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Association of Vitamin D Status with Acute Respiratory Infection and Diarrhoea in Children Less Than Two Years of Age in an Urban Slum of Bangladesh

A. M. Shamsir Ahmed

MBBS

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Abstract

Background and aims: Diarrhoea and pneumonia are leading causes of morbidity and mortality in children under two years of age, and micronutrients have been shown to play an important role in the prevention of these conditions. The immune-modulatory functions of vitamin D in infectious diseases are well known; however, its role in childhood conditions such as diarrhoeal and acute respiratory infections (ARI) is limited and contradictory. Additionally, there are no studies reporting the role of vitamin D on pathogen specific diarrhoeal diseases.

I aimed to quantify the prevalence and identify the socioeconomic predictors of vitamin D status; evaluate the association of vitamin D status with diarrhoeal incidence and severity and whether vitamin D status confounded the association between other micronutrients and diarrhoeal incidence and severity; explore the role of vitamin D in diarrhoeal events with enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC); and investigate the association of vitamin D, retinol and zinc status with ARI in underweight and normal-weight children aged 6–24 months in urban slum of Bangladesh.

Methods: I analysed data from the Bangladeshi component of the Malnutrition & Enteric Diseases (Mal-ED) project, which is a multisite project concerned with malnutrition and diarrhoeal diseases in early childhood. As part of the Bangladeshi Mal-ED project, a prospective case-control study was conducted in children aged 6–24 months at the urban Mirpur field site in Dhaka. From November 2009 to February 2012, 500 cases and 480 controls were enrolled and matched for sex and area of residence. Cases were defined as children who were severely to moderately underweight (weight-for-age Z, WAZ, score < –2.00 SD) and controls were defined as well-nourished or normal-weight children (WAZ > –1.00). Serum vitamin D and other micronutrients were measured at baseline and children were followed for five months with active biweekly surveillance for common infectious diseases. Diarrhoeal stool samples were collected for isolation and characterisation of causative organisms. Data on household socio-economic status, and dietary intake were also collected. Data for underweight and normal-weight children were analysed separately. Multinomial logistic regression was used to identify risk indicators of vitamin D status. Multivariable generalised estimating equations (GEE) were used to estimate the incidence rate ratios of diarrhoea and ARI. Cox proportional hazard models of unordered failure events of the same type were used to determine risk factors for ETEC, EPEC and EAEC diarrhoeal incidence.

Results: Among underweight children only 23.1% were vitamin D sufficient (≥75 nmol/L), 42.3% insufficient (50-74.99 nmol/L) and 34.6% deficient (<50 nmol/L), In normal-weight children 14.8% were vitamin D sufficient, 39.6% insufficient and 45.6% were deficient. Risk factors [ORs (95%

CIs)] for vitamin D deficiency in underweight children were: older age group (18–24 months) [2.9 (1.5–5.7)] compared to younger age group (6-11 months); measurement of vitamin D status during winter [3.0 (1.4–6.4)] and spring [6.9 (3.0–16.1)] compared to during summer; and maternal education (≥6 years of institutional education) [2.2 (1.0–4.9)] compared to illiterate mothers. Risk factors in normal-weight children were: older age group [3.6 (1.2–10.6)] and being from a household in the wealthiest quintile [3.7 (1.1–12.5)] compared to being from lowest quintile".

Normal-weight and underweight children contributed 62,117 and 62,967 days observation, with 14.2 and 12.8 days/child/year of diarrhoea, respectively. None of the multivariable models (GEE) showed significant associations between vitamin D status and diarrhoeal morbidity. Zinc-insufficient normal-weight children had 1.3 times more days of diarrhoea and 1.8 times more risk of severe diarrhoea than zinc-sufficient children ($p < 0.05$). Vitamin D status was not independently associated with the risk of ETEC, EPEC and EAEC diarrhoea in underweight children but moderate to severe retinol deficiency was associated with a reduced risk for EPEC diarrhoea. Among normal-weight children, insufficient vitamin D status and moderate to severe retinol deficiency were independently associated with 44% and 38% reduced risk of incidence of EAEC diarrhoea, respectively.

Underweight children with insufficient and deficient vitamin D status had 20% and 23–25% reduced risk of upper respiratory infection (URI) respectively compared with children with sufficient vitamin D status. Underweight children, with retinol deficiency, were at 1.8 (1.4–2.4) times higher risk of acute lower respiratory infection (ALRI) than those with retinol sufficiency. Normal-weight children with zinc insufficiency and those with retinol deficiency had 1.2 (1.0–1.5) times higher risk of URI and 1.9 (1.4–2.6) times higher risk of ALRI respectively.

Conclusion: These findings have important public health implications. Given the significant burden of vitamin D insufficiency and deficiency, intervention programs for accessing natural sources of vitamin D need to be prioritised urgently to prevent chronic diseases such as bone disease, diabetes mellitus, hypertension, and many common cancers during adulthood. My findings demonstrate that vitamin D did not confound the effect of zinc and vitamin A in childhood diarrhoea or ARI, and thus supplementation programs with vitamin D could be recommended for children under two years during winter and spring. However, findings also indicate that vitamin D induced immunomodulatory functions of innate and adaptive immunity in infectious disease morbidity warrants further exploration.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Published manuscripts incorporated in this thesis

1. Ahmed AMS, Ahmed T, Long ZK, Magalhaes RJS, Hossain MI, Islam MM, Mahfuz M, Gaffar SMA, Sharmeen A, Haque R, Guerrant RL, Petri WA, Mamun AA. Prevalence and risk factors of vitamin D insufficiency and deficiency among 6-24 months old underweight and normal weight children living in an urban slum of Bangladesh. *Public Health Nutrition*, 1-11. doi:10.1017/S1368980015003353
2. Ahmed AMS, Magalhaes RJS, Ahmed T, Long ZK, Hossain MI, Islam MM, Mahfuz M, Gaffar SMA, Sharmeen A, Haque R, Guerrant RL, Petri WA, Mamun AA. Vitamin D status is not a confounder of the relationship between Zinc and diarrhoea: a study in 6-24 months old underweight and normal weight children of urban Bangladesh. *Eur J Clin Nutr*. 2016 May;70(5):620-8.
3. Ahmed AMS, Magalhaes RJS, Long ZK, Ahmed T, Alam MA, Hossain MI, Islam MM, Mahfuz M, Mondal D, Haque R, Mamun AA. Association of vitamin D status with incidence of pathogen specific diarrhoea in underweight and normal weight under 2 years old urban children of Bangladesh. *Tropical Medicine & International Health*, 2 June 2016, doi/10.1111/tmi.12731/pdf
4. Ahmed AMS, Ahmed T, Magalhaes RJS, Long ZK, Alam MA, Hossain MI, Islam MM, Mahfuz M, Mondal D, Haque R, Mamun AA. Association between serum vitamin D, retinol and zinc status, and acute respiratory infections in 6-24 months old underweight and normal weight children living in an urban slum in Bangladesh. Accepted in *Epidemiology Infection*, 20 July, 2016

1. Publications included in this thesis

Publication citation 1 – incorporated as Chapter 4

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Author contribution	*Development of research question:* Ahmed AMS (80%), Ahmed T (5%), Long ZK (5%), Magalhaes RJS (5%), Mamun AA (5%) *Data collection and quality assurance:* Ahmed AMS (20%), Ahmed T (25%), Hossain MI (10%), Islam MM (10%), Mahfuz M (5%), Gaffar SMA (5%), Sharmeen A (5%), Haque R (10%), Guerrant RL(5%), Petri WA (5%), *Data Management:* Ahmed AMS (80%), Ahmed T (10%), Mamun AA (10%) *Statistical analysis and interpretation:* Ahmed AMS (80%), Long ZK (10%), Magalhaes RJS (5%),Mamun AA (5%) *Wrote the manuscript:* Ahmed AMS (100%) *Edited the manuscript:* Ahmed AMS (80%), Ahmed T (5%), Long ZK (5%), Magalhaes RJS (5%),Mamun AA (5%)

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Author contribution	*Development of research question:* Ahmed AMS (80%), Magalhaes RJS (5%), Ahmed T (5%), Long ZK (5%), Mamun AA (5%) *Data collection and quality assurance:* Ahmed AMS (20%), Ahmed T (25%), Hossain MI (10%), Islam MM (10%), Mahfuz M (5%), Gaffar SMA (5%), Sharmeen A (5%), Haque R (10%), Guerrant RL(5%), Petri WA (5%), *Data Management:* Ahmed AMS (80%), Ahmed T (10%), Mamun AA (10%) *Statistical analysis and interpretation:* Ahmed AMS (80%), Magalhaes RJS (10%), Long ZK (5%), Mamun AA (5%) *Wrote the manuscript:* Ahmed AMS (100%) *Edited the manuscript:* Ahmed AMS (80%), Ahmed T (5%), Long ZK (5%), Magalhaes RJS (5%), Mamun AA (5%)

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Contributions by others to the thesis

All contributions have been in statement for jointly-authored articles above.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

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Keywords

diarrhoea, acute respiratory infections, vitamin d, zinc, retinol, under two children, undernutrition, risk factors, urban slum, bangladesh

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FoR code: 1103, Clinical Sciences, 20%

Contents

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List of Abbreviations

ALRI	Acute lower respiratory infections
AMP	Antimicrobial peptides
ARI	Acute respiratory infections
BDHS	Bangladesh Demographic and Health Survey
BMI	Body Mass Index
CI	Confidence intervals
DALYs	Disability adjusted life years
DC	Dendritic cells
DEC	Diarrhoeagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FFQ	Food frequency questionnaire
GEE	Generalized estimating equation
HIV	Human immune-deficiency virus infection
HPLC	High-performance liquid chromatography
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
Ig	Immunoglobulin
MAL-ED	Malnutrition and Enteric Diseases
NNP	National Nutrition Program
OR	Odds ratio
PCR	polymerase chain reaction
RCT	Randomised control trial
URI	Upper respiratory tract infections
UV	ultraviolet
UVB	ultraviolet-B
VDR	Vitamin D receptor
WAZ	Weight-for-age Z score,
WHO	World Health Organisation

CHAPTER 1 INTRODUCTION AND OVERVIEW OF THESIS

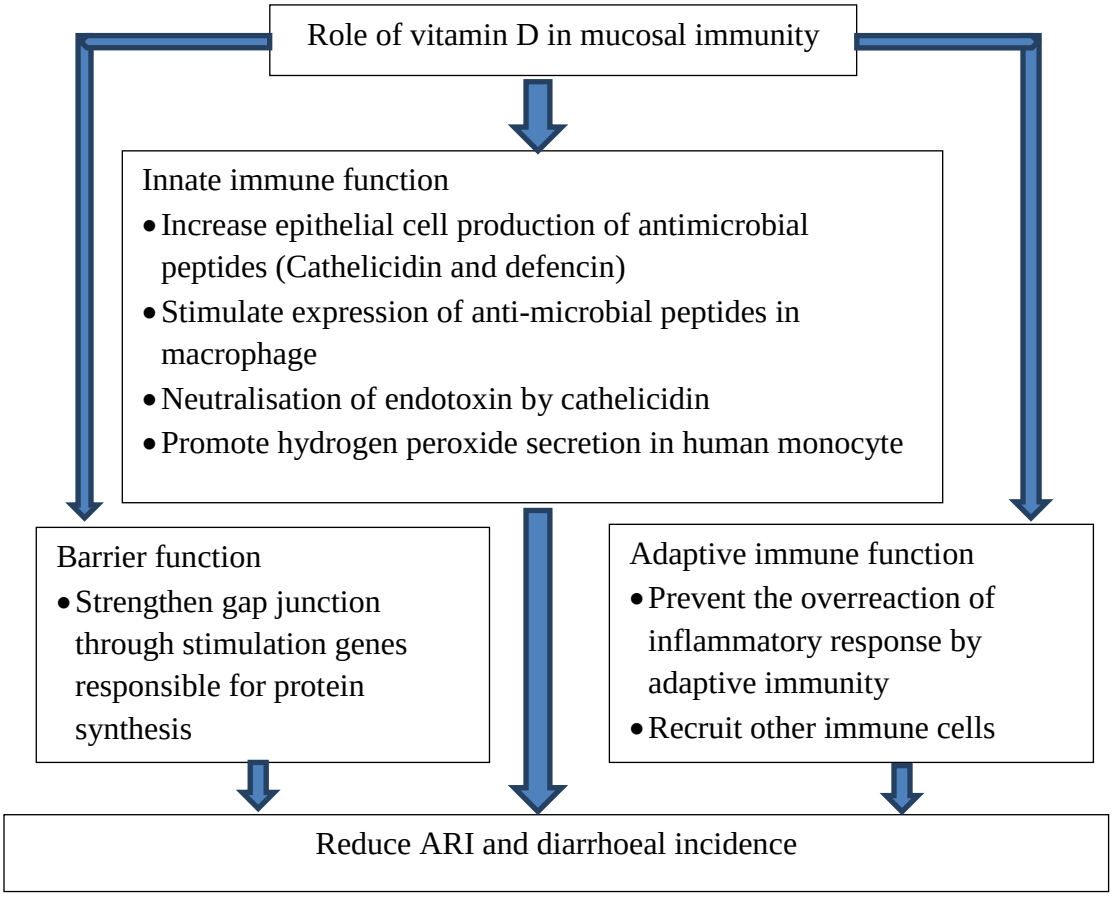
1.1 Background

Diarrhoea and pneumonia continue to be major health problems among children under five years of age and contribute to much of the disease burden found in developing countries [1-3]. It is estimated that around 1.731 billion episodes of diarrhoea and 120 million episodes of pneumonia occurred globally in children under five years old during 2010 [1]. It is also estimated that globally 0.71 million deaths were associated with diarrhoea and 1·26 million with pneumonia during 2011, with 72% deaths resulting from diarrhoea and 81% of deaths from pneumonia occurring during the first two years of life. In Bangladesh, the overall incidence of diarrhoea among preschool children in 2007, was 3.8–4.3 episodes per child per year, while 6% of deaths among preschool children were due to diarrhoeal diseases in 2010 [4]. Additionally, a recent national survey reported 4.6% of children under five years old were suffering from diarrhoea during the preceding two weeks of the survey [5]. Acute respiratory infections (ARI) are leading cause of health consultations and hospitalisations in Bangladesh [6]. A study estimated that the global median incidence of clinical pneumonia was 0.28 episodes per child per year among children 0–4 years of age [7]. The recent Bangladesh Demographic and Health Survey (BDHS), 2011 reported that 5.8% of children suffered from symptoms of ARI in the two weeks prior to the survey [5]. Two studies from rural and urban Bangladesh reported an estimated 0.2–0.5 episodes of acute lower respiratory infection (ALRI) per child per year among children under five years old [8, 9]. It is evident that both these diseases are the most frequent childhood diseases and causes of attendance at health services and hospital admission for severe cases in developing countries [1]. There have been substantial reductions in the number of deaths due to pneumonia and diarrhoea globally, but these diseases are still the leading causes of morbidity and mortality in children under two years old [1].

Researchers are continuously developing new treatment modalities or strengthening established programs for the management of ARI and diarrhoea in children that include nutritional interventions [10, 11]. As part of this effort, researchers in the last few decades have been evaluating the prophylactic and therapeutic use of different micronutrients to reduce infectious disease including diarrhoea and ARI among children [12, 13]. Recently there has been a renewed interest in the role of vitamin D in infectious, inflammatory and neoplastic disease outcomes throughout the life course [14]. Two key factors have contributed to this interest: first, the growing evidence of the link between vitamin D status and immune functions and infectious diseases [15-23]; and second, the controversies concerning the optimal cut-off level of serum vitamin D for maintenance of good health [24-27]. Few studies have presented a framework for understanding the mechanisms through which vitamin D plays a role in the prevention of diarrhoea or ARI among

children. Here I introduce a framework that illustrates the role of vitamin D in mucosal immunity that is essential in protecting children against diarrhoeal disease and ARI [16, 19, 28-32] (Figure 1.1).

Figure 1.1: Conceptual framework for role of vitamin D in the mucosal immune response and reduction of ARI and diarrhoeal incidence



*Adapted from Schwalfenberg, 2011 and modified for the current dissertation

In summary, vitamin D upregulates genes to strengthen the barrier function of the epithelial membrane of the gastrointestinal tract and respiratory tract. Vitamin D also induces the production of antimicrobial peptides that have a broad spectrum of antimicrobial activity against viruses, bacteria and fungi. Additionally, it prevents the overreaction of the inflammatory response by adaptive immunity and recruits immune cells to fight against infection if necessary. In this framework, production of antimicrobial peptides is the key pathway to prevent ARI and diarrhoea since these peptides appear to play a role in the regulation of innate and adaptive immunity in ARI as well as in gastrointestinal infection [33, 34].

This growing realisation of the greater role of vitamin D in health and disease has led to an increased interest in determining the vitamin D status of previously uncharacterised populations

including children. Recent reviews of the literature suggest that there is a significant burden of vitamin D deficiency and insufficiency among preschool children from developed and developing countries including Bangladesh [35-40]. Furthermore, it is not clear what risk factors contribute to this burden and, in turn, how deficiency is increasing the risk of major morbidities associated with infectious diseases such as ARI and diarrhoea.

The aims of this thesis are to evaluate the vitamin D status of urban preschool children in Bangladesh, identify risk factors associated with vitamin D deficiency or insufficiency, and determine the association of vitamin D status with infectious disease morbidity (diarrhoea and acute respiratory tract infections) among this vulnerable group. Data collected from a multi-site project entitled: 'Comprehensive investigation into the risk factors of malnutrition and its consequences for child health' and 'Study of risk factors for childhood malnutrition using molecular and genomic tools' were used in this thesis. These are key projects of the global network for the study of Malnutrition and Enteric Diseases (MAL-ED).

These projects are being carried out at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), *Mirpur* field site in Dhaka. One of the components of the Mal-ED study is an interventional case-control study of underweight and normal-weight children which evaluated the micronutrient status of participants at baseline and then followed them for five months with active bi-weekly morbidity surveillance. Field workers are also collecting diarrhoeal stool samples during diarrhoeal episodes in children for isolation and characterisation of causative organisms. Additionally, data on household socio-economic status, and dietary intake as well as other relevant data has been collected from these children. A total of 500 cases and 480 controls were enrolled during the period of February 2010 to February 2012. Cases were severe to moderately underweight children (weight-for-age Z score, WAZ, < -2.00 SD) and controls were well-nourished or normal-weight children (WAZ ≥ -1.00) matched for sex and area of residence. Enrolled children received two different intervention packages according to the children's nutritional status (underweight or normal-weight) for five months. The supplementary food given to underweight children was not enriched with vitamin D nor was the micronutrient supplement given to both groups of children.

The data collected in the MAL-ED study gives us the opportunity to determine the prevalence of vitamin D deficiency among underweight and normal-weight children in urban Bangladesh aged 6–24 months, and to clarify what socio-economic, demographic, dietary, health and community factors are associated with their vitamin D status. The cases and controls were considered as two different cohorts for the analyses of data for this PhD dissertation. As such the morbidity surveillance data were used to evaluate associations of ARI and diarrhoeal incidence among the underweight and normal-weight children who vary in vitamin D status. It is hypothesised that

vitamin D status could be linked with the prevalence of pathogen specific diarrhoeal disease among the children, given that vitamin D influences both the innate and adaptive immune responses [15, 17, 18, 20, 21, 41]. Finally, additional analyses will be carried out to evaluate the prevalence of pathogen-specific diarrhoeal diseases among children who differ in vitamin D status.

1.2 Research aims and objectives

Aims

The primary aims of this research work are:

1. Quantify the prevalence and factors associated with childhood vitamin D status.
2. Estimate the association between vitamin D status with acute respiratory tract infection and diarrhoeal incidence among underweight and normal-weight children.
3. Investigate the association between vitamin D status and incidence of common pathogen specific diarrhoeal episodes among underweight and normal weight children.

Objectives

1. Determine the vitamin D status of underweight and normal-weight children aged 6–24 months in urban Bangladesh; and identify the socio-economic and dietary predictors of status.
2. Determine how differences in status of vitamin D are associated with the incidence and severity of diarrhoea in underweight and normal-weight children aged 6–24 months.
3. Investigate which pathogen-specific diarrhoeal infections are prevalent among children with vitamin D sufficiency compared with children who are not vitamin D deficient or insufficient.
4. Determine how differences in status of vitamin D are associated with the incidence of ARI in underweight and normal-weight children aged 6–24 months.

Hypotheses

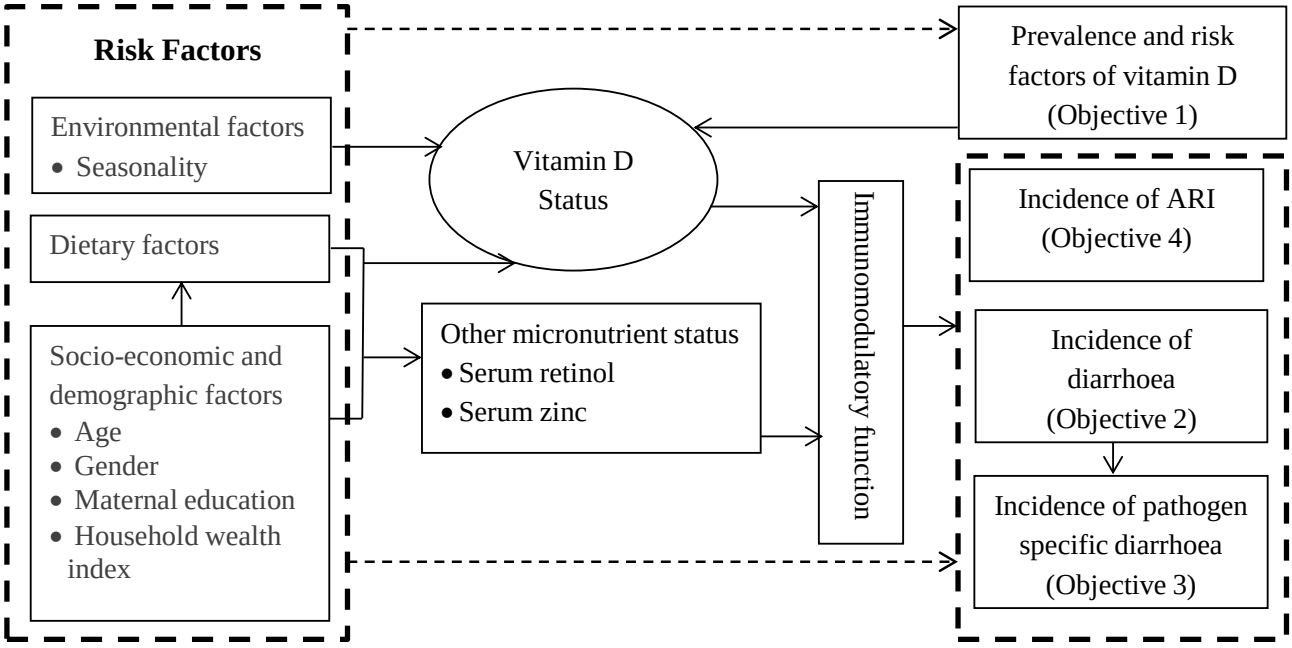
1. Vitamin D status is associated with socio-economic, demographic and dietary factors in children aged 6–24 months (Objective 1).
2. Incidence of diarrhoea and acute respiratory tract infections in children aged 6–24 months is associated with vitamin D deficiency or insufficiency (Objective 2 and 4).
3. Underweight or normal-weight children with vitamin D deficiency or insufficiency are at increased risk of pathogen-specific diarrhoeal morbidity (Objective 3).

1.3 Structure and scope of the thesis

Figure 1.2 provides an overall summary of the structure of the thesis as well as each of the proposed objectives. From the literature review, it is evident that socio-demographic factors, intake of dairy products, the provision of fortified complementary foods to children and the duration of breastfeeding play an important role in determining vitamin D status among children. Moreover, environmental factors such as living at high latitude or in polluted areas were also found to be associated with the vitamin D status of children.

Vitamin D regulates the immune system and as a result influences the susceptibility of children to infection leading to diarrhoea and ARI. The immune-modulatory properties of vitamin D in turn, depend on the status of vitamin D in the children. Additionally, the effect of vitamin D on the occurrence of infectious diseases is also partly determined by socio-demographic [42, 43], dietary, and environmental factors and the status of other micronutrients which all can influence the immune system and determine the transmission and incidence of diseases like diarrhoea and ARI. In this thesis I have considered the interactions of the factors mentioned in the conceptual framework while evaluating the association of vitamin D status with the incidence of diarrhoea and ARI as well as pathogen-specific diarrhoea.

Figure 1.2: Conceptual framework of the research



Prevalence and risk factors for vitamin D status

Recently conducted national micronutrient survey of Bangladesh reported a high prevalence of vitamin D deficiency among children under five years of old (44). Same study also reported 47.9%

children under five years of old living in urban slum had vitamin D deficiency (<50 nmol/L). However, measurement of vitamin D conducted in sub-sample (n=461) of children of national representative estimated participants (44). Two studies reported a high prevalence of rickets in the south-eastern sub-district of *Chakaria* (35, 45). Another study found a high prevalence of deficiency among children with pneumonia and matched healthy controls (38, 39). The research will report prevalence of vitamin D deficiency among a population of urban slum children and will also examine the influences of socio-economic, demographic and dietary factors in determining vitamin D status among urban children under two years of age.

Association of vitamin D status with the incidence of diarrhoea

Evidence on the role of vitamin D in diarrhoeal morbidity among children under two years of age is minimal. Studies carried out among school age children have reported reduced severity of diarrhoea with vomiting among vitamin D sufficient children (46, 47). Another study from Tanzania reported no increased risk in the incidence of diarrhoea among children under five years of age who were born to mothers with serum 25(OH)D <80 nmol/L and HIV infection during pregnancy (48). Recently published results from a randomised control trial (RCT) of quarterly vitamin D supplementation of children 1 to 30 months old found no effect on diarrhoea incidences (49). The heterogeneous results of studies and the lack of studies among younger children warrant further research to evaluate the relationship between vitamin D status and incidence of diarrhoea.

Association of vitamin D status with incidence of pathogen- specific diarrhoea

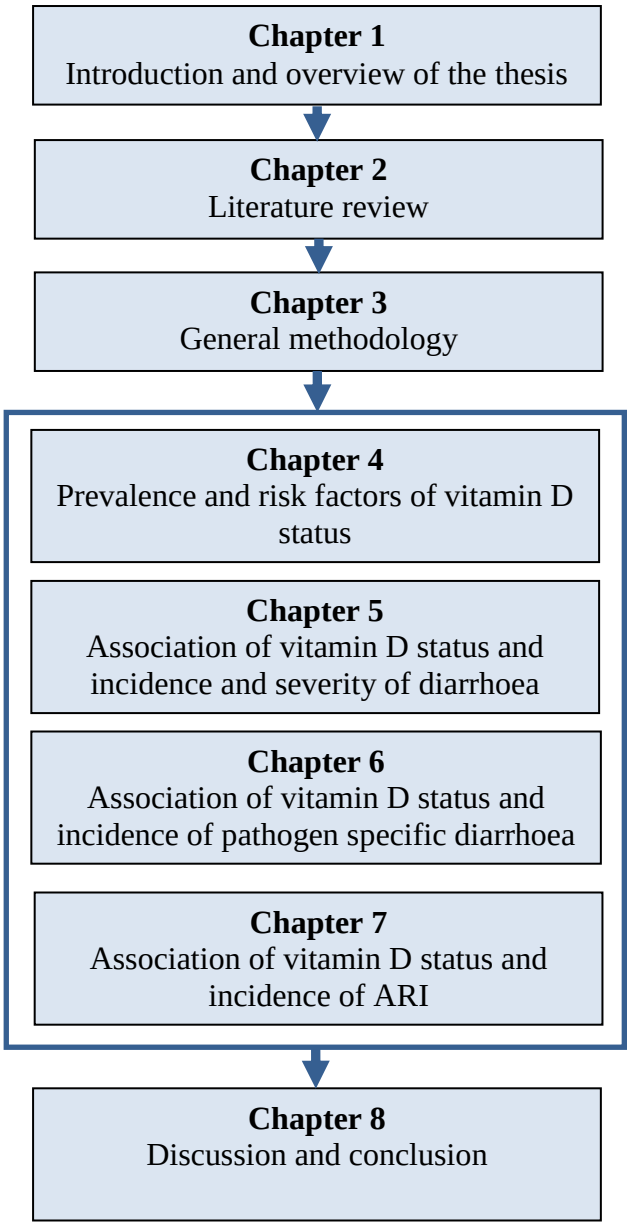
Laboratory studies and animal models have shown that vitamin D regulates anti-microbial peptides that have bactericidal effects on *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, S. typhimurium, Salmonella* and *Shigella* (50-54). Vitamin D induces the production of antimicrobial peptides through the arm of the innate immune system that has a broad spectrum of antimicrobial activity. On the other hand vitamin D prevents the overreaction of the inflammatory response by adaptive immunity and recruits immune cells to fight against infection if necessary. Therefore, there could be higher prevalence of certain pathogen specific diarrhoeal illnesses with vitamin D deficiency or insufficiency.

Association of vitamin D status with incidence of ARI

It is clear from the literature review that vitamin D plays an important role in childhood ARI. Case control studies conducted in India and Bangladesh (39, 55) reported significant difference in mean concentration of vitamin D in children with respiratory tract infections compared with healthy controls. However, similar studies carried out in developed countries (56-58) have not found such

differences. Due to the design constraints, it is difficult to explain whether low vitamin D status is causally associated with disease or disease itself causes the deficiency. On the other hand most of the longitudinal studies had reported that adequate serum vitamin D concentrations in maternal or cord blood is associated with reduced risk of lower respiratory tract infections among children in the first year of life (59, 60). A prospective cohort study from Saudi Arabia (61), reported associations between low cord vitamin D levels and an increased risk of developing ALRI during the first two years of life. A review of RCTs of vitamin D supplementation found only one study reporting a beneficial effect on repeated episodes of lower respiratory tract infection during follow-up period (62). The well designed study done in Kabul, Afghanistan (63) showed no beneficial

Figure 1.3: Diagrammatic overview of the thesis



effect of vitamin D supplementation on incidence of ARI. However, issues were raised about the dose of supplementation that probably impairs the modulatory function of vitamin D, the confounding effect of high rates of undernutrition and the presence of other micronutrient deficiency among the participants (64). All three factors may be modifying the beneficial effects of vitamin D. The heterogeneous results of studies and issues raised by researchers warrant further studies to evaluate the relationship between vitamin D status and incidence of ARI more carefully. Thus, I proposed to conduct a prospective study to investigate the association between the serum concentrations of vitamin D status and ARI among underweight and normal-weight urban children in urban Bangladesh aged 6-24 months.

There are eight chapters in the thesis (figure 1.3). Chapter 1 provides an introduction to the research with an outline of the thesis chapters. The literature review (Chapter 2) comprises reviews of prevalence and risk factors of vitamin D deficiency among preschool children; vitamin D and diarrhoea; and evidences of association of vitamin D status among preschool children with ARI. Chapter 3 describes the general methodology of the thesis. Chapter 4 and 5 consist of manuscripts on prevalence and risk factors for vitamin D deficiency and association of vitamin D status with incidence of diarrhoea, accepted for publication in peer reviewed journals. Chapter 6 provides information on the association of vitamin D status with incidence of pathogen specific diarrhoea. Chapter 7 consists of a manuscript regarding the association of vitamin D status with the incidence of ARI. Finally, the results are summarised and discussed in Chapter 8, followed by a discussion of implications, limitations of the thesis, and conclusions.

1.4 Public health significance

The proposed research will establish the prevalence of the deficiency, insufficiency, and sufficiency of vitamin D among urban Bangladeshi children aged 6-24 months. It will also identify the risk factors for deficiency and insufficiency and the association of vitamin D status with childhood diarrhoeal disease and ARI. These findings will highlight the need for health and education programs for vitamin D and also enable researchers to design better research projects on the role of vitamin D for different health outcomes among children in this age group.

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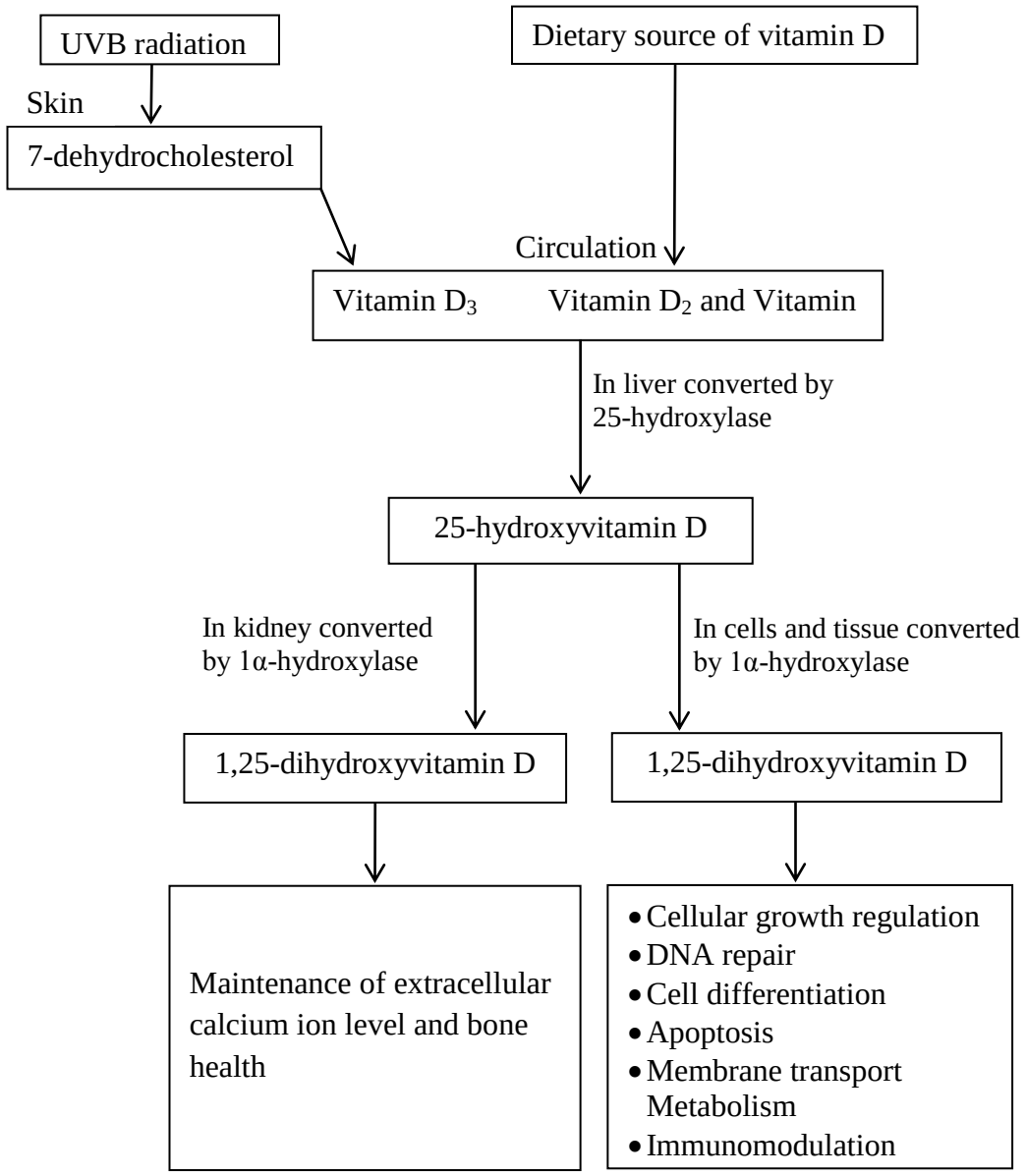
CHAPTER 2 LITERATURE REVIEW

2.1 Health benefits of vitamin D

Vitamin D belongs to a group of several related sterols that plays a vital role in the maintenance of the extracellular calcium ion level in the human body through absorption of calcium from the intestine and from the bone [1]. In the human body, vitamin D occurs in two forms. Vitamin D_3 or cholecalciferol is produced by skin as a result of ultraviolet-B (UVB) irradiation of 7-dehydrocholesterol or by digestion of animal products that are absorbed by the intestine. Vitamin D_2 or ergocalciferol can also be obtained from plant products. However, it is almost impossible to obtain sufficient vitamin D from diet alone [2]. Vitamin D_3 and D_2 are biologically inactive and require metabolism in the liver to form the main circulating form of vitamin D, 25 hydroxyvitamin D (25(OH)D or calcidiol), which is used to measure vitamin D status. Serum 25 hydroxyvitamin D concentration is the best indicator for vitamin D status since it is not influenced by dietary intake of vitamin D_2 (calciferol) and cutaneous production of vitamin D_3 (cholecalciferol) [3]. To be activated, 25 hydroxyvitamin D needs to be converted in the kidney or the cells and tissues of the human body to the form, 1,25-dihydroxyvitamin D ($1,25(OH_2)D$, or calcitriol), which is the biologically active hormone responsible for its physiological actions (Figure 2.1). Vitamin D binding protein is the major carrier of vitamin D and its metabolites 25 hydroxyvitamin D and 1,25-dihydroxyvitamin D. All the biological actions of the active form of vitamin D (1,25-dihydroxyvitamin D) are mediated through the vitamin D receptor (VDR).

Currently, there is no agreement regarding the cut-off level of the serum vitamin D or 25 hydroxyvitamin D concentrations among children for maintenance of good health [4-7]. Most clinicians define vitamin D deficiency using a cut-off point of serum 25 hydroxyvitamin D less than 25 nmol/L or 10ng/ml, [8, 9] which is the cut-off point associated with the occurrence of rickets and osteomalacia. The US Endocrine Society guideline has suggested cut-off levels for serum vitamin D deficiency as less than 20 ng/ml (<50 nmol/L), insufficiency between 21–29 ng/ml (50–75 nmol/L), and the safety margin to minimise the risk of hypercalcaemia equal to 100 ng/ml (250 nmol/L) of serum *25* hydroxyvitamin D [10]. In contrast, the US IOM report has suggested that serum vitamin D concentrations equal to 16 ng/ml (40 nmol/L) covers the requirements of approximately half the population, serum vitamin D equal to 20 ng/ml (50 nmol/L) covers the requirements of 97.5% of the population, and serum vitamin D >50 ng/ml (125 nmol/L) should raise concerns about potential adverse effects. However, studies among adults showed that parathyroid hormone concentration is at the ideal physiologic level when concentration of 25 hydroxyvitamin D is equal to 32ng/ml (80 nmol/L) or above [11, 12].

Figure 2.1: Metabolism and physiological actions of vitamin D



*Adapted from Burgaz A, 2011 and modified for the current dissertation

Various studies have used these different cut-off points of serum vitamin D for optimal bone mineral density, bone turnover, muscle strength, and immune function. One author has proposed a cut-off point of >50 nmol/L for optimal bone mineral density, bone turnover, and muscle strength, while a cut-off point of >75 nmol/L has been proposed for maintaining an adequate immune response [13, 14]. The difference in these recommendations may reflect the different actions of vitamin D in the physiological processes.

2.2 Vitamin D in infectious disease immunity

Role of vitamin D in innate immunity

The new perspective on the immunomodulation of vitamin D started from the detection of the localised conversion of vitamin D to its active form $1,25(OH)_2D$. The active metabolite of vitamin D, $1,25(OH)_2D$ plays a central role in the immune modulation function of vitamin D in the human body. The immune modulatory functions of vitamin D are mediated through the VDR which is expressed in many cells of the immune system, including T and B lymphocytes, neutrophils, monocytes, macrophages, and dendritic cells [15-19]. Circulating 25 hydroxyvitamin D bound to plasma vitamin D binding protein (DBP) enters macrophages and is converted to $1,25(OH)_2D$ by mitochondrial CYP27B, and then bound to the VDR in the cell. Once bound to VDR, $1,25\ (OH)_2D$ is able to initiate transcriptional factors in epithelial cells including enterocytes and those found in the respiratory tract that induce the expression of the antimicrobial peptides (AMP) cathelicidins and defencins [20]. These AMP are a component of the innate immune system and are important in killing and clearing both gram-positive and gram-negative bacteria, virus, fungi and mycobacteria in the skin and the mucosal linings of the respiratory and gastrointestinal systems [19, 21-29]. Additionally $1,25(OH)_2D$ promotes hydrogen peroxide secretion in human monocyte and recruits other immune cells to fight against infection [30-32] .

Role of vitamin D in adaptive immunity

Monocyte/Macrophage and dendritic cells (DC) are the two principle cells in the innate immune system which are important in the recognition, inactivation or killing of microorganisms or invader agents. These cells also present antigens from the agent/pathogen to resting T and B lymphocyte which leads to activation and concomitant development of an adaptive immune response. This autocrine innate antimicrobial function by macrophage also induces monocytes, and T or B lymphocytes as a consequence of $1,25(OH)_2D$ secretion in paracrine fashion. The activated macrophages and mature DCs also express the vitamin D activating enzyme CYP27B and are thus able to synthesise $1,25(OH)_2D$ from precursor 25 hydroxyvitamin D. The $1,25(OH)_2D$ synthesised in this way acts in a paracrine fashion on activated B lymphocytes and activated T lymphocytes, which are expressing abundant VDR and initiate several functions by those cells. Under the influence of $1,25(OH)_2D$, activated B lymphocytes reduce proliferation, Immunoglobulin (Ig) production, memory, and plasma cell differentiation. Additionally the activated $1,25(OH)_2D$ promotes inhibition of T-cell differentiation and proliferation, Th1 cell immunoactivity and interleukin 2-driven B-cell immunoglobulin production [20].

The overall summary of the role of vitamin D in innate and adaptive immunity is $1,25(OH)_2D$ stimulates the innate immune response in antigen-presenting cells and on the other hand same time it inhibits any overzealous responsivity in the adaptive immune response to the offending infection/antigen.

2.3 Prevalence of vitamin D status among preschool children

A literature review was carried out on the vitamin D status of children under five years old—globally and specifically in Asia and Bangladesh. The risk factors associated with the deficiency of vitamin D status were also considered. The literature search was carried out using the PubMed database to identify studies concerned with vitamin D status among preschool children. Manual searches of retrieved articles were also carried out to identify additional articles addressing the status of vitamin D among children. A summary of the findings of the selected articles is presented in Appendix tables 1 to 3 of Appendix A. Details of the mean concentration of serum vitamin D, cut-off levels used by different studies to report vitamin D deficiency, settings, age of the participants also incorporated in the Appendix tables (1-3).

From the literature review, it is evident that there is a huge burden of vitamin D deficiency and insufficiency among preschool children from developed and developing countries (33, 34). However, there is a wide range (30–80%) in the prevalence of vitamin D deficiency among children and adults worldwide (1, 35, 36). This is compounded by the relatively few national representative surveys of vitamin D status that have been collected at a population level or surveys identifying risk factors for vitamin D deficiencies (37). Few studies from Bangladesh have reported the vitamin D status of preschool children although there is a high prevalence of rickets among children in specific regions of Bangladesh. Recently conducted national micronutrient survey in Bangladesh have reported mean concentration of serum vitamin D 56.3 (50.6, 62.1) nmol/L and 39.6% children aged under five years of age had vitamin D deficiency (<50 nmol/L) (38). Similar study also reported 47.9% of children under 5 years of age from urban slum had vitamin D deficiency. However, national micronutrient survey reported findings among the sub-sample of national representative population of under five children of Bangladesh. Studies carried out in *Chakaria*, *Coxesbazar* found that 11% of children had active rickets and that vitamin D deficiency ranges from 6–21% among children under five years old in this community (39-41). Recently a case-control study concerned with ALRI was carried out among children aged 1–24 months in north-eastern rural Bangladesh (42, 43). Approximately 32% of all children (including case and control) were vitamin D deficient (<25 nmol/L) and 70% had serum 25 hydroxyvitamin D <40 nmol/L. interestingly, mothers of children with serum vitamin D >25 nmol/L consumed more milk, meat and eggs during the week

prior to the interview. The same study also found that vitamin D deficient children are from the lower socio-economic group and are more stunted or have a lower Body Mass Index (BMI).

Several studies from India have demonstrated low serum vitamin D levels among preschool children (44-47). Agarwal *et. al.* (44)reported that children from communities with high levels of atmospheric pollution, had significantly lower mean serum vitamin D concentrations compared with those living in the less polluted area. A longitudinal cohort study among pregnant women and their offspring found that 50% of mothers had insufficient vitamin D during the birth of children while 36% and 62% neonates were found to be vitamin D deficient or insufficient respectively (45). Another study in impoverished areas of Delhi, India, found that the prevalence of low-serum vitamin D status among children ranged from 2–84% but could not explain this wide variation in prevalence (46). A hospital-based case–control study revealed that a significant proportion of children with severe ALRI had vitamin D insufficiency or deficiency compared with the control children (47).

Vitamin D deficiency is also a public health issue among Pakistani mothers and infants. In Karachi, severe vitamin D deficiency was found among all of the nursing mothers while 52% of healthy breastfed infants were found to be vitamin D deficient (48, 49). Researchers reported that low vitamin D status in children was associated with poor maternal vitamin D status, low vitamin D intake, and reduced exposure to sunlight. More than half of the studied children were introduced at four months of age to complementary food made of lentils and rice which is not fortified with vitamin D. These authors also found significantly low vitamin D concentrations among children from the upper socio-economic strata and infants of educated mothers, which could be due to their reduced exposure to sunlight as these infant are living in flats and villas. A study from Saudi Arabia supports the results found among Pakistani infants (50).

There is high prevalence of vitamin D deficiency among all age groups in Iran, especially among women. This deficiency could result from social and cultural practices that require women in Iran to cover themselves completely thus restricting their exposure to sunlight that would enable synthesis of vitamin D (51). Among neonates, 75% were found to be vitamin D deficient during the winter while 35% were deficient in the summer. There was positive correlation of serum vitamin D status between cord blood and maternal blood in this study (51).

More than half of the preschool children of Ankara, Turkey were found to be vitamin D insufficient while 46% of neonates were vitamin D deficient (52, 53). The authors identified low sunshine exposure, skin pigmentation, air pollution, skin covering, and low vitamin D intake as risk factors for deficiency and insufficiency. Similar results were also reported by the studies carried out in Jordan (54).

Vitamin D deficiency is an important problem in industrialised countries as well. Analysis of data from the US NHANES survey found that 63% preschool children were vitamin D insufficient (55). The lowest mean serum concentrations (58 nmol/L) were found among black non-Hispanic children. Overall, children who had <75 nmol/L of serum 25-hydroxyvitamin D, which is the cut-off level for maintenance of good health, were 85% black non-Hispanic, 73% Hispanic, 61% others and 54% White. In New Zealand approximately 10% of urban children were found to be severely vitamin D deficient with vitamin D status varying with age, ethnicity, and season. Children who had not received any formula had a 4.8 times greater risk of being vitamin D deficient (56). Similarly, children who received home-made complementary food were at a greater risk of vitamin D deficiency compared with children fed commercially prepared complementary food while children who did not eat eggs, meat or fish are at increased risk of vitamin D deficiency. In the same study, household expenditure to income ratio, number of household members, and crowding were found to be the risk factors for vitamin D deficiency among children aged 6–23 months (56). Studies carried out among immigrants in Europe have found that children of immigrants from Asia as well as adolescents and adults were at greatest risk of being vitamin D deficient than native Europeans (57-61).

Overall, there is no clear picture of the burden of vitamin D deficiency among preschool children. Part of the problem results from studies using different cut-off points when reporting prevalence of deficiency or insufficiency. As a result, the overall prevalence of deficiency ranges from 0% to 92% among preschool children globally, whereas insufficiency ranges from 5% to 95%. There is a greater prevalence of deficiency among children from South Asia compared with the rest of the world, despite the extensive exposure to sunlight in that region. Most likely the unplanned urbanisation and environmental pollution, less physical and outdoor activities, skin pigmentation, and cultural barriers put South Asian populations, including Bangladesh, at greater risk of vitamin D deficiency.

2.4 Vitamin D and ARI among children under 5 years of age

A review of the global literature on the relationship between vitamin D status and ARI among children under five years of age was carried out. For this review, a literature search was carried out in the PubMed database using vitamin D and acute respiratory tract infections as keywords. Summary of the findings of the selected articles are presented in the Appendix tables 4–6, of Appendix A. A total of 16 studies have been selected for this review. A recently published systematic review was also used for identifying studies among children under five years of age (62). Out of the 16 studies, one is cross-sectional, seven are case-control studies, five are cohort, and three are RCTs.

All the cross-sectional and case-control studies were carried out among hospitalised children. In case-control studies, controls were selected from hospitalised children with the exception of studies done in India and Bangladesh (43, 47). A case-control study from Ethiopia reported that the prevalence of vitamin D deficiency was significantly greater among children with rickets and pneumonia (63). Several other studies from developing countries have suggested an association between nutritional rickets and pneumonia (64-67).

The case-control studies carried out in India and Bangladesh reported significant differences in vitamin D status between patients with pneumonia and healthy controls (42, 47). In contrast, studies carried out in Canada (68-70) have not found any significant differences in mean serum vitamin D concentration between children with lower respiratory tract infection and hospitalised or healthy control children. The heterogeneity in findings between Asian and Canadian studies is probably due to the different socio-economic and environmental background of participants, different burden of causative organisms, and severity of disease.

Most of the longitudinal studies have focused on either maternal or cord blood concentration of serum vitamin D and incidence of ARI in the children during infancy to preschool period. A study carried out by Morales et al. (71) found a reduced risk of lower respiratory tract infections among children in the first year of life if the mother had high serum vitamin D concentrations during pregnancy (71). In a cohort of children from New Zealand, newborns with cord blood vitamin D concentration less than 25 nmol/L were twice as likely to develop respiratory infection within three months of age compared with those with concentration of 75 nmol/L or higher (72). A study in the Netherland reported an increased risk of ALRI due to respiratory syncytial virus among infants with low plasma concentrations of vitamin D at birth (73). A prospective cohort study from Saudi Arabia (74), reported associations between low cord vitamin D levels and an increased risk of developing ALRI during the first two years of life. In this study there was a significant inverse relationship

between circulating levels of serum vitamin D and the severity of the ALRI. This finding could open new arenas for the prognosis of ALRI among preschool children.

A study from Afghanistan showed the beneficial effect of vitamin D supplementation during treatment of pneumonia following episodes of ALRI or any acute respiratory infection (75). Another RCT that followed low birth-weight children for up to six months after birth, found no effect of weekly vitamin D supplementation on mortality or the incidence of pneumonia (76). Another well-designed RCT performed in Kabul, Afghanistan concerned with evaluating the efficacy of a quarterly bolus dose of vitamin D in prevention of radiologically proven pneumonia in children found no effect on its incidence or on the incidence of only pneumonia (77). However, an excess of repeated episodes of pneumonia was recorded in the intervention group (0.60 vs. 0.04 episodes per child per year).

2.5 Vitamin D and diarrhoea

Few studies were found in the literature search concerned with the relationship between vitamin D status and diarrhoea. A recently published study from Columbia reported increased risk of vomiting with diarrhoea (adjusted IRR: 2.05, 95% CI: 1.19, 3.35) among school-aged children with vitamin D deficiency (78). A study from Qatar reported significant higher prevalence of gastrointestinal infections among vitamin D deficient children (79). The only study from Tanzania among children under five years of age reported no risk of increased incidence of diarrhoea in children who were born to mothers with serum 25(OH)D <80 nmol/L and HIV infection during pregnancy (80). Evidence about the role of vitamin D in diarrhoeal morbidity among children under two years of age is limited. Recently an RCT reported no effect with six quarterly bolus doses of vitamin D supplementation on diarrhoeal incidence among children aged 1–30 months (81).

Studies of the role of vitamin D in the regulation of gut function and health suggests that vitamin D status may contribute to a host's ability to resist or limit diarrhoeal disease as outlined below. Briefly, laboratory studies and animal models have shown that vitamin D regulated anti-microbial peptides that have bactericidal effects on *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, S. typhimurium, Salmonella* and *Shigella* (15-19). These are the micro-organisms responsible for diarrhoea among children. Vitamin D mediated strengthening of gap junction could provide protection against viral infection (82, 83) as well invasive organisms like shigella and salmonella. Finally vitamin D can recruit other immune cells to fight against infection if required and also regulates the inflammatory response. Thus, vitamin D deficient or insufficient children are more susceptible to diarrhoea as well as severity of the infection.

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CHAPTER 3 GENERAL METHODOLOGY

3.1 Background

The Aetiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) Study, led by the Fogarty International Center of the National Institutes of Health and the Foundation for the National Institutes of Health, was conducted during 2009–2014. The aims of the MAL-ED study were to evaluate the complex interrelationships between gut microbial ecology, enteropathogen infection, dietary intake, nutritional status, gut physiology, growth, immune function and vaccine response, and cognitive development, and childhood undernutrition. Thus, the MAL-ED study was focused on birth cohorts followed longitudinally, in eight countries including Bangladesh, where the incidence of diarrhoeal disease and undernutrition are high among children. The details of the MAL-Ed study are described elsewhere [1]. The intensive biweekly household surveillance and efforts to collect clinical specimens in birth cohorts may create a Hawthorne effect that dramatically reduces diarrhoea rates and malnutrition. To overcome the Hawthorne effect, the MAL-ED network also conducted a case-control study in children, 6–24 months old, at the urban Mirpur field site in Dhaka, Bangladesh [2]. Data and biological samples collected in the case-control interventional study were analysed for this PhD dissertation.

3.2 The thesis

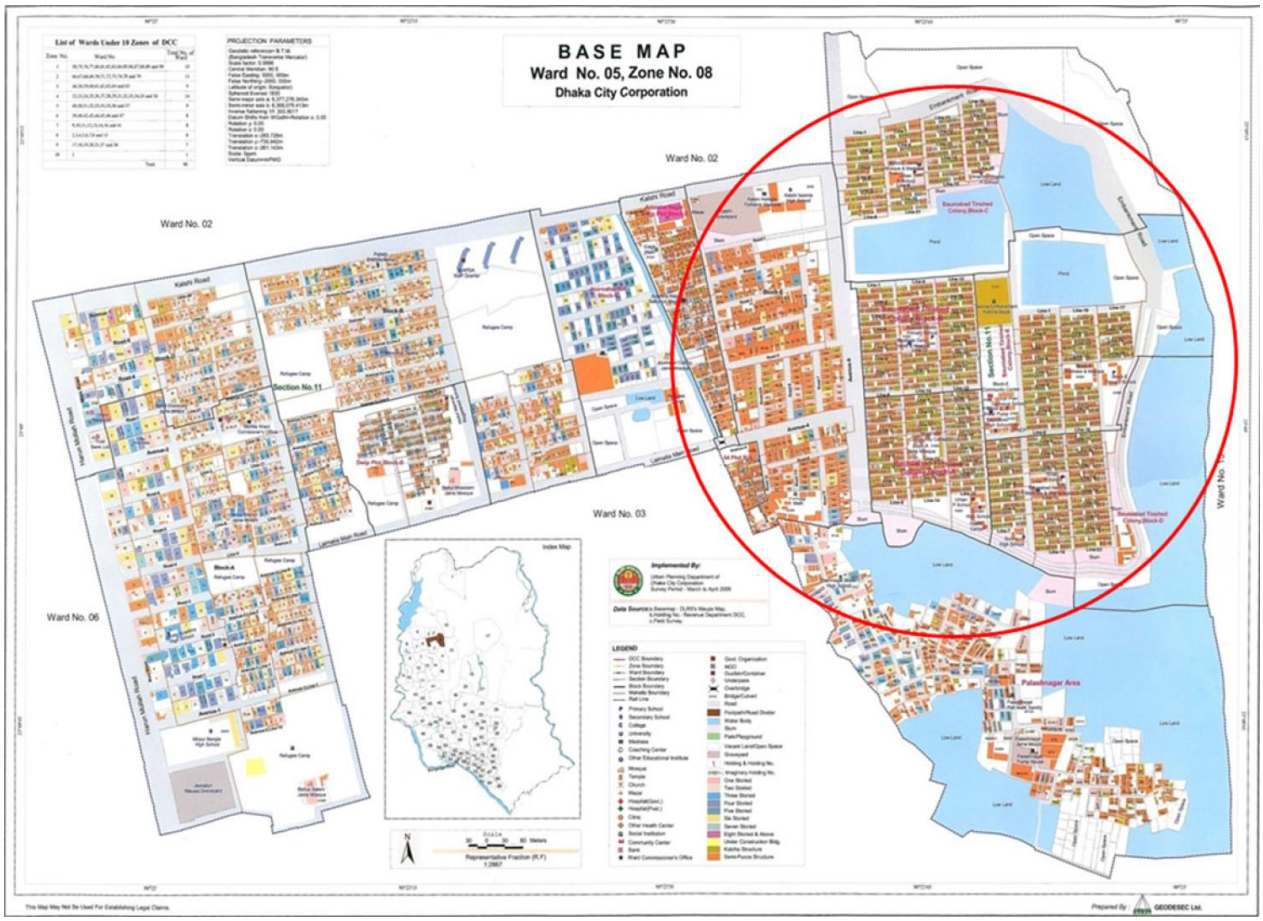
The study conception and design of this doctoral thesis was based on the case-control study conducted as a part of the MAL-ED network at the Mirpur field site of Dhaka, Bangladesh. I was one of the co-investigators for the MAL-ED Bangladesh site studies. Accordingly, I was directly involved with the inception of the case-control study design, development of data and sample collection tools, training of the field workers, supervision and quality assurance of data and sample collection, supervision of sample assays, and management of data entry and quality assurance until January 2012. I have received a complete clean dataset in 2013 and 2014 for my PhD dissertation. In the following section I describe the case-control study methods specifically relating to this thesis.

3.3 Case-control study

The Mal-ED study (proposal # 2008-020) was approved by the Research Review Committee and the Ethical Review Committee of icddr,b in 2008. Parents/caregivers of eligible children provided informed written consent before collection of data and all biological samples at the time of enrolment. Before enrolment parents/caregiver were informed about the objectives, procedures, and

potential benefits and risks involved in participation with this study, as well as the right of withdrawal or the decision to not provide data or biological samples.

Figure 3.1: MAL-ED study site, Mirpur, Dhaka, Bangladesh.



Source: Urban planning department, Dhaka City Corporation North. Available at: http://www.dncc.gov.bd/departments-with-function/mayor-other/urban-planning.html

Study site and participants: The case-control study was conducted among residents of an under-privileged community in Mirpur, Dhaka, Bangladesh. Mirpur has been selected as the site of the study because it is inhabited by poor and middle-class families; residential and sanitary conditions are typical of any congested urban settlement, and the investigators have ongoing field research activities in the area. Mirpur is one of the 14 Thanas of Dhaka City with a population of about one million in an area of 59 square kilometres. Mirpur Thana is divided into several sections and we conducted the project activities in Section 11 of Mirpur (Figure 3.1). The coordinates of Mirpur are 23.8042° N 90.3667° E. Initially, the majority of the inhabitants were of Bihari ethnic origin, who settled in Mirpur long before the war with Pakistan in 1971; however, intensive cultural and social integration has occurred since then and the population was representative of any typical urban area in Bangladesh. The population of Section 11 was approximately 50,000, and was stable with low socio-economic conditions and sub-optimal sanitation. The average family size was 4.8 and

monthly family income was below TK. 10,000 (about US$ 123) for 83% of the inhabitants in the study area. About 68% of mothers did not have any formal education [2].

Mal-ED study conducted biannual surveillance of every household of *Bauniabadh* area of section 11 of *Mirpur t*o identify eligible participates for the study. Participants (children aged 6–24 months) were screened for eligibility using weight measurement during household surveys. Cases were identified if they were severe to moderately underweight (weight-for-age Z, WAZ, score <–2.00 SD) and controls were well-nourished/normal-weight children (WAZ >–1.00 SD) matched for area of residence and sex. Upon selection the caregivers/guardians of children were invited to participate in the study through signing an informed voluntary consent form. A total of 1129 children were eligible for enrolment and 149 children's Parents/caregivers were refusing to participate in the study (figure 3.2). Overall, 500 cases and 480 controls were enrolled during November 2009 through December 2012.

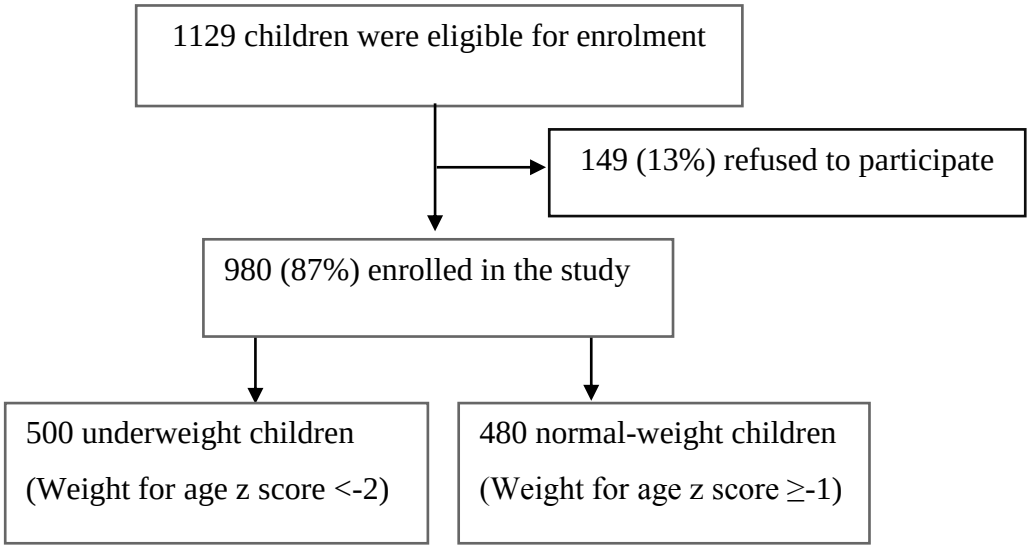


Figure 3.2: Study profile

Exclusion criteria: Children were excluded from enrolment if they had any features suggestive of illnesses that impact on nutritional status as well as on response to treatment of malnutrition (e.g. severe diarrhoea or pneumonia at the time of enrolment, persistent diarrhoea, cleft lip/palate, blindness, tuberculosis, jaundice, renal or cardiac disease, cerebral palsy and any chromosomal disorders).

Intervention package: All cases (underweight) received standard of care nutritional supplementation as recommended by the National Nutrition Program (NNP) of Bangladesh (Table 3.1). The NNP aims at reducing the prevalence of child malnutrition in the country through behaviour change communication, supplemental feeding (which actually serves the purpose of

demonstrative feeding), and provision of vitamin A at six-monthly intervals. The supplementary food given is called *Pushti* Packet (PP) which means nutrition packet in Bangla language. It is composed of roasted rice and lentil powder, molasses and vegetable oil; each packet giving 150 kcal (3). In this study, to ensure adequacy of nutritional support, children with severe malnutrition (WAZ <–3.00 SD) received three packets daily (450 kcalories) as opposed to the NNP guidelines of two packets, while moderately malnourished children received the NNP-recommended two packets daily (300 kcalories) for five months or until graduation by achieving WAZ –2.00 SD (or WAZ –1.00 SD for children enrolled with moderate malnutrition). The packets were given to the children daily (6 days a week) when they visited one of the four outposts established for the study. The outposts were within walking distance of all participants. The caregivers (mothers, grandmothers or an older sibling) were asked to sit down on a mat and feed the children the contents of the packet.

Table 3.1: Interventions given to the enrolled children

Interventions	Moderate or severe underweight children (WAZ <–2) (case)					Well-nourished children (WAZ >–1) (control)				
	Months					Months				
	1	2	3	4	5	1	2	3	4	5
Supplementary feeding * (locally made)	Receiving at least 2 packs 6 days a week for 5 months					Not receiving				
Multiple micronutrient powder**	One sachet daily for 4 months					One sachet daily for 4 months				
Health and nutritional education for care givers	Six days weekly for 5 months					Once weekly for 5 months				
Vitamin A supplementation (6-12 months old 100,000 IU and >12 months old 200,000 IU)	At enrolment if not received in previous 6 months					At enrolment if not received in previous 6 months				
Deworming***	At enrolment					At enrolment				
Ensure immunization¥	At enrolment					At enrolment				

* Each sachet contains roasted rice powder 20g, roasted lentil powder 10g, molasses 5g and vegetable oil 5ml providing approximate 150 kcals. Severe malnourished (WAZ <–3) received three packets daily (450 kcals) and moderate malnourished children received 2 packets daily (300 kcals) for 5 months or until graduation by achieving WAZ –2 for severe malnourished children and WAZ –1 for moderate malnourished children).

** Each sachet contains: 12.5 mg elemental iron, 5 mg elemental zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C.

***200 mg **a**lbendazole syrup was given orally as a single dose to all children more than one year old. In children under one year old, pyrantel pamoate was given 10 mg/kg as a single dose as well.

¥ Immunisation covers BCG, DPT, OPV, measles, hepatitis B, and Hib vaccines

Both case and control children received high potency vitamin A supplementation every six months (100,000 international units for children 6–12 months old and 200,000 units for older children). Both case and control children received multiple micronutrient powder supplementation (MNP)

without any vitamin D. Field workers demonstrated, how to use MNP with the regular diet. Each sachet of MNP contained: 12.5 mg iron, 5 mg zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C. Children more than one year old received albendazole, 200 mg, at the time of enrolment. Diarrhoeal episodes were treated with oral rehydration solution and oral zinc treatment as per WHO/UNICEF recommendations (20 mg zinc sulfate daily for 10 days; half the dose for infants). The caregivers were encouraged to have their children vaccinated at the nearest Expanded Program on Immunization (EPI) centre against the six EPI diseases—poliomyelitis, TB, diphtheria, pertussis, tetanus, and measles. Caregivers of all case and control children were strongly encouraged to attend health and nutrition sessions taking place in the outposts every week. Each session was centred on selected health and nutrition issues, preferably one issue at each session. Issues included preparation of nutritious food using family food ingredients, importance of hand washing and sanitation, immunisation, home management of diarrhoea, recognition of danger signs of common childhood illnesses, etc. Pre-tested information, education and communication (IEC) materials were used during these sessions. Active involvement of the caregivers in the discussions was ensured. Inter-current illnesses including diarrhoea, dysentery, mild pneumonia, fever, etc. experienced by case and control children were treated at the community clinic established and maintained by the investigators in Mirpur. All episodes of severe illnesses were referred to an appropriate hospital.

Table 3.2: Timeline/schedule for data and specimen collection from enrolled children

	Months						
	0	1	2	3	4	5	End of 5 months
Incidence and prevalence of enteric pathogens (diarrhoeal samples during 5 months of follow up) (monthly sample at enrolment, end of 3 and 5 months)	×			×		×	
Morbidity surveillance	×	×	×	×	×	×	
Anthropometry	×	×	×	×	×	×	×
Nutrition (FFQ)	×	×	×	×	×	×	
Micronutrients assay	×						×
SES data	×						×

Sample types
Stool
Blood

Both case and control children received high potency vitamin A supplementation every six months (100,000 international units for children 6–12 months old and 200,000 units for older children).

Both case and control children received multiple micronutrient powder supplementation (MNP) without any vitamin D. Field workers demonstrated, how to use MNP with the regular diet. Each sachet of MNP contained: 12.5 mg iron, 5 mg zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C. Children more than one year old received albendazole, 200 mg, at the time of enrolment. Diarrhoeal episodes were treated with oral rehydration solution and oral zinc treatment as per WHO/UNICEF recommendations (20 mg zinc sulfate daily for 10 days; half the dose for infants (≤ 12 months of age). The caregivers were encouraged to have their children vaccinated at the nearest Expanded Program on Immunization (EPI) centre against the six EPI diseases—poliomyelitis, TB, diphtheria, pertussis, tetanus, and measles. Caregivers of all case and control children were strongly encouraged to attend health and nutrition sessions taking place in the outposts every week. Each session was centred on selected health and nutrition issues, preferably one issue at each session. Issues included preparation of nutritious food using family food ingredients, importance of hand washing and sanitation, immunisation, home management of diarrhoea, recognition of danger signs of common childhood illnesses, etc. Pre-tested information, education and communication (IEC) materials were used during these sessions. Active involvement of the caregivers in the discussions was ensured. Inter-current illnesses including diarrhoea, dysentery, mild pneumonia, fever, etc. experienced by case and control children were treated at the community clinic established and maintained by the investigators in Mirpur. All episodes of severe illnesses were referred to an appropriate hospital.

Quality assurance of data and sample collection: Before implementation of the study in the field site all the questionnaires were field tested. Field staff members received standard training for data and sample collection before the implementation of the study at the field site. Twice yearly refresher training courses were also conducted for quality assurance of data collection. Standard operating procedures (SOP) for each of the questionnaires and sample collection were also developed. Field staff members involved with data and sample collection used the SOPs for standardised data collection. Finally, all field staff received training for weight and length measurement of children.

Demographic characteristics, socio-economic status, and food access insecurity: The Demographic and Health Survey (DHS) questionnaires on household socio-economic and demographic status were adapted for data collection (4). Household food insecurity was assessed with a questionnaire based on the one used for the Food and Nutrition Technical Assistance project (5).

Assessment of nutritional status: Nutritional status of the children assessed with anthropometry. Children's naked body weight was taken using the digital baby and toddler scale (Seca 354) to

nearest 10 g and length by the Infantometer/length measuring board (Seca 416) to nearest 0.1 cm. Linear growth was measured as supine length in children less than two years of age and as stature (standing height) in older children. Anthropometry was done at the time of enrolment and on monthly basis for five months. The weight-for-age, length-for-age, and weight-for-length *z* scores are calculated using the World Health Organization (WHO) Multicentre Growth Reference Study Group program (6). Dietary intake data of the child was collected using a food frequency questionnaire (FFQ) at enrolment and every month for 5 months. The questionnaire was based upon the DHS questionnaires (4), and were analysed in a similar fashion to derive recommended indicators of infant feeding. Adaptation to local names for common examples of the food items had been carried out before the data collection. It was also designed to capture dietary diversity and an index of food adequacy. Field staff received standard training on data and sample collection including information regarding food frequency before the implementation of the study at the field site.

Morbidity surveillance: A structured standardised questionnaire was designed to collect a daily record of symptoms of cough, fever, vomiting, diarrhoea, and medication use from the caregivers of the enrolled children. The daily data of morbidity was collected through twice weekly home visits by trained field workers. If the caregiver was absent during the home visit, field workers visited the home at least three times on that day, and then at least once a day on all following days until the fieldworker was able to contact the caregiver to collect the information. If participants were absent more than seven days from the study site, field workers collected morbidity information about the last seven days at the first successful visit. If the fieldworkers were unable to make contact with the caregiver for 60 consecutive days, the child was then dropped from the study.

Standard case definitions were used which are as follows:

Diarrhoea: Passage of three or more loose/watery stools in 24 hours except in breastfed infants where diarrhoea will be defined by the mother as an abnormal stool (7, 8).

Dysentery: Passage of blood or mucus in diarrhoeal stools with/without abdominal pain, tenderness, or fever (8).

Acute Lower Respiratory Infection (ALRI): Age-specific fast breathing (more than 60, 50, 40 breaths per minute for infants less than 2 months old, 2–12 months old, and more than one year old respectively) with cough, fever, and with/without auscultatory chest findings (8).

Stool enteropathogens: Stool samples were collected during diarrhoeal episodes (diarrhoeal episodes were defined as three or more loose/watery stools in 24-hours followed by two consecutive days with fewer than three loose/watery stools passed by the children) and monthly samples were

collected at enrolment and at the end of the three and five months of follow-up. Caregivers were provided with two stool-collecting containers and requested to collect diarrhoeal stool samples during the episode of diarrhoea. Caregivers were also instructed about the urgency of collection of a fresh sample and transporting that sample as soon as possible to increase the likelihood of identifying causative pathogens. Moreover fieldworkers visited the household of enrolled children every day and enquired whether the child was suffering from diarrhoea, and when the definition of diarrhoea was met every attempt was made to to collect a sample before the episode ended, according to standard sample collection protocol.

Within two hours of collection of stool samples a swab was transferred into Cary Blair media (transport media) and the rest of the sample refrigerated in cold packs for transport to the laboratory and processing. All stools were analysed for the presence of 15 most common enteropathogens in children under age two (*Ascaris*, *Trichuris*, Amoebiasis, Giardiasis, Cryptosporidiosis, Rotavirus, enterotoxigenic *E. coli* (ETEC), enterpathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), Cholera, *Salmonella*, *Shigella*, *Campylobacter*, and *Aeromonas*) using traditional methods of microscopy, culture, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (9).

Micronutrient assays: A 5ml blood sample was collected in trace element free tubes with all aseptic precautions from children upon enrolment and after completion of five months follow-up for determination of haemoglobin, ferritin, and plasma transferrin receptor, retinol, zinc and vitamin D. Processing of the collected samples and micronutrient assays were performed at the nutritional biochemistry laboratory of icddr,b.

Serum retinol was measured using the high performance liquid chromatography (HPLC) method. An aliquot of serum/plasma is deproteinized with methanol containing 50 μg % retinyl acetate, and retinol is extracted into hexane. The hexane layer is transferred to a clean vial, evaporated under nitrogen, re-dissolved in mobile phase (95% methanol), and injected into an HPLC column. Two plasma pool samples with assigned value set against standard serum from the National Institute of Science and Technology were run with each set of samples, and the concentration of retinol was calculated based on known concentration of retinol in the pool samples. This pool was stored in aliquots at −80 °C and used as a secondary calibrator/QC in each run/day. NIST Standard Reference Material 968 was used to assign value of retinol to that pool. This pool was stored in aliquots at −80 °C and used as a secondary calibrator/QC in each run/day. Co-efficient of variation (CV %) range from 1.0% to 4.0%.

Serum zinc concentration was determined by diluting the sample twelve times with deionised water and by an air-acetylene flame atomic absorption spectrophotometer at 213.9 nm. Accuracy and

precision of analysis was ensured by using the bi-level serum trace element control from UTAK Laboratories Inc. (Valencia, CA). Bi-level trace elements serum toxicology control (normal and high range, UTAK Laboratories Inc, USA) was used with each set of samples to check the accuracy for serum zinc. Pooled serum was used in each lot to check for both accuracy and precision. Within-day CVs for pooled sera, normal and high range of zinc QC sera were 3.7%, 2.9% and 2.2%, respectively. Between-day CVs for pooled sera, normal and high range of zinc QC sera were 4.9%, 5.0% and 4.1%, respectively.

Serum vitamin D was measured using the IDS 25-Hydroxy Vitamin D Enzyme immunoassay (EIA) Kit (10) (Source: IDS Ltd, 10 Didcot Way, Boldon Business Park, Boldon, UK). Two levels of controls (REF AC-5705A - AC-5705B) were included in the each kit. These two controls were run in each plate/run for monitoring accuracy and precision. The co-efficient of variation was (3.8-11.8) % for control 1 and (5.2-10.7) % for control 2. Moreover, the same ELISA kit was used to participate in the different EQAS programs like DEQAS (Vitamin D External Quality Assessment Scheme), NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP), and Vitamin A Laboratory - External Quality Assurance (VITAL-EQA) program. The icddr,b biochemistry laboratory has always received certificates for successful participation. The results of micronutrient status were adjusted for subclinical infections (11, 12).

3.4 Measurements

Demographic and socio-economic variables were recoded to generate age groups (6–11, 12–17 and 18–24 months), maternal education (illiterate, 1–5 years and <5 years of institutional education), and other socio-economic parameters with information from the database. Household wealth index was constructed with principle component analysis as described in the DHS (13). Four seasons—summer (May to July), autumn (August to October), winter (November to January) and spring (February to April)—were created from the date of the blood sample collection. There is a lack of consensus and definitions regarding the cut-off level for the determination of serum vitamin D concentrations among children for maintenance of good health (14-17). Different studies have given rise to a range of terminology and associated values to describe vitamin D status (18). In this study, initially serum vitamin D was recoded using standard serum cut-off points: severe deficiency (<25 nmol/L), deficiency (25–49.99 nmol/L), insufficiency (50–74.99 nmol/L) and sufficiency (≤75 nmol/L). However, to increase the statistical precision for multivariable analyses we combined both severe deficiency and deficiency categories into one category termed “deficient status” (<50 nmol/L). Thus, finally serum vitamin D status was categorised into deficient (<50 nmol/L), insufficient (≥50 and <75 nmol/L) and sufficient (≥75 nmol/L) status as per the recommendations of The US Endocrine Society guideline (19). Serum retinol status was categorised as moderate to

severe deficiency (<0.7μmol/L) and mild deficiency to normal status (≥0.7μmol/L) (20). Serum zinc insufficiency was defined as serum zinc <9.9 μmol/L and categorised into insufficient and sufficient (≥9.9 μmol/L) status (21).

Diarrhoea was defined as three or more loose stools in 24 hours, or one loose stool with visible blood (22). A new diarrhoeal episode was defined as three or more loose/watery stools in 24-hours followed by two consecutive days with fewer than three loose/watery stools passed by the child. The rate of diarrhoea was calculated by dividing the number of days with diarrhoea by total number of days of observation (23). The severity of diarrhoeal episodes were categorised as 'low', 'medium' and 'high' severity according to the method described in Lee *et al*. (24).

Standard case definitions were used to identify URI and ALRI from collected information. Upper respiratory tract infection (URI) was defined as 'presence of cough in absence of World Health Organization defined clinical signs of pneumonia and severe pneumonia' while, ALRI was defined as 'presence of cough and/or respiratory difficulty plus rapid respiratory rate at the time of household visits (cut-off for age: ≥50 breaths per minute in children aged 2 to 11 months and ≥40 breaths per minute in children aged 12 months to two years)' (8). If any child was diagnosed with ALRI during episodes of URI, then all days reported with cough were considered as an ALRI episode. Rate of URI and ALRI was calculated by dividing the number of days with URI and ALRI by total number of days of observation.

3.5 Statistical analyses

Objective 1 (Chapter 4): Determine the vitamin D status of underweight and normal-weight children aged 6–24 months in urban Bangladesh; and identify the socio-economic and dietary predictors of status.

- Study design: Cross-sectional as baseline data were analysed.
- Method summary: Vitamin D status presented with mean, median, range and standard deviation, and quartile, then I categorised severe deficiency, deficiency, insufficiency and sufficiency to present the prevalence. Socio-economic and demographic variables, maternal education status, qualitative dietary intake and vitamin A and zinc status were compared according to vitamin D status using ANOVA for continuous variables and chi square test of independence for categorical variables. Multinomial logistic regression was used to estimate the odds of being vitamin D deficient or insufficient with the reference being vitamin D sufficient children. A probability of less than 0.05 was considered a statistically significant association. Strength of association was measured by estimating odds ratio (OR) and 95% confidence intervals (CI). Details are presented in the statistical analysis section of the publication in Chapter 4.

Objective 2 (Chapter 5): Determine how differences in status of vitamin D are associated with the incidence and severity of diarrhoea in underweight and normal-weight children aged 6–24 months.

- Study design: Two separate prospective cohorts of underweight and normal-weight children
- Exposure: Baseline vitamin D status
- Outcome: Incidence and severity of diarrhoea during the five month follow-up period
- Method summary: Rates of diarrhoea were estimated according to socio-economic and demographic variables, maternal education status, vitamin A and zinc status, then compared by generalised estimate equation model with a Poisson distribution and p value was tested for linear trend. For dichotomous predictor, p value was tested using the Wald test with robust standard errors in a generalised estimate equation model. Multivariable generalised estimating equations were used to estimate incidence-rate ratios for incidence (Poisson) and severity (binomial) of diarrhoea. Details are presented in the statistical analysis section of the manuscript in Chapter 5.

Objective 3 (Chapter 6): Investigate which pathogen-specific diarrhoeal infections are prevalent among children with vitamin D sufficiency compared with children who are not vitamin D deficient or insufficient.

- Study design: Two separate prospective cohorts of underweight and normal-weight children
- Exposure: Baseline vitamin D status
- Outcome: Incidence of ETEC, EPEC and EAEC diarrhoeal episodes during the five month follow-up period. Children were censored if ETEC, EPEC, and EAEC were not isolated from the collected sample or children did not experience any diarrhoeal episodes during the follow-up period or the field worker was unable to collect samples during a diarrhoeal episode.
- Method summary: Cumulative hazard curves were presented with different vitamin D for incidence of ETEC, EPEC and EAEC diarrhoeal episodes and tested with the Log-rank test. Cox proportional hazard models were used to estimate the relative hazards of vitamin D status and incidence of ETEC, EPEC and EAEC diarrhoeal episodes. Details are presented in the statistical analysis section of the manuscript in Chapter 6.

Objective 4 (Chapter 7): Determine how differences in status of vitamin D are associated with the incidence of ARI in underweight and normal-weight children aged 6–24 months.

- Study design: Two separate prospective cohorts of underweight and normal-weight children
- Exposure: Baseline vitamin D status
- Outcome: Incidence of URI and ALRI during the five month follow-up period

- Method summary: Rates of URI and ALRI were estimated according to socio-economic and demographic variables, maternal education status, vitamin A and zinc status, then compared by the generalised estimate equation model with a Poisson distribution, and p value was tested for linear trend. For the dichotomous predictor, p value was tested using the Wald test with robust standard errors in the generalised estimate equation model. Multivariable generalised estimating equations were used to estimate incidence rate ratios for incidence (Poisson) of URI and ALRI among children. Details are presented in the statistical analysis section of the manuscript in Chapter 7.

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CHAPTER 4 PREVALENCE AND RISK FACTORS OF VITAMIN D STATUS

4.1 Context

The important role of vitamin D in health and disease has led to an increased interest in measuring vitamin D status and its risk factors among children under two years of age. There is a lack of evidence about the prevalence of vitamin D status and risk factors in children under two years of age in Bangladesh. Moreover, studies conducted in preschool children were not considering nutritional status in reporting prevalence and risk factors of vitamin D status.

In this study, I compiled and analysed the most-up-to-date data on underweight and normal-weight children aged under two years residing in an urban slum area of Bangladesh. It merits a new way to look at the prevalence of vitamin D and its risk factors among children according to their nutritional status. The research presented in this chapter identifies a high burden of vitamin D insufficiency and deficiency in both underweight and normal-weight children, who are living in an impoverished, resourced constrained slum area. In this Chapter, I also demonstrate that the risk factors for vitamin D deficiency and insufficiency differed between normal-weight and underweight children.

The findings reported in this study are significant in a number of ways. First, the huge burden of vitamin D deficiency and insufficiency among urban children under two years old is not recognised or reported. Thus, no program exists in Bangladesh to address this important public health problem among children under two years. Second, identification of risk indicators for vitamin D deficiency and insufficiency could be useful for designing and implementing integrated national control programs for vitamin D deficiency and insufficiency. It has been shown in Chapter 1 and 2 that vitamin D is important in calcium homeostasis and bone health and also maintaining the integrity of the innate immune system and protection against infections. In Chapter 5, 6 and 7, the findings of association of vitamin D status with diarrhoea and ARI among children where vitamin D deficiency and insufficiency are highly prevalent are presented.

This Chapter forms a manuscript which has been published in *Public Health Nutrition.*

4.2 Prevalence and risk factors of vitamin D insufficiency and deficiency among 6-24 months old underweight and normal weight children living in an urban slum of Bangladesh

A. M. Shamsir Ahmed, Tahmeed Ahmed, Kurt Z Long, Ricardo J. Soares Magalhaes, Md. Iqbal Hossain, Md. Munirul Islam, Mustafa Mahfuz, S. M. Abdul Gaffar, Atiya Sharmeen Rashidul Haque, Richard L. Guerrant, William A Petri Jr, Abdullah Al Mamun

Abstract

Objective: We quantified the prevalence of vitamin D status in underweight and normal-weight children aged 6–24 months old and identified the socio-economic and dietary predictors.

Design: Cross-sectional, baseline data from a nutritional intervention study were analysed. Multinomial logistic regression was used to estimate the odds of being vitamin D deficient or insufficient with the reference being vitamin D sufficient.

Setting: Urban slum area of *Mirpur* field site, Dhaka, Bangladesh.

Subjects: underweight (WAZ <–2.00 SD) and normal-weight children (WAZ ≥–1.00 SD) children aged 6–24 months.

Results: Among 468 underweight children vitamin D status was 23.1% sufficient, 42.3% insufficient, 31.2% deficient and 3.4% severely deficient. Among 445 normal-weight children vitamin D status was 14.8% sufficient, 39.6% insufficient, 40.0% deficient, and 5.6% severely deficient. With adjusted multinominal regression analysis, risk factors [ORs (95% CIs)] for vitamin D deficiency in underweight children were: older age group (18–24 months old) [2.9 (1.5-5.7)]; measurement of vitamin D status during winter [3.0 (1.4-6.4)] and spring [6.9 (3.0–16.1)]; and maternal education (≥6 years of institutional education) [2.2 (1.0–4.9)]. In normal-weight children, being in an older age group [3.6 (1.2–10.6)] and living in the richest quintile [3.7 (1.1-12.5)] were found to be significantly associated with vitamin D insufficiency.

Conclusions: This study demonstrates a significant burden of vitamin D insufficiency and deficiency in both underweight and normal-weight children under two years of age from an urban slum of Bangladesh. Identification of risk factors of insufficiency and deficiency may help in mitigating the important burden of such children.

Introduction

The primary role of vitamin D in the human body is to maintain the extracellular calcium levels but recently it has been implicated in a non-skeletal role including protection from infectious, inflammatory and neoplastic disease outcomes [1-4]. In humans most of vitamin D is primarily

synthesised by the skin through exposure to sunlight (ultraviolet B radiation, wavelength, 290 to 315 nm) while only a small fraction (5–10%) comes from diet [1]. Recently a study estimated 4 billion cases of bone disease globally (rickets, osteomalacia and osteoporosis) and 3.3 billion disability adjusted life years (DALYs) lost due to vitamin D deficiency that resulted from reduced UV ray exposure [5].

The prevalence of vitamin D deficiency among children and adults varies significantly worldwide due to variation in sunlight exposure around the year and presence of insufficient corrective programs [1, 6-9]. Several small studies in the Indian subcontinent, including Bangladesh, reported wide variation (as low as 2% to 84%) in the prevalence of vitamin D deficiency and insufficiency among preschool children [10-17]. Use of a different serum cut-off point for vitamin D deficiency and insufficiency is also another important factor in the reporting of the wide range of variations in the prevalence [7]. Several studies in rural Bangladesh have reported low vitamin D status of children under 5 years and under 10 years old, although there is a high prevalence of rickets among the children [13, 18]. Another study found a high prevalence of severe deficiency (<25 nmol/L) among children under two years old with pneumonia who were matched with healthy controls [14].

There have been few empirical studies aimed at identifying risk factors among preschool children for vitamin D deficiency in the Indian subcontinent and Bangladesh [12, 14-16, 19, 20]. Studies conducted among the children identified age, reduced intake of vitamin D enriched food, low sunshine exposure, skin covering, skin pigmentation, ethnicity, maternal vitamin D status, household crowding, and air pollution as risk factors for vitamin D deficiency and insufficiency [12, 15, 16, 21-28]. A study in rural Bangladesh among children under two years of age found that vitamin D deficient children were more likely to live in households of lower socio-economic status and were more stunted than vitamin-sufficient children [14]. However, a study among Pakistani infants found significantly lower vitamin D concentration among children from the upper socio-economic strata and among infants of educated mothers [19]. Thus, risk indicators for vitamin D deficiency need to be explored more carefully among children under two years of age.

The important role of vitamin D in health and disease has led to increased interest in measuring vitamin D status among children under two years of age. There is lack of evidence about the prevalence of vitamin D deficiency in children under two years of age in Bangladesh. Most of the studies in Bangladesh reported prevalence of vitamin D deficiency in rural areas. We were unable to identify studies reporting vitamin D deficiency among urban Bangladeshi children or what risk factors are associated with deficiency and insufficiency. Additionally, most of the studies did not consider nutritional status, which may play a role in the rate of prevalence as well as risk factors for insufficiency and deficiency among children under two years old. In this study we aimed to

determine the prevalence of vitamin D insufficiency and deficiency among underweight and normal-weight urban-slum children aged 6–24 months as well as to examine the socio-economic and dietary risk indicators.

Methods

Study design, setting and subjects

We used data from the Bangladesh component of the Malnutrition & Enteric Diseases (Mal-ED) consortium [29], which is a multisite research project concerned with malnutrition and diarrhoeal diseases in early childhood. One of the components of the Mal-ED study was an intervention study carried out at the urban *Mirpur* field site in Dhaka. The coordinates of Mirpur are 23.8042° N 90.3667° E. Children aged 6 to 24 months with severe to moderate underweight (weight-for-age Z, WAZ, score <–2.00 SD) were selected as cases for enrollment in the study through biannual household demographic surveillance of the community. Controls were well-nourished, normal-weight children ((WAZ ≥–1.00 SD) matched for area of residence only. The detail of the study design and site has been reported elsewhere [30]. From November 2009 to February 2012, 500 cases and 480 controls were enrolled. Children were assigned to receive either one of two different intervention packages according to their nutritional status (underweight or normal-weight), for five months while enrolled in the study. To achieve our proposed objectives only the baseline data were analysed from the cross-sectional study design. Thus intervention packages and follow-up procedures are not described in this study.

Data collection

Trained field workers collected household socio-economic information and qualitative dietary intake information from mothers through a structured questionnaire at the time of enrolment. Demographic and Health Survey (DHS) questionnaires on household socioeconomic and demographic status were adapted for data collection [31]. Similarly dietary intake data were also collected using a food frequency questionnaire (FFQ) based upon the DHS questionnaires which had been previously adapted (local names for common examples of the food items) and field tested by our team prior to data collection [31]. Field staff received standard training for data and sample collection before the implementation of the study at the field site. Trained field workers measured children's weight using a digital baby or toddler scale (Seca 354) at the time of enrolment and on a monthly basis throughout the study period. Twice yearly refresher-training courses for staff were also conducted for quality assurance of data collection.

Laboratory procedure

A sample of 5 ml of venous blood was collected from the children at the time of enrolment. Samples were collected in trace element free containers for micronutrient assay. All the assays for micronutrients including vitamin D were performed at the nutritional biochemistry laboratory of the icddr,b. Serum vitamin D was measured using the IDS 25-Hydroxy Vitamin D Enzyme immunoassay (EIA) Kit [32] (Source: IDS Ltd, 10 Didcot Way, Boldon Business Park, Boldon, UK). Two levels of controls (REF AC-5705A - AC-5705B) were included in each kit. These two controls were run in each plate/run for monitoring accuracy and precision. The coefficient of variation was (3.8–11.8) % for Control 1 and (5.2–10.7) % for Control 2. Serum retinol was measured using the HPLC method described elsewhere [33]. Serum/plasma zinc concentration was determined by air-acetylene flame atomic absorption spectrophotometer at 213.9 nm following dilution of the sample twelve times with deionised water. Accuracy and precision of analysis is ensured by using a bi-level serum trace element control provided by UTAK Laboratories Inc. (Valencia, CA).

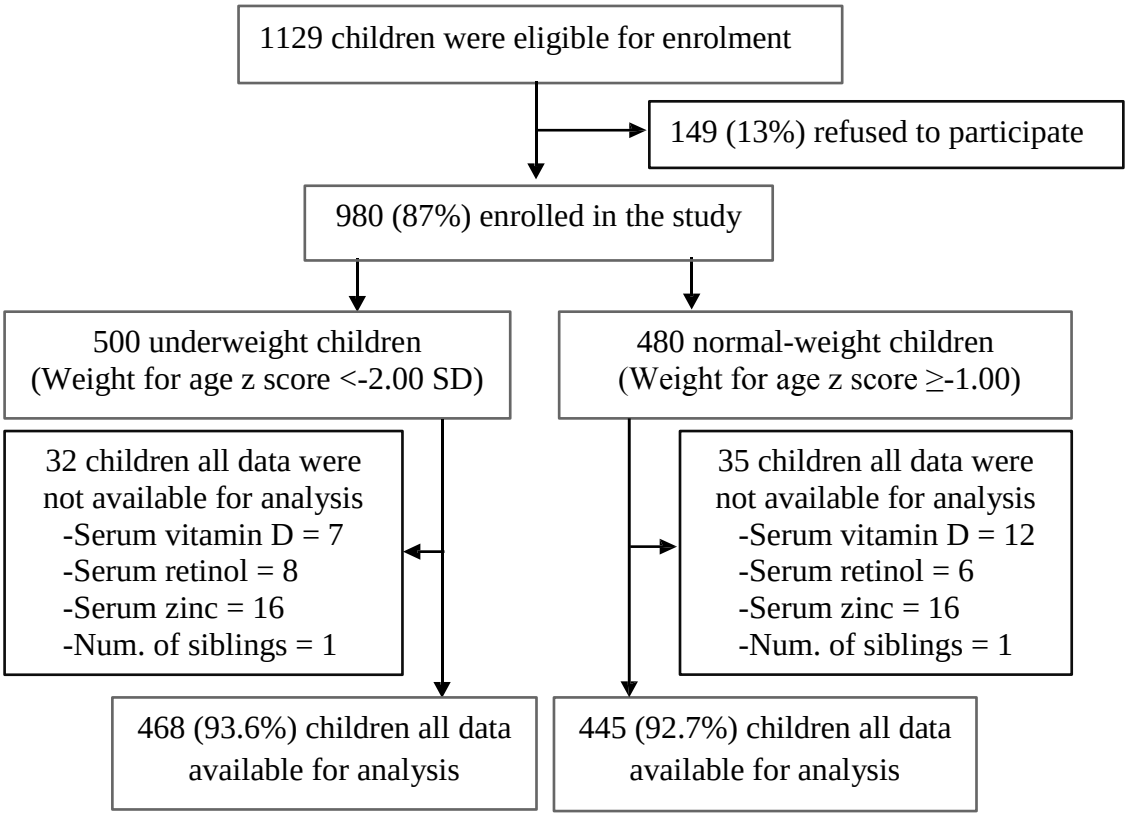
Sample size and measurements

The study profile is described in Figure 4.1. Complete data of 468 underweight and 445 normal-weight children were available for the final analysis. The primary outcome of the study is the prevalence of vitamin D insufficiency and deficiency among children aged 6–24 months. Serum vitamin D was recoded using standard serum cut-off points: severe deficiency (<25 nmol/L), deficiency (25–49.99 nmol/L), insufficiency (50–74.99 nmol/L) and sufficiency (≤75 nmol/L). The available exposure variables comprised personal characteristics, measures of socio-economic status (SES), environmental factors, seasonality, and vitamin A and zinc status. Construction of a household asset index was done from the household asset information with principle component analysis described for the Demographic and Health Survey [34]. The UV –index is usually high in Dhaka during April to September and lowest from November to January [35]. As ultraviolet B radiation is essential for synthesis of vitamin D in human skin, four seasons— summer (May to July), autumn (August to October), winter (November to January), and spring (February to April)— were identified from the date of blood sample collection to measure the seasonal variation. Child's age (6–11, 12–17 and 18–24 months), maternal education (illiterate, 1–5 years and ≥6 years of institutional education) and other relevant variables were created with recoding of information in the database. Vitamin A deficiency was defined as serum retinol level of <0.7μmol/L [36] and serum zinc deficiency was defined as serum zinc level of <9.9 μmol/L in preschool children [37].

Statistical analysis

Socio-economic and demographic variables, qualitative dietary intake, and vitamin A and zinc status were compared among individuals with vitamin D sufficiency, insufficiency and deficiency using ANOVA for continuous variables and chi square test of independence for categorical variables. For multivariable analyses, we combined both severe deficiency and deficiency into one category of deficient status (<50 nmol/L) to increase the statistical precision. A probability of less than 0.05 was considered a statistically significant association. Strength of association was measured by the estimating odds ratio (OR) and 95% confidence intervals (CI). Multinomial logistic regression was used to estimate the odds of being vitamin D deficient or insufficient with the reference being vitamin D sufficient children. Analyses were then done separately for underweight and normal-weight children. The variables that were statistically significant in the univariate analysis or deemed physiologically important factors or reported risk indicators in the published literature were subsequently included in multivariable models to determine their independent association with the outcome variable. Analyses were carried out in the statistical software STATA (version 12.0; Stata Corp, College Station, TX).

Figure 4.1: Study profile



Ethical Statement

The study (proposal # 2008-020) was approved by the Research Review Committee and the Ethical Review Committee of icddr,b. Informed, voluntary written consent was obtained from the parents

or guardian for the participation of their child in the study. Parents or caregivers were assured about the non-disclosure of information collected from them, and were also informed about the use of data for analysis and using the results for improving health and nutritional care activities as well as publication without disclosing the name or identity of their children.

Results

Vitamin D status

The vitamin D statuses of underweight and normal-weight children are presented in Table 4.1. The median (inter-quartile range) vitamin D concentration was 57.5 (45.7, 73.6) nmol/L among underweight children and 51.8 (39.9, 65.9) nmol/L among normal-weight children. Only 23.1% of underweight children and 14.8% of normal-weight children were vitamin D sufficient.

Table 4.1: Serum vitamin D status among underweight and normal-weight children aged 6–24 months

Serum vitamin D (nmol/L)	Underweight children (Weight for age z score <-2)	Normal-weight children (Weight for age z score ≥-1)
	(n=468)	(n=445)
Mean ± SD	60.6±23.2	54.1±20.8
Median (25 and 75 percentile)	57.5 (45.7, 73.6)	51.8 (39.9, 65.9)
Range	12.3-188.9	10.6-188.7
Sufficient (≥75 nmol/L) % (n)	23.1 (108)	14.8 (66)
Insufficient (50-74.99 nmol/L) % (n)	42.3 (198)	39.6 (176)
Deficient (25-49.99 nmol/L) % (n)	31.2 (146)	40.0 (178)
Severe deficient (<25 nmol/L) % (n)	3.4 (16)	5.6 (25)

Factors associated with underweight children

In the unadjusted analysis, the odds of being serum vitamin D deficient was significantly increased among underweight children in the age groups 12–17 and 18–24 months compared with children in the 6–11 months age group (Table 4.2). Similarly, underweight children were significantly at a higher risk of vitamin D deficiency during winter and spring. Underweight children who had not consumed any animal protein in the last 24 hours had 40% lower risk of vitamin D deficiency than underweight children who consumed animal protein. There was a 50% lower risk of vitamin D deficiency among the serum zinc-sufficient underweight children than the serum zinc-deficient group in comparison with vitamin D sufficient children.

In the adjusted model, children who were ≥18 months of age were found to have a significantly greater risk of being vitamin D deficient than children in the younger age group (6–11 months) when compared with the vitamin D sufficient group. Similarly, children were 3.0 times at greater

risk of vitamin D deficiency in winter than summer. Likewise, in spring the risk increased up to 6.9 times when compare with vitamin D sufficient underweight children (Table 4.2). The probability of the children having vitamin D sufficient or insufficient status decreased during the spring time. On the other hand, the probability of being vitamin D deficient increased among the underweight children during winter and spring (Figure 4.2). Children, whose mothers had six years or more of institutional education, were found to be at 2.2 times greater risk of vitamin D deficiency and at 2.6 times greater risk of vitamin D insufficiency than illiterate mother's children. Children of mothers, who had one to five years of institutional education, were found to be at 1.9 times greater risk of vitamin D insufficiency than children of illiterate mothers when compared with children with vitamin D sufficiency after adjustment of other variables (Table 4.2).

Factors associated with normal-weight children

The risk of being serum vitamin D insufficient was greater among normal-weight children aged 18–24 months than among children aged 6–11 months in the unadjusted analysis (Table 4.3). However, there was no association of season with vitamin D insufficiency or deficiency in the unadjusted analyses. Among normal-weight children, those whose mothers had six years or more of institutional education were approximately at three fold greater risk of being vitamin D deficiency than those whose mothers were illiterate. And similar results were found when comparing children from the highest quintile of the household wealth index with children from the lowest quintile. There was 2.4 and 2.2 times greater risk of vitamin D insufficiency and deficiency respectively among zinc-sufficient normal-weight children when compared to vitamin D sufficient children (Table 4.3).

In the adjusted model, children 18–24 months of age were found to be 3.6 times more vitamin D insufficient than children in the younger age group (6–11 months) when compared with the serum vitamin D sufficient group. Autumn was found to be associated with significantly lower risk of vitamin D insufficiency among children than the summer months. Winter and spring were not associated with either deficiency or insufficiency of serum vitamin D status among the normal-weight children (Table 4.3). Among the normal-weight children the probabilities of vitamin D deficiency and insufficiency were high (40%–50%) even during the summer (Figure 4.3). Maternal education was not associated with vitamin D deficiency or insufficiency after adjusting for other variables. On the other hand, normal-weight children from richest quintile found to be 3.7 times more vitamin D insufficient than the lower quintile of the household wealth index in compare to children with sufficient vitamin D status (Table 4.3).

A detail description of consumption of vitamin D rich food in last 24 hours by vitamin D status among underweight and normal-weight children has been presented in Table 4.4.

Table 4.2: Characteristics and factors associated with vitamin D deficiency and insufficiency among underweight children aged 6–24 months; multinomial logistic regression analysis with sufficient serum vitamin D status as reference (n=468)

Indicators	Serum vitamin D status						
	Sufficient % (n)	Insufficient % (n)	Deficient % (n)	Insufficient Odds ratio (95% CI)		Deficient Odds ratio (95% CI)	
				Unadjusted	Adjusted	Unadjusted	Adjusted
Age group in months*							
6-11	49.1 (53)	39.9 (79)	27.2 (44)	1	1	1	1
12-17	27.8 (30)	30.8 (61)	34.6 (56)	1.3 (0.8, 2.3)	1.1 (0.6, 2.1)	2.2 (1.2, 4.1)*	1.7(0.9, 3.4)
18-24	23.1 (25)	29.3 (58)	38.3 (62)	1.5 (0.9, 2.8)	1.4 (0.7, 2.6)	3.0 (1.6, 5.5)*	2.9 (1.5, 5.7)*
Female	43.5 (47)	49.0 (97)	53.7 (87)	1.2 (0.8, 2.0)	1.3 (0.8, 2.1)	1.5 (0.9, 2.4)	1.6 (0.9, 2.7)
Season of vitamin D measurement*							
Autumn	28.7 (31)	22.2 (44)	11.7 (19)	1.1 (0.5, 2.1)	1.0 (0.5, 2.1)	1.0 (0.4, 2.2)	0.9 (0.4, 2.3)
Winter	29.6 (32)	40.4 (80)	38.3 (62)	1.9 (1.0, 3.6)	1.8 (0.9, 3.4)	3.0 (1.5, 6.4)*	3.0 (1.4, 6.4)*
Spring	14.7 (16)	18.2 (36)	38.9 (63)	1.7 (0.8, 3.7)	1.6 (0.7, 3.5)	6.3 (2.8, 14.2)*	6.9 (3.0, 16.1)*
More than one siblings	58.3(63)	64.1 (127)	53.7 (87)	1.3 (0.8, 2.1)	1.4 (0.8, 2.4)	0.8 (0.5, 1.3)	0.8 (0.5, 1.4)
Family size > 5 family members	25.0 (27)	30.3 (60)	22.8 (37)	1.3 (0.8, 2.2)	1.4 (0.8, 2.5)	0.9 (0.5, 1.6)	0.9 (0.5, 1.7)
Mother's education							
One to five years	45.4 (49)	50.0 (99)	40.7 (66)	1.6 (0.9, 2.8)	1.9 (1.0, 3.6)*	1.1 (0.6, 2.0)	1.1 (0.6, 2.2)
Six and more than six years	23.1 (25)	28.3 (56)	33.9 (55)	1.8 (0.9, 3.4)	2.6 (1.2, 5.5)*	1.8 (0.9, 3.5)	2.2 (1.0, 4.9)*
Household wealth index							
Second	21.3 (23)	23.7 (47)	23.5 (38)	1.1 (0.6, 2.1)	1.0 (0.5, 2.0)	1.0 (0.5, 2.0)	1.0 (0.5, 2.1)
Third	22.2 (24)	20.2 (40)	17.9 (29)	0.9 (0.5, 1.8)	0.7 (0.3, 1.4)	0.7 (0.4, 1.5)	0.6 (0.3, 1.4)
Fourth	13.9 (15)	18.2 (36)	18.5 (30)	1.3 (0.6, 2.7)	0.9 (0.4, 2.1)	1.2 (0.6, 2.6)	1.1 (0.5, 2.9)
Highest	13.9 (15)	9.1 (18)	8.6 (14)	0.6 (0.3, 1.5)	0.4 (0.1, 0.98)	0.6 (0.2, 1.3)	0.4 (0.1, 1.2)
Did not drink any animal or powder milk in last 24 hours	63.0 (68)	68.2 (135)	72.8 (118)	1.3 (0.8, 2.1)	1.2 (0.7, 2.0)	1.6 (0.9, 2.7)	1.3 (0.7, 2.3)
Did not consume any animal protein in last 24 hours	55.6 (60)	47.0 (95)	42.0 (68)	0.7 (0.5, 1.2)	0.8 (0.5, 1.3)	0.6 (0.4, 0.9)*	0.7 (0.4, 1.2)
Serum retinol mild deficiency or normal status (>=0.7 µmol/L)	64.8 (70)	58.1 (115)	56.8 (92)	0.7 (0.5, 1.2)	x	0.7 (0.4, 1.2)	x
Serum zinc sufficiency (>= 9.9 µmol/L)*	85.2 (92)	82.3 (163)	74.1 (120)	0.8 (0.4, 1.5)	x	0.5 (0.3, 0.9)*	x

*p <0.05, x = not included in the model

Reference values for independent variable : 6–11 months, male, summer, number of siblings <=1, illiterate mother, family size <=5 members, lowest asset quintal, consumption of animal or power milk, consumption of any animal protein, serum retinol moderate to severe deficiency and serum zinc insufficiency

Adjusted for child age group, child sex, season of vitamin D measurement, number of siblings, family size, mother's education, household wealth index, consumption of any diary product and animal protein

Table 4.3: Characteristics and factors associated with vitamin D deficiency and insufficiency among normal-weight children aged 6–24 months; multinomial logistic regression analysis with sufficient serum vitamin D status as reference (n = 445)

Indicators	Serum vitamin D status						
	Sufficient % (n)	Insufficient % (n)	Deficient % (n)	Insufficient Odds ratio (95% CI)		Deficient Odds ratio (95% CI)	
				Unadjusted	Adjusted	Unadjusted	Adjusted
Age group in months (p = 0.055)							
6–11	66.7 (44)	50.0 (88)	52.7 (107)	1	1	1	1
12–17	25.8 (17)	27.8 (49)	31.5 (64)	1.4 (0.7, 2.8)	1.2 (0.6, 2.5)	1.5 (0.8, 2.9)	1.1 (0.5, 2.2)
18–24	7.6 (5)	22.2 (39)	15.8 (32)	3.9(1.4, 10.6)*	3.6 (1.2, 10.6)*	2.6 (0.96, 7.2)	2.2(0.8, 6.4)
Female	47.0 (31)	50.6 (89)	47.3 (96)	1.1 (0.6-2.0)	1.3 (0.7, 2.4)	1.0 (0.6-1.8)	1.1 (0.6, 1.9)
Season of vitamin D measurement							
Autumn	25.8 (17)	14.8 (26)	12.3 (25)	0.5 (0.2, 1.2)	0.4 (0.1, 0.9)*	0.5 (0.2, 1.3)	0.4 (0.2, 1.1)
Winter	31.8 (21)	43.2 (76)	42.9 (87)	1.1 (0.5, 2.5)	1.0 (0.4, 2.4)	1.4 (0.6, 3.3)	1.3 (0.5, 3.2)
Spring	25.8 (17)	21.6 (38)	29.1 (59)	0.7 (0.3, 1.6)	0.6 (0.2, 1.5)	1.2 (0.5, 2.8)	1.1 (0.5, 2.8)
More than one siblings	66.7 (44)	60.2 (106)	62.1 (126)	0.8 (0.4, 1.4)	0.6 (0.3, 1.2)	0.8 (0.4, 1.5)	0.8 (0.4, 1.5)
Family size >5 family members	27.3 (18)	23.3 (41)	22.2 (45)	0.8 (0.4, 1.5)	0.6 (0.3, 1.3)	0.8 (0.4, 1.4)	0.6 (0.3, 1.3)
Mother's education							
One to five years	51.5 (34)	43.7 (77)	38.9 (79)	1.1 (0.5, 2.4)	0.8 (0.4, 1.9)	1.2 (0.6, 2.7)	1.1 (0.5, 2.5)
Six and more than six years	27.3 (18)	40.3 (71)	48.3 (98)	2.0 (0.9, 4.5)	1.0 (0.4, 2.8)	2.9 (1.3, 6.7)*	2.1 (0.8, 5.5)
Household wealth index							
Second	19.7 (13)	15.3 (27)	17.2 (35)	1.0 (0.4, 2.9)	1.2 (0.4, 3.8)	1.3 (0.5, 3.7)	1.3 (0.4, 4.0)
Middle	28.8 (19)	14.8 (26)	21.2 (43)	0.7 (0.2, 1.8)	0.8 (0.3, 2.3)	1.1 (0.4, 3.0)	1.2 (0.4, 3.3)
Fourth	21.2 (14)	26.1 (46)	21.2 (43)	1.6 (0.6, 4.5)	2.0 (0.7, 6.1)	1.5 (0.6, 4.2)	1.4 (0.5, 4.4)
Highest	16.7 (11)	33.5 (59)	31.5 (64)	2.7 (0.9, 7.5)	3.7 (1.1, 12.5)*	2.9 (1.0, 8.1)*	2.7 (0.8, 8.8)
Not drinking any animal or powder milk in last 24 hours	47.0 (31)	55.0 (97)	56.2 (114)	1.4 (0.8, 2.4)	1.6 (0.9, 3.1)	1.4 (0.8, 2.5)	1.7 (0.9, 3.1)
Did not consume any animal protein in last 24 hours	60.6 (40)	51.7 (91)	48.8 (99)	0.7 (0.4, 1.2)	1.0 (0.5, 2.0)	0.6 (0.3, 1.1)	0.9 (0.5, 1.6)
Serum retinol mild deficiency or normal status (>= 0.7 µmol/L)	68.2 (45)	67.0 (118)	64.0 (130)	0.9 (0.5, 1.7)	x	0.8 (0.5, 1.5)	x
Serum zinc sufficiency (>= 9.9 µmol/L)	72.7 (48)	86.4 (152)	85.2 (173)	2.4 (1.2, 4.7)*	x	2.2 (1.1, 4.2)*	x

*p <0.05, x = not included in the model

Reference values for independent variable : 6-11 months, male, summer, number of siblings <=1, illiterate mother, family size <=5 members, lowest asset quintal, consumption of animal or power milk, consumption of any animal protein, serum retinol moderate to severe deficiency and serum zinc insufficiency

Adjusted for child age group, child sex, season of vitamin D measurement, number of siblings, family size, mother's education, household wealth index, consumption of any diary product

Figure 4.2: Adjusted predictions of vitamin D sufficiency, insufficiency and deficiency by season with 95% CIs among underweight children

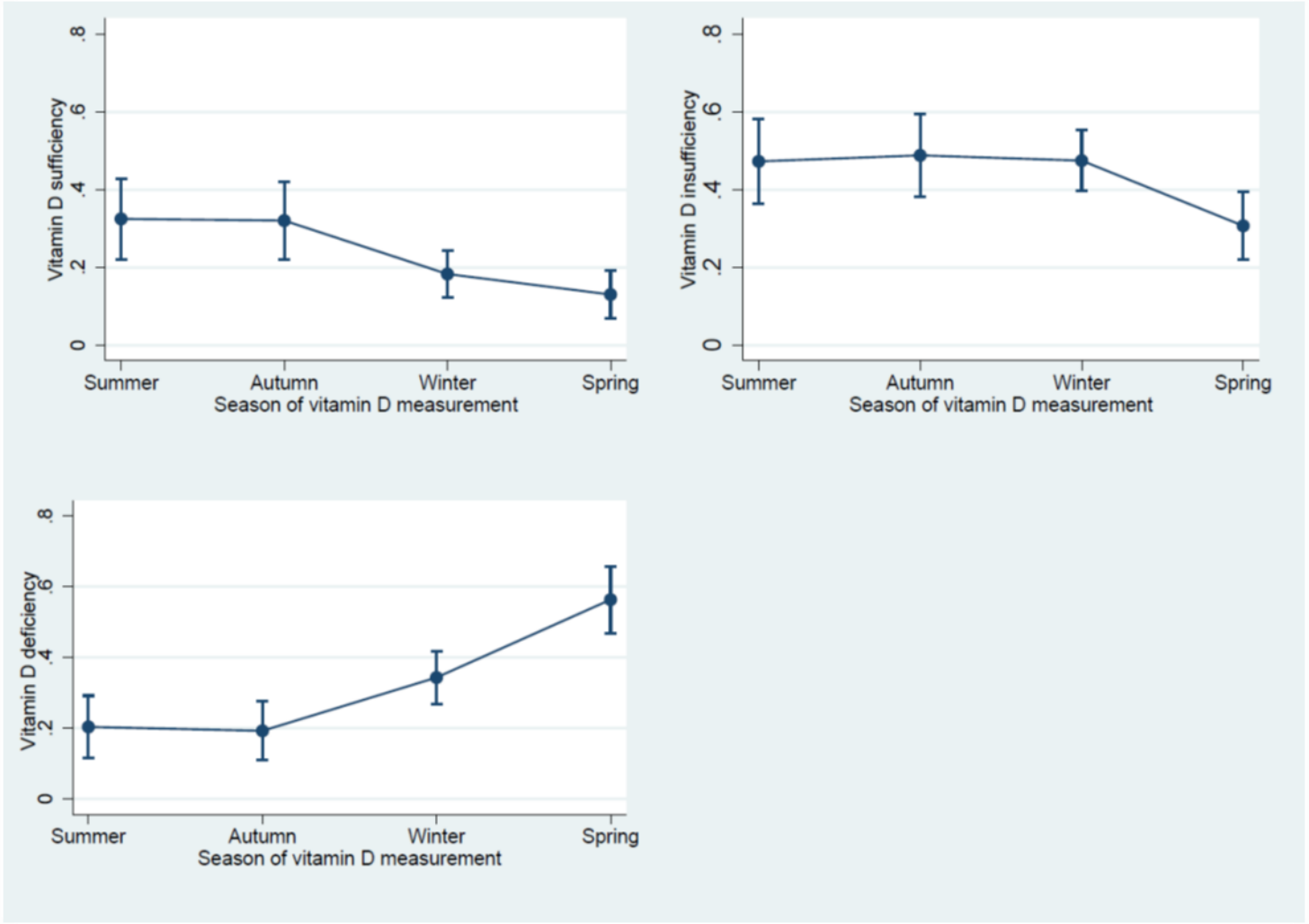


Figure 4.3: Adjusted predictions of vitamin D sufficiency, insufficiency and deficiency by season with 95% CIs among normal-weight children

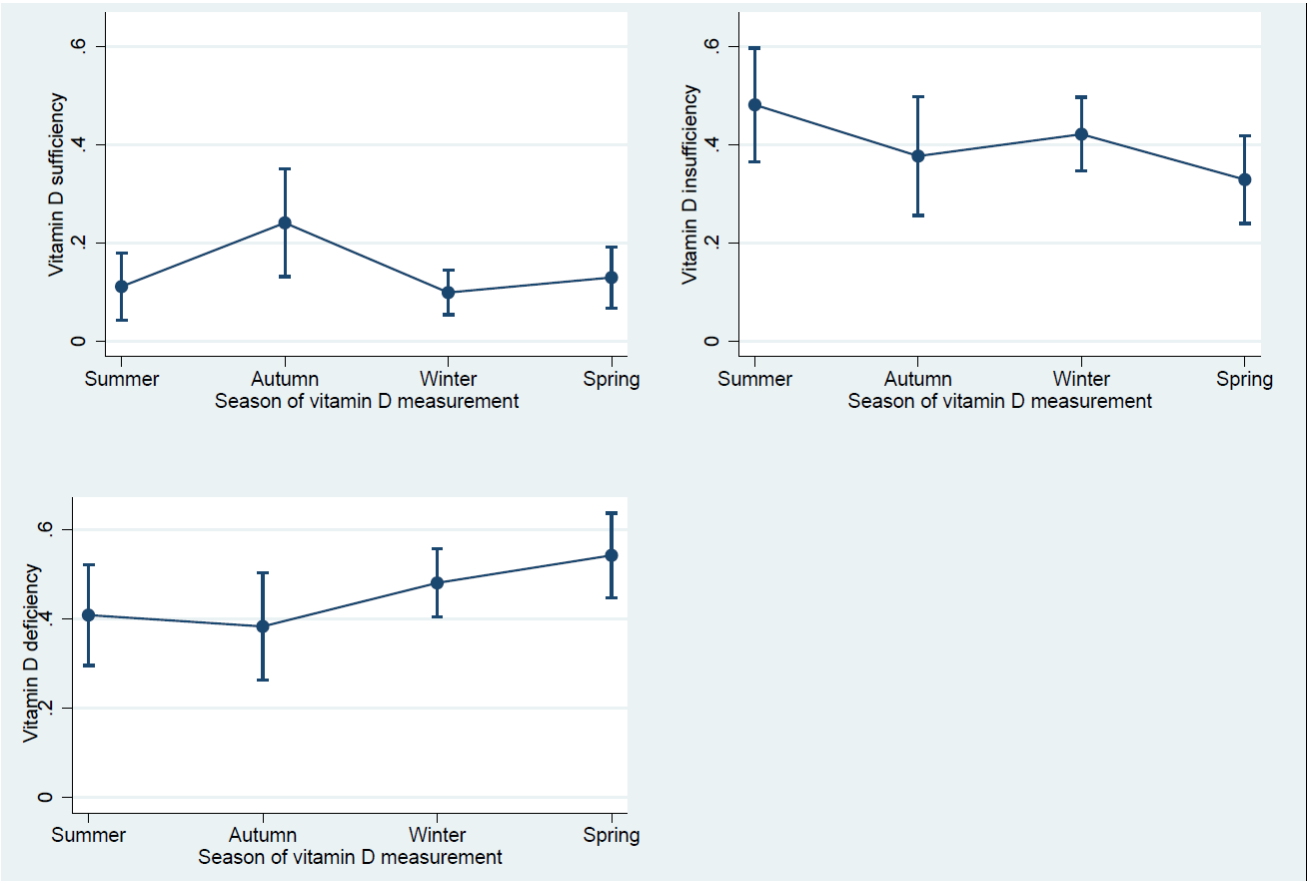


Table 4.4: Consumption of vitamin D rich food in last 24 hours by vitamin D status among underweight and normal-weight children

Food items	Underweight children			Normal-weight children		
	Vitamin D status			Vitamin D status		
	Sufficient % (n)	Insufficient % (n)	Deficient % (n)	Sufficient % (n)	Insufficient % (n)	Deficient % (n)
Currently breastfeeding [1, 2]	93.5 (101)	96.0 (190)	89.5 (145)	98.5 (65)	94.3 (166)	93.6 (190)
Infant formula [1, 2]	6.5 (7)	10.1 (20)	5.6 (9)	27.2 (18)	15.3 (27)	8.9 (18)
Powder or fresh animal milk [1, 2]	24.1 (26)	20.7 (41)	18.5 (30)	22.7 (15)	28.4 (50)	30.1 (61)
Dairy product (cheese, yoghurt) [3,4]	8.5 (9)	3.6 (7)	3.2 (5)	7.7 (5)	6.6 (11)	10.0 (20)
Organ meat (liver, kidney, heart) [3,4]	4.7 (5)	4.1 (8)	3.2 (5)	0.0 (0)	4.8 (8)	4.0 (8)
Any meat (chicken, beef, lamb, goat, duck) [3,4]	13.2 (14)	9.7 (19)	9.6 (15)	9.2 (6)	12.0 (20)	13.0 (26)
Fresh or dried fish [3,4]	10.4 (11)	26.0 (51)	33.8 (53)	13.9 (9)	27.5 (46)	24.0 (48)
Eggs [3,4]	29.3 (31)	28.1 (55)	26.8 (42)	26.2 (17)	23.4 (39)	27.0 (54)

[1] Number of underweight children (sufficient = 108, insufficient = 198, deficient = 162)
[2] Number of normal-weight children (sufficient = 66, insufficient = 176, deficient = 203)
[3] Number of underweight children (sufficient = 106, insufficient = 196, deficient = 157)
[4] Number of normal-weight children (sufficient = 65, insufficient = 167, deficient = 200)

Discussion

We have found remarkable vitamin D insufficiency and deficiency among underweight as well as normal-weight urban slums children aged 6–24 months, indicating that vitamin D insufficiency and deficiency are important health problems especially among young children living in Bangladesh. Importantly the factors associated with vitamin D insufficiency and deficiency were different between normal-weight children and underweight children.

One of the important drawbacks of available studies reporting prevalence of vitamin D status is the use of different cut-off levels by researchers. Most clinicians define vitamin D deficiency using a cut-off point of serum vitamin D <25 nmol/L, which is the cut-off point associated with the occurrence of rickets and osteomalacia [38, 39]. One author has proposed a cut-off point of >50 nmol/L for optimal bone mineral density, bone turnover and muscle strength, while a cut-off point of >75 nmol/L has been proposed for maintaining an adequate immune response [40, 41]. The difference in these recommendations may reflect the different actions of vitamin D in physiological processes. Regardless of the different cut-off levels used for reporting status of serum vitamin D, all studies [13-15, 18] including our study showed a significant burden of vitamin D deficiency and insufficiency among children under two years old in Bangladesh.

The tropical geographical location of Bangladesh means that vitamin D synthesis is possible all year long due to the intense UVB radiation in the country compared with other zones of the globe [35]. Our study shows that despite its geographical setting, vitamin D insufficiency and deficiency is

quite prevalent in children 6–24 months of age. This study demonstrates that this is a significant issue in normal-weight children since we found a high prevalence of vitamin D insufficiency and deficiency among normal-weight children aged 6-24 months. Studies carried out in *Chakaria, Coxesbazar* have found that 11% of children had active rickets and that vitamin D deficiency ranges from 6–21% among children under 5 years old in that community [18, 20]. Additionally a study from same area reported only 6% (10 out of 158 participants) of children were suffering from severe vitamin D deficiency [13] which support our finding (Table 4.1). Recently a case-control study [15] was carried out among children aged 1–24 months in north-eastern rural Bangladesh and it found 32% of all children (including case and control) were severely vitamin D deficient (<25 nmol/L) and 70% had serum vitamin D less than 40 nmol/L. However, the study was conducted during winter season (January–February) when children had the significant risk of severe deficiency of vitamin D. Unreported results of our study also correlate with the finding of high prevalence of severe deficiency during winter and spring.

Several studies from India also have demonstrated low serum vitamin D levels among preschool children. A longitudinal study conducted among pregnant women found that 36% and 62% of neonates were vitamin D deficient or insufficient respectively [12]. Another study in impoverished areas of Delhi, India, found that prevalence of low serum vitamin D status among children ranged from 2–84% but could not explain this wide variation in prevalence [16]. In Karachi, Pakistan severe vitamin D deficiency was found among 52% of healthy breastfed infants [11]. Thus, our findings from urban Bangladesh in combination with earlier findings from infants in urban Pakistan and India have demonstrated that there is remarkable vitamin D deficiency and insufficiency among young infants in South Asia and that a tropical climate with adequate sunshine all year long does not necessarily protect against low vitamin D status in the first two years of life. In regions such as Bangladesh, vitamin D levels can be low due to skin pigmentation [25, 42]; air pollution (by preventing the penetration of ultraviolet- B rays) [43]; clothing covering practises of children [44]; less physical and outdoor activities; maternal vitamin D deficiency [45]; inadequate or very little intake of liver, egg, and dairy products; inadequate or very little intake of sea fish or fish oil which are rich sources of vitamin D; and also the absence of any supplementary food fortification program for this vulnerable population group.

Our study demonstrated that the vitamin D deficiency and insufficiency in young children increases with age, which is in agreement with previous studies [8, 21, 26]. The high level of vitamin D in our youngest age group can partially be explained by the consumption of dairy products and the almost universal rate of breastfeeding. Again underweight children had the significant risk of vitamin D deficiency during winter and spring, and this was also observed by studies conducted elsewhere

among children in the general population [7, 9, 10, 12, 16, 21, 22, 24, 46]. However in our study, autumn was found to be protective for vitamin D insufficiency in normal-weight children. The higher probabilities (40%–50%) of normal-weight children being vitamin D insufficient and deficient in the summer could influence the results in other seasons—autumn, winter and spring (Figure 4.3). Perhaps for this reason autumn was found to be protective, and there were no significant differences of vitamin D deficiency or insufficiency during winter and spring among normal-weight children.

Among underweight children, those whose mothers had six years or more of school instruction were found to be significantly more vitamin D insufficient and deficient than those whose mothers were illiterate. Studies from Pakistan, Jordan, and Saudi Arabia reported similar findings for children in general populations [11, 23, 47]. Educated mothers may confine their infants indoors due to the polluted, congested and heavily populated slum environment, which ultimately leads to reduced exposure to sunlight, and this could explain the high prevalence of vitamin D deficiency among them. Maternal education did not predict the vitamin D deficiency in normal-weight children. In an unreported analysis of our study, we found that mothers of normal-weight children are significantly more literate than mother of underweight children. Homogeneity in the higher educational status of the mothers of normal-weight children probable plays the role in such findings among normal-weight children.

In our study we did not find any association between dietary intake of milk products or animal protein and vitamin D deficiency or insufficiency. Breastfed infants are often at a greater risk of developing deficiency and this might be due to the low vitamin D status of women of child bearing age [45, 48]. However, we didn't collect breast milk or blood samples from mothers for estimation of serum vitamin D status. Moreover, the breast feeding rate was almost universal in our study participants, thus we unable to explore the role of breast feeding for serum vitamin D status.

Vitamin D and zinc both play important roles in human health and don't interact directly. Both play an important role in the immune function. In our study we found a positive relationship between insufficient serum zinc status and deficient status of vitamin D in underweight weight children but an inverse relationship in normal-weight children in unadjusted analysis. A possible explanation for this finding is that underweight children were suffering from multiple micronutrient deficiencies [49, 50] especially serum zinc and D deficiency coexisting.

Limitations

The results of the study need to be interpreted in light of the study's limitations. First, we used data from a MAL-ED community-based prospective study with a case-control design in an urban setting;

results do not represent children in rural settings or in the general population. Second, the main source of vitamin D in humans is exposure to sunlight on bare skin. We did not collect any information about the frequency and duration of sunlight exposure, clothing covering children, cultural belief, and outdoor activities of the children; all of which could be weaknesses of this study. Third, the supporting information related to vitamin D and calcium homeostasis such as measurement of serum intact parathyroid hormone levels, alkaline phosphatase, bone markers or bone parameters were not measured and these would have added more strength to the study.

Conclusions

This study provides important information about the significant burden and associated risk factors of vitamin D insufficiency and deficiency in both underweight and normal-weight children in urban Bangladesh. Our study demonstrates that the risk factors for vitamin D deficiency and insufficiency differed between normal-weight and underweight children highlighting the need for interventions—including nutritional education regarding spending time outdoors in sunshine for 10–15 minutes at least 3–4 times per week, and supplementation—to be tailored to the specific needs of particular subgroups. Importantly, the burden and risk factors of vitamin D deficiency in both underweight and normal-weight children aged 6–24 months warrants the need for the design and implementation of a vitamin D specific health and nutritional program in Bangladesh.

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CHAPTER 5 ASSOCIATION OF VITAMIN D STATUS AND INCIDENCE AND SEVERITY OF DIARRHOEA

5.1 Context

Diarrhoea continues to be a leading cause of morbidity and mortality among children under two years in developing countries [1]. While the immune-modulatory function of vitamin D in infectious disease morbidity is a well-known phenomenon, studies related to vitamin D status and incidence of childhood diarrhoea is limited and controversial [2-9]. In this chapter, I investigated the association of micronutrient status especially vitamin D and zinc with incidence and severity of diarrhoeal diseases among underweight and normal-weight children aged 6–24 months in a resource-constrained urban slum setting. Moreover, documentation of diarrhoeal events on a daily basis through twice weekly home visits provides high resolution information on the community-based incidence and severity of diarrhoea among children under two years.

Following a longitudinal design, I demonstrated that vitamin D status is not associated either with incidence or severity of diarrhoea among children under two years living in an urban slum where basic hygienic conditions and caring practices are far below those of a common urban residential area. However, the beneficial role of zinc in preventing diarrhoea is still evident among the normal-weight children who are living in a polluted and hygiene-constrained area.

The adapted framework showing the immunomodulatory functions of vitamin D in prevention of diarrhoea in Chapter 1 is not effective in this study population. The lack of basic hygienic facilities, feeding and caring practices (introduction of complementary food exposed children to the unhygienic environment as well as abundance of pathogenic microorganisms), and the resource-constrained polluted environment of the slum resulted in repeated and overwhelming infections by enteric pathogens and could be masking the protective role of vitamin D in incidences of diarrhoea among children under two years of age. The analysis presented in this chapter is a new way to look at the association between multiple micronutrient status and the incidence and severity of diarrhoeal diseases in children. In this chapter, I demonstrate that vitamin D status did not confound the beneficial effect of zinc even in a resource-constrained setting. The results of this study are significant for supporting the ongoing zinc supplementation program in management and prevention of acute diarrhoeal disease among children.

Infectious childhood diarrhoea can be caused by a broad spectrum of pathogens which can be influenced by vitamin D status. In the next chapter, the role of vitamin D status in the incidence of diarrhoeagenic *E.coli* diseases is explored.

This Chapter forms a manuscript which has been accepted for publication in the *European Journal of Clinical Nutrition*.

5.2 Vitamin D status is not a confounder of the relationship between Zinc and diarrhoea: a study in 6-24 months old underweight and normal weight children of urban slum of Bangladesh

A. M. Shamsir Ahmed, Ricardo J. Soares Magalhaes, Tahmeed Ahmed, Kurt Z Long, Md. Iqbal Hossain, Md. Munirul Islam, Mustafa Mahfuz, S. M. Abdul Gaffar, Atiya Sharmeen, Rashidul Haque, Richard L. Guerrant, William A Petri Jr, Abdullah Al Mamun

Abstract

Background: The role of micronutrients particularly zinc in childhood diarrhoea is well established. Immunomodulatory functions of vitamin D in diarrhoea and its role in the effect of other micronutrients are not well understood. This study aimed to investigate whether vitamin D directly associated or confounded the association between other micronutrients status and diarrhoeal incidence and severity in underweight and normal-weight children aged 6–24 months in urban Bangladesh.

Methods: Multivariable generalised estimating equations were used to estimate incidence rate ratios for incidence (Poisson) and severity (binomial) of diarrhoea on cohorts of 446 normal-weight and 466 underweight children. Outcomes of interest included incidence and severity of diarrhoea, measured daily during a follow-up period of five months. The exposure of interest was vitamin D status at enrolment.

Results: Normal-weight and underweight children contributed 62,117 and 62,967 days observation, with 14.2 and 12.8 days per child per year of diarrhoea respectively. None of the models showed significant associations of vitamin D status with diarrhoeal morbidity. In the final model, zinc-insufficient normal-weight children had 1.3 times more days of diarrhoea than zinc-sufficient children ($p<0.05$). Again zinc insufficiency and mothers education had more risk of severe diarrhoea (1–5 years of education 1.8 times; >5 years 2.3 times). In underweight children, those who were of an older age had 24–63% fewer days of diarrhoea and 52%–54% fewer chances of severe diarrhoea; those who were female had 17% fewer days of diarrhoea and 31% fewer chances of severe diarrhoea.

Conclusion: Vitamin D status was not associated with incidence and severity of diarrhoea in study children. Role of zinc in diarrhoea was only evident in normal-weight children. Our findings demonstrate that vitamin D is not a confounder of the relationship between zinc and diarrhoea.

Background

Diarrhoea remains one of the leading causes for childhood morbidity and mortality among preschool children of developing countries [1]. Despite reductions in diarrhoeal-disease related mortality in preschool children over the last 20 years, globally morbidity continues to remain high with 1.73 billion diarrhoea episodes annually [10]. In Bangladesh, the overall incidence of diarrhoea among preschool aged children in 2007 was 3.8–4.3 episodes per child per year, while in 2010, 6% of deaths among preschool children were due to diarrhoeal diseases [11].

A recent meta-analysis of vitamin A supplementation programs reported a 15% reduction of diarrhoeal incidence in children [12]. Similarly, another meta-analysis of zinc supplementation showed 13% reduction of all forms of diarrhoea [13]. On the other hand, immune-modulatory function of vitamin D [4-6, 8, 9, 14] has led researchers to investigate its role in infectious disease associated morbidity such as diarrhoea. A study in school age children showed a reduction in the severity of diarrhoea and vomiting in vitamin D-sufficient children [7]. Evidence on the role of vitamin D in diarrhoeal morbidity among children aged under two years is limited. Recently a randomized control trial (RCT) reported no effect of six quarterly bolus doses of vitamin D supplementation on diarrhoeal incidence among children aged 1–30 months [3]. The heterogeneous findings and lack of studies among younger children warranted a comprehensive evaluation of the association of vitamin D status with incidence and severity of diarrhoea.

Experimentally it was shown that zinc binds with the vitamin D receptors (VDR), and the activity of vitamin D dependent genes in cells is influenced by the intracellular concentration of zinc [15]. Yet again, the active form of vitamin D bound to VDR, then initiates transcriptional factors for immune modulatory functions within different immune cells [16-18]. Thus, existing evidence indicates that vitamin D can be an important confounder of the effect of zinc for incidence and severity of diarrhoea in younger children.

In this study, we aimed to evaluate whether vitamin D was associated with diarrhoeal incidence and severity, or confounded the association of other micronutrients status and diarrhoeal incidence and severity in underweight and normal-weight children aged 6–24 months in urban Bangladesh.

Materials and methods

Study design, participants and site

The Mal-ED (Malnutrition & Enteric Diseases) consortium [19] is a multisite research project that conducted an interventional case-control study in underweight and normal-weight children aged 6–24 months in an urban slum area of the Mirpur field site in Dhaka, Bangladesh. The description of the study design and study site has been reported elsewhere.[20] One of the objectives of the MAL-

ED study is to evaluate the interaction of enteric infection and malnutrition in children under two years. The status of vitamin D and other micronutrients were determined at baseline among children enrolled in the case-control study. Additionally, their nutritional status and diarrhoeal morbidity data collected prospectively for five months gives us the opportunity to achieve the study objectives by employing longitudinal study designs for both underweight and normal-weight children.

The Research Review Committee and the Ethical Review Committee of icddr,b approved the study. Caregivers of eligible children provided voluntary, informed written consent at the time of enrolment for collection of data and biological samples for the purpose of the study.

Children were selected for enrolment into the study based on their nutritional status which was assessed during household surveillance of the study site. Cases were severe to moderately underweight children (weight-for-age Z, WAZ, score <–2.00 SD) and controls were well-nourished or normal-weight children (WAZ >–1) matched for sex and area of residence. A total of 500 cases and 480 controls were enrolled from November 2009 to December 2012. Complete data of 466 underweight and 446 normal-weight children were available for the final analysis (Figure 5.1). Both case and control children received nutritional intervention packages (without any vitamin D supplementation) which included: health and nutritional education for the caregivers; a high potency vitamin A capsule at 6-month intervals; deworming at enrolment; vaccines from the Expanded Program on Immunization; and supplementation with multiple micronutrient powder (MNP). Only underweight children (cases) received supplementary food, which is popularly known as *Pushti* packet. Details of the supplementary food (*Pushti* packet) and MNP are described elsewhere [20] and in Figure 5.1. . Fieldworkers demonstrated how to use MNP with the regular diet. For supplementary feeding, underweight children visited one of the four outposts established for the study six days a week. The caregivers were asked to sit down on a mat and feed the children the contents of the *Pushti* packet. Children were visited twice weekly for five months to collect information on morbidity. Standardised, previously validated morbidity questionnaires were used to interview caregivers regarding the presence of diarrhoeal symptoms. Fieldworkers collected morbidity information for the last seven days, if any child was absent for more than seven days from the household. Participants were dropped out of the study if absent from the study site for more than 60 days. The study physician collected 5 ml of venous blood form the children at enrolment for quantification of serum vitamin D [21], retinol [22] and zinc [23]. Sample processing and assays were performed at the Nutritional Biochemistry Laboratory at icddr,b. The IDS 25-Hydroxy Vitamin D Enzyme immunoassay (EIA) Kit (Source: IDS Ltd, 10 Didcot Way, Boldon Business Park, Boldon, UK) was used to measure serum vitamin D status. Results for micronutrient status were adjusted for C-reactive protein and and α1-acid glycoprotein [23, 24].

Measurements

Standard case definitions were used for diarrhoea, which included passage of three or more loose/watery stools in the last 24 hours. A new diarrhoeal episode was defined as three or more loose/watery stools in 24 hours followed by two consecutive days with fewer than three loose/watery stools passed by the child. Rate of diarrhoea was calculated by dividing the number of days with diarrhoea by total number of days of observation [7]. The diarrhoeal episodes were categorised as 'low', 'medium' and 'high' severity according to the method described in Lee *et al*. [25]. Only a small number of episodes were low-severe episodes (7–8%) and thus low and medium-severe episodes were combined, then compared with high-severe episodes to increase the statistical precision. The incidence (rate of diarrhoea) and severity of diarrhoea during the five months of active surveillance were primary outcomes in all analyses. The exposure of interest, vitamin D status at enrolment, was categorised as deficient (<50 nmol/L), insufficient (≥50 and <75 nmol/L) and sufficient (≥75 nmol/L) [26]. Serum retinol status was categorised as severe to moderate (<0.7 µmol/L) and mild deficiency to normal (≥0.7 µmol/L) status [27] , and serum zinc insufficiency was defined as <9.9 µmol/L [23]. Age group, maternal education, household hygiene and sanitation characteristics (source of drinking-water, toilet facility, toilet sharing, hand washing practices following defecation or prior to cooking/nursing of the child) and other socioeconomic parameters were created by recoding information in the database. Principle component analysis was used to construct a household wealth index [28]. Four seasons: summer (May-July), autumn (August-October), winter (November-January) and spring (February-April) were created using the date of blood sample collection.

Statistical analyses

The estimated rates of diarrhoea were compared with baseline child and maternal characteristics, and micronutrient status. Appropriate statistical tests were performed and p-value <0.05 was considered a statistically significant association. Generalized estimating equation (GEE) models with Poisson distribution were built to estimated incidence rate ratios (IRRs) and 95% CIs for diarrhoea incidence; these models included an exchangeable correlation structure to account for within-child and within-family correlations. Similarly, GEE models with binomial distribution were used to estimate IRRs and 95% CIs for comparing low and medium-severe episodes with high-severe episodes. Finally, adjusted IRRs were estimated from multivariable GEE models that included predictors of socio-demographic, and micronutrient status that were significantly associated with the outcomes in the univariable analyses or physiologically important factors or reported risk indicators in published literature. Four models were tested: in Model 1, the association between rate of diarrhoea or severity of diarrhoea and vitamin D status was adjusted for age group

and sex of the participants; in Model 2, the relationship was then adjusted for serum retinol and zinc status; in Model 3, the relationship was further adjusted for maternal education and household wealth index. Finally, in Model 4 the relationship was also adjusted for the season. All analyses were conducted using the STATA-12.0 software (StataCorp, College Station, TX).

Results

Characteristics of study children

Complete data of 466 underweight and 446 normal-weight children were available for final analysis (detail in Figure 5.1). Fieldworkers collected more than 120 days of morbidity information from 87.1% underweight and 90.1% normal-weight children. Vitamin D concentrations (mean ± SD) were 60.7 ± 23.3 nmol/L in underweight children and 54.2 ± 20.8 nmol/L in normal-weight children; 34.8% and 45.5% of underweight and normal-weight children were vitamin D deficient (<50 nmol/L), and 41.8% and 39.5% were insufficient (50–75 nmol/L). Breastfeeding was almost universal in both underweight and normal-weight groups thus it was not included in the analyses. We did not find any significant effect of hygiene practices (hand washing after defecation/helping child after defecation/before preparation of food, boiling or other drinking-water purification) in descriptive analysis so these were not included in the final analyses (results not shown).

Association of diarrhoeal incidence and severity of episodes with vitamin D status in normal-weight children

Normal-weight children contributed 62,117 total days of observation and had a diarrhoeal incidence rate of 14.2 days per child per year. Children who were vitamin D deficient (15.2 days per child per year) had more days of diarrhoea than children with vitamin D insufficiency (13.1 days per child per year) or sufficiency (14.2 days per child per year) but results were not statistically significant (Table 5.1).

The lack of significance for serum vitamin D remained in all the models after adjusting for confounding variables (Table 5.2). Older children (12–17 months and 18-24 month) had 34%–61% fewer days of diarrhoea than younger children; serum zinc-insufficient children had 1.3 times more days of diarrhoea than serum zinc sufficient children in the fully adjusted model. In Models 2 and 3, mild deficiency of serum retinol had 1.4 times more days of diarrhoea than serum retinol-sufficient children (Table 5.2).

Approximately 18% of all diarrhoeal episodes were classified as severe. Vitamin D statuses were not associated with severity of diarrhoeal episode (Table 5.3). None of the models showed any significant differences in the risk of high-severe diarrhoea for vitamin D status (Table 5.4). After adjusting for all confounding variables, older children had a 49%–56% reduced risk of high-severe

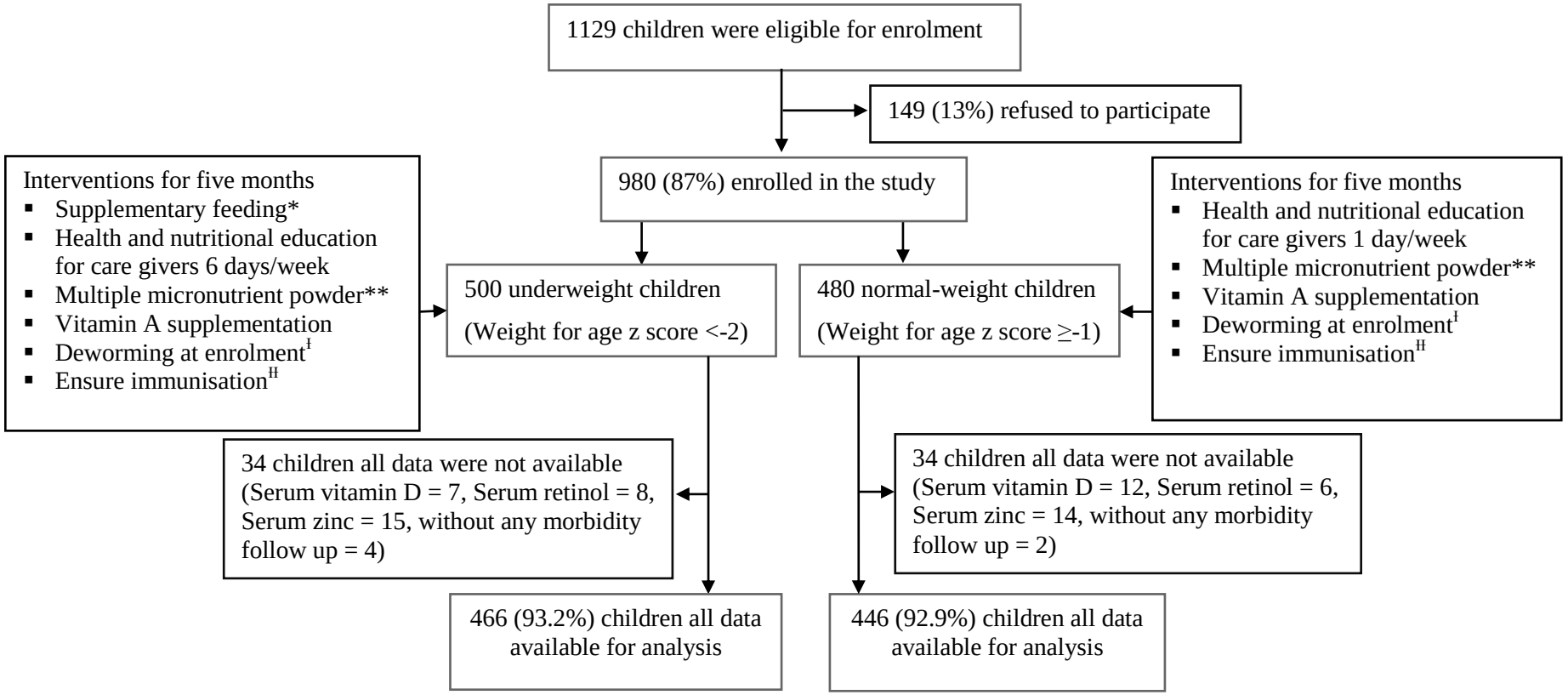
diarrhoea than younger children; female children had 38% reduced risk of high-severe diarrhoea than boys; serum zinc-insufficient children had 1.8 times greater risk of high-severe diarrhoea compared to zinc sufficient children; children of educated mothers (1–5 and >5 years of institutional education) had 2.3 great risk of high-severe diarrhoea than children of illiterate mothers (Table 5.4).

Association of diarrhoeal incidence and severity of episodes with vitamin D status in underweight children

Underweight children contributed 62,967 total days of observation and had 12.8 days per child per year of diarrhoea. Underweight children who were vitamin D insufficient had more days of diarrhoea (14.2 days per child per year) than deficient (11.0 days per child per year) or sufficient (13.0 days per child per year) groups but the results were not statistically significant (Table 5.1). Associations between vitamin D status and incidence of diarrhoea remained non-significant in the all adjusted models (Table 5.2). In the final model, older children had 24%–63% fewer days of diarrhoea than younger children and female children had 17% fewer days of diarrhoea than male children (Table 5.2).

Approximately 17% of all diarrhoeal episodes were classified as high-severe (Table 5.3). Vitamin D statuses were not associated with high-severe diarrhoea. In the final adjusted model, older age children had a 52%–54% reduced risk of a severe diarrhoeal episode compared with younger children; girls had a 31% reduced risk of a severe diarrhoeal episode than boys; children of mothers with more than five years of institutional education had 1.8 times more risk of a severe diarrhoeal episode than children of illiterate mothers (Table 5.4).

Figure 5.1: Study profile and children selected for longitudinal studies



* Each sachet of supplementary food (*Pushti* packet) contains roasted rice powder 20 g, roasted lentil powder 10 g, molasses 5 g and vegetable oil 5 ml providing approximate 150 kcals. Severely underweight (WAZ <–3) and moderately underweight child were receiving three packets and two packets respectively, 6 days/week for five month or until graduation by achieving WAZ –1.

** Each sachet of multiple micronutrient powder contains: 12.5 mg elemental iron, 5 mg elemental zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C, given for 2 months from enrolment in first 392 children enrolled, then given for 4 months in remainder of the children.

[Ɨ] 200 mg albendazole syrup was given orally as a single dose to all children more than 1 year old. In children aged under 1 year, pyrantel pamoate was given 10 mg/kg as single dose

[Ħ] Immunisation covers BCG, DPT, OPV, measles, hepatitis B, and Hib vaccines

Table 5.1: Diarrhoeal incidence according to socio-demographic characteristics and micronutrient status (Vitamin A and D, and zinc) in normal-weight and underweight children aged 6–24 months[1]

	Diarrhoea							
	Normal-weight children (n = 446)				Underweight children (n = 466)			
Child characteristics	n	Days of follow-up	Days of morbidity	Rate per child-year	n	Days of follow-up	Days of morbidity	Rate per child-year
All children	446	62,117	2,422	14.24	466	62,967	2,209	12.81
Age group (in months)[2]								
6–11	239	33,566	1,603	17.44	174	23,965	1,081	16.48
12–17	132	17,920	508	10.35	148	19,897	660	12.12
18–24	75	10,631	311	10.69	144	19,105	468	8.95
Sex								
Boys	229	31,505	1,238	14.35	234	31,433	1,212	14.08
Girls	217	30,612	1,184	14.13	232	31,534	997	11.55
Serum vitamin D at enrolment								
Sufficiency (≥75 nmol/L)	67	9,129	356	14.24	109	14,317	511	13.04
Insufficiency (≥50 and <75 nmol/L)	176	25,132	904	13.14	195	27,145	1,053	14.17
Deficiency (<50 nmol/L)	203	27,856	1,162	15.24	162	21,505	645	10.95
Serum zinc at enrolment								
Sufficiency (≥9.9 µmol/L)	374	51,981	1,934	13.59	373	50,480	1,693	12.25
Insufficiency (< 9.9 µmol/L)	72	10,136	488	17.59	93	12,487	516	15.09
Serum retinol at enrolment								
Normal to mild deficiency (≥0.7 µmol/L)	294	41,241	1,615	14.30	277	38,280	1304	12.44
Moderate to severe deficiency (<0.7 µmol/L)	152	20,514	807	14.37	189	24,687	906	13.40
Mother's institutional education								
Illiterate	68	9,556	345	13.19	116	15,551	589	13.83
1–5 years	191	26,624	1,061	14.56	215	29,391	930	11.56
>5 years	187	25,934	1,016	14.31	135	18,025	690	13.98
Household wealth index								
Lowest	45	5,924	253	15.60	139	18,196	715	14.35
Second	74	10,162	440	15.81	107	14,238	487	12.49
Third	88	11,928	505	15.46	91	12,701	435	12.51
Fourth	104	14,928	552	13.51	81	10,944	333	11.11
Highest	135	19,175	672	12.80	48	6,888	239	12.67
Season of vitamin D measurement								
Summer	79	10,897	440	14.75	85	11,635	430	13.50
Autumn	68	9,416	376	14.59	93	12,529	467	13.61
Winter	184	25,284	919	13.28	173	22,890	721	11.50
Spring	115	16,520	687	15.19	115	15,913	591	13.57

[1]For ordinal predictors, the categories of the predictor were introduced as continuous into the GEE model with a Poisson distribution and p value was a test for linear trend. For dichotomous predictor, p value is from Wald test with robust standard errors in the GEE model.

[2] p <0.001 for age in both normal-weight and underweight children

Table 5.2: Incidence rate ratios (IRRs) of diarrhoeal incidence in underweight and normal-weight children aged 6–24 months

	Normal-weight children (n = 446)				Underweight children (n = 466)			
Child characteristics	Model 1	Model 2	Model 3	Model 4	Model 1	Model 2	Model 3	Model 4
Age group (in months)								
12–17	0.59 (0.46, 0.75)**	0.59 (0.46, 0.75)**	0.58 (0.46, 0.74)**	0.58 (0.45, 0.74)**	0.76 (0.61, 0.94)*	0.78 (0.62, 0.96)*	0.75 (0.61, 0.93)*	0.76 (0.61, 0.95)*
18–24	0.66 (0.50, 0.89)*	0.66 (0.49, 0.88)**	0.66 (0.49, 0.88)*	0.66 (0.49, 0.89)*	0.55 (0.43, 0.70)**	0.56 (0.43, 0.71)**	0.56 (0.44, 0.72)**	0.57 (0.44, 0.73)**
Sex								
Girls	0.97 (0.80, 1.18)	0.97 (0.80, 1.18)	0.96 (0.79, 1.17)	0.96 (0.79, 1.17)	0.84 (0.69, 1.01)	0.84 (0.70, 1.02)	0.83 (0.69, 1.00)^	0.83 (0.69, 1.00)^
Serum vitamin D at baseline								
Insufficiency (≥50 and <75 nmol/L)	1.01 (0.75, 1.38)	1.07 (0.79, 1.45)	1.10 (0.81, 1.49)	1.10 (0.81, 1.50)	1.11 (0.87, 1.40)	1.09 (0.86, 1.38)	1.10 (0.87, 1.39)	1.11 (0.87, 1.40)
Deficiency (<50 nmol/L)	1.17 (0.87, 1.57)	1.23 (0.92, 1.65)	1.24 (0.92, 1.66)	1.23 (0.91, 1.66)	0.93 (0.72, 1.20)	0.90(0.70, 1.17)	0.90 (0.69, 1.16)	0.89 (0.68, 1.16)
Serum zinc status at baseline								
Insufficiency (< 9.9 µmol/L)		1.33 (1.04, 1.70)*	1.34 (1.04, 1.71)*	1.32 (1.03, 1.70)*		1.14 (0.91, 1.44)	1.11 (0.89, 1.40)	1.11 (0.88, 1.39)
Serum retinol status at baseline								
Moderate to severe deficiency (<0.7 µmol/L)		0.93 (0.75, 1.14)	0.92 (0.74, 1.13)	0.91 (0.74, 1.13)		0.99 (0.91, 1.43)	1.00 (0.82, 1.21)	1.00 (0.82, 1.21)
Mother's institutional education								
1–5 years			1.25 (0.92, 1.69)	1.26 (0.93, 1.71)			0.88 (0.69, 1.11)	0.87 (0.69, 1.10)
>5 years			1.36 (0.98, 1.88)	1.37 (0.99, 1.91)			1.10 (0.85, 1.43)	1.09 (0.84, 1.42)
Household wealth index								
Second			0.88 (0.60, 1.29)	0.88 (0.60, 1.29)			0.91 (0.71, 1.17)	0.91 (0.71, 1.18)
Third			0.92 (0.64, 1.33)	0.92 (0.64, 1.33)			0.88 (0.67, 1.16)	0.88 (0.67, 1.16)
Fourth			0.79 (0.54, 1.15)	0.79 (0.54, 1.15)			0.78 (0.58, 1.05)	0.79 (0.59, 1.06)
Highest			0.72 (0.49, 1.05)	0.71 (0.49, 1.04)			0.92 (0.66, 1.30)	0.93 (0.66, 1.30)
Season of vitamin D measurement								
Autumn				1.04 (0.74, 1.46)				0.87 (0.66, 1.16)
Winter				1.05 (0.79, 1.39)				0.87 (0.67, 1.13)
Spring				1.13 (0.84, 1.52)				0.99 0.75, 1.30)

^p = 0.05, *p < 0.05 and **p <0.001

Reference values for variables: 6–11 months, boys, sufficient vitamin D status, sufficient serum zinc status, normal or mild deficient serum retinol status, illiterate mother, lowest wealth quintal and summer season for vitamin D measurement

Model 1: Adjusted for age and sex; Model 2: Model 1 + adjusted for serum retinol and zinc; Model 3: Model 2 + adjusted for maternal education and household wealth index

Model 4: Model 3 + adjusted for season of vitamin D measurement

Table 5.3: Severity of diarrhoeal episode according to socio-demographic characteristics and micronutrient status (vitamin A and D, and zinc) in normal-weight and underweight children aged 6–24 months[1]

	Normal-weight children (n = 446)			Underweight children (n = 466)		
Child characteristics	Number of episodes	Mild to moderate episode % (n)	Severe episode % (n)	Number of episodes	Mild to moderate episode % (n)	Severe episode % (n)
Total number of episodes	826	82.2 (679)	17.8 (147)	802	82.8 (664)	17.2 (138)
Age group (in months)[2]						
6–11	517	59.9 (407)	74.8 (110)	348	40.7 (270)	56.5 (78)
12–17	193	25.3 (172)	14.3 (21)	260	34.0 (226)	24.6 (34)
18–24	116	14.7 (100)	10.9 (16)	194	25.3 (168)	18.8 (26)
Sex[3]						
Boys	418	49 (333)	57.8 (85)	423	51.4 (341)	52.7 (82)
Girls	408	51 (346)	42.2 (62)	379	48.6 (323)	47.3 (56)
Serum vitamin D at enrolment						
Sufficiency (≥75 nmol/L)	121	14.7 (100)	14.3 (21)	186	23.6 (157)	21.0 (29)
Insufficiency (≥50 and <75 nmol/L)	337	41.1 (279)	39.5 (58)	369	45.3 (301)	49.3 (68)
Deficiency (<50 nmol/L)	368	44.2 (300)	46.3 (68)	247	31.0 (206)	29.7 (41)
Serum zinc at enrolment[4]						
Sufficiency (≥9.9 µmol/L)	670	82.8 (562)	73.5 (108)	626	79.1 (525)	73.2 (101)
Insufficiency (< 9.9 µmol/L)	156	17.2 (117)	26.5 (39)	176	20.9 (139)	26.8 (37)
Serum retinol at enrolment						
Normal to mild deficiency (≥0.7 µmol/L)	557	66.7 (453)	70.8 (104)	469	57.4 (381)	63.8 (88)
Moderate to severe deficiency (<0.7 µmol/L)	269	33.3 (226)	29.2 (43)	333	42.6 (283)	36.2 (50)
Mother's institutional education[5]						
Illiterate	122	16.0 (109)	8.8 (13)	204	26.5 (176)	20.3 (28)
1–5 years	259	42.6(289)	47.6 (70)	366	47.0 (312)	39.1 (54)
>5 years	345	41.4 (281)	43.5 (64)	232	26.5 (176)	40.6 (56)
Household wealth index						
Lowest	86	10.8 (73)	8.8 (13)	266	34.8 (231)	25.4 (35)
Second	144	17.5 (119)	17.0 (25)	176	21.2 (141)	25.4 (35)
Third	164	19.9 (135)	19.7 (29)	163	20.9 (139)	17.4 (24)
Fourth	194	22.7 (154)	27.2 (40)	114	13.4 (89)	18.2 (25)
Highest	238	29.2 (198)	27.2 (40)	83	9.6 (64)	13.7 (19)
Season of vitamin D measurement						
Summer	147	17.4 (118)	19.7(29)	138	16.1 (107)	22.5 (31)
Autumn	117	13.7 (93)	16.3 (24)	163	20.6 (137)	18.8 (26)
Winter	330	41.7(283)	32.0 (47)	285	36.5 (242)	31.2 (43)
Spring	232	27.2 (185)	32.0 (47)	216	26.8 (178)	27.5 (38)

[1] For ordinal predictors, the categories of the predictor was introduced as continuous into the GEE model with a Poisson distribution and p value was a test for linear trend. For dichotomous predictor, p value is from Wald test with robust standard errors in the GEE model.

[2] p <0.01 for age in both normal-weight and underweight children

[3] p = 0.05 for sex in normal-weight children

[4] p <0.01 for serum zinc status in normal-weight children

[5] p <0.01 for mother's institutional education in underweight children

Table 5.4: Incidence rate ratios (IRRs) for severe form of diarrhoeal episode compared with mild and moderate form of diarrhoeal episode in underweight and normal-weight children aged 6–24 months

	Normal-weight children				Underweight children			
Child characteristics	Model 1	Model 2	Model 3	Model 4	Model 1	Model 2	Model 3	Model 4
Age group (in months)								
12–17	0.46 (0.28, 0.75)*	0.45 (0.27, 0.76)*	0.43 (0.25, 0.73)*	0.44 (0.26, 0.77)*	0.49 (0.31, 0.77)*	0.48 (0.30, 0.75)*	0.46 (0.29, 0.73)*	0.46 (0.29, 0.73)*
18–24	0.49 (0.27, 0.89)*	0.49 (0.27, 0.89)*	0.50 (0.27, 0.93)*	0.51 (0.27, 0.96)*	0.57 (0.35, 0.93)*	0.49 (0.30, 0.81)*	0.47 (0.29, 0.77)*	0.48 (0.29, 0.78)*
Sex								
Girls	0.63 (0.44, 0.92)*	0.64 (0.44, 0.93)*	0.62 (0.42, 0.92)*	0.62 (0.42, 0.93)*	0.73 (0.50, 1.06)	0.72 (0.49, 1.05)	0.70 (0.48, 1.02)	0.69 (0.47, 1.00)^
Serum vitamin D at baseline								
Insufficiency (≥50 and <75 nmol/L)	1.17 (0.66, 2.09)	1.29 (0.72, 2.33)	1.25 (0.67, 2.32)	1.27 (0.68, 2.37)	1.33 (0.81, 2.18)	1.40 (0.86, 2.29)	1.35 (0.83, 2.19)	1.32 (0.80, 2.16)
Deficiency (<50 nmol/L)	1.18 (0.67, 2.09)	1.38 (0.77, 2.47)	1.32 (0.72, 2.42)	1.32 (0.71, 2.47)	1.34 (0.79, 2.27)	1.21 (0.70, 2.09)	1.10 (0.64, 1.89)	1.11 (0.63, 1.96)
Serum zinc status at baseline								
Insufficiency (< 9.9 µmol/L)		1.92 (1.21, 3.04)*	1.89 (1.17, 3.05)*	1.82 (1.12, 2.96)*		1.31 (0.83, 2.06)	1.32 (0.84, 2.06)	1.29 (0.82, 2.02)
Serum retinol status at baseline								
Moderate to severe deficiency (<0.7 µmol/L)		0.71 (0.47, 1.09)	0.73 (0.47, 1.14)	0.73 (0.47, 1.13)		0.65 (0.44, 0.98)*	0.76 (0.51, 1.14)	0.77 (0.51, 1.17)
Mother's institutional education								
1–5 years			2.27 (1.14, 4.51)*	2.26 (1.13, 4.52)*			1.03 (0.62, 1.72)	0.99 (0.59, 1.66)
>5 years			2.22 (1.07, 4.60)*	2.27 (1.09, 4.73)*			1.85 (1.07, 3.22)*	1.84 (1.05, 3.22)*
Household wealth index								
Second			0.99 (0.44, 2.24)	0.97 (0.43, 2.20)			1.64 (0.98, 2.74)	1.65 (0.99, 2.77)
Middle			1.00 (0.45, 2.21)	0.99 (0.45, 2.21)			1.12 (0.63, 1.97)	1.13 (0.64, 2.00)
Fourth			1.13 (0.52, 2.47)	1.12 (0.51, 2.45)			1.64 (0.91, 2.95)	1.71 (0.95, 3.08)
Highest			0.87 (0.39, 1.92)	0.83 (0.37, 1.86)			1.66 (0.86, 3.20)	1.71 (0.88, 3.33)
Season of vitamin D measurement								
Autumn				1.12 (0.58, 2.18)				0.57 (0.32, 1.02)
Winter				0.90 (0.51, 1.59)				0.73 (0.43, 1.26)
Spring				1.16 (0.66, 2.07)				0.76 (0.43, 1.34)

^p = 0.05 and *p <0.05

Reference values for variables: 6–11 months, boys, sufficient vitamin D status, sufficient serum zinc status, normal or mild deficient serum retinol status, illiterate mother, lowest wealth quintal and summer season for vitamin D measurement

Model 1: Adjusted for age and sex; Model 2: Model 1 + adjusted for serum retinol and zinc; Model 3: Model 2 + adjusted for maternal education and household wealth index

Model 4: Model 3 + adjusted for season of vitamin D measurement

Discussion

It was hypothesised that vitamin D may have a protective role against childhood diarrhoea; however, following a longitudinal design, no association was found between vitamin D status and childhood diarrhoeal disease indicators among underweight and normal-weight children aged 6–24 months.

Previous studies have presented frameworks for understanding the mechanisms through which vitamin D plays a role in the activation of innate and adaptive immunity for infectious diseases including diarrhoea [6, 14, 29-32]. Vitamin D has demonstrated a protective effect against infection through the up-regulation of genes involved in strengthening the barrier function of the epithelial membrane including gastrointestinal tract. A strengthened barrier function would inhibit the attachment of entry of microorganisms to the epithelial and subsequent infection [30]. It also induces production of the antimicrobial peptides, cathelicidin, that have a broad spectrum of antimicrobial activity against viruses, bacteria and fungi [33-35]. Production of antimicrobial peptides (cathelicidin) is the key pathway to preventing diarrhoea since these peptides appear to play a role in the regulation of innate and adaptive immunity in gastrointestinal infection [36, 37]. We assume that similar protective mechanisms would be induced in our study participants with better vitamin D status. However, a recently conducted randomized controlled trial with quarterly bolus dose of vitamin D at high-risk population aged 1–29 months did not find any effect on first or recurrent incidence of diarrhoea [3]. In a longitudinal study among school-going children (mean age 8.9 years) in Colombia, researchers found vitamin D deficient children were at greater risk of gastrointestinal illness [7]. Our study consisted of children aged 6–24 months living in urban slums, where the burden of disease is highest due to poor sanitation and low socio-economic status. They reside in an overcrowded unhygienic environment where most households have only one room for living and daily life activities [20]. These conditions may result in constant exposure to pathogenic organisms and subsequently infections by multiple enteric pathogens and increased diarrhoeal disease incidence [38, 39]. Continuous and overwhelming infections by enteric pathogens could be masking the role of vitamin D in this population. Poor caregiving could be another potential explanation for no association of vitamin D for reducing incidence and severity of diarrhoea in our children. Probably for this reason, our study findings were concordant with the finding of an RCT with vitamin D supplementation conducted in a similar setting in Afghanistan [3].

Population-based studies conducted to determine the effect of serum retinol or zinc in incidence and severity of childhood diarrhoea, did not consider the nutritional status of children, nor targeted the population most at risk, especially undernourished children [12, 13]. To that extent, our finding that

normal-weight children with insufficient serum zinc had an increased incidence and severity of diarrhoea compared with children with sufficient serum zinc status, is a novel finding. In all the models we tested for normal-weight children, it is evident that the role of zinc in prevention of diarrhoea was not confounded by vitamin D or vitamin A status. A recent RCT among children aged 6–60 months receiving zinc alone also reported similar findings after comparing children who received other multi-nutrients with or without zinc [40]. We also didn't find that zinc played a role in incidence or severity of diarrhoea among undernourished or underweight children. The lack of catalysing enzymes and compromised immunity in undernourished children may resulted in non-activation of the immune response that is initiated by serum zinc, and this could be the explanation for such findings [41].

Similar to other studies [10, 42], we also found that the risk of incidence and severity of diarrhoea were significantly less among older children in all models tested. Children aged 6–11 months are in a critical transitional period due to introduction of complementary food that leads them to be exposed to the wider environment as well as pathogenic microorganisms [43], which increases the risk of diarrhoeal diseases among this young age group. In the final model, girls had significantly less risk of diarrhoea than boys after adjusting for all other potential confounders. It is reported in several studies that both mortality and morbidity are higher in boys than girls, but without appropriate explanations [44-46]. It is argued that male vulnerability in response to environmental stress in early life is predicted by natural selection and can result in greater morbidity and mortality than females [47]. Our results demonstrate that children of more educated mothers had greater risk of severe forms of diarrhoea in both normal-weight and underweight children. However, reporting of severity of diarrhoea might be influenced by several factors including education of mother/caregiver and socio-economic conditions [48]. In our study, educated mothers might be reporting more competently about the number of stool frequency or other symptoms of diarrhoeal illness than illiterate mothers, which resulting in the higher number of severe diarrhoea episodes in the children of educated mothers. This result indicates that future studies should measure differences in mothers' perception of diarrhoeal illness in their children according to their educational status, and this will enable researchers to obtain validated data on childhood diarrhoeal illnesses.

The results of this study should be interpreted in the light of its limitations. First, both underweight and normal-weight children received multiple micronutrient powder supplementation with vitamin A and zinc but no vitamin D, as per recommendation of National Nutrition Program of Bangladesh. We did not consider the effect of the multiple micronutrient supplementations for incidence of diarrhoeal morbidity, which may confounded the findings of our results. Second, continuous health

and nutritional education, and provision of clinical services for common childhood illness at the field site could have promoted over-reporting of illnesses, ultimately resulting in reporting bias by caregivers. Thirdly, study findings cannot be generalized due to inclusion of only urban 6-24 months old children. Feeding and feeding practices of complementary food are known to modify the risk of diarrhoea among children. However, the collected data were inadequate to assess the risk of diarrhoea in relation to feeding practices.

The strengths of the study include the twice-weekly follow-up and the low rate in the loss to follow-up. The documentation of diarrhoeal events on a daily basis through twice-weekly home visits by trained field workers also enhanced disease identification and provided validity for the results [49]. The overall and and age-specific incidences of diarrhoea is also concordant with other studies conducted in resource-limited settings.[10, 50]

In conclusion, this study demonstrated that serum vitamin D status was neither associated in reducing the incidence or the severity of diarrhoeal episodes in normal-weight and underweight children aged 6–24 months living in the urban slums of Dhaka. The beneficial effects of serum zinc in diarrhoeal morbidity were only apparent in normal-weight children. Importantly, the results of this study indicate that vitamin D did not confound the relationship between serum zinc and diarrhoeal morbidity in both underweight and normal-weight children.

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CHAPTER 6 ASSOCIATION OF VITAMIN D STATUS AND INCIDENCE OF PATHOGEN SPECIFIC DIARRHOEA

6.1 Context

Diarrhoea is usually caused by a broad range of extensive and diverse group of gastrointestinal pathogens and among them diarrhoeagenic *Escherichia coli (*DEC*)* encompasses an important group of bacteria, which is highly prevalent in our study population [1]. Each species of DEC has distinct mechanisms of disease pathogenesis [2]. There is lack of epidemiological evidence related to the role of vitamin D status in the incidence of DEC diarrhoea. In this chapter, I explore the role of vitamin D status and incidence of ETEC, EPEC and EAEC diarrhoea in both underweight and normal-weight children aged 6–24 months.

Using a community-based collection of diarrhoeal stool samples and microbiological analyses for the presence common enteropathogens, I revealed that, the incidence of ETEC, EPEC and EAEC diarrhoea is high in both underweight and normal-weight children. Although, I found that there is a significantly lower cumulative incidence of EAEC diarrhoea among vitamin D deficient underweight children, vitamin D deficient status was not independently associated after adjusting for other covariates. However, vitamin D insufficient normal-weight children experienced significantly lower risk of EAEC diarrhoea than children with sufficient status. Similarly, moderate to severe vitamin A deficient children had a reduced risk of EPEC diarrhoeal incidence compared with children with normal to mild vitamin A deficiency status. Vitamin D deficient normal-weight children were also at higher risk of ETEC diarrhoea after adjustment with other micronutrient status only.

The results of the study are significant in many ways. First, it provides a community-based incidence rate of ETEC, EPEC and EAEC diarrhoea. Second, the findings reported in this chapter raise important research questions about the effect of multiple micronutrient supplementation programs increasing the risk of incidence of certain pathogen-specific diarrhoea. Thus, further research on the micronutrients mediating innate and adaptive immune responses in pathogen-specific diarrhoea is warranted.

In the next Chapter, I further evaluated the association between vitamin D and other micronutrient status with URI and ALRI, which are the leading cause of childhood mortality and morbidity among the children of low-income and middle-income countries.

This chapter forms a manuscript which has been submitted to *Tropical Medicine &International Health.*

6.2 Association of vitamin D status with incidence of enterotoxigenic, enteropathogenic and enteroaggregative *Escherichia Coli* diarrhoea in 6-24 months old underweight and normal weight children of urban slum of Bangladesh

A. M. Shamsir Ahmed, Ricardo J. Soares Magalhaes, Kurt Z Long, Tahmeed Ahmed, Md. A. Alam, Md. Iqbal Hossain, M. Munirul Islam, Mustafa Mahfuz, Dinesh Mondal, Rashidul Haque, Abdullah Al Mamun

Abstract

Background: Experimental evidence indicates that vitamin D can regulate the gut immune response affecting infection caused by intestinal organisms. This study aims to evaluate the association between vitamin D status and diarrhoeal episodes by enterotoxigenic (ETEC), enterpathogenic (EPEC) and enteroaggregative (EAEC) *E. coli* in underweight and normal-weight children aged 6–24 months in urban Bangladesh.

Methods: Cohorts of 446 normal-weight and 466 underweight children were analysed separately for the presence of ETEC, EPEC, and EAEC from diarrhoeal stool samples collected during five months follow-up, while considering vitamin D status at enrolment as the exposure. Cox proportional hazards models with unordered failure events of the same type were used to determine diarrhoeal risk factors after adjusting for sociodemographic and serum retinol and zinc status.

Results: Vitamin D status was not independently associated with the risk of incidence of ETEC, EPEC and EAEC diarrhoea in underweight children but moderate to severe retinol deficiency associated with reduce risk for EPEC diarrhoea upon adjustment. Among normal-weight children, insufficient vitamin D status and moderate to severe retinol deficiency were independently associated with 44% and 38% reduced risk of incidence of EAEC diarrhoea respectively. Vitamin D deficient normal-weight children were also at higher risk of ETEC diarrhoea after adjustment with age, sex, and other micronutrient status.

Conclusion: This study demonstrates for the first time that normal-weight children with insufficient vitamin D status have a reduced risk of EPEC diarrhoea compared with those with sufficient status. This study also shows that moderate to severe deficiency of serum retinol is associated with reduced risk of EPEC and EAEC diarrhoea in underweight and normal-weight children.

Background

Childhood diarrhoea continues to be an important public health problem with 1.73 billion diarrhoea episodes reported annually in developing countries [3]. Diarrhoea is usually caused by a broad range of extensive and diverse group of gastrointestinal pathogens including bacteria, virus and protozoa [1]. Among them diarrhoeagenic *Escherichia coli (*DEC*)* encompasses an enormous population of bacteria that exhibit a very high degree of both genetic and phenotypic diversity [2]. DEC can cause diarrhoea through three major paradigms: (i) enterotoxin production: enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC); (ii) invasion: enteroinvasive *E. coli* (EIEC); and/or (iii) intimate adherence with membrane signalling: enteropathogenic *E. coli* (EPEC), and enterohemorrhagic *E. coli* (EHEC) [2]. EHEC diarrhoea is rare in children of both developed and developing countries and usually causes an epidemic outbreak because of contaminated food [4]. EIEC diarrhoea is usually documented with a foodborne or waterborne outbreak and endemic sporadic disease occurs in some areas [2]. ETEC strains are associated with weanling diarrhoea among children and traveller's diarrhoea among adults. In a recent multicounty study, ETEC was found to be one of the top four most important pathogens for moderate-to-severe childhood diarrhoea in developing countries including Bangladesh [1]. A cohort study among children under two years of age in Bangladesh showed that ETEC was the most common pathogen and was isolated in 19.5% of cases, with an incidence of 0.5 episodes per child per year [5]. EPEC is primarily a disease of children aged under two years [6] causing diarrhoea in both the community and hospital settings [7]. EAEC is detected in hospitalised patients with persistent diarrhoea [8], outpatients clinics [9] and during household surveillance [10] and also during sporadic diarrhoeal outbreaks [11, 12].

Recent experimental evidence supports a positive effect of vitamin D on the immunomodulation of infectious diseases which has led researchers to investigate its effect on diarrhoea [13-17]. A study among school-aged children has shown that sufficient vitamin D status had a protective effect on the severity of diarrhoea and vomiting [18]. Findings are not consistent with the results from a RCT among children aged 1–30 months, which reported no effect with six quarterly bolus doses of vitamin D supplementation on diarrhoeal incidence [19]. These results may be confounded by the type of pathogen underlying the diarrhoeal episode. Studies concerned with the role of vitamin D in the regulation of gut function and health suggest that vitamin D status may contribute to a host's ability to produce antimicrobial peptide–cathelicidin and defensin–to resist or limit pathogen responsible for diarrhoeal diseases [17, 20, 21]. Laboratory studies and animal models have shown that anti-microbial peptides have bactericidal effects on intestinal pathogens including *Escherichia coli* [22-24] which is also regulated by vitamin D.

The MAL-ED network [25], conducted a case-control study on the aetiology, risk factors, and interactions of enteric infections and malnutrition in children aged 6–24 months at the urban Mirpur field site in Dhaka, Bangladesh [26]. The study also evaluated micronutrient status including serum vitamin D at baseline and followed the children for five consecutive months with active biweekly morbidity surveillance including diarrhoea. Causative organisms were isolated and characterised from stool samples collected during a diarrhoeal episode in children. Thus, the data generated by the MAL-ED study is ideal to investigate the association of vitamin D status with the incidence of ETEC, EPEC and EAEC specific diarrhoea which has not been reported previously.

In this study, I aim to investigate the association of vitamin D status (controlling for other micronutrients status and household/socioeconomic variables) with ETEC, EPEC, EAEC diarrhoea episodes among underweight and normal-weight children aged 6–24 months residing in the urban slum community of Mirpur, Bangladesh.

Methods

Subjects and study design

The details of the case-control interventional study of the Mal-ED network at the Bangladesh site has been described elsewhere [26]. The study has been approved by the Research Review Committee and the Ethical Review Committee of icddr,b (proposal # 2008–020). Underweight (weight-for-age Z (WAZ) score < –2.00 SD) case children and normal-weight (WAZ score > –1.00) control children were identified through biannual demographic surveillance of the field site. Eligible children were enrolled in the study after their parents/guardians were invited to participate in the study and signed informed, voluntary consent forms. A longitudinal designed study with two different cohorts of underweight and normal-weight children was used to achieve my study objectives.

Data collection and morbidity surveillance

During November 2009 through to December 2012, 500 underweight and 480 normal-weight children were enrolled and matched for sex and area of residence. At the time of enrolment, information on socio-demographic factors and household economic conditions were recorded using standard DHS household questionnaires [27]. Trained health fieldworkers collected morbidity information including diarrhoea via home visits every third or fourth day for five months using standardised, previously validated morbidity questionnaires. If any participants were absent more than seven days, field workers collected morbidity information f or the last seven days during the first available successful visit. Any children absent from their household or study site for more than 60 days were dropped from the study.

Sample collection and assays

Fieldworkers and caregivers were instructed to collect diarrhoeal stool specimens within 48 hours of an episode. Diarrhoea was defined as maternal report of three or more loose stools in 24 hours, or one loose stool with visible blood [28]. Discrete episodes were defined as three or more loose/watery stools in 24 hours followed by two consecutive days with fewer than three loose/watery stools passed by the children. Fieldworkers visited households of enrolled children every day to enquire about whether the child had a diarrhoeal episode. By using standard sample collection protocol, every attempt was made to collect diarrhoeal samples. Fieldworkers also left sterile extra stool containers with the family with instructions to collect at least 10 g of the stool sample when a child has diarrhoea. Within two hours of collection of stool samples a swab was transferred into Cary Blair medium and the rest of the sample refrigerated in cold packs for transport to the laboratory for processing

Collected stool samples were analysed with a standardised microbiology protocol described in detail elsewhere [29]. In brief, conventional stool culture methods were used to identify bacterial pathogens. For *Escherichia coli* identification and characterisation, five lactose-fermenting colonies were pooled from the culture for testing of DEC, multiplex PCR was then used for detection of toxin-encoding genes stx1, stx2, eae, bfpA, ipaH, aatA, and aaiC, as well as those encoding heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). ETEC, EPEC, and EAEC patho-types were classified [30] as per toxin-encoding genes detected by using a multiplex PCR, but adapted for the purpose of this study. The minimum criteria for determination of the diarrhoea genic *E. coli* were: the presence of *eltB* and/or *estA* for ETEC; the presence of *bfpA* and/or *eaeA* for EPEC; and the presence of *aatA* and/or *aaiC* for EAEC [31].

For determination of serum vitamin D and other micronutrient status of the children, a 5 ml blood sample was collected in trace element-free tubes upon enrolment. Processing of the collected samples and micronutrient assays was performed at the nutritional biochemistry laboratory of icddr,b. Serum vitamin D (25-Hydroxy vitamin D) was measured by using IDS 25-Hydroxy Vitamin D Enzyme immunoassay (EIA) Kit [32] (Source: IDS Ltd, 10 Didcot Way, Boldon Business Park, Boldon, UK). Serum retinol was measured using the HPLC method described elsewhere [33]. Serum/plasma zinc concentration was measured with air-acetylene flame atomic absorption spectrophotometer at 213.9 nm following dilution of the sample twelve times with deionised water. The result of serum retinol and zinc were adjusted for subclinical infections with C-reactive protein and α1-acid glycoprotein [34, 35].

Measurements

Demographic and socio-economic variables were recoded to generate age group (6–11, 12–17 and 18–24 months), maternal education (illiterate, 1–5 years and >5 years of institutional education), and other socio-economic parameters using variables in the database. A household wealth index was constructed with principal component analysis as described in the DHS [36]. Four seasons—summer (May to July), autumn (August to October), winter (November to January) and spring (February to April)—were created using the date of blood sample collection. Serum vitamin D status were categorised into deficient (<50 nmol/L), insufficient (≥50 and <75 nmol/L) and sufficient (≥75 nmol/L) status as per recommendation of the US Endocrine Society guideline [37]. Serum retinol status was categorised as moderate to severe deficiency (<0.7μmol/L) and mild deficiency to normal status (≥0.7μmol/L [38]. Serum zinc <9.9 μmol/L was categorised as having insufficiency status and ≥9.9 μmol/L was defined as sufficient status [34].

Statistical analyses

Isolation of the ETEC, EPEC, and EAEC from collected diarrhoeal stool samples were considered as the events of interest for the analysis. All children were included in the analysis regardless of their duration of follow-up, in order to minimise selection bias. Children were censored if ETEC, EPEC, and EAEC were not isolated from the collected sample, or children did not experience any diarrhoeal episodes during the follow-up period, or fieldworkers were unable to collect samples during a diarrhoeal episode. Gap time method was used to estimate the risk interval to take account of multiple failure events for the same children [39]. Cumulative hazard curves were drawn to display the proportion of children with different vitamin D status who had recurrent events over time after enrolment. Cox proportional hazards models were used with unordered failure events of the same type to determine risk factors of ETEC, EPEC and EAEC diarrhoeal incidence [40]. This model was used to take into account the occurrence of two or more events (failures) of the same type (i.e. isolation of ETEC at different points in time during the enrolment period) for the same subject. Unadjusted hazard ratios were estimated for the predictor variables including child, maternal and socio-economic characteristic as well as serum retinol and zinc statuses measured at baseline. A probability of ≤0.05 was considered statistically significant. Multivariable Cox proportional hazards models were built for each of the pathogen-specific diarrhoeal incidence (ETEC, EPEC and EAEC) separately with predictor variables to estimate adjusted hazard ratios. Model 1 was adjusted for vitamin D status, age group and sex of children. Model 2 was additionally adjusted for serum retinol and zinc status of the children. Model 3 was additionally adjusted for maternal education, household wealth index and season of serum-D measurement. All analyses were conducted by using the STATA software (version13.0; StataCorp, College Station, TX).

Results

Dataset for analysis

Complete data on 466 underweight and 446 normal-weight children were available for analysis (Figure 6.1). A total of 786 and 800 diarrhoeal episodes were recorded among underweight and normal-weight children respectively during the five-month follow-up period. Fieldworkers successfully collected 527 (67%) and 560 (70%) diarrhoeal stool samples in underweight and normal-weight children. ETEC was isolated in 17% and 15% of collected stool samples in underweight and normal-weight children respectively. EPEC and EAEC were found in 14% and 17% of stool samples collected in underweight children and 14% and 22% in normal-weight children respectively (Figure 6.1). Prevalence of vitamin D deficiency and insufficiency were 34.8% and 41.8% respectively for underweight children. Prevalence of vitamin D deficiency and insufficiency were 45.5% and 39.5% respectively for normal-weight children (result not presented).

Association of incidence of ETEC, EPEC and EAEC diarrhoeal episodes with vitamin D status in underweight children

Figure 6.2, presents Kaplan–Meier hazard function curves of cumulative incidence of ETEC, EPEC and EAEC diarrhoea for different vitamin D statuses in underweight children. Children with deficient vitamin D status had a significantly lower cumulative incidence of EAEC diarrhoea compared with children with sufficient vitamin D status (Log-rank test, $p < 0.05$). There were no significant differences in cumulative hazards of ETEC or EPEC diarrhoeal incidence among children who differed by vitamin D status.

In unadjusted Cox proportional hazard models, older children (12–17 and 18–24 months) had significantly reduced risks of ETEC, EPEC and EAEC diarrhoeal incidence over time (Table 6.1, 6.2 and 6.3). Children with vitamin D deficiency status were significantly associated with a reduced hazard risk of EAEC diarrhoeal incidence while children with vitamin D sufficient status were not (unadjusted HR 0.47; 95 % CI 0.24–0.90; $p < 0.05$). Children with mothers who had >5 years of institutional education were more likely to be associated with reduced risk of EAEC diarrhoeal incidence than children with illiterate mothers.

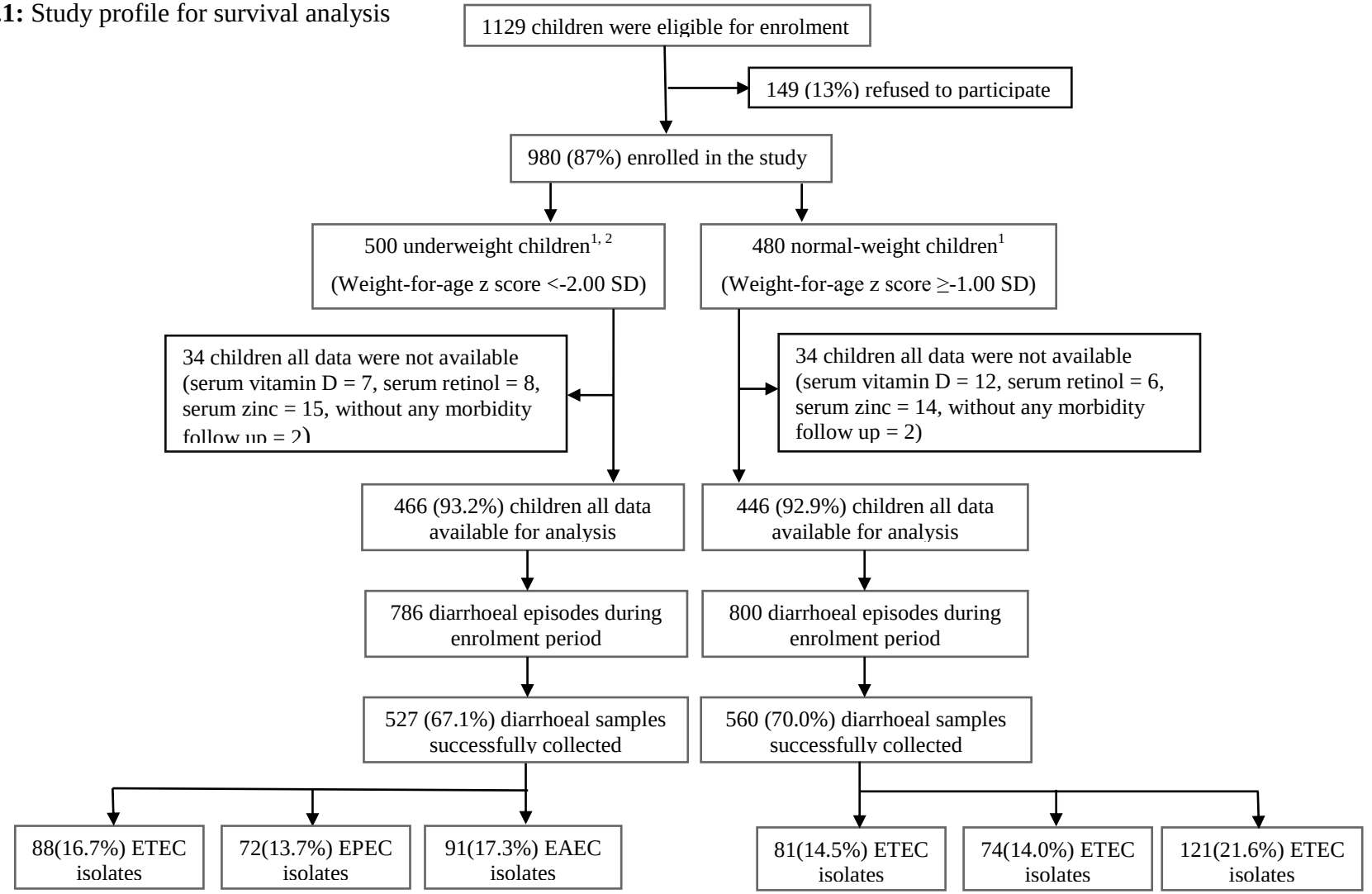
After adjustment of other covariates, in the final models, vitamin D status was not independently associated with the risk of incidence of EAEC diarrhoea. On the other hand, children with moderate to severe retinol deficiency had a reduced risk of EPEC diarrhoeal incidence when compared with children with normal to mild retinol deficiency. In all the models tested for ETEC, EPEC and EAEC, older-age children were independently associated with relative reduced risk of ETEC, EPEC and EAEC diarrhoea (Table 6.1, 6.2 and 6.3).

Association of incidence of ETEC, EPEC and EAEC diarrhoeal episodes with vitamin D status in normal-weight children

Among normal-weight children, Kaplan–Meier hazard function curves for cumulative incidence showed a significantly lower cumulative incidence (Log-rank test, $p < 0.05$) of EAEC diarrhoea among children with vitamin D insufficiency compared with children with sufficient vitamin D status (Figure 6.3). In unadjusted Cox proportional hazard models, older children (12–17 months and 18–24 months) had a significantly reduced risk of EPEC and EAEC diarrhoeal incidence over time (Table 6.1, 6.2 and 6.3). Children aged 12–17 months had a significantly reduced hazard rate for ETEC diarrhoeal incidence compared with children aged 6–11 months. Children with insufficient vitamin D status had significantly reduced risk of EAEC diarrhoeal incidence than children with sufficient vitamin D status. The measurement of vitamin D status during winter was significantly associated with a reduced risk of ETEC, EPEC and EAEC diarrhoea than the measurement of vitamin D status during summer.

Deficient vitamin D status was independently associated with 2.0 times more risk of ETEC diarrhoea (adjusted HR 2.04; 95 % CI 1.00–4.18; $p = 0.05$) only in Model 3. After adjustment of other covariates, in the final models, insufficient vitamin D status was independently associated with a 44% reduced risk of EAEC diarrhoea (adjusted HR 0.56; 95 % CI 0.33–0.95; $p < 0.05$). Similarly, children with moderate to severe retinol deficiency had a reduced risk of EAEC diarrhoeal incidence when compared with children with normal to mild retinol deficiency. Measurement of vitamin D status during winter was also independently associated with a reduced risk of EPEC and EAEC diarrhoeal than the measurement of vitamin D status during summer.

Figure 6.1: Study profile for survival analysis

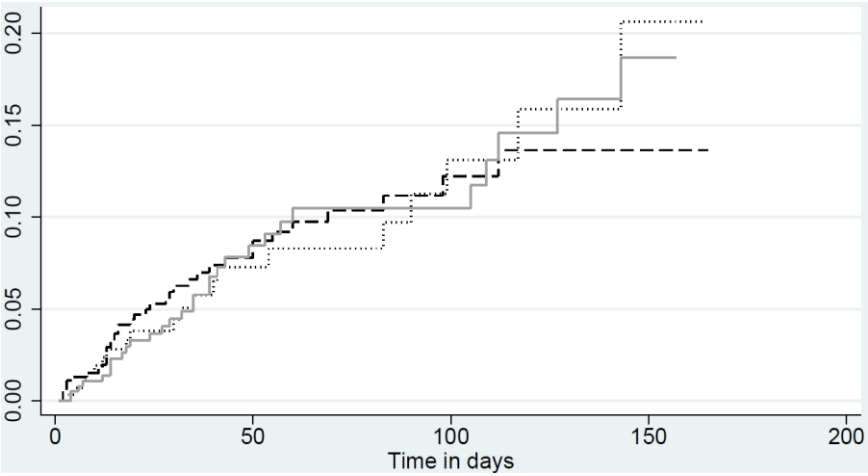


[1]Both underweight and normal-weight children received interventions for five months with 1) health and nutritional education for caregivers, 2) multiple micronutrient powder for two months (12.5 mg elemental iron, 5 mg elemental zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C), 3) vitamin A supplementation, 4) deworming at enrolment (200 mg albendazole syrup was given orally as a single dose to all children aged more than one year. In children aged under one year, 10 mg/kg pyrantel pamoate was given as a single dose), 5) immunisation (BCG, DPT, OPV, measles, hepatitis B, and Hib vaccines)

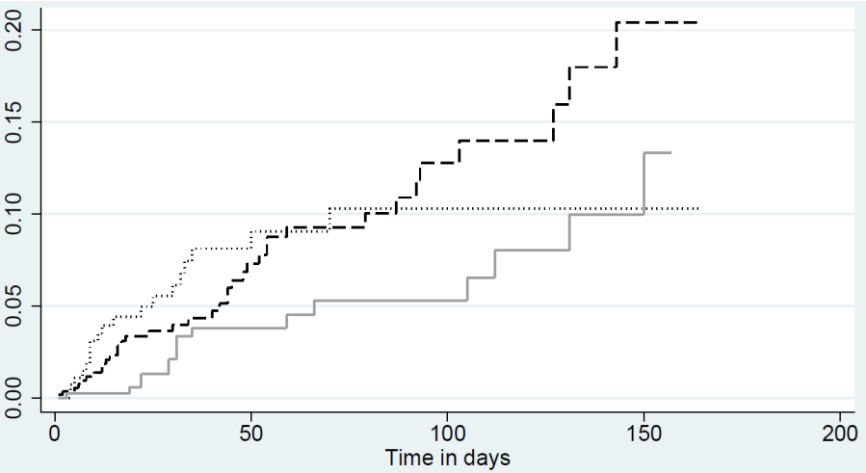
[2]Only underweight children received supplementary feeding. Each sachet of supplementary food contains roasted rice powder 20 g, roasted lentil powder 10 g, molasses 5 g and vegetable oil 5 ml providing approximate 150 kcals. Severely underweight (WAZ <–3) and moderately underweight child were receiving three packets and two packets respectively, 6 days/week for five month or until graduation by achieving WAZ –1.

Figure 6.2: Cumulative hazard graph for ETEC, EPEC, EAEC diarrhoeal episodes according to vitamin D status at baseline among underweight children aged 6–24 months

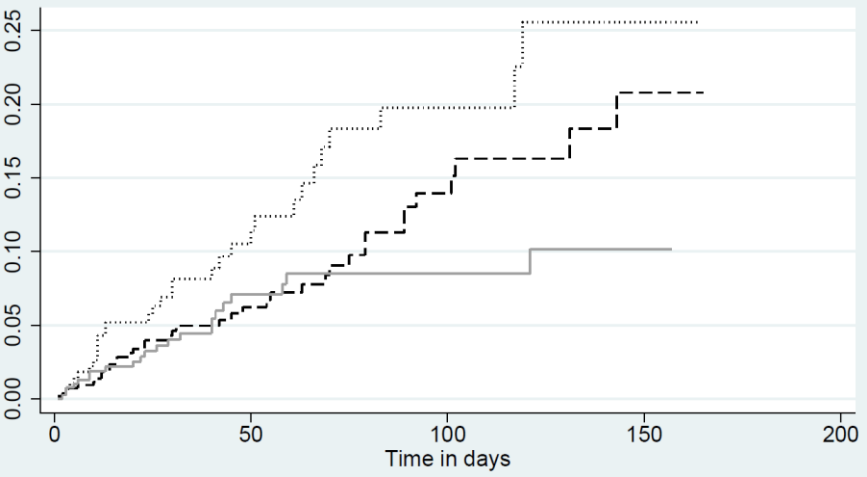
ETEC

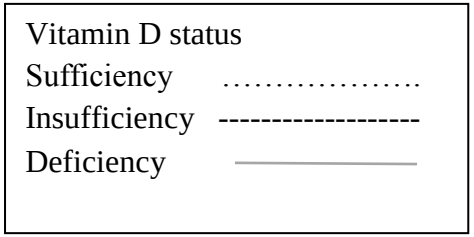


EPEC



EAEC

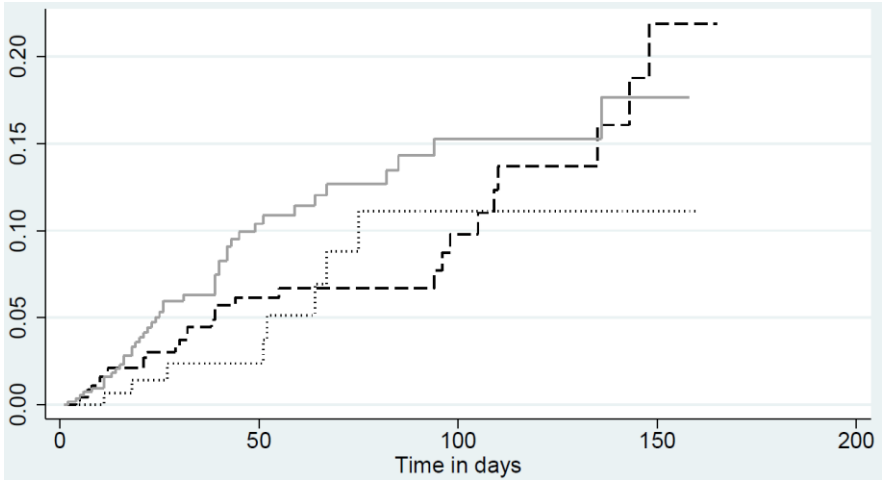




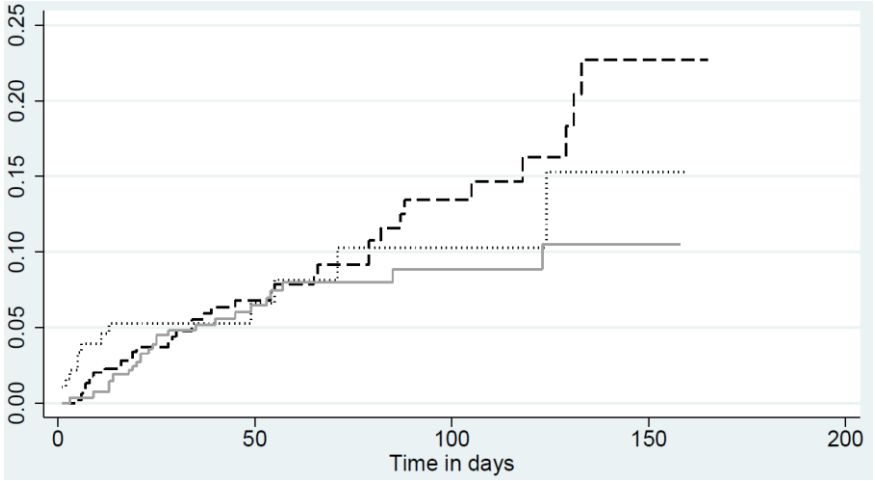
Log-rank test p<0.05 for EAEC diarrhoea

Figure 6.3: Cumulative hazard graph for ETEC, EPEC, EAEC diarrhoeal episodes according to vitamin D status at baseline among normal-weight children aged 6–24 months

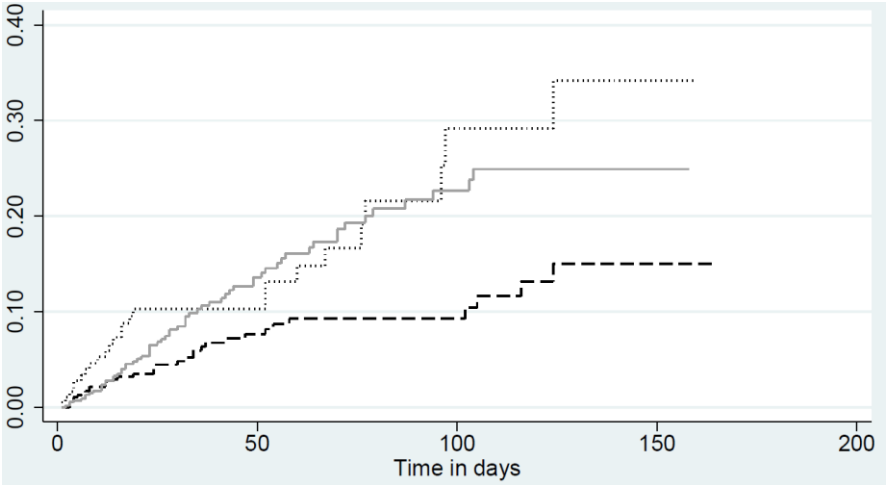
ETEC



EPEC



EAEC



Vitamin D status
Sufficiency .....
Insufficiency ----------
Deficiency ———

Log-rank test p<0.05 for EAEC diarrhoea

Table 6.1: Unadjusted and adjusted hazard ratios from Cox's proportional hazard models for ETEC diarrhoeal episodes in underweight and normal-weight children aged 6–24 months

	Normal-weight children (n = 446)				**Underweight children (n = 466)**			
	Unadjusted	Model 1	Model 2	Model 3	Unadjusted	Model 1	Model 2	Model 3
Age group (in months)								
12–17	0.36(0.20, 0.67)*	0.35 (0.19, 0.66)*	0.36 (0.19, 0.66)*	0.39 (0.21, 0.72)*	0.61 (0.38, 098)*	0.59 (0.37, 0.95)*	0.60 (0.38, 0.95)*	0.61 (0.38, 0.99)*
18–24	0.79 (0.44, 1.41)	0.77 (0.43, 1.39)	0.77 (0.43, 1.37)	0.89 (0.50, 1.60)	0.46 (0.27, 0.81)*	0.45 (0.25, 0.81)*	0.47 (0.26, 0.84)*	0.49 (0.27, 0.90)*
Sex								
Girls	0.88 (0.56, 1.36)	0.87 (0.56, 1.35)	0.87 (0.57, 1.35)	0.86 (0.56, 1.34)	0.81 (0.54, 1.22)	0.78 (0.51, 1.19)	0.78 (0.52, 1.17)	0.78 (0.51, 1.18)
Serum vitamin D at baseline								
Insufficiency (≥50 and <75 nmol/L)	1.35 (0.64, 2.84)	1.42 (0.67, 3.01)	1.52 (0.70, 3.33)	1.31 (0.60, 2.90)	1.00 (0.60, 1.67)	1.09 (0.65, 1.82)	1.06 (0.63, 1.77)	1.17 (0.69, 1.99)
Deficiency (<50 nmol/L)	1.80 (0.89, 3.64)	1.90 (0.94, 3.84)	2.04 (1.00, 4.18)^	1.76 (0.85, 3.64)	0.99 (0.57, 1.73)	1.18 (0.66, 2.12)	1.16 (0.65, 2.05)	1.31 (0.69, 2.50)
Serum retinol status at baseline								
Moderate to severe deficiency (<0.7 µmol/L)	1.21 (0.78, 1.89)	x	1.09 (0.70, 1.69)	1.10 (0.71, 1.69)	1.40 (0.94, 2.10)	x	1.32 (0.86, 2.01)	1.32 (0.86, 2.03)
Serum zinc status at baseline								
Insufficiency (< 9.9 µmol/L)	1.46 (0.88, 2.44)	x	1.51 (0.91, 2.53)	1.40 (0.85, 2.30)	1.20 (0.71, 2.03)	x	0.97 (0.60, 1.59)	0.89 (0.56, 1.44)
Mother's institutional education								
1–5 years	1.47 (0.74, 2.92)	x	x	1.27 (0.65, 2.48)	0.63 (0.40, 1.01)	x	x	0.71 (0.44, 1.16)
>5 years	1.52 (0.76, 3.04)	x	x	1.22 (0.61, 2.43)	0.78 (0.46, 1.31)	x	x	0.91 (0.50, 1.64)
Household wealth index								
Second	1.52 (0.51, 4.55)	x	x	1.38 (0.48, 3.99)	0.79 (0.46, 1.35)	x	x	0.81 (0.47, 1.39)
Middle	1.69 (0.58, 4.90)	x	x	1.83 (0.66, 5.08)	0.69 (0.38, 1.24)	x	x	0.75 (0.40, 1.39)
Fourth	2.50 (0.90, 6.92)	x	x	2.49 (0.93, 6.62)	0.58 (0.30, 1.10)	x	x	0.60 (0.30, 1.19)
Highest	2.47 (0.90, 6.77)	x	x	2.41 (0.91, 6.40)	0.68 (0.33, 1.40)	x	x	0.79 (0.37, 1.71)
Season of vitamin D measurement								
Autumn	0.60 (0.28, 1.29)	x	x	0.58 (0.27, 1.27)	1.13 (0.64, 2.00)	x	x	1.08 (0.61, 1.90)
Winter	0.41 (0.22, 0.76)*	x	x	0.49 (0.27, 0.89)	0.65 (0.37, 1.13)	x	x	0.66 (0.37, 1.19)
Spring	1.18 (0.68, 2.04)	x	x	1.20 (0.71, 2.03)	0.88 (0.49, 1.58)	x	x	0.79 (0.42, 1.47)

Total time at risk, normal-weight children = 62 290 days, underweight children = 64 254 days
^ p = 0.05, *p < 0.05 , **p <0.001
Reference values for variables: sufficient serum vitamin D status, male, 6–11 months, normal to mild deficiency of serum retinol, sufficient serum zinc status, illiterate mother, lowest wealth quintal and summer season for vitamin D measurement
Model 1: Adjusted for age, sex and vitamin D status at enrolment
Model 2: Model 1 + adjusted for serum retinol and zinc status at enrolment
Model 3: Model 2 + adjusted for maternal education, household wealth index and season of vitamin D measurement

Table 6.2: Unadjusted and adjusted hazard ratios from Cox's proportional hazard models for EPEC diarrhoeal episodes in underweight and normal-weight children aged 6–24 months

	Normal-weight children (n = 446)				**Underweight children (n = 466)**			
	Unadjusted	Model 1	Model 2	Model 3	Unadjusted	Model 1	Model 2	Model 3
Age group (in months)								
12–17	0.38 (0.21, 0.69)*	0.39 (0.22, 0.70)*	0.39 (0.21, 0.70)*	0.44 (0.24, 0.80)*	0.53 (0.30, 0.92)*	0.57 (0.32, 1.01)^	0.57 (0.32, 1.01)^	0.55 (0.31, 0.98)*
18–24	0.38 (0.19, 0.80)*	0.37 (0.17, 0.80)*	0.36 (0.17, 0.79)*	0.44 (0.21, 0.94)*	0.44 (0.24, 0.80)*	0.47 (0.25, 0.87)*	0.45 (0.24, 0.84)*	0.44 (0.23, 0.82)*
Sex								
Girls	1.22 (0.77, 1.93)	1.17 (0.75, 1.85)	1.18 (0.75, 1.84)	1.13 (0.73, 1.74)	1.13 (0.72, 1.77)	1.13 (0.72, 1.79)	1.19 (0.76, 1.87)	1.21 (0.78, 1.87)
Serum vitamin D at baseline								
Insufficiency (≥50 and <75 nmol/L)	1.03 (0.54, 1.94)	1.15 (0.62, 2.16)	1.26 (0.63, 2.51)	1.45 (0.77, 2.72)	1.03 (0.59, 1.79)	1.09 (0.62, 1.92)	1.11 (0.63, 1.94)	1.21 (0.67, 2.18)
Deficiency (<50 nmol/L)	0.71 (0.37, 1.37)	0.78 (0.41, 1.48)	0.85 (0.43, 1.68)	0.94 (0.49, 1.79)	0.55 (0.29, 1.07)	0.64 (0.31, 1.29)	0.61 (0.30, 1.24)	0.72 (0.34, 1.52)
Serum retinol status at baseline								
Moderate to severe deficiency (<0.7 µmol/L)	0.93 (0.57, 1.50)	x	0.84 (0.51, 1.38)	0.77 (0.48, 1.24)	0.64 (0.39, 1.05)	x	0.55 (0.33, 0.92)*	0.56 (0.33, 0.92)*
Serum zinc status at baseline								
Insufficiency (< 9.9 µmol/L)	1.41 (0.79, 2.52)	x	1.45 (0.79, 2.66)	1.30 (0.74, 2.28)	1.27 (0.77, 2.11)	x	1.42 (0.83, 2.42)	1.47 (0.86, 2.49)
Mother's institutional education								
1–5 years	1.65 (0.78, 3.50)	x	x	2.03 (0.95, 4.35)	0.78 (0.45, 1.38)	x	x	0.72 (0.42, 1.23)
>5 years	1.17 (0.53, 2.54)	x	x	1.92 (0.85, 4.34)	0.92 (0.50, 1.68)	x	x	0.74 (0.40, 1.36)
Household wealth index								
Second	1.08 (0.49, 2.38)	x	x	0.71 (0.30, 1.67)	0.70 (0.34, 1.44)	x	x	0.71 (0.35, 1.44)
Middle	1.38 (0.62, 3.03)	x	x	1.11 (0.50, 2.45)	0.96 (0.50, 1.81)	x	x	0.93 (0.51, 1.68)
Fourth	1.21 (0.56, 2.63)	x	x	0.80 (0.35, 1.84)	1.00 (0.53, 1.90)	x	x	1.03 (0.53, 1.97)
Highest	0.49 (0.21, 1.18)	x	x	0.36 (0.15, 0.89)*	1.27 (0.60, 2.67)	x	x	1.36 (0.67, 2.77)
Season of vitamin D measurement								
Autumn	0.85 (0.45, 1.63)	x	x	0.93 (0.51, 1.70)	1.00 (0.53, 1.87)	x	x	0.97 (0.51, 1.85)
Winter	0.31 (0.17, 0.59)*	x	x	0.41 (0.22, 0.76)*	0.71 (0.39, 1.30)	x	x	0.88 (0.47, 1.65)
Spring	0.72 (0.39, 1.34)	x	x	0.91 (0.51, 1.63)	0.45 (0.22, 0.92)	x	x	0.53 (0.26, 1.09)

Total time at risk, normal-weight children = 62 290 days, underweight children = 64 254 days

^ p = 0.05, *p < 0.05 , **p <0.001

Reference values for variables: sufficient serum vitamin D status, male, 6–11 months, normal to mild deficiency of serum retinol, sufficient serum zinc status, illiterate mother, lowest wealth quintal and summer season for vitamin D measurement

Model 1: Adjusted for age, sex and vitamin D status at enrolment

Model 2: Model 1 + adjusted for serum retinol and zinc status at enrolment

Model 3: Model 2 + adjusted for maternal education, household wealth index and season of vitamin D measurement

Table 6.3: Unadjusted and adjusted hazard ratios from Cox's proportional hazard models for EAEC diarrhoeal episodes in underweight and normal-weight children aged 6–24 months

	Normal-weight children (n=446)				**Underweight children** (n=466)			
	Unadjusted	Model 1	Model 2	Model 3	Unadjusted	Model 1	Model 2	Model 3
Age group (in months)								
12–17	0.32 (0.19, 0.55)**	0.33 (0.19, 0.56)**	0.33 (0.19, 0.56)**	0.39 (0.22, 0.69)**	0.57 (0.35, 0.95)*	0.60 (0.36, 1.00)^	0.61 (0.36, 1.00)^	0.62 (0.37, 1.05)
18–24	0.35 (0.16, 0.76)*	0.38 (0.18, 0.83)*	0.37 (0.18, 0.76)*	0.41 (0.19, 0.89)*	0.19 (0.09, 0.37)**	0.19 (0.09, 0.40)**	0.19 (0.09, 0.39)**	0.21 (0.10, 0.44)**
Sex								
Girls	1.18 (0.80, 1.75)	1.15 (0.80, 1.67)	1.14 (0.79, 1.64)	1.11 (0.76, 1.64)	0.67 (0.42, 1.06)	0.67 (0.43, 1.04)	0.68 (0.44, 1.06)	0.70 (0.48, 1.09)
Serum vitamin D at baseline								
Insufficiency (≥50 and <75 nmol/L)	0.47 (0.27, 0.82)*	0.53 (0.31, 0.90)*	0.59 (0.34, 0.99)*	0.56 (0.33, 0.95)*	0.65 (0.39, 1.08)	0.72 (0.43, 1.21)	0.73 (0.44, 1.21)	0.82 (0.48, 1.37)
Deficiency (<50 nmol/L)	0.83 (0.51, 1.34)	0.90 (0.58, 1.42)	0.98 (0.61, 1.57)	0.96 (0.61, 1.52)	0.47(0.24, 0.90)*	0.62 (0.32, 1.19)	0.62 (0.31, 1.20)	0.65 (0.32, 1.32)
Serum retinol status at baseline								
Moderate to severe deficiency (<0.7 µmol/L)	0.67 (0.44, 1.04)		0.64 (0.42, 0.99)*	0.62 (0.41, 0.95)*	0.87 (0.56, 1.39)	x	0.79 (0.50, 1.22)	0.73 (0.47, 1.13)
Serum zinc status at baseline								
Insufficiency (< 9.9 µmol/L)	1.26 (0.73, 2.16)		1.30 (0.74, 2.27)	1.37 (0.82, 2.32)	1.22 (0.74, 2.04)	x	1.20 (0.72, 2.01)	1.14 (0.69, 1.87)
Mother's institutional education								
1–5 years	1.43 (0.78, 2.63)			1.46 (0.79, 2.71)	0.63 (0.38, 1.06)	x	x	0.68 (0.41, 1.12)
>5 years	1.40 (0.76, 2.60)			1.56 (0.82, 2.95)	0.53 (0.30, 0.94)*	x	x	0.55 (0.30, 1.01)^
Household wealth index								
Second	1.50 (0.73, 3.08)			1.09 (0.66, 2.92)	1.14 (0.63, 2.05)	x	x	1.20 (0.69, 2.08)
Middle	1.09 (0.50, 2.37)			0.82 (0.40, 1.84)	1.04 (0.56, 1.96)	x	x	1.15 (0.63, 2.07)
Fourth	1.18 (0.58, 2.42)			0.89 (0.43, 1.96)	0.77 (0.35, 1.72)	x	x	0.88 (0.40, 1.93)
Highest	0.88 (0.42, 1.85)			0.74 (0.44, 2.15)	0.82 (0.39, 1.72)	x	x	1.17 (0.53, 2.56)
Season of vitamin D measurement								
Autumn	0.73 (0.43, 1.24)			0.71 (0.42, 1.34)	1.63 (0.87, 3.05)	x	x	1.47 (0.80, 2.72)
Winter	0.37 (0.22, 0.62)**			0.46 (0.27, 0.78)*	0.79 (0.41, 1.53)	x	x	0.96 (0.50, 1.88)
Spring	0.54(0.33, 0.88)*			0.57 (0.35, 0.92)*	1.39 (0.74, 2.61)	x	x	1.58 (0.83, 3.04

Total time at risk, normal-weight children = 62 290 days, underweight children = 64 254 days
^ p = 0.05, *p < 0.05 , **p <0.001
Reference values for variables: sufficient serum vitamin D status, male, 6–11 months, normal to mild deficiency of serum retinol, sufficient serum zinc status, illiterate mother, lowest wealth quintal and summer season for vitamin D measurement
Model 1: Adjusted for age, sex and vitamin D status at enrolment
Model 2: Model 1 + adjusted for serum retinol and zinc status at enrolment
Model 3: Model 2 + adjusted for maternal education, household wealth index and season of vitamin D measurement

Discussion

This study presents results of a comprehensive longitudinal assessment of the cumulative incidence of diarrhoeal episodes by ETEC, EPEC and EAEC among children aged under two years. The study design yielded high quality data due to twice-weekly follow-up of two separate cohorts of children with different nutritional statuses, and a low rate of attrition. Furthermore, this study is different in the way it documented any type (mild to severe) of diarrhoeal events at community level through twice-weekly home visits by trained fieldworkers which also enhanced disease identification [41]. The resulting overall incidences of pathogen-specific diarrhoea are also concordant with other studies conducted in resource-limited settings [2, 5].

We hypothesised that improved vitamin D status can result in a protective effect for early childhood diarrhoeal episodes by ETEC, EPEC and EAEC. This was based on previous studies which presented frameworks for understanding the mechanisms through which vitamin D plays an important role in activation of innate and adaptive immunity for infectious diseases including diarrhoea [14, 15, 20]. Laboratory studies and animal models have shown that vitamin D regulated anti-microbial peptides have bactericidal effects on *Escherichia coli* [22, 24]. In the present study, we found that normal-weight children with vitamin D insufficiency and severe to moderate retinol deficiency had a reduced risk of EAEC diarrhoea. On the other hand, underweight children with severe to moderate serum retinol deficiency showed a reduction in the cumulative incidence of EPEC diarrhoea compared to children with mild deficiency to normal status.

Retinol and vitamin D have received particular attention in recent years due to these vitamins having been shown to have an important effect on the immune response. The metabolites of vitamin D, (i.e. $1,25(OH)_2$ vitamin D_3) and serum retinol (i.e. retinoic acid) both use retinoid x receptors (RXRs) to affect their immunomodulatory activity and can potentially antagonise each other's effects due to using a similar pathway [42]. This is supported by a recent study which showed that vitamin A supplementation showed an increase in duration of EPEC infection among children aged under two years [43]. The authors of that study also argued that with vitamin A supplementation the pro-inflammatory responses may play different roles in different pathogen-specific infections. We do not have any explanation for our finding of the protective effects of vitamin D insufficiency and severe to moderate serum retinol deficiency on pathogen-specific diarrhoea among the two groups of children who differed by nutritional status. Among underweight children vitamin D might be more involved in the immune response and maintenance and less so for bone growth. In normal-weight children there may be greater use of vitamin D in bone metabolism and skeletal growth. These differences may have an effect on the immune response to the different pathogens. The

immunomodulatory function for both vitamin A and vitamin D in infectious diarrhoea needs to be explored further among children who differ in nutritional status including EPEC and EAEC diarrhoea.

We also found that, after adjusting for age, sex and concurrent micronutrient status—children with vitamin D deficiency were associated with an increased risk of ETEC diarrhoea in normal-weight children. ETEC causes diarrhoea through enterotoxin production while EPEC causes diarrhoea through the pathogenesis induced by its intimate adherence and modification of enterocyte membranes [2]. Mechanisms of diarrhoea in EAEC infection result from its ability to adhere to intestinal cells, as well as producing enterotoxins and cytotoxins [44, 45]. Thus vitamin D may have a protective effect against the organisms those produce only enterotoxins. This is consistent with the effect of vitamin D on the adaptive T cell response which includes a shift from a Th1 to Th2 response, inhibition of Th17 cell development and facilitation of T regulatory cell [46-48] . All of these effects lead to down regulation of an inflammatory response and up-regulation of a regulatory response. These arms of the adaptive immune responses play different roles in pathogen-specific outcomes leading to infection resolution and reduced severity for some enteric pathogens [49]. However, there may be no beneficial effect where an inflammatory response may be necessary for infection resolution. These different effects may underlie the distinct associations between vitamin D and pathogen-specific outcomes as well as overall diarrhoea and acute respiratory infection.

Our results also demonstrate that the risk of ETEC, EPEC and EAEC diarrhoea was significantly less among older children in all models similar to what has been reported in previous studies [2, 5]. A study carried out at the same site reported the highest incidence of ETEC diarrhoea during the second six months of a child's life with the incidence remaining unchanged during the second year of life [5]. This pattern may result from the development of mucosal immunity due to the exposure of a relative high infectious dose of pathogen during and following the weaning period [50]. The reasons for lower risk of EPEC in older children is not known; it is assumed that loss of a specific receptor with age may contribute to relative resistance against EPEC infection [2]. The development of a protective mucosal immune response due to exposure to the pathogen may also play a significant role. In a community-based study in India, researchers found higher prevalence of EAEC diarrhoea among younger children which also supports our finding [9]. However, it is not known why EAEC is prevalent in young children.

In the current study we found that normal-weight children had a reduced risk of EPEC diarrhoea, and underweight children had a reduced risk of EAEC diarrhoea if they came from households with the highest socio-economic status and had well-educated mothers. The household wealth index and maternal education were not found to play a significant role in any of the other models tested. These

findings can be partly explained by different patterns of transmission and distinct mechanisms of pathogenesis of each of the pathogens.

The results of this study should be interpreted in light of its limitations. First, despite our intense follow-up design we only collected 70% of diarrhoeal samples from the study participants; this was due to the shorter duration of diarrhoeal episodes of only one day or the absence of the child from the study site. Second, as per the recommendation of the National Nutrition Program of Bangladesh, a multiple micronutrient powder supplement containing vitamin-A and zinc but no vitamin D was also made available to the study participants , and as this was not included in the analysis, it could have confounded the findings of our study. Third, co-infections with other pathogens have been shown to play an important role in the ecology of diarrhoea; unfortunately, we have not been able to account for this in this analysis because of an insufficient number of co-infections.

In conclusion, the results of our study demonstrate that insufficient vitamin D status is associated with reduced EAEC diarrhoeal risk among normal-weight children. It also shows that moderate to severe retinol deficiency is associated with a reduced risk of EPEC and EAEC diarrhoea in underweight and normal-weight children respectively. It is known that the pathophysiology of ETEC, EPEC and EAEC diarrhoea varies and that innate and adaptive immune responses modified by micronutrient status, thus role of vitamin D and vitamin A status in incidence of pathogen-specific diarrhoea is distinct and can depend on the child's nutritional status. There is a need for further research on how vitamin D and vitamin A modifies the immune response pathways and how these results in protection from ETEC, EPEC and EAEC diarrhoea in children aged under two years.

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CHAPTER 7 ASSOCIATION BETWEEN VITAMIN D STATUS AND ACUTE RESPIRATORY INFECTIONS

7.1 Context

The results of RCTs with vitamin D supplementation in prevention and treatment of pneumonia among children are inconsistent [1, 2]. The heterogeneous results of studies and issues raised by researchers such as the dose of supplementation, the confounding effect of high rates of undernutrition, and the presence of other micronutrient deficiency among the participants have highlighted the need for further studies to evaluate the relationship between vitamin D status and incidence of ARI more carefully [3].

In this chapter, I evaluate the association between micronutrient status, including vitamin D, and the incidence of both mild form (common cough and cold or URI) and severe form (ALRI or pneumonia) of ARI among underweight and normal-weight children aged 6–24 months old. Using data on respiratory morbidity events collected on a daily basis through biweekly active surveillance, I demonstrate that insufficient and deficient vitamin D status reduced the risk of URI among underweight children. I also demonstrate the beneficial role of retinol at reducing the risk of ALRI in underweight children. The results also showed that the beneficial role of serum zinc in URI was only evident among normal-weight children.

The results of the study were significant in many ways. First, the analyses provided a new way to look at the association of multiple micronutrient status and incidence of ARI according to a child's nutritional status. Second, vitamin D status did not confound the beneficial effect of retinol and zinc even in a resource-constrained setting with a high rate of both URI and ALRI. Third, the findings show that among children aged under two years living in a resource-constrained slum area, multiple micronutrient supplementation programs with zinc and vitamin A contribute to reducing the incidence of two major childhood infectious diseases—diarrhoea and ARI, thus providing support for continuing the programs.

This chapter forms a manuscript which has been submitted to *Epidemiology and Infection.*

7.2 Association between serum vitamin D, retinol and zinc status, and acute respiratory infections in underweight and normal weight children aged 6–24 months living in an urban slum in Bangladesh

A. M. S. Ahmed, T. Ahmed, R. J. Soares Magalhaes, K. Z. Long, Md. A. Alam, Md. I. Hossain, M. M. Islam, M. Mahfuz, D. Mondal, R. Haque, A. Al Mamun

Summary

We conducted a longitudinal assessment among 466 underweight and 446 normal-weight children aged 6–24 months living in the urban slum of Dhaka, Bangladesh to determine the association between vitamin D and other micronutrient status with upper respiratory tract infection (URI) and acute lower respiratory infection (ALRI). Incidence rate ratios of URI and ALRI were estimated using multivariable generalized estimating equations. Our results indicate that underweight children with insufficient and deficient vitamin D status were associated with 20% and 23%–25% reduced risk of URI respectively compared with children with sufficient status. Among underweight children, those with serum retinol moderate to severe deficiency were at 1.8 (95% CI:1.4–2.4) times higher risk of ALRI than those with mild retinol deficiency to normal status. Among normal-weight children there were no significant differences between vitamin D status and the incidence of URI and ALRI. However, normal-weight children with zinc insufficiency and those that were moderate to severe serum retinol deficient had 1.2 (1.0–1.5) times higher risk of URI and 1.9 (1.4–2.6) times higher risk of ALRI respectively. Thus, our results should encourage efforts to increase the intake of retinol-enriched food or supplementation in this population. However, the role of vitamin D on the incidence of childhood respiratory tract infection still needs further research to draw any conclusion.

Introduction

Globally, acute respiratory infections (ARI) are the leading cause of childhood morbidity and mortality [4]. ARI include the common cough and cold or URI, the mild form of the disease and the severe form—ALRI. Children in developing countries experience three to eight episodes of URI annually [5]. In 2011, ALRI were estimated to be responsible for 1.3 million deaths globally among children aged under five years [4]. The most recent Bangladesh Demographic and Health Survey reported that 5.8% of children under five years old experienced ARI [6]. Two studies from rural and urban Bangladesh reported estimated 0.2–0.5 episodes of ALRI per child per year among children under five years old [7, 8]. ARI are the most frequent cause of health consultations and hospitalisations for children under five years old in Bangladesh [9].

In the last few decades, researchers have been evaluating the prophylactic and therapeutic use of different micronutrients to reduce infectious disease, including ARI among children [10]. The first studies investigating the role of vitamin D in pneumonia reported an association between nutritional rickets and pneumonia [11, 12]. Case-control studies conducted later found a significantly lower mean concentration of vitamin D in children with pneumonia than in healthy children [13, 14]. Longitudinal studies reported reduced risk ALRI among infants, if the mother had higher serum vitamin D concentrations during pregnancy or in cord blood [15, 16]. A study from Saudi Arabia [17] reported associations between low vitamin D levels in cord blood with increased risk of developing ALRI during the first two years of life. A well-designed randomized controlled trial (RCT) done in Kabul, Afghanistan [1] reported no beneficial effect of vitamin D supplementation on the incidence of pneumonia in children aged under two years. However, it has been suggested that the lack of any effect of vitamin D reported in previous studies may be due to the supplementation dose that could have impaired the modulatory function of vitamin D, the confounding effect of high rates of undernutrition, and the presence of other micronutrient deficiency among study participants [3]. These study limitations warrant the development of longitudinal studies to more systematically evaluate the relationship between serum vitamin D, retinol and zinc status with incidence of ARI.

Our analysis of longitudinal data from two parallel cohorts of children (underweight and normal-weight), aged 6–24 months from an urban slum of Bangladesh, investigates the association between serum concentrations of vitamin D, retinol and zinc with ARI (including URI and ALRI), adjusted for socio-economic factors.

Methods

Study design, site and participants

We used data from an interventional case-control study in an urban slum of the *Mirpur* field site in Dhaka, Bangladesh, which was one of the field sites of the Mal-ED research project [18]. The study was approved by the Research Review Committee and the Ethical Review Committee of icddr,b. Eligible children were enrolled in the study after their parent/guardian signed the consent form.

Descriptions of the Mal-ED study design, site, participants and interventional packages have been reported in detail elsewhere [19]. In brief, participants (children aged 6–24 months) were screened for eligibility using weight measurement during household surveys. Severe to moderately underweight children (weight-for-age Z score, WAZ < –2.00 SD) were considered as cases and controls were well-nourished/normal-weight children (WAZ ≥ –1.00 SD) matched for area of residence and sex. Overall, 500 cases and 480 controls were enrolled during November 2009

through to December 2012. Two different intervention packages were given for five months to enrolled children based on their nutritional status (underweight or normal-weight). Underweight children received sachets of supplementary food while both groups were given micronutrient supplements (Figure 7.1). Both interventions did not contain vitamin D supplementation.

Data collection and morbidity surveillance

A longitudinal study design was used to follow both normal-weight and underweight children for a period of 5 months. At the time of the enrolment, information on socio-demographic factors and household economic conditions were collected using a standard Demographic and Health Survey (DHS) household questionnaire. Trained health fieldworkers collected information regarding common infectious disease illnesses including sign and symptoms of ARI through home visits every third or fourth day for five months, using previously validated standardised, morbidity questionnaires. At the time of home visits, caregivers were asked about signs and symptoms of any illness experienced by the child since the last visit. The fieldworkers also measured the respiratory rate of any child suffering from cough with fever or any sign of respiratory distress. Children with signs of cough and respiratory distress, such as difficulty or rapid breathing and chest in-drawing, were referred to the study physicians for evaluation of ALRI and treatment. If any study participants were absent from the study site for more than seven days, during the next available visit fieldworkers would then collect morbidity information for the last seven days only. Any children absent from the household or study site for more than 60 consecutive days were dropped from the study.

Assessment of micronutrient status

The determination of serum vitamin D and other micronutrients in each child was conducted at enrolment using a 5 ml blood sample collected into trace element-free tubes. Processing of the collected samples and micronutrient assays were performed at the nutritional biochemistry laboratory of icddr,b. Serum vitamin D (25-Hydroxy vitamin D) was measured by using IDS 25-hydroxy vitamin D EIA kit [20] (Source: IDS Ltd, 10 Didcot Way, Boldon Business Park, Boldon, UK). Serum retinol was measured with the HPLC method described elsewhere [21]. Serum/plasma zinc concentration was measured with air-acetylene flame atomic absorption spectrophotometer at 213.9 nm following dilution of the sample twelve times with deionised water. The results for serum retinol and zinc status were adjusted for subclinical infections with C-reactive protein and α1-acid glycoprotein.

Measurements

We used standard case definitions to identify URIs and ALRI from collected information. For the purposes of this study, a URI was defined as the 'presence of cough in absence of World Health Organization defined clinical signs of pneumonia and severe pneumonia' while, ALRI was defined as 'presence of cough and/or respiratory difficulty plus rapid respiratory rate at the time of household visits (cut-off for age: ≥50 breaths per minute in children aged 2 to 11 months and ≥40 breaths per minute in children aged 12 months to two years)' [22]. If any child was diagnosed with ALRI during episodes of URI, then all days reported with cough were considered as an ALRI episode.

Demographic individual-level variables included age group (6–11, 12-17 and 18-24 months) and maternal education (illiterate, 1–5 years and >5 years of institutional education). A household wealth index was constructed using socio-economic variables created using information from the database which was analysed using principle component analysis as described in the DHS [23]. Four seasons—summer (May to July), autumn (August to October), winter (November to January) and spring (February to April)—were created using the date of blood sample collection. Children's vitamin D status were then categorised into deficient (<50 nmol/L), insufficient (≥50 and <75 nmol/L) and sufficient (≥75 nmol/L) as per recommendation of The US Endocrine Society guideline [24]. Serum retinol status was categorised as moderate to severe deficiency (<0.7 μmol/L) and mild deficiency to normal status (≥0.7 μmol/L) [25]. Serum zinc insufficiency was defined as serum zinc <9.9 μmol/L while sufficient status was defined as ≥9.9 μmol/L [26].

Complete data was available from 466 underweight and 446 normal-weight children for the analysis (Figure 7.1). Study physicians successfully collected blood samples from all the children at the time of enrolment. A total of 64 assay results for serum vitamin D, retinol and zinc among underweight and normal-weight children were not completed due to sample haemolysis, coagulation or not enough serum available from collected blood samples to perform the assays. Additionally, parent/caregivers of six participants (four in the underweight group and two in the normal- weight group) did not agree to provide morbidity surveillance data after enrolment in the study so were dropped from the study.

Statistical analyses

The primary outcomes in all analyses were the rates of URI and ALRI among children during the five months of the active surveillance period. Rates of URI and ALRI were calculated as the number of days with URI or ALRI by the total number of days that a child was observed. Rates of URI and ALRI were then annualised as rate per child per year. In all the analyses, the main exposure of interest was serum vitamin D status at the time of the enrolment. The rates per child

per year of URI and ALRI were estimated as per baseline child, maternal and socio-economic characteristic as well as serum retinol and zinc status including serum vitamin D status. Generalized estimating equation (GEE) models with a Poisson distribution were built to estimate incidence rate ratios (IRRs) and 95% CIs for URI and ALRI incidence; an exchangeable correlation structure was specified to account for within-child and within-family correlations of outcome measures. For the multivariable GEE modelling, potential confounders included measures of socio-demographic status and micronutrient status indicator, which were physiologically deemed important factors or reported risk indicators in the published literature [8, 27]. A probability of less than 0.05 was considered for a statistically significant association for all the analyses. Adjusted IRRs of URI and ALRI were estimated from multivariable models. Model 1 was adjusted association between rate of URI or ALRI with vitamin D status, age group, and sex of children. Model 2 comprised all variables in Model 1 and was additionally adjusted for serum retinol and zinc status of the children. Model 3 comprised all variables in Model 2 and was additionally adjusted for maternal education and household wealth index. Model 4 comprised all variables in Model 3 and was additionally adjusted for season of serum D measurement. All analyses were conducted in STATA software (version13.0; StataCorp, College Station, TX).

Results

The serum concentration of vitamin D in underweight children was 60.4 ± 23.9 nmol/L (mean ± SD); 23.4% were classified as vitamin D sufficient while 41.8% and 34.7% were insufficient and deficient respectively; mean concentrations of serum retinol and zinc were 21.8 ± 7.6 µmol/L and 0.75 ± 0.14 µg/dl respectively, with 41% and 19.7% of underweight children having moderate to severe serum retinol deficiency and serum zinc insufficiency respectively. Similarly, serum vitamin D concentration (mean ± SD) was 54.2 ± 20.8 nmol/L in normal-weight children; 45.5% of normal-weight children were vitamin D deficient, and 39.5% were insufficient. Mean concentrations of serum retinol and zinc (mean ± SD) were 23.6 ± 7.8 µmol/L and 0.76 ± 0.13 µg/dl in normal-weight children; 34.1% of normal-weight children had moderate to severe serum retinol deficiency, while 16.1% of normal-weight children had serum zinc insufficiency (results not shown).

Association of URI and ALRI with vitamin D status in underweight children

Underweight children contributed 62,988 total days of observation and experienced 69.1 days per child-year of URI (Table 7.1). Among underweight children, those who were vitamin D deficient ($p < 0.001$) and insufficient ($p < 0.05$) experienced significantly fewer days of URI than those who were vitamin D sufficient. Younger-aged children (6–11 months) experienced more days of URI than older-aged children (12–17 months, $p < 0.05$; and 18–24 months, $p < 0.001$).

Underweight children experienced 10.8 days per child-year of ALRI. There was no significant association between vitamin D status and ALRI in underweight children. Children aged 12–17 months ($p<0.001$) had significantly higher rates of ALRI than children in the younger group (6–11 months). Underweight children whose mothers had 1–5 years and >5 years of institutional education were found to have significantly lower ALRI rates than children of illiterate mothers. Household wealth index was inversely associated with the number of days with ALRI in underweight children. Moderate to severe serum retinol-deficient children ($p<0.001$) had experienced more days of ALRI than children with mild to normal retinol-status (Table 7.1).

Among underweight children, there was 20% lower risk of URI across all the models among vitamin D insufficient children when compared with vitamin D sufficient children (Table 7.2); similarly, there was 25-27% lower risk of URI among vitamin D deficient children when compared with vitamin D sufficient children. Older-aged children (18–24 month) had 33% lower risk of URI than younger-aged children after adjusting for vitamin D status, serum retinol and zinc status, age group, sex, maternal education, household wealth index, and season of vitamin D measurement.

No significant differences were found in ALRI risk among underweight children who differed in vitamin D status (Table 7.2). After adjustment of confounders in the final model for underweight children, older -aged children (12–17 months) had 1.8 times higher risk of ALRI than younger-aged children (6-11 month). Similarly, those with severe to moderate serum retinol deficiency had 1.8 times higher risk of ALRI compared with those with mild to normal retinol status. Underweight children of educated mothers (1–5 years and >5 years of institutional education) had 43% to 38% lower risk of ALRI than underweight children whose mothers were illiterate. Also underweight children from the low-middle and middle stratum of the household wealth index had 52% lower risk of ALRI compared with underweight children from the lowest stratum.

Association of URI and ALRI with vitamin D status in normal- weight children

Normal-weight children contributed 62,133 total days of observation and had 78.4 days per child-year of URI (Table 7.3). Older-aged children (12–17 months and 18–24 months) had fewer days of URI than younger-aged children (6–11 months) ($p<0.05$). Children from the highest stratum of the wealth index had significantly fewer days with URI than children from the lowest stratum. Among normal-weight children, those who were serum zinc insufficient had a greater number of days of URI than those who were zinc sufficient ($p = 0.05$).

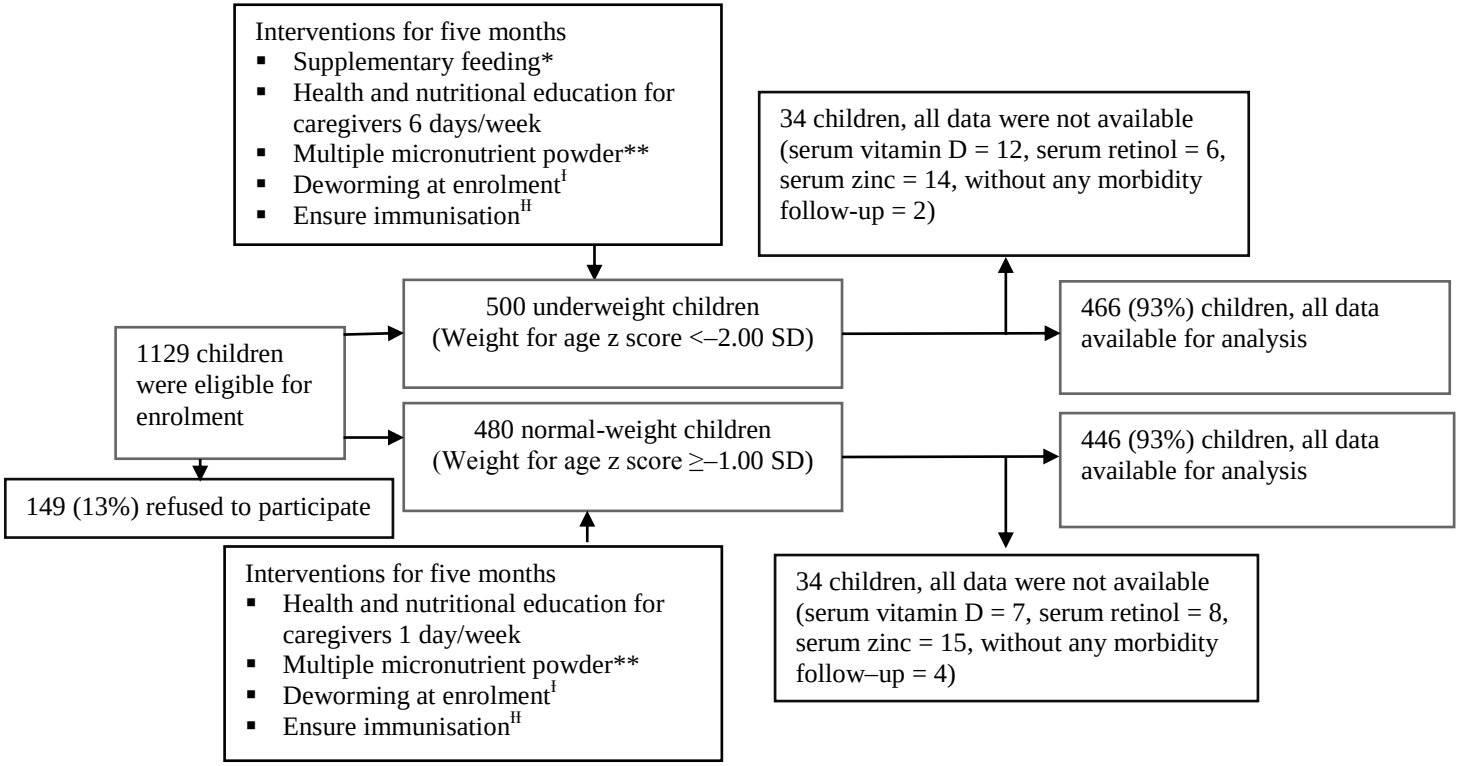
Normal-weight children had 8.3 days per child-year of ALRI. Normal-weight girls had fewer days with ALRI than boys ($p <0.001$). Among normal-weight children, measurement of vitamin D status during spring was associated with reduced ALRI compared with summer. Normal-weight children

from middle and high-middle stratums of the household wealth index had fewer days with ALRI than normal-weight children from the lowest stratum of the index. Also among normal-weight children, those with moderate to severe serum retinol deficiency had more days with ALRI than those with mild to normal serum retinol status ($p<0.001$) (Table 7.3).

Vitamin D status in normal-weight children was not associated with URI across all the models. There were 20% and 31% reduced risk of URI among children aged 12-17 months and 18-24 months respectively compared with younger aged children (6-11 months). Normal-weight children with insufficient zinc status had 1.2 times greater risk of URI compared with normal-weight children with sufficient serum zinc status. There was 26% lower risk of URI among normal-weight children from the highest wealth stratum compared with normal-weight children from the lowest wealth stratum. Among normal-weight children, measurement of vitamin D status during autumn indicated a 1.3 greater risk of URI compared with measurement of vitamin D status during summer (Table 7.4).

No significant differences in the IRRs of ALRI were found between normal-weight children who differed in vitamin D status in any of the models. After adjustment of confounders in the final model, girls had 43% lower risk of ALRI than boys. There were 1.9 times greater risk of ALRI among normal-weight children with severe to moderate serum retinol deficiency compared with normal-weight children with mild deficiency and normal serum retinol status. Normal-weight children from the low-middle and high-middle stratum of the household wealth index had 44% and 50% lower risk of ALRI respectively than normal-weight children from the lowest stratum. Measurement of vitamin D status during spring had 44% lower risk of ALRI among the normal-weight children compared with measurement of vitamin D status during summer (Table 7.4).

Figure 7.1: Study profile



* Each sachet contains roasted rice powder 20 g, roasted lentil powder 10 g, molasses 5 g and vegetable oil 5 ml providing approximate 150 kcals. Severely underweight (WAZ<–3.00) and moderately underweight children were receiving three packets and two packets respectively, 6 days/week for five month or until graduation by achieving WAZ –1.00

** Each sachet contains: 12.5 mg elemental iron, 5 mg elemental zinc, 300 µg vitamin A, 150 µg folic acid, and 50 mg of vitamin C, given for 2 months from enrolment in first 392 children enrolled, then given for 4 months in remainder of the children.

[I]200 mg albendazole syrup was given orally as a single dose to all children more than one year old. In children under one year old, 10 mg/kg pyrantelpamoate was given as a single dose

[H]Immunisation covers BCG, DPT, OPV, measles, hepatitis B, and Hib vaccines

Table 7.1: Upper respiratory tract infection (URI) and acute lower respiratory tract infection (ALRI) incidence according to socio-demographic characteristics and micronutrient status (serum vitamin D, retinol and zinc) in underweight children aged 6–24 months[1]

Underweight children (n = 466)						
Child characteristics	n	Days of follow-up	Days with URI	URI rate per child-year	Days with ALRI	ALRI rate per child-year
All children	466	62,988	11,921	69.13	1,865	10.81
Age group (in months)						
6–11	174	23,973	5,530	84.25	490	7.47
12–17	148	19,903	3,664	67.24*	844	15.49**
18–24	144	19,112	2,727	52.12**	513	9.80
Sex						
Boys	234	31,446	6,108	70.95	909	10.56
Girls	232	31,542	5,813	67.31	956	11.07
Serum vitamin D						
Sufficiency	109	14,320	3,380	86.21	390	9.95
Insufficiency	195	27,162	4,966	66.78*	857	11.52
Deficiency	162	21,506	3,575	60.72**	614	10.43
Serum retinol						
Normal or mild deficiency	277	38,295	7,099	67.71	901	8.59
Moderate or severe deficiency	189	24,693	4,822	71.33	964	14.26**
Serum zinc						
Sufficiency ≥9.9 µmol/L	373	50,501	9,425	68.17	1,541	11.15
Insufficiency < 9.9 µmol/L	93	12,487	2,496	73.01	324	9.48
Mother's institutional education						
Illiterate	116	15,556	3,178	74.62	748	17.56
1-5 years of education	215	29,402	5,480	68.08	691	8.58**
>5 years of education	135	18,030	3,263	66.10	426	8.63**
Household wealth index						
Lowest	139	18,210	3,515	70.50	803	16.11
Lower-middle	107	14,243	2,846	72.98	316	8.10**
Middle	91	12,702	2,407	69.21	233	6.70**
Higher-middle	81	10,944	1,947	64.98	326	10.88*
Highest	48	6,889	1,206	63.94	187	9.91*
Season of vitamin D measurement						
Summer	85	11,644	2,320	72.77	268	8.41
Autumn	93	12,534	2,358	68.71	316	9.21
Winter	173	22,894	4,227	67.44	905	14.44*
Spring	115	15,916	3,016	69.21	376	8.63

[1]For ordinal predictors, the categories of the predictor were introduced as continuous into the generalized estimate equation model with a Poisson distribution and p-value was a test for linear trend. For dichotomous predictor, p-value is from Wald test with robust standard errors in generalized estimate equation model
*p<0.05, **p<0.001

Table 7.2: Incidence rate ratios of upper respiratory tract infection (URI) and acute lower respiratory tract infection (ALRI) in underweight children aged 6–24 months

	Underweight children (n=446)							
	Upper respiratory tract infection				Acute lower respiratory tract infection			
	Model 1	Model 2	Model 3	Model 4	Model 1	Model 2	Model 3	Model 4
Age group (in months) (Ref: 6–11)								
12–17	0.88 (0.75, 1.03)	0.89 (0.76, 1.04)	0.88 (0.75, 1.03)	0.87 (0.74, 1.02)	2.07 (1.45, 2.96)**	2.12 (1.50, 2.99)**	1.92 (1.38, 2.66)**	1.77 (1.27, 2.48)**
18–24	0.67 (0.56, 0.79)**	0.68 (0.57, 0.81)**	0.68 (0.57, 0.81)**	0.67 (0.56, 0.80)**	1.15 (0.76, 1.73)	1.25 (0.84, 1.86)	1.34 (0.92, 1.96)	1.25 (0.86, 1.84)
Sex (Ref: Boys)								
Girls	0.93 (0.82, 1.07)	0.94 (0.82, 1.07)	0.94 (0.82, 1.07)	0.94 (0.82, 1.07)	1.08 (0.81, 1.46)	1.07 (0.81, 1.42)	1.08 (0.83, 1.41)	1.09 (0.84, 1.43)
Serum vitamin D at baseline (Ref: Sufficiency)								
Insufficiency	0.80 (0.68, 0.94)*	0.80 (0.68, 0.94)*	0.80 (0.68, 0.95)*	0.80 (0.68, 0.94)*	1.00 (0.68, 1.47)	0.95 (0.66, 1.36)	1.02 (0.72, 1.44)	1.01 (0.71, 1.44)
Deficiency	0.75 (0.63, 0.89)**	0.73 (0.61, 0.88)**	0.74 (0.62, 0.89)**	0.73 (0.61, 0.89)**	0.95 (0.63, 1.42)	0.88 (0.60, 1.30)	0.88 (0.61, 1.27)	0.95 (0.65, 1.40)
Serum retinol status at baseline (Ref: Mild deficiency to normal)								
Moderate to severe deficiency		1.05 (0.91, 1.20)	1.03 (0.90, 1.19)	1.03 (0.90, 1.18)		1.95 (1.46, 2.60)**	1.86 (1.41, 2.45)**	1.84 (1.40, 2.42)**
Serum zinc status at baseline (Ref: Sufficiency)								
Insufficiency		1.08 (0.91, 1.28)	1.06 (0.90, 1.26)	1.06 (0.90, 1.26)		0.94 (0.65, 1.36)	0.83 (0.59, 1.18)	0.86 (0.60, 1.22)
Mother's institutional education (Ref: Illiterate)								
1-5 years of education			0.91 (0.77, 1.08)	0.91 (0.77, 1.08)			0.57 (0.41, 0.78)**	0.57 (0.41, 0.78)**
>5 years of education			0.93 (0.76, 1.13)	0.93 (0.77, 1.13)			0.61 (0.41, 0.89)*	0.62 (0.42, 0.90)*
Household wealth index (Ref: Lowest)								
Lower-middle			1.11 (0.93, 1.33)	1.12 (0.93, 1.34)			0.48 (0.33, 0.71)**	0.48 (0.33, 0.71)**
Middle			0.96 (0.79, 1.18)	0.96 (0.79, 1.18)			0.46 (0.30, 0.73)**	0.48 (0.31, 0.75)**
Higher-middle			0.96 (0.77, 1.18)	0.96 (0.78, 1.19)			0.76 (0.51, 1.15)	0.75 (0.50, 1.14)
Highest			0.95 (0.73, 1.24)	0.95 (0.73, 1.24)			0.76 (0.45, 1.26)	0.76 (0.46, 1.28)
Season of vitamin D measurement (Ref: Summer)								
Autumn				0.94 (0.76, 1.16)				1.13 (0.70, 1.83)
Winter				1.00 (0.83, 1.22)				1.40 (0.94, 2.09)
Spring				0.99 (0.81, 1.23)				0.88 (0.55, 1.40)

*p < 0.05 and ** p<0.001
Model 1: Adjusted for age and sex
Model 2: Model 1 + adjusted for serum retinol and zinc
Model 3: Model 2 + adjusted for maternal education and household wealth index
Model 4: Model 3 + adjusted for season of vitamin D measurement

Table 7.3: Upper respiratory tract infection (URI) and acute lower respiratory tract infection (ALRI) incidence according to socio-demographic characteristics and micronutrient status (serum vitamin D, retinol and zinc) in normal-weight children aged 6–24 months[1]

Normal-weight children (n = 446)						
Child characteristics	n	Days of follow-up	Days with URI	URI rate per child-year	Days with ALRI	ALRI rate per child-year
All children	446	62,133	13,344	78.44	1,416	8.32
Age group (in months)						
6–11	239	33,577	8,072	87.81	719	7.82
12–17	132	17,924	3,492	71.16*	456	9.29
18–24	75	10,632	1,780	61.15*	241	8.28
Sex						
Boys	229	31,519	7,063	81.85	950	11.01
Girls	217	30,614	6,281	74.94	466	5.56**
Serum vitamin D						
Sufficiency	67	9,130	2,093	83.73	165	6.60
Insufficiency	176	25,144	5,328	77.40	590	8.57
Deficiency	203	27,859	5,923	77.65	661	8.67
Serum retinol						
Normal or mild deficiency		41,246	8,831	78.20	717	6.35
Moderate or severe deficiency		20,877	4,513	78.96	699	12.23**
Serum zinc						
Sufficiency ≥9.9 µmol/L	374	51,995	10,760	75.59	1,228	8.63
Insufficiency < 9.9 µmol/L	72	10,138	2,584	93.10^	188	6.77
Mother's institutional education						
Illiterate	68	9,564	2,029	77.49	313	11.95
1-5 years of education	191	26,628	5,988	82.14	484	6.64
>5 years of education	187	25,914	5,327	75.08	619	8.72
Household wealth index						
Lowest	45	5,929	1,430	88.09	233	14.35
Lower-middle	74	10,164	2,223	79.88	223	8.01
Middle	88	11,928	2,744	84.02	211	6.46*
Higher-middle	104	14,934	3,357	82.10	296	7.24*
Highest	135	19,178	3,590	68.37*	453	8.63
Season of vitamin D measurement						
Summer	79	10,905	2,171	72.72	334	11.19
Autumn	68	9,418	2,416	93.70	254	9.85
Winter	184	25,289	5,616	81.11	598	8.64
Spring	115	16,521	3,141	69.44	230	5.08*

[1]For ordinal predictors, the categories of the predictor was introduced as continuous into the generalized estimate equation model with a Poisson distribution and p value was a test for linear trend. For dichotomous predictor, p value is from the Wald test with robust standard errors in generalized estimate equation model.
^p=0.05, *p<0.05, **p<0.001

Table 7.4: Incidence rate ratios of upper respiratory tract infection (URI) and acute lower respiratory tract infection (ALRI) in normal-weight children aged 6–24 months

	Normal-weight children (n = 446)							
	Upper respiratory tract infection				Acute lower respiratory tract infection			
	Model 1	Model 2	Model 3	Model 4	Model 1	Model 2	Model 3	Model 4
Age group (in months) (Ref: 6–11)								
12–17	0.82 (0.69, 0.97)*	0.82 (0.70, 0.97)*	0.82 (0.69, 0.96)*	0.80 (0.68, 0.94)*	0.91 (0.62, 1.32)	0.92 (0.63, 1.33)	0.92 (0.64, 1.33)	0.96 (0.67, 1.38)
18–24	0.72 (0.58, 0.90)*	0.72 (0.58, 0.89)*	0.72 (0.58, 0.89)*	0.69 (0.56, 0.85)**	0.79 (0.48, 1.28)	0.81 (0.50, 1.31)	0.76 (0.47, 1.22)	0.72 (0.45, 1.15)
Sex (Ref: Boys)								
Girls	0.90 (0.78, 1.04)	0.90 (0.78, 1.03)	0.90 (0.78, 1.03)	0.89 (0.77, 1.02)	0.54 (0.38, 0.76)*	0.55 (0.39, 0.77)**	0.57 (0.41, 0.80)**	0.57 (0.41, 0.80)**
Serum vitamin D at baseline (Ref: Sufficiency)								
Insufficiency	1.00 (0.80, 1.24)	1.03 (0.83, 1.27)	1.05 (0.85, 1.31)	1.08 (0.87, 1.34)	1.09 (0.66, 1.83)	1.08 (0.65, 1.79)	1.08 (0.66, 1.78)	1.13 (0.69, 1.86)
Deficiency	1.00 (0.81, 1.23)	1.03 (0.83, 1.27)	1.05 (0.85, 1.30)	1.09 (0.89, 1.34)	1.11 (0.67, 1.83)	1.06 (0.65, 1.75)	1.04 (0.64, 1.69)	1.15 (0.71, 1.86)
Serum retinol status at baseline (Ref: Mild deficiency to normal)								
Moderate to severe deficiency		0.98 (0.84, 1.13)	0.96 (0.82, 1.11)	0.97 (0.83, 1.12)		1.81 (1.31, 2.52)**	1.86 (1.34, 2.57)**	1.88 (1.37, 2.59)**
Serum zinc status at baseline (Ref: Sufficiency)								
Insufficiency		1.21 (1.01, 1.46)*	1.21 (1.00, 1.45)*	1.22 (1.02, 1.46)*		0.95 (0.61, 1.48)	0.93 (0.60, 1.44)	0.98 (0.64, 1.50)
Mother's institutional education (Ref: Illiterate)								
1-5 years of education			1.13 (0.91, 1.40)	1.13 (0.92, 1.39)			0.76 (0.48, 1.20)	0.73 (0.47, 1.14)
>5 years of education			1.12 (0.89, 1.42)	1.11 (0.88, 1.40)			1.13 (0.69, 1.84)	1.06 (0.65, 1.71)
Household wealth index (Ref: Lowest)								
Lower-middle			0.84 (0.64, 1.11)	0.84 (0.64, 1.10)			0.73 (0.42, 1.28)	0.72 (0.42, 1.25)
Middle			0.89 (0.68, 1.15)	0.85 (0.66, 1.10)			0.58 (0.33, 1.03)	0.56 (0.32, 0.98)*
Higher-middle			0.87 (0.67, 1.13)	0.86 (0.66, 1.11)			0.52 (0.30, 0.92)*	0.50 (0.28, 0.87)*
Highest			0.75 (0.57, 0.98)*	0.74 (0.57, 0.96)*			0.66 (0.38, 1.15)	0.68 (0.39, 1.17)
Season of vitamin D measurement (Ref: Summer)								
Autumn				1.34 (1.06, 1.69)*				1.29 (0.79, 2.11)
Winter				1.21 (0.99, 1.49)				0.87 (0.57, 1.33)
Spring				0.95 (0.76, 1.19)				0.56 (0.34, 0.93)*

*p < 0.05 and ** p<0.001
Model 1: Adjusted for age and sex
Model 2: Model 1 + adjusted for serum retinol and zinc
Model 3: Model 2 + adjusted for maternal education and household wealth index
Model 4: Model 3 + adjusted for season of vitamin D measurement

Discussion

The strength of our study is that we documented the respiratory morbidity events through twice-weekly home visits which allowed us more accurate disease identification by minimising reporting bias and allowing for adequate documentation of the duration of the morbidity events [28]. Provision of clinical care at the study site and the referral of children to specialised hospitals for severe illness may have resulted in better identification of ARI events but also may have promoted over reporting of illnesses, ultimately resulting in reporting bias by the caregiver. Longitudinal study designs, moderate sample size, and a low rate of loss to follow-up also contributed to the strength of the study. Furthermore, the incidence rate of URI and ALRI reported in this study is concordant with other studies conducted in resource-limited settings[8].

The findings of the present study demonstrate that underweight children with vitamin D deficiency or insufficiency had reduced risk of URI than underweight children with sufficient vitamin D status. This is the first reported finding indicating that vitamin D insufficiency or deficiency among underweight children might protect children aged 6–24 months against URI. Results of RCTs in children examining the effect of vitamin D supplementation on respiratory tract infection have not been consistent [1, 2]. One study found no association in the incidence of ARI with vitamin D supplementation but did report that supplementation was associated with an increase in repeated episodes [1]. A recent RCT among older adults also reported increased risk and duration of URI with intermittent bolus dose supplementation of vitamin D [29]. These results support our finding about underweight children. Moreover, the high burden of air pollution, crowding of the household, and the unhygienic environment characteristic of an urban slum could also be contributing factors for these findings [19]. Recently, a study conducted in the same study area also reported a higher incidence of ARI associated with indoor air pollution and crowding of the households [30]. Additionally, vitamin D may not play a significant role in the prevention of URI, when the burden of the disease is high. In our study, children had been suffering 69 to 78 days with URI annually in an environment with poor caring practices and low socio-economic status. Continuous and overwhelming air pollution, both indoors and outdoors, could be masking the role of vitamin D in this population [27, 30, 31]. Furthermore, the role of vitamin D in triggering the immune system among undernourished children is yet to be explored.

We found that the risk of URI in children was significantly lower among both underweight and normal-weight older children (12–24 months) in all multivariable models which contrasts with findings from a previous study carried out in rural Bangladesh [8]. Our study was based in an urban slum setting leading to participants living in a more polluted environment. A study recently

conducted in the same field site reported exposure to high indoor particulate matter (PM 2.5), associated with childhood respiratory tract infections [27]. Moreover, children aged 6–11 months are in a critical transitional period in their development of immunity. Lack of immunity and the high burden of a polluted environment simultaneously are likely to contribute to young children (6–11 months) being more vulnerable to URI than older children.

In a recent review, it was reported that among preschool children, those receiving vitamin A supplementation had no significant difference in incidence of pneumonia compared with those in a placebo group [32]. However, in our study we found that both normal-weight and underweight children with moderate to severe serum retinol deficiency had a higher risk of ALRI. These findings could be explained by the known role of serum retinol in enhancing the regeneration and integrity of respiratory and gastrointestinal epithelia and also in modulating the immune function [33].

We did not find any difference in risk of ALRI incidence among children with insufficient zinc status compared with children with adequate zinc status; which is also supported by a recent review done among children aged 6 months to 12 years old [34]. However, normal-weight children classified as zinc insufficient were found to have a greater risk of URI than zinc-sufficient children. This finding is in line with a study on adults, reporting a reduction in symptoms of common cold among adults supplemented with zinc [35].

Underweight children aged 12–17 months had significant risk of ALRI compared with no risk for those in the 6–11 months age group. In developing countries, WAZ score is stable during first year of life but is followed by a period of progressive and slow faltering up until children are five years of age [36]. This critical period of progressive faltering may lead to a reduced immune response and so increase the risk of ALRI. Female normal-weight children had significantly less risk of ALRI than boys after adjusting for all other potential confounders. A similar finding for URI among children aged 5–11 years was also reported in a study conducted in rural Bangladesh [37].

A study done at the same site did not find any association between maternal elementary education and the risk of ALRI [27]. However, in our study, underweight children of mothers with institutional education showed a lower risk of ALRI than children of illiterate mothers. Possible explanations for this finding are that educated mothers have better childcare practices [38] thus, preventing children from progressing to more severe forms of the disease. Among both underweight and normal-weight children, those from higher socio-economic households had a lower risk of ALRI than those from lower socio-economic households. These findings are supported by two small studies conducted in urban slums and rural settings in Bangladesh [39, 40].

Limitations

The findings of this study need to be interpreted in light of its limitations. First, both groups of children received multiple micronutrient powder supplementations, as per recommendations of the National Nutrition Program of Bangladesh, which included vitamin A and zinc, but no vitamin D. The role of supplementation with vitamin A and zinc were not considered for incidence of ARI in these analyses due to fewer number of serum collected for micronutrient assays at the end of follow-up, which may confound the findings of our results. Second, the diagnosis of URI in this study relied on the presence of cough but did not consider other signs such as nasal discharge. Similarly, for ALRI events we did not consider central cyanosis or oxygen saturation. Third, we missed some of the episodes of ARI due to absence of the child and care giver from the household. At least 120 days of morbidity surveillance information was successfully collected from 87.1% and 90.1% underweight and normal-weight children respectively. Finally, we used data from a MAL-ED community-based study with a prospective case-control design and urban setting, results may not be representative of the general population of children in Bangladesh, and specifically those in rural areas.

Conclusions

The roles of serum vitamin D on the incidence of childhood respiratory tract infections among underweight and normal-weight children, especially considering immunomodulatory functions, still need further research to draw any final conclusion. On the other hand, our study demonstrated that serum retinol deficiency has significant association with the increased incidence of ALRI in both underweight and normal-weight children aged 6–24 months indicating that efforts to increase the intake of retinol-enriched food or the implementation of supplementation programs in this population should be encouraged.

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CHAPTER 8 DISCUSSION AND CONCLUSION

8.1. Introduction

Recently it has been estimated that around 11% of all deaths for children under five years old are related to four micronutrient deficiencies—vitamin A, zinc, iron and iodine [1]. However, the magnitude of vitamin D deficiency or insufficiency and the implications for the health and nutrition of children under two years of age is little known [2]. It is noteworthy that most infectious morbidity and mortality events occur during the first two years of life [3]. Although widespread vitamin D deficiency among children in developing countries has been reported, [4] corresponding data on the associated risk of morbidity such as diarrhoea or ARI are inconsistent [2]. Moreover, data on the prevalence of vitamin D deficiency among children under two years old are lacking and none of the studies look at its role in diarrhoea and ARI incidence among children living in the impoverish, resource–constrained, polluted environment of urban slum areas where basic hygienic conditions and childcare practices are sub-optimal. The research in this thesis revolved around the specific objectives outlined in Chapter 1. The body of research comprised in this Thesis aimed to address the burden of vitamin D status and its association with the incidence of the two most common infectious disease morbidities, diarrhoea and ARI, among children residing in urban slum areas where basic hygienic conditions and childcare practices are far below standard.

The research has important practical implications for the control of vitamin D deficiency and insufficiency, and for identifying the role of vitamin D in diarrhoea and ARI among children: firstly, it showed there is a significant burden of vitamin D deficiency and insufficiency irrespective of the nutritional status of children under two years of age who were residing in the urban slums of Dhaka, Bangladesh; secondly, the identification of risk indicators for vitamin D deficiency and insufficiency could be used when implementing national control programs for mitigating the huge burden of vitamin D deficiency and insufficiency; thirdly, it provides practical insights regarding the roles of vitamin D status alone, as well as in combination with other micronutrients status and socioeconomic indicators, in incidence and severity of childhood diarrhoeal diseases and ARI; fourthly, it identifies the role of vitamin D status on the diarrhoeagenic *E.coli* diseases among children under two years old; and finally, it is one of the leading studies reporting findings about vitamin D and other micronutrients and, therefore, will be useful for devising multiple micronutrient supplementation programs to combat childhood infectious disease morbidity.

8.2. Key research findings

The research detailed in Chapter 4 has provided the prevalence rate and risk factors of vitamin D deficiency and insufficiency among children under two years of age residing in the resource-poor

settings of urban slums of Bangladesh. Despite the geographical location and temperate climate, vitamin D synthesis by the skin with ultra-violate radiation from sun exposure is possible within any area of Bangladesh. However, the study identified 35% of underweight children and 45% of normal-weight children had vitamin D deficiency (<50 nmol/l), and their insufficiency status (50-75 nmol/l) was 42% and 40% respectively. Recently conducted national micronutrient survey in Bangladesh have also reported 47.9% children aged under five years of age living in slum area had vitamin D deficiency (<50 nmol/L) and supported our findings [5]. Thus, the study demonstrates a significant burden of vitamin D insufficiency and deficiency in both underweight and normal-weight children under two years old living in a slum area. Thus, vitamin D deficiency and insufficiency is an important public health problem among young children living in urban slum area of Bangladesh.

The primary role of vitamin D is to maintain extra cellular calcium ion and bone health [6]. The deposition of calcium in the bone occurs during the early stages of life in humans, starting prenatally and continuing during childhood and adolescence in order to prevent osteoporosis-related bone disease in adulthood [7, 8]. The key factors for optimal bone mineral density or bone health during childhood are vitamin D, calcium, and weight-bearing activity [8]. As the huge burden of vitamin D insufficiency and deficiency was not reported in earlier publications, there is no existing program addressing this important public health problem among the children of Bangladesh. Children who live in resource-constrained settings are vulnerable to both macro and micro nutritional deficiency which warrants the introduction of programs to reduce such deficiency. Since the primary source of vitamin D is sunlight, behaviour change communication could be effective means for alleviating deficiency and insufficiency. As such, educational interventions that centred on exposure to sunlight on bare skin for 10–15 minutes, 4–5 times per week could be incorporated in the current government and Non-Government Organizational community health education program.

The risk factors for vitamin D deficiency and insufficiency identified in Chapter 4 —older age, higher maternal education, living in a household with the highest wealth quintal, measurement of vitamin D during winter and spring—are supported by other studies conducted elsewhere among children under two years old [9, 10]. The identification of common risk factors across different studies enables researchers, academicians, and program managers to design effective interventional programs for combating vitamin D deficiency and insufficiency. Skin pigmentation [11, 12], air pollution [13], and maternal vitamin D deficiency [14] are the well-established risk factors that were not measured in the current study. However, these are not amenable to change with an educational intervention to address vitamin D deficiency and insufficiency. Therefore, a

supplementation program with vitamin D and calcium during prenatal periods and during the winter and spring seasons (when the exposure to sunlight is low) could be another way of mitigating such a burden. Currently, there is no vitamin D supplementation program in Bangladesh for pregnant women and children. A vitamin D supplementation program could be introduced within the national Maternal and Child Health program and the Expanded Program on Immunization during winter and spring—when exposure to sunlight is low.

It is evident from Chapter 1 and 2 that vitamin D is important in calcium homeostasis and bone health and also for maintaining the integrity of the innate immune system and protection against infections. The identified higher rate of vitamin D insufficiency and deficiency among children under two years old, may lead to increased incidence of childhood infectious diseases morbidity. The research detailed in Chapter 5, 6 and 7 have presented findings of the association of vitamin D status with the leading infectious disease morbidity—diarrhoea and ARI among children under two years of age.

The analyses were conducted according to the research framework presented in Chapter 1. Following a longitudinal design and inclusion of modifiable factors such as personal, socio-economic and other micronutrient status (Vitamin A and zinc), no associations were found between vitamin D status and childhood diarrhoeal disease and ALRI among underweight and normal-weight children aged 6–24 months. Rather, underweight children who were vitamin D deficient or insufficient had experienced significantly fewer days of URI than underweight children who were vitamin D sufficient. Children with insufficient vitamin D status had a significantly reduced hazard of EAEC diarrhoeal incidence than children with sufficient vitamin D status.

The immunomodulatory roles of vitamin D in infectious diseases need to be investigated more carefully for the explanations for such findings. In Chapter 1, a framework has been presented and explaining how vitamin D influences both the innate and adaptive immunity for infectious disease morbidity, including diarrhoea and ARI. In Chapter 2, the mechanisms induced by the vitamin D for both innate and adaptive immunity in infectious disease morbidity have been explained elaborately. From those descriptions, it is evident that vitamin D upregulates innate immunity for killing and clearing of microorganisms but inhibits any overzealous responsivity in the adaptive immune response to the offending infection and antigen.

In the human body, the innate immune system provides a first line of defence and is essential for the control of common bacterial infections. Firstly, the innate immune system cannot eliminate infectious organisms all the time; secondly, it is unable to recognise some pathogens; and finally the innate immune systems does not have any memory of a previous infection [15]. On the other

hand, the adaptive immune system provides a more versatile means of defence by differentiation and recruitment of different types of immune cells as well as by production of antibodies, and provides protection against subsequent reinfection with the same pathogen [15]. Accordingly, vitamin D potentiates the weaker innate immune response and inhibits the action of the stronger adaptive immune response in infectious disease immunity. Perhaps this could be the foremost possible explanation for the findings of Objectives 2, 3 and 4. This explanation is also supported by the findings of the recently conducted RCTs which provided vitamin D supplements to impoverished children in developing countries for the treatment and prevention of diarrhoea and ARI [16-18].

In this study, the participant children were 6–24 months of age and were living in urban slums, where the burden of infectious disease like diarrhoea and ARI are highest due to poor sanitation, crowding of the household, air pollution, poor socio-economic status, and unhygienic environment [19-23]. Continuous and overwhelming infections by pathogens are common in a slum environment and immature immune responses among this age group could be masking the role of vitamin D in diarrhoea and ARI. The findings of this study do not support the findings about the school-aged children from Columbia, where the burden of diarrhoea was low [24].

The protective role of deficient and insufficient vitamin D status for URI among underweight children could be explained by findings of the recently conducted RCT among children aged 1–30 months, which found no association in the incidence of ARI with vitamin D supplementation but did report that supplementation was associated with an increase in repeated episodes [16]. Furthermore, when intention-to-treat analysis was used, the incidence of the first episode of pneumonia was greater in the vitamin D supplementation group than the placebo group. Additionally, in a recent RCT among older adults researchers also reported increased risk and duration of URI with intermittent bolus dose supplementation of vitamin D [25]. These results support our finding among underweight children. The findings of Chapter 5, 6 and 7 contradict previous evidence suggesting an association between vitamin D and the childhood infectious diseases—diarrhoea and ARI [24, 26-29]. However, our findings are supported by previous studies conducted on the effect of supplementation of vitamin D on diarrhoeal disease and respiratory infection among children under two years old [16-18].

Like vitamin D, other micronutrients are important for health and growth in children but widespread deficiencies of essential vitamins and minerals are common among children of low-income and middle-income countries [30]. Vitamin A and zinc are two of the most important micronutrients implicated in childhood mortality and morbidity [31, 32]. Recent systematic reviews on supplementation of zinc and vitamin A showed significant reduction in mortality and morbidity

related to diarrhoea and ARI among children [33, 34]. Supplementation of vitamin A (in the neonatal period and late infancy) and preventive zinc supplements are recommended interventions among children for reduction of mortality and morbidity as well as promoting growth [1]. As described in Chapter 5, 6 and 7, the association of serum retinol (vitamin A) and zinc, along with vitamin D status, were evaluated with incidence of diarrhoea or pathogen-specific diarrhoea and ARI among underweight and normal-weight children under two years old.

Vitamin A and zinc supplementation among children are recommended by the Government of Bangladesh [35, 36]. Thus, we evaluated whether the beneficial role of zinc and vitamin A was confounded by the vitamin D status. It is evident from the interpretation of the results of all the models we constructed for analyses in Chapter 5, 6 and 7, that the role of zinc and vitamin A in prevention of diarrhoea and ARI were not confounded by vitamin D status.

The results of Chapter 5 demonstrate that the beneficial effect of sufficient serum zinc status in the incidence and severity of diarrhoea is only evident with normal-weight children. This is a novel finding which can be partly explained by the lack of catalysing enzymes and compromised immunity in undernourished children which can result in non-activation of the immune response initiated by serum zinc [37]. The results of Chapter 7 indicated that there is no association between incidence of ALRI with the serum zinc status among both cohorts of children, a finding that is supported by a recently published systematic review [34].

It is reported in Chapter 7 that both normal-weight and underweight children with moderate to severe serum retinol deficiency had a higher risk of ALRI. In a recent review, it was reported that supplementation with vitamin A showed no significant differences in incidence of pneumonia among preschool children when compared with a placebo group [38]. These findings could be explained by the known role of serum retinol in enhancing the regeneration and integrity of respiratory and gastrointestinal epithelia and also modulating immune function [39].

The results of Chapter 6 reported the severe to moderate vitamin A deficiency had a reduced risk of EPEC diarrhoea than mild to normal vitamin A status among the underweight children, which is supported by a recent study reporting vitamin A supplementation increases the duration of EPEC infection among children under two years old [40]. The pro-inflammatory responses may play different roles in different pathogen specific infections with vitamin A supplementation. However, inflammatory markers were not measured in this study. Thus, we were unable to evaluate further effect of vitamin A or other micronutrients in diarrhoeagenic *E.coli diseases*.

Previous studies reported the role of vitamin D, vitamin A, and zinc discretely in childhood infectious disease morbidity. To that extent, the studies included in this thesis are unique in

highlighting that the role of zinc and vitamin A is not confounded by vitamin D status. The major findings relating to vitamin A and zinc status along with vitamin D status in incidence of diarrhoea or pathogen specific diarrhoea and ARI in Chapter 5, 6 and 7 are supported by the published scientific literature. In Chapter 6, severe to moderate vitamin A deficiency had a reduced risk of EPEC diarrhoea may raise questions about supplementation programs with vitamin A. However, considering the greater role of vitamin A and zinc in childhood mortality, morbidity, and growth, my finding support continuation and strengthening of the multiple micronutrient supplementation program for this age group

8.3. Strengths and limitations

Strengths

Data from a prospective case-control study was analysed for this thesis. Cohort study designs were used which were optimal for evaluating the incidence and severity of diarrhoea and ARI among children over time. Participants were selected through biannual demographic surveillance and this reduces selection bias. Longitudinal study designs, moderate sample size and a low rate of loss to follow-up also contributed to the strength of the study.

The documentation of common illnesses on a daily basis through twice-weekly home visits by trained fieldworkers also enhanced disease identification and provided validity for the results because of adequate documentation of the duration of illnesses [41]. The overall and and age-specific incidences of diarrhoea and ARI are also concordant with other studies conducted in resource-limited settings [3, 42, 43]. The design of the study is one of its kind in the way it reported community-level incidence of diarrhoea and ARI (mild to severe) due to documentation of any morbidity events on a daily basis. The quality assurance of data and sample collection was ensured through standardisation and validation of questionnaires, development of a manual of procedure for data and sample collection, training and refresher training of data and sample collectors, re-interviewing and anthropometric measurement of 5% of participants by a quality assurance team on a monthly basis and double entry of data.

Generalized estimating equations (GEE) models, which control for the inherent correlation of observations within each subject, were used to estimate the incidence ratio of diarrhoea and ARI according to the vitamin D status of the children. Moreover, Kaplan-Meier curves and Cox hazard models were used to analyse pathogen-specific risk of diarrhoeal illness with censored time-to-event data. Thus, all the statistical methods used for analysis of data also contributed in the strength of the study.

Limitations

Diagnostic uncertainty

Determination of serum 25-hydroxyvitamin D is of diagnostic importance for the exploration of vitamin D insufficiency and deficiency. The IDS 25-hydroxy vitamin D EIA kit (enzyme immunoassay) was used for the quantitation of serum vitamin D. A recent report showed that the specificity of this kit for 25-hydroxyvitamin D measurement is 75% but the precision of immunoassay, high performance liquid chromatography and liquid chromatography–mass spectrometry are comparable [44]. However, continuing efforts to improve laboratory performance and vigilance with quality assurance programs are required for any methods used for vitamin D measurement [45]. All the assays for micronutrients including vitamin D, were performed at the nutritional biochemistry laboratory of the icddr,b which regularly participates with international organisations and laboratories for quality assurance of these assays. Moreover, two controls were run in each plate/run for monitoring accuracy and precision of vitamin D measurements. The co-efficient of variation was 3.8-11.8 % for control 1 and 5.2-10.7 % for control 2.

Bias

The main source of vitamin D in humans is exposure to sunlight on bare skin. Information about the frequency and duration of sunlight exposure, clothing worn by children, cultural beliefs, and children's outdoor activities were not collected in the Mal-ED study, and these could be weaknesses in this research.

The diagnosis of diarrhoea and ARI were made from the maternal report of the symptoms in a child which may cause reporting bias. Previous studies found 97% agreement with maternal reporting of diarrhoea [46]. However, reporting of severity of diarrhoea might be influenced by several factors including education of mother/caregiver and socio-economic conditions [47]. Before the implementation of the Mal-ED study, the questionnaires were standardised and validated for the field site to minimise mis-classification errors for diarrhoea and ARI [48]. Data collection through twice-weekly home visits also minimises recall bias.

Continuous health and nutritional education, and provision of clinical services for common childhood illnesses at the field site could have promoted over-reporting of illnesses, ultimately resulting in reporting bias by caregivers. Feeding and feeding practices of complementary food are known to modify the risk of diarrhoea among children. However, the collected data were inadequate to assess the risk of diarrhoea in relation to feeding practices.

Data collectors missed some of the episodes of diarrhoea and ARI due to absence of the child and caregiver from the household. At least 120 days of morbidity surveillance information was

successfully collected from 87.1% and 90.1% of underweight and normal-weight children respectively. Finally, study findings cannot be generalised due to inclusion of only urban children aged 6–24 months old.

Confounding

Both underweight and normal-weight children received multiple micronutrient powder supplementations with vitamin A and zinc but without vitamin D, as per recommendation of the National Nutrition Program of Bangladesh. The role of supplementation with vitamin A and zinc were not considered for incidence of diarrhoeal and ARI morbidities in these analyses due to fewer numbers of serums collected for micronutrient assays at the end of follow-up, which may confound the findings of our results.

8.4. Future research

There are considerable opportunities for further research about the burden and risk factors of vitamin D status among children and for in-depth assessment of immunomodulatory function of vitamin D in childhood infectious-disease immunity.

Future studies could include:

- Prevalence of vitamin D status in older children and adolescents could be explored.
- The efficacy and effectiveness of intervention programs (behaviour change communication and supplementation with vitamin D) for better vitamin D status among children under two years old in Bangladesh could be evaluated.
- Assessment of bone density and risk factors among children of urban Bangladesh could be undertaken.
- In-depth assessment of how vitamin D status modulates the innate and adaptive immune functions of infectious diseases and role of nutritional status of children could be undertaken.
- Association of vitamin D status with the incidence of other pathogen-specific diarrhoea such as rota and shigellosis in children could be investigated.
- Role of vitamin D status in gastrointestinal infection to disease spectrum could be explored

8.5. Conclusions

The research included in this Thesis, quantified the burden of vitamin D status and risk factors among children under two years of age living in an urban slum, according to their nutritional status; explored the association between vitamin D status and leading causes of childhood infectious-disease morbidity and pathogen-specific common *Escherichia coli* diarrhoea; and it also

investigated whether vitamin D status confounded the association between other micronutrients status and these conditions.

The studies included in this thesis have important public health implications. Research detailed in Chapter 4 shows a high burden of vitamin D deficiency and insufficiency which is an important public health problem in urban slums of Bangladesh, highlighting the need for intervention programs (behaviour change communication and supplementation with vitamin D) in current government and non-government community health education programs, national maternal and child health programs and in the Expanded Program on Immunization.

The estimation of vitamin D status allowed for the evaluation of the association of vitamin D status and incidence of childhood infectious disease in a poor socio-economic and unhygienic environment, where burden of infectious disease is high among children under two years old. The findings reported in Chapter 5 and 7 demonstrate a lack of association between vitamin D status and incidence of childhood diarrhoeal disease and ALRI. Somewhat perplexing, vitamin D deficient and vitamin D insufficient underweight children had experienced significantly fewer days of URI than vitamin D sufficient underweight children. Thus, vitamin D supplementation programs to prevent infectious disease morbidity in children might not be recommended according to the findings of this thesis. However, vitamin D did not confound the beneficial role of zinc and vitamin A in childhood diarrhoea and ARI, supporting the continuation and strengthening of the multiple micronutrient supplementation program for the study age group.

The findings in Chapter 6 show that insufficient vitamin D status and moderate to severe vitamin A status is associated with lower incidence of EAEC diarrhoea among normal-weight children. This finding may raise questions about the effect of multiple micronutrient supplementation programs in children aged under two years. However, considering the greater role of vitamin A and zinc in childhood mortality, morbidity and growth, the recommendation to continue and strengthen the multiple micronutrient supplementation program for the study age group is upheld.

The findings of this thesis may raise question about the immunomodulatory role of vitamin D in childhood infectious disease morbidity. Thus, further research should look at how vitamin D status modulates the innate and adaptive immune functions for infectious diseases and the role of the nutritional status of children; empirical evidence for the possible mechanisms underlying such complex relationships of infectious diseases and vitamin D status children under two year old; and identifying factors that determine the immunological effectiveness of vitamin D status.

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Appendix A: Literature review tables

Appendix Table 1: Vitamin D status/prevalence among children aged under five years in North America, Australia and Europe

Study	Country	Study design	Participants (n)	Age	Serum Vitamin D (*25 hydroxyvitamin D*)		
					Mean ± SD or 95% CI (nmol/L)	**% <25 nmol/L**	**% <50 nmol/L**
Bodner, 2007 [1]	Pittsburgh, Pennsylvania USA	Cohort study	Newborn White: 200 Black: 200	Newborn	67.4 (63.8–71.3) 39.0 (36.3–41.8)	9.7 (<37.5 nmol/L) 45.6 (<37.5 nmol/L)	NA
Hintzpeter, 2008 [2]	Germany	Cross-sectional	Children Immigrant Non- immigrant Total: 10,015	1–17 yrs	NA	Non- immigrant Boys: 7.1 Girls: 7.1 Immigrant Boys: 10.8 Girls: 17.2 (1–2 yr-age grp)	Non- immigrant Boys: 31.2 Girls: 36.4 Immigrant Boys: 41.5 Girls: 45.5 (1–2 yrs-age- grp)
Mansbach, 2009 [3]	US Nationally representative sample (NHANES)	Cross-sectional	Children: 1799	1–5 yrs	70 (68–73)	<1	63 (<75.0 nmol/L)
Grant, 2009 [4]	Auckland, New Zealand	Cross-sectional	Urban children: 353	6–11 months 12–17 months 18–23 months	62 (42–78) 58 (44–70) 49 (39–61)	10 (<27.5 nmol/L)	NA
Cole, 2010 [5]	Atlanta Georgia USA	Cross-sectional	Low-income minority children Hispanic : 141 Non-Hispanic Black: 149	1–5 yrs	64.7 ± 14.8 66.3 ± 22.3	NA	18.1 26.3
Merewood, 2010 [6]	Boston, Massachusetts USA	Cross-sectional	Primarily low-income Black and Hispanic: 376	Newborn	43 (40–47)	38.0 (<37.5 nmol/L)	58.0

Appendix Table 2: Vitamin D status/prevalence among children aged under five years in Asia

Study	**Country**	**Study design**	**Participants (n)**	**Age**	**Serum Vitamin D (*25 hydroxyvitamin D*)**		
					Mean ± SD or 95% CI (nmol/L)	**% <25 nmol/L**	**% <50 nmol/L**
Andiran, 2002 [7]	Ankara, Turkey	Cross-sectional	Paediatric Hospital Newborn: 54	7–28 days	18.6 ± 8.0	46	NA
Strand, 2007 [8]	Yuci, Shanxi, China	Cross-sectional	Rural Children : 200	12–24 months	NA	65.3 (<37.0 nmol/L) during spring	
Gharaibeh, 2009 [9]	Northern Jordan	Cross-sectional	Children : 93	4–5 yrs	55.8 ± 19.8	NA	39
Kazemi, 2009 [10]	Zanjan, Iran	Cross-sectional	Newborn: 61	Newborn	16.7 ± 2.9	75.5 (during winter) 35.0 (during summer)	NA
Andiran, 2012 [11]	Ankara, Turkey	Cross-sectional	Paediatric Hospital Children: 440	1–16 yrs	85.5 ± 40.5 (0–5 yrs) 51.3 ± 21.8 (5–10 yrs) 46.8 ± 28.8 (11–16 yrs)		52.1 (Boys) 64.8 (Girls)
Zhu, 2012 [12]	Hangzhou, China	Cross-sectional	Paediatric Hospital Children : 4385	0–5 yrs	98.7 ± 47.1 (0–1 yrs) 69.6 ± 30.4 (2–5 yrs)	0.4 (0–1 yrs) 1.1 (2–5 yrs)	5.4 (0–1 yrs) 21.9 (2–5 yrs)

Appendix Table 3: Vitamin D status/prevalence among children aged under five years of South Asia

Study	Country	Study design	Participants (N)	Age	Serum Vitamin D (*25 hydroxyvitamin D*)		
					Mean ± SD or 95% CI (nmol/L)	**% <25 nmol/L**	**% <50 nmol/L**
Atiq, 1998 [13]	Karachi, Pakistan	Immunisation clinic	Nov–Mar: 23 Apr–Oct: 48	1.5–11 months	Nov-Mar: 24.5 Apr- Oct: 40.7	52.0 (throughout the year)	NA
Fischer, 1999 [14]	Chakaria, Bangladesh	Case-control	Case: 14 Control: 13	10–120 months	Case: 50.0 Control: 62.5	Case: 14.0 Control: none (<35.0 nmol/L)	NA
Agarwal, 2002 [15]	Delhi, India	Cross-sectional	High pollution area: 26 Low pollution area: 31	9–24 months	High pollution: 31.0 Low pollution: 68.0	High pollution: 35.0 Low pollution: none (<30.0 nmol/L)	NA
Tiwari, 2004 [16]	Delhi, India	Cross-sectional	Sundernagari: 47 in January Rajiv Colony : 49 in February Rajiv Colony: 48 in August Gurgaon: 52 in August	9–30 months	Sundernagari: 96.3 Rajiv Colony : 23.8 Rajiv Colony: 17.8 Gurgaon: 19.2	Sundernagari: 2.0 Rajiv Colony : 82.9 (Feb) Rajiv Colony: 84.0 (Aug) Gurgaon: 82.0 (Aug) (<35.0 nmol/L)	NA
Wayse, 2004 [17]	Indapur, India	Case-control	Children with ARI: 80 Healthy controls: 70	<5 years		Case: 80.0 Control: 32.0 (<22.5 nmol/L)	Case: 95 Control: 61
Bhalala, 2007 [18]	Mumbai, India	Cohort study	Newborn: 42 3 months old: 35	Neonate and 3 months old	19.4 ± 9.6 at birth 18.2 ± 9.7 at 3 months	36% at birth 51% at 3 months (<30.0 nmol/L)	62.0 at birth 80.0 at 3 months of age
Combs, 2008 [19]	Chakaria, Cox's Bazar, Bangladesh	RCT	Children from rural and low income families: 158	1–5 yrs	68.3	6.0 (<25.0) 21.0 (<37.5)	NA
Roth, 2010 [20]	Zakiganj, Sylhet, Bangladesh	Case-control	Case: 39 Control: 35	1–24 mths	32.6 (29.1-36.2)	32% considering both case and control children	70% (<40.0)

Appendix Table 3 (continued)

Study	Country	Study design	Participants (N)	Age	Serum Vitamin D (*25 hydroxyvitamin D*)		
					Mean ± SD or 95% CI (nmol/L)	% <25 nmol/L	% <50 nmol/L
National Micronutrient Survey, 2011-12 [21]	Bangladesh	Survey	Children from National: 461 Rural: 141 Urban: 127 Slum: 193	<5 years	National: 56.3 (50.6-62.1) Rural: 56.5 (49.1-63.9) Urban: 56.2 (47.5-64.9) Slum: 54.6 (48.8-60.4)	National: 39.6 (24.2-55.1) Rural: 38.0 (18.0-57.9) Urban: 44.6 (30.3-58.9) Slum: 47.9 (34.9-61.0)	National: 7.5 (1.0-14.0) Rural: 7.0 (0.0-15.5) Urban: 8.9 (3.3-14.6) Slum: 10.1 (3.5-16.6

Appendix Table 4: Association between vitamin D status and acute respiratory infections (ARI) among children aged under 5 years, results from cross-sectional and case-control studies

Author, year	Type of study	Setting and study population	Vitamin D status	Findings
Muhe, 1997 [22]	Case- control	Total 1000 children 500 rickets cases and 500 healthy controls Ethiopia Mean age: 13 months	Not measured	Rickets (vitamin D deficiency) associated with susceptibility to pneumonia (adjusted OR 13·37; 95% CI, 8·08–24·22; p <0·001)
Najada 2004 [23]	Case- control	Total 443 hospitalised children 47 rickets cases and 396 controls (without rickets) Jordan Age: 3–24 months	Not measured	Rickets associated with ALRI (85% with rickets vs. 30% without rickets, had ALRI) (p <0.01)
Wayse, 2004 [17]	Case- Control	Total 150 children 80 cases with ALRI and 70 healthy controls India Mean age: 23.9 months	Cases: mean 9.1 ng/ml Controls: mean 15.4 ng/ml Vitamin D < 50 nmol/L: Cases 95% and controls 61% Vitamin D < 22.5 nmol/L: Cases 80% and control 32%	Serum vitamin D significantly low in cases than control (p <0.001) Serum vitamin D >9.0 ng/ml associated with decreased risk of ALRI (adjusted OR 0.09; 95% CI, 0.03–0.24; (p < 0.001)

Appendix Table 4 (continued)

Author, year	Type of study	Setting and Study population	Vitamin D status	Findings
Roth, 2009 [24]	Case- control	Total 129 children 64 hospitalised children 65 healthy controls, Canada Mean age 13 months	Cases: 30.9 ng/ml Controls: 30.8 ng/ml	No significant difference in mean serum vitamin D cases vs. controls (p = 0.96) Inadequate vitamin D status was not associated with the risk of ALRI at 16 ng/ml or 32 ng/ml (p ≥0.37)
McNally, 2009 [25]	Case-control	Total 197 children Hospitalised with ALRI (case) = 105 Attending hospital with other diagnosis (control) = 92 Canada Mean age 14 months	Cases: 32.5 ng/ml Controls: 33.3 ng/ml	No significant difference in mean serum vitamin D cases vs. controls (p = 0.71) <20 ng/ml associated with increased risk of admission to the intensive care unit in cases (adjusted OR 8.23, 95% CI, 1.4–48.0, p = 0.02)
Jarrti, 2010 [26]	Cross-sectional	284 hospitalised children, Finland	Mean 27.2 ng/ml 31% (<50 nmol/L) 6% (<25 nmol/L)	Vitamin D concentration inversely associated viral ARI Respiratory syncytial virus infection: (OR per 10 nmol/L increase, 0.91; 95% CI 0.83–0.99) Rhinovirus infection: (OR per 10 nmol/L increase, 0.92; 95% CI 0.85–0.99) Multiple viral cause: (OR per 10 nmol/L increase, 0.91; 95% CI 0.84–0.99)
Roth, 2010 [27]	Case-control	Total 50 children Hospitalised with ALRI (case) = 25 Healthy (controls) = 25 Bangladesh Aged 1–18 months	Cases: 11.7 ng/ml Controls: 15.7 ng/ml	Mean serum vitamin D concentration significantly lower in ALRI cases (p = 0.015) ALRI reduced 4.3-fold for every 4 ng/ml (adjusted OR 0.23; 95% CI, 0.06–0.81; p = 0.02)
Leis, 2012 [28]	Case-control	Total 197 children Hospitalised with ALRI (case) = 105 Attending hospital with other diagnosis (control) = 92 Canada Age < 5 years	Not measured	Vitamin D intake <80 IU/kg/day associated with increased risk of LRTI (adjusted OR 4.9, 95% CI 1.5–16.4, p = 0.01)

Appendix Table 5: Association between vitamin D status and acute respiratory infection among children aged under 5 years, results from longitudinal studies

Author, year	Type of study	Setting and study population	Vitamin D status	Findings
Gale, 2008 [29]	Prospective birth cohort	466 infants Duration of follow-up: 9 months United Kingdom	Mean maternal vitamin D level at late pregnancy: 20 ng/ml • 21.2% with <11 ng/ml • 28.3% with 11–20 ng/ml • 50.4% with >20 ng/ml	Maternal serum vitamin D concentration in the top quartile (>30 ng/ml) vs. bottom quartile (<12 ng/ml): Increased risk of pneumonia or bronchiolitis in children (OR 4.80, 95% CI 1.01–22.72) Maternal serum concentration not associated with risk of ARI in offspring
Camargo, 2011 [30]	Prospective birth cohort	922 children Duration of follow-up: 6 years New Zealand	Cord blood concentrations: Median, 17.6 ng/ml • 19.5% with <10 ng/ml • 53.3% with 10–29 ng/ml • 27.2% with 30 ng/ml	Serum vitamin D concentration inversely associated with risk of ARI in children 3 months of age: (OR 1.0 for ≥30 ng/ml, OR 1.39 for 10–30 ng/ml, OR 2.16 for <10 ng/ml,) (p for trend 0.004) Vitamin D levels inversely associated with risk of wheezing by 15 months, 3 years and 5 years of age (p <0.05)
Belderbos, 2011 [31]	Prospective birth cohort	156 infants Duration of follow-up: 1 years Netherland	Mean, 32.9 ng/ml • 23.1% with <20 ng/ml • 30.8% with 20–29 ng/ml • 46.1% with ≥30 ng/ml	Vitamin D concentration at birth inversely associated with risk of respiratory syncytial virus ALRI over 1st year of life (adjusted relative risk 6.2, 95% CI 1.6 to 24.9, p = 0.01 for neonates with 25(OH)D <20 ng/ml vs. ≥ 30 ng/ml
Morales, 2012 [32]	Prospective birth cohort	1724 children Duration of follow-up: 6 years Spain	Median maternal vitamin D level at 12 weeks of gestation: 29.5 ng/ml	Maternal serum vitamin D concentration inversely associated with risk of ALRI by 1st year of life (OR 0.67, 95% CI 0.50–0.90, p = 0.02, for highest vs. lowest quartile of maternal 25(OH)D)
Mohamed 2012 [33]	Prospective birth cohort	206 infants Duration of follow-up: 6 years Saudi Arabia	Cord blood concentration: Mean, 24.1 ng/ml • 12% with <12 ng/ml • 18% with 12–19 ng/ml • 26% with 20–29 ng/ml • 44% with ≥30 ng/ml	Mean cord blood vitamin D concentration was lower among infants who developed ALRI in the first 2 years of life vs. those who did not (13.6 ng/ml vs. 28.6 ng/ml, p <0.0001) In multivariate analysis, low cord blood vitamin D concentration independently associated with subsequent risk of ALRI (OR 1.08; 95% CI 1.05–1.10; p <0.001)

Appendix Table 6: Efficacy of the vitamin D supplementation on incidence of ARI among children aged under 5 years

Author, year	Type of study	Setting and study population	Vitamin D status	Findings
Manaseki-Holland, 2010 [34]	RCT	Total 453 children with pneumonia, aged 1–36 months 224 allocated to intervention group single bolus dose of 100,000 IU vitamin D 229 allocated to placebo Duration of follow up: 3 months Afghanistan	Not presented	In vitamin D supplementation group reduced risk of repeat ALRI episode within 90 days (RR 0.78; 95% CI, 0.64–0.94; p = 0.01) Intervention did not affect the mean number of days to recovery (4.7 days in intervention arm vs. 5.0 days in placebo arm; p = 0.17)
Kumar, 2011 [35]	RCT	Total 2079 low birth weight infants 1039 allocated to intervention with 1400 IU/week of vitamin D 1040 allocated to placebo Duration of follow-up: 6 months India	Intervention group At 6 months 22.0 ng/ml Placebo group At 6 months 14.4 ng/ml	Intervention did not affect incidence of pneumonia or incidence of all-cause hospital admission or death (adjusted rate ratio 0.98, 95% CI 0.70–1.38, p = 0.92)
Manaseki-Holland, 2012 [36]	RCT	Total 3046 children, aged 1–11 months 1524 allocated to intervention with 3-monthly bolus dose of 100,000 IU vitamin D 1522 allocated to placebo Duration of follow up: 18 months Afghanistan	Intervention group At 1 wk: 51.9 ng/ml At 1.5 months: 30.6 ng/ml At 3 months: 22.2 ng/ml At 6.5 months: 42.0 ng/ml At 22 months: 20.8 ng/ml Placebo group At 1 wk: 17.2 ng/ml At 1.5 months: 13.2 ng/ml At 3 months: 15.9 ng/ml At 6.5 months: 21.2 ng/ml At 22 months: 20.1 ng/ml	Intervention did not affect the incidence of first or only pneumonia. (incidence rate ratio 1.06, 95% CI 0.89–1.27) (p = 0.48) Increase incidence of repeat episodes of pneumonia (incidence rate ratio 1.69, 95% CI 1.28–2.21) (p < 0.0001)

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