SUPPLEMENT ARTICLE

Microbiologic Methods Utilized in the MAL-ED Cohort Study

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A central hypothesis of The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) study is that enteropathogens contribute to growth faltering. To examine this question, the MAL-ED network of investigators set out to achieve 3 goals: (1) develop harmonized protocols to test for a diverse range of enteropathogens, (2) provide quality-assured and comparable results from 8 global sites, and (3) achieve maximum laboratory throughput and minimum cost. This paper describes the rationale for the microbiologic assays chosen and methodologies used to accomplish the 3 goals.

Keywords. culture; ELISA; enteropathogen; microscopy; PCR.

A central hypothesis of The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) study is that enteropathogens participate in and drive a vicious cycle of malnutrition and diarrhea. It has been proposed that enteric infection can lead directly to malnutrition, but existing data are surprisingly scarce. The vast majority of data on enteropathogens derive from studies of diarrhea, usually of severe forms from inpatient studies [1, 2]. This is partially informative to our question, in that diarrhea of a chronic or recurrent nature can lead to malnutrition, as illustrated by the classic studies of Guatemalan children described by Mata [3]. The corollary is also true, that malnutrition predisposes to diarrhea incidence, persistence, and severity [4]. One of the primary outcomes of the MAL-ED study is linear growth (ie, stunting) as the primary outcome of interest. Thus, a major question shifts to whether carriage of particular enteropathogens or enteropathogen profiles is associated with stunting independent of diarrhea.

Associations Between Particular Enteropathogens and Malnutrition

Bacteria

The studies by Mata [3] and Dale and Mata [4] from Guatemala in the 1960s utilized a laboratory setting that emphasized bacterial culture. In those case-control studies of malnourished children vs village controls, it was observed that *Shigella* infection, burden, and duration were associated with the malnourished children [4]. Interestingly, presence of fecal *Escherichia coli* and *Candida albicans* were associated with the controls. Notably, in 1 malnourished child followed serially, a shift in intestinal flora on day 2 prior to a diarrheal episode (marked by fewer *E. coli*, lactobacilli, and streptococci, replaced by slow lactose fermenters and staphylococci) was a harbinger for *Shigella* detection (day –1) followed by diarrhea (day 0). In Bangladesh during the 1970s, the effect of diarrhea caused by *Shigella*, enterotoxigenic

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E. coli (ETEC), and rotavirus were compared in a longitudinal community-based birth cohort, and it was observed that these different pathogens had different effects on child growth. *Shigella* affected linear growth and ETEC weight gain, whereas rotavirus was not found to have significant effects on either [5]. These early studies that compared different pathogens in the same samples added important information to support the rationale of the MAL-ED study.

In a prospective study of diarrhea in Brazil, Steiner et al [6] found that control children who were infected with enteroaggregative *E. coli* (EAEC) exhibited significant growth impairment independent of diarrhea. In the Gambia and Colombia, rates of *Helicobacter pylori* infection in infancy were high (30%–38%) and were associated with malnutrition as measured by weight and height faltering [7–9]. Asymptomatic and symptomatic *Campylobacter* infections have been demonstrated to attenuate weight gain over a subsequent 3-month period, whereas symptomatic *Campylobacter* infection, especially severe forms, was associated with linear growth deficits over a subsequent 9-month period [10].

Protozoa

In a Peruvian study, asymptomatic infection with *Cryptosporidium* had an adverse effect on weight gain, and infected infants did not catch up at 1 year postinfection [11]. In Brazil, an association has been observed between *Cryptosporidium* and growth shortfalls and long-term deficits in fitness and cognitive function [12, 13]. Additionally, in Egypt, *Cryptosporidium parvum*, *Giardia lamblia*, *Entamoeba histolytica*, and *Blastocystis hominis* were overrepresented in stool samples from immunocompromised children, including many with protein-calorie malnutrition, marasmus, and marasmic kwashiorkor without diarrhea [14].

Helminths

An enormous disability-adjusted life-year burden has been ascribed to hookworm, *Ascaris*, and *Trichuris* due to their effect on undernutrition [15]; however, much of the underpinning prevalence data come from decades-old stool microscopy surveys. In a study of children in the Democratic Republic of the Congo, stunting was significantly associated with *Ascaris* infection, whereas wasting was associated with *Ascaris* or *Trichuris* [16]. Meanwhile, another study in Ethiopia showed no association with *Ascaris* and malnutrition [17]. A study in Jamaica found that treatment of *Trichuris* with albendazole in 9- to 12-year-olds led to a significant improvement in tests of short-term memory and scanning and retrieval of long-term memory [18]. Deworming interventions with albendazole or tetramisole have led to significant improvements in malnutrition in certain studies [19]. However, a large meta-analysis by Dickson et al [20] concluded that the evidence was "inconsistent and limited" that routine antihelminthic treatment had positive effects on weight gain in children.

Possible Mechanisms Whereby an Enteropathogen, Independent of Diarrhea, Could Lead to Malnutrition

Kosek and colleagues [21] address other mechanisms that an enteropathogen could lead to malnutrition independent of diarrhea. Windle et al [8] have postulated that *H. pylori* initiates a vicious cycle that begins with hypochlorhydria, which predisposes to infection with additional enteropathogens (eg, *Vibrio cholerae*, *Salmonella*, *Giardia*) as well as iron deficiency anemia, and then leads to malnutrition. In some settings, *Giardia* infection, but not *Ascaris*, *Trichuris*, hookworm, or tapeworm has correlated with a higher intestinal permeability as measured by the lactulose:mannitol ratio [22]. The lactulose:mannitol ratio is a measurement that has correlated with malnutrition and severe kwashiorkor in several other studies [23, 24].

Protein loss could also result from infection; for example, *Strongyloides* has been associated with elevated fecal α-1 antitrypsin levels and protein-losing enteropathy [25]. Additionally, subclinical intestinal inflammation due to enteropathogens may also play a role in protein loss. For example, growth-impaired, EAEC-infected children exhibited high fecal lactoferrin and interleukin 1β concentrations [6].

Most of these data are indirect and subject to confounding by mixed infections and incomplete microbiologic studies that focus on selected enteropathogens. Therefore, the MAL-ED Microbiology Technical Subcommittee adopted an agnostic approach toward determining which enteropathogens play a role in malnutrition. MAL-ED investigators strived to detect as many enteropathogens present in our study samples as feasible within our laboratory and budget constraints, balancing cost, cross-comparability, and throughput.

MICROBIOLOGICAL METHODS

Start-up

The MAL-ED study investigators agreed that the microbiologic protocols must be standardized and harmonized across the 8 study sites. All sites performed their own assays to take advantage of local capacity and avoid onerous shipping of samples to central laboratories. We used widely accepted references such as *The Manual of Clinical Microbiology* [26] and published literature to assist in selecting microbiologic methods best practices. The MAL-ED Microbiology Technical Subcommittee then developed the Standard Operating Procedures (SOPs) through collaboration with the larger set of researchers in the MAL-ED network. All 8 of the MAL-ED sites contributed their expertise, explained the challenges unique to their site, debated vigorously, compromised, and created 1 set of consensus SOPs, which was then implemented at the sites prior to study recruitment.

Specimen Collection

Specimen collection procedures demanded strict transport and processing times, as specimen integrity is critical to organism

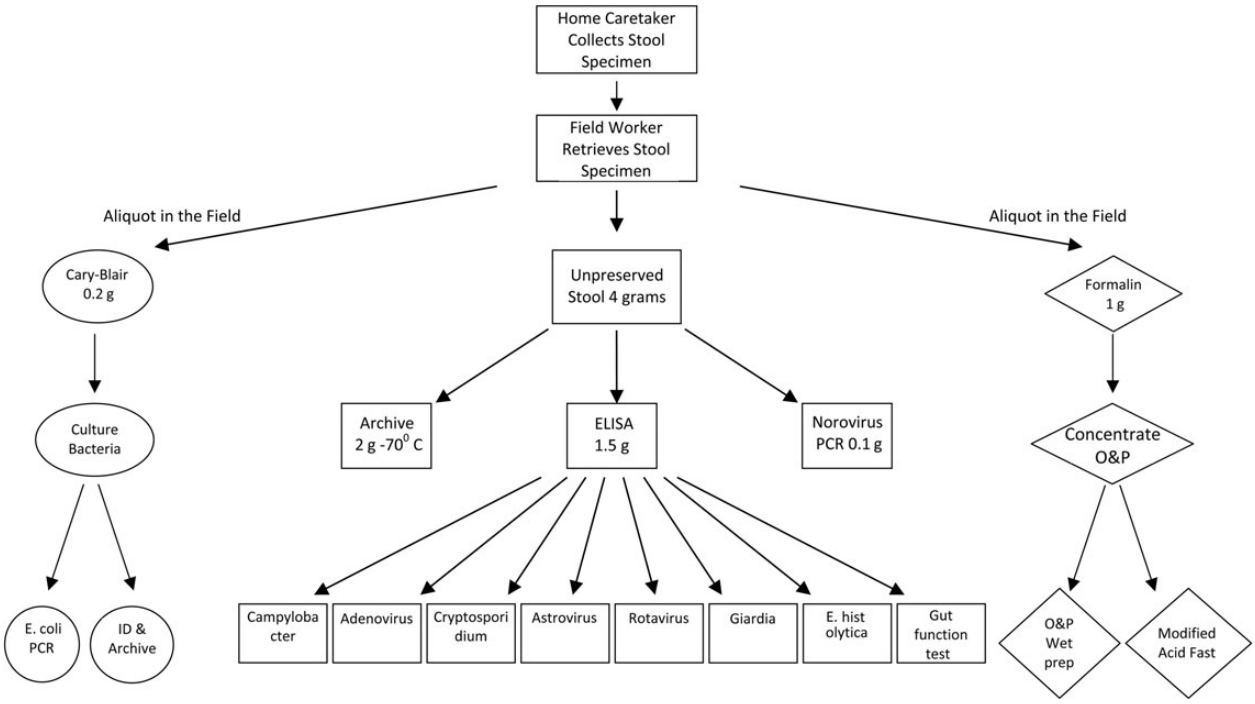

Figure 1. Workflow for stool specimen from collection to assay. Abbreviations: ELISA, enzyme-linked immunosorbent assay; O&P, ova and parasites; PCR, polymerase chain reaction.

recovery (Figure 1). Surveillance stool samples were collected on the monthly anniversary of the child's birth ±2 days. Additionally, we sought to collect from enrolled children a specimen from every diarrheal episode. For the densely urban sites, meeting the time collection windows was relatively straightforward. However, at the rural sites the participant's home could be several hours from the laboratory. Therefore, we enlisted the assistance of the mother or other caregiver and equipped them with materials to collect stool the evening before a planned collection. At some sites, mothers or caregivers were introduced to the use of disposable diapers worn absorbent side out, which was often not the local custom, while other sites used plastic sheets. Mothers or caregivers were instructed to collect 3–4 spoonfuls of stool and place it within 1 hour in a labeled stool container, seal it in a plastic bag, and to then place it inside a transport box with cold packs. The stool collection usually occurred in the morning, such that the study researcher, nurse, or field-worker processed the specimen shortly thereafter. In all study sites, we required that stool samples be placed into Cary-Blair transport media within 2 hours of production. The maximum allowed time from placement of stool swab in Cary-Blair to receipt of all the specimens in the laboratory was 18 hours. Due to the long distances often involved, a few study sites opted to train and leave the Cary-Blair transport media with the mother or other caregiver to use; those trained in its use managed without difficulty. Cary-Blair was chosen as the sole transport media for culture because it facilitates the recovery of all of the bacteria the MAL-ED study sought to isolate [26]. If <4 g of specimen was received, a standardized priority testing protocol was followed and the recollection of additional specimen was attempted for 48 hours.

Specimen Management in the Laboratory

Specimens were processed in the laboratory the same day as collected, such that during peak specimen collection the laboratory was staffed evenings and weekends, and arrangements were made for staff to travel home safely. To complete all the required tests and to archive specimens for future studies, a minimum of 4 g of stool was required. We anticipated that some specimen quantities would be insufficient, and developed a priority test list and recollection protocol whereby the field-worker returned to the household the next day to attempt another specimen collection. Because 30 000–50 000 cryovials of stool specimens was collected and processed at each study site, a specimen-tracking database using barcode labels was essential. An in-house tracking system initially developed by the Brazil MAL-ED site was modified and successfully deployed to all other sites.

Bacteriology

We optimized our bacteriology protocol to detect the major bacterial enteropathogens: *Salmonella*, *Shigella*, *Vibrio*, *Yersinia*, *Aeromonas*, and *Plesiomonas*. Culture media from BD

Table 1. Bacterial Culture Methods Used in the MAL-ED Study

Medium	Pathogens	Suspect Colonies
MacConkey agar	*Salmonella*, *Shigella*, *Aeromonas*, *Plesiomonas*, *Yersinia*, *Vibrio*, *E. coli*	Nonlactose fermenter, colorless or transparent, *E. coli*–polymerase chain reaction archive
XLD	*Salmonella*, *Shigella*, *Aeromonas*, *Plesiomonas*, *Yersinia*, *Vibrio*	Transparent, red with or without H2S
TCBS	*Vibrio*, *Aeromonas*	Yellow, green, transparent blue-green (any growth)

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose agar; XLD, xylose lysine desoxycholate.

(Sparks, Maryland) was purchased prepared or prepared in-house by the study sites and were quality controlled. Media used are indicated in Table 1. Up to 5 suspect colony morphologies were selected for screening, and each colony was inoculated into a set of biochemicals: Kligler iron agar slants, lysine decarboxylase tubes, motility indole ornithine medium tubes, and urea slants. Study sites without in-house media preparation chose to screen colonies using the Analytical Profile Index (API) 20E (bioMérieux, Craponne, France) identification system or MicroScan Identification System (Siemens Corporation, Washington, D.C.). All presumptive *Salmonella*, *Shigella*, and *Vibrio* were confirmed by serotyping with Difco (BD) or Denka-Seiken (Tokyo, Japan) antisera. *Yersinia*, *Aeromonas*, and *Plesiomonas* were confirmed by API 20E or the MicroScan Identification System.

Escherichia coli

Escherichia coli is a normal constituent of human intestinal flora, but pathogenic strains arise when they acquire toxin genes or other virulence factors. Detection of diarrheagenic *E. coli* is therefore based on detecting these factors directly in a subset of a patient's *E. coli*. For the MAL-ED study, we elected to pick and pool 5 lactose-fermenting colonies resembling *E. coli*, and characterize them for virulence genes using a multiplex polymerase chain reaction (PCR) assay. Studies have revealed greater detection of *E. coli* with greater screening of colonies [27]. The testing of pools has been shown to reduce the cost and workload, yet maintained good sensitivity and specificity compared with testing isolates individually [28]. We chose to use multiplex PCR to characterize *E. coli* isolates over more conventional phenotypic methods (eg, enzyme-linked immunosorbent assay [ELISA] or cell culture assay), for throughput and cross-comparability. We adapted PCR assays from the literature [29–31] to create a single 9-plex PCR. Detection of amplicons was performed by gel electrophoresis because this technology was in place at all sites, and was less expensive than utilizing

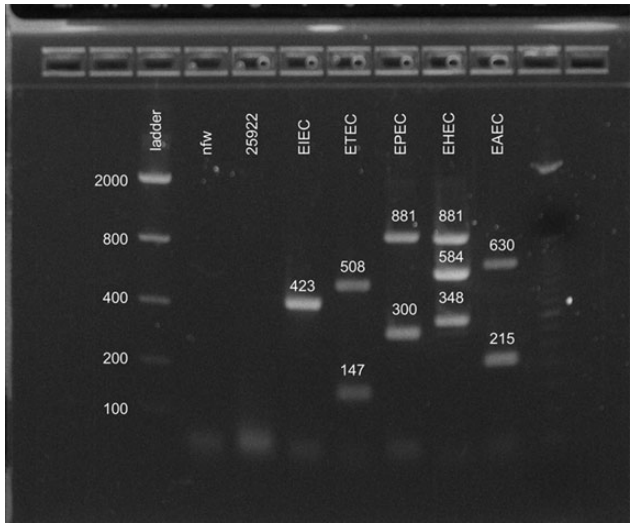

Figure 2. Multiplex polymerase chain reaction (PCR) gel electrophoresis assay for diarrheagenic *Escherichia coli*. The *E. coli* isolates underwent DNA extraction and multiplex PCR for virulence genes as described in the Methods. Virulence genes are discriminated by band length. Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; EIEC, enteroinvasive *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli;* nfw, nuclease free water.

real-time PCR probes, but this required some assay redesign to create amplicons of visually distinct band sizes. Ultimately, our assay detected the 5 major diarrheagenic *E. coli* pathotypes as follows (band sizes in parentheses):

- Shiga toxin–producing *E. coli*: *stx1* (348 bp) and *stx2* (584 bp)
- ETEC: *LT* (508 bp) and *ST* (147 bp)
- Enteropathogenic *E. coli* (EPEC): *eae* (881 bp) and *bfpA* (300 bp)
- Enteroinvasive *E. coli* (EIEC): *ipaH* (423 bp)
- EAEC: *aatA* (630 bp) and *aaiC* (215 bp)

DNA template from reference *E. coli* strains—EAEC O42; enterohemorrhagic *E. coli* (EHEC) O157:H7; EIEC O124; EPEC 2348/69; and ETEC H10407—were used as positive controls for every PCR run. Negative controls included nuclease-free water and DNA template from ATCC standard strain *E. coli* ATCC 25922 (no virulence genes). SYBR Safe gel stain (LifeTechnologies, Grand Island, New York) was encouraged to avoid ethidium waste. Gels were UV illuminated and photographed. A typical gel is shown in Figure 2.

Full details of the assay and primer sequences used can be found in the supplemental material of Taniuchi et al [32]; however, some details are worth further mentioning. The *E. coli* heat-stable toxin (ST) is a challenging target. Two main subtypes of ST have been found in humans: STp (or STIa) and STh (or STIb), with the latter more diarrhea-associated in

some studies [33]. Our assay is based on the primers of Nguyen et al [29], which amplify STh. We emphasize that sequence data in GenBank are variable, such that the forward primer may have 2 mismatches with certain STh sequences; specifically, the forward primer 5′-GCTAAACCAG**T**AR**G**GTCTTCAAAA-3′ may have mismatches with some GenBank sequences at the 2 positions in boldface type. Despite these mismatches of unclear significance, our assay amplified the desired target from reference materials, and clinical samples confirmed the targets by sequencing (data not shown), so we proceeded with its use.

Campylobacter

Campylobacter culture was preferred by some sites that had experience with this method and desired isolates for further investigation. However, other study sites were not equipped for the elevated temperatures and microaerophilic requirements and thus preferred ELISA. Because the published literature reported the functional equivalence of ProSpecT *Campylobacter* ELISA with culture, we initially allowed either culture or ELISA [34]. However, after preliminary analysis of the site data showed lower *Campylobacter* detection with culture vs ELISA (data not shown), we elected to perform *Campylobacter* ELISA at all study sites with an option to add culture if desired. We have subsequently learned in the MAL-ED study that the *Campylobacter* ELISA had vast detection over culture, and secondary PCR revealed that many (27.6%) of these detections are likely due to non-*jejuni*/*coli Campylobacter* species [35].

Immunoassays for Viruses and Protozoa

The MAL-ED study's SOP emphasized ELISA methodology as it simplified procurement, streamlined workload, and was relatively easy to quality control. At peak sample collection, >1600 tests were performed per month. This was a workload that ELISA made possible by batch testing, as all assays allowed storage at −20°C. A single aliquot was thawed only once and placed into the appropriate test diluents. Once the fecal specimen was suspended in diluent, the *Cryptosporidium*, *E. histolytica*, and *Giardia* ELISAs allowed testing within 24 hours; *Campylobacter* within 72 hours; and adenovirus, astrovirus, and rotavirus within 1 week. This allowed testing to be spread out over several days. We used ProSpecT kits (Oxoid Ltd, Ely, United Kingdom) for rotavirus (VP6), adenovirus (detects all human adenovirus serotypes via a genus-specific adenovirus hexon antigen), and astrovirus kits. Protozoa kits were from Techlab (Blacksburg, Virginia). The *E. HISTOLYTICA II kit* detects a surface adhesin molecule of *E. histolytica* that does not cross-react with *Entamoeba dispar*, the GIARDIA II kit detects a *G. lamblia* cyst wall protein, and *CRYPTOSPORIDIUM* II kit detects an oocyst protein. Performance of these protozoa ELISAs is excellent and comparable to other commercial kits [36].

Norovirus Quantitative Reverse Transcription PCR

Norovirus is an RNA virus with extensive genetic diversity and is divided into at least 2 genogroups, I (GI) and II (GII). IDEIA Norovirus kit ELISAs (Oxoid Ltd) are available, but sensitivity is poor compared with reverse transcription (RT) PCR [37–39] thus, we chose the latter. Quantitation of norovirus has been reported to correlate with symptoms [40]; therefore, we chose to amplify by quantitative (real-time) RT-PCR. For RNA extraction, we used the QIAamp Viral RNA kit (Qiagen, Valencia, California) to promote reproducibility. The same primers and probes were used at all sites (based on Kageyama et al [41]). Four different real-time PCR platforms were available across the 8 sites (Qiagen Rotor-Gene, BioRad CFX, Roche Lightcyler, and ABI 7500). Therefore, prior to testing the study specimens, a 15-sample panel was sent to each site. Performance of the assay at the 8 sites on the 4 different platforms revealed acceptable performance levels across platforms: 86% consensus for norovirus GI and 96% consensus for norovirus GII. Therefore as the performance levels were acceptable, we did not feel it was of monetary value to purchase a common identical RT-PCR platform.

Microscopy

We used formalin ethyl acetate sedimentation to separate parasites from fecal debris (FPC Fecal Parasite Concentration kit, Evergreen Scientific, Los Angeles, California). Organisms were identified morphologically by wet prep and modified acid-fast stain. The study sites were required to have the capacity to photograph parasites for verification purposes.

Quality Assurance/Quality Control

To address the central hypotheses of the MAL-ED study, we required that accurate and complete microbiology data be obtained through harmonized protocols. The microbiology Quality Assurance (QA)/Quality Control (QC) activities were designed to support the site laboratories and to verify data quality, but not to impose an excessive financial or time burden.

Preenrollment Quality Assurance Site Visit

Most microbiology procedures were routine and easily implemented at the MAL-ED study sites. Therefore, the goal of a preenrollment QA site visit (J. G.) was to confirm and document the capacity of each site to adhere to the standardized protocols and deliver reliable microbiology data. The QA site visit included (1) review of all microbiology SOPs and data forms, (2) specimen dry run (collection or receipt to testing with all assays to archive), (3) identification and resolution of any site-specific obstacles to compliance, (4) site-specific training assistance if needed, and (5) International Air Transport Association specimen shipping certification.

Quality Control

Each microbiology SOP specified QC measures to ensure that all kits and reagents were performing to their design and/or operational specifications.

External Quality Assurance

Commercially available proficiency test panels are expensive and not relevant to our testing menu. Thus, the University of Virginia (author E. H.'s laboratory) prepared custom external quality assurance (EQA) panels that were sent to site laboratories both prior to enrollment and then twice per year thereafter. The custom EQA panels were as follows: (1) 5 formalin stool concentrates for wet prep microscopy and modified acid-fast staining, (2) 5 *E. coli* isolates for identification of diarrheagenic *E. coli* by multiplex PCR, (3) 5 blinded bacteria isolates for identification, and (4) 5 frozen undiluted stool specimens (previously characterized) for norovirus quantitative RT-PCR testing. Per usual routines, an 80% consensus was required between sites, and each site needed to have a minimum score of 80% to pass QA. Any discrepant results were investigated, first by sites and then centrally, and corrective action was taken in the form of retesting, which to date has resolved all discrepancies; there have been no reoccurring trends. The summary of all MAL-ED site EQAs to date is as follows:

- Microscopy: 50 challenges, 95% consensus, site scores 92%–100%.
- Bacteriology: 50 challenges, 93% consensus, site scores 80%–100%.
- Diarrheagenic *E. coli* PCR: 50 challenges, 97% consensus, site scores 92%–100%.
- Norovirus PCR: 40 challenges, 80% consensus GI, 96% consensus GII, site scores 80%–100%.

Internal Quality Assurance

Internal quality assurance includes annual competency assessment of staff, continuing education, and investigation of internally discrepant results and unusual rates. MAL-ED study sites shared tools with the other site laboratories (eg, training checksheets, annual competency tests), which served to further build capacity. The collaborative relationships between the microbiologists at all sites allowed for free and open discussions of challenges and concerns. For example, in February 2011, the Bangladesh MAL-ED site observed that a higher rate of specimens than expected tested positive by 1 ELISA assay. Although all kits passed QC, an investigation with the assay manufacturer revealed a possible problem with 1 specific ELISA lot number. All sites were then instructed to retest the positives obtained from the defective ELISA lot, and this retesting corrected a 10%–30% false-positive rate across all sites from the errant lot.

Central Procurement

The geographic distribution of study sites required the procurement of a suite of reagents to work up a single stool specimen. This included media for culture, consumables for microscopy, ELISA kits, and molecular diagnostic reagents. Generally, sites were able to cost-effectively procure the media and microscopy consumables locally. However, we elected to procure the ELISA kits and molecular diagnostic reagents centrally through the University of Virginia and make periodic shipments of supplies to sites, typically twice a year. Centralized procurement minimized shipping costs by consolidating packages, ensured consistency in product and lot numbers, and maximized efficiency in ordering and communicating with vendors. For example, upon placement of these large ELISA kit orders, the manufacturers went into special production for MAL-ED, which enabled the manufacture of a single lot with maximum shelf life (typically 12 months or longer). We also were able to negotiate bulk purchase pricing that further reduced overall costs. The PCR master mix necessitated a dry-ice shipment, which we were able to bundle with the norovirus EQA samples. There were also site-specific needs for materials that could not be sourced locally. Reagents for gut function testing were also procured through this central scheme, which allowed us to rapidly distribute new developmental assays to 8 distant sites.

Overall, the MAL-ED central procurement program purchased 12 ELISA kits, 1 common PCR master mix, nucleic acid extraction kits, and other supplies. We received discounts on average of 49% (range, 9%–84% discount from list price). Given total central procurement supply purchases of $1 963 969.52, this amounted to approximately $2 million in savings, not including additional savings in staff time and shipping, which usually adds approximately 30% to ship to these countries, but only 13% in our scheme ($254 575.05).

CONCLUSIONS

The microbiology efforts of the MAL-ED Network were significant and included the development of harmonized protocols for a diverse range of enteropathogens, which resulted in quality-assured and -controlled results from 8 disparate sites, at maximum throughput and minimum cost. MAL-ED sites and their laboratories, staff, and capabilities were diverse and ranged from world-class centers with decades of experience to untouched rural communities with laboratories constructed out of shipping containers. The atmosphere of MAL-ED was one of collaboration, sharing, and capacity building, with everyone involved learning through the experiences of others. Through this array of diagnostic methods, MAL-ED will offer an unprecedented look at the timing, duration, and burden of enteropathogen carriage in children around the world. Our microbiological approach will capture with great sensitivity the major viruses,

protozoa, and bacteria, with much more sensitive methods than those performed in classic studies [3, 5]. This approach will allow the MAL-ED study to draw new and confirmatory inferences on enteropathogens and diarrheal disease, and their relationship to child growth and immune response. A review of the multiple methods that will be used to analyze the results of MAL-ED's microbiological findings is included in this supplement [42].

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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References

1. Kotloff KL, Blackwelder WC, Nasrin D, et al. The Global Enteric Multicenter Study (GEMS) of diarrheal disease in infants and young children in developing countries: epidemiologic and clinical methods of the case/control study. Clin Infect Dis **2012**; 55(suppl 4):S232–45.
2. Huilan S, Zhen LG, Mathan MM, et al. Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries. Bull World Health Organ **1991**; 69:549–55.
3. Mata L. Diarrheal disease as a cause of malnutrition. Am J Trop Med Hyg **1992**; 47:16–27.
4. Dale DC, Mata LJ. Studies of diarrheal disease in Central America. XI. Intestinal bacterial flora in malnourished children with shigellosis. Am J Trop Med Hyg **1968**; 17:397–403.
5. Black RE, Brown KH, Becker S. Effects of diarrhea associated with specific enteropathogens on the growth of children in rural Bangladesh. Pediatrics **1984**; 73:799–805.
6. Steiner TS, Lima AA, Nataro JP, Guerrant RL. Enteroaggregative *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. J Infect Dis **1998**; 177:88–96.
7. Mera RM, Correa P, Fontham EE, et al. Effects of a new *Helicobacter pylori* infection on height and weight in Colombian children. Ann Epidemiol **2006**; 16:347–51.
8. Windle HJ, Kelleher D, Crabtree JE. Childhood *Helicobacter pylori* infection and growth impairment in developing countries: a vicious cycle? Pediatrics **2007**; 119:e754–9.
9. Thomas JE, Dale A, Bunn JE, et al. Early *Helicobacter pylori* colonisation: the association with growth faltering in The Gambia. Arch Dis Child **2004**; 89:1149–54.
10. Lee G, Pan W, Penataro Yori P, et al. Symptomatic and asymptomatic campylobacter infections associated with reduced growth in Peruvian children. PLoS Negl Trop Dis **2013**; 7:e2036.
11. Checkley W, Gilman RH, Epstein LD, et al. Asymptomatic and symptomatic cryptosporidiosis: their acute effect on weight gain in Peruvian children. Am J Epidemiol **1997**; 145:156–63.
12. Guerrant DI, Moore SR, Lima AA, Patrick PD, Schorling JB, Guerrant RL. Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four–seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg **1999**; 61:707–13.
13. Agnew DG, Lima AA, Newman RD, et al. Cryptosporidiosis in northeastern Brazilian children: association with increased diarrhea morbidity. J Infect Dis **1998**; 177:754–60.
14. Noureldin MS, Shaltout AA, El Hamshary EM, Ali ME. Opportunistic intestinal protozoal infections in immunocompromised children. J Egypt Soc Parasitol **1999**; 29:951–61.
15. Stephenson LS, Latham MC, Ottesen EA. Malnutrition and parasitic helminth infections. Parasitology **2000**; 121(suppl):S23–38.
16. Tshikuka JG, Gray-Donald K, Scott M, Olela KN. Relationship of childhood protein-energy malnutrition and parasite infections in an urban African setting. Trop Med Int Health **1997**; 2:374–82.
17. Freij L, Meeuwisse GW, Berg NO, Wall S, Gebre-Medhin M. Ascariasis and malnutrition. A study in urban Ethiopian children. Am J Clin Nutr **1979**; 32:1545–53.
18. Nokes C, Grantham-McGregor SM, Sawyer AW, Cooper ES, Bundy DA. Parasitic helminth infection and cognitive function in school children. Proc Biol Sci **1992**; 247:77–81.
19. Gupta M, Arora KL, Mithal S, Tandon BN. Effect of periodic deworming on nutritional status of ascaris-infested preschool children receiving supplementary food. Lancet **1977**; 2:108–10.
20. Dickson R, Awasthi S, Williamson P, Demellweek C, Garner P. Effects of treatment for intestinal helminth infection on growth and cognitive performance in children: systematic review of randomised trials. BMJ **2000**; 320:1697–701.
21. Kosek M, Guerrant RL, Kang G, et al. Assessment of environmental enteropathy in the MAL-ED cohort study: theoretical and analytic framework. Clin Infect Dis **2014**; 59(suppl 4):S239–47.
22. Goto R, Panter-Brick C, Northrop-Clewes CA, Manahdhar R, Tuladhar NR. Poor intestinal permeability in mildly stunted Nepali children: associations with weaning practices and *Giardia lamblia* infection. Br J Nutr **2002**; 88:141–9.
23. Lunn PG, Northrop-Clewes CA, Downes RM. Intestinal permeability, mucosal injury, and growth faltering in Gambian infants. Lancet **1991**; 338:907–10.
24. Brewster DR, Manary MJ, Menzies IS, O'Loughlin EV, Henry RL. Intestinal permeability in kwashiorkor. Arch Dis Child **1997**; 76:236–41.
25. Sullivan PB, Lunn PG, Northrop-Clewes CA, Farthing MJ. Parasitic infection of the gut and protein-losing enteropathy. J Pediatr Gastroenterol Nutr **1992**; 15:404–7.
26. Murray PR, Baron EJ. Manual of clinical microbiology. Washington, DC: ASM Press, **2007**.
27. Galbadage T, Jiang ZD, DuPont HL. Improvement in detection of enterotoxigenic *Escherichia coli* in patients with travelers' diarrhea by increasing the number of *E. coli* colonies tested. Am J Trop Med Hyg **2009**; 80:20–3.
28. Barletta F, Ochoa TJ, Ecker L, Gil AI, Lanata CF, Cleary TG. Validation of five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic *Escherichia coli*. J Clin Microbiol **2009**; 47:1915–7.

29. Nguyen TV, Le Van P, Le Huy C, Gia KN, Weintraub A. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. J Clin Microbiol **2005**; 43:755–60.
30. Panchalingam S, Antonio M, Hossain A, et al. Diagnostic microbiologic methods in the GEMS-1 case/control study. Clin Infect Dis **2012**; 55(suppl 4):S294–302.
31. Cebula TA, Payne WL, Feng P. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. J Clin Microbiol **1995**; 33:248–50.
32. Taniuchi M, Walters CC, Gratz J, et al. Development of a multiplex polymerase chain reaction assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool. Diagn Microbiol Infect Dis **2012**; 73:121–8.
33. Steinsland H, Valentiner-Branth P, Perch M, et al. Enterotoxigenic *Escherichia coli* infections and diarrhea in a cohort of young children in Guinea-Bissau. J Infect Dis **2002**; 186:1740–7.
34. Tolcin R, LaSalvia MM, Kirkley BA, Vetter EA, Cockerill FR, Procop GW. Evaluation of the Alexon-Trend ProSpecT *Campylobacter* microplate assay. J Clin Microbiol **2000**; 38:3853–5.
35. Platts-Mills JA, Liu J, Gratz J, et al. Campylobacter detection and significance in stool by culture, enzyme immunoassay, and PCR in developing country settings. J Clin Microbiol **2014**; 52:1074–80.
36. Garcia LS, Shimizu RY. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. J Clin Microbiol **1997**; 35:1526–9.
37. Okitsu-Negishi S, Okame M, Shimizu Y, et al. Detection of norovirus antigens from recombinant virus-like particles and stool samples by a commercial norovirus enzyme-linked immunosorbent assay kit. J Clin Microbiol **2006**; 44:3784–6.
38. Burton-MacLeod JA, Kane EM, Beard RS, Hadley LA, Glass RI, Ando T. Evaluation and comparison of two commercial enzyme-linked immunosorbent assay kits for detection of antigenically diverse human noroviruses in stool samples. J Clin Microbiol **2004**; 42: 2587–95.
39. Costantini V, Grenz L, Fritzinger A, et al. Diagnostic accuracy and analytical sensitivity of IDEIA norovirus assay for routine screening of human norovirus. J Clin Microbiol **2010**; 48:2770–8.
40. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, Gray J. Diagnosing norovirus-associated infectious intestinal disease using viral load. BMC Infect Dis **2009**; 9:63.
41. Kageyama T, Kojima S, Shinohara M, et al. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol **2003**; 41:1548–57.
42. Platts-Mills JA, McCormick BJJ, Kosek M, Pan W, Checkley W, Houpt ER. Methods of analysis of enteropathogen infection in the MAL-ED cohort study. Clin Infect Dis **2014**; 59(suppl 4): S233–8.