SUPPLEMENT ARTICLE

Methods of Analysis of Enteropathogen Infection in the MAL-ED Cohort Study

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Studies of diarrheal etiology in low- and middle-income countries have typically focused on children presenting with severe symptoms to health centers and thus are best equipped to describe the pathogens capable of leading to severe diarrheal disease. 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 evaluate, via intensive community surveillance, the hypothesis that repeated exposure to enteropathogens has a detrimental effect on growth, vaccine response, and cognitive development, which are the primary outcome measures for this study. In the setting of multiple outcomes of interest, a longitudinal cohort design was chosen. Because many or even the majority of enteric infections are asymptomatic, the collection of asymptomatic surveillance stools was a critical element. However, capturing diarrheal stools additionally allowed for the determination of the principle causes of diarrhea at the community level as well as for a comparison between those enteropathogens associated with diarrhea and those that are associated with poor growth, diminished vaccine response, and impaired cognitive development. Here, we discuss the analytical methods proposed for the MAL-ED study to determine the principal causes of diarrhea at the community level and describe the complex interplay between recurrent exposure to enteropathogens and these critical long-term outcomes.

Keywords. birth cohort study; enteropathogens; diarrhea etiology; growth; MAL-ED.

The 2010 Global Burden of Disease study found that diarrhea-associated years of life lost were roughly halved from 1990 to 2010, contributed to by improvements in supportive care, including oral rehydration therapy, and pathogen-specific interventions, including the oral rotavirus vaccine [1]. Diarrhea and malnutrition combined remain responsible for 5% of all disability-adjusted life years lost [2]. However, the long-term morbidity associated with enteropathogen infections is much harder to estimate. The primary rationale behind The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) study was that the determinants of the long-term complications of enteric infection, including poor growth, diminished vaccine response, and impaired cognitive development, have not been well described, as the majority of research has focused on the outcome of infectious diarrhea, a syndrome for which both the pathogen-specific causes and risk factors might be different. While estimates of the pathogen-specific burden of acute diarrhea that requires care at a health center may be an appropriate guide for interventions designed to reduce diarrhea-associated mortality, it is unclear whether the same pathogen hierarchy is relevant for reducing the long-term morbidity associated with recurrent symptomatic and asymptomatic enteropathogen infection in the community [3, 4]. For example, though rotavirus is clearly documented to be a principal driver of diarrhea-associated mortality in children aged <5 years in low- and middle-income countries, a recent follow-up study of a clinical trial of rotavirus vaccination in

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Bangladesh found that there was no effect of rotavirus vaccination on malnutrition, as measured by weight gain or linear growth in that population [5]. Meanwhile, there is evidence that the adverse effect on growth of asymptomatic infection with *Cryptosporidium* and *Campylobacter* spp. may exceed that of symptomatic infection [6, 7]. Here, we discuss the benefits of a longitudinal cohort study design, outline analyses for relating diarrhea to pathogen infection, briefly discuss the analysis of symptomatic and asymptomatic enteropathogen infections as determinants of malnutrition, and introduce the potential for reanalysis of MAL-ED samples with novel molecular diagnostics.

Impact of Study Design and Definitions

Case-control and cohort studies are commonly used epidemiological study designs. While case-control studies enroll patients based on a disease state, cohort studies enroll patients based on membership in a defined population and are then followed for the outcomes of interest. For studies of pathogen-specific causes of severe diarrhea, case-control studies are a frequently used, efficient approach due to the relative ease of case enrollment upon presentation to a health facility [8]. Case-control studies typically provide more statistical power than cohort studies of the same size to detect differences in outcome across different levels of exposure, especially when the outcome under study is rare [9]. However, a prospective cohort design offers substantial advantages for understanding the broader burden of enteropathogen infection in the community for several reasons. Most important, case ascertainment of mild diarrhea is difficult and ascertainment of asymptomatic infection is impossible without intensive community surveillance. Indeed, the line between the 2 is not completely clear and may vary from population to population. It has been observed that the more common diarrhea is in a community, the less likely it is to be reported as abnormal [10]. Thus, the case definition is less clear than for a study of more severe disease. Additionally, it is difficult to prospectively select controls. Diarrhea is a common and often underreported phenomenon, which in case-control studies could result in controls being overmatched for the risk factors of interest (ie, presence of pathogens in the stool sample). Even if it is assumed that we can accurately distinguish between symptomatic and asymptomatic stools, the increased detection of many pathogens in peridiarrheal sampling makes it challenging to identify "pristine" controls that represent the true baseline asymptomatic incidence of pathogens. Finally, because enrollment in case-control studies is done on the basis of the specific outcome in question (eg, diarrhea), these studies cannot be used to analyze the association between recurrent enteropathogen infection and multiple long-term outcomes.

Consistent with World Health Organization guidelines, the a priori definition of diarrhea in the MAL-ED study is ≥3 loose stools in 24 hours or presence of blood in stool [11]. This threshold was derived from a single community-based study and was chosen on the basis of test performance (sensitivity 77.8% and specificity 96.3%) in comparison to a gold standard of maternal perception of diarrhea. The analysis was not stratified by age, and a higher threshold for stool frequency may be appropriate for infants, given that they produce higher rates of loose stool than older children [12]. In the MAL-ED study, in addition to querying the frequency of stools during surveillance, mothers were also asked directly whether or not they believed that their child was having diarrhea. Thus, our prospective cohort design allows us to evaluate the Baqui et al [11] assessment across age strata as well as the 8 MAL-ED study sites. In addition, we can compare maternal report of diarrhea to the study definition as a marker of the presence of specific pathogens in stools.

The biased selection of controls is a common pitfall for case-control studies [13]. In the MAL-ED longitudinal cohort, monthly surveillance stools were collected in the context of intensive surveillance for diarrheal symptoms, which allowed us to test the sensitivity of assumptions that assign a stool as a control. For example, when determining the pathogen-specific burden of diarrhea, all asymptomatic stools might be considered as controls. However, asymptomatic infection may be temporally associated with symptomatic infection [14]. For example, the median duration of carriage of *Campylobacter* spp. after an associated episode of diarrhea is 1 month [15]. Thus, controls selected during this period will bias the results toward a conclusion that *Campylobacter* spp. are not associated with diarrhea. Our prospective cohort design will allow us to evaluate the effect on our burden estimates of limiting control stools to those with varying windows of diarrhea-free days before and after collection. There is evidence that such an approach in the context of a longitudinal study can reveal associations with diarrhea that will be missed by a case-control approach [16, 17].

Estimating the Burden of Pathogen-Specific Diarrhea in the MAL-ED Cohort

Whether or not diarrhea is an important mediator of the association between enteropathogen infection and the primary study outcomes, it is still crucial to determine the relative burden of pathogen-specific diarrhea for the following reasons: it allows for comparison with other studies undertaken in these settings; it makes it possible to determine whether the principal etiologies of diarrhea in the community are similar to those for more severe disease; it helps in determining whether diarrhea is a helpful marker of enteropathogen infection in terms of the primary outcome measures; and it provides a logistically convenient outcome for understanding the importance of mixed infections.

Common approaches for estimating the strength of association between putative risk factors and health outcomes in observational studies are the odds ratio and relative risk. In the case of

analyzing the association between enteropathogens and diarrhea, they serve as a measure of pathogenicity. Given that the same children are followed for the duration of the MAL-ED study, we will calculate pathogen-specific risk estimates via a mixed model with random effects per child to account for child-specific biases [18]. There remains some concern that this approach can overestimate the importance of individual pathogens despite adjusting for mixed infections. For this reason, we intend to apply an additional approach using multiple response variables—1 for each of a number of pathogens—and examine whether the same set of independent variables, including whether or not the stool was an asymptomatic or diarrheal sample, can better quantify the potential overestimation of risk.

As a measure of the strength of association between detection of an enteropathogen and diarrhea, the risk estimate is independent of the prevalence of the associated risk factor in diarrheal stools. Of greater use to direct intervention measures is the population attributable fraction (AF), which incorporates the strength of association between a pathogen and diarrhea as well as the prevalence of that pathogen in diarrheal stools. The AF approach allows for the possibility that weaker but more ubiquitous risk factors will be identified as more efficient targets for interventions than rarer risk factors with a stronger disease association. Following Bruzzi et al [19], it is convenient to estimate the AF from a logistic regression that is also capable of controlling for a number of potentially confounding factors (eg, study site, age of the child, breastfeeding status).

Rückinger et al [20] observed that the estimation of the AF is sensitive to the inclusion of cofactors and that the order of their inclusion (or exclusion) can change the overall AF. Instead, robust estimates can be calculated in the presence of other possible risks by averaging the AF across models that each removes a given risk factor. Rückinger et al [20] suggest that their approach is both different and arguably more robust than sequential models (ie, models that are sensitive to the sequence of cofactors). Also, their approach has the advantage that the sum of all attributable risks will be, at most, 100% rather than exceeding 100% because of contributions not only in isolation but in combination with other risks. Because our intention is to establish an association between many co-occurring pathogens and diarrheal symptoms, this approach has a substantial benefit over standard approaches [21]. In reality, each specific mixed-infection combination is comparatively uncommon and thus unlikely to change the AF substantially. This finding appears to be supported by the Global Enterics Multi-Center Study (GEMS), a case-control study of moderate to severe diarrhea in Africa and Asia [21].

At present, the estimation of AF using the averaging method of Rückinger et al [20] does not allow the inclusion of continuous variables. This is a notable deficiency when one considers longitudinal data, the purpose of which is to account for changing patterns of infection as children age. Thus, the AFs in the MAL-ED cohort study will be estimated for age categories. Another commonly reported limitation of the AF is the reliance on the point estimate of the risk without reference to the degree of confidence in this estimate. When establishing a hierarchy of pathogens, this limitation can be problematic if more common pathogens are excluded on the basis of a nonsignificant risk estimate, while rare but more strongly diarrhea-associated pathogens are included. This analytical challenge can be resolved through estimation of upper and lower confidence limits for the AF in addition to the mean.

While the above approach for generating estimates of the pathogen-specific burden of disease is a well-developed method for attributing diarrheal etiology using clearly phenotyped cases and controls, there is some concern that it may miss important associations between diarrhea and pathogens that have a high prevalence of asymptomatic infection. Lopman et al [22] applied mathematical modeling to demonstrate this problem using the example of norovirus, which has frequently not been clearly associated with diarrhea in well-designed etiological studies of children in the developing world [23]. They show that despite a similar incidence of associated diarrheal disease, settings in which the point prevalence of asymptomatic norovirus infection is high will show a lower burden of disease in comparison to settings in which the point prevalence of asymptomatic infection is low. This phenomenon may be responsible for why longitudinal studies in which the distinction between symptomatic and asymptomatic infection can be more clearly made can show a higher burden of norovirus diarrhea in similar settings [16, 17]. Another approach is to distinguish clearly between first and subsequent infections with each pathogen, such that significant early associations with diarrhea can be identified despite the rapid development of natural immunity [24]. In the context of the MAL-ED study, it will be important to leverage the longitudinal nature of the data to most clearly reveal pathogen-specific burdens of diarrheal disease.

Another critical element of the analysis of enteropathogen infection in the MAL-ED cohort study is between-country comparisons. One of the interesting findings from cross-country surveys of severe diarrhea is the consistency of the top pathogens implicated between populations [23, 25]. However, the incidence of asymptomatic or mildly symptomatic infections may very well prove to be population specific. We will add an indicator for study site to capture and test cross-site differences. Ecological and sociodemographic factors can also be added. Additionally, the use of simple mathematical models such as the per capita rate of infection (ie, the force of infection) may help to uncover biological similarities in the response to infection. For example, Gupta et al [26] used the rate of cerebral malaria in 5 villages to estimate both the village-specific infection

rates and the immune response to malaria exposure that could be considered a common human physiological parameter. The challenge when analyzing MAL-ED data in this way is distinguishing between new infections and persistent carriage, which is part of the rationale for the MAL-ED study's goal of intensive community surveillance with standardized specimen collection and high rates of completion.

Enteropathogen Infection as a Determinant of Long-Term Outcomes

The longitudinal design of the MAL-ED study allows for a measurement of the cumulative effect of enteropathogen infection both with and without acute manifestations of disease. As an illustration of the sort of analysis that is possible with this longitudinal data, the cumulative effect of enteric infection can be evaluated on physical growth through a linear mixed effects model that uses regression splines to model short- and long-term effects of multiple exposures over time [27]. The total number of pathogens experienced during each period can be included, and not only the effect of current but also the lagged impact of historic infection can be quantified. Lee et al [28] used a similar longitudinal model to assess short- and long-term changes in growth metrics in relation to different diseases. Similar approaches could be developed to compare the impact of enteropathogen infection both broadly and by specific etiologies [29].

Much work has been predicated on enteric pathogens as part of a vicious cycle of infection and suppressed immunity [30, 31]. The analytical treatment of pathogens as a predictor of health outcomes ignores the feedback that infections reduce resistance and resilience to future infection (eg, by diverting metabolic activities). Structural equations are a possible solution that could be used to test hypothesized relationships between infection, symptoms, and long-term outcomes. Unobserved latent variables can be constructed from observed data and then fed into a system of interacting equations [32]. The strength of this approach is that it allows predicted structures to be assessed and can enable tests of causal, rather than correlative, relationships [33, 34]. However, the inherent weakness of this approach is the assumption of structural relationships between components of the disease system [35]. This issue can be avoided by applying Bayesian network analysis to derive both the structure and the parameter values from empirical data. In both cases, a systems approach to gauging combinations of risk factors may enable a more holistic description of the interdependency of enteric infection and other health-related exposures.

Future Analytical and Methodological Opportunities for the MAL-ED Cohort Study

The MAL-ED microbiological methods were selected with the goal of harmonized protocols for a diverse range of enteropathogens in order to provide quality-assured and controlled results from 8 disparate sites while maintaining laboratory throughput and minimum cost [36]. While this protocol was chosen to represent the gold-standard diagnostic panel, it was understood that diagnostic methodology is an evolving field and banking of stool specimens is performed to allow for future reevaluation with novel methods in order to better understand the influence of enteropathogen infection on these children. As an example, the choice of diagnostic method for detection of *Campylobacter* infections can have a substantial influence on our understanding of the prevalence and clinical significance of this pathogen [37]. As we have discussed, a principal challenge for studies of diarrhea causation is the high rate of asymptomatic pathogen detection. Conventional diagnostics, which generally yield a qualitative result, may obscure meaningful differences between low-burden carriage and high-burden infection. Though molecular diagnostic strategies undoubtedly increase the amount of asymptomatic pathogen detection [38], the quantitative nature of polymerase chain reaction (PCR)–based testing allows for analysis of the association between pathogen burden and diarrhea [39–44]. A TaqMan array card that simultaneously detects the stable of common enteropathogens would serve this purpose well [45, 46]. Because the association between the quantity of enteropathogens detected and symptoms may vary substantially from individual to individual and because it is otherwise difficult to interpret the detection of pathogens associated with prior episodes of diarrhea, the collection of peridiarrheal surveillance specimens is particularly valuable for a quantitative analysis of enteropathogen infection.

The prospective cohort design of MAL-ED also allows for the development of nested case-control studies for evaluating the relationship between specific outcomes, such as diarrhea and malnutrition, and additional covariates including quantitative real-time PCR, strain-specific studies, detection of newly described pathogens, and next-generation sequencing. For example, real-time PCR can be used to assess whether stool pathogen quantity is associated with diarrhea [46]. The approach to calculating a pathogen-specific attributable fraction outlined above can be applied once adapted for continuous variables [47]. Similarly, for analysis of the multiple long-term outcomes of enteropathogen infection, a case-cohort design can be used to incorporate additional pathogen testing where it may not have been feasible to test all samples [48]. Also, the longitudinal study design of MAL-ED will allow us to investigate the persistence of enteropathogen carriage after episodes of diarrhea, a question that is well suited to molecular detection methods.

CONCLUSIONS

The MAL-ED cohort study is uniquely situated to provide a more accurate and comprehensive understanding of the myriad consequences of enteropathogen infection for children

in low- and middle-income countries. Though acute severe diarrhea is a critical and clearly defined problem, the broader morbidity associated with enteropathogen infection is harder to characterize. This intensive community-based study will help evaluate whether similar pathogens, alone or in concert with others, are relevant to the more common problems of mild diarrhea and poor growth. It is possible that these syndromes are distinct and that the pathogens most strongly associated with them may differ. Therefore, identifying the causes of these syndromes and their variability in different populations will be important to inform the next generation of preventive 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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