the possibility to detect whether mild or severe VPD was present in individuals or the occurrence of a VPD outbreak. Wild polioviruses were not known to be circulating in any of our study sites, with the possible exception of the Pakistan site. We were not able to ascertain whether wild viruses were circulating in Pakistan but had not received any reports of poliomyelitis in our study population.

Blood Sample Collection

Blood collection was scheduled for each child at 7 and 15 months of age. On the day of blood collection, the child had to be free of symptoms of illness (ie, fever, acute lower respiratory infection, diarrhea, or dehydration). To accommodate scheduling and illness episodes, the blood collection window extended from 2 days before to 12 days after the target collection date. A maximum of 5 mL of blood was drawn from each child by MAL-ED study staff trained in phlebotomy at the child's home or in a health clinic. Samples were centrifuged at 2000*g* for 10–15 minutes, and aliquoted and frozen at −70°C prior to shipping and testing.

Vaccine Response Determination

Enzyme-linked immunosorbent assays (ELISA) were used to quantitatively measure the immunoglobulin G (IgG) antibody levels to poliovirus, measles virus, tetanus toxoid, and pertussis

Table 1. MAL-ED Site Vaccination Schedules

Vaccine and Dose No.	BGD	BRF	INV	NEB	PEL	PKN	SAV	TZH
Bacillus Calmette-Guerin (BCG)								
BCG1	Birth	Birth	Birth	Birth	Birth	Birth	Birth	Birth
Oral Polio (OPV)								
OPV1	6w	2m	Birth	6w	2m	Birth	Birth	Birth
OPV2	10w	4m	6w	10w	4m	6w	6w	1m
OPV3	14w	6m	10w	14w	6m	10w		2m
OPV4	9m	15m	14w			14w		3m
OPV5			16-24m					
Inactivated Polio (IPV)								
IPV1							6w	
IPV2							10w	
IPV3							14w	
IPV4							18m	
Diphtheria/Pertussis/Tetanus (DPT)								
DPT1	6w	2m	6w	6w	2m	6w	6w	1m
DPT2	10w	4m	10w	10w	4m	10w	10w	2m
DPT3	14w	6m	14w	14w	6m	14w	14w	3m
DPT4		15m	16-24m		18m		18m	
Hepatitis B (HepB)								
HepB1	6w	Birth	6w	6w	Birth	6w	6w	1m
HepB2	10w	1m	10w	10w	2m	10w	10w	2m
HepB3	14w	6m	14w	14w	4m	14w	14w	3m
HepB4					6m			
Heamophilus Influenzae B (HiB)								
HiB1	6w	2m	6w	6w	2m	6w	6w	1m
HiB2	10w	4m	10w	10w	4m	10w	10w	2m
HiB3	14w	6m	14w	14w	6m	14w	14w	3m
HiB4					18m		18m	
Measles								
Measles1	9m	12m	9-12m	9m	12m	9m	9m	9m
Measles2	15-18m	15m	12-15m	15m		15m	18m	
Rotavirus								
Rotavirus1		2m			2m		6w	
Rotavirus2		4m			4m		14w	
Yellow Fever								
YF1		9m			12m			
Pneumococcal Conjugate (PCV)								
PCV1		2m			3m		6w	
PCV2		4m			5m		14w	
PCV3		6m			7m		9m	
PCV4		12m						
Vitamin A								
Vitamin A1	9m	6m	9m	6m	6m		6m	9m
Vitamin A2		12m	18m	12m	12m		12m	15m
Vitamin A3		18m	24m	18m	18m		18m	21m
Vitamin A4		24m					24m	

Abbreviations: m, month; w, weeks; BGD, Dhaka, Bangladesh; BRF, Fortaleza, Brazil; INV, Vellore, India; NEB, Bhaktapur, Nepal; PEL, Loreto, Peru; PKN, Naushahro Feroze, Pakistan; SAV, Venda, South Africa; TZH, Haydom, Tanzania.

toxoid, and the immunoglobulin A (IgA) and IgG antibody levels to rotavirus. Paired samples (at 7 and 15 months) were run on the same plate for all ELISAs. For 6 study sites (BGD, BRF, NEB, PEL, SAV, and TZH), assays for all vaccines other than OPV were performed centrally at the Armed Forces Research Institute for Medical Studies (AFRIMS) in Bangkok, Thailand; INV and PKN study sites performed the assays in-country. For the poliovirus (types 1, 2, and 3) antibody neutralization assay, samples from all 8 sites were evaluated at WHO reference laboratories (Centers for Disease Control and Prevention [CDC], Atlanta, Georgia, for 7 sites and the Enterovirus Research Centre, Mumbai, India, for INV).

Measles, Tetanus, Pertussis, and Poliovirus IgG Assays

Quantitative antimeasles, anti–tetanus toxoid, and anti–pertussis toxin IgG ELISAs (Euroimmun, Lubeck, Germany) and antipoliovirus IgG ELISAs (Genway, San Diego) were centrally procured and run at AFRIMS for 6 sites; kits and supplies were provided and the assays performed on-site in INV and PKN. Assays were conducted according to the manufacturer's instructions.

Rotavirus Assays

Quantitative antirotavirus serum IgG and IgA ELISAs were conducted following previously published methods [28–30]. These assays were performed centrally for 6 of the sites, and on site in INV and PKN. In brief, microplates were coated with rabbit antirotavirus IgG, and after washing, either cell lysate or virus preparation was added to alternating rows. Eight 2-fold dilutions starting with 1:80 dilutions of the serum IgA and IgG standards were prepared. Four 2-fold dilutions of 1:20 dilutions of known reference IgA and IgG and unknown serum or plasma samples were prepared. After washing, the serum standard dilutions and serum sample dilutions were added to the microplates. After washing again, biotinylated rabbit antihuman IgA (for the IgA plates) or IgG (for the IgG plates) was added and followed by washing and addition of avidin-biotin-peroxidase complex. After the final wash, O-phenylenediamine dihydrochloride substrate was added to each well and the reaction stopped with sulfuric acid. The plates were read at 492 nm, and the titers were computed from a 4-parameter fit of the transformed optical density values.

Poliovirus Neutralizing Antibody Assays

Randomized and blinded serum samples from BGD, BRF, NEB, PEL, PKN, SAV, and TZH were shipped frozen to the CDC, where neutralization titers were determined. The serum or plasma samples were tested to detect neutralizing antibody to poliovirus types 1, 2, and 3 [31]. In brief, to conduct the microneutralization assay, a 4-fold dilution of each sample ranging from 1:4 to 1:512 was prepared and 25 μL was distributed in each of 6 wells of 96-well microtiter plates. Approximately 100 median tissue culture infective doses ($TCID_{50}$) of Sabin poliovirus strains were added to replicate wells at each dilution and the mixtures incubated at 37°C for 1 hour before the addition of 50 000 cells/mL of Vero cells. Following further incubation at 37°C for 3 days, the cells were fixed, stained, and examined for cytopathic effect. Positive controls were set up in parallel for each poliovirus as "back titrations" to ensure that an adequate amount of virus was added, with back titrations required in the range of 30–300 $TCID_{50}$ of input virus. The endpoint was defined as the highest dilution of serum that showed 50% or greater reduction in cytopathic effect. A neutralizing antibody titer of $\geq$1:8 was considered protective.

Quality Control

The quality control systems established at study sites and laboratories for data collection and sample evaluation were designed to facilitate cross-site comparison. The protocols used for data and sample collection and processing were uniform across all sites. Quality control performed on the vaccine coverage data focused on minimizing discrepancies and inconsistencies between recorded vaccines and vaccination dates. Discrepancies in vaccine names or vaccination dates between the 2 vaccine data collection forms (the monthly surveillance form MOA/MOB and the quarterly VIF) were identified and reported back to the sites for correction. Data from the assays for antibodies were generated centrally at AFRIMS for 6 of the 8 sites. As international shipment of biological samples is not permitted in India and Pakistan, the same kits, controls, and protocols were used to decrease the risk of variability. The poliovirus neutralizations assays were performed at WHO reference laboratories.

CHALLENGES

Data Collection

The vaccine information was ideally obtained from vaccination cards that were legible and fully completed by a health worker at the time of vaccine administration. However, if vaccination cards were not available, study staff relied on other clinical records or the memory of the caregiver for information on the specific vaccines received and dates. A common problem encountered was the inconsistent naming of vaccines on the vaccination cards (eg, brand names and antigens were often used interchangeably). This was a particular concern with the use of combination vaccines. However, as vaccines were mainly provided by the public sector at the MAL-ED study sites, verification of a particular brand distributed from local clinics helped to clarify which vaccines were administered.

Sample Collection

Obtaining 5-mL blood volume collection from infants with limited phlebotomy attempts was a significant challenge. Although

adequate blood samples were important to complete all assays prescribed by protocol, field-workers sought to maintain a balance between needing multiple attempts to collect the full volume of blood with minimizing the burden on the children and inducing study dropouts. Participants were allowed to refuse blood collection without sacrificing their participation in other areas of the MAL-ED study.

Data Analysis

This is the first study that seeks to evaluate immune responses to routine childhood vaccines in lower socioeconomic settings across 3 continents using unified assessments. The scope of MAL-ED posed significant analytic challenges. The analysis of vaccine response has 2 primary objectives: (1) to identify and quantify factors associated with vaccine titers, and (2) to examine factors that influence the trajectory of vaccine titers within the first 2 years of life. Both objectives pose challenges that require particular statistical care to achieve proper inference due to the variability that exists between and within sites. Perhaps most challenging is modeling the distributional variability of titers that exists due to the variation in vaccine schedules, diarrheal (and other) disease burden, and childhood nutrition. Such factors are often controlled for in a mixed-effects regression framework [32, 33] that includes a site-specific random effect; however, some vaccine titers exhibit distributions that vary across the longitudinal sample, possibly requiring a mixture modeling approach. For example, pertussis titers have a zero-inflated distribution during the first sampling round (mostly due to lack of vaccination, but can also be a result of a limit to detection in the ELISA), while the 15-month sample distribution appears more Gaussian.

Another important challenge is related to the estimation and adjustment of correlation. We have alluded to the site-level random effects that adjust for correlation within sites, but we also must account for correlation due to repeated measures. Although this is relatively straightforward from a statistical point of view, the small number of sites may require specifying them as fixed effects or using Bayesian methods to improve inference. It is also important to note that parameterization of the model is difficult given the variability in data such as age at sample collection and age at vaccination. We have explored several creative approaches to parameterize variables that describe the impact of hypothesized effects on vaccine response.

This was the first study examining these relations; thus, reasonable effect sizes to detect factors related to vaccine response were not readily available. In addition, because the goal of this project was observational, we estimated that 1600 children pooled across 8 sites were sufficient.

DISCUSSION

Factors that modulate the response to vaccines are diverse and complex. MAL-ED, as a longitudinal birth cohort study harmoniously performed at 8 diverse sites with detailed study of enteropathogen exposure, gut inflammation and permeability, and nutritional indices, will offer important insights into the prevalence and extent of hyporesponsiveness to the principal oral and parenteral vaccines. Collection of the vaccine data, as well as data regarding the determinants (nutrition, breastfeeding practices, socioeconomic factors) were obtained using common protocols and thereby were fundamentally comparable across sites and will greatly add to the strength of the analysis of factors underlying differences in vaccine responsiveness.

The vaccination exposures of the MAL-ED cohort varied significantly in terms of vaccination schedules, vaccines used, and the number and the timing of doses. This heterogeneity was unavoidable given the observational nature of the study and the different geographic locations of the study sites. For example, in some countries, OPV was given at birth as a part of EPI, whereas in others, administration was started at 6 weeks or 2 months of age. Additionally, OPV was administered as part of national and subnational immunization campaigns (eg, India and Pakistan and others). The increasing number of OPV doses may lead to higher seroconversion rates, as has been noted in Jordan [34]. Nonetheless, given the rigor of data collection and the quality of the testing, the MAL-ED study should help elucidate biologically relevant links between nutrition, infection, and the immune response.

The innate and adaptive immune responses to vaccines have shown significant variation between populations [35]; therefore, responses ideally should be further evaluated based on genetic background [36]. In addition, analytic methods must take into account the effect of microbial exposure, nutrition, gut function, and the microbiome on the developing immune system [37, 38] and on its response to specific antigens. In terms of microbial exposure, distinguishing responses induced by natural infection rather than vaccination posed an additional challenge in the evaluation of some vaccines, such as rotavirus or pertussis. Although possible immune modulatory effects of the microbiome have been proposed [9], the role of a functional gut in the quality of immune response has not been evaluated for orally delivered vaccines prior to the initiation of this study.

It is challenging to draw conclusions from single-site studies using a variety of different assays as shown in earlier studies. A study addressing anti–measles antibody production showed a significant delay in anti–measles antibody production in malnourished children compared with well-nourished children [39]; however, the malnourished children caught up over time. Additionally, an observational study in Ecuador found low mean IgG and IgM antibody titers to tetanus toxoid in stunted children, but there was no difference in antibodies to diphtheria toxin [3], whereas a small study in Egypt described low diphtheria toxoid antitoxin levels in severely malnourished children assessed 21 days after vaccination [40]. The use of multiple assay

formats complicates the comparison of these studies in different settings. The MAL-ED study design created a scenario where it is possible to compare antibody responses across sites in a reliable way. Other factors adding to the level of complexity of the MAL-ED data included prior exposure to infectious agents and the influence of maternal antibodies. Previous studies have shown that prior exposure to influenza and measles vaccines negatively influences vaccine response [41, 42]. Furthermore, the presence or absence of maternal antibodies in young children may affect immune response to both parenteral and oral vaccines [43, 44]. However, because the data reflect the complexity of the real-world setting of intense exposure to pathogens, the conclusions reached are highly relevant to policy and delivery program decisions.

CONCLUSIONS

The complex data sets of the MAL-ED study, which included repeated measures of anthropometry, enteric infections, intestinal function, and antibody estimations to multiple vaccines at 2 time points, create the potential for data that can, for the first time, conduct cross-country comparisons of immune response to oral and parenteral vaccines. These data could potentially clarify associations between host and environmental factors that influence the development and duration of a protective vaccine response, especially relevant in the setting of a global program to eradicate poliomyelitis. The MAL-ED study is critical to developing mechanistic insights, which may further the understanding of the biology of vaccination and lead to the design of better vaccines and/or vaccination strategies.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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