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21st Century Approaches To Addressing Childhood Diarrhea In Low And Middle-Income Countries: Zinc As A Cornerstone Of New Prevention Strategies

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21ST CENTURY APPROACHES TO ADDRESSING CHILDHOOD DIARRHEA IN LOW AND MIDDLE-INCOME COUNTRIES: ZINC AS A CORNERSTONE OF NEW PREVENTION STRATEGIES

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ABSTRACT

During the 20th century, significant strides were made in curtailing the burden of childhood diarrhea, including advances in vaccine research, the advent of antibiotics, improved water and sanitation, and expanded access to health information across the globe. Despite this progress, today diarrhea ranks second only to pneumonia as a leading cause of mortality in children under five years, with a disproportionate burden of 90% of diarrheal deaths in South Asia and Sub-Saharan Africa. Additionally, substantial morbidity due to diarrhea persists in young children, with more than 45 million disability-adjusted life years (DALYs) lost due to diarrhea in 2015. Long-term consequences of childhood diarrhea include undernutrition, impaired gut function, altered gut microbiota, and compromised cognitive development.

The 21st century presents an opportunity to eliminate the health disparity affecting millions of children suffering disproportionately from preventable diarrheal diseases. Recent advances in molecular laboratory technology have enabled detailed assessment of diarrheal burden and etiology, illuminating the highest burden pathogens for focused interventions. Among the top diarrheal pathogens, rotavirus (RV) is the leading cause of diarrhea-attributable death in the first year of life. While we have vaccines against RV, these vaccines consistently underperform in low and middle-income countries (LMICs) with efficacy of 18% to 61% compared to > 85% efficacy in high income countries. Reasons for rotavirus vaccine underperformance remain unclear, and no vaccines are available for other high burden diarrheal pathogens. This requires consideration of complementary and alternative interventions for diarrhea prevention.

To assess factors related to rotavirus vaccine performance, we enrolled a 700-infant birth cohort in an urban slum of Dhaka, Bangladesh, in the Performance of Rotavirus and Oral Polio Vaccines in Developing Countries (PROVIDE) study: a randomized controlled trial of a 2-dose monovalent oral rotavirus vaccine (RV1). With a primary outcome of any rotavirus diarrhea (RVD) post-vaccination to one year, we conducted biweekly home-based diarrhea surveillance with rotavirus antigen detection in diarrheal stools by ELISA. We found RV1 efficacy of 51% (95% CI 33.8–63.7) in per protocol analysis. Importantly, among 12 explanatory variables tested for association with RVD, serum zinc concentration (SZC) in infants at week 18 associated with risk of RVD up to one year (OR 0.77, 95% CI 0.66–0.91), independent of vaccination status. This finding led to broader investigation of the relationship between zinc status and diarrhea in the PROVIDE cohort.

Among 577 PROVIDE infants, 16.5% were zinc deficient at week 18 (SZC < 65μg/dL). By logistic regression, zinc deficient infants had increased odds of diarrhea in the first year of life compared to zinc replete infants (OR 2.76, 95% CI 1.08–7.04), and they were nearly 4 times more likely to have diarrhea of viral etiology (OR 3.94, 95% CI 1.55–10.03). Furthermore, in Kaplan Meier analysis we found a strong correlation between zinc deficiency and time to first episode of viral diarrhea (median survival 27 vs 33 weeks in zinc deficient vs non-deficient infants, $p < 0.0001$), with zinc deficient infants at 55% greater risk of viral diarrhea (HR 1.55, 95% CI 1.21 – 1.99).

Our results indicate further consideration of zinc as a critical and modifiable co-factor in ameliorating the burden of childhood viral diarrhea. Carefully designed trials of zinc supplementation interventions could determine whether zinc may fill the gap in protection against childhood viral diarrhea, and inquiries into the zinc-diarrhea molecular pathway could elucidate mechanisms for focused development of future interventions.

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This is dedicated to my son, Soitmatua, whose intelligence, curiosity, support and understanding inspires me every day. He earned this as much as I did.

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1.1. Introduction

During the 20th century, significant strides were made in curtailing the burden of childhood diarrhea, including advances in vaccine research, the advent of antibiotics, improved water and sanitation, and expanded access to health information across the globe. Despite this progress, today over half a million children die from diarrhea-associated complications before their fifth birthday [1]; almost all of them in South Asia and Sub-Saharan Africa. The 21st century presents an opportunity to incorporate past successes with cutting-edge basic science and excellent clinical research to drive our understanding and elimination of the persistent public health challenge of childhood diarrhea.

To that end, the objective of this review is to identify optimal preventive interventions to address the health disparity affecting millions of children suffering disproportionately from preventable diarrheal diseases. This work constitutes a fully translational inquiry beginning with a review of the global epidemiology of childhood diarrheal morbidity and mortality, including our recently expanded understanding of diarrheal burden and etiology. This brings into sharp relief questions about effective prevention strategies against lead diarrheal pathogens. Review of currently available diarrhea interventions follows, with particular emphasis on zinc for diarrhea prevention based on results from

original research in Bangladesh on rotavirus vaccine efficacy (Chapter 2), which led to observations correlating zinc deficiency with increased risk of viral diarrhea (Chapter 3). Review of the zinc literature culminates with hypotheses on the molecular mechanisms involved in the zinc-diarrhea pathway and evaluation of evidence on the effectiveness of zinc supplementation for diarrhea prevention. The review ends with discussion of unanswered questions and future directions.

1.2. Epidemiology of childhood diarrhea

In 2000, the global community set ambitious priorities for ameliorating poverty and public health disparities in the 21st century with the declaration of the United Nations' (UN) Millennium Development Goals. One of the eight targets agreed by UN member states was a two-thirds reduction in child mortality by 2015. This goal brought into focus the leading causes of mortality in children under 5 years, among which diarrhea ranks second only to pneumonia in the post-neonatal period [2]. Diarrhea-attributable mortality can result from either acute or chronic diarrhea, with dehydration and malnutrition as the immediate cause of death from each condition, respectively.

Despite significant progress, the target reduction in child mortality is yet to be met, with overall deaths declining one third from 9.9 million in 2000 to 6.3 million in 2013. Diarrhea-attributable mortality has decreased at an annual rate of reduction of 6.5% since 2000; however diarrhea still accounts for over half a million child deaths annually, with a disproportionate burden of 90% of diarrheal mortality in South Asia and Sub-Saharan Africa [1-3], as shown in **Figure 1.1.**.

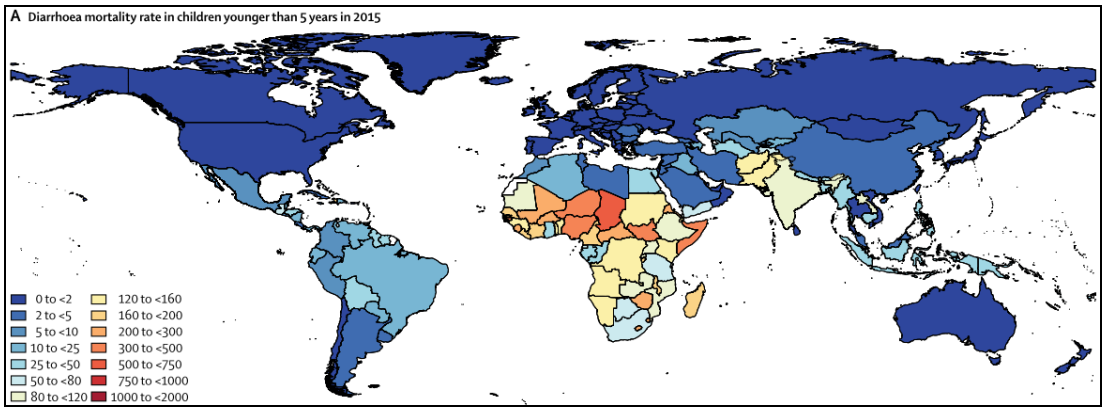

Figure 1.1. Disproportionate burden of diarrheal mortality in South Asia and Sub-Saharan Africa, 2015 (From: Global Burden of Diseases Diarrhoeal Diseases Collaborators [3])

In addition to the challenge of reducing diarrhea-attributable mortality, substantial morbidity due to diarrhea persists in young children. Recent analysis estimates more than 45 million disability-adjusted life years (DALYs) lost due to diarrhea and approximately one billion episodes of diarrhea in children under 5 in 2015 [3]. Again, this burden falls disproportionately on children in South Asia and Sub-Saharan Africa. Diarrheal incidence decreased more slowly than mortality over the past few decades, with a reduction from 3.4 to 2.9 episodes per child-year between 1990 and 2010 [4].

The long-term consequences of childhood diarrhea are just beginning to be understood and include undernutrition (linear growth faltering and underweight), impaired gut function, dysregulation of inflammatory and immune responses, altered microbiota, and compromised cognitive development [5-8]. Collectively, these sequelae contribute to decreased productivity and poor health outcomes into adulthood.

1.2.1. Epidemiology of viral diarrhea

Very little has been published on the epidemiology of viral diarrhea as a discrete etiology. Diarrheas of viral etiology share a few key features. First, viral diarrhea knows no boundaries. Rotavirus, the leading cause of childhood diarrhea globally (see below), accounted for the highest burden of childhood diarrhea in the United States and other high income countries (HICs) prior to vaccine introduction in the 2000's [9]. Similarly, other top viral pathogens are found to cause diarrhea across the globe, regardless of national income or infrastructure. Diarrheal viruses tend to infect humans early in life, with the highest rates of viral diarrhea due to rotavirus, adenovirus, norovirus, sapovirus and astrovirus all occurring in children < 2 years [9]. Clinically, viral diarrheas are often grouped together as relatively short duration and self-limiting; these clinical observations from HICs may not be relevant in LMICs where children develop under different living conditions and may be pre-disposed to longer duration, greater severity or increased risk of viral diarrhea.

1.3. Etiology of diarrhea

In order to identify optimal interventions to address the continued burden of diarrheal morbidity and mortality, scientists recognized the need to better understand the etiology of childhood diarrhea, particularly in low and middle-income countries (LMICs). To this end, two landmark, multi-site studies were conducted between 2007–2014, enrolling over 24,000 children across 12 countries in South Asia, Africa and South America: the Global Enteric Multicenter Study (GEMS), and the Malnutrition and Enteric Disease Study (MAL-ED). GEMS was a case-control study of moderate-to-severe diarrhea in children

age 1-59 months, while MAL-ED was a longitudinal birth cohort with community-based diarrheal detection (i.e. milder cases) in which children were enrolled at birth and followed for 2 years including collection of both diarrheal and asymptomatic stools. Despite different study designs, the top pathogens contributing to attributable incidence of diarrhea in GEMS and MAL-ED were similar, particularly in the first year of life.

Statistical methods for determining attributable incidence in GEMS and MAL-ED are extensively detailed elsewhere [10, 11], and include first estimation of pathogen-specific attributable fractions by Quantification Cycle (Cq) read-outs from quantitative polymerase chain reaction (PCR) TaqMan Array Card (TAC) assay (detailed below). Attributable fractions per pathogen were adjusted for prevalence of asymptomatic carriage by odds of detection in cases vs controls, Cq value, quantity of co-pathogens, study site, age and sex. Attributable incidence was then calculated from attributable fraction estimates based on previous methods [12].

Rotavirus (RV) accounted for the highest attributable incidence of diarrhea during infancy in all study sites where RV vaccines had not been introduced, and RV remained the fifth leading cause of diarrhea in countries with RV vaccines [10, 11]. This aligns with estimates from the World Health Organization's (WHO) Global Rotavirus Surveillance Network that approximately 40% of hospitalizations for acute diarrhea in children under 5 in LMICs are due to rotavirus [13].

Although the GEMS and MAL-ED studies showed some geographic heterogeneity in pathogen prevalence, other important diarrheal agents in the first year of life were adenovirus 40/41, *Shigella* spp, *Campylobacter* spp, heat-stable enterotoxigenic *E. coli* (ST-ETEC), and *Cryptosporidium* spp. In GEMS, 77.8% of attributable diarrhea was due to RV and these five pathogens [10]. Notably, in MAL-ED, norovirus GII was the highest burden diarrheal pathogen in countries with RV vaccine, a trend also seen in the United States after introduction of RV vaccines in 2006 when norovirus GII replaced rotavirus as the leading cause of acute gastroenteritis in children [1, 14].

1.3.1. Next generation diagnostic tools

The findings in both the GEMS and MAL-ED studies were facilitated by a next generation molecular laboratory assay, the quantitative polymerase chain reaction (PCR) TaqMan Array Card (TAC). TAC originated as a tool for gene expression studies and was adapted for detection of respiratory and bioterror pathogens in 2010-2012. In 2012, the assay was developed for stool-based enteropathogen detection at the University of Virginia [15]. The TAC platform consists of a set of individual, microbe-specific, probe-based assays assembled on a single card for simultaneous nucleic acid detection of multiple pathogens per stool sample for up to 42 pathogen targets per card at present. The assay read-out is a quantification cycle value (Cq) indicating the amplification cycle number at which flouresced pathogen-specific nucleic acid is detectable above background. Similar to C_T values for other PCR-based platforms, the positive detection range for TAC Cq has been established as $0 < Cq < 35$ based on comparator studies with conventional and other PCR methods [15, 16].

The TAC assay addresses some of the shortcomings of conventional lab methods for pathogen detection (i.e. culture, microscopy, ELISA and PCR), including: time and cost associated with multiple tests on individual stools, limited comparability across sites using different detection methods for specific pathogens, risk of contamination of specimens or assays when moving between platforms, and most importantly, poor sensitivity for detection of several diarrheal pathogens. The TAC assay has undergone extensive optimization and validation across international sites, as well as head-to-head comparisons for pathogen-specific specificity and sensitivity compared to conventional methods [16, 17]. Importantly, with the exception of ELISA for rotavirus detection, conventional methods generally showed poor sensitivity and similar specificity compared to TAC [16].

The TAC assay has limitations. Notably, near the TAC assay's lower limit of detection (Cq = 30-35) the odds ratios of pathogen detection in cases vs controls approaches 1 for most pathogens, possibly indicating less clinically meaningful results at this end of the detection range [16]. In this case, an odds ratio of 1 indicates no difference between cases and controls, while an odds ratio < 1 suggests greater pathogen carriage in children without overt diarrhea (controls). Additionally, with all pathogen targets assembled on a single card, re-running a whole card for repeat tests of individual pathogens is relatively expensive, and back-up conventional methods for single-pathogen repeats are necessary. Finally, for labs without PCR capacity, substantial initial investment in a PCR platform is

required, though once established, TAC is less expensive per specimen compared to running multiple conventional methods.

As applied to the GEMS and MAL-ED studies, TAC has illuminated two important aspects of diarrheal disease previously obscured by conventional lab methods. First, due to its high sensitivity (median 90.2% [IQR 84.2 – 95.1]), TAC detected a high prevalence of enteric co-infections in both diarrheal and control specimens [16]. For example, in GEMS, ≥ 2 pathogens were detected *at diarrhea-associated Cq values* in nearly 40% of diarrheal stools (e.g. low Cq indicating high pathogen load with an odds ratio > 1 for detection in cases vs controls). This finding revolutionizes our understanding of diarrheal etiology, moving us past the model of single-pathogen diarrhea causation. Secondly, analytic methods applied to TAC data suggest high levels of subclinical carriage of many pathogens in controls based on the odds of pathogen presence in diarrheal vs control specimens [10, 16].

These findings challenge the field to define a clinically-meaningful TAC Cq cut-off for each pathogen that associates with diarrhea, and to grapple with the biologic relevance and consequences of 1) multiple pathogens present at diarrhea-associated levels per stool (how do we determine attribution?), and 2) the substantial prevalence of pathogenic microbes found in children without overt diarrhea. Interpretation of TAC data and the biologic implications of TAC findings is a work-in-progress, but this new tool has already boosted our understanding of the complexity of diarrheal disease in the 21st century.

1.4. Prevention of diarrhea

Armed with a better understanding of diarrheal etiology and the complex mix of co-infections in which pathogen exposure either does or does not lead to diarrhea, we turn to the question of prevention. How do we leverage emerging knowledge and tools to address the persistent burden of childhood diarrheal morbidity and mortality in LMICs? **Table 1.1.** summarizes the current prevention (vaccines) and treatment options for the top seven diarrhea-associated pathogens among infants in LMICs.

Table 1.1. Summary of prevention and treatment options for key pathogens (immunocompetent infants)

Pathogen	***Pathogen Class***	***Transmission route***	***Licensed vaccine(s)?***	***Treatment options***
Rotavirus	Virus	Fecal-oral Possibly respiratory (limited evidence [18])	Yes, with 18-61% efficacy in LMICs	ORS and zinc
Norovirus GII	Virus	Fecal-oral	No	ORS and zinc
Adenovirus 40/41	Virus	Fecal-oral	No	ORS and zinc
***Campylobacter* spp**	Bacteria	Fecal-oral	No	ORS and zinc Fluoroquinolone and azithromycin, antibiotic resistance is common (severe disease only)
***Shigella* spp/EIEC**	Bacteria	Fecal-oral	No	ORS and zinc Fluoroquinolone and azithromycin, antibiotic resistance is common
ST-ETEC	Bacteria	Fecal-oral	No	ORS and zinc Fluoroquinolones
Cryptosporidium	Parasite	Fecal-oral Zoonosis	No	ORS Nitazoxanide

Abbreviations: spp=species; EIEC=Enteroinvasive *Escherichia coli*; ST-ETEC=Enterotoxigenic *Escherichia coli;* ORS=oral rehydration solution.
Table compiled from pathogen-specific information available from the Centers for Disease Control and Prevention (CDC) [19]

Our focus is on prevention of diarrhea, as opposed to treatment, particularly given the limited treatment options against viral enteropathogens and the problematic development of antimicrobial resistance against antibiotics for the leading causes of bacterial diarrhea in LMICs (an issue beyond the scope of this inquiry), as indicated in **Table 1.1.**.

1.5. Vaccines for diarrhea prevention

Rotavirus (RV) is the only leading diarrheal pathogen for which licensed vaccines are currently available. Vaccine development for *Shigella* spp, ST-ETEC, *Campylobacter*, and norovirus is ongoing, though no vaccine candidates are near licensure [14, 20]. Significant challenges to vaccine development remain for adenovirus 40/41 and *Cryptosporidium* spp, and vaccines for these enteric pathogens are not under clinical investigation.

1.5.1. Rotavirus vaccines

Given the high relative contribution of RV to diarrheal morbidity and mortality in infancy, substantial efforts focused on RV vaccine development in the 1990s. To date, five live, oral RV vaccines have been licensed, and two are pre-qualified by the WHO and available in over 100 countries: Rotarix™ (RV1) and Rotateq® (RV5). Several other live, oral RV vaccines are under development, as well as next generation inactivated parenteral and subunit vaccines (subunit vaccines consist of viral protein fragments, as opposed to whole virus) [21, 22].

In high-income countries (HICs), where RV was the leading cause of childhood diarrhea prior to vaccine introduction in the mid-2000's, RV1 and RV5 demonstrated efficacy exceeding 95% against severe RV gastroenteritis [23, 24]. Following vaccine introduction in the US in 2006, hospitalizations for RV decreased by up to 94%, and the Centers for Disease Control and Prevention (CDC) reported up to 89.9% declines in

laboratory detection of RV compared to pre-vaccination years [25, 26]. Furthermore, cost savings from reductions in health care utilization for RV gastroenteritis in the four years following vaccine introduction (2007-2011) were estimated at $924 million. Vaccine effectiveness studies have shown sustained protection against RV hospitalizations in HICs using RV vaccines [27].

In contrast, efficacy and effectiveness of the same oral RV vaccines ranges from 18─61% in LMICs, leaving a gap in vaccine protection against the highest-burden diarrheal pathogen, for reasons that remain unclear [28-31]. Further complicating interpretation of low vaccine efficacy, assessment of the standard immunogenicity measure for RV vaccines, anti-RV IgA, has found mixed results for correlation with vaccine efficacy in LMICs [32-35]. For example, RV vaccine efficacy trials in Malawi and South Africa found only 43.6% of the vaccine group (treatment) effect explained by post-vaccination RV IgA seropositivity [34]. Additionally, post-vaccination IgA seroconversion and geometric mean concentrations among infants in countries with high child mortality rates (LMICs) fall significantly below those of vaccinated infants in countries with low child mortality (HICs): 53% vs 87% and 47 U/mL vs 236 U/mL respectively [35]. Determination of improved correlates of protection for RV is currently underway in our research group.

Substantial research efforts by our team and others have aimed to identify factors that explain RV vaccine underperformance in LMICs, including: maternal breast milk and transplacental RV antibody inhibiting vaccine take [36-38]; delayed dosing of RV

vaccine to improve immune responses [39, 40]; RV strain diversity and homotypic vs heterotypic vaccine protection [41]; RV seasonality and timing of vaccine dosing [42]; co-administration of oral polio vaccine and its effect on RV vaccine immunogenicity [43, 44]; and systemic inflammation association with reduced RV vaccine immunogenicity [6]. Additional recent studies have found associations between histo-blood group antigen polymorphisms (Lewis and secretor status) and risk of RV diarrhea, and microbiome composition correlations with RV vaccine immunogenicity [45-48]. While these studies offer insight into aspects of this complex problem, considerably more work must be done to identify discrete factors that, collectively or individually, explain RV vaccine performance.

Despite the remaining questions around RV vaccine efficacy in LMICs, the global community, supported by the WHO and the Global Alliance for Vaccines and Immunizations (GAVI), are proceeding with global roll-out of the currently licensed oral RV vaccines on the premise that protection of ~50% is better than none, particularly in countries with high diarrhea-attributable child mortality [49]. Given that RV is the highest burden diarrheal pathogen in countries without the vaccines, vaccine roll-out can be expected to have a substantial impact on diarrheal morbidity and mortality; however, a significant gap remains in protection requiring consideration of complementary interventions.

1.6. Non-vaccine diarrhea prevention measures

Given that RV is the only high-burden pathogen for which licensed vaccines are available, as well as the efficacy issues with RV vaccines raised above, what other preventive interventions may contribute to the goal of reducing childhood diarrheal morbidity and mortality? Decades of public health research have tried to address this question, with three interventions consistently mentioned in the literature on diarrhea prevention: 1) household-level improvements in water, sanitation and hand washing; 2) promotion of exclusive breastfeeding; and 3) micronutrient supplementation, specifically zinc and vitamin A.

1.6.1. WASH interventions

The axiom that improved water, sanitation and handwashing (WASH) ameliorates diarrheal burden has persisted; however, recent evidence challenges this canon. Pickering *et al* found no difference in diarrhea prevalence in children from cluster-randomized villages with and without a community-led total sanitation intervention [50]. Baker *et al*, reporting on handwashing practices among caregivers in the GEMS study, found no difference in the incidence of diarrhea among children whose caregivers washed their hands with soap vs water and ash [51]. In the case of RV, the field has long rejected a significant role for WASH in risk of RV diarrhea due to the ubiquitous prevalence of RV in HICs with improved sanitation prior to vaccine introduction [18, 27]. We are awaiting the results of the multi-site WASH Benefits trial enrolling a birth cohort of nearly 14,000 children in Kenya and Bangladesh with a primary outcome of diarrhea across six randomization arms with different sets of WASH interventions [52]. Interesting

hypotheses to pursue with next generation tools such as TAC would be whether the effectiveness of WASH interventions is pathogen-specific, if there is greater impact on bacterial diarrhea vs viral diarrhea, and whether success of WASH interventions associates with inoculum size or pathogen mode of transmission (i.e. waterborne transmission is more likely addressed by WASH interventions).

1.6.2. Promotion of exclusive breastfeeding

The benefits of exclusive breastfeeding (EBF) have been well characterized and reviewed numerous times [53]. In short, infants, particularly in the first 6 months of life, have immature and developing immune systems. During this period, breast milk not only provides essential fats and micronutrients for survival and optimal growth, but also transfers immune components, specifically IgA, to protect the infant during immune maturation. As reviewed by Duijts *et al*, interventional and observational studies in high income countries consistently found reduced risk of diarrheal disease with increased duration of exclusive breastfeeding, often in a dose-dependent manner with each additional month of exclusive breastfeeding [54]. Similar to other studies, results from the Southhampton Women's Survey showed a relative risk of diarrhea from birth to 6 months of RR 0.43 (95% CI 0.30–0.61) in infants breastfed for 6+ months vs those never breastfed [55]. In LMICs with high levels of infectious disease exposure, breastfeeding is critical for early protection from diarrheal (and other) pathogens.

1.6.3. Micronutrient supplementation

Among micronutrients, deficiencies of zinc and vitamin A have been implicated for association with increased diarrheal risk in the literature [3]. Among interventions for the prevention of diarrhea, zinc prophylactic supplementation has shown positive results in reducing the burden of diarrhea in LMICs [56-59]; however, clinical findings in numerous trials have not elucidated a mechanism(s) by which zinc may affect diarrheal risk, nor examined zinc associations with specific pathogens or class of pathogens. As reported in Chapter 2, we found no correlation between vitamin A (measured as retinol binding protein) and RV diarrhea in the PROVIDE Study; however infant serum zinc level was inversely associated with risk of RV diarrhea.

1.7. The PROVIDE Study

From 2010-2014, we conducted the "Performance of Rotavirus and Oral Polio Vaccines in Developing Countries" (PROVIDE) Study in a high-density urban slum in Dhaka, Bangladesh to better understand why oral vaccines (rotavirus and polio) perform sub-optimally in LMICs. The study's central hypothesis was that failure of oral vaccines is due to environmental enteric dysfunction (EED), a subclinical condition common in LMICs characterized by gut barrier dysfunction, malabsorption and permeability of the small intestine. The study design was 2x2 factorial with two interventional randomized controlled trials (RCT): a RV vaccine efficacy trial, reported here and in Chapter 2 [60], and a trial of the impact on mucosal immunity of an inactivated polio vaccine (IPV) boost on the standard oral polio vaccine (OPV) series. Results of the polio trial are reported elsewhere [61], and exploratory analysis of select biomarkers of EED and associations

with vaccine performance in the first year of life are published [6]. We enrolled 700 infants in the first week of life and their mothers and followed them for two years.

With the 2013 WHO recommendation for global roll-out of RV vaccines into all national immunization programs, PROVIDE was one of the last RCTs of RV vaccine efficacy in the pre-vaccination era. To date, Bangladesh has not added RV vaccine to the national program; however, GAVI has committed funding support for RV vaccine introduction, which is scheduled to begin in 2018.

For the PROVIDE RV vaccine efficacy trial, infants were randomized 1:1 to receive RV1 or no RV1 on a delayed dosing schedule of 10 and 17 weeks (vs 6 and 10 weeks on the standard WHO schedule). To better understand factors associated with RV vaccine efficacy, we collected extensive socio-economic, clinical and biologic data, including baseline household surveys, maternal surveys, comprehensive diarrhea surveillance, feeding practices, regular anthropometry, cognitive function assessments, blood, diarrheal and non-diarrheal stools, urine, saliva, and breast milk. Infants received all Bangladesh national Expanded Programme on Immunization (EPI) vaccines through the study clinic, which also provided free, high-quality primary care to both mothers and infants throughout study participation. Detailed study methods are published [62].

As described in Chapter 2, in PROVIDE we found similar RV vaccine efficacy results compared to other trials in LMICs: 51% (95% CI, 33.8%–63.7%) against RV diarrhea (RVD) of any severity and 73.5% (95% CI, 45.8%–87.0%) against severe RVD in the

per-protocol analysis [60]. We modeled risk of RV diarrhea using multivariable logistic regression with 12 co-variates based on the literature and biologic plausibility, including RV vaccine, socio-demographic and household variables, sex, breastfeeding, RV IgA seroconversion and baseline infant health indicators (linear growth, retinol binding protein, vitamin D and serum zinc).

In the final model, only lack of RV vaccine (OR 2.84, 95% CI 1.87–4.30) and serum zinc status (OR 0.77, 95% CI 0.66─0.91) correlated with risk of RV diarrhea in the first year of life: for every 10 µg/dL increase in serum zinc concentration (SZC), the odds of RV diarrhea decreased 23%. No interaction was detected between SZC and RV vaccine; the association between RV1 and RV diarrhea did not depend on zinc level (interaction term $p > 0.33$). For severe RV diarrhea, only vaccination correlated with risk of disease.

Building on these results, Chapter 3 reports detailed analysis of the association between zinc deficiency in the PROVIDE cohort and risk of pathogen-specific diarrhea, as well as any viral diarrhea, using data from TAC analysis of PROVIDE diarrheal stool specimens. Compared to the current literature, this is the most complete evaluation of the association between zinc deficiency and specific pathogens to date.

In PROVIDE, 16.5% of the per-protocol infant population (N=577) were serum zinc deficient at 18 weeks. These infants were 2.76 times more likely to have diarrhea in the first year compared to zinc replete infants (OR 2.76, 95% CI 1.08 – 7.04), and zinc deficient infants were nearly 4 times more likely to have viral diarrhea compared to non-

zinc deficient infants (OR 3.94, 95% CI 1.55 – 10.03). Furthermore, in Kaplan Meier analysis, we found a strong correlation between zinc deficiency and time to first episode of viral diarrhea, with median survival time of 27 weeks among zinc deficient infants compared to 33 weeks in non-zinc deficient infants (p<0.0001). Infants with zinc deficiency were at 58% greater risk of viral diarrhea compared to non-deficient infants (HR 1.58, 95% CI 1.24 – 2.02).

The strength of these findings, combined with the results of our RV vaccine efficacy trial (Chapter 2), led to an in-depth review of the zinc literature to better understand the clear association between zinc deficiency and diarrhea, and to generate hypotheses to interrogate the mechanism(s) of this association.

1.8. Zinc as an essential trace metal

Elemental zinc is essential to maintaining overall health and metabolism in humans as well as plants, invertebrates, vertebrates and microbes. Known as a Type II nutrient due to its ubiquitous roles, zinc catalyzes more than 300 metalloenzymes, anchors the structural integrity of the genome, and regulates gene expression. Zinc also plays crucial roles in cellular proliferation, differentiation and apoptosis, as well as in cell signaling, response to infection, and inflammatory processes [56, 63-65].

1.9. Zinc bioavailability

The richest sources of zinc are animal products: shell fish, red meats, and dairy products [66]. Zinc prolifically binds protein thereby facilitating zinc absorption in the intestines

during protein digestion. High-protein diets are uncommon in LMICs where plant-based diets are prevalent [67, 68]. In contrast to animal products, plant-based food sources are high in phytate, especially legumes and grains, which are the mainstays of the Bangladeshi diet (dal and rice). Phytate, the salt molecule of phytic acid found in plants, binds both dietary and endogenous zinc, reducing zinc's bioavailability, blocking effective zinc absorption, and disrupting endogenous zinc homeostatic mechanisms [69]. While homeostatic regulation of zinc can cope with low zinc bioavailability in the absence of phytate, there does not seem to be an adaptation in adult humans to increase zinc absorption when faced with high phytate diets [70]. In this setting, reducing the ratio of phytate to zinc in dietary intake, development and distribution of zinc-fortified foods, fermentation to reduce phytate content in plant-based foods, or zinc supplementation are the available options for addressing this physiologic limitation [56, 71, 72].

Interestingly, phytate inhibition of zinc absorption may be particular to adults. A compelling modeling exercise incorporating data from 8 studies showed a lack of effect of dietary phytate consumption on absorbed zinc among children aged 6 months – 13 years [73], a finding supported by several studies in diverse geographic locations [74-76]. The authors hypothesized that phytate is hydrolyzed in the upper gastrointestinal tract of infants and children, who naturally have a higher stomach pH than adults, inducing enzymatic activity aimed at degrading phytate during early digestion.

Relevant to this inquiry, the sole source of zinc nutrition for infants during EBF is from breast milk. Poor zinc nutrition in infants during EBF therefore is either a result of

insufficient zinc in breast milk or poor absorption of zinc in the infant. One recent study in Thailand found correlations between zinc deficiency in infants and both maternal zinc status and zinc levels in breast milk [77]; however the same result was not found in several studies conducted 10 to 30+ years ago, as reviewed by Donangelo *et al* [78]. Zinc levels in breast milk have been shown to be conserved, even under maternal zinc stress, in a murine model, and one zinc supplementation trial of lactating human mothers showed no effect of supplementation on breast milk zinc levels [79, 80]. A comprehensive review by Brown *et al* concluded the most important determinant of breast milk zinc concentration is length of time post-partum (with breast milk zinc declining over time), as opposed to maternal zinc status [81].

Despite naturally declining zinc levels over the first 6 months post-partum, breast milk continues to be an important source of zinc after cessation of EBF, particularly where dietary zinc in complementary foods is suboptimal [81]. In the PROVIDE study, mean duration of EBF was 17 weeks, short of the WHO-recommended 24 weeks, after which the nutritional content in complementary foods becomes critical. In Bangladesh only 40-45% of the recommended zinc intake for infants is met through breastfeeding plus typical complementary foods [82].

1.10. Zinc homeostatic regulation

Given the intrinsic importance of zinc in a multitude of cellular processes, zinc in the body is subject to tight homeostatic regulation. Zinc homeostasis is achieved through the balancing effects of zinc absorption and excretion, which maintain zinc equilibrium

across a broad range of dietary zinc intake levels. Both absorption and excretion take place primarily in the intestines, with some urinary excretion, and both are responsive to zinc status and changing physiologic needs [83, 84]. The highest rate of zinc absorption in the small intestine takes place in the jejunum, but the greatest volume in the duodenum [56]. Endogenous excretion of zinc into the intestinal lumen adjusts most rapidly to changes in zinc intake, with marked decreases in excretion under conditions of low dietary intake [85]. Zinc excretion occurs primarily via cellular release of zinc into the intestinal lumen followed by fecal excretion.

In the setting of very low zinc intake, small zinc reserves in specific organs (liver, kidney, spleen and pancreas) or minute cellular reserves in organelle compartments may be tapped to maintain essential functions [56]. Bones hold approximately 30% of whole body zinc, and bone reserves seem to provide a zinc reservoir during very low intake, though the mechanism has not been characterized. It has been hypothesized that during low intake, bones forego their usual uptake of zinc from the serum to sustain other tissues (i.e. heart and muscles) [85].

Serum zinc level, which represents only approximately 1% of whole body zinc, is less responsive to changes in dietary zinc intake, except at very low levels of zinc availability, presumably to preserve circulating zinc for distribution to body tissues. Serum zinc concentration (SZC) does, however, respond quickly to initiation and cessation of zinc supplementation [86, 87]. Generally, clinical signs and symptoms of zinc deficiency are not detectable in advance of a precipitous drop in SZC. (Note: Although plasma versus

serum zinc concentrations (SZC) can differ slightly, likely due to differences in blood processing, they are both considered valid measurements of zinc levels in individuals [88].) Standard reference ranges for SZC, based on data from the National Health and Nutrition Examination Survey (USA), are published [56, 89]. Reference ranges for populations in LMICs are not available.

At the cellular level, zinc homeostasis is mediated by methallothionein (MT) and zinc transporter proteins. Synthesis of MT, an intracellular zinc-binding protein discussed in detail below, is upregulated in the setting of increased intracellular zinc (i.e. through supplementation or dietary manipulation), as well as during acute phase responses, inflammation and oxidative stress. Zinc also signals MT for degradation. Zinc transporters have 2 distinct families: 14 Zrt- and Irt-like proteins (ZIP) import extracellular zinc and zinc held in the lumen of cellular organelles into the cytoplasm, and 9 known zinc transporter proteins (ZnT) move zinc from the cytoplasm into organelles or export it across the plasma membrane [90].

1.11. Epidemiology and consequences of zinc deficiency

Zinc deficiency (ZD) is highly prevalent in LMICs with 17.3% of the world's population at risk [91]. Infants, children, and pregnant or lactating women have higher zinc requirements, putting them at increased risk of ZD and its sequelae. The global distribution of ZD resembles the geographic spread of highest risk for diarrheal morbidity and mortality; South Asia and Africa are at greatest risk with an estimated 30% and 25%

of the populations, respectively, consuming inadequate zinc. Among children under 5 years, 14.5% of diarrheal deaths in Asia and Africa are attributable to ZD [92].

Recent data from a cross-sectional study in Bangladesh showed an overall ZD prevalence of 44.6% in children age 6-59 months, edging up to 51.7% among children sampled from urban slums, like the PROVIDE population [68]. ZD prevalence in non-pregnant, non-lactating women age 15-49 was 57.3% nationally and 66.4% in slums, with an astonishing 91% of women consuming inadequate dietary zinc to meet basic physiologic needs. Pervasive high-phytate, low animal-source diets were reported across the socio-economic spectrum, but with pronounced imbalance among the lowest income participants. A separate study found a similar rate of ZD, 55%, in Bangladeshi women during early pregnancy (mean gestation, 3.6 months) [93].

Consequences of zinc deficiency include increased susceptibility to infectious diseases such as diarrhea, stunting, impaired cognitive development, and in extreme cases, severe skin lesions [56, 89, 94]. Evidence from *in vivo* experiments of zinc restriction in adults demonstrated extensive dysfunction of the immune system in zinc deficiency, including dysregulated natural killer cell and TNF-α activity, thymic atrophy, aberrant lymphoid hematopoiesis, dysregulated NF-κB signaling, reduced CD4+ T cell function, and reduced immunoglobulin (Ig) responses [65, 95-99]. Zinc deficiency may disrupt the structural integrity of enzymes critical to V(D)J recombinase events, such as recombinase activating genes 1 and 2 (RAG1, RAG2), potentially limiting the repertoire of Ig's and T cell receptors and leading to increased susceptibility to infections [100-102]. From

murine models, immunologic consequences of gestational zinc deficiency have been suggested, with pups born from zinc-deprived dams exhibiting impaired humoral and cell-mediated immune responses for up to three generations [103, 104].

1.11.1. Zinc deficiency and gut epithelial health and function

Strong evidence, from both animal and human models, indicates a role of zinc deficiency in disrupting gut epithelial barrier function, which may have particular relevance to diarrheal risk [99]. Chai *et al* conducted a compelling study in a porcine model in which they demonstrated that piglets on a low zinc diet challenged with a porcine GI virus exhibited significant small intestine villous atrophy and reduced jejunal surface area compared to piglets on medium or high zinc diets with no post-challenge alteration in epithelial structure [105]. Furthermore, piglets on the high zinc diet experienced increased MT production and had higher and earlier antibody responses to viral challenge compared to low zinc piglets.

The histologic changes described in low zinc piglets are consistent with environmental enteric dysfunction (EED), a pathologic spectrum of structural and functional abnormalities of the small intestine common in LMICs [106]. While the porcine study showed zinc deficiency contributing to altered gut epithelium, the direction of this relationship may not be straightforward. Evidence from a study among children in Malawi concluded that EED disrupts zinc homeostasis by inhibiting zinc absorption in the small intestine resulting in higher-than-expected fecal zinc excretion based on dietary intake and physiologic requirements [107]. Lindenmayer *et al* describe the complex

interplay between EED and zinc deficiency and develop a model of a vicious cycle in which both conditions contribute to intestinal permeability and malabsorption in the small intestine leading to immunologic dysregulation and increased susceptibility to infections, particularly by diarrheal pathogens [108]. Attributing cause and effect in the EED – zinc deficiency nexus, and its effect on risk of diarrhea in children, is complicated and requires detailed interrogation of basic mechanisms as well as translational studies in humans.

1.12. Possible mechanisms for the association of zinc deficiency and viral diarrhea

Findings from clinical research on zinc have contributed to characterizing the signs, symptoms and consequences of zinc deficiency; however; research specifically designed to test biologic pathways leading to increased risk of viral diarrhea is lacking. While many questions remain unanswered, two intriguing areas for future mechanistic investigation are highlighted here: dysregulated metallothionein transcription and application of the concept of nutritional immunity to zinc regulation as an innate defense against pathogens. Given the strong correlation found in PROVIDE between zinc deficiency and specifically viral diarrhea (Chapter 3), as well as the limited treatment options for viral diarrhea compared to other etiologies and the underperformance of rotavirus vaccines, emphasis is placed on the relationship of these mechanisms to viruses.

1.12.1. Dysregulated metallothionein transcription in zinc deficiency

Metallothionein (MT) is a low molecular weight, intracellular, cysteine-rich metal-binding protein. A single MT protein can bind up to 7 zinc molecules, though MT has a

higher affinity for cadmium and copper. Increased free zinc levels in the cytoplasm induce metal regulatory transcription factor 1 (MTF-1) to initiate MT transcription [109-111]. Strong evidence from porcine and murine models demonstrate MT transcription is initiated by increased intracellular zinc as an acute phase response (APR) to infection or injury (i.e. lung epithelial insult from mechanical ventilation), indicating a central role for MT in regulating zinc homeostasis and mediating the APR [111-113].

Zinc deficiency has been shown to correlate with reduced MT expression [65, 113, 114]. One small study in humans (N=13) found, despite only a marginal decrease of 7% in plasma zinc concentration on a 7-day zinc-restricted diet, mean MT level fell by 68% in zinc-restricted human volunteers. By contrast, the same researchers found a 7-fold increase in mean MT concentration from 40 ± 6 μg/g protein to 273 ± 85 μg/g protein within 7 days on zinc supplementation (effect of supplementation on plasma zinc not reported) [115]. Two additional small studies in humans corroborate these results, particularly when measuring MT expression in monocytes [116, 117]. Due to its sensitivity to whole-body zinc levels, MT has been suggested for evaluation as a biomarker of zinc status [63].

Based on these findings, it would be particularly interesting to evaluate whether MT expression differs in zinc deficient vs non-zinc deficient infants particularly during the acute phase of viral diarrheal illness (i.e. Day 1 of a diarrheal episode), which could illuminate critical pathways to preventing viral diarrheal disease.

1.12.2. Zinc and nutritional immunity

The concept of "nutritional immunity" emerged out of the iron literature, but has since expanded to examine the role of other essential trace metals, including zinc, in regulating host response to pathogen presence as an innate defense against infection [118, 119]. Regarding zinc, substantial literature has described the role of nutritional immunity in the clearance of bacterial infections, in which intracellular zinc accumulation, either for zinc sequestration away from invading bacteria or zinc intoxication, particularly in phagocytes, leads to bacterial death [120-123]. The rationale for the intoxication defense involves the reliance of bacteria on zinc to support their own metabolic processes, similar to humans, making bacteria vulnerable to zinc toxicity. Few studies have investigated nutritional immunity as a defense strategy against viruses, which lack metabolic functions. In this case, zinc sequestration away from intracellular viral pathogens, as opposed to zinc accumulation for intoxication, may be the relevant nutritional immunity defense strategy.

As described by Chaturvedi *et al*, zinc ions and zinc finger proteins (structural motifs with 4 cysteines facilitating zinc-binding [56]) are intrinsic to many viruses and play critical roles in viral transcription, replication, structural conformation and in counter-defense to host immune responses [124]. On the host side, evidence from one small human study and one murine model showed increased MT expression specifically in response to viral infections (hepatitis C and coxsackievirus) [125, 126]. Given its strong binding affinity for zinc, increased intracellular MT would facilitate zinc sequestration by binding labile zinc. This has not been examined for viral diarrheal pathogens; however,

rotavirus provides a good model for consideration of how nutritional immunity may mediate the host:virus interface in virus-infected cells.

Rotavirus is a triple-layered particle, which sheds its outmost layer of VP7 and VP4 upon cell entry, exposing the VP6 layer. Rotavirions rely on zinc ions to anchor the structure of the VP6 layer; however, the zinc recruitment strategy of rotavirus for this critical conformational element has not been characterized [127, 128]. Rotavirus also relies on zinc binding to interfere with host defense. Rotavirus non-structural protein 1 (NSP1) has been identified as an E3 ubiquitin ligase depending on an N-terminal zinc-binding domain to bind and target host interferon (IRF3) for proteasome degradation, thereby disrupting the host's immune response to rotavirus infection [129].

The importance of zinc in the "life cycle" of viruses suggests nutritional immunity, in this case the MT-mediated sequestration of zinc away from pathogens in the intracellular environment, may be important for clearance of viral infection. I hypothesize that reduced MT expression, as demonstrated in zinc deficiency, inhibits innate nutritional immunity against viral pathogens by reducing the cell's ability to effectively sequester zinc leaving labile intracellular zinc available for recruitment by viruses. Furthermore, perhaps zinc supplementation in this context would promote adequate MT expression to restore nutritional immunity sequestration functions and provide protection from viral pathogens. These are areas for further investigation.

Notably, the ability of MT to effectively utilize zinc in nutritional immunity may be inhibited by heavy metal contamination. MT preferentially binds cadmium; however in the absence of sufficient MT expression, as in zinc deficiency, cells become vulnerable to cadmium toxicity, with consequences for zinc homeostasis [109, 130, 131]. This may be particularly problematic in populations subjected to high levels of environmental heavy metal contamination, such as in Bangladesh, and children may be at particularly high risk for heavy metal toxicity [132, 133]. The relevance of heavy metal contamination, particularly cadmium, to MT expression, zinc deficiency and risk of viral diarrhea remains unstudied.

1.13. Zinc interventions: benefits and risks of supplementation for diarrhea prevention

Finally, the potential for zinc supplementation interventions to positively impact diarrheal morbidity and mortality in children in LMICs must be addressed, with consideration of both infant and maternal supplementation strategies.

1.13.1. Infant supplementation

Several randomized clinical trials have found significant benefits of preventive zinc supplementation in children in ameliorating the burden of diarrhea [58, 134-136]; however zinc supplementation is not a panacea. Some trials have not found an effect of zinc supplementation on diarrheal prevalence or incidence, particularly among infants < 6 months [87, 137, 138]. Furthermore, open debate remains regarding appropriate dosage, timing and delivery method for zinc supplementation, which may depend on the goal of

supplementation: correcting the micronutrient deficiency vs preventing diarrhea associated with low zinc [139-141].

On the vaccine front, Habib *et al* found no effect of zinc supplementation on oral polio vaccine immunogenicity, even though they demonstrated a significant difference in zinc levels between the intervention and control groups [142]. No similar study of the effect of zinc supplementation on RV vaccine immunogenicity has been published. The only clear effect of supplementation on vaccine response was in Bangladesh with a killed, subunit oral cholera vaccine [143], though results of this trial showed mixed effects: while the vibriocidal antibody response to vaccine was enhanced among zinc supplemented children, the cholera-toxin antibody response was suppressed. Finally, it is worth noting that many studies of zinc supplementation and vaccine response were conducted in HICs where zinc deficiency is less prevalent and therefore supplementation may be less effective in boosting vaccine responses.

1.13.2. Maternal supplementation

Evidence on the effects of maternal zinc supplementation on infant zinc status or diarrhea is scant. Regarding maternal supplementation and reduced diarrheal morbidity in infants, one double-blind, randomized controlled trial of maternal zinc supplementation (pre-natal through neonatal) in Peru found significant reductions in total days with diarrhea and incidence of diarrheal episodes lasting longer than 7 days among infants of supplemented mothers [118]. Two additional studies, in Bangladesh and Indonesia, found similar

benefits of maternal supplementation on infant diarrheal risk; this effect was only seen in low-birth weight infants [144, 145].

Regarding maternal immunization, the limited data available showed no effect of zinc supplementation during pregnancy on infant responses to BCG or Hib vaccines; no results were reported for maternal immunization against diarrheal pathogens [146]. The potential for maternal zinc supplementation to positively impact infant diarrheal burden requires further investigation.

For breastfed infants, important questions include whether maternal zinc supplementation improves zinc availability in breast milk and, if yes, whether this translates into improved zinc status in infants. A detailed review of the literature by Donangelo and King concluded the only consistently demonstrated effect of maternal zinc supplementation during lactation has been a slower rate of decline in breast milk zinc concentration compared to non-supplemented mothers [78]. No trials have tested whether zinc supplementation of mothers during breastfeeding correlates with improved zinc status in infants.

Perhaps the strongest role for mothers in diarrhea prevention is through continued exclusive breastfeeding through the entire first six months of life. Public health programs should continue to promote EBF, particularly in populations tending to cease EBF prior to six months. The immunologic and nutritional benefits of EBF through six months cannot be overstated. With the introduction of complementary foods, community

education programs should emphasize locally-available and affordable options to ensure adequate zinc nutrition throughout early childhood.

1.13.3. Risks of zinc supplementation

Risks associated with zinc supplementation are rare, but include nausea, vomiting and diarrhea, and are usually seen only with zinc supplementation far exceeding the Tolerable Upper Intake Level for extended periods [56, 141, 147].

1.14. Conclusions and future directions

The mandate to effectively curb the excess burden of childhood diarrhea in LMICs persists as a top global public health priority in the 21st century. To reach the goal of reducing diarrhea-associated child mortality from 4.1 per 1000 live births (2013) to 1 per 1000 live births by 2030, investments in new technology, clinical research and vaccine science must continue. However, complementary interventions are required as the research community continues to try to understand the challenges with vaccine underperformance (rotavirus), accelerating the pace of vaccine development for enteric diseases, and leveraging new technologies to increase our understanding of the complex biologic pathways contributing to risk of death or disability due to diarrhea in early life.

Recent large-scale trials, including the PROVIDE Study, have clarified several important insights into the etiology of diarrhea in LMICs and possible interventions for ameliorating this burden. We now understand a single-pathogen model for diarrhea causation is too simplistic. The TAC platform illuminated pervasive carriage of

enteropathogens in both symptomatic children and children without diarrhea; the implications of which are actively under investigation. What is the optimal strategy for addressing the multiple co-pathogen burden, and how does this impact the efficacy of pathogen-specific vaccines or other diarrheal interventions? These are open questions in the field.

Among currently available interventions for diarrhea prevention, we have vaccines for only one high-burden pathogen, rotavirus. While rotavirus vaccines have had a tremendous impact, they leave a gap in protection, particularly against diarrhea-attributable morbidity. WASH interventions have not consistently demonstrated efficacy in preventing diarrhea, although the WASH Benefits Study may provide greater insight into optimizing WASH interventions for this goal. Promotion of exclusive breastfeeding (EBF) through 6 months and education on balanced complementary foods should continue to receive attention, particularly in populations tending to stop EBF prior to 6 months and where traditional complementary foods offer sub-optimal nutrition.

The PROVIDE Study also provided evidence that infant zinc level plays a significant role in risk of diarrhea, in particular from viruses, although this finding requires validation in larger cohorts, possibly GEMS and MAL-ED. There is also significant geographic overlap between risk of diarrhea and risk of zinc deficiency. However, many questions remain regarding associations and mechanistic pathways between zinc and particularly viral diarrhea that require synergistic, translational research to fully address.

The role of zinc in pathogen propagation and infection remains understudied. From the rotavirus model, we know zinc is intrinsic to the virus' life cycle; the extent to which other high-burden diarrheal pathogens rely on zinc and specifically how viruses recruit and integrate zinc is uncharacterized. Does nutritional immunity with zinc extend to viruses, and what are the underlying mechanisms of this evolutionary arms race between host and pathogen? Does viral success in establishing infection relate to host metallothionein (MT) levels and the ability of the host to sequester zinc away from viral pathogens? Answers to these questions could elucidate mechanisms for focused development of effective interventions to prevent the establishment of diarrhea-associated viral infections.

Clinical investigations (i.e. observational and case-control studies) are required to determine whether MT transcription differs between zinc deficient and non-zinc deficient infants, particularly during the acute phase of infection. If yes, how does this relate to viral diarrheal risk? Can insufficient MT expression be corrected through zinc supplementation? Specific to LMICs with high rates of environmental enteric dysfunction (EED), untangling the cause-and-effect relationship between EED and zinc deficiency may contribute to our understanding of the determinants of infant zinc status and how this relates to viral diarrhea. Also relevant to many populations in LMICs, the role of heavy metal contamination in MT function and zinc homeostasis as it relates to risk of viral diarrhea requires further investigation with potential policy implications for affecting clean water and environmental standards.

Finally, carefully designed trials of zinc supplementation have the potential to determine whether zinc, as a complementary intervention in both infants and mothers, may fill the gap in protection against childhood diarrhea. In particular, zinc supplementation in infants < 6 months is understudied, and appropriate preventive dosages, delivery methods and timing of administration for optimal impact on viral diarrhea must be carefully considered. Furthermore, it remains unclear whether maternal zinc supplementation during lactation contributes to increased bioavailability of zinc in breast milk, and whether this could affect protective differences in infant zinc status between supplemented vs non-supplemented mothers. As future enteric vaccines become available, and for the current rotavirus vaccines, there are no data on the effect of infant or maternal zinc supplementation on infant vaccine outcomes, specifically in LMICs with highest risk of diarrhea.

In conclusion, despite many unanswered questions, great progress has occurred and we now have additional tools at our disposal to attempt to reduce the continued burden of childhood diarrhea in the 21st century. This review delineates several key areas for future investigation to solidify our understanding of diarrhea and expand the available set of evidence-based interventions. The goals of elimination of childhood diarrheal mortality and reduced morbidity are within reach and must be achieved.

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CHAPTER 2: DELAYED DOSING OF ORAL ROTAVIRUS VACCINE DEMONSTRATES DECREASED RISK OF ROTAVIRUS GASTROENTERITIS ASSOCIATED WITH SERUM ZINC: A RANDOMIZED CONTROLLED TRIAL

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2.2. Abstract

Background. Rotavirus is the world's leading cause of childhood diarrheal death. Despite successes, oral rotavirus vaccines are less effective in developing countries. In an urban slum of Dhaka, we performed active diarrhea surveillance to evaluate monovalent G1P[8] rotavirus vaccine (RV1) efficacy and understand variables contributing to risk of rotavirus diarrhea (RVD).

Methods. We performed a randomized controlled trial of monovalent oral rotavirus vaccine (RV1). Seven hundred healthy infants received RV1 or no RV1 (1:1) using delayed dosing (10 and 17 weeks) and were followed for 1 year. Intensive diarrhea surveillance was performed. The primary outcome was ≥1 episode of RVD. Nutritional, socioeconomic, and immunologic factors were assessed by logistic regression best-subsets analysis for association with risk of RVD and interactions with vaccine arm.

Results. Incidence of all RVD was 38.3 cases per 100 person-years. Per-protocol RV1 efficacy was 73.5% (95% confidence interval [CI], 45.8%–87.0%) against severe RVD

and 51% (95% CI, 33.8%–63.7%) against all RVD. Serum zinc level (odds ratio [OR], 0.77; P = .002) and lack of rotavirus immunoglobulin A (IgA) seroconversion (OR, 1.95; P = .018) were associated with risk of RVD, independent of vaccination status. Water treatment and exclusive breastfeeding were of borderline significance. Factors not associated with RVD included height for age at 10 weeks, vitamin D, retinol binding protein, maternal education, household income, and sex.

Conclusions. In an urban slum with high incidence of RVD, the efficacy ofRV1 against severe RVD was higher than anticipated in the setting of delayed dosing. Lower serum zinc level and lack of IgA seroconversion were associated with increased risk of RVD independent of vaccination.

Clinical Trials Registration. NCT01375647.

Keywords. rotavirus diarrhea; zinc; oral vaccine; underperformance; developing countries.

2.3. Introduction

Rotavirus is the leading cause of child death from diarrhea. In 2008, prior to vaccine introduction, rotavirus diarrhea (RVD) caused approximately 453 000 deaths, most in South Asia and sub-Saharan Africa [1]. Three oral live attenuated rotavirus vaccines are now licensed and having tremendous impact: the 3-dose pentavalent human-bovine vaccine (RV5), the 2-dose monovalent G1P[8] vaccine (RV1), and the 3-dose human bovine G9P[11] vaccine (116E). In phase 3 trials, RV5 and RV1 have robust efficacy of >85% in high-income countries, as measured by protection from severe RVD or related hospitalization [2, 3]. In contrast, efficacy is markedly lower in developing countries: in

multiple clinical trials, oral rotavirus vaccine efficacy ranges from 18% to 61% in Africa and Asia [4–7].

Although lower rotavirus vaccine efficacy in developing countries is well established, little is understood about the biologic basis of vaccine underperformance. Previous efforts have postulated mechanisms related to the vaccine itself, including dosing schedule and inoculum, and factors impacting the child's ability to respond to vaccination [8]. The latter includes factors that prevent the vaccine from replicating in the intestine or blunt infant immune responses, such as breast milk and maternal antibody interference, enteropathy, and enteric coinfections [9–11]. Micronutrient deficiencies may also contribute; zinc specifically plays an extensive role in host defense and gut health, and deficiency has been associated with diarrheal morbidity and mortality [12, 13].

To assess factors related to RV1 performance, we enrolled a 700-child birth cohort in an urban slum of Dhaka, Bangladesh, in the Performance of Rotavirus and Oral Polio Vaccines in Developing Countries (PROVIDE) study: a randomized controlled trial of 2-dose RV1 vaccine using a delayed dosing schedule at 10 and 17 weeks of age (compared to the Expanded Programme on Immunization [EPI]–recommended schedule of 6 and 10 weeks). With a primary outcome of any RVD post-vaccination to 1 year, we conducted biweekly home-based diarrhea surveillance for RVD. To inform public health interventions and vaccine development efforts, we determined RV1 efficacy in this population, assessed additional factors for associations with risk of RVD, and examined possible interactions with the vaccine.

2.4. Methods

2.4.1. Study design and participants

As part of the PROVIDE study, we performed a randomized, open-label, controlled trial of live oral G1P[8] rotavirus vaccine (RV1) in a birth cohort of 700 children from the Mirpur urban slum in Dhaka, Bangladesh. Infants meeting inclusion and exclusion criteria were enrolled in the first week of life. Detailed study methods including consenting and eligibility criteria, as well as results from PROVIDE on the association of environmental enteropathy, enteric infection, and small intestinal bacterial overgrowth on health outcomes, are published elsewhere [11, 14–16]. The study was approved by the ethical review boards of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), the University of Vermont, and the University of Virginia. The study was registered at Clinicaltrials.gov (NCT01375647).

2.4.2. Randomization and masking

Children were randomized using permuted blocks with random block size selection (4 or 8) and assigned to 1 of2 treatment groups: 50% (n = 350) to receive RV1 vaccine at weeks 10 and 17, and 50% no rotavirus vaccine. All clinical investigators and laboratories were masked to vaccine arm, but medical officers were not.

2.4.3. Procedures

The study was conducted from May 2011 through November 2013. Children were enrolled from birth to age 7 days in the home by trained field research assistants,

following comprehensive consenting procedures according to International Council on Harmonisation Good Clinical Practice guidelines. Mothers were administered a baseline survey at enrollment for demographics, household socioeconomic, water, and sanitation data.

There were 10 clinic visits in the first year of life for anthropometry, phlebotomy, and vaccinations [14]. Children received the Bangladesh EPI vaccines, including trivalent oral polio vaccine at weeks 6, 10, and 14. All acute illnesses were evaluated by medical officers, and RV1 dosing was delayed in children presenting with fever at scheduled vaccination visits (n = 1). Breastfeeding was not withheld. Vaccine cold chain was reviewed before administration. Children with severe malnutrition (weight-forage z score < -3 SD) were referred for specialized care.

Complete diarrhea surveillance was conducted throughout the first year of life [14]. Field research assistants visited households twice weekly to determine diarrheal episodes through a structured questionnaire. Diarrhea was defined as ≥3 abnormally loose stools in 24 hours, per the mother, with distinct episodes separated by >72 hours diarrhea-free. Severe diarrhea was defined as Vesikari score ≥11 [17]. One diarrheal stool sample was collected during each episode. Mothers brought children into the clinic for further assessment and treatment of diarrheal illness. Diarrheal stool specimens were tested for rotavirus antigen by PosSpecT enzyme-linked immunosorbent assay (ELISA; Oxoid Ltd, Hampshire, United Kingdom).

Blood specimens for immunogenicity and micronutrients were collected at weeks 6 and 18 into trace-metal free Vacutainer tubes and cryovials (Grenier Bio-One and ThermoScientific/Nunc, respectively). Plasma was evaluated for rotavirus-specific immunoglobulin A (IgA) antibodies as described [18]. In brief, a capture enzyme immunoassay (EIA) was performed using the rotavirus SA11 antigen; results were expressed as units per milliliter determined by positive control reference serum. Seropositive was defined as rotavirus IgA≥ 20 U/mL; seroconversion was defined as seropositivity at week 18 following a seronegative result pre-vaccination (week 6). Vitamin D and retinol binding protein were assessed using commercial ELISA kits (Immunodiagnostics Systems Ltd, Tyne, United Kingdom and R&D Systems, Minneapolis, Minnesota), and serum zinc was analyzed by flame atomic absorption spectrophotometer using WinLab32 software.

2.4.4. Outcomes

The primary outcome for intention-to-treat (ITT) efficacy analysis was 1 or more episodes of RVD from birth to 1year. RVD was defined as diarrhea positive for rotavirus antigen by ELISA. Secondary outcomes were severe RVD, all-cause diarrhea (diarrhea of any etiology) and all-cause severe diarrhea in the first year of life, and any and severe RVD post-vaccination, from 18 to 52 weeks of age. Missed diarrheal stool specimens were assumed negative for rotavirus. Rates of missingness are reported [14].

2.4.5. Statistical analysis

The trial was designed with at least 90% power to detect 50% vaccine efficacy at $\alpha = .05$, assuming rotavirus infection in 26% of non-vaccinated children by 1 year. Primary analysis was by ITT: all randomized subjects were included regardless of whether they adhered to the protocol vaccine regimen or terminated study participation prior to 1 year. Secondary per-protocol (PP) analyses were performed including all children who had 365 days of follow-up and, if assigned to the vaccine arm, received both doses of rotavirus vaccine within the protocol-specified window.

Vaccine efficacy was calculated using the standard formula:
$(AR_{UNVAX} - AR_{VAX}) / (AR_{UNVAX} \times 100)$, with AR_{VAX} the attack rate in vaccinated and AR_{UNVAX} the attack rate in unvaccinated individuals. For dichotomous clinical outcomes, proportions of children with diarrhea along with Wilson 95% confidence intervals (CIs) and absolute risk differences in vaccinated and unvaccinated groups were calculated [19, 20].

Considering the study size and limitation on outcome events, a limited pool of 12 explanatory variables was selected a priori for analysis by best-subsets multivariable logistic regression to determine a model for risk of RVD based on the literature, biologic plausibility, and data availability. The best subset of p variables was chosen for the minimum Mallows' Cp statistic for which $Cp < p$ [21]. From this main effects model, all 2-way interactions were tested by change in deviance from the reduced model (likelihood ratio test). Model diagnostics included inspection of residuals, influential cases, and

linearity in the logit for continuous variables. The Hosmer-Lemeshow goodness of fit test was performed. Raw *P* values (Wald test) for regression coefficients were adjusted for multiplicity post hoc using a stepdown Holm procedure [22]. The final model for RVD was then applied to the clinical outcome of severe RVD. Analyses were performed using SAS software version 9.3 (SAS Institute, Cary, North Carolina).

2.5. Results

2.5.1. Study population

After a community-wide survey of Mirpur, Dhaka, Bangladesh, 700 mother-child pairs were consented and enrolled within 7 days of birth (median age, 5 days [range, 1–7 days]) (**Figure 2.1.**). Children were randomized to receive the 2-dose RV1 vaccine at 10 and 17 weeks of age or not. Population characteristics by randomization arm are shown in **Table 2.1.**. There were no differences in characteristics between arms, except in rotavirus IgA seroconversion (26.8% seroconverted among vaccinated vs 17.2% in unvaccinated children; P = .005) and rotavirus IgA geometric mean titer (GMT) at week 18 (12.2 U/mL in vaccinated vs 4.3 U/mL in unvaccinated children; P = 2.4 × 10−8). There were 92 dropouts, 12% (n = 43) in the RV1 arm and 14% (n = 49) in the control arm (P = .53).

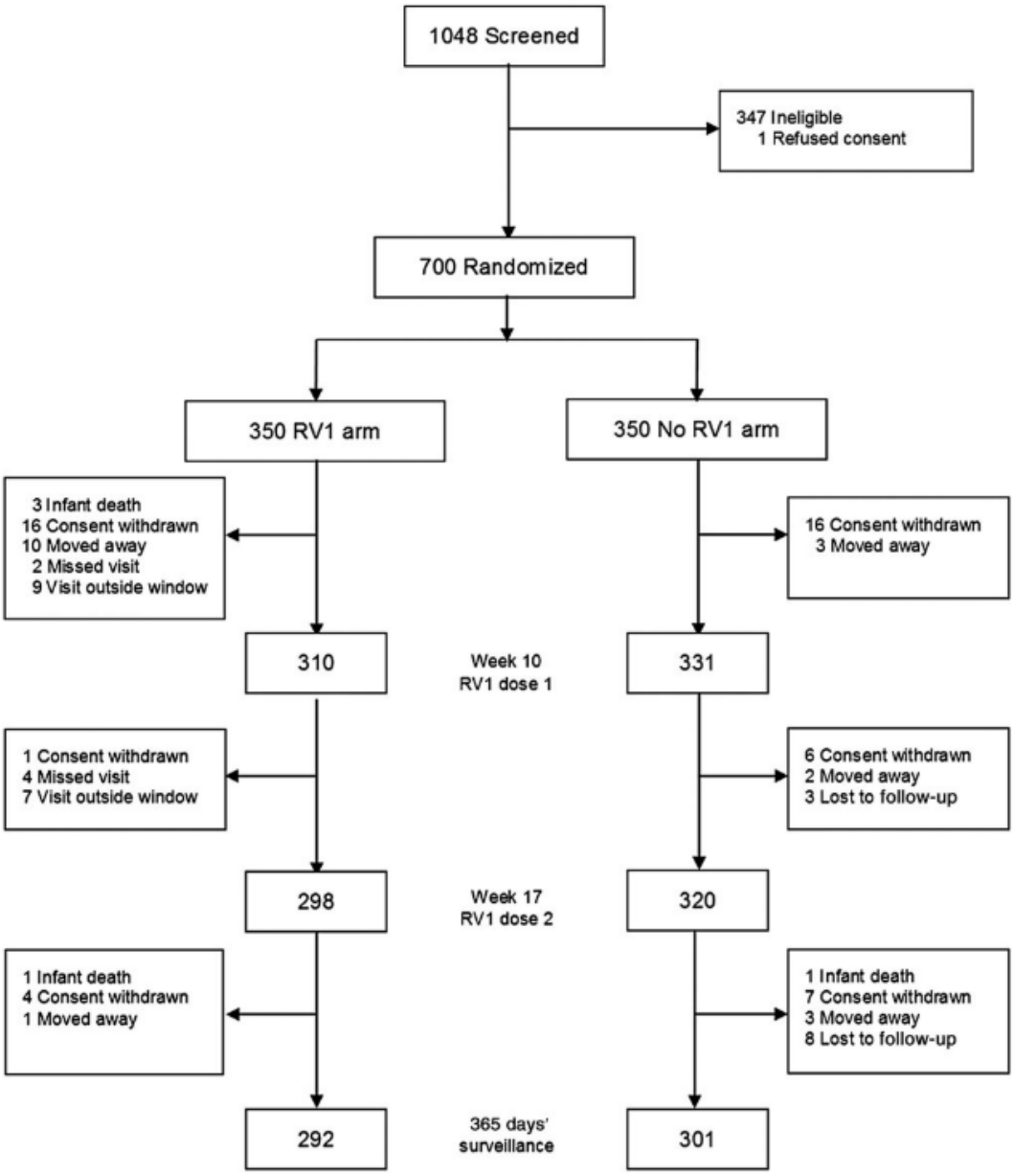

Figure 2.1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram.
Abbreviation: RV1, monovalent G1P[8] rotavirus vaccine.

Table 2.1. Characteristics by randomization arm

	Randomization Arm	
	RV1 arm (n=350)	**No RV1 arm (n=350)**
Child features		
Sex, male	182 (52.0)	186 (53.1)
Median age at enrollment, d	5 (1 – 7)	5 (1 – 7)
Median weight at enrollment, kg	2.7 (1.7 – 4.1)	2.8 (1.9 – 4.0)
Median length at enrollment, cm	48.5 (43.1 – 55.4)	48.8 (44.5 – 54.6)
Median height-for-age z score at 10 wk[a]	-0.98 (-3.54 – 2.67)	-0.89 (-4.36 – 1.69)
Median weight-for-age z score at 10 wk [a]	-0.91 (-4.53 – 1.83)	-0.86 (-3.61 – 1.40)
Exclusive breastfeeding at 18 wk[b]	154 (50.0)	161 (53.5)
Home birth	100 (28.6)	81 (23.1)
Maternal features		
Median age at enrollment, y	24 (18 – 40)	24 (18 – 41)
Vaginal delivery	277 (79.1)	263 (75.1)
Median height, cm[c]	150 (137– 187)	150 (134 – 167)
Median post-partum weight, kg[c]	48.0 (30.2 – 80.0)	47.0 (30.0 – 77.0)
Other children ≤5 y old in home	92 (26.3)	96 (27.4)
Mother education class 9+	54 (15.4)	55 (15.7)
Household and Socioeconomic features		
Median total monthly income, 1000 taka	10 (3 – 77)	10 (3 – 70)
Piped municipal water	339 (96.9)	339 (96.9)
Toilet or septic tank	195 (55.7)	172 (49.1)
One-room home	251 (71.7)	256 (73.1)
Median No. of household members	5 (1 – 16)	4 (2 – 18)
Any water treatment	209 (59.7)	211 (60.3)
Immunogenicity		
RV IgA seroconversion [d]	80 (26.8)*	50 (17.2)*
RV IgA geometric mean titer, U/mL[e]	12.2**	4.3**
Serum Micronutrients at 18 weeks		
Zinc, µg/dL [f]	75 (44 – 173)	77 (55 – 150)
Vitamin D, nmol/L [g]	58.8 (15.1 – 146.1)	58.1 (13.2 – 139.9)
Vitamin A, µg/ml [g]	26.1 (6.0 – 99.8)	25.8 (4.8 – 99.9)

Data are presented as No. (%) or median (range).
Abbreviations: IgA=immunoglobulin A; RV=rotavirus; RV1=monovalent G1P rotavirus vaccine.
[a] N=318 RV1 arm, N=326 No RV1 arm
[b] N=308 RV1 arm, N=301 No RV1 arm
[c] N=330 RV1 arm, N=339 No RV1 arm
[d] N=299 RV1 arm, N=291 No RV1 arm
[e] N=289 RV1 arm, N=269 No RV1 arm
[f] N=300 RV1 arm, N=301 No RV1 arm
[g] N=305 RV1 arm, N=294 No RV1 arm
*P = 0.005, Chi-square test. ** $P = 2.4 \times 10^{-8}$

2.5.2. Rotavirus diarrhea incidence and vaccine efficacy

Under intense surveillance, incidence of RVD in unvaccinated children in the densely populated urban slum was 38.3 cases per 100 person-years, higher than previously reported in developing countries [4, 5, 7] (**Table 2.2.**). In the primary analysis, vaccinated children vs unvaccinated had significantly less RVD (19.1% vs 32.6%) and severe RVD (4% vs 11.1%) in the first year of life (**Table 2.3.**). Overall, by ITT analysis, vaccine efficacy was 41.2% (95% CI, 23.6%–54.8%) against all RVD and 64.1% (95% CI, 35.1%–80.1%) against severe RVD. The number of children needed to treat to prevent 1 case of RVD, derived as the reciprocal of the absolute risk difference, was 8 (95% CI, 6–15). There were 3 cases of RVD and 2 cases of severe RVD between week 6, first dose of the EPI-recommended RV1 dosing regimen, and week 10 when the first dose ofRV1 was given in PROVIDE. Efficacy estimates against all-cause diarrhea were 1.3% (95% CI, −4.8% to 7.1%) for any diarrhea and 12.7% (95% CI, −7.5% to 29.1%) for severe diarrhea.

Table 2.2. Incidence of rotavirus diarrhea in "Performance of Rotavirus and Oral Polio Vaccines in Developing Countries" Study compared with other cohorts

	Rotavirus Diarrhea			Severe Rotavirus Diarrhea		
	Cases (N)	Person Years	Incidence [a]	Cases (N)	Person Years	Incidence [a]
PROVIDE (urban, unvaccinated)	121	315.9	38.3	41	315.9	13
Rural Bangladesh and Vietnam [4]	109	1143.4	9.5	71	1156.9	6.1
Sub-Saharan Africa [5]	294	2556.3	11.5	129	2585.9	5.0
South Africa and Malawi [7]	NA	NA	NA	70	NA	8.0

Abbreviations: NA, not available; PROVIDE, Performance of Rotavirus and Oral Polio Vaccines in Developing Countries study. [a] Incidence per 100 person-years.

PP analysis of efficacy post-vaccination (18–52 weeks) was performed. PP efficacy against all RVD was 51%(95%CI, 33.8%–63.7%) and 73.5% (95% CI, 45.8%–87.0%)

against severe RVD (**Table 2.3.**), while efficacy against any and severe all-cause diarrhea was −3.1% (95% CI, −9.2% to 2.7%) and 22.1% (95% CI, −3.0% to 41.1%), respectively.

Table 2.3. Rotavirus diarrhea incidence and vaccine efficacy, intention-to-treat and per-protocol analyses

Analysis	All Subjects, % (95% CI)	RV1 Arm, % (95% CI)	No RV1 Arm, % (95% CI)	Risk Difference, % (95% CI)	*P* Value	RR (95% CI)	Efficacy % (95% CI)
Year 1 ITT analysis (n = 700)							
RVD	25.8 (22.7–29.2)	19.1 (15.3–23.6)	32.6 (27.8–37.6)	13.4 (7.0–19.8)	4.0×10^{-5}	1.70 (1.31–2.21)	41.2 (23.6–54.8)
Severe RVD	7.6 (5.8–9.8)	4.0 (2.4–6.6)	11.1 (8.2–14.9)	7.1 (3.2–11.2)	3.0×10^{-4}	2.78 (1.54–5.02)	64.1 (35.1–80.1)
All-cause diarrhea	85.7 (82.9–88.1)	85.1 (81.0–88.5)	86.3 (82.3–89.5)	1.1 (−4.1 to 6.4)	.66	1.01 (.95–1.08)	1.3 (−4.8 to 7.1)
Severe all-cause diarrhea	33.7 (30.3–37.3)	31.4 (26.8–36.5)	36.0 (31.1–41.2)	4.6 (−2.4 to 11.5)	.20	1.14 (.93–1.41)	12.7 (−7.5 to 29.1)
Post-vaccination per-protocol analysis (n = 593)							
RVD	25.6 (22.3–29.3)	16.8 (12.9–21.5)	34.2 (29.1–39.8)	17.4 (10.5–24.2)	6.6×10^{-7}	2.04 (1.51–2.75)	51.0 (33.8–63.7)
Severe RVD	7.4 (5.6–9.8)	3.1 (1.6–5.8)	11.6 (8.5–15.7)	8.5 (4.4–12.9)	5.0×10^{-5}	3.77 (1.85–7.71)	73.5 (45.8–87.0)
All-cause diarrhea	88.7 (85.9–91.0)	90.1 (86.1–93.0)	87.4 (83.1–90.7)	−2.7 (−7.8 to 2.5)	.30	0.92 (.92–1.03)	−3.1 (−9.2 to 2.7)
Severe all-cause diarrhea	25.5 (22.1–29.1)	22.3 (17.9–27.4)	28.6 (23.8–33.9)	6.3 (−.71 to 13.2)	.08	1.28 (.97–1.70)	22.1 (−3.0 to 41.1)

Abbreviations: CI, confidence interval; ITT, intention-to-treat; RR, relative risk; RV1, monovalent G1P[8] rotavirus vaccine; RVD, rotavirus diarrhea.

2.5.3. Best subset of factors associated with rotavirus diarrhea and vaccine interactions

There were 555 children with complete data for all 12 explanatory variables in the best-subsets modeling of risk of RVD post-vaccination to 1 year. The explanatory variable pool consisted of rotavirus vaccine arm, household income, sex, delivery status (cesarean vs vaginal), mother's education (below class 9 vs class 9 and above), household water treatment (yes/no), exclusive breastfeeding at 18 weeks (yes/no), rotavirus IgA seroconversion (IgA < 20 U/mL at 6 weeks and ≥20 U/mL at 18 weeks), height-for-age z score (HAZ) at 10 weeks, and retinol binding protein, vitamin D, and serum zinc at 18 weeks.

In the multivariable best-subsets logistic regression analysis, the most parsimonious main effects model (minimum Cp < p) was the 5-variable model including vaccination arm, serum zinc, IgA seroconversion, exclusive breastfeeding, and water treatment. No 2-way interactions were statistically significant; no variable impacted the effect of rotavirus vaccine on risk of RVD (interactions with vaccine arm P > .33). The Hosmer-Lemeshow goodness-of-fit test for the main effects model was performed (P = .67). Based on model diagnostics, removing the 5 cases most poorly fit by the model (change in deviance P < .005 with largest leverage) strengthened the odds ratio (OR) point estimates by approximately 10% for vaccination arm, seroconversion, and serum zinc. There was no expectation these cases were different from the study population, so results include all cases.

Variables most strongly associated with risk of RVD were not receiving RV1 (OR, 2.84 [95% CI, 1.87–4.30]), serum zinc level (OR, 0.77 [95% CI, .66–.91]), and lack of IgA seroconversion (OR, 1.95 [95% CI, 1.12–3.39]). Absence of water treatment and not exclusively breastfeeding were also associated with increased risk of RVD (ORs of 1.50 and 1.46, respectively; **Table 2.4.**). The effects of RV1 and serum zinc retained statistical significance after adjusting for multiple testing.

The final model selected for the RVD outcome was applied to severe RVD. Only lack of vaccine was significantly associated with increased risk of severe RVD (OR, 3.81 [95% CI, 1.78– 8.82]; P = .0008; **Table 2.4.**). The coefficients and effect size estimates for all

variables were comparable to the all-severity RVD model, although cases of severe RVD (incidence rate, 7.4%) were insufficient to meet statistical significance.

Table 2.4. Multivariable logistic regression main effects model for risk of rotavirus diarrhea and severe rotavirus diarrhea

Variable	Coefficient (SE)	Odds Ratio (95% CI)	Main effect raw *P* value	Main effect adj. *P* value
Rotavirus Diarrhea post week 18 in year 1				
No RV1 arm (control)	1.04 (0.68)	2.84 (1.87 – 4.30)	9.6×10^{-7}	1.4×10^{-5}
Zinc, 18 weeks (x 10ug/dL)	-0.26 (0.08)	0.77 (0.66 – 0.91)	0.002	0.024
Lack of RV IgA seroconversion	0.67 (0.28)	1.95 (1.12 – 3.39)	0.018	0.228
Absence of water treatment	0.40 (0.40)	1.50 (0.99 – 2.24)	0.051	0.612
Stopped excl. breast feeding by week 18	0.38 (0.20)	1.46 (0.97 – 2.18)	0.066	0.726
Severe Rotavirus Diarrhea post week 18 in year 1				
No RV1 arm (control)	1.37 (0.39)	3.93 (1.83 – 8.47)	0.0005	0.008
Zinc, 18 weeks (x 10ug/dL)	-0.12 (0.13)	0.88 (0.69 – 1.13)	0.325	1.000
Lack of RV IgA seroconversion	0.59 (0.50)	1.81 (0.68 – 4.81)	0.233	1.000
Absence of water treatment	0.54 (0.33)	1.72 (0.92 – 3.30)	0.099	1.000
Stopped excl. breast feeding by week 18	0.47 (0.33)	1.59 (0.83 – 3.07)	0.163	1.000

Raw P values from Wald test and adjusted using Holm stepdown procedure.
Abbreviations: CI, confidence interval; IgA, immunoglobulin A; RV, rotavirus; RV1, monovalent G1P[8] rotavirus vaccine; SE, standard error.

2.6. Discussion

Using a clinical primary endpoint of RVD, we performed a controlled efficacy trial of oral rotavirus vaccine given at 10 and 17 weeks after birth. The study was performed in a highly rotavirus-endemic, densely populated, urban setting in Bangladesh. PP analysis allowed comparison of our vaccine efficacy estimates with large phase 3 trials ofRV1 [4, 7]. Following a delayed dosing regimen, the efficacy of RV1 vaccine to prevent severe RVD was 73.5% (95% CI, 45.8%–87.0%), higher than previously reported in developing countries, including 45.7% efficacy against severe RVD in Bangladesh [4]. Vaccine efficacy against RVD of any severity was 51% (95% CI, 33.8%–63.7%). Although past

studies have suggested that rotavirus vaccine may protect against diarrhea from all etiologies [2, 4], we saw no impact of rotavirus vaccination on all-cause diarrhea.

We postulate our improved efficacy was due to several factors in our study design. Intense community-based diarrhea surveillance captured higher than expected incidence of RVD (including mild cases not requiring medical attention). This high incidence exposes the large burden of rotavirus disease and contributes toward higher vaccine efficacy. Additionally, although not directly tested, our delayed dosing schedule at 10 and 17 weeks may have minimized maternal antibody interference with RV1 vaccine. Although previous efficacy trials [4, 7] and immunogenicity studies [23, 24] have tested 2–3 doses of rotavirus vaccine administered between 6 and 16 weeks, this work is the only efficacy trial with delayed dosing (10 and 17 weeks). An additional trial, using a clinical endpoint to compare early vs late dosing schedules, would be necessary to confirm superiority of delayed dosing. With only 5 cases of RVD between the currently EPI-recommended start of vaccination (week 6) and week 10 (used here), the added risk of delaying dosing appears minimal.

To further understand risk of RVD, we used multivariable best-subsets analysis to evaluate factors for association with risk of RVD, including nutritional, socioeconomic, hygiene, and immunologic variables. Beyond vaccination, only serum zinc and IgA seroconversion were strongly associated with protection from RVD, and both were independent of vaccination status. This suggests that, regardless of vaccine performance,

improvement in these variables would decrease risk and overall burden of RVD. In the final model, only vaccination and serum zinc level retained significance.

The observation that serum zinc is associated with decreased risk of RVD (OR, 0.77; P = .002) recalls the importance of zinc in intestinal epithelial repair and immunologic response mechanisms critical for mucosal protection [12, 25]. Previous studies have demonstrated a clear benefit of both supplemental and therapeutic zinc in protection from diarrheal disease in infants [13, 26]; however, there have been mixed results regarding the benefits of zinc specifically in RVD [27, 28]. We did not find an association between zinc and all-cause diarrhea.

Relevant to interpretation of our zinc findings, there has been controversy about the value of serum zinc level as a biomarker of individual zinc status; however, the recently published review of zinc under the National Institutes of Health's Biomarkers of Nutrition for Development (BOND) program [29] supports the use of serum zinc for this purpose as it relates to clinical signs of zinc deficiency, is responsive to zinc supplementation interventions, and can predict functional responses to supplementation, particularly in populations where functional bioindicators (i.e., low HAZ scores) suggest risk of zinc deficiency. With a stunting prevalence exceeding 20% by 1 year of age, the PROVIDE cohort meets BOND's recommended threshold for populations in which serum zinc may be a particularly strong biomarker of zinc status, lending confidence to the interpretation of our findings.

As in the PROVIDE study, a disconnect has been noted in several trials in developing countries between development of rotavirus-specific IgA and vaccine efficacy [9]; however, our findings regarding immunologic responses raise concern. Less than one-third of PROVIDE children seroconverted following vaccination, and IgA GMTs were markedly lower in PROVIDE, even compared to another cohort in Bangladesh that found GMTs of47 U/mL among vaccinated children [30]. Importantly, immunogenicity in PROVIDE was measured 1 week after the second dose of vaccine (week 18), whereas other studies examined IgA 1–2 months after the second dose. Another possible contributing factor may be the difference in antigenic lysate used in the capture EIA assay in PROVIDE (rotavirus SA11 antigen) vs other studies.

Although not reaching statistical significance at the $P \leq .05$ level, other potential risk factors for RVD warrant further investigation in larger studies. Lack of exclusive breastfeeding and absence of water treatment in particular, together with zinc supplementation and vaccination, might be envisioned as the core of a focused diarrhea-prevention public health plan offering both vaccination and improved baseline health to reduce the burden of RVD.

Our work has several limitations. Although we performed intense diarrhea surveillance, results may underreport the incidence of short-duration diarrheal episodes. We assumed these episodes were negative for rotavirus, which may downwardly bias our efficacy estimates. The Hawthorne effect, in which enrolled children receive higher-standard primary care, may be present and upwardly bias efficacy estimates. Our sample size and

number of outcome events limited the explanatory variables in our best-subsets analysis; our results need confirmation in larger studies. Additionally, we measured post-vaccination immunogenicity earlier than comparable trials. Although our results may reflect poor primary response or poor boosting after the second dose of RV1, our data could have underestimated immunogenicity based on this earlier time point.

Additional data are necessary to fully understand the limitations of oral vaccine efficacy in developing countries and delineate the optimal response. Previously we described the association of concurrent enteroviruses, as well as environmental enteropathy (measured by fecal reg1B, neopterin, serum soluble CD14, and enteric infection), on RV1 failure [11, 16]. Future work will focus on the impact of maternal antibodies and blood group antigens, the role of enteric co-pathogens, the effect of zinc supplementation and whether zinc has a pathogen-specific effect in protection from diarrheal disease, and the role of asymptomatic infection in IgA seroconversion and rotavirus vaccine performance. Research toward the identification of more predictive immune correlates of protection for rotavirus is also under way (B. D. Kirkpatrick, personal communication). With high efficacy against severe RVD, our work demonstrates the importance of oral rotavirus vaccines in highly endemic settings and suggests that support of baseline health and sanitation are still critical components of a public health approach, including vaccination, to prevent morbidity and mortality due to rotavirus diarrhea.

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CHAPTER 3: ZINC DEFICIENT INFANTS AT HIGH RISK OF VIRAL DIARRHEA IN A BANGLADESHI BIRTH COHORT

3.1. Introduction

Diarrhea remains the second leading cause of child mortally globally. A significant proportion of infectious diarrhea in infants is due to viral pathogens, which now can be detected more readily with the use of quantitative multiplex PCR technologies [1]. Among all diarrheal pathogens, rotavirus (RV) accounts for the highest attributable incidence of diarrheal disease and mortality in the first year of life [2]. Prevention of RV and other viral diarrhea may be best addressed by vaccination. However, oral RV vaccines consistently show sub-optimal efficacy of 18-61% in low and middle-income countries (LMICs) with further evidence of waning effectiveness in year 2 [3-6], and no vaccines are available for other leading viral diarrheal pathogens, specifically norovirus and adenovirus 40/41. Furthermore, unlike bacterial infections for which antibiotics are a treatment option, treatment of viral diarrhea is limited to oral rehydration solution and zinc. A gap in protection against viral diarrhea exists in LMICs, and complimentary strategies for diarrhea prevention are critical.

Zinc deficiency (ZD) is prevalent in LMICs with a high burden of RV and other diarrhea, particularly South Asia and Sub-Saharan Africa [7, 8]. ZD has been linked to dysregulation of biologic processes that may lead to increased risk of diarrhea, including: poor gut epithelial repair and barrier function, immune and inflammatory dysfunction, and aberrant microbiota composition [9-11]. Zinc deficiency in LMICs is complex. In

these settings, children often are infected with multiple co-pathogens and experience high rates of gut and systemic inflammation [12, 13], complicating interpretations of zinc deficiency with zinc biomarkers responsive to acute-phase inflammatory processes [14]. Zinc deficiency in infants also may be tightly linked to maternal zinc nutrition, obscuring the optimal target population for possible zinc interventions [15].

A significant body of evidence from LMICs demonstrates beneficial effects of zinc interventions in children to treat diarrhea; however, less conclusive evidence from randomized controlled trials is available for zinc in diarrhea prevention. One meta-analysis estimated preventive zinc supplementation of ≥ 3 months was associated with 14% reduction in diarrheal incidence (RR 0.86, 95% CI: 0.79–0.93) [16], while other trials have not demonstrated an effect, particularly among infants < 6 months [17, 18]. Most trials of zinc supplementation have not distinguished diarrheal risks or responses between children with vs without zinc deficiency at baseline.

In our past work evaluating rotavirus vaccines in Dhaka, Bangladesh ("Performance of Rotavirus and Oral Polio Vaccines in Developing Countries" (PROVIDE) Study) [19, 20], we demonstrated that week 18 serum zinc concentration correlated with risk of subsequent RV diarrhea from week 18–52 of life (OR 0.77, 95% CI (0.66-0.91), independent of RV vaccination. This surprising correlation suggests focused consideration of of zinc supplementation in populations at high risk of rotavirus diarrhea to address the gap in vaccine protection.

However, this observation also illustrates gaps in knowledge regarding the relationship between infant zinc status and infectious diarrhea, which require better understanding to guide the use of preventive zinc supplementation. Of interest, previous studies have not assessed whether zinc is particularly effective against specific diarrheal pathogens, and whether additional risk factors impact pathogen-specific diarrheal risk related to zinc deficiency.

With the ultimate goal of optimizing preventive strategies against childhood diarrhea, herein we seek to more comprehensively understand the important associations of zinc deficiency and infectious diarrhea, and to evaluate co-factors including inflammatory markers, breastfeeding practices, breast milk composition and demographics for possible effects on the zinc-diarrhea relationship. Given the high burden of RV and other viral pathogens in the first year of life, a unique relationship between higher zinc levels and protection against viral diarrhea would support consideration of zinc interventions to address the gap in protection against childhood diarrhea.

3.2. Methods

3.2.1. Study design and population

We conducted a formal randomized controlled trial of oral rotavirus vaccine efficacy under the PROVIDE Study from May 2011 – November 2013 in a birth cohort of 700 infants in an urban, high-density, low-income section of Dhaka, Bangladesh, where rotavirus vaccine is not routinely administered. Eligible infant-mother pairs were enrolled in the first week post-partum following consent of the mother, and infants were

randomized 1:1 to receive oral Rotarix™ vaccine (RV1) or no RV1 at weeks 10 and 17. Infants were followed for one year in the RV1 trial. Detailed eligibility criteria and consenting and randomizations procedures are described elsewhere [19, 20]. The study was conducted per Good Clinical Practice guidelines, and the study was approved by ethical review boards at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and the Universities of Vermont and Virginia. Clinicaltrials.gov registration is NCT01375647.

3.2.2. Clinical procedures

Mothers' study participation included completing detailed questionnaires on household demographics, water and sanitation, socioeconomic status, and personal reproductive history, as well as breast milk specimen collection within 6 weeks of enrollment. Infants were scheduled for 10 study visits in one year for receipt of routine childhood immunizations (per Bangladesh's national program) and RV1 vaccine, anthropometry and blood draws. Interim clinic visits for acute illness and diarrheal assessments occurred as needed. All acute care treatments prescribed through the study clinic were recorded.

Active, community-based diarrhea surveillance consisted of twice weekly home visits by trained Field Research Assistants to capture infants' history of diarrhea, fever, and vomiting, home use of antibiotics and oral rehydration solution, and breastfeeding practices. Infants with diarrhea were referred to the study clinic for assessment. Mothers or study field assistants collected one diarrheal specimen per episode, defined as ≥ 3

loose stools in 24 hours per the mother with three diarrhea-free days distinguishing episodes.

3.2.3. Laboratory procedures

Each diarrheal specimen was analyzed by quantitative real-time PCR, TaqMan Array Card (TAC), for 35 enteropathogen targets. Detailed methods for development, validation and optimization of the TAC assay have been published [21-23]. Briefly, QIAamp Fast DNA Stool mini kits were used for nucleic acid extractions, which were then run in individual pathogen-specific probe-based assays compiled on a single card, including two external controls. The assay read-out was a Quantification Cycle (Cq) value, representing the amplification cycle number at which flouresced pathogen-specific nucleic acid was detectable above background, with a positive range of $0 < Cq < 35$.

For this analysis, the top seven pathogens identified in previous studies for diarrhea attributable incidence in the first year of life in LMICs were selected from the TAC panel: rotavirus, adenovirus 40/41, norovirus, *Campylobacter jejuni*, *Shigella* spp/enteroinvasive *E. coli* (EIEC), heat-stable enterotoxigenic *E. coli* (ST-ETEC), and *Cryptosporidium* spp [1, 24, 25]. Non-polio enterovirus (NPEV) was also included based on previously reported high prevalence of NPEV infection in the PROVIDE cohort [26]. Published diarrhea-associated TAC Cq cut-offs for each pathogen were applied for assigning pathogen etiology in diarrheal specimens; extensive methods for determining diarrhea-associated TAC Cq's are described elsewhere [1, 21, 23, 25, 27]. No diarrhea-associated Cq threshold has been defined for NPEV, $Cq < 35$ was used.

Blood specimens for serum zinc concentration (SZC) and acute phase inflammatory markers interleukin-6 (IL-6) and C-reactive protein (CRP) were collected at week 18 in trace metal-free Vacutainer tubes and cryovials (Grenier Bio-One and ThermoScientific/Nunc). Specimens were centrifuged and 25-30 μL of serum aliquoted for SZC measurement and stored at < -70^{o} C for 4 to 28 months prior to analysis by flame atomic absorption spectrophotometer usingWinLab32 software. Zinc deficiency was defined as SZC < 65μg/dL based on suggested SZC cut-offs from the BOND Zinc Expert Panel Review and sensitivity analysis in the PROVIDE dataset [28]. Serum IL-6 was analyzed by BioRad's 17-plex Bio-Plex Cytokine Assay kit (Cat # M50-00031YV). Serum CRP was assessed by ALPCO ELISA kit assay per the manufacturer's instructions (Cat # K-9710s).

Detailed methods for breast milk specimen collection, processing and analysis are published [29]. Briefly, within six weeks of enrollment specimens were expressed directly into 5mL falcon tubes at the mother's convenience after instruction on manual expressing by field staff. Samples were collected from mothers and transported to the icddr,b laboratory at 4^{o} C where dried milk spot cards were prepared with 50μL breast milk and anti-oxidant pre-treatment prior to shipping to OmegaQuant Analytics, Sioux Falls, SD. The cards were analyzed by gas chromatography for 26 breast milk fatty acids, including linoleic acid (LA) and dihomo-γ-linoleic acid (DGLA). The ratio of LA:DGLA in breast milk was included as a potential marker of maternal zinc status [30, 31].

3.2.4. Statistical analysis

Frequencies for select enteropathogens in diarrheal specimens were determined as total number of pathogen-specific positive TAC results (0 < Cq < diarrhea-associated Cq cut-off) over total number of evaluated stools (N=1,367). Incidence per infant was calculated as ≥ 1 pathogen-specific episode of diarrhea from weeks 18-52, using the same diarrhea-associated Cq cut offs. Diarrheal stools or episodes with more than one select pathogen present at diarrhea-associated levels were counted for each pathogen(s). Infants were excluded from pathogen-specific analysis if they had missing stools plus only negative TAC results for a pathogen in evaluated stools. Infants with a positive TAC result in any evaluated stool were included in analysis for the detected pathogen(s), regardless of missing stools.

The primary analysis was a set of independent univariate logistic regression models each with an outcome of proportion of infants with incidence of ≥ 1 pathogen-specific diarrheal episode from week 18-52 and zinc deficiency (yes vs no) as predictor. To control for infections or inflammatory processes coinciding with the week 18 SZC measurement that may have effected SZC [14], acute phase pro-inflammatory markers IL-6 and CRP at week 18 were tested for possible confounding on the relationship between SZC and diarrheal outcomes. Spearman's correlation was used to determine relationships between IL-6 and CRP with SZC (continuous). IL-6 and CRP were assessed for confounding in bivariate and multivariable models by > 10% change in the zinc beta coefficient over the primary models, and model fit was assessed by Akaike information criterion (AIC) [32].

Given high rates of co-infection with multiple pathogens per diarrheal specimen, including at diarrhea-associated TAC Cq's as well as sub-clinical carriage (TAC Cq > diarrhea-associated cut off), co-pathogen burden in rotavirus positive stools was evaluated to determine whether SZC at week 18 differed between specific combinations of co-pathogens. The Mann-Whitney test compared mean week 18 SZC in infants with rotavirus positive diarrhea plus vs minus individual co-pathogens, determined by presence or absence of each select enteropathogen at any positive Cq value, $0 < Cq < 35$, in rotavirus diarrheal stools.

Kaplan-Meier (KM) time-to-event analysis compared time to first viral diarrhea in zinc deficient vs not deficient infants, assessed for each virus separately and any viral diarrhea. Infants with no viral diarrhea detected from week 18-52 were censored at Day 365. Cox proportional hazards modeling was used to assess candidate confounders of the KM time series results, applying the same assessment criteria described above for multivariable logistic regression models. Candidate confounders for the Cox proportional hazards model were tested for association with zinc deficiency by Chi Square for dichotomous predictors and Wilcoxon Rank Sum for continuous predictors.

Analyses were performed in SAS software version 9.3 (SAS Institute, Cary, North Carolina).

3.3. Results

3.3.1. Diarrhea and zinc descriptive data

Out of 700 infants enrolled in PROVIDE, 608 completed one year of follow up per protocol contributing 1,773 reported diarrheal episodes between weeks 18 and 52. Diarrheal specimens were collected for 1,434 episodes (81%). The majority of missed specimens were from short duration episodes lasting 1-2 days. 88% (N=535) of infants had zero or only one missing specimen. Of the collected specimens, 1,367 (95%) were evaluable by TAC.

As shown in **Table 3.1.**, non-polio enterovirus (NPEV) and adenovirus 40/41 were the most prevalent pathogens in diarrheal stools and had the highest incidence in infants, although positive results for NPEV were based on TAC Cq < 35 in the absence of a published (and perhaps lower) diarrhea-associated cut-off. Of note, rotavirus accounted for the highest attributable incidence of diarrhea in the first year of life in PROVIDE based on attributable fraction calculation [1, 33]. High rates of sub-clinical carriage were detected for several pathogens, in particular norovirus, *Campylobacter jejuni*, and ST-ETEC, which were detected in diarrheal stools but at > diarrhea-associated Cq's in 13%, 26% and 18% of stools respectively, in addition to the prevalences reported in **Table 3.1.**.

Table 3.1. Prevalence and incidence of pathogen-specific diarrhea, weeks 18–52

Pathogen (diarrhea-associated Cq)	Pathogen prevalence (% positive in 1,367 diarrheal stools)[a]	Percent of infants with ≥ 1 pathogen-specific diarrheal episode (N=608)[a]
Rotavirus (35)	19.9	47.6
Norovirus (27.6)	12.7	34.7
Adenovirus 40/41 (35)	39.9	66.0
Non-polio enterovirus (35[b])	47.9	69.4
Any viral diarrhea	78.5	82.2
Campylobacter jejuni (19.7)	3.4	11.0
Shigella spp/EIEC (33.1)	11.1	29.5
ST-ETEC (26.2)	11.9	33.3
Any bacterial diarrhea	23.1	51.1
Cryptosporidium (29.1)	6.0	15.5

[a] Columns sum to > 100% due to co-pathogens
[b] No published diarrhea-associated Cq cut-off; 0<Cq<35 used to determine positive stools.
Abbreviations: spp=species; EIEC=Enteroinvasive *Escherichia coli*; ST-ETEC=Enterotoxigenic *Escherichia coli*

At week 18, 577 infants had serum zinc concentration (SZC) measurements. 16.5% of the cohort (N=95) was at or below the threshold for zinc deficiency at week 18. The range of SZC was 44–173µg/dL, with a mean of 77µg/dL and median of 76µg/dL. **Table 3.2.** summarizes population characteristics for key variables, comparing infants with vs without zinc deficiency.

Table 3.2. Key variables in infants with vs without zinc deficiency

Variable	Zinc deficient infants	Non-zinc deficient infants	*P*-value
Sex (% female)	36.8	50.2	0.02
Lack of exclusive breast feeding at week 18 (%)	52.6	50.8	0.75
Lack of household water treatment (%)	44.2	38.2	0.27
Birth delivery method (% cesarean)	25.3	23.2	0.67
Any pre-week 18 diarrhea (%)	63	61	0.66
Week 18 IL-6 inflammatory marker[a]	64.0 (83.8)	50.5 (104.5)	0.0003
Week 18 CRP inflammatory marker[a]	5.8 (12.2)	2.5 (6.4)	0.01
LA:DGLA in breast milk[a]	20.0 (10.8)	22.0 (10.0)	0.004

[a] Mean (SD). Wilcoxon Rank Sum P-values based on log transformed data.
All other P-values from Chi Square.

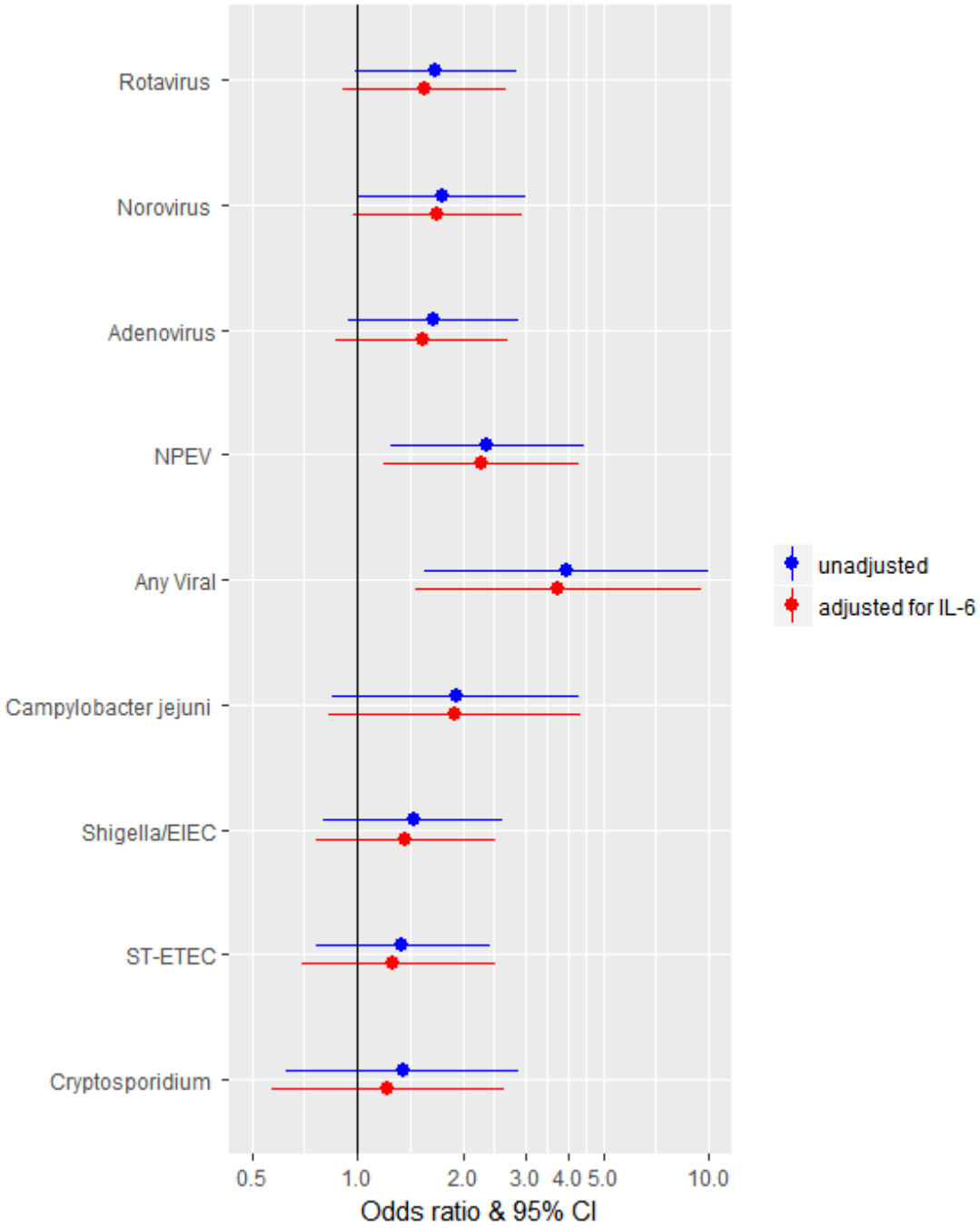
3.3.2. Logistic models for diarrheal outcomes and zinc deficiency

Univariate logistic regression models tested the association between zinc deficiency and pathogen-specific diarrheal outcomes. As shown in **Figure 3.1.**, the odds ratio point estimates and confidence intervals for each viral pathogen were similar, with increased odds among zinc deficient vs non-deficient infants for rotavirus OR 1.67 (95% CI 0.99 – 2.83), norovirus OR 1.74 (95% CI 1.01 – 3.01) and adenovirus OR 1.64 (95% CI 0.94 – 2.86). The odds of NPEV diarrhea among zinc deficient infants was highest OR 2.34 (95% CI 1.24 – 4.41).

Grouping viral pathogens into any viral diarrhea, zinc deficient infants had nearly four times higher odds of viral diarrhea compared to non-zinc deficient infants, OR 3.94 (95% CI 1.55 – 10.03, p=0.004), see **Figure 3.1.**. The wide confidence interval for any viral diarrhea was likely due the low frequency of zinc deficient infants with no viral diarrhea (6%). Zinc deficiency was associated with having any diarrhea OR 2.76 (95% CI 1.08 – 7.04), regardless of etiology. Zinc deficiency did not associate with pathogen-specific bacterial or parasitic diarrheal outcomes.

Both inflammatory biomarkers, IL-6 and CRP, correlated with zinc deficiency at week 18 (**Table 3.2.**); however, neither independently associated with pathogen-specific diarrheal outcomes or any viral diarrhea in univariate tests. Inclusion of IL-6 in multivariable models did, however, improve model fit and change the zinc beta coefficients by > 10% for any viral diarrhea, as well as most individual pathogens. IL-6-adjusted point estimates and confidence intervals are shown in **Figure 3.1.**.

Figure 3.1. Odds ratios with 95% CI for univariate diarrheal outcomes in infants zinc deficient vs non-deficient at week 18



3.3.3. Kaplan-Meier analysis and Cox proportional hazards

Figure 3.2. Kaplan-Meier curves for time to first episode of diarrhea weeks 18–52 in infants zinc deficient vs non-deficient at week 18

Figure 3.2A: *Any* viral diarrhea

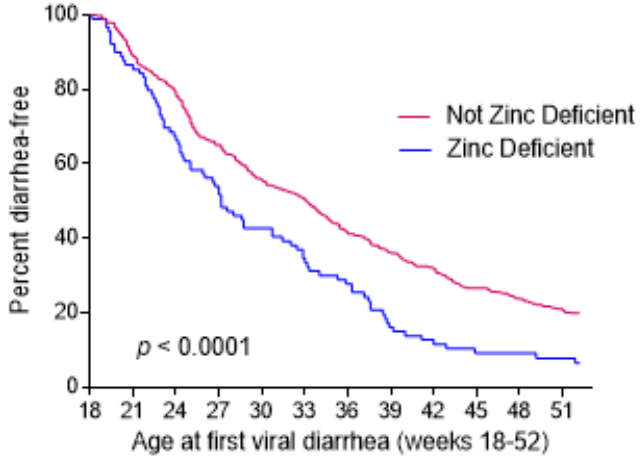


Figure 3.2B: Rotavirus diarrhea

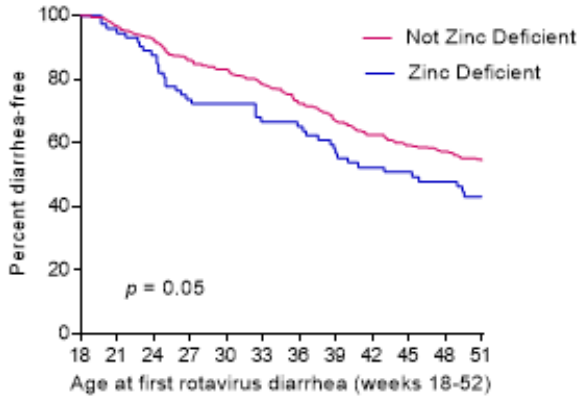
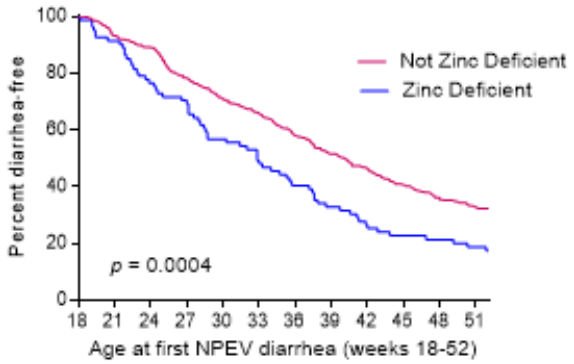


Figure 3.2C. Non-polio enterovirus (NPEV) diarrhea



To determine whether zinc deficient infants were at risk for viral diarrheas earlier in the first year of life compared to non-zinc deficient, Kaplan-Meier analyses were done for each viral pathogen, as well as any viral diarrhea. On time-to-event curves, a strong correlation was found between zinc deficiency and time to first episode of any viral diarrhea, with median time diarrhea-free of 27 weeks in zinc deficient infants vs. 33 weeks in non-deficient infants ($p<0.0001$). At the individual pathogen level, significant associations were seen between zinc deficiency and shortened time to rotavirus (log rank $p=0.05$) and NPEV diarrhea (log rank $p<0.001$), with no significant difference at $p\leq0.05$ for adenovirus or norovirus diarrhea ($p=0.12$ and $p=0.08$ respectively). Kaplan-Meier curves for any viral diarrhea, rotavirus and NPEV are shown in **Figure 3.2.**.

Given strong correlations found throughout the analyses between zinc deficiency and any viral diarrhea, Cox proportional hazards modeling focused on this outcome. Eight potential risk factors were assessed for association with time to first viral diarrhea, **Table 3.3.**. Adding these step-wise into a multivariable model resulted in the most parsimonious proportional hazards model including zinc deficiency, infant's sex, lack of household water treatment, LA:DGLA ratio in mother's breast milk, and lack of exclusive breastfeeding at week 18. In the final model, zinc deficient infants were at 45% greater risk of viral diarrhea compared to non-deficient infants (HR 1.45, 95% CI 1.13–1.87, $p=0.003$) with evidence of confounding due primarily to the addition of LA:DGLA to the model (18% change in zinc beta coefficient over univariate zinc model). Females had reduced risk of viral diarrhea compared to males (HR 0.84, 95% CI 0.69 – 1.02), and the

effect sizes for lack of both exclusive breastfeeding and household water treatment were very similar (HR 1.20, 95% CI 0.98─1.45 and 1.46 respectively).

Table 3.3. Cox proportional hazards model with candidate confounders of association between week 18 zinc deficiency and time to first viral diarrhea, week 18-52

Variable	Hazard ratio (95% CI)	*P*-value
Univariate Hazards Ratios (main effect p-values)		
Zinc deficient at week 18	1.58 (1.24 – 2.02)	0.0003
Sex (female)	0.79 (0.65 – 0.96)	0.02
No household water treatment	1.25 (1.03 – 1.53)	0.02
LA:DGLA (log, continuous)	0.78 (0.62 – 0.98)	0.03
Lack of exclusive breast feeding at week 18	1.23 (1.02 – 1.50)	0.03
Birth delivery method (cesarean)	0.85 (0.67 – 1.07)	0.16
Week 18 IL-6 (log, continuous)	1.05 (0.98 – 1.13)	0.20
No rotavirus vaccine	1.07 (0.89 – 1.30)	0.47
Multivariable Model (adjusted p-values)		
Zinc deficient at week 18	1.45 (1.13 – 1.87)	0.003
Sex (female)	0.83 (0.68 – 1.01)	0.06
No household water treatment	1.20 (0.98 – 1.46)	0.08
LA:DGLA (per standard deviation of log, continuous)	0.93 (0.84 – 1.02)	0.13
Lack of exclusive breast feeding at week 18	1.20 (0.98 – 1.45)	0.07

3.3.4. Co-pathogens

PROVIDE infants had a mean of 2.74 enteropathogens detected per diarrheal stool (range, 0-9 pathogens per stool at any positive Cq value). Although rotavirus (RV) was considered diarrhea-causing when present, 95% of RV positive stools were co-infected. The highest rates of co-infection were with other viruses: 40% with NPEV, 38% with adenovirus and 12% with norovirus, as compared to 4% – 12.5% co-infection with bacterial or parasitic pathogens. Despite high rates of co-infection, there was no difference in mean week 18 SZC between infants who had RV diarrhea +/- co-infection with other pathogens (all p-values for paired comparisons > 0.20, Mann-Whitney test, data not shown). Separate exploratory analyses showed pervasive carriage of non-ST-ETEC *E. coli* spp, particularly enteroaggregative *E. coli* (EAEC), which was detected in

68% of all diarrheal stools, nearly 80% of infants, and co-infected with 71% of RV diarrheal stools.

3.4. Discussion

Previous work from the PROVIDE Study suggested a relationship between serum zinc concentration (SZC) and risk of rotavirus diarrhea in the first year of life [19]. Here, we have shown a broader association with a significant relationship between infant zinc deficiency at week 18 and risk of any viral diarrhea up to one year. While the association appears not to be specific to rotavirus, the trends of similar risks across viruses in the state of zinc deficiency may suggest a common mechanism for the protective effect of zinc against viral diarrhea. The association of SZC with any diarrhea in the first year also is likely driven by the high prevalence of viruses in the cohort: for diarrheal attributable incidence, five out of the top 6 diarrheal pathogens in PROVIDE are viruses [33]. These findings have particular importance for populations in LMICs affected by high rates of zinc deficiency and heavy childhood diarrheal burdens in the setting of moderate rotavirus vaccine efficacy and no vaccines for other viral diarrheal pathogens.

Given that breast milk is the sole source of zinc nutrition for most infants in the first months of life, understanding the role of maternal zinc nutrition is critical to optimizing zinc interventions for diarrhea prevention. The extent to which zinc deficiency in mothers translates into zinc insufficiency in breast milk is unclear; however, associations have been shown between maternal plasma zinc and breast milk zinc concentration, as well as between maternal and infant plasma zinc [15]. Furthermore, consequences of maternal

zinc deficiency during gestation have been shown to persist for three generations of offspring [34]. While we did not directly measure mothers' zinc, we measured the breast milk fatty acid ratio of linoleic acid to dihomo-γ-linoleic acid (LA:DGLA). This ratio, when measured in erythrocytes, correlates with individual zinc status [30, 31]. We have not found literature demonstrating a similar association in breast milk, however, to the extent this association may be found, we could hypothesize that reduced breast milk LA:DGLA may indicate maternal zinc deficiency which in turn may negatively impact zinc nutrition in infants, contributing to increased risk of viral diarrhea. The confounding effect shown here of LA:DGLA on the association of infant zinc deficiency with earlier viral diarrhea supports further investigation of this hypothesis.

Once infants cease exclusive breastfeeding (EBF), other factors become important in both zinc nutrition and diarrheal pathogen exposures. We found infants not exclusively breastfed by week 18 were at significantly higher risk of earlier viral diarrhea. Only approximately half the PROVIDE cohort maintained EBF through week 18 [19], which may be particularly problematic in Bangladesh where only 40-45% of the recommended zinc intake for infants is met through breastfeeding plus typical complementary foods [35]. A longer duration of EBF in this context has greater potential to meet infants' zinc requirements [36], perhaps affording better protection against viral diarrhea through the first year of life. Lack of household water treatment also correlated with earlier viral diarrhea; household-level interventions of water purification methods may improve diarrhea-related outcomes.

In terms of biologic mechanisms, zinc deficiency has been linked to dysregulated metabolic processes that may lead to increased risk of viral diarrhea, in particular poor gut epithelial repair and barrier functions. In a clear example, Chai et al (2014) demonstrated that piglets on a low zinc diet challenged with a porcine GI virus exhibited significant small intestine villous atrophy and reduced jejunal surface area compared to piglets on medium or high zinc diets with no post-challenge alteration in epithelial structure [9]. The histologic changes described in low-zinc piglets are consistent with environmental enteric dysfunction (EED), a pathologic spectrum of structural and functional abnormalities of the small intestine common in LMICs. Given the high co-pathogen burden and high levels of systemic inflammation (IL-6) in the PROVIDE cohort, EED is likely prevalent [13, 37]. As hypothesized by Lindenmayer et al (2014), in this context, a vicious cycle of zinc deficiency and EED may contribute to increased susceptibility to enterpathogen infection and risk of diarrhea [12]. Future research may elucidate additional mechanisms linking zinc deficiency to viral diarrhea, including immune-mediated and microbiome-related.

Limitations of the present analysis include the PROVIDE study sample size and single measurements of serum zinc and breast milk. Our results require validation in larger cohorts, ideally including serial zinc and breast milk measurements over the first year, temporally related to diarrheal episodes. Collection of data on maternal zinc status would also enhance our analysis. Interpretation of TAC data, particularly related to diarrhea attribution, is a work-in-progress, and assay results near the limit of detection (Cq 30─35) may be less clinically meaningful [23].

In summary, as oral rotavirus vaccines are adopted globally in national immunization programs, complementary interventions to address the excess burden of rotavirus diarrhea must be considered. The evidence provided here supports further assessment of targeted zinc interventions for the prevention of not only rotavirus diarrhea, but all viral diarrhea. Future directions should include targeted zinc supplementation trials designed to test the public health value of zinc supplementation, in both infants and mothers, for the prevention of viral diarrheas. Additionally, continued promotion of exclusive breastfeeding through six months and improved sanitation will contribute to a comprehensive approach to reducing diarrheal disease burden due to viruses. Finally, further interrogation of the complex biologic mechanisms relating zinc deficiency to viral diarrhea will contribute to enhanced interventions moving forward.

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