NATURAL HISTORY OF CRYPTOSPORIDIAL INFECTION IN A BIRTH COHORT IN A SOUTH INDIAN SEMI-URBAN SLUM

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIDS - Acquired immunodeficiency syndrome

ANC – Antenatal check-up

AU - Arbitrary Units

BLAST - Basic Local Alignment Search Tool

CDC - Centers for Disease Control and Prevention, Atlanta

CHAD - Community Health and Development

CI - Confidence Interval

CMC - Christian Medical College, Vellore

DNA - Deoxyribonucleic acid

ELISA - Enzyme-Linked Immunosorbent Assay

GI - Gastrointestinal

GIS - Geographic Information System

gp - Glycoprotein

GPS - Global Positioning System

HAZ - Height-for-Age Z-score

Hb - Haemoglobin

HCl – Hydrochloric Acid

HIV - Human Immunodeficiency Virus

HR - Hazard Ratio

IgG - Immunoglobulin G

IQR – Inter-Quartile Range

IRR - Incidence Risk Ratio

KOH - Potassium hydroxide

LRI - Lower Respiratory Tract Infection

MDG - Millennium Development Goals

MUSCLE - Multiple Sequence Comparison by Log-Expectation

NIH - National Institutes of Health, USA

OR – Odds ratio

PCR - Polymerase Chain Reaction

RFLP - Restriction Fragment Length polymorphism

RNA - Ribonucleic Acid

RR - Rate Ratio

SD - Standard Deviation

SSU rRNA – Small-Subunit Ribosomal Ribonucleic Acid

UHC – Urban health centre

UN - United Nations

URI – Upper Respiratory Tract Infection

WAZ – Weight-for-Age Z-score

WHO – World health organisation

WHZ – Weight-for-Height Z-score

CHAPTER 1
INTRODUCTION

Infectious diseases are a leading cause of childhood mortality. Five of the top 10 causes of death among children living in middle and low income countries are attributed to diseases of infectious origin [1]. Although during the past few decades (between 1970 and 2013), the number of deaths among under-five children worldwide has reduced by more than 64%, an estimated 6.3 million children under the age of five were reported to have died in 2013 [2] and 64% of all childhood deaths have been attributed to infectious causes [3]. In order to reach United Nations Millennium Development Goal (MDG) 4 of decreasing childhood mortality by two thirds by 2015, there is a drive to control and prevent deaths from preventable infectious diseases by prioritizing them in various child health programmes [4].

Diarrhoea still remains one the most important infectious causes of childhood mortality accounting for 0.7 million child deaths in 2011 and contributing 28.5% of total mortality in children younger than five years [2]. Almost 88% of diarrhoea in the world is attributable to unsafe water, inadequate sanitation or improper hygiene [5]. A systematic review of community based cohort studies of diarrhoea in under-five children living in middle and low income countries published between 1980 and 2010 was done, and it estimated that each child in these countries experienced a median of 2.9 episodes of diarrhoea per year [6].

Cryptosporidium spp., an apicomplexan protozoan parasite, is one of the important causes of parasitic diarrhoea throughout the world. *Cryptosporidium* spp. oocysts are ubiquitous in nature. They survive in any environment for a long time when conditions are suitable. They are hardy and resistant to chemical disinfectants [7-8]. This infectious parasite can cause infection even with a low dose of 9-10 oocysts in healthy volunteers [9-10]. It causes self limiting diarrhoea in immunocompetent persons. However in the immunocompromised it can

cause severe and persistent diarrhoea, as seen in patients with AIDS and malnourished children, indicating the role of the immune status of an individual in determining the manifestation of the disease.

Cryptosporidium spp. has multiple routes of transmission – infection can spread through contaminated water and food [11] or direct contact, either with an infected person [12] or animals [13]. In developed countries, it has been associated with many water related outbreaks [7, 14-15]. The National Institutes of Health (NIH) and Centers for Disease Control and Prevention (CDC) have listed *Cryptosporidium* as a Category B pathogen for biodefense. This is due to the possibility of intentional contamination of drinking water supplies [16].

In many of the developing countries, cryptosporidial infections are endemic and cryptosporidiosis is one of the important causes of parasite associated diarrhoea in children [17]. The recent Global Enteric Multicenter Study (GEMS) reported *Cryptosporidium* spp. as one of the five top pathogens causing moderate to severe diarrhoea in children [18]. In India alone, cryptosporidiosis is estimated to cause 3.9 to 7.1 million diarrhoeal episodes, 66.4 to 249 thousand hospitalizations and 5.8 to 14.6 thousand deaths in children under the age of two years [19]. A large proportion of children, especially those living in resource-limited settings, have also been found to be asymptomatically infected [20-21]. In a recently concluded study from Vellore, 64.4% of the *Cryptosporidium*-infected children developed only asymptomatic infections during the first two years of life [22].

The impact of cryptosporidiosis is not limited to the diarrhoeal episode alone but has been associated with long term sequelae, with several studies suggesting that both asymptomatic

and symptomatic cryptosporidial infections have a significant adverse effect on nutritional status, cognitive development, increased overall diarrhoeal burden and mortality in children [18, 23-26]. These data support the importance of prevention of cryptosporidial infection as a more significant public health measure than the management of disease.

Although there are studies showing high cryptosporidial disease burden in developing countries, resulting in immediate and prolonged morbidity, the epidemiology of cryptosporidial infections in humans is not clearly understood. Moreover, there is a dearth of longitudinal data on the course of infection in the absence of overt disease. A clear understanding of the natural history of cryptosporidiosis and correlates of protection are essential in developing effective, efficient and sustainable disease control and preventive measures, or for designing an effective vaccine. In order to address these lacunae, we conducted intensive active surveillance of a cohort of children from birth till three years of age to study the natural history of cryptosporidiosis in an semi-urban slum in southern India by harnessing the synergistic benefits of a birth cohort study design in a community setting and new efficient molecular approaches to detect cryptosporidial infections.

CHAPTER 2

AIMS AND OBJECTIVES

The study aimed to investigate the natural history of cryptosporidial infection in children and correlates of protection against cryptosporidial infection.

Specific objectives

To describe the natural history of cryptosporidial infection, a birth cohort of children living in a southern Indian semi-urban slum were monitored for a period of 3 years.

1. To measure the incidence of species-specific symptomatic and asymptomatic cryptosporidial infection during 0 - 3 years of age.

2. To assess the effect of selected risk factors including age, breast feeding, hygiene practices and socioeconomic status on risk of cryptosporidial infection and disease.

3. To assess the effect of cryptosporidial infection/disease on the growth of the child.

4. To measure the homotypic and heterotypic protection offered by natural infection(s) with *Cryptosporidium* spp. against future infection and disease.

CHAPTER 3

REVIEW OF LITERATURE

3.1. Natural history of infectious diseases

3.1.1. Background

Infectious diseases are a leading cause of morbidity and mortality globally. Children under the age of five are especially vulnerable, accounting for almost 20% of the deaths worldwide [1] and more than 99% of these deaths occur in middle and low income countries [27-28].

Pneumonia, diarrhoea, neonatal disorders, malaria, under-nutrition and HIV are the important causes of mortality in children [28]. Diarrhoea and lower respiratory infections contributed 3.6% and 4.6% of global disability-adjusted life years (DALYs), respectively [29]. Studies by the World Health Organization (WHO) have attributed various environmental and behavioural factors, such as poor sanitation and hygiene, unsafe drinking water, poor living conditions as overcrowding and indoor and outdoor air pollution to the burden of various diseases in the developing countries [30]. In order to achieve MDG 4, there is a drive to control and prevent communicable disease by making policies for restructuring the social environment and advocating vaccines for vaccine preventable diseases. Many models of vaccine preventable diseases are derived from determining the susceptible fraction of the population. Susceptible fraction is estimated from natural immunity from presumed past infections in areas without previous vaccination coverage, attack rates of disease, disease sequelae, and case fatality rates. This information is best obtained by studying the natural history of the disease [31]. The complexity and expense of such studies mean that they are available only for a limited number of infectious diseases. Nonetheless, for development of

effective, efficient and sustainable control and preventive measures, it is of importance to have an understanding of the natural history of a disease.

3.1.2. Natural history of disease

The natural history of disease generally refers to the process of how a disease evolves and progresses over time from a pre-pathogenesis phase through a symptomatic phase of manifestation of disease to its cessation as recovery, life with disability or death in a given individual, in the absence of prevention or treatment. Every disease is unique and has its own natural history, which may not necessarily be similar in each individual [32]. The phases involved in the natural history of disease are pre-clinical/ pre-pathogenesis phase followed by clinical/pathogenesis phase.

3.1.2.1. Pre-clinical/ Pre-pathogenesis phase/ stage of susceptibility

This is a stage in which the disease has not developed yet but is marked by the presence of risk factors which favour the occurrence of the disease. The host in this stage is exposed to the risk of disease.

3.1.2.2. Pathogenesis phase

This stage begins when the disease agent enters the susceptible host and multiplies to produce tissue and physiological changes. It is to be noted that there is high variability of host response to infection with a pathogen. The host may have a clinical or subclinical infection.

Hosts may become carriers, with some having developed clinical disease and some who have not. The pathogenesis phase is divided into subclinical, clinical and disability stages.

Stage of subclinical disease: A period which begins with exposure and ends with onset of symptoms, without overt pathological changes.

Stage of clinical disease: This stage begins with onset of symptoms. It indicates the transition from the subclinical to the clinical disease stage. Clinically evident disease may be mild, severe or fatal. The clinical spectrum of the disease depends on infectivity, pathogenicity, the virulence of the causative organism and the host immune response to the organism.

Stage of disability: Some diseases resolve completely but some may leave residual effects of short or long duration, leaving a person disabled to a lesser or greater extent.

Knowledge of these different disease stages is essential for planning prevention at different levels. Targeting preventive measures at different stages of a disease can alter the natural course of disease.

3.1.3. Epidemiological triad model of disease causation

The epidemiological triad model comprises of the agent, the host, and the environment. The mere presence of this triad is not sufficient to initiate the disease in humans but interaction between them is vital to start the disease process. Hence the emergence and spread of infectious diseases in a population cannot be attributed to a single factor alone, but results from the dynamic interaction of a complex set of agent, host and environmental factors.

Presence of a critical number of susceptible hosts, presence of reservoirs and vectors, and favourable environmental conditions are the essential and necessary factors for the multiplication and propagation of the disease agent and disease in a population. The chain of infectious disease transmission consists of the infectious agent, reservoirs of infection, portal of exit and entry, the mode of transmission, and host susceptibility [33]. Altering any one of these pathways will disrupt the chain of transmission and change the course of the disease. The success or failure of any infectious disease prevention and control program will depend on a proper understanding of the interaction between these factors which determine or alter the natural history of a disease.

3.1.3.1. Disease agents

The disease agent is defined as a substance which may be tangible or intangible; non living or living, the relative lack or excessive presence of which may initiate the process of a disease. Development of a disease can be brought about by a single agent or a number of other independent agents or a combination of two or more factors which are all essential [34]. Disease agents can be biological, nutrients, physical, chemical, mechanical and social in nature.

Biological agents are macro- or micro-organisms, such as viruses, bacteria, parasites or fungi or prions (self-propagating proteins that can cause diseases), which are capable of causing disease in a susceptible host [35-36]. Infectivity, pathogenicity and virulence of the micro-organisms determine the propagation and severity of the disease [37].

The degree of pathogenicity of a disease agent can be determined by the pathogenicity index, defined as the ratio of the isolation of a specific agent from individuals with and without the disease [38]. Based on the degree of pathogenicity, an infectious agent can be classified as a primary or an opportunistic pathogen. Infectious doses of different pathogens show wide variability depending on the strain of the organism and the physical condition and age of the host [39]. The biochemical mechanisms of pathogenesis also seem to play a role on determining the infectious dose; pathogens with locally acting mechanisms requiring lower infectious doses [40]. The process by which pathogens enter a new host population or spread from a localised population group to newer populations is called microbial traffic [41-42]. A key factor influencing microbial traffic is the capability of a pathogen to effectively shift from one host species to another. Research has shown that majority of the emerging diseases were caused by the introduction of zoonotic pathogens into the human population [43]. Microbial adaptation is another aspect of an infectious agent that allows it to survive and propagate in the host population [41]. Pathogens can mutate easily, possibly due to their simpler genetic makeup, thereby producing new strains to which the host is susceptible.

3.1.3.2. Host factors

These are intrinsic factors like age, race, gender, behaviours, etc which influence a host's susceptibility, exposure and response to a disease agent. Hosts can also act as sources or reservoirs of infection, either as cases or carriers. Cases can be clinical, subclinical or latent. Subclinical and latent cases play a vital role in the transmission of disease as they often remain undetected. A carrier can serve potentially as a source of infection to others, although their infectivity is often lower than that of cases. Under certain circumstances, the carrier

state can become chronic and persist for years. The most famous example is that of Typhoid Mary, the New York cook who was responsible for at least 51 cases of typhoid fever, 3 of whom died [44].

Demographic characteristics of the host such as age, gender and ethnicity play a role in the host's susceptibility. For example, men are more vulnerable to major life-threatening chronic diseases, including cerebrovascular disease, ischaemic heart disease, neoplasms, atherosclerosis, chronic liver disease, emphysema and renal disease. Women, on the other hand, suffer usually from a different set of chronic disorders, such as iron deficiency anaemia, thyroid dysfunction, gall stones, vascular headache, joint pains, bowel disturbances, and skin allergies [45]. Gender differences in infectious differences could also be related to differential exposure to vectors like mosquitoes or Tse-tse flies or occupation related [46]. Analysis of mortality and morbidity data for acute respiratory infections suggest a higher burden of disease in young children and the elderly [47-49].

Biological characteristics can determine immunological responses of a host and could predispose to certain diseases or conditions. Genetic variations can have small or large effects on the likelihood of developing a disease. Advances in molecular techniques have now made it possible for researchers to identify genes that modulate host susceptibility to infectious diseases. Consequently, more and more linkages between host gene polymorphisms and infectious disease agents are being discovered [50-51].

Socio-demographic and behavioural factors such as race, socioeconomic status, sexual practices, lifestyle and eating habits also affect host susceptibility. The seroprevalence of

certain infectious disease agents were found to be higher among the racial and ethnic minorities in the United States [52]. Prevalence of fatal Kuru disease among the communities who practiced cannibalism was high [53]. The rapid transmission of HIV in northeast India was attributed to the high prevalence of injecting drug users in those states [54].

Nutritional status of the host is another factor that affects the acquisition and progression of infection. It has been observed that malnourished children are more likely to die from infectious diseases such as diarrhoea and acute respiratory infections [55]. The vicious cycle of malnutrition and infection is now well established - malnutrition causing an increased susceptibility to infections, which in turn, intensifies the nutritional deficiency of the host [56]. In most situations, the interaction between host malnutrition and infection is synergistic, although antagonistic effects have also been documented [57-58].

3.1.3.3. Environmental factors

Environmental changes can occur naturally and through human intervention, and their impact can be felt both globally and locally. The environment has a complex relationship with disease, influencing both survival and growth of the disease agent and contact of the host with the agent. Environmental factors can be broadly classified as biological, social and physical factors.

Biological environment include infectious disease agents, their reservoirs and vectors. Reservoirs are essential components of the infectious disease cycle, maintaining the agent in nature and helping it survive and thrive. Apart from human beings who serve as the primary

reservoir for many common infectious diseases, a wide range of animals, domesticated as well as wild, may also act as reservoirs of infectious disease agents [59]. Soil and water can also act as a reservoir of infectious agents. Some infectious diseases have more complex cycles involving multiple reservoirs [60-62]. Therefore, an understanding of the reservoir structure and dynamics is important for formulating effective control strategies [63].

Where people live, work and play results in the creation of social groups and a social environment which influences the health of individuals and groups within society [64]. Rural-to urban migration and rapid urbanisation have altered the dynamics of host-pathogen interaction resulting in epidemiological transition. A large proportion of the population in the urban areas, especially in developing countries live in slums with poor housing, overcrowding, and lack of basic facilities such as potable drinking water and good sanitation. Consequently, people living under such conditions suffer from a wide range of diseases, predominantly respiratory and gastrointestinal infections [65-66].

The physical environment has profound effects on human health. Temperature, humidity and precipitation not only affect the survival of pathogens but also influence the vector population as well as animal and environmental reservoirs [67-69]. Although a large number of infectious diseases show seasonality [67], the seasonal patterns tend to vary with geographic location. For example, rotavirus shows a distinct winter peak in the Americas, but in other parts of the world, autumn or spring peaks have been observed [70]. It was noticed that the magnitude and intensity in the incidence of poliomyelitis varies by latitude [67-68]. Thus, a proper understanding of the seasonal drivers of infectious diseases is necessary to be on the

alert and devise early outbreak warning systems and improve existing disease control strategies [67].

3.1.4. Epidemiologic studies for characterizing natural history of a disease

3.1.4.1. Cohort studies

The term cohort originates from the Latin word *cohors*, referring to a group of warriors in the Roman army [71].They are also called follow-up or incidence studies. Cohort studies are designed to capture information about aetiology of disease. They also measure the risk of disease directly. A cohort study comprises of a group of disease free healthy persons who are recruited and followed up for a certain period of time to identify the occurrence of health related outcomes like disease, recurrence, recovery and death [72]. In simple terms, a cohort study compares outcomes in two groups; one in which individuals are exposed to a certain factor with another group in which individuals are not exposed to that factor. The association between exposure and outcome becomes evident if the group which was exposed has a different frequency of the outcome, in comparison to the unexposed [73]. Measurement of multiple outcomes and time relationships are advantages of cohort studies. However, these studies are not suitable for rare diseases and diseases with long latent periods. A basic assumption in a cohort study is that the exposed subjects are representative of all exposed persons with respect to the risk of disease, and likewise, the unexposed subjects are representative of all unexposed persons in the population [37]. In addition to these, there is a low possibility of selection bias, recall bias and confounding.

The strengths of a cohort study lie in its ability to record the natural history of diseases over a period of time and to demonstrate a temporal, and possibly causal, relationship between exposure and outcome variables [74]. Cohort studies can be used to estimate rates, average risks, and times of occurrence, especially when there is little or no depletion of the cohort from competing risks. The longitudinal nature of follow-up allows for the estimation of incidence rates with person-time as the unit of measurement. The focus on time rather than individuals in the denominator allows flexibility in that each unit of person-time contributed to follow-up by a given individual possesses its own classification with respect to exposure.

Cohort studies are expensive, require lengthy follow-up periods and are often undermined by attrition, which limits the feasibility of conducting such studies [75]. Follow-up studies that go on for many years, present problems of logistics and can adversely influence validity. The core problem is to trace the subjects. A substantial number of losses to follow up can raise serious doubts about the validity of the study. Follow-up studies in which less than 60% of the subjects are traced, are generally regarded with scepticism. Sometimes follow-up of even 70 or 80% can be considered low for not providing reassurance against bias that there may be a differential loss to follow-up [35].

3.1.4.2. Organizing and following population-based cohorts

A population-based cohort study uses a sample from a defined population, or the entire population, for longitudinal assessment of the relationship between exposure and outcome. It provides an ideal setting for unbiased assessment of the exposure-outcome association, and allows the researcher to estimate the distribution and prevalence rates of relevant exposure

variables in the reference population, which can then be used to compute population attributable risks. It also allows one to assess trends in risk factors over time [76]. The major advantage of a population-based cohort study is its external validity, which, in turn, depends on the representativeness of the cohort of the entire population.

A factor that can severely affect the external validity of the cohort study is non-response or refusal at baseline. Active engagement of members of the community in the initial planning and organization of the study can reduce the non-response rate [77]. Another method of improving response rates is to allow potential participants, especially women, time to discuss the study with their family members prior to enrolment [78].

Losses to follow-up/attrition can affect the cohort's representativeness [78]. Procuring contact addresses of friends and relatives, maintaining regular contacts with participants, providing incentives periodically, collecting data through telephonic interviews or mailed questionnaires and conducting proxy interviews to obtain information on important exposure and outcome variables are some of the ways of retaining and following information of cohort participants [79].

Other reasons for participant refusal include fatigue, lack of interest or resistance by other members of the family. Death of the study participant can also be a cause of attrition. Establishing a community advisory board to act as a link between study participants, investigators and the community at large help promote bonding and ensure long-term study participation [79].

3.1.4.3. Bias in cohort studies

A major focus in any epidemiologic study is to identify and minimise biases that might occur during different stages of the study process [80]. This task can be accomplished at two levels: (a) by choosing an appropriate study design to answer a research question, and (b) by establishing and carefully monitoring valid and reliable data collection procedures [72]. Even though many types of biases have been described in literature, they are commonly classified into three general categories - information bias, selection bias and confounding [81].

A common source of information bias among the participants of a cohort study is the misclassification bias, which results when a study participant is misclassified in terms of either their exposure or outcome status. Misclassification bias can be categorized into differential and non-differential. In general, non-differential misclassification is considered to bias the risk estimate towards the null value in case of dichotomous exposures; and in situations when the exposure is a continuous variable, it produces a reduction of the dose-response slope [81]. On the other hand, in the case of differential misclassification bias, the association can be influenced in either direction- away from or towards the null [82]. The bias due to misclassification depends on the complex interplay involving differences in specificity and sensitivity of the classification method, and the frequency of exposure and outcome. This bias is mainly dependent on the specificity in the case of cohort studies, and is greater in rare diseases [83]. Surveillance bias, a non-random type of information bias, refers to the idea that "the more you look, the more you find". It occurs when some patients are followed up more closely or have more diagnostic tests performed than others, often leading to an outcome diagnosed more frequently in the more closely monitored group [84].

The regression dilution bias is another form of information bias created in longitudinal studies analyzing the effect of baseline determinants of a continuous exposure variable to an outcome. This is associated with the concept of regression to the mean. This is seen as a trend wherein a variable with an extreme value on its first assessment tends to be nearer to the measure of central tendency of its distribution on later measurements, which results in an underestimation of the association between an outcome and exposure [82].

Selection bias is a systematic error resulting from the selection of study participants in a manner that deviates from random selection so that some individuals have a higher or a lower probability of being included in the study sample. This results in a sample that is not representative of the population that is targeted [82]. Non-response and loss to follow-up are two of the greatest concerns in the conduct of a cohort study, both of which can result in a selection bias. A high rate of non-response and/or loss to follow up can severely undermine the validity of study findings, more so if such losses are different in both the exposure and outcome groups. Even if losses are evenly distributed with respect to either exposure or outcome, it might still result in an exaggerated or a diminished association between the two without significantly altering the prevalence of exposure or the disease incidence [85].

The term confounding refers to a condition wherein the observed association between an exposure and outcome variable is due to the influence of a third variable (or a group of variables) which can either spuriously strengthen or weaken the true association [72]. The effect of confounding can be minimized during the stage of designing the study or during the time of analysis, or both. At study design matching the study participants on potential

confounders is commonly done. Another way to control for confounding is to adjust for the confounding variables at the time of data analysis [81].

3.1.4.4. Quality assurance and quality control in cohort studies

The reliability and accuracy of the conclusions inferred from any study, to a great extent is dependent on the quality of the data that is collected. Poor quality data, or data biased due to the use of faulty instruments or errors in protocol implementation may bring about either type I or type II errors. The completeness and clarity of questionnaires, interview technique, accuracy of the instruments used and measurement errors can all affect the quality of collected data. The longitudinal dimension of cohort data can result in situations related to passing over time, such as a change in equipment and staff turnover. The large volume of collected data in itself can pose challenges to quality control [86].

Quality assurance activities are undertaken prior to the collection of data, thereby ensuring that the collected data is of highest possible quality. An important component of such activity is to create a proper manual of operations documenting the study protocol, which can be used as a reference document by all data collection staff. Training and certification of the study personnel as per the protocol is another vital component of quality assurance, and such procedures should be created before the study begins. Procedures should also be developed to ensure maintenance and recalibration of equipment regularly and these efforts should be documented well. Data collection instruments should be pilot-tested to ensure collection of reliable and valid data on an appropriate scale. Similarly, electronic databases need review and editing before entering any data and, wherever possible, double-entry process should be

used [86]. Soon after the commencement of data collection, quality control procedures need to be implemented in order to identify and correct sources of either bias or excessive errors in the data, both during and after the data collection. It includes periodic and timely reports on meeting the recruitment goals and collection of data on key study parameters.

Analysis of data should be performed at regular intervals to assess the performance of field staff, and significant deviations from other members of the group should be reported. In addition, data should be analysed by study staff to identify drifts over time. Frequent training and retraining of the staff members should be carried out in order to ensure quality and minimise variability. Routine analysis of data should also be undertaken to identify problems such as extreme or inconsistent values. For data collected longitudinally, the quality control procedures should be able to recognise inconsistent answers by participants, who can then be revisited by the field staff for clarifications. In case of equipment, they should be maintained and calibrated on a regular basis in order to minimise measurement errors resulting due to faulty instruments. If older equipment needs to be replaced, both the old and the new equipment should be used to the collect data to ensure comparability [86].

3.1.4.5. Analysis of cohort data

The advantage of a cohort study is that it allows the researcher to directly calculate the incidence of a particular outcome and to compare these rates across different exposure categories. If complete follow-up data is available for all members of the cohort, the incidence can be measured by simply adding the number of events that occur during the follow-up period and dividing it by the total number of subjects recruited. However, in a

typical cohort study, each subject might have different follow-up periods, due to loss to follow-up or shorter follow up because they were recruited later in the accrual period for the study [72]. Hence, it is possible that the outcome of interest might not have occurred in some subjects till the end of follow-up. In addition, survival data are not normally distributed, but are usually skewed, typically with many events which happen early and relatively fewer ones happening late depending on the condition being investigated. These characteristics of the data require the use of special analytical techniques such as survival analysis [87].

3.1.4.6. Survival analysis

The primary variable in a survival analysis is the calculation of survival time. This is defined as a "non-negative random variable measuring the time interval from an origin to the occurrence of a given event" [88]. The observation of survival time requires two components to be defined clearly: (a) the beginning point or origin of the study, and (b) the endpoint, i.e. the occurrence of the event of interest. There are two ways which can lead to an incomplete observation of the survival time. They are censoring and truncation. If the value of an observation is incomplete due to factors that are random for each subject, it is called a censored observation. A truncated observation, on the other hand, is incomplete because of a selection process inherent in the study design [89]. These require special methods of data exploration and analysis as, under such circumstances, the standard statistical methods cannot be used [87].

In general, survival data are described and modelled in terms of two related probabilities, they are survival and hazard. The survival function (alternatively called survival probability),

typically denoted as *S(t)*, is the probability of a subject surviving up to a specified future time *t*, from the time of origin. This is important in survival analysis as the survival probabilities for various values of *t* directly describe the survival experience of the cohort [87].The hazard function, commonly denoted by *h(t)*, is the instantaneous rate of a specified outcome in an individual at time *t*. In other words, it is the probability that an individual, under observation at a specified time *t*, has an event at that time. The cumulative hazard function is the sum (or integral) of hazard functions over time. The instantaneous and cumulative hazard functions are of interest because they provide an insight into the conditional failure rates and help select the most appropriate survival model [87, 90].

3.1.4.7. Estimating survival and hazard functions

The estimation of survival probabilities from the observed survival time can be done using different methods, both parametric and non-parametric. The simplest method of summarising survival data is through the direct method, i.e. by estimating the survival probability at a given time by calculating the ratio between the number of survivors at the end of a specified time interval and the number of participants at the start of the study. However, in this method, censored observations are included only from those people who have completed the specified follow-up time [91].

Another method used to calculate the survival probabilities is the life-table (actuarial) method devised by Cutler and Ederer. In this method, the total duration of follow-up is divided into short time intervals, the length of which is set a priori based on the distribution of events over the period of study. The cumulative probability of survival at the end of a given interval can

be calculated by multiplying the conditional probabilities over all intervals up to the specified time point [91]. The life table method utilises all available survival information in computing survival rates by including data on subjects with partial follow-up information, in addition to those with complete information.

A commonly used method to calculate survival probability is a non-parametric estimation method suggested by Kaplan and Meier, also known as the product-limit (PL) estimate. Unlike the life table method, which has pre-specified time intervals, in the PL method each outcome, by itself, occupies a time interval. The time intervals are then ranked in ascending order of magnitude. Thus, the estimated probability of survival, *S(t)*, is considered to be a step-function which changes at the time of observed outcomes. The cumulative probabilities are calculated as the product of a series of estimated conditional probabilities [92]. The cumulative survival curve, which plots the survival probabilities against time, can be used to calculate the median survival time, which is defined as the time at which the probability of survival is equal to 50% [87].

The difference in survival curves between two or more groups can be compared using nonparametric techniques. A commonly used method for such comparisons is the log-rank test, which is designed to detect the difference in survival experience between two groups considering the rate of difference to be constant over time, i.e. the survival probability in one group remains higher or lower than the other, throughout the duration of follow-up [91]. The observed number of events at any time point is compared with the expected number of events at that point, which follows the χ^2 distribution with (g-1) degrees of freedom, where g is the number of groups [87].

3.1.4.8. Regression models in survival analysis

Although significance tests, such as the log-rank test, compare the difference in survival probabilities between two groups (thereby providing a *P*-value to suggesting whether or not the differences are statistically significant), they do not provide an estimate of the magnitude of difference. Further, they cannot adjust for the effect of covariates that can potentially impact the observed association between the exposure and outcome variables. Thus, in order to estimate the actual effect size of an exposure variable with respect to other potentially confounding factors, regression methods need to be used. In survival analysis, the regression methods used can broadly be divided into two broad categories - parametric and semi-parametric models.

Parametric models assume the hazard follows a specific statistical distribution. Some of the well known distributions used in parametric models include exponential, Weibull, lognormal, gamma, Gompertz and log-logistic. Of these distributions, the ones commonly used are the exponential and the Weibull regression models. The exponential model assumes a constant hazard rate. On the other hand, the Weibull distribution has broader applications as it can be additionally used to model survival distributions with either increasing or decreasing risk over time [88].

A semi-parametric method of analysing survival data is the Cox proportional hazards (PH) model, which assumes a multiplicative relationship between the hazard function and a set of covariates. Another assumption of this model is that the effect of covariates on the hazard function is log-linear. The advantage of the Cox PH model is that, being a semi-parametric

method, no assumption about the distribution of the observed events over time is required; although the hazard ratio should be constant over time [91]. If the hazard is not constant over time, i.e. if proportional hazards assumption is not fulfilled, a possible solution to overcome this problem is to perform a stratified analysis by breaking the data into different subgroups and then applying the model to each stratum.

3.1.4.9. Cross-sectional and case-control study designs

To look at the natural history of a disease, cross-sectional and case-control study designs can also be used. The disadvantage of both the designs is that they cannot capture the incidence of a disease and cannot establish the temporal association of exposure and the disease. This is because cross sectional studies look at exposure and disease in a population at given point of time, whereas case-control studies retrospectively explore the causal association of a disease with the exposure. Both the study designs have the advantages of involving less time and money, and provide vital information for hypothesis generation for further exploration.

3.1.5. Epidemiology of enteric infections

Enteric infections, can exist even without overt diarrhoea, but can have serious effects on absorption of nutrients, and child development and mortality worldwide. In 2011, globally about 0.71 million children died from diarrheal disease annually, before reaching their fifth birthday [93]. The proportion contributed by Africa and South Asia was 25% and 31% respectively [94-95].

As mentioned earlier, in developing countries children less than five years of age are the more affected, suffering a median of 2.9 episodes of diarrhoea per child-year [6]. Children living in poor resource settings also have higher chance of death when they develop diarrhoea due to lack of access to health care facilities and timely interventions with effective treatment [96]. Diarrhoeal diseases have been attributed to unsafe drinking water, poor sanitation and unhygienic practices [97]. In addition, recurrent diarrhoea leads to poor absorption of nutrients resulting in chronic malnutrition and developmental delay in children [98-99].

In India, about 20% of all post-neonatal deaths in children under the age of 5 years are due to diarrhoea, resulting in an estimated 391,000 deaths annually [100]. In birth cohort studies in Vellore, south India, gastrointestinal illnesses comprised of 18.4% - 26.8% of all morbidities experienced by children during the first three years of their life. The incidence of gastrointestinal morbidity was highest during infancy, thereafter decreasing rapidly [40, 101].

Amongst the bacterial, viral and parasitic causes of acute diarrhoea studied till date, the most frequently reported aetiological agents in children in the developing countries are diarheagenic *Escherichia coli*, rotavirus, *Shigella* spp, *Campylobacter jejuni, Giardia lamblia*, non-typhoidal *Salmonella*, *Entamoeba histolytica* and *Cryptosporidium* spp. astrovirus, enteric adenovirus and norovirus have also been reported. The relative contribution of different pathogens vary by location; children living in areas of poor sanitation having a higher risk of acquiring bacterial and parasitic infections [102].

In the recent Global Enteric Multicenter Study, *Cryptosporidium* was one of the top five pathogens identified to cause moderate to severe diarrhoea in children [18]. *Cryptosporidium*

is a parasite responsible for 2-6% of all diarrhoeal disease in immunocompetent people and 14-24% of diarrhoea in people with HIV [103].Childhood cryptosporidiosis is not limited to diarrhoea alone, but has long-term sequelae like growth impairment and cognitive deficiency [24-25].

3.1.6. *Cryptosporidium* and Cryptosporidiosis

3.1.6.1. Cryptosporidiosis

Cryptosporidiosis is caused by *Cryptosporidium* spp., intracellular protozoan parasite belonging to the family Cryptosporidiiae within the phylum Apicomplexa [104]. *Cryptosporidium* spp. was first reported by Tyzzer in 1907. Oocysts of *Cryptosporidium* are environmentally resistant and often retain their infectivity for a protracted period of time [105] and survive most water disinfection treatments [106]. It is an ubiquitous parasite and triggers infection in volunteers with a minimal dose of 9 to 10 oocysts [107]. Cryptosporidial infections are self limiting in immunocompetent hosts and can be asymptomatic on repeated exposure, but may have devastating effects on immunocompromised patients like ones with severe malnutrition and those with AIDS [108]. The disease came to notice of the healthcare community in the first few years of the AIDS pandemic and afterwards in 1993 with the epidemic in Milwaukee, Wisconsin [109].

3.1.6.2. Molecular epidemiology of Cryptosporidiosis

There are presently 16 species of *Cryptosporidium*, identified from a large diversity of hosts in all five groups of vertebrates, including humans [110]. The anthroponotic species *C. hominis* which was until recently called *C. parvum* genotype I almost exclusively infects humans. *C. parvum*, is the zoonotic species of genotype II, which infects humans and animals [110-111]. Other *Cryptosporidium* spp. known to infect humans include *C. felis, C. canis, C. meleagridis* and *C. muris* [110]. Predominantly the infections in humans are caused by *C. hominis,* including those in middle and low-income countries [112-113]. In order to plan for preventive measures, it is vital to identify the species and sub-genotypes to differentiate between anthroponotic and zoonotic strains.

3.1.6.3. Transmission of cryptosporidiosis

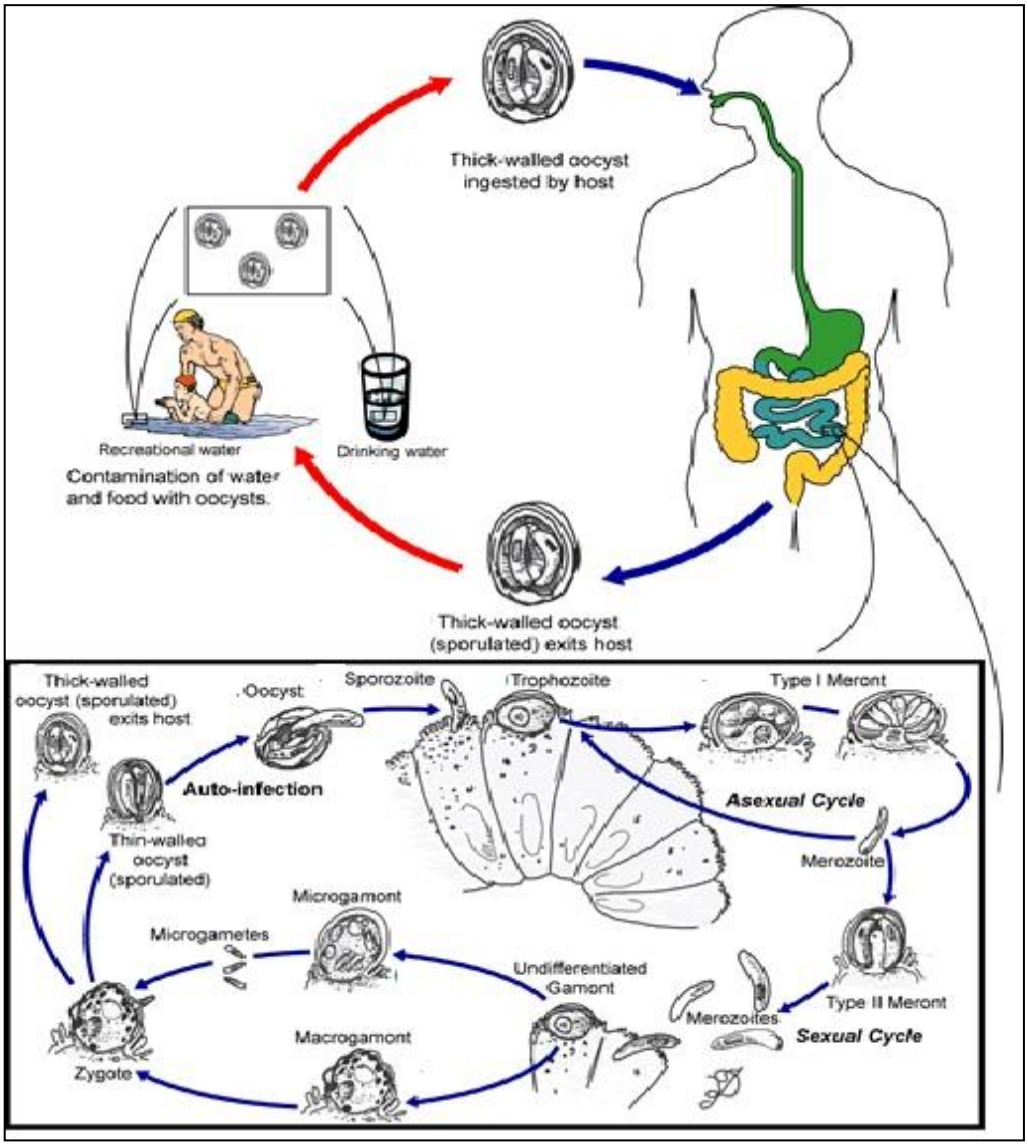
The common reservoirs for *Cryptosporidium* are humans, cattle, and sheep. Birds (hens, parrot), reptiles and other domestic animals (cats, dogs, pigs) and rarely, wild animals, also act as reservoirs for this protozoan parasite [114-115]. Manure used as fertiliser for growing vegetable can also be a source of infection [115]. Cryptosporidiosis in humans has multiple routes of transmission, predominantly through the faecal-oral route due to ingestion of contaminated water and food [116]. Most outbreaks that have been documented have been attributed to contamination of water supplies used for drinking and recreational activities [116]. The other modes of transmission are through direct contact with animals which are infected (zoonotic), person to person contact and mechanical transport through soil and insects [117] and possibly air borne [118].

3.1.6.4. Life-cycle of *Cryptosporidium*

A host is required for the survival and reproduction of *Cryptosporidium* [119-120]. The pathogen has a complex life cycle. It comprises of a sexual and an asexual reproductive stages [121-122]. All the *Cryptosporidium* species undergo endogenous development which results in the formation of a cyst which is excreted in the host's faeces [116].

Infection starts with the ingestion of oocysts. Once ingested, cysts undergo a process of excystation in the small bowel [107]. Released sporozoites invade intestinal epithelial cells, where the parasite replicates within a parasitophorous vacuole via asexual (schizogony or merogony) and sexual cycles (gametogony). In the asexual cycle, merozoites get released into the lumen of the intestine from where they are able to re-invade adjacent epithelial cells. After 2 cycles of merogony, microgametes which are released into the intestinal lumen undergo fusion with macrogamonts to form zygotes. These mature to form oocysts which are excreted in the faeces. Thus, at some point in the life cycle, microgametes, merozoites, sporozoites, and oocysts, are present in the intestinal lumen where they are potentially accessible to mucosal antibodies. The life cycle of *Cryptosporidium* is shown in Figure 3.1.

Figure 3.1.: The life cycle of *Cryptosporidium*



Source: http://www.dpd.cdc.gov/dpdx

3.1.6.5. Pathogenesis of the infection and clinical features

Viable oocysts release sporozoites after ingestion, which later attach to the intestinal epithelial cell and invade it. Sporozoites contain secretory apical organelles with distinct structures and functions essential for host cell invasion [123]. Pathogenesis results from changes in the structure and function of the intestine. Infection results in the loss of microvillus border, shortening and fusion of the villi, and lengthening of the crypts, resulting in malabsorption because of loss of membrane-bound digestive enzymes, reduced absorption, decreased glucose-NaCl absorption, and increased secretion of chloride anion. Proinflammatory cytokines like TNFα and IFNγ, which are produced, contribute to cryptosporidial pathogenesis [107, 124].

The infection presents in a highly variable fashion. It is usually characterized by watery diarrhoea. Abdominal pain usually accompanies it. Occasionally constitutional symptoms like malaise, fever, loss of appetite, nausea and vomiting also occur. These symptoms usually begin approximately 5 days to 2 weeks after the ingestion of oocysts. In an immunocompetent host the duration of illness can last from a few days to 5 weeks. It is to be noted that in many exposed hosts, particularly in adults, infection is frequently asymptomatic [125].

3.1.6.6. Diagnosis

Microscopy has remained the standard tool for diagnosis of intestinal cryptosporidiosis in humans. The most commonly used technique is the modified acid-fast stain, which is carried

out on concentrated stool samples. Cryptosporidial oocysts appear acid fast and irregularly stained and are in the size range of 4-6µ. Other non-morphological tests include immunofluorescence assay (IFA) and ELISA generally based on the oocyst wall protein, and immunochromatography for detection of oocysts or antigen in the stools [126]. Flow cytometry based tests using standardized methods are used to detect oocysts in large volume of samples such as sewage or water, but are not done on a routine basis. PCR based tests are also used in surveillance and environmental samples [108] and have better sensitivity and specificity in the detection of infection [127-129].

3.1.6.7. Immune response in cryptosporidiosis

The immune status of the host determines the infection outcome and severity. The nature of response of immune system in human cryptosporidiosis is incompletely understood. Data from immunodeficient animal models have shown that a Th_1 response involving primarily TCR αb + CD4 + lymphocytes with IFNγ, and IL-12 playing a major role [130]. Experimental animal studies with mice and calves demonstrate that immunity is dependent on the number of the CD4 T cells increasing within the intestinal intraepithelial lymphocytes and generating IFNγ, IL-12 may play a role, possibly through its ability to induce IFNγ production [131]. The susceptibility of HIV/AIDS patients to this pathogen and resolution of cryptosporidiosis following immune restoration emphasizes the importance of CD4 + T cells [132]. Innate immune mechanisms might play an important role in resistance to cryptosporidial infection. In AIDS patients, mutations in genes coding for a component of complement cascade, the mannose-binding lectin were associated with the increased risk of cryptosporidiosis [133]. It is likely that the adaptive immune response is required for

infection resolution and innate immune mechanisms play a role in resisting the infection [133].

3.1.6.8. Treatment

There are no consistently effective chemotherapeutic agents for the treatment of human cryptosporidiosis although many studies have evaluated therapy for cryptosporidiosis in patients with AIDS [131]. Nitazoxanide has been licensed as effective treatment for use in immunocompetent patients, but was ineffective in HIV patients in a meta-analysis [134]. Anti-retroviral therapy has great impact in the outcome of cryptosporidiosis in HIV patients. It indirectly restores immunity by increasing the CD4 counts and the protease inhibitors also have a direct effect on oocyst shedding, thereby providing a sustained therapeutic effect [124].

3.1.6.9. Cryptosporidiosis in individuals with HIV/AIDS

Diarrhoea is the most common gastrointestinal symptom reported in HIV infected patients. Patients with CD4 T-cell counts of <150/ml who become exposed to *Cryptosporidium* spp., are most likely to develop a persistent infection and their diarrhoea could become life-threatening [135]. Patients with acquired [136] or congenital [137] immunodeficiencies can have protracted infections, lasting months or even years, involving the entire gastrointestinal tract in addition to the hepatobiliary and respiratory tracts [138]. They can have chronic watery diarrhoea and excrete oocysts in their stools during this period, resulting in severe dehydration, loss of weight and malnutrition, prolonged hospitalizations and death [131,

134]. AIDS patients had a significantly shorter life span from time of diagnosis of cryptosporidiosis [139]. However, anti-retroviral therapy (ART) restores immune function and positively affects the outcome of cryptosporidial infections in HIV patients. Prior to ART being used, cryptosporidiosis accounted for 10 to 20% of diarrheal cases in HIV-infected individuals living in developed countries, and as much as 50% in developing countries [140]. In Asia, cryptosporidial infection rates ranged from 0.5% to 56.5% in HIV-infected individuals with or without diarrhoea [140]. India accounts for approximately 50% of the HIV infections in Asia [141]. Reports on the prevalence of *Cryptosporidium* infections from different parts of India range from 4.7% to 56.5% in HIV-infected individuals [142-148].

3.1.6.10. Cryptosporidiosis among children in developing countries

Cross sectional surveys in the developing world report that cryptosporidiosis is endemic in children, with high rates of asymptomatic infection and low symptomatic infection rates [149-150], indicating that not every infected child would have diarrhoea or develop gastrointestinal symptoms. However, longitudinal studies from Brazil and Israel and cross sectional studies on children with diarrhoea from Uganda and Mexico have reported a greater prevalence of symptomatic infection than asymptomatic infection, in contrast to a study done in Peru which identified more asymptomatic infections [149, 151-152].

Cryptosporidiosis is more common in malnourished children in developing countries with more severe consequences, possibly due to impaired T cell responses. Studies of Haitian children with cryptosporidial infection reported that malnourished children had increased levels of systemic and faecal proinflammatory cytokines [153]. Studies from Brazil suggest

that a single episode of cryptosporidial infection predicts an increased risk of diarrhoeal disease in future [25]. Persistent diarrhoea has been frequently reported from developing countries [151]. A study from Guinea Bissau reported cryptosporidiosis in infancy was associated with mortality [26].

When cryptosporidial infection takes place in early childhood, long-term ill effects on the cognitive function, and growth faltering and stunting may occur. The risk of stunting increases with number of episodes of cryptosporidiosis per year. Cryptosporidiosis, even at a subclinical level, has been found to have a significant negative effect on the growth of children, both in terms of weight and height [24, 154-155]. Studies from Peru suggest that both symptomatic and asymptomatic cryptosporidiosis were associated with growth faltering and that recovery is slower in children with symptomatic infection [24, 98, 154].

Studies in different settings have identified male gender, low birth weight, presence of animals (pigs, cats and dogs) in the household, overcrowding, storage of cooked food, drinking non-potable water, lack of breast feeding, low socioeconomic status, rainy season and diarrhoea in the family as potential risk factors for cryptosporidiosis [151, 156-159].

3.1.6.11. Cryptosporidiosis among Indian children

Community and hospital based studies from India have demonstrated *Cryptosporidium* to be one of the important causes of infectious diarrhoea in children, with estimated positivity rates ranging from 1.1% to 34% [124, 160]. A high proportion of asymptomatic cryptosporidiosis has also been reported in Indian children. Studies on healthy children from Kolkata and

southern India showed an asymptomatic carriage rate of 2.3% and 3% on examination of faecal samples [161-162]. The prevalence rates identified in these studies are not directly comparable, because studies differed in their methods, patient recruitment and diagnostic tests employed in detection of the pathogen, as shown in Table 3.1.

A birth cohort from southern India showed *Cryptosporidium* to be one of the common parasitic causes of diarrhoea in children under the age of 3 years [163]. In a subset of infected children, 40% experienced multiple infections. The study also showed that about 50% of the children had prolonged asymptomatic shedding of the oocysts before and after a *Cryptosporidium* associated diarrhoea [164].

A quasi-experimental study from Vellore in southern India showed that almost 67% of the children had cryptosporidiosis during a two-year follow-up period, most of these being asymptomatic infections. It also demonstrated that protected/packaged water was not associated with a decreased risk of cryptosporidiosis [22].

Table 3.1.: Cryptosporidiosis among Indian Children

Location	Year	Study design	Study setting	Sample size	Age group	*Cryptosporidium* detection method	% Symptomatic	% Asymptomatic	Reference
Vellore	2014	RCT	Community/ Hospital	124	0.5-5	Microscopy, PCR	33.8		[160]
Vellore	2013	Quasi experimental	Community	176	<2	PCR, ELISA serum	23.8	67	[22]
Aligarh	2012	Cross sectional	Hospital	250	<12	Microscopy, ELISA	11.6		[165]
Vellore	2010	Cross sectional	Hospital	2579	<2	Microscopy	2.7		[128]
New Delhi	2008	Cross sectional	Hospital	50	<12	Microscopy	14		[166]
Secunderabad	2007	Cross sectional	Hospital	681	<12	Microscopy, ELISA, PCR	7.6		[167]
Kolkata	2006	Cross sectional	Hospital	1338	<5	Microscopy, IFA, PCR	4.6	1.2	[168]
Kolkata	2005	Cross sectional	Community	609	<12	Microscopy		2.3	[161]
Delhi	2002	Cross sectional	Hospital	127	<5	Microscopy	18.9		[157]
Manipal	2002	Cross sectional	Hospital	780	<5	DFA	6.4	3	[169]
Vellore	2001	Cross sectional	Hospital	249	<3	Microscopy		7.2	[170]
Secunderabad	2001	Cross sectional	Hospital	1002	0.25-3	Microscopy	6		[171]
Chandigarh	1999	Cross sectional	Hospital	355	<12	Microscopy	1.4		[172]
Manipal	1995	Cross sectional	Hospital	106	<5	Microscopy	1.8		[173]
Amritsar	1995	Cross sectional	Hospital	150	<3	Microscopy	1.3		[174]
Varanasi	1993	Case control	Community	1136	<5	Microscopy	3.8	1.3	[175]
Kolkata	1993	Cross sectional	Hospital	1337	<15	Microscopy	7.4		[176]
New Delhi	1991	Cross sectional	Hospital	380	<10	Microscopy	4.9		[177]
Delhi	1991	Cross sectional	Hospital	100	<2	Microscopy	5		[178]
Idukki	1989	Cross sectional	Hospital	560	<10	Microscopy, IFA	6	3	[162]
Kolkata	1989	Cross sectional	Hospital	566	<5	Microscopy	5.6	1.2	[179]
Bhubaneswar	1989	Cross sectional	Hospital	77	<8	Microscopy	13		[180]
Mumbai	1989	Cross sectional	Hospital	180	<5	Microscopy	4.4		[181]
Chandigarh	1987	Cross sectional	Hospital	375	<12	Microscopy	1.3		[182]
Vellore	1985	Not available	-	-	<3	-	13.1	9.8	[183]

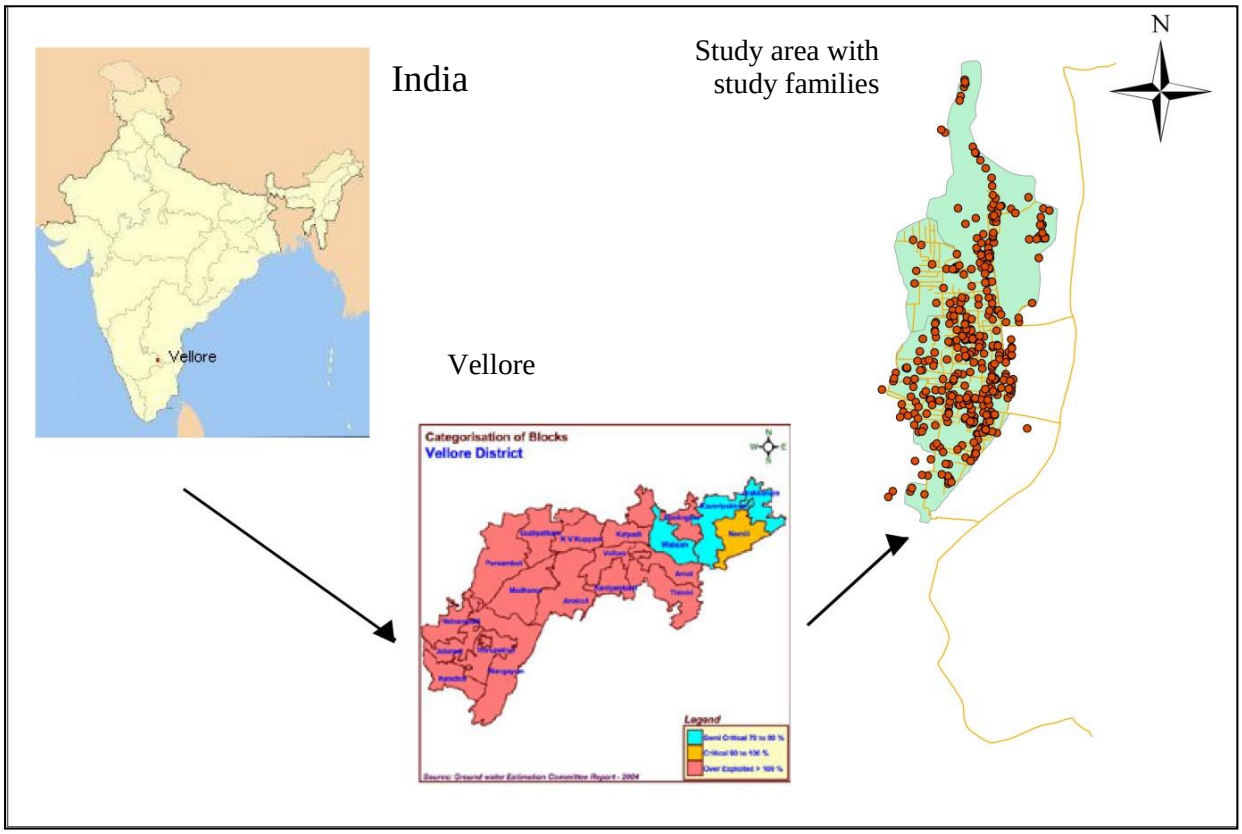
CHAPTER 4
MATERIALS AND METHODS

4.1. Study area and population

The study site is located in four geographically adjacent semi-urban slum areas, Chinnallapuram, Ramnaickapalayam, Kaspa and Vasanthapuram, on the western outskirts of Vellore, Tamil Nadu, India (Figure 4.1). The area is called a semi-urban slum, as it partially fulfils the definition of slum by the Government of India (compact settlement of at least 20 household with a collection of poorly built tenements, crowded together, with inadequate sanitary and drinking water facilities in unhygienic conditions) [184]. Vellore is located at 12.55 N latitude and 79.08 E longitude and is approximately 220 m above the mean sea level. Vellore has a dry and hot climate, with a temperature range from 18°C in December to 44°C in May. It receives its maximum rainfall during the Northeast monsoon (September – November) with a mean annual rainfall of 996.7 mm (http://vellorecorp.tn.gov.in).

The total population in the area was approximately 43000 during a census in 2010. There were 9170 families residing in the area with a median (IQR) family size of 4(3-6). A majority, 76%, of the population live in nuclear families. Predominantly, people (67%) live in "pucca" (concrete roof and floor)/permanent houses and 10% live in hut/ "kutcha" (thatched roof, with mud or concrete floor)/semi-permanent or temporary houses. The area has an almost equal proportion of Hindus (52%) and Muslims (43%).

Figure 4.1.: Map and location of the study area



The most common occupation of the families is to make homemade “beedis” (indigenous cigarettes made of tobacco wrapped in dried tendu leaves) and followed by unskilled labour (45%). The population is predominantly of low and middle socioeconomic strata. Almost 60% of the families belonged to low socio-economic strata (SES), and 4% belong to high SES. Roads are paved, but have open drains that are cleaned manually by the city administration. The local administration supplies piped drinking water to domestic taps and street stand-pipes at intervals ranging from 2 to 28 days. The water is sourced from deep bore wells in a dry river bed about 8 kilometers away and was rarely chlorinated before distribution. The common practice is to collect and store water in multiple wide mouthed plastic/steel containers. People usually do not boil water before consumption.

Free government health facilities are available at the Urban Health Centre (UHC), located in the study area, and at a 500-bed government general hospital located five kilometers away. There are also large numbers of private clinics, nursing homes and traditional medicine clinics. The 2500-bed Christian Medical College (CMC), Vellore and its extensions – the Community Health and Development (CHAD) and the Low Cost Effective Care Unit (LCECU) hospitals, are located within a few kilometers of the study area. The area has a vast network of paved roads and streets and public and private transport facilities.

4.2. Study design and recruitment

Prior to the commencement of the study, a three day workshop was conducted by experienced field supervisors and study coordinators for the field team. All the questionnaires (forms on census, delivery details, morbidities, clinical profile of diarrhoea, breast feeding

practices, growth and hygiene practices) were discussed and explained in detail. Practical training was conducted regarding collection of data, interviewing the participants, daily house visits and importance of participant-field worker rapport. Post training period, a pilot study was conducted where field workers were asked to interview the mothers visiting the study clinic located in the study area after obtaining an informed consent. The responses were cross-validated by the field supervisors and the study coordinators to check for uniformity and consistency. Discrepancies were corrected, clarified and a feedback was given to the trainees. At the start of the study, house visits and surveillance data were collected in the presence of the supervisors. Every six months the field team received retraining. All this was done in order to standardise the way the data was collection and reduced the inter-observer variations.

After the training, a door-to-door survey was conducted by trained field staff from March 2009 to May 2010 to identify new pregnancies by interviewing women of childbearing age. Children of pregnant women intending to stay in the study area for a period of 3 years were eligible to participate. Recruitment commenced after written informed consent was obtained from the parents or legal guardians of the eligible children. Infants with very low birth weight (less than 1500 gm) and major congenital malformations were excluded from participation and data were collected on pregnancy outcomes where ever available. The study was approved by the Institutional Review Boards of the Christian Medical College, Vellore, India and the Tufts University Health Sciences campus, Boston, USA.

Baseline information on demography, socioeconomic factors, delivery and birth, post-delivery details were obtained from each household after the child was born, by the trained

field staff using a structured questionnaire. Socioeconomic status was assessed using a five point scale, which is a modification of the Kuppuswamy scale, and includes highest education, occupation, possessions and type, ownership and number of rooms in a house [185-186].

Each of the enrolled infants had two surveillance visits a week. During the surveillance visits, the child was observed, and the caregiver was interviewed about any illness (diarrhoea, vomiting, fever, cold/cough or any other illness) experienced by the child during each day since the last visit, using a structured questionnaire. Field workers were trained to use standard definitions to identify illness and follow standard protocol for data collection (Table 4.1). The data collected by the field workers was validated weekly by a field supervisor in a 10% random subsample.

Table 4.1.: Definitions used by the field workers in assessing morbidity in the cohort

Symptom/Illness	**Description**
Diarrhoea	Three or more watery stools per day or a change in number or consistency reported by the mother and which she considers indicative of diarrhoea.
Vomiting	Three or more episodes of vomiting
Fever	Increased temperature of the body as perceived by primary caregiver
Cold/ cough/ runny nose	Cough/ runny nose with or without fever
Wheezing *	Difficult breathing with an audible sound
Pneumonia *	Physician based diagnosis
Tuberculosis (primary) *	Physician based diagnosis
Typhoid	Physician based diagnosis
Exanthematous fever	Fever with visible eruptive lesions of the skin marked by redness, rash and prominence
Jaundice	Yellowish discoloration of the white of eyes and skin
Acute central nervous system infections *	Physician based diagnosis
Skin infections	Any skin associated lesions including infections, rashes, itching
Eye infections	Painful conditions of the eyes, swelling and discharge from the eyes
Ear nose and throat infections	Painful conditions, swelling and discharge from the ears, other conditions of the nose and throat
Convulsions due to non-infectious causes *	Physician based diagnosis
Incessant crying	Prolonged crying, due to irritation or pain associated with any abnormal condition, as reported by the mother.
Injuries, stings & bites	includes injuries, scalds, burns, stings, bites
Others	Miscellaneous
* Diagnoses to be confirmed by a physician	

During an event of diarrhoea (defined as 3 or more loose, watery stools in a 24-hour period [187], details regarding the colour, consistency, frequency of passing of stool, dehydration and treatment were recorded every day until the end of the episode. An episode was defined as at least one day of diarrhoea, preceded and followed by minimum of two days without diarrhoea. Severity of the diarrhoeal episode was assessed using the Vesikari scoring system, which is used extensively to assess severity of rotavirus diarrhoea in children. An episode was considered mild for a score ≤ 5, moderate for a score of 6-10 and severe for scores >10 [188].

A total of three diarrhoeal stool samples were collected for every episode. The diarrhoeal samples were tested for the presence of *Cryptosporidium* spp. by PCR, and bacteria by culture. The child was monitored daily until the end of the diarrhoeal episode. Surveillance stool samples were collected twice monthly for screening for *Cryptosporidium* spp. by PCR from each study child.

As mentioned earlier, at the beginning of the study, the field team also received training on measuring the height and weight of children during the protocol training. Inter- and intra-observer standardizations were carried out during these training workshops. Every month, weight and height/length were measured for all children at the study clinic. Only in situations where the child could not be brought to the clinic, field workers took the anthropometric measurements at home. Electronic weighing scales were used to weigh the children and a length board/infantometer was used to measure the length of the children**.** The instruments were calibrated every 6 months.

A month prior to delivery, a maternal blood sample was collected and a cord blood where possible, or a blood sample from the infant within 45 days after birth was collected from the infant. Every 6 months blood samples were collected from the study children for IgG antibody level determination by ELISA.

Table 4.2.: Definitions used in the study:

End of diarrhoeal episode	Absence of diarrhoea for 48 hours following a diarrhoeal episode
Acute diarrhoea	Diarrhoeal episode lasting ≤ 14 days
Persistent diarrhoea	Diarrhoeal episode lasting > 14-30 days
Chronic diarrhoea	Diarrhoea lasting >30 days
Malnutrition	Graded by weight for age z-score (WAZ), height for age z-score (HAZ), and weight for height z-score (WHZ)
Cryptosporidium infection	Detection of *Cryptosporidium* by PCR in a stool specimen or fourfold increase in IgG in sequential serological samples
Absence of *Cryptosporidium* infection	No detection of *Cryptosporidium* by PCR in any stool specimen or no four-fold increase in IgG between two sequential serological samples
Asymptomatic cryptosporidiosis	Presence of *Cryptosporidium* by PCR and no diarrhoea for a period of two weeks before and two weeks after the sample was collected
Symptomatic cryptosporidiosis	Diarrhoea with *Cryptosporidium* detected by PCR at any time 1 week before and 1 week after the sample was collected
Diarrhoea in the absence of *Cryptosporidium*	Diarrhoea with no *Cryptosporidium* detected by PCR in diarrhoeal samples
Primary cryptosporidiosis /first infection	First documented cryptosporidial infection as defined above
Cryptosporidium species	*Cryptosporidium* species identified by PCR-RFLP of the *Cryptosporidium* spp. small subunit ribosomal RNA (SSU rRNA) on stool DNA
Serum IgG levels to gp15	Serum IgG levels to gp15, expressed in ELISA units.

4.3. Sample collection and testing

Collection and the transportation of samples were done as early as possible during the day. Stool samples were transported to the study clinic located within the study area, and blood samples were collected at the clinic and refrigerated on collection. The samples were then transported to the laboratory in an ice-box within 3-4 hours after collection. Empty stool containers were left with the study families in order to facilitate collection of fresh stool samples in the morning.

4.3.1. Laboratory methods

4.3.1.1. DNA Extraction, PCR-RFLP and sequencing

Stool samples were tested for *Cryptosporidium* spp. by PCR. DNA was extracted from stool samples using the QIAamp DNA stool kit (Qiagen Inc, Valencia, CA). DNA extracts were then screened by a nested PCR at the SSU rRNA locus. PCR-restriction fragment length polymorphism (RFLP) using enzymes SspI and VspI for species determination were carried out among the PCR positive samples using a previously described protocol [163, 189]. In samples from children identified as zoonotic species (not *C. hominis* or *C. parvum*) identified by RFLP, sequencing of the PCR amplicon was carried out followed by a BLAST analysis to confirm the species.

C. hominis and *C. parvum* isolates were also subgenotyped at the polymorphic gp60 locus. PCR using previously published primers followed by sequencing of purified PCR products by

the Big dye-terminator method was carried out [190]. Multiple sequence alignment was carried out using MUSCLE followed by phylogenetic analysis by the Maximum Likelihood method with PhyML[191-192] and tree construction with TreeDyn [193] using default "oneclick" settings in the Phylogeny.fr server (Version 2) [194]. Reference sequences for each subgenotype family were included as previously described [128, 195].

4.3.1.2. Identification of bacteria by stool culture

Diarrhoeal stool samples were tested for the presence of bacterial pathogens such as *Salmonella, Shigella, Vibrio, Yersinia, Aeromonas* and *Plesiomonas* by bacterial culture method using selective and differential media (MacConkey Agar, Xylose-Lysine-Deoxycholate agar, Thiosulphate Citrate Bile Salt Sucrose Agar). Further confirmation of the suspected pathogens was carried out by using the semi automated analytical profile index (API) biochemical systems and anti sera [196].

4.3.1.3. Anti gp15 IgG Levels in Serum by ELISA

Serum IgG levels to *C. hominis* gp15 were quantified by ELISA using a recombinant (r) form of the antigen. The quantity of anti gp15 IgG was determined by comparison of the optical density (OD) from sample wells to a standard curve generated by a human serum pool standard. Briefly, the rgp15 protein from *C. hominis* cloned in a pET-46 LIC vector (Novagen), over-expressed in *E. coli* and purified by metal-affinity chromatography was obtained from the Ward Lab (Tufts Medical Center, Boston) and coated on 96-well microtiter plates (Nunc, Rochester, NY) at a concentration of 0.5µg protein/well. Excess antigen was

washed off with phosphate buffered saline (PBS) containing 0.05% Tween 20 and nonspecific binding was blocked with 200µL of 0.25% of bovine serum albumin (BSA) in PBS. Wells were then incubated with 50µL of serum diluted at 1:100 and 1:200 in 0.25% BSA/PBS for 1 hour at 37°C along with standards. The standards used for quantification were serial dilutions of pooled human immunoglobulin from 1:100 to 1:1600. After washing 5 times, wells were incubated with 50µL of alkaline phosphatase-conjugated goat-antihumanchain-specific IgG (Sigma, St. Louis, MO), diluted 1:2000 in 0.25% BSA/PBS.

After washing, wells were incubated with 50µL substrate solution (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM MgCl2 containing p-nitrophenyl phosphate at 1 mg/ml, Sigma, St. Louis, MO) at room temperature, the reaction stopped after 15 min and absorbance read at405 nm (A405). The same negative control sera (sera which were negative by ELISA and Western blot analysis using *C. parvum* oocyst lysate as antigen) were run on each plate. All samples and standards were run in 2 replicates and the average OD of the blank wells in the periphery of the plate was subtracted from all wells. The OD of all standards, controls and samples was considered valid if the difference between the 2 replicates was <0.1, following which the mean OD was calculated. Additionally, the sample replicates were considered valid only if they had a coefficient of variation of <15%. Each point on the standard curve was considered valid if the mean OD value was within a predetermined range and each plate was considered valid if at least 5 points of the standard curve were available and the mean negative control OD was <0.1. The standards were assigned arbitrary values ranging from3200 to 200 and a standard curve was modelled using a four-parameter logistic regression function. The linear part of the sigmoidal dose response curve was used to assign values to the samples using by GraphPad Prism, Version 4.0. The value obtained was

multiplied by the dilution factor and the results were expressed as arbitrary units (AU). The mean AU of 1:100and 1:200 dilutions for each sample was then calculated and used for all further analysis. Samples with positive AU values were considered as seropositive.

4.4. Sample size calculation

The sample size was calculated to detect a protective effect of symptomatic infection against subsequent symptomatic infection, with statistical significance of 95% and statistical power of 80%. From the previous study done in the same community, the incidence of diarrhoea in Vellore on average was 1.44 episodes per child year (1949/452/3=1.44) of which 10% diarrheal episodes were positive for cryptosporidial infection detected by PCR. Hence the average number of cryptosporidial diarrheal episodes expected was 0.43 per child during 3 years (1.44 x 0.10 x 3). Assuming the incidence of cryptosporidial diarrhoea to be high around the median age of 18 months with 50% protection offered following a primary infection and expecting incidence of diarrhoea to follow a Poisson distribution, taking variance (V1) and mean (M1) of cryptosporidial diarrheal episodes at median age (18 months) as 0.216 and variance (V2) and mean (M2) of cryptosporidial diarrheal incidence after median age is 0.108, the sample size was calculated to be 490 after accounting for a 10% drop out rate. The formula used to compute sample size is given below [197].

$$N = \frac{(V_1 + V_2) * (Z_\alpha + Z_\beta)^2}{(M_1 - M_2)^2}$$

4.5. Data entry, management and statistical analysis

Baseline and surveillance data were collected using structured questionnaires. All the questionnaires were piloted in the community before the commencement of the study. Weekly schedules with the scheduled date of collection of a particular sample or anthropometric measurement for all the children from whom a sample was due for that week were provided to the field workers, in order to maximise the sample collection rate. To reduce the discrepancies between the field and laboratory teams, all the samples were accompanied by a sample transfer log which was signed by the field supervisor in the field and counter signed by the laboratory staff after receiving the samples at the laboratory.

All data were double entered using the Epi- Info 2002 (CDC, Atlanta, GA, USA) software, following which each item in the dataset were compared subject by subject, item by item, to detect missing values or discrepancies between the two data entry sets. Missing values and discrepancies were sent to the field site for resolution. Identical values were saved to a master database in a central server, with a network backup system. In order to check the quality of the entered data, 10% validation of the data was conducted independently by a data manager. Data were analysed using STATA 10.1 for Windows (StataCorp, College Station, TX, USA) software. Statistical analyses are described in detail in each individual sections; 5.1- 5.5.

CHAPTER 5

RESULTS AND DISCUSSION

CHAPTER 5.1

RISK FACTORS AFFECTING MORBIDITY IN THE FIRST THOUSAND DAYS OF LIFE IN THE BIRTH COHORT

5.1.1. Introduction

The UN MDGs target a decrease in mortality among under five children by two thirds by the year 2015 and the strategy for such a reduction aims at decreasing deaths caused by pneumonia, diarrhoea and malnutrition [4]. In developing countries with rapid growth of urban slums with overcrowding [198-199], malnutrition [200], infectious diseases [201] leading to poor growth and development of the young child touches upon a number of social, environmental and economic risk factors, all of which have a bearing on whether the MDGs would be achieved or not.

National health planning requires a multitude of information that includes mortality and morbidity indicators and the myriad risk factors alluded to above. Information could be gathered through population based longitudinal/cohort studies that capture information prospectively, allow documentation of the different risk factors that could affect the development and growth of a child including exposure to one or more risk factors of disease. Thus, such studies help to establish temporal relationships between exposure and illnesses and overcome shortcomings of cross-sectional studies, especially those that are hospital-based.

Although data on all morbidity were collected for a three-year period, previous analyses of birth cohort data from the same area have shown that the second and third years of life are very similar in terms of the illness experience of the cohort. Hence, additional data from the pre-natal period was analysed with the data on the first two years of life to examine the effects of pre- and post-natal risk factors on the birth outcomes and morbidity in children.

5.1.2. Methods

5.1.2.1. Establishment of cohort and follow up

Study area and the study design, data collection methods and operational definitions have been described in chapter 4. A total census of the study area was conducted by trained field staff employed for this study and they identified 561 pregnant women who fulfilled the eligibility criteria for inclusion. They read and signed consent to participate in the study using an institutional IRB approved consent in the vernacular. Weekly visits to the pregnant women by the field staff helped to build rapport. During the antenatal and perinatal follow-up period, some (64) pregnant women became unavailable (details in Figure 5.1.1). Baseline information on demography, socioeconomic factors, delivery and birth details were obtained, post-delivery, from each household. The pre-natal/antenatal data were obtained from the antenatal cards of mothers. Gestation period was calculated from the last menstrual period (LMP) and the date of delivery recorded on the antenatal cards. Risk factors included number of antenatal care (ANC) visits [202], maternal anaemia (moderate-severe) with a cut off of <10 gm% [202] and exclusive breastfeeding for less than six months [203] as specified by WHO. A median cut off of five was used to categorize households as those with equal to or lesser and greater than five members. The maternal age was categorized into teenage mothers (<20 years) or those at least or older than 20 years. Socioeconomic status was assessed using a five point scale, which is a modification of the Kuppuswamy scale, and includes highest education, occupation, possessions and type, ownership and number of rooms in a house [185-186]. Flow charts of the cohort recruitment, cumulative recruitment and follow-up of the cohort are depicted in Figure 5.1.1 and Figure 5.1.2.

Figure 5.1.1.: Flowchart of cohort recruitment and follow-up

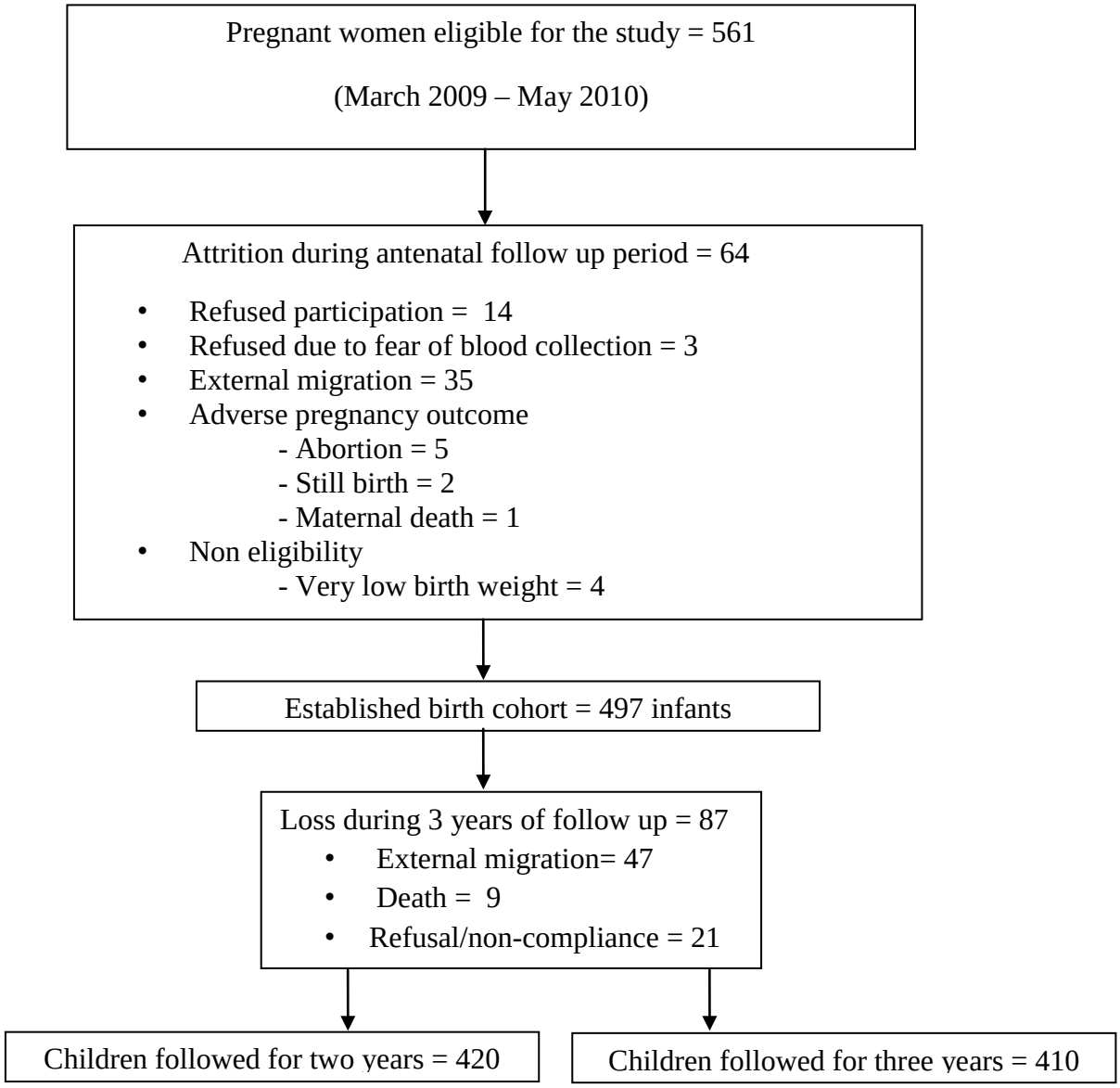
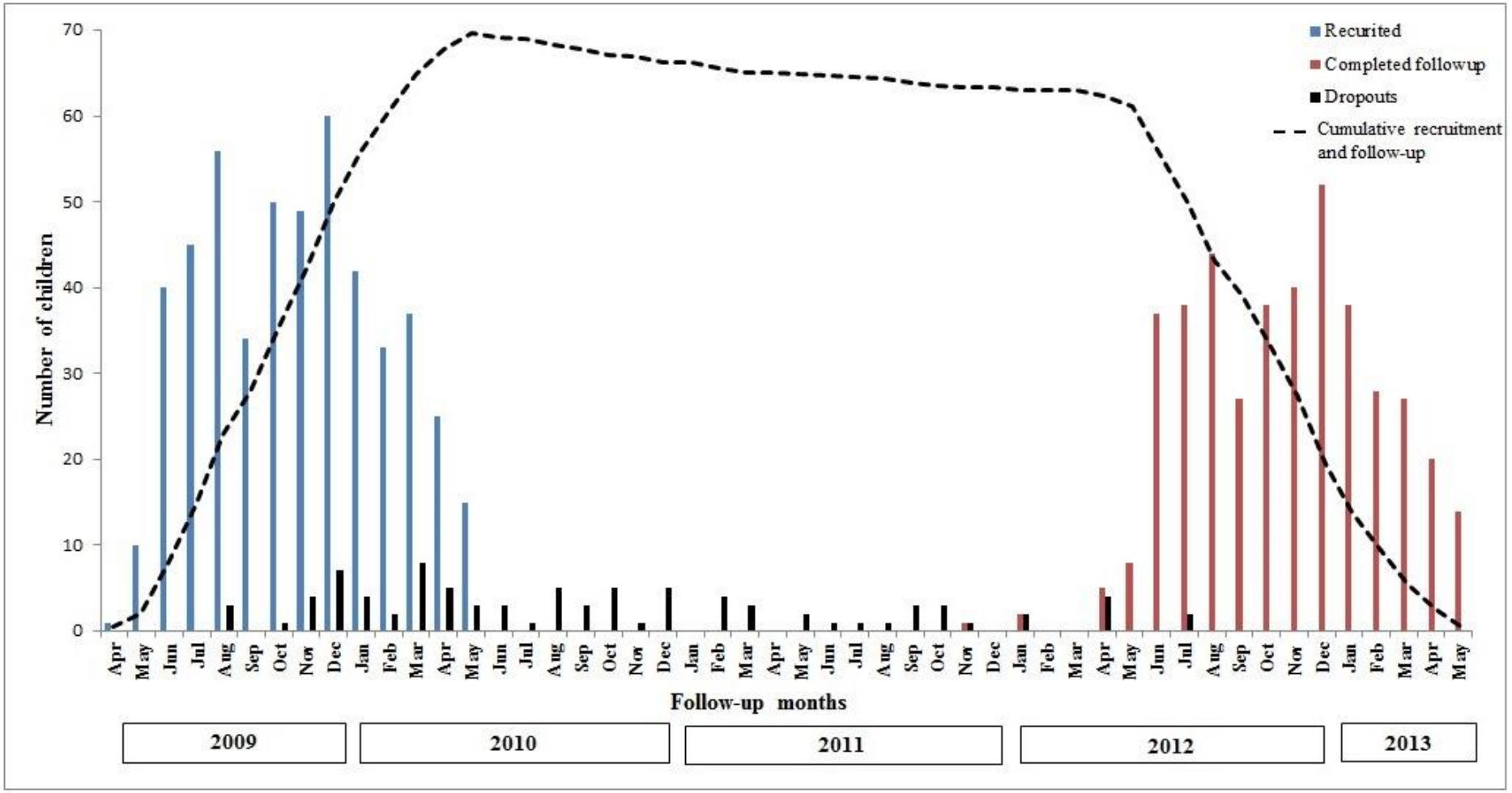


Figure 5.1.2.: Recruitment and follow-up of birth cohort (n=497): April 2009 to May 2013



5.1.2.2. Surveillance and data collection

Every enrolled infant was visited twice a week with 463,653 observations among 497 infants. Of the 497, 444 (89.33%), 420 (84.5%) and 410 (82.5%) children completed one, two, three years of follow up respectively. Migration out of the study area was the commonest reason for loss to follow up. On an average, each child was followed for 31(0.46 to 36) months and adding up to 15707 child months of follow-up. Those who dropped out remained in the study for an average of 10 months (0.46 to 34). Baseline socio-demographic characteristics of those lost to follow-up were similar to those who remained in the study (presented in chapter 5.3).

Field workers had been trained in standard case definitions and in the use of a structured questionnaires and this was used by them at each visit to the child to observe the child and interview the caregiver about any illness experienced by the child for each day since the last visit.

A physician-run study clinic was established within the study area to cater to the health needs of all under five children living in the area. These children were either managed at the clinic or referred to the CHAD (secondary care) or CMC hospital (tertiary care) depending on the severity of the disease. All hospital visits and hospitalizations were recorded.

Illness episodes experienced by the study children were classified as either hospital reports or summaries of hospitalization or reports from caregivers, usually the mother. Gastrointestinal (GI) illnesses were defined as diarrhoea (three or more watery stools in a 24 h period) or vomiting lasting for more than 24 hours. Upper respiratory illnesses (URI) were defined as

runny nose or cough, either with or without fever, lasting for five or more days. Undifferentiated fever was defined as fever not associated with other symptoms lasting at least 48 hours. Skin lesions were defined as rashes, vesicles, pustules, cysts, ulcerations and traumatic ulcers. Other infections included infections of the eyes, ears or any other localized infection with or without fever. Non-infectious morbidities included non-specific swellings, surgical conditions such as hernia, congenital diseases, fractures, injuries, insect bites and accidents. A new episode for GI morbidities was defined as illness occurring at least 48 hours after cessation of the previous episode. For all other illnesses, an interval of 72 hours separated two episodes. All severe illnesses were assessed and managed by the physicians/ paediatricians at the study clinic or at the hospital where children were referred.

Descriptive analysis of baseline socio-demographic results are presented for all the 561 pregnant women enrolled in the study; maternal and delivery details are presented for the 497 infants who were recruited into the birth cohort. The baseline demographic comparison between children with and without prenatal maternal data was done using χ^2 test or Fisher's exact test for categorical variables and two-tailed *t*-tests or Wilcoxon rank sum tests for continuous variables, depending on the distribution of the data. Incidence rates were calculated as the number of episodes divided by the child-years of follow-up. The total person-time at risk was calculated as the total days under surveillance minus days of missing surveillance data (if ≥one week). Multiple failures within a child were accounted for using frailty Poisson Survival models, which were fitted to obtain the variance corrected incidence rates and rate ratios to assess the factors associated with overall (summation of GI, respiratory, undifferentiated fever, skin, non-infectious, other infectious morbidity), GI and respiratory morbidities and the risk is presented as hazard ratio (HZ) with 95% confidence

interval (CI). Logistic regression was performed to identify the factors associated with the risk of low birth weight and are presented as odds ratio (OR) with 95% CI.

The explanatory variables used in the regression models were time independent socio-demographic variables such as religion, maternal education, type of family, socioeconomic status, and antenatal/delivery/post-natal variables such as maternal anaemia, hypertension, diabetes, preterm birth, parity, history of abortion/still birth, duration of breast feeding. Factors identified in the univariate analysis at the significance level of 0.30 and clinically relevant variables were considered for inclusion in the full multivariate models. Multivariate analysis was performed using backward stepwise method. A parsimonious regression model was chosen considering the significance of predictors in the full model. For biological comparisons some non-significant variables, such as socio-economic status were retained in the final model where considered relevant. Risk factor analyses for low birth weight and morbidities were restricted to a subgroup of 216 children, because of the availability of complete prenatal data.

5.1.3. Results

5.1.3.1. Baseline demographics of enrolled pregnant women in the study

Among the 561 pregnant women enrolled in the study, 54.2% were Hindus, 41.5% were Muslims. Families were largely nuclear families (60.3%) with a median a family size of five and IQR ranged from 4 to 7. A majority (60%) belonged to a low socio-economic stratum

(Table 5.1.1). Mothers of children enrolled in the cohort were not socio-demographically different from those not recruited.

Table 5.1.1.: Baseline characteristics of pregnant women enrolled in the study (n=561)

Variable	Number	Percentage
Religion		
Hindu	304	54.2%
Muslim	233	41.5%
Christian	24	4.3%
Type of family		
Joint	111	19.8%
Extended	112	20.0%
Nuclear	338	60.2%
No. of household members		
<= 5	295	52.6%
>5	266	47.4%
Ownership of house		
Ownership of house	354	63.1%
Rented/lease/govt	207	36.9%
Primary mode of cooking		
Firewood	225	40.1%
Kerosene	124	22.1%
Gas stove/other	212	37.8%
Socioeconomic status		
Low	338	60.3%
Middle	197	35.1%
High	26	4.6%

5.1.3.2. Baseline characteristics of the children, antenatal and delivery information (n=497)

The study children numbered 263 males (53%) and 234 females (47%) and were from largely nuclear families (293, 59%). Hindus were the majority (264, 53.1%), followed by Muslims (214, 43%) and a small proportion (19, 3.8%) were Christians. The majority of the families resided in a pucca house (306, 61.6%) made of concrete roof and floor, and 63 (12.7%) families resided in a kutcha house (thatched roof, with mud or concrete floor). Firewood was the main cooking fuel in 199 (40%) of the study families and more than half the families (328, 66%) belonged to the low socio-economic status by the modified Kuppusamy scale (Table 5.1.2).

Of the 490 children for whom birth weight data was available, the average (SD) birth weight was 2.9 (0.4) kg and 84 (17.1%) children were considered low birth weight (<2500gms). The mean gestational age was 39.6 weeks. The average (SD) age of the mother at the time of delivery was 24 (3.6) years and 196 (39.4%) of them were primiparous. Almost 184 (37%) of the mothers were illiterate and only 17 (3.4%) had more than 12 years of education. The median (IQR) duration of exclusive breast feeding was 4.09 (2.36-5.24) months (Table 5.1.3). Subgroup analyses between children with and without complete pre-natal maternal information demonstrated both groups did not differ socio-demographically (Table 5.1.4).

Table 5.1.2.: Baseline demographic description of birth cohort (n=497)

Variable	Number	Percentage
Gender of child		
Male	263	53%
Female	234	47%
Religion		
Hindu	264	53.1%
Muslim	214	43.1%
Christian	19	38.2%
Type of family		
Joint	102	20.5%
Extended	102	20.5%
Nuclear	293	59.0%
House ownership		
Own	315	63.4%
Rented/Government	182	36.6%
Mode of cooking		
Firewood	199	40.0%
Kerosene	111	22.3%
Gas	172	34.7%
More than one	15	3%
Socioeconomic status		
Low	328	66%
Middle	160	32.2%
Upper	9	1.8%
Presence of sibling		
Yes	278	56%
No	219	44%

Table 5.1.3.: Maternal and delivery description of the cohort

Variable	Number	Percentage	Variable	Number	Percentage
Maternal age			***Maternal hypertension* (>120/80) ¶¶**	44	17.5%
≤ 23 yr	256	51.5%			
> 23 yr	241	48.5%	***Vaccination for Tetanus toxoid*** ‡	244	96.1%
Maternal Education			***Mode of delivery***		
Illiterate	184	37.0%	Normal	387	77.9%
Primary/middle (1-9 years of education)	167	33.6%	Instrumental	20	4.0%
High/higher secondary (10-12 years of education)	129	26%	Caesarean	90	18.1%
College (15 years of education)	17	3.4%	***Place of delivery***		
			Hospital	487	98%
Consanguineous marriage*	47	20.6%	Home	10	2%
Median(IQR) days gestation period †	278 (269-286)		***Birth weight in Kg*** ‡‡		
Gravida			< 2.5	84	17.1%
Primiparous	196	39.4%	>= 2.5	406	82.9%
2	154	31%	***Mean (SD) Height of mother (cms)****	153.03 (6.4)	
> 2	147	29.5%			
			BMI of mother ‡‡‡		
History of abortion	67	13.4%	< =18.5 (underweight)	60	15.5%
History of stillbirth	18	3.6%	18.6 -24.99 (normal)	184	47.4%
			25 -29.99 (overweight)	107	27.6%
Median(IQR) ANC visit ††	7 (6 -9)		>=30 (obese)	37	9.5%
Maternal haemoglobin ¶					
			Median (IQR) duration of exclusive breastfeeding in months	4.09 (2.36-5.24)	
< 10gm%	66	29.6%			
10 -12gm%	128	57.4%			
>12 gm%	29	13%			

** n= 228, †n = 470, ††n= 242, ¶n = 223, ¶¶n = 251, ‡n =254, ‡‡n= 490, *n = 388, ‡‡‡n= 388

Table 5.1.4.: Comparison of demographics between children with and without complete maternal information

Socio-demographic Variable	Children with Maternal Information (n=216)	Children without Maternal information (n =281)	*P*-value
Female	110 (47%)	124 (53%)	0.13
Religion			
Hindu*	111 (42%)	153 (58%)	
Christian	4 (21%)	15 (79%)	
Muslim	101 (47%)	113 (53%)	0.07
Family Type			
Joint	42 (41%)	60 (59%)	
Extended	43 (42%)	59 (58%)	
Nuclear	131 (45%)	162 (55%)	0.78
Socioeconomic status			
Low SES	141 (43%)	187 (57%)	0.76
Firewood	96 (48%)	103 (52%)	0.08
Beedi work	71 (55%)	59 (45%)	0.003
Crowding (>5 people per room)	35 (41%)	51 (59%)	0.54
Household ownership	146 (67%)	169 (60%)	0.08
Illiteracy of mother	85 (46%)	99 (54%)	0.34
Morbidity Details (incidence/child year)			
Overall Morbidity	14.9	14.65	0.15
Respiratory Morbidity	7.07	7.08	0.82
GI Morbidity	2.95	3.12	0.7

5.1.3.3. Factors associated with low birth weight

In the multivariate analysis, preterm birth (OR=3.31, 95% CI=1.12-9.78), less than four ANC visits (OR=6.88, 95% CI=2.10-22.51), and anaemia defined as haemoglobin <10 gm/dL during pregnancy (OR=2.36, 95% CI=1.08-5.18) were independent risk factors associated with low birth weight (LBW). Although beedi making at home was associated with risk of LBW, it did not remain significant in the multivariate model (Table 5.1.5).

Table 5.1.5.: Risk factors associated with low birth weight in univariate and multivariate analyses in 216 infants in a birth cohort

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Female child	2.45 (1.50-4.00)	<0.0001	1.73 (0.80-3.73)	0.16
Preterm (<37 wks)*	3.45 (1.81-6.57)	<0.0001	3.31 (1.12-9.78)	0.03
Less than four antenatal care visits†	3.11 (1.19-8.11)	0.001	6.88 (2.10-22.5)	0.001
Anaemia during pregnancy (Hb<10)‡	2.00 (0.98-4.06)	0.05	2.36 (1.08-5.18)	0.03
Beedi work at home	1.79 (1.08-2.96)	0.02	1.66 (0.76-3.63)	0.19
Maternal illiteracy	1.18 (0.73-1.91)	0.48		
Maternal age <20 yrs	1.37 (0.67-2.81)	0.37		
Low SES	1.38 (0.82-2.32)	0.21		
History of still birth	2.27 (0.76-6.72)	0.13		

* n = 467, † n = 241, ‡n = 222

5.1.3.4. Illness in the birth cohort in the first 1000 days

A total of 12,803 episodes of illness which included all episodes of gastrointestinal illnesses, upper and lower respiratory tract illnesses, undifferentiated fever, skin lesions, non-infectious illnesses and other infections such as infections of the eyes, ears or any other localized infection with or without fever were recorded during the first two-years of follow-up period, resulting in an incidence of 14.77 (95% CI, 14.26-15.30) episodes of illness/child-year. Children had more illnesses during infancy (16.04 episodes of illness/child year) than during the second year of life (13.45 episodes of illness/child year) (P<0.001).

Respiratory infections were the most common, accounting for 47.8% (6122/12803) of all morbidities, with an incidence of 7.06 (95% CI, 6.86-7.27) episodes/child-year. The median (IQR) duration of an episode of respiratory illness was 14 (9-24) days, since all uncomplicated upper respiratory infections (e.g. runny nose or cold with no other associated symptoms) of less than five days were not included.

GI illness accounted for 20.6% (2634/12803) of all reported illnesses, with an incidence of 3.04 (95% CI, 2.82-3.28) episodes of GI illness/child-year. The median (IQR) duration of an episode of GI illness was 4 (3-6) days. Incidence of diarrhoea was 2.12 (95% CI, 1.96-2.30) episodes/child-year with a median (IQR) duration was 3 (2-5) days. Severity of diarrhoeal episodes was recorded using Vesikari scale; 845 (47.5%) were mild (score ≤5), 719 (40.5%) were moderate (score 6–10), and 213 (12%) were severe (score ≥11).

The overall incidence of undifferentiated fever during the two years of follow up was 0.80 episodes/child-year, decreasing from 0.94 episodes/child-year in the first year to 0.66 episodes/child-year in the second year. Incidence of other infectious illnesses was 0.96 episodes/child-year. In general, the incidence of skin and other infectious illnesses was less among children during the second year. The overall incidences of non-infectious illness were 1.03 episodes/ child-year, increasing from 0.40 episode/ child-years during infancy to 1.68 in the second year. Table 5.1.6 presents the incidence rates of different illnesses during the first and second year of life.

A large proportion of morbidities (7681/12803, 60%) resulted in clinic or hospital outpatient visits. Among all morbidity reported, healthcare (clinic/hospital visits) was sought most frequently for respiratory illnesses (4346/6122, 71%), followed by GI (1524/2634, 57.9%) and other infectious (470/835, 56.3%) morbidities.

Overall, 2.27% (290/12803) of illnesses required hospitalization, at a rate of 0.33/child-year. A slightly larger proportion of GI illnesses (72/2634, 2.7%) required hospitalization than respiratory (149/6122, 2.4%), skin (37/1622, 2.3%) and non-infectious (14/891, 1.6%) illnesses. The rate of hospitalization in the first year was higher (0.46/child-year) than the rate (0.19/child-year) in the second year (Table 5.1.6).

Table 5.1.6.: Incidence rates of illness and proportion of clinic visits and hospitalizations during first 730 days of post-natal life in the birth cohort

Type of illness	Overall	First year	Second year
Number of children at start of follow up	497	497	444
Child years of follow up	866.58	443.04	423.54
All cause morbidity			
Number of episodes	12803	7107	5696
Rate (95% CI) per child year	14.77 (14.26-5.3)	16.04 (15.45-6.66)	13.45 (12.89-14.03)
Number of episodes requiring clinic visits (%)	7681 (60%)	4538 (63.85%)	3143 (55.18%)
Number of episodes requiring hospitalization (%)	290 (2.27%)	207 (2.91%)	83 (1.46%)
Gastrointestinal Illness			
Number of episodes	2634	1935	699
Rate (95% CI) per child year	3.04 (2.82-3.28)	4.36 (4.06-4.70)	1.65 (1.46-1.87)
Number of episodes requiring clinic visits (%)	1524 (57.86%)	1126 (58.19%)	398 (56.94%)
Number of episodes requiring hospitalization (%)	72 (2.73%)	52 (2.69%)	20 (2.86%)
Respiratory Illness			
Number of episodes	6122	3283	2839
Rate (95% CI) per child year	7.06 (6.86-7.27)	7.41 (7.17-7.65)	6.70 (6.44-6.97)
Number of episodes requiring clinic visits (%)	4346 (70.99%)	2509 (76.42%)	1837 (64.71%)
Number of episodes requiring hospitalization (%)	149 (2.43%)	105 (3.20%)	44 (1.55%)

Table 5.1.6.(contd) Incidence rates of illness and proportion of clinic visits and hospitalizations during first 730 days of post-natal life in the birth cohort

Type of illness	Overall	First year	Second year
Undifferentiated fever			
Number of episodes	699	416	283
Rate (95% CI) per child year	0.80 (0.73-0.89)	0.94 (0.83-1.06)	0.66 (0.58-0.77)
Number of episodes requiring clinic visits (%)	255 (36.48%)	125 (30.05%)	130 (45.94%)
Number of episodes requiring hospitalization (%)	5 (0.72%)	5 (1.20%)	0
Skin Infections			
Number of episodes	1622	843	779
Rate (95% CI) per child year	1.87 (1.73-2.02)	1.90(1.74-2.08)	1.83 (1.67-2.03)
Number of episodes requiring clinic visits (%)	796 (49.08%)	435 (51.60%)	361 (46.34%)
Number of episodes requiring hospitalization (%)	37 (2.28%)	28 (3.32%)	9 (1.16%)
Other Infections			
Number of episodes	835	451	384
Rate (95% CI) per child year	0.96 (0.86-1.08)	1.02 (0.90-1.15)	0.90 (0.79-1.05)
Number of episodes requiring clinic visits (%)	470 (56.29%)	259 (57.43%)	211(54.95%)
Number of episodes requiring hospitalization (%)	13 (1.56%)	9 (2.00%)	4 (1.04%)
Non-infectious Morbidity			
Number of episodes	891	179	712
Rate (95% CI) per child year	1.03 (0.91-1.16)	0.40 (0.32-0.51)	1.68 (1.49-1.90)
Number of episodes requiring clinic visits (%)	290 (32.55%)	84 (46.93%)	206 (28.93%)
Number of episodes requiring hospitalization (%)	14 (1.57%)	8 (4.47%)	6 (0.84%)

5.1.3.5. Factors associated with overall morbidity in the first 1000 days of life (n=216)

In univariate Poisson regression analysis, factors such as older age (two years) of the child (HZ=0.88, 95% CI=0.80-0.97), exclusive breast feeding for six months (HZ=0.75, 95% CI=0.65-0.87) were associated with protection, whereas using firewood as the primary fuel (HZ=1.15, 95% CI=1.01-1.30) was a risk factor for overall morbidity.

In the multivariate Poisson regression model, factors such as older age (second year of life) of the child (HZ=0.87, 95% CI=0.79-0.95) female gender (HZ=0.88, 95% CI=0.79-0.99), exclusive breasting feeding for six months (HZ=0.76, 95% CI=0.66-0.88) exhibited significant protective effect against overall morbidity. Using firewood as the primary fuel (HZ=1.12, 95% CI=1.00-1.27) was associated with an increase in overall morbidity (Table 5.1.7).

Table 5.1.7.: Factors associated with episodes of overall morbidity in univariate and multivariate analyses of longitudinal data from 216 infants

Variable	Number of episodes	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)					
One *	3390	1			
Two	2880	0.88 (0.80-0.97)	0.01	0.87 (0.79-0.95)	0.002
Female	3014	0.89 (0.78-1.01)	0.07	0.88 (0.79-0.99)	0.03
Religion					
Muslim/Christian*	3007	1			
Hindu	3263	1.02 (0.90-1.17)	0.65	-	-
Illiteracy of mother	2556	1.07 (0.94-1.22)	0.24	-	-
Family Type					
Joint/extended	2400	1			
Nuclear	3870	1.05 (0.92-1.19)	0.45	-	-
Socioeconomic status					
Low SES	4217	1.10 (0.96-1.26)	0.13	1.06 (0.93-1.21)	0.31
Firewood as primary fuel	2953	1.15 (1.01-1.30)	0.02	1.12 (1.00-1.27)	0.05
Beedi work	2075	1.01 (0.87-1.16)	0.87	-	-
Maternal factors					
Hypertension during pregnancy	733	1.16 (0.95-1.43)	0.13	-	-
Anaemia in pregnancy (Hb <10)	1731	0.99 (0.86-1.14)	0.93	-	-
Preterm (<37 wks) delivery	500	1.03 (0.85-1.25)	0.69	-	-
Primiparous	2584	0.94 (0.82-1.07)	0.39	-	-
Mother with history of abortion	710	1.07 (0.88-1.30)	0.45	-	-
Mother with history of stillbirth	322	1.27 (0.98-1.65)	0.06	1.15 (0.89-1.48)	0.27
More than four antenatal visits	5667	1.08 (0.89-1.31)	0.38		
Exclusive breast feeding for six months	905	0.75 (0.65-0.87)	<0.0001	0.76 (0.66-0.88)	<0.0001

* Reference category

5.1.3.6. Factors associated with gastrointestinal and respiratory illness in the first 1000 days of life (n=216)

In univariate Poisson regression analysis for GI illness, factors such as older age/second year of life (HZ=0.42, 95% CI=0.34-0.53) was associated with protection, whereas using firewood as the primary fuel (HZ=1.58, 95% CI=1.12-2.24) was a risk factor for GI illnesses. Exclusive breast-feeding for six months (HZ=0.73, 95% CI=0.48-1.10) was associated with protection, but was not significant. In the multivariate analysis also age was associated with protection and usage of firewood as the primary fuel was a significant risk factor (Table 5.1.8).

In multivariate analysis for respiratory illness, children of a primiparous mother (HZ=0.85, 95% CI=0.77-0.95), and exclusively breast fed for six months (HZ=0.81, 95% CI=0.71-0.93) were protected against respiratory illness, whereas usage of firewood as the main fuel, a proxy for low socioeconomic status, was associated with increased risk of respiratory illness (HZ=1.10, 95% CI=1.00-1.21) (Table 5.1.9).

Table 5.1.8.: Factors associated with gastrointestinal illnesses in univariate and multivariate analyses in 216 infants

Variable	Number of episodes	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)					
One*	906	1			
Two	335	0.42 (0.34-0.53)	<0.0001	0.44 (0.34-0.54)	<0.0001
Female	570	0.77 (0.54-1.09)	0.14	0.76 (0.57-1.02)	0.07
Religion					
Christian/ Muslim	549	1			
Hindu	692	1.27 (0.89-1.82)	0.23	-	-
Illiteracy of mother	509	1.14 (0.80-1.63)	0.46	-	-
Family Type					
Joint/Extended *	470	1			
Nuclear	771	1.20 (0.84-1.72)	0.30	-	-
Socioeconomic status					
Low SES	840	1.29 (0.89-1.85)	0.17	1.09 (0.76-1.57)	0.6
Firewood as primary fuel	618	1.58 (1.12-2.24)	0.009	1.40 (1.05-1.88)	0.02
Beedi work	423	1.17 (0.78-1.74)	0.43		
Crowding (>5 people per room)	174	0.66 (0.40-1.09)	0.11	0.68 (0.45-1.04)	0.08
Maternal factors					
Hypertension during pregnancy	143	1.24 (0.76-2.00)	0.37	-	-
Anaemia in pregnancy	336	0.94 (0.63-1.41)	0.79	-	-
Preterm (<37 wks)	107	1.20 (0.59-2.42)	0.60	-	-
Primiparous	514	0.97 (0.68-1.38)	0.87	-	-
Mother with history of abortion	145	1.23 (0.75-2.00)	0.39	-	-
Mother with history of stillbirth	71	1.52 (0.71-3.21)	0.27	-	-
More than four antenatal visits	1118	1.14 (0.64-2.01)	0.64	-	-
Exclusive breast feeding for 6 months	189	0.73 (0.48-1.10)	0.14	0.76 (0.52-1.10)	0.15

* Reference category

Table 5.1.9.: Factors associated with respiratory illnesses in univariate and multivariate analyses in 216 infants

Variable	Number of episodes	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)					
One*	1581	1			
Two	1390	1.17 (0.98-1.40)	0.07	1.15 (0.98-1.37)	0.08
Female	1485	0.97 (0.88-1.07)	0.59	-	-
Religion					
Christian/Muslim	1408	1		-	-
Hindu	1563	1.06 (0.96-1.16)	0.24	-	-
Illiteracy of mother	1175	1.01 (0.92-1.12)	0.72	-	-
Family Type					
Joint/ extended*	1164	1			
Nuclear	1807	1.00 (0.90-1.11)	0.93	-	-
Socioeconomic status					
Low SES	1988	1.09 (0.97-1.21)	0.11	-	-
Firewood	1358	1.07 (0.97-1.18)	0.13	1.10 (1-1.21)	0.05
Beedi work	974	0.99 (0.89-1.10)	0.93	-	-
Crowding (>5 people per room)	503	1.05 (0.91-1.21)	0.46	-	-
Maternal factors					
Hypertension during pregnancy	305	0.98 (0.86-1.12)	0.84	-	-
Anaemia in pregnancy	796	0.94 (0.84-1.06)	0.36	-	-
Preterm (<37wks)	235	1.03 (0.86-1.23)	0.74	-	-
Primiparous	1174	0.71 (0.78-0.96)	0.007	0.85 (0.77 -0.95)	0.005
Mother with history of abortion	332	1.04 (0.89-1.22)	0.56	-	-
Mother with history of stillbirth	130	1.04 (0.84-1.28)	0.67	-	-
More than four antenatal visits	2676	1.04 (0.89-1.22)	0.57	-	-
Exclusive breast feeding for six months	458	0.83 (0.73-0.95)	0.007	0.81 (0.71 - 0.93)	0.004

* Reference category

5.1.3.7. Mortality in the cohort

Seven deaths were reported during the antenatal follow-up period, of which five were spontaneous abortions and two were stillbirths. In the cohort during two years of follow-up, nine deaths were reported; three children died of diarrhoea, two due to lower respiratory infections, two due to congenital metabolic conditions, and two due to unknown causes.

5.1.4. Discussion

Tamilnadu has seen a marked increase in institutional deliveries from 79.3% in 1998-99 to 90.4% in 2005-06 [204-205]. This increase has been attributed to the conditional cash transfers for institutional deliveries under the Janani Suraksha Yojana or JSY scheme in 2005. This study found that 98% of the mothers delivered in an institution. The JSY scheme has led to a change in the health seeking behaviour during pregnancy and has resulted in more antenatal visits, better awareness and an improvement in maternal and neonatal survival. In the study cohort, there were no early neonatal deaths and the low birth weights (17%) in this study is much lower than the national average of 22%, but identical to the Tamilnadu average, 17.2% [204].

Periodic antenatal check-up is important for identification of high risk mothers, to monitor weight gain during pregnancy, screen for anaemia; provide nutritional and iron supplements, all of which are vital for a good pregnancy outcome. These also help to reduce and/or prevent maternal and neonatal complications and mortality. Studies have demonstrated that increasing the number of ANC visits coupled with good quality antenatal care decreases low

birth weight babies [206-208]. This study found that children born to the mothers with less than 4 antenatal visits had a six time higher odds of having a low birth weight child and this is similar to earlier reports from Tanzania and India [209-211]. Low birth weight, being an important indicator of vulnerability to childhood illnesses and survival, has been shown to be linked to higher mortality and morbidity [212], impaired mental development [213], and risk of chronic adult diseases such as cardiovascular diseases and diabetes in later life [214-215].

Over 50% of pregnant women are anaemic in developing countries [216] and this is as a result of inadequate maternal nutrition, particularly micronutrients. Routine and early ANC visits result in early detection of anaemia with the possibility of introduction of supplemental iron and folic acid and appropriate nutritional advice. In this study, mothers with a haemoglobin <10 gm/dL during pregnancy had twice the risk of having a low birth weight baby. Studies elsewhere have also demonstrated the negative effect of maternal anaemia on birth weight [217-219]. A study from Pakistan reported 64% low birth weight among anaemic mothers compared to only 10% in non-anaemic mothers [220]. Taken together, these findings highlight the importance of at least four regular antenatal check-ups, as recommended by WHO [202].

Respiratory and diarrhoeal diseases are the major causes of morbidity among children in India [101, 186, 221] and other developing countries [222-223]. Children in this cohort also suffered predominantly from respiratory and GI illness with estimates very similar to earlier studies conducted in the same study area over the past decade for all illnesses, respiratory and GI disease [186,101]. Widespread faecal contamination of drinking water supply is one of the suggested reason for the high disease burden of GI illness in this area [224]. Improving

quality and quantity of drinking water and its delivery to homes as well as the use of sanitary latrines are often the suggested public health measures in the study area and elsewhere in India.

The age of the child is inversely related to the burden of GI tract infections while respiratory illnesses increase with age [101, 225]. Children from low socio-economic strata families have been documented to have a higher risk of morbidity [226-227]. Children from households where firewood is the main fuel also have a higher risk of illness in this study. Firewood could be an indicator of low socio-economic status besides a greater exposure to smoke and an increased of respiratory illness [228-231]. Indoor and outdoor air quality could be improved by the provision of alternate fuel and properly ventilated homes in marginalized communities such as this study area.

Biological differences because of gender seemed to protect girls from GI and respiratory illnesses as compared to boys. Other studies have reported similar findings from India and elsewhere [226,101, 232- 233].

WHO recommends exclusive breast feeding for six months of life for child survival [203]. Human milk glycans are part of the natural immunological mechanism that offers protection against diarrhoeal diseases in breastfed infants [234]. Breast feeding also reduces exposure to contaminated foods and liquids besides contributing to adequate nutrition and non-specific immunity. About 20-25% protection was seen against overall morbidity and acute respiratory illness among children who were exclusively breastfed for six months. Although it offered some protection against GI morbidity, this was not statistically significant. The lack of

protection in this study may be a reflection of overall high rates of breastfeeding. Reviews on breastfeeding have demonstrated a protection offered by exclusive breastfeeding against diarrhoea [234-235] respiratory infections [236] and its importance for child survival [233, 237].

A lack of complete data on antenatal care and pregnancy outcome of all enrolled women was one limitation of this study but there were no significant differences between children with and without complete maternal data. Studies have shown the first two years of life to be crucial for cognitive development in children [238], but cognitive function was not assessed in this study.

5.1.5. Conclusion

Provision of good quality health care coupled with better health seeking behaviour can overcome economic barriers in society that limit the quality of the health of people, particularly that of young children. A high proportion in this study had antenatal care and institutional deliveries and the outcome in terms of early child survival was shown to be good. At times of illness, early intervention can prevent or slow the disease process, which in turn reduces complications and death. Hospitalization rates in this and earlier community-based surveillance studies from Vellore [101, 186] possibly reflect an unmet need for hospitalization in resource-constrained settings.

While there is increasing evidence that the first 1000 days of life influences health and intellectual capacity, this study shows that antenatal factors influence birth outcomes in

children, and children from impoverished urban slum communities in developing countries continue to have a high burden of morbidity predominantly respiratory and GI, in the first two years of life. National policy makers need to understand the natural history of diseases that are prevalent, target the vulnerable groups (pregnant women and young children) in the community with health-care provision or interventions.

CHAPTER 5.2

CRYPTOSPORIDIOSIS IN THE BIRTH COHORT

5.2.1. Introduction

Cryptosporidium spp. is abundant in nature and the survival of the oocysts in different environments, its ability to retain infectivity for prolonged periods of time [105] and survive most water disinfection treatments [106] makes it an important pathogen.

Notable events in the history that led to the health care community's interest in *Cryptosporidium* and its association with high morbidity and mortality among children in developing countries have been highlighted in section 3.6.1. Much of the information about cryptosporidiosis is from hospital based cross sectional studies, but there are limited studies on infection in the community or in the absence of overt illness.

Cryptosporidium has been identified from a large diversity of hosts in all five groups of vertebrates [110]. Human infections are predominantly caused by *C. hominis*. This is so both in developed and developing countries [112-113]. A parasite with multiple routes of transmission and with no standard treatment requires community-based studies to obtain estimates of the burden of infection, identify susceptible populations and to understand the pattern of infection and disease. There are very few community based studies on infection or disease rates in specific populations. Cohort or longitudinal studies to study the incidence and the natural history of infection of *Cryptosporidium* spp. have been limited. This study was designed to describe cryptosporidiosis in a birth cohort followed up until the age of 3 years in a semi-urban slum community in south India.

5.2.2. Methods

A total of 497 children who reside in four semi urban slum which are located in the western suburbs of Vellore formed the birth cohort. Description of the study design, monitoring of the study children, data collection and faecal sample collection are provided in Chapter 4. The stool samples were tested for the presence of *Cryptosporidium* spp. by PCR. The determination of *Cryptosporidium* species for PCR positive samples was done using standard protocols as described in Chapter 4. Severity of diarrhoea was assessed using the Vesikari scoring system [188].

A diarrhoeal episode was considered to be associated with cryptosporidiosis if a stool sample collected within ± 7 days of that episode was positive. Asymptomatic cryptosporidial infections were based on PCR and serology. An infection identified by stool examination was considered to be asymptomatic cryptosporidiosis if a stool sample tested positive for *Cryptosporidium*.spp but the child did not have a diarrhoeal episode within a range of two weeks before and after the sample was collected. A new episode of cryptosporidiosis was considered if there was at least one negative intervening stool sample with a minimum two week interval. Asymptomatic infection identified by serology was considered when there was a fourfold increase in IgG titre between the two serum sample collections with no history of diarrhoea or stool positivity for *Cryptosporidium* spp. during that period. Where a child was positive for asymptomatic infection by both stool and serology during a defined time interval, the child was considered to have asymptomatic infection by stool and was not double counted as asymptomatic infection by serology for analysis.

Symptomatically undifferentiated cryptosporidial infection was considered to have occurred when there was a fourfold increase in IgG titres between the two serum samples with a history of diarrhoea during the period, but the stool was negative for *Cryptosporidium*.spp. Here the infection was considered detected only on the basis of serologic testing.

The incidence rates of cryptosporidial infections were obtained by Poisson regression equations and calculated estimates, adjusting for clustering of disease/infection within a child. Cumulative incidence of cryptosporidial infection was studied using survival analysis techniques. Kaplan-Meier estimates of median time to infection were calculated and compared between groups. All Kaplan-Meier curves were plotted to assess the median time to first infection as well as to the survival probabilities of children in the cohort. The time point for defining infection was the time at which the stool testing confirmed cryptosporidial infection. We did not define time for infections identified on serology because the exact time of seroconversion could not be ascertained. Hence for estimation of proportion of children acquiring cryptosporidial infection, both stool and serology results were considered, but all the time to event analysis such as incidence rates, Kaplan-Meier curves and seasonality were restricted to only stool results.

The clinical features, severity, treatment and progress of *Cryptosporidium* associated diarrhoea are reported. The number of episodes and the duration of each episode, age at the time of infection, severity of diarrhoeal episodes and clinical profile associated with *Cryptosporidium* spp. and non *Cryptosporidium* spp. in the cohort were compared using the Fisher's exact test or Chi square test for categorical variables and for continuous variables Wilcoxon rank sum test and two-tailed *t*-test were used.

The calendar months of the year were grouped into four broad seasonal categories - the cold and dry period (January to March), hot and dry period (April to June), southwest monsoon season (July to September) and northeast monsoon season (October to December). The incidence rate of infection for each month was calculated accounting for the number of children being followed during that month as the denominator. The magnitude of association between seasons and infection was assessed by Poisson regression analysis.

5.2.3. Results

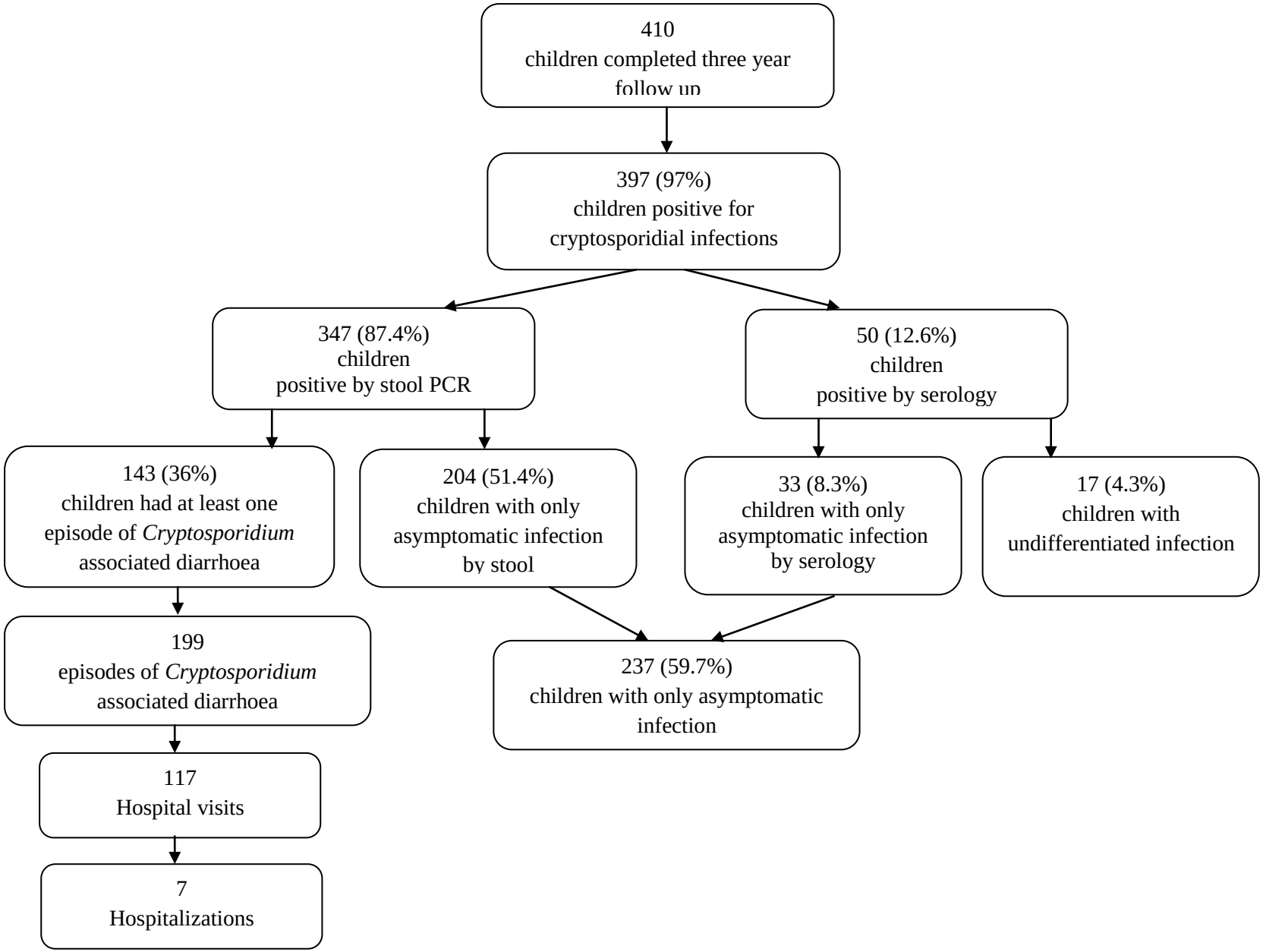
5.2.3.1. Burden of cryptosporidiosis

Among 497 children in the birth cohort, 410 children completed three years of follow-up contributing 1218 child years. Of the 410, 397 (97%) acquired cryptosporidiosis in the first three years of life. Of all children with cryptosporidiosis, 237 children (59.7%) had only asymptomatic infections and 119 (30%) developed both symptomatic/*Cryptosporidium* associated diarrhoea and asymptomatic cryptosporidiosis. Among the remaining children, 24 (6.0%) children had one or more episodes of *Cryptosporidium* associated diarrhoea but no asymptomatic infection and 17 (4.3%) children had only undifferentiated cryptosporidial infection. There was no gender difference between children with and without cryptosporidiosis (P=0.10). Similarly, cryptosporidial diarrhoea had equal distribution amongst males and females (P=0.17).

Among all the children in the cohort confirmed positive for cryptosporidial infection either by stool or serology, 165 (40.2%) of acquired infection during the first six months, 379

(92.4%) by two years and 397 (97%) by three years. Among symptomatic cryptosporidiosis, 24 (5.8%) experienced *Cryptosporidium* associated diarrhoea by six months, 55 (13.4%) by one year, 116 (28.3%) by two years and 143/410 (35%) by three years. The burden of cryptosporidiosis in the cohort based on testing by stool and serology is shown in Figure 5.2.1.

Figure 5.2.1.: Cryptosporidial infections in 410 children completing three years of follow-up



5.2.3.2. Age at first cryptosporidial infections

In the cohort, the median (IQR) age at first infection among children with cryptosporidiosis was 12 (6-20) months. Symptomatic infections/*Cryptosporidium* associated diarrhoea occurred a little earlier (median age=11.4, IQR=4.4-18.5 months) than asymptomatic infections (median age=12.4, IQR=6-20.4 months), which was statistically similar (*P*=0.12). The Kaplan-Meier survival curves for the first cryptosporidial infections in the cohort are presented in Figure 5.2.2.1 and 5.2.2.2 for overall, asymptomatic and symptomatic infections.

Figure 5.2.2.: Kaplan Meier survival curves for cryptosporidial first infections in the birth cohort (n=347)

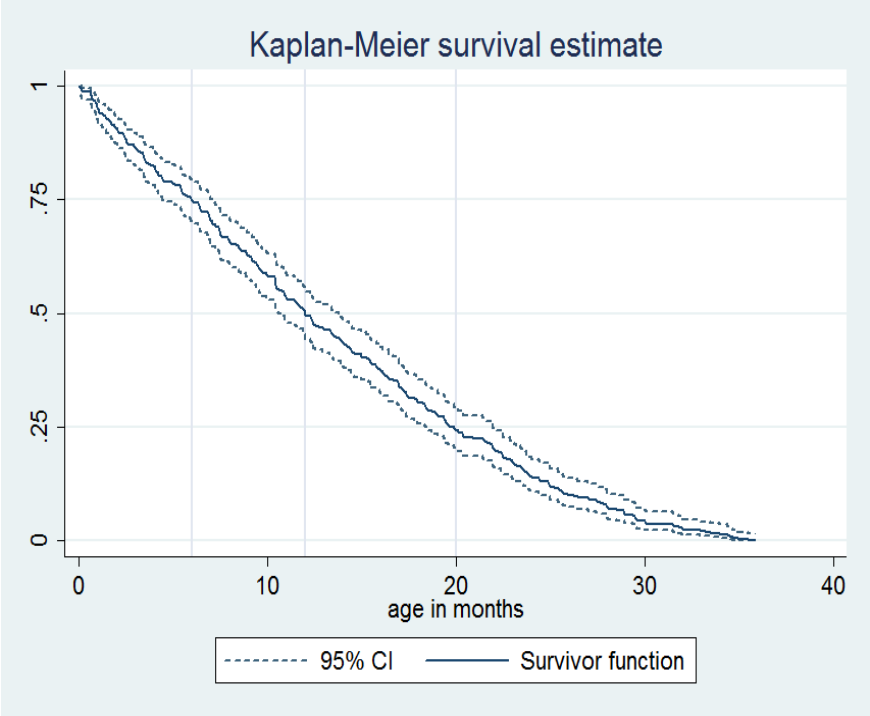


Figure: 5.2.2.1.: All infections in the cohort (n=347)

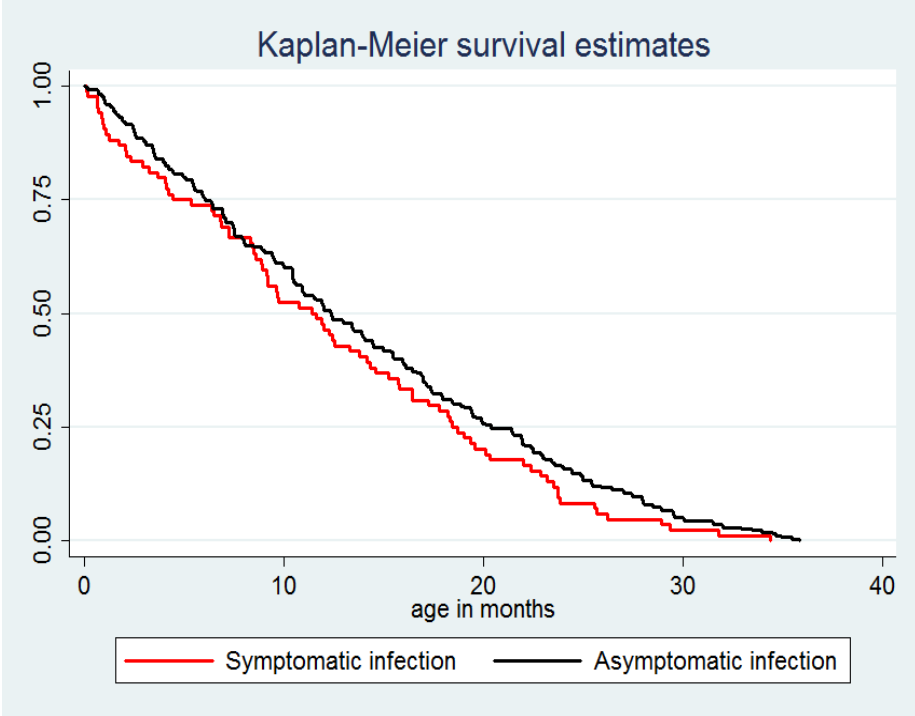


Figure: 5.2.2.2.: Symptomatic (n=84) and Asymptomatic infection (n=263)

5.2.3.3. Incidence rates of cryptosporidial infection in the birth cohort

Among children who completed three years of follow up, 347 experienced 733 episodes of cryptosporidiosis, of which 199 (27%) episodes were symptomatic and 534 (73%) were asymptomatic infections.

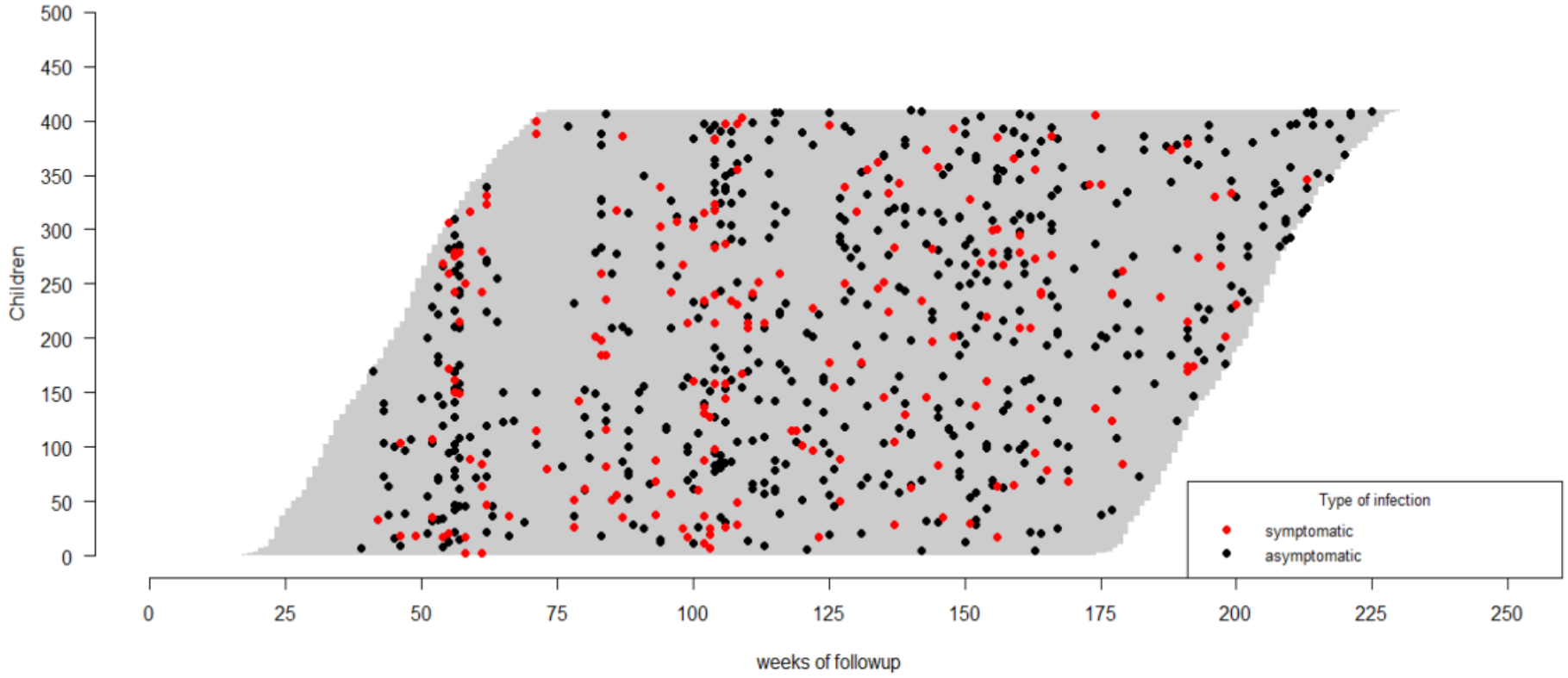
Overall, the incidence of cryptosporidial infection was 0.60 episodes per child year, with the highest incidence (0.72 episodes per child-year) in the second year of life. The overall incidence of symptomatic and asymptomatic infection was 0.16 episodes per child year and 0.44 episodes per child year respectively. The incidence of both symptomatic and asymptomatic infections seemed to increase during the second year of life and was followed by a minimal reduction by three years of age. The age-wise distribution of incidence of cryptosporidial infection is presented in Table 5.2.1.

Figure 5.2.3 shows the distribution by symptomatic and asymptomatic infections for each week of the study for each child. The grey zone shows the recruitment and follow-up of each child. Red and black dots indicate the symptomatic and asymptomatic infections respectively.

Table 5.2.1.: Age-wise distribution of the incidence of cryptosporidiosis in the birth cohort (n=410)

Cryptosporidial infection	0 to 3 years	0 to 1 year	1.1 to 2 year	2.1 to 3 year
Child follow-up years	1218.1	403.46	406.09	408.55
Overall Infection				
Episodes	733	225	292	216
Incidence rate (95% CI) per child-year	0.60 (0.56-0.64)	0.56 (0.49-0.63)	0.72 (0.64-0.80)	0.52 (0.46-0.60)
***Cryptosporidium* associated diarrhoea**				
Episodes	199	66	86	47
Incidence rate (95% CI) per child-year	0.16 (0.14-0.19)	0.16 (0.12-0.21)	0.21 (0.16 -0.26)	0.11 (0.08-0.15)
Asymptomatic infection				
Episodes	534	159	206	169
Incidence rate (95% CI) per child-year	0.44 (0.40-0.47)	0.39 (0.34-0.46)	0.51 (0.44-0.57)	0.41 (0.35-0.47)

Figure 5.2.3.: Scatter plot showing weekly distribution of symptomatic and asymptomatic cryptosporidial infection



5.2.3.4. Repeated cryptosporidial infections

Among 347 children with cryptosporidiosis identified based on stool microbiology, 118 (28.8%) had only one episode of infection, 126 (30.7%) had two episodes, 63 (15.4%) had three episodes, 29 (7.0%) had four episodes and 11 (2.7%) had five or more episodes, indicating a sizeable proportion of children with repeated infections. Median (IQR) duration between two consecutive cryptosporidial infections was 8 (3-13) months. During the three year follow up period, each child had an average of 1.78 episodes of cryptosporidiosis identified based on stool microbiology. Based on this observation and on the assumption that cryptosporidiosis should follow a Poisson distribution, the number of children expected to have 0, 1, 2, 3, 4, 5 and 6 episodes of cryptosporidiosis was calculated and compared with the observed frequency to verify if there was clustering of cryptosporidiosis in children. The formulae used to test the distribution for this computation was [197]:

$$f(x) = \frac{e^{-\lambda}\lambda^{x}}{x!}$$

λ =Mean
X= number of events
e =base of natural

This comparison did not show any evidence of clustering (χ^2 test for goodness of fit =3.08, degrees of freedom= 6, *P*= 0.70, Table 5.2.2).

Table 5.2.2.: Observed and expected frequency of Cryptosporidial infections in the birth cohort

Number of infections	Number of children (n=410)	
	Observed	Expected
0	63	69.14
1	118	123.07
2	126	109.53
3	63	64.99
4	29	28.92
5	8	10.29
6	3	3.05

Similarly, clustering of *Cryptosporidium* associated diarrhoea/symptomatic infection in children was tested. Among the 143 children with symptomatic infection, 99 (69.2%) had only one symptomatic episode, 36 (25.2%) had two episodes, while 8 (5.6%) had three or more episodes. A child in the study developed an average of 0.48 episodes of symptomatic infection. The number of children expected to have 0, 1, 2, 3, 4 and 5 episodes of symptomatic infection was calculated and compared with the observed frequency. The results of this comparison showed evidence of clustering of symptomatic cryptosporidial infection in children (χ^2 test for goodness of fit=25.04, degrees of freedom=5, P<0.0001, Table 5.2.3).

Table 5.2.3.: Observed and expected frequency of symptomatic cryptosporidial infections among the study children.

Number of Symptomatic infections	Number of children (n=410)	
	Observed	Expected
0	267	251.17
1	99	123.07
2	36	30.15
3	5	4.92
4	2	0.6
5	1	0.05

5.2.3.5. Cryptosporidiosis and diarrhoea

Of the 2134 episodes of diarrhoea among the study children (n=410), at least one stool sample could be obtained for 2121 (99.4%) episodes, of which 199 (9.4%) were associated with cryptosporidiosis. Almost 99% of cryptosporidial diarrheal episodes were associated with acute diarrhoea (≤ 14 days duration). A cryptosporidial diarrheal episode had a median (IQR) duration of 3 (2-4) days, which was similar to the duration of non-cryptosporidial diarrhoea.

Of the 199 *Cryptosporidium* associated diarrheal episodes, 45 (22.6%) and 40 (20.1%) episodes were associated with vomiting and fever respectively (Table 5.2.4). The presence of vomiting (*P*=0.86) or fever (*P*=0.29) was not significantly associated with *Cryptosporidium* associated diarrhoea. Twenty four (12.1%) of 199 episodes were classified as severe diarrhoea by Vesikari scoring system, although episodes were predominantly mild or moderate (n=175, 88%). There was no association between disease severity and diarrhoea associated with *Cryptosporidium* (*P*=0.76). There were 48 (24%) episodes of *Cryptosporidium* associated diarrhoea where other co-pathogens, predominantly *Giardia* (27, 13%) and *Shigella* (12, 6%), were also found.

Table 5.2.4.: Comparison of clinical characteristics of diarrhoeal episodes associated with *Cryptosporidium* spp. and non-cryptosporidial diarrhoea

Clinical Characteristics	*Cryptosporidium* associated diarrhoea (n=199)	Non-cryptosporidial diarrhoea (n=1922)	*P*-value
Median (IQR) age in months	16 (9-24)	10 (6-20)	<0.0001**
Mean (IQR) duration in days	3 (2-4)	3 (2-4)	-
Accompanying symptoms			
Vomiting (%)	45 (22.6%)	455 (23.6%)	0.86#
Fever (%)	40 (20.1%)	450 (23.4%)	0.29#
Treatment required			
Clinic visits (%)	117 (58.8%)	1235 (64.3%)	0.12#
Hospitalization (%)	7 (3.5%)	47 (2.4%)	0.36#
Intravenous fluids (%)	1 (0.5%)	13 (0.7%)	0.77+
*Severity of diarrhoeal episodes**			
Mild (%)	95 (47.7%)	955 (49.8%)	
Moderate (%)	80 (40.2%)	758 (39.5%)	0.76#
Severe (%)	24 (12.0%)	203 (10.6%)	

*Data not available for 6 episodes, *P*-values calculated using: ** Wilcoxon rank sum test, # χ^2 test, + Fisher exact test

5.2.3.6. Cryptosporidiosis and seasonality

A plot of the incidence of cryptosporidial infections by calendar time showed peaks during December and January, which are cooler months, and a reduction of infection rates during April to July which are warmer months (Figure 5.2.4). Consistent patterns were observed when the rates were compared between all the 3 years as shown in Figure 5.2.5 (years with 12 months data were considered). The risk (incidence risk ratio-IRR) of acquiring an infection in the cold/dry season was three times higher than in the hot/dry season (Table 5.2.5).

Table 5.2.5.: Estimation of risk of cryptosporidial infection across different seasons during three year follow-up

Seasons	IRR (95% CI)
Dry/Hot (April to June)*	1
South West monsoon (July to Sept)	1.66 (1.28-2.15)
North East monsoon (Oct to Dec)	2.54 (2.00-3.23)
Dry/Cold (Jan to Mar)	3.34 (2.65-4.21)

* Reference category

Figure 5.2.4.: Monthly distribution of cryptosporidial incidence rates during the study period: April 2009 to May 2013

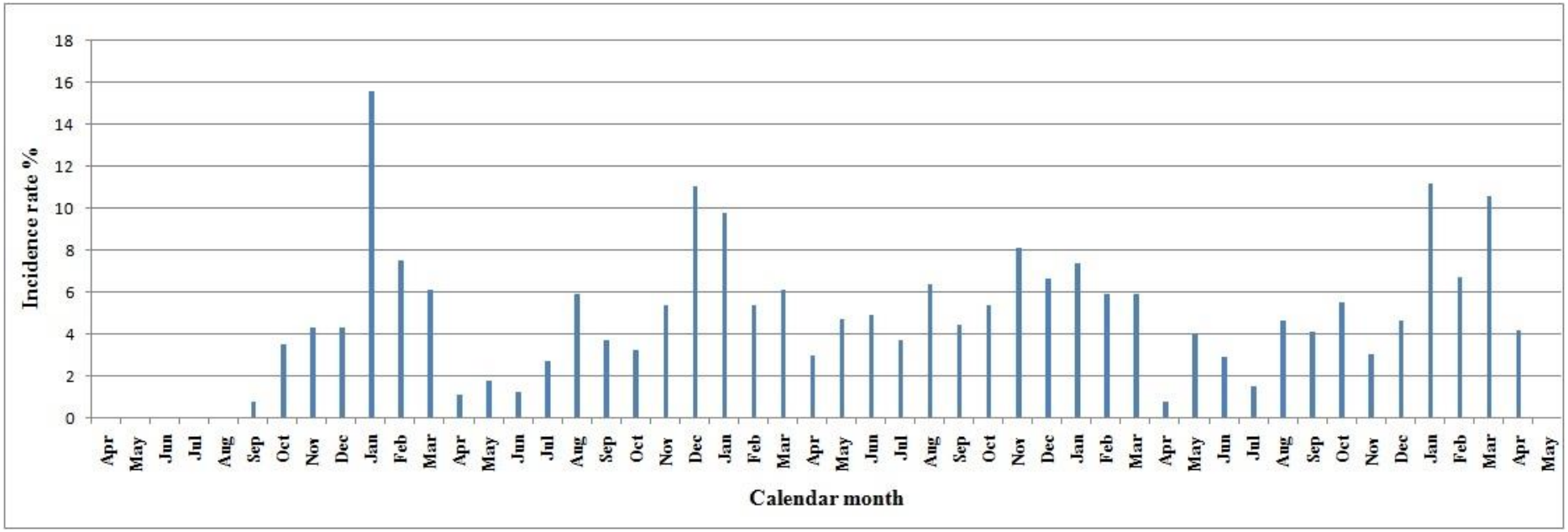


Figure 5.2.5.: Comparison of monthly incidence rate of cryptosporidial infection across three years (2010, 2011 and 2012)

5.2.3.7. Molecular epidemiology

Genotypes were determined for 473 (64.5%) of 733 episodes of cryptosporidiosis which were positive by stool examination. *C. hominis* was the predominant species and was associated with 347 (73.3%) of all speciated infections, followed by C. *parvum* in 81 (17.1%) infections. Other species isolated from stool samples were *C. meleagridis* (5.2%), *C. felis* (1%), *C. andersoni/muris* (0.2%). Mixed infection by both *C. parvum* and *C. hominis* as identified in 14 (2.9%) episodes. The frequency of infections caused by the different species of *Cryptosporidium* spp. is presented in Table 5.2.5.

Table 5.2.5.: Frequency of infections caused by the different species of *Cryptosporidium*.spp among study children

Species distribution	All infections	*Cryptosporidium* associated diarrhoea	Asymptomatic infection
Total no. of episodes	733	199	534
No. Genotyped	473	138	335
C. hominis	347 (73.3%)	99 (71.7%)	248 (74.0%)
C. parvum	81 (17.1%)	20 (14.5%)	61 (18.2%)
C. meleagridis	25 (5.2%)	6 (4.3%)	19 (5.6%)
C. felis	5(1%)	1 (0.7%)	4 (1.2%)
Mixed Infections			
C. hominis & C. parvum	14 (2.9%)	12 (8.7%)	2 (0.6%)
C. andersoni & C. muris	1 (0.2%)	0 (0%)	1 (0.3%)

The incidence rate of *C. hominis and C. parvum* among the children infected with *Cryptosporidium* was 0.50 (0.46-0.55) episodes per child year and 0.11 (0.09-0.15) episodes per child year respectively.

Confining the analysis to the first infection only, median (IQR) age of *C. hominis* infection was 11.6 (7.2-19) months, and for those infected with *C. parvum*, the median age was 13.2 (10-18) months, not significantly different (*P*=0.38, Wilcoxon rank sum). The median age of first infection for *C. meleagridis* was 11 (10-19) months.

5.2.3.8. Association of clinical characteristic with *Cryptosporidium* species

Of 199 cryptosporidial diarrhoea, 138 (69.3%) could be genetically characterised to species level. Ninety-nine episodes were associated with *C. hominis.* There was no statistically significant difference in the clinical profile of *C. hominis* diarrhoeal episodes when compared to diarrhoea caused by other species (Table 5.2.6).

Table 5.2.6.: Clinical features associated with *Cryptosporidium* species

Clinical profile	*C. hominis* (n=99)	Other species (n=39)	*P*-value
Associated with vomiting	19 (19.2%)	8 (20.5%)	0.86#
Associated with fever	20 (20.2%)	8 (20.5%)	0.96#
Episodes of severe diarrhoea	10 (10.1%)	4 (10.3%)	0.59+
Clinic visits	56 (56.6%)	23 (58.9%)	0.79#
Median (IQR) duration per episodes (in days)	2 (2-4)	3 (2-6)	0.13**

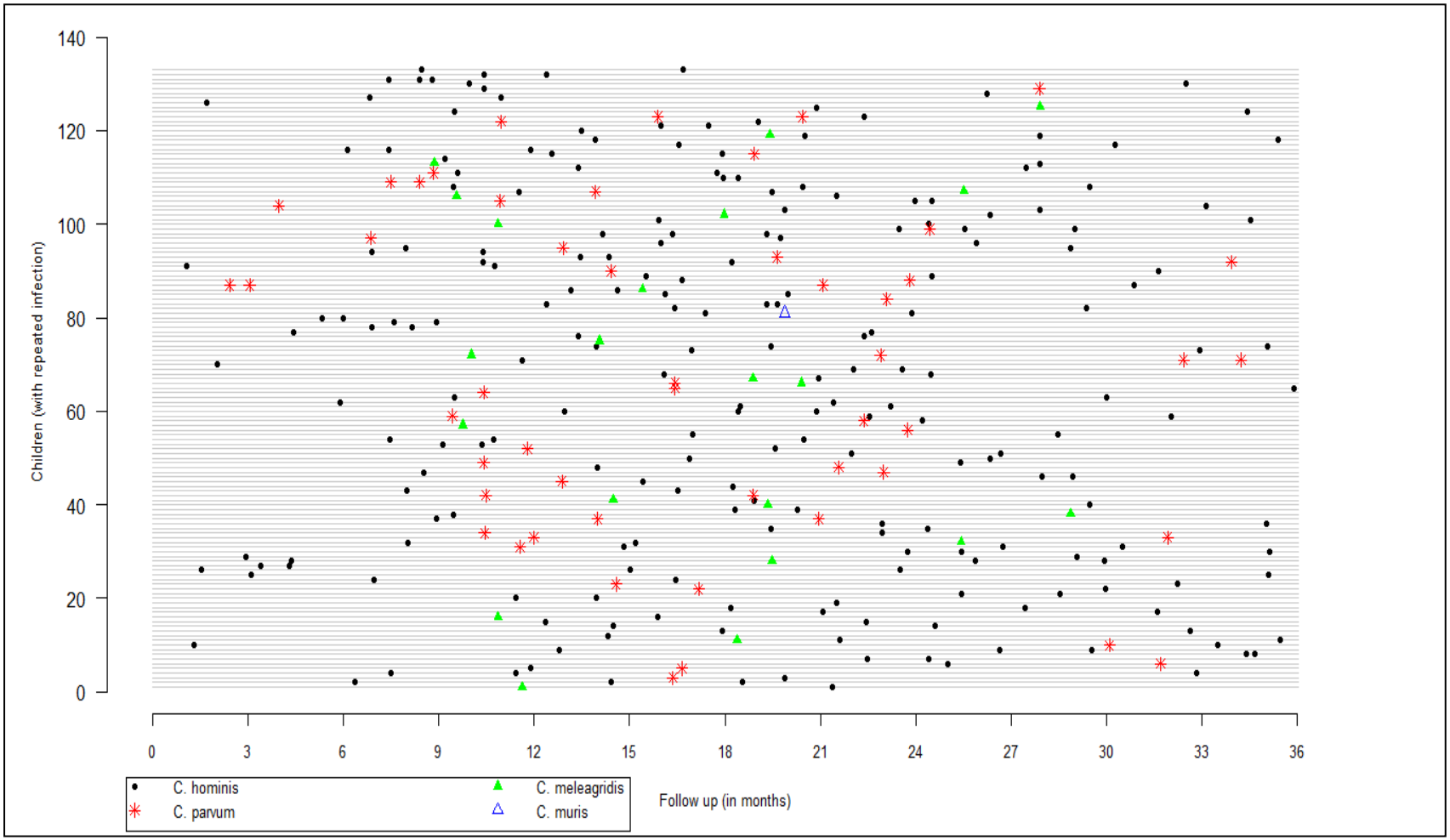
P-values calculated using: ** Wilcoxon rank sum test, # χ^2 test, + Fisher exact test

5.2.3.9. Repeated infections with *Cryptosporidium* species

Of 347 children with cryptosporidiosis by stool examination, species data were available for 298 (86%) children. Of these, 133 (44.6%) had two or more symptomatic or asymptomatic episodes of cryptosporidiosis during their follow-up period, resulting in a total of 308 episodes. *C. hominis* was the commonest, accounting for 220 (71.4%), followed by *C. parvum* in 50 (16.2%) of the 308 episodes (Figure 5.2.5).

Of 217 children with *C. hominis* during the primary infection, 90 (86%) had re- infections with *C. hominis* and 8% with *C. parvum*. Fifty five children had primary infection with *C. parvum*. Of these, 24 experienced re-infections with *C. parvum* (52.6%) and with *C. hominis* (38.6%).

Figure 5.2.5.: Distribution of the different *Cryptosporidium* species among children with multiple infections (n=133):



5.2.4. Discussion

Although *Cryptosporidium* spp. was discovered many decades ago, it became a public health concern only after the AIDS pandemic and the Milwaukee outbreak in 1993. Even after recognition of its importance in immunocompromised individuals and as a water-borne pathogen resistant to many methods of water purification, its role in childhood infections has not received the attention it deserved, with most infectious disease physicians, paediatricians and public health specialists unaware of its importance as a pathogen in children. The recent multi-centric GEMS study reported *Cryptosporidium* as one of the five top pathogens causing moderate to severe diarrhoea in children [18], thus bringing it to the attention of the global health community. Although GEMS identified the importance of cryptosporidiosis in diarrhoeal disease, little is known about the natural history of infection and about the ability of prior infections to protect against subsequent disease. This cohort study reported here is a major effort to address these lacunae.

There was a high burden of cryptosporidiosis reported in this study with 97% of children being infected by *Cryptosporidium* spp. by the age of 3 years Almost 85% of the children were positive by stool examination and an additional 12% positivity was contributed by serology. The incidence of cryptosporidiosis was 0.60 episodes/child-year, which is much higher than reported from cohort studies among children in Peru (0.22 episodes/child year) [239] and Guinea Bissau (0.33 episodes/child year) [240]. Among urban Brazilian children followed–up for four years and Israeli Bedouin children followed-up for two years, about 31% and 49% developed cryptosporidial infection respectively [151-152]. The high rates of infection being reported in our study can be attributed to the intensive surveillance and use of sensitive diagnostic methods such as PCR and complementation by serology. It has been

established that the use of molecular techniques significantly increases the rates of detection of enteric pathogens [127] and seroepidemiological studies have documented a higher prevalence of enteric infection than parasitological studies relying on stool examination alone [21, 241-243]. The determination of infection rates and hence of the real burden of cryptosporidial infections and disease in a population requires a longitudinal design, and molecular and serological techniques for diagnosis to yield more sensitive estimates.

In this study, a large proportion of cryptosporidiosis was contributed by asymptomatic infections, with the majority (60%) of children developing asymptomatic cryptosporidial infections. Our findings were similar to Peruvian and Indian cohorts of children where high prevalence of asymptomatic infections of 67% and 64.4% has been reported previously [22, 149]. High asymptomatic infection rates have also been reported from cross-sectional studies in Venezuela (86.3%) [244] and Thailand (64.2%) [21]. In contrast, longitudinal studies from Brazil [151] and Guatemala [245] and a cross-sectional study from Uganda [246] have reported 79%, 65% and 72.7% cryptosporidial diarrhoea, respectively. A reason for the high proportion of asymptomatic infections in the community may be the infectious nature of the parasite and constant and prolonged exposure and re-exposure to the parasite resulting in subclinical infections, especially in endemic areas where there is transmission within communities through multiple routes of entry [12].

Although *Cryptosporidium* spp. infects children throughout the first three years of life, infection starts early with 40% of the children infected in the first six months. An explanation for these findings could be that infants are exposed early to an environment saturated with the parasite and have a higher probability of acquisition of infection because of poor hygiene,

water and sanitation in urban slums. Another reason could be waning maternal antibodies which may be protective against early infection and exposure to contaminated environmental sources at a period corresponding to weaning [247-250].

In this cohort, 66% of children had more than one episode of cryptosporidiosis during the three-year follow-up period. There was significant clustering observed in children with *Cryptosporidium* associated diarrhoea, but not for cryptosporidial infections. This indicates that children living in an endemic area may acquire repeated infections due to constant exposure to this parasite and risk factors associated with this parasitic infection, but some children seem to be predisposed to become symptomatic when infected. This could be because of host immunologic factors and genetic susceptibility in these children. The finding of mannose binding lectin levels being associated with cryptosporidiosis, as shown in Bangladesh and Haiti, supports the role of innate immune factors in the host determining susceptibility to symptomatic infection [153, 251].

In the study cohort, 9.4% of diarrhoeal episodes were associated with cryptosporidiosis, quite similar (8.4%) to a study reported by Sarkar *et al.* among children in the same community [22]. In a cohort of Guatemalan infants, 8.3% of the diarrhoeal episodes were associated with *Cryptosporidium* spp. [245]. In a Brazilian cohort, *Cryptosporidium* oocysts were isolated from 8.4% and 16.5% of samples obtained from episodes of acute and persistent diarrhoea respectively [151]. Only 24 (1.1%) episodes of persistent diarrhoea were observed in our study, of which 2 episodes were attributed to *Cryptosporidium*. Longitudinal studies from Brazil [151] and Guinea–Bissau [155] have reported persistent diarrhoea in children; however in this study, *Cryptosporidium* was predominantly associated with acute diarrhoea.

This study did not demonstrate any clinical differences between cryptosporidial diarrhoea and diarrhoea caused by other pathogens. Studies from Uganda [252] and Bangladesh [253] also showed no clinical differences between *Cryptosporidium* associated diarrhoea and non-cryptosporidial diarrhoea, except that the duration of cryptosporidial diarrhoea was longer in the Bangladesh study.

This study found that *Cryptosporidium* spp. peaks during cooler months. Studies from Spain [254], Kenya [112], Malawi [255], Rwanda [256] also had similar peaks of infection in the colder season, whereas peaks were observed in New Zealand during spring (September) [257] and summer to fall in the United States [258-259]. Longitudinal studies from Peru showed higher infections during the warm season (December to May) [149], whereas in Brazil, high infection rates were reported during the rainy season (April) and reduced infection rates during the driest months (August to December) [151]. A possible explanation could be that during cooler moist conditions/seasons, oocysts can persist for months in the environment and remain viable [260-262]. Factors such as extremes of temperature, exposure to ultraviolet radiation, and desiccation can substantially reduce the number of infective oocysts [263-265], which could be a reason for the reduced infections in summer (April to June) in our study.

The majority of cryptosporidial infections in this study were associated with *C. hominis* (73%), although infections with *C. parvum* and other zoonotic species were also observed. *C. hominis* had been identified as the most common (ranging from 79-88%) species in children from previous community and hospital based studies from the same region [22, 128, 163-164] and elsewhere in India [128, 167, 266]. Developing countries such as Brazil [267],

Bangladesh [268], Malawi [255], Peru [239], Nigeria [112] have also reported the predominance of *C. hominis* among children, whereas higher prevalence of *C. parvum* was identified in Kuwaiti [269] and Nicaraguan [270] children. The predominance of *C. hominis* among children may indicate the primary role of person-to-person transmission of infection in this community. A high proportion of re-infection, with *C. hominis* was observed in this study. In this region, previous reports also demonstrated a large proportion of children were re-infected by *C. hominis* [22, 164]. It is possible that some of the re-infection episodes could be seen as prolongation of a single infection, as studies have shown that children with cryptosporidial disease may shed oocysts even (2 months) after the infection [164, 271], but more detailed subtyping may also be applied to determine whether or not re-infection with a new subtype had occurred.

5.2.5. Conclusion

This study provides important insights into the natural history of cryptosporidiosis in an endemic semi-urban slum community in southern India. The fact that almost all children in the study acquired cryptosporidial infection by three years of age indicates a high rate of transmission in the community. The high proportion of asymptomatic infections in the community also suggests constant exposure to this parasite. Almost two-thirds of children had repeated cryptosporidial infections. Children with symptomatic infection tended to have a higher probability of repeated diarrhoea, suggestive of genetic and host susceptibility. The predominance of *C. hominis* supports predominantly person-to-person transmission. These findings could be attributed to the poor hygiene and unsanitary living conditions in slum communities.

CHAPTER 5.3

RISK FACTORS FOR CRYPTOSPORIDIOSIS IN THE BIRTH COHORT

5.3.1. Introduction

Cryptosporidium is ubiquitous and has multiple modes of transmission such as person-to-person, zoonotic, contaminated food and water [272]. There is lack of effective treatment available for cryptosporidiosis in vulnerable populations [273]. The long term sequelae of early childhood cryptosporidiosis have been associated with growth retardation and cognitive deficit [23-24, 26]. Host, socio-economic and environmental factors such as low birth weight, age of the child, male gender, lack of breast feeding, low socioeconomic status, presence of animals in the same household, food storage practices, diarrhoea in the family, rainy season been described as risk factors for cryptosporidial infections in various countries (Table 5.3.1). The data from these countries suggests that risk factors vary from place to place. Hence it is essential to identify the factors associated with cryptosporidial infection which are socially and geographically relevant, in order to design strategies to prevent or control disease caused by this protozoan parasite.

This analysis was undertaken to identify risk and protective factors associated with cryptosporidial infection in the birth cohort.

Table 5.3.1.: Studies on the risk and protective factors of cryptosporidial infections in developed and developing countries

Location	**Year(s)**	**Study design**	**Study setting**	**Sample size**	**Target population**	***Cryptosporidium* detection method**	**Risk and protective factors**	**Reference**
Guatemala	1985--1986	Cohort	Community	130	< 1 year	Microscopy	***Risk:*** Liquid or solid foods in the diet; presence of domestic animals (dogs, cats, or poultry); absence of toilet facilities	[245]
Guinea-Bissau	1988--1990	Case-control	Community	250	0--37 months	Microscopy	***Risk:*** Presence of pigs and dogs in the household; male gender, storage of cooked food for later consumption ***Protection:*** Breast feeding	[274]
Mexico	1988--1989	Cross-sectional	Community	403	< 5 years	IFA	***Risk:*** Malnutrition; non breast-fed children	[275]
Brazil	1989--1993	Cohort	Community	189	≤ 4 years	Microscopy	***Risk:*** Low birth weight; crowded living conditions	[151]
Bangladesh	1991--1994	Case-control	Hospital	272	< 5 years	Microscopy	***Risk:*** Age below two years; non-breastfed children; stunting	[156]
Indonesia	1992--1993	Cross-sectional	Community	4368	All age groups	Microscopy	***Risk:*** contact with cats; crowded living conditions, rainy season; flooding	[158]

Table 5.3.1(contd): Studies on the risk and protective factors of cryptosporidial infections in developed and developing countries

Location	**Year(s)**	**Study design**	**Study setting**	**Sample size**	**Target population**	***Cryptosporidium* detection method**	**Risk and protective factors**	**Reference**
Peru	1995--1998	Cohort	Community	368	< 12years	Microscopy	***Risk:*** Houses without a latrine or toilet	[149]
Zambia	1995--1996	Cross-sectional	Hospital	222	< 12years	Microscopy	***Risk:*** Rainy season; breast feeding; living in households that owned their house	[276]
USA	1996--1997	Cross sectional	Hospital	285	6 months--13 years	Serum ELISA	***Risk:*** Consumption of municipal water; increased age of the child; lower annual household income	[277]
Brazil	1998--1999	Cross-sectional	Hospital	445	≤ 10 years	Direct immunoflurescent assay	***Risk:*** Age less than two yrs; male gender; day care attendance; having children with diarrhoea in the household	[278]
USA	1999--2001	Case-control	Community*	282	All age groups	Fluorescent microscopy	***Risk:*** International travel; contact with cattle; contact with persons >2 to 11 years of age with diarrhoea; freshwater swimming; ***Protection:*** Eating raw vegetables	[279]
Mexico	---	Cross-sectional	Community	132	1--15 years	Microscopy	***Risk:*** Diarrhoea in the family; crowded living conditions; drinking non-potable water	[159]

Table 5.3.1.(contd) Studies on the risk and protective factors of cryptosporidial infections in developed and developing countries

Location	**Year(s)**	**Study design**	**Study setting**	**Sample size**	**Target population**	***Cryptosporidium* detection method**	**Risk and protective factors**	**Reference**
UK	2000--2003	Case-control	Community*	6736	All age groups	PCR	***Risk:*** High socioeconomic status; age less than four years; residing in areas with poor water treatment	[280]
UK	2001--2002	Case-control	Community*	854	All age groups	PCR	***Risk:*** Travel outside of the country; contact with another person with diarrhoea; touching cattle; ***Protection:*** Eating ice cream and raw vegetables	[281]
Malaysia	2004	Cross-sectional	Community	276	2--15 years	Microscopy	***Risk:*** Low birth weight; large family size; breast feeding	[282]
Iran	2005--2006	Cross-sectional	Hospital	171	< 5years	Stool ELISA	***Risk:*** Low birth weight; breast feeding for less than one month	[283]
Nigeria	2006--2007	Cross-sectional	Community	692	19.5--72 months	PCR	***Risk:*** Stunting; younger age	[284]
Venezuela	2008	Cross-sectional	Community	536	All age groups	Microscopy	***Risk:*** Living in a hut or small residence; extreme poverty; open air defecation; crowded living condition	[244]

5.3.2. Data and analytic methods

To identity risk factors associated with cryptosporidiosis, cases were defined as children who developed one or more episodes of cryptosporidiosis at any time during the follow-up and controls were defined as children with no evidence of cryptosporidial infection (as detected by stool PCR). Analysis was restricted to the 410 children who had completed three years of follow-up.

Household hygiene was assessed using an 18-point scale, which covered aspects of hygiene in domains of personal, water and food. The questionnaire has been used in this community earlier [185, 224]. The hygiene measurement closest to the time of child's weaning was used for the present analysis, and families with a score of ≥12 (upper tertile of the hygiene score) were considered to have good household hygiene.

In study houses, presence of potential environmental contaminants such as cow or other animal sheds, garbage dumping sites, sewage channels and open-air defecation fields, within a specified perimeter of the study house was assessed using GIS data collected through Garmin GPS V receivers (Garmin International Inc., Olathe, KS, USA) and mapped using ArcGIS 10 software (Environmental Systems Research Institute Inc., Redlands, CA, USA). Nearest distances between study households and the potential environmental contaminant was calculated using the "distance between points (between layers)" feature in the Hawth's Analysis Tools 3.26 (http://www.spatialecology.com/htools), an extension of the ArcGIS software. Occupants of houses within a specified distance (50 m for open-air defecation fields, 10 m for other attributes) of a potential environmental contaminant were considered to have an increased risk of acquiring cryptosporidial infection. Maps depicting the distribution

of cryptosporidial infections among the children in the study were created in ArcGIS 10.0 by overlaying layers with the study area boundaries and by categorising the study households as either "with infection" or "without infection" as presented in the two panels. Figure 5.3.1 shows the distribution of overall cryptosporidial infections among study children, whereas Figure 5.3.2 is restricted to the distribution to children with "species" data available, i.e. 298 out of the 410 children followed for the study.

Nutritional deficiency in children was assessed by computing the weight-for-height (WHZ), height-for age (HAZ) and weight-for-age (WAZ) z-scores. The WHO child growth standards of 2006 were used as the reference [285]. Children were categorized as wasted (WHZ < -2 SD), stunted (HAZ < -2 SD, and underweight (WAZ < -2 SD) or normal based on their z-scores. Children who were stunted, wasted and underweight at 6 months were considered for the analysis.

The socio-demographic and other baseline characteristics of children who had completed the study and the children who dropped out were compared using the Fisher's exact test or Chi square test for categorical variables and Mann-Whitney U test and the two tailed t test for continuous variables, depending on the distribution of data.

A Poisson survival analysis with robust standard errors was performed to identify factors associated with overall cryptosporidiosis. Univariate survival analysis was performed at first for all exposure variables, and crude hazard ratios (HR) and 95% confidence interval (95% CI) calculated. The variables significant at $P \leq 0.2$ level and/or those that were known risk

factors for childhood cryptosporidiosis were then included in the multivariate analysis and a final model built using the backward stepwise method.

A nested case-control analysis was performed to ascertain factors associated for *Cryptosporidium* associated diarrhoea, asymptomatic and multiple cryptosporidial infections. Univariate logistic regression analysis was performed at first for all exposure variables, and crude odds ratios (OR) and 95% confidence interval (95% CI) calculated. The variables significant at $P \leq 0.2$ level and/or those that were known risk factors for childhood cryptosporidiosis were then included in the multivariate analysis and a final model built using the backward stepwise method. The factors associated with *Cryptosporidium* associated diarrhoea and asymptomatic cryptosporidiosis were assessed by separately comparing the control children with those having *Cryptosporidium* associated diarrhoea (defined as cryptosporidial infection detected by stool PCR within ±7 days of a diarrheal episode) and asymptomatic (defined as cryptosporidial infection detected by stool PCR on biweekly or monthly surveillance samples) infections, respectively. Similarly, children with multiple cryptosporidial infections (defined as more than two asymptomatic infections or *Cryptosporidium* associated diarrhoea) were compared separately with the controls.

In order to ascertain factors associated with species-specific cryptosporidiosis, a logistic regression was done on a subgroup of children where species data were available. To identify factors associated with *C. hominis* species, children infected with only *C. hominis* species were considered as cases, whereas for *C. parvum*, children who were ever positive for *C. parvum* during follow-up were considered as cases. Controls in both cases were children without cryptosporidiosis. The factors associated with *C. hominis* and *C. parvum* species were assessed separately.

5.3.3. Results

5.3.3.1. Baseline comparison of study children

The baseline socio-demographic characteristics of children who stayed in the study till the end of study period did not differ significantly from those who dropped out (Table 5.3.2). Of the 410 children who completed the follow-up, 347 (84.6%) developed one or more episodes of cryptosporidiosis, 229 (66%) had multiple infections, ranging from 2 to 6 episodes.

5.3.2. Comparison of baseline characteristics between children who completed the follow-up (n=410) and those who dropped out (n=87)

Characteristic	3 yrs of follow-up (n=410)	< 3yr of follow-up (n=87)	*P*-value
Gender of child			
Male	225 (54.9%)	38 (43.7%)	
Female	185 (45.1%)	49(56.3%)	0.06
Birth weight			
Missing	6 (1.5%)	1 (1.1%)	
<2.5kg	69 (16.8%)	15 (17.2%)	
≥ 2.5 kg	335 (81.7%)	71 (81.6%)	0.93
Mode of delivery			
Normal vaginal	323 (78.8%)	64 (73.6%)	
Instrument aided	17 (4.1%)	3 (3.4%)	0.42
Caesarean	70 (17.1%)	20 (23%)	
Place of the birth			
Hospital or health center	402 (98%)	85 (97.7%)	
Home	8 (2%)	2 (2.3%)	0.83
Maternal age			
≤ 23 yr	220 (53.7%)	36 (41.4%)	
>23 yr	190 (46.3%)	51 (58.6%)	0.39
Maternal literacy			
Illiterate	147 (35.9%)	37 (42.5%)	
Literate	263 (64.1%)	50 (57.5%)	0.24
Presence of sibling			
Yes	230 (56.1%)	48 (55.2%)	
No	180 (43.9%)	39 (44.8%)	0.87
Religion			
Hindu	219 (53.4%)	45 (51.7%)	
Muslim	175 (42.7%)	39 (44.8%)	0.92
Christian	16 (3.9%)	3 (3.4%)	
Type of family			
Joint	84 (20.5%)	18 (20.7%)	
Extended	86 (21%)	16 (18.4%)	0.85
Nuclear	240 (58.5%)	53 (60.9%)	
House ownership			
Own	257(62.7%)	58 (66.7%)	
Rented/Government	153 (37.3%)	29(33.3%)	0.48
Mode of cooking			
Firewood	169 (41.2%)	30 (34.5%)	
Kerosene	87 (21.2%)	24 (27.6%)	
Gas	144 (35.1%)	28 (32.2%)	0.17
More than one	10 (2.4%)	5 (5.74%)	
Socioeconomic status			
Low	265 (64.6%)	63 (72.4%)	
Middle	139 (33.9%)	21 (24.1%)	0.11
Upper	6 (1.5%)	3 (3.5%)	
Exclusively breastfed for 6 months	65 (15.8%)	9 (10.3%)	0.19

The factors associated with acquisition of cryptosporidial infections in the study children were assessed over a wide range of demographic, socio-economic, nutritional, hygiene and environmental variables.

In the univariate analysis, children in the age group 1 to 2 years were found to have higher risk of cryptosporidial infections compared to infancy (HR=1.28, 95% CI=1.08-1.53). Maternal age <23 years (HR=1.21, 95% CI=1.05-1.38), living in a hut/kutcha house (HR=1.18, 95% CI=0.99-1.41), presence of a cow in the house (HR=1.17, 95% CI=1-1.36), and presence of any domestic animals in the house (HR=1.16, 95% CI=1.01-1.35) were associated with an increased risk of childhood cryptosporidiosis in the univariate analysis. Always boiling drinking water (HR=0.80, 95% CI =0.64-1.00) and usage of toilet by all the members of the household (HR=0.83, 95% CI=0.73-0.96) showed some degree of protection against cryptosporidial infections. No associations between childhood cryptosporidiosis and presence of potential environmental contaminants such as sewage channels, garbage dumps, animal sheds or open-air defecation areas near the house were observed (Table 5.3.3).

In the multivariate analysis, children within the age of 1-2 years (HR=1.29, 95% CI=1.08-1.54), presence of one or more older siblings in the house (HR=1.16, 95% CI=1.01- 1.34), maternal age <23 years (HR=1.26, 95% CI=1.09-1.46), presence of an animal in the house (HR=1.16, 95% CI=1.01-1.35) were associated with an increased risk of cryptosporidial infections, whereas always boiling drinking water (HR=0.77, 95% CI=0.61-0.96) and use of toilet by all the household members (HR=0.87, 95% CI=0.75-1.00) were found to be protective. The results of the multivariate analysis are presented in Table 5.3.4. Figure 5.3.1 shows the distribution of cryptosporidiosis in the birth cohort along with the distribution of animals in the study houses.

Table 5.3.3.: Univariate survival regression analysis for risk factors of cryptosporidiosis

Variable	Univariate Analysis	
	Hazard Ratio (95% CI)	*P*-value
Age of the child		
0-1 year*	1	
1-2 year	1.28 (1.08-1.53)	0.004
2-3 year	0.94 (0.79-1.12)	0.54
Male child	0.95 (0.83-1.09)	0.53
Low birth weight	1.12 (0.93-1.33)	0.21
Presence of older sibling	1.05 (0.91-1.20)	0.46
Breast feeding over 6months	1.16 (0.97-1.39)	0.09
Maternal factors		
Illiterate mother	0.95 (0.82-1.10)	0.52
Maternal age <23 years	1.21 (1.05-1.38)	0.006
Economic and living conditions		
Low socioeconomic status	1.05 (0.9-1.21)	0.45
Hut/kutcha house	1.18 (0.99-1.41)	0.05
Firewood as main cooking fuel	1.01 (0.88-1.15)	0.87
Crowding (>5 persons per room)	1.01 (0.87-1.17)	0.85
Hygiene and sanitation		
Good hygiene	0.94 (0.82-1.08)	0.38
Always boiling drinking water	0.80 (0.64-1.00)	0.05
Presence of toilet ‡	0.85 (0.74-0.98)	0.03
Use of toilet by all members ‡	0.83 (0.73-0.96)	0.01

Table5.3.3.(contd) Univariate survival regression analysis for risk factors of cryptosporidiosis

Variable	Univariate Analysis	
	Hazard Ratio (95% CI)	*P*-value
Animal and environmental factors		
Presence of cow at home ‡	1.17 (1.00-1.36)	0.04
Presence of animal at home ‡	1.16 (1.01-1.35)	0.03
Contact with the animal ‡	1.14 (0.98-1.33)	0.08
Cow or other animal shed within 10 m of the house	0.98 (0.81-1.18)	0.86
Garbage dump within 10 m of the house	1.01 (0.88-1.16)	0.81
Open sewage channel within 10m of the house	1.01 (0.87-1.17)	0.83
Open-air defecation area within 50 m of the house	0.94(0.74-1.19)	0.63
Nutritional status of the child		
Stunted at 6 months of age	1.13 (0.97-1.31)	0.1
Wasted at 6 months of age	0.91 (0.73-1.12)	0.4
Underweight at 6 months of age	1.05 (0.90-1.22)	0.5

* Reference category

‡ missing data from 13 children

$ “kutcha” house: A house thatched roof, with mud or concrete floor

Table 5.3.4.: Multivariate survival regression analysis for risk factors of cryptosporidiosis

Variable	Multivariate Analysis	
	Hazard Ratio (95% CI)	*P*-value
Age of the child		
0-1 year*	1	
1-2 year	1.29 (1.08-1.54)	0.004
2-3 year	0.94 (0.79-1.12)	0.50
Presence of older sibling	1.16 (1.01-1.34)	0.04
Maternal factors		
Maternal age <23 years	1.26 (1.09-1.46)	0.002
Economic and Living conditions		
Hut/kutcha house [$]	1.18 (1.00-1.41)	0.05
Hygiene and sanitation		
Always boiling drinking water	0.77 (0.61-0.96)	0.02
Use of toilet by all members [‡]	0.87 (0.75-1.00)	0.05
Animal and environmental factors		
Presence of animal at home[‡]	1.16 (1.01-1.35)	0.04

* Reference category

‡ missing data from 13children

$ “kutcha” house: A house with thatched roof, with mud or concrete floor

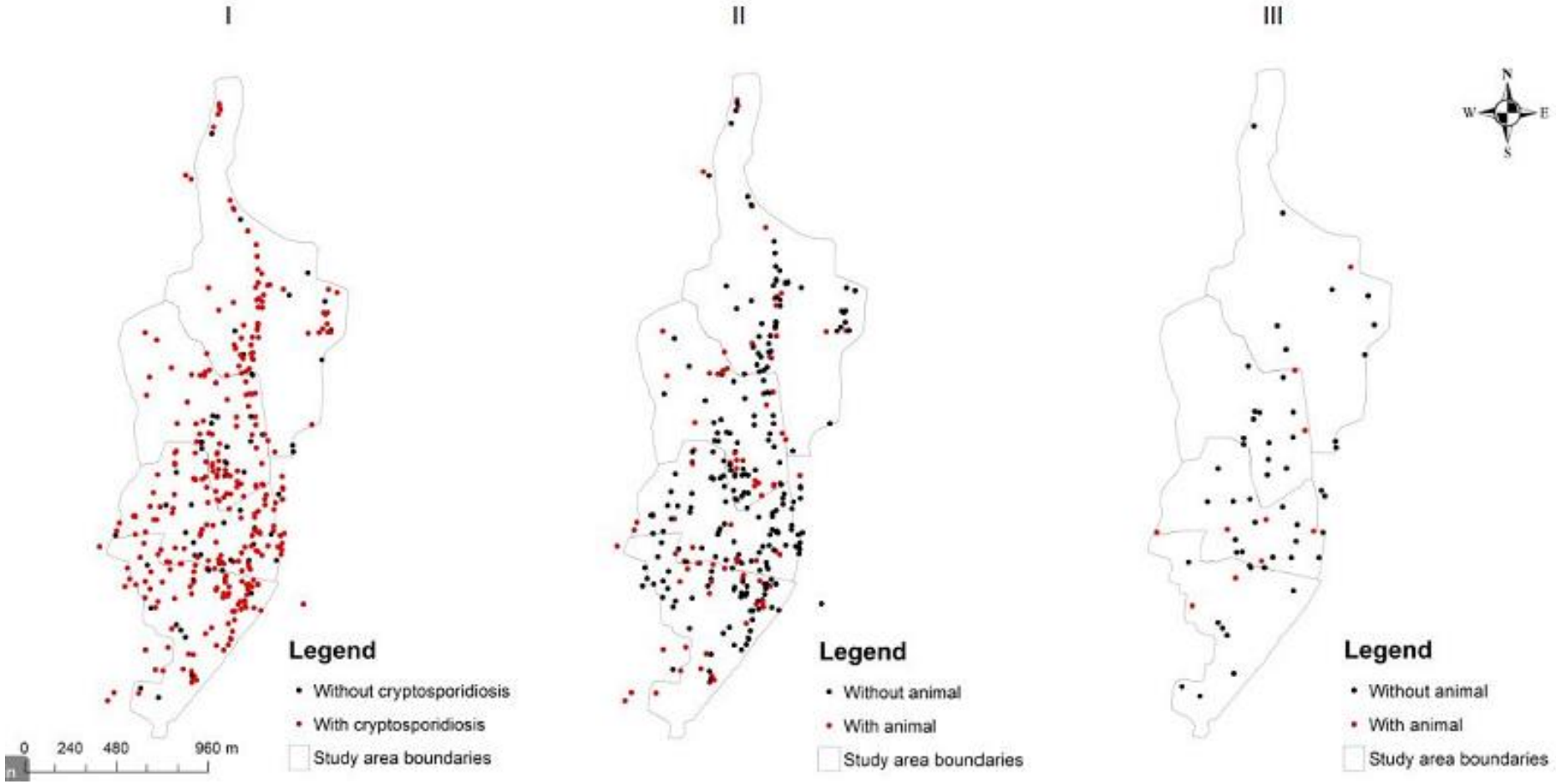


Figure 5.3.1: (I) Spatial distribution of the houses of study children with and without cryptosporidial infections, (II) Children with cryptosporidial infections with animals in and around household, (III) Children without cryptosporidial infections with animals in and around household

5.3.3.3. Factors associated with *Cryptosporidium*-associated diarrhoea and asymptomatic infections

In the univariate analysis, maternal age <23yrs was associated with higher odds for both *Cryptosporidium*-associated diarrhoea (OR=2.26, 95% CI=1.19-4.28) and asymptomatic (OR=2.22, 95% CI=1.20-4.09) cryptosporidial infections and contact with domestic animals in the house was also associated with the risk of both *Cryptosporidium*-associated diarrhoea (OR=2.72, 95% CI=1.13-6.53) and asymptomatic (OR=2.35, 95% CI=1.00-5.53) infections. A child living in a hut/kutcha house was a risk factor for asymptomatic infection (OR=3.31, 95% CI=0.97-11.27). The use of a toilet by all members in the household, always boiling drinking water before consumption conferred protection against both *Cryptosporidium*-associated diarrhoea and asymptomatic infections in the univariate analysis, this was not statistically significant (Table 5.3.5).

In the multivariate analysis, maternal age <23 was significantly associated with higher risk for both *Cryptosporidium* associated diarrhoea (OR=2.89, 95% CI=1.40-5.95) and asymptomatic (OR=3.72, 95% CI=1.79-7.70) cryptosporidial infections. Children who had contact with animals had significantly increased odds of *Cryptosporidium* associated diarrhoea (OR=2.96, 95% CI=1.16-7.54). There was significantly higher odds of acquisition of asymptomatic infections in children who had one or more older siblings in the house (OR= 2.08, 95% CI=1.05-4.14) and also for children residing in a hut/kutcha house (OR=4.01, 95% CI=1.12-14.26) in the multivariate model (Table 5.3.6).

Table 5.3.5.: Univariate logistic regression analysis for comparison of demographic and maternal factors between cases and controls

Variable	Control (n=63)	*Cryptosporidium* associated diarrhoea			Asymptomatic infections		
		Cases (n=143)	Odds Ratio (95% CI)	*P*-value	Cases (n=204)	Odds Ratio (95% CI)	*P*-value
Male child	38 (60.3%)	85 (59.4%)	0.96 (0.52-1.76)	0.9	102 (50%)	0.65 (0.37-1.16)	0.15
Low birth weight	7 (11.1%)	27 (18.8%)	1.86 (0.74-4.53)	0.17	35 (17.2%)	1.65 (0.69-3.93)	0.25
Presence of older sibling	33 (52.4%)	81 (56.6%)	1.18 (0.65-2.15)	0.57	116 (56.8%)	1.19 (0.67-2.11)	0.53
Exclusive breast feeding more than over 6 months	8 (12.7%)	27 (18.8%)	1.60 (0.68-3.75)	0.27	30 (14.7%)	1.18 (0.51-2.73)	0.69
Maternal factors							
Illiterate mother	24 (38.1%)	55 (38.4%)	1.01 (0.55-1.86)	0.96	68 (33.3%)	0.81 (0.45-1.45)	0.48
Maternal age <23 years	18 (28.6%)	68 (47.5%)	2.26 (1.19-4.28)	0.01	96 (47.1%)	2.22 (1.20-4.09)	0.01

Table 5.3.5.(contd) Univariate logistic regression analysis for comparison of economic, living conditions and household hygiene factors between cases and controls

Variable	Control (n=63)	*Cryptosporidium* associated diarrhoea			Asymptomatic infections		
		Cases (n=143)	Odds Ratio (95% CI)	*P*-value	Cases (n=204)	Odds Ratio (95% CI)	*P*-value
Economic and living conditions							
Low socioeconomic status	40 (63.5%)	87 (60.8%)	0.89 (0.48-1.64)	0.71	138 (67.6%)	1.20 (0.66-2.17)	0.54
Hut/kutcha house[$]	3 (4.7%)	16 (11.2%)	2.51 (0.70-8.97)	0.15	29 (14.2%)	3.31 (0.97-11.27)	0.05
Firewood as main fuel	23 (36.5%)	60 (42%)	1.25 (0.68-2.31)	0.46	86 (42.2%)	1.26 (0.70-2.27)	0.42
Crowding (>5 persons per room)	15 (23.8%)	34 (23.8%)	0.99 (0.49-2)	0.99	67 (32.8%)	1.56 (0.81-3)	0.17
Hygiene and sanitation							
Good hygiene	35 (55.5%)	68 (47.5%)	0.72 (0.39-1.31)	0.29	109 (53.4%)	0.91 (0.52-1.61)	0.76
Always boiling drinking water	10 (15.8%)	13 (9.1%)	0.53 (0.21-1.28)	0.15	30 (14.7%)	0.91 (0.41-2)	0.82
Presence of toilet[‡]	48 (77.4%)	96 (70.6%)	0.70 (0.34-1.41)	0.31	137 (70.2%)	0.68 (0.35-1.34)	0.27
Use of toilet by all members[‡]	43 (69.3%)	86 (63.2%)	0.76 (0.39-1.44)	0.4	112 (57.4%)	0.59 (0.32-1.09)	0.09

‡ missing data from 13 children

Table 5.3.5.(contd) Univariate logistic regression analysis for comparison of environmental factors between cases and controls

Variable	Control (n=63)	*Cryptosporidium* associated diarrhoea			Asymptomatic infections		
		Cases (n=143)	Odds Ratio (95% CI)	*P*-value	Cases (n=204)	Odds Ratio (95% CI)	*P*-value
Animal and environmental factors							
Presence of cow at home‡	12 (19.3%)	36 (26.4%)	1.5 (0.72-3.13)	0.28	48 (24.6%)	1.36 (0.66-2.76)	0.39
Presence of animal at home‡	10 (16.1%)	40 (29.4%)	2.16 (1.00-4.68)	0.04	54 (27.6%)	1.99 (0.94-4.19)	0.07
Contact with the animal‡	7 (11.3%)	35 (25.7%)	2.72 (1.13-6.53)	0.02	45 (23.0%)	2.35 (1.00-5.53)	0.04
Cow or other animal shed within 10 m of the house	7 (11.1%)	19 (13.3%)	1.22 (0.48-3.08)	0.66	29 (14.2%)	1.32 (0.55-3.19)	0.52
Garbage dump within 10 m of the house	31 (49.2%)	71 (49.6%)	1.01 (0.56-1.84)	0.95	82 (40.2%)	0.69 (0.39-1.22)	0.2
Open sewage channel within 10m of the house	13 (20.6%)	35 (24.5%)	1.24 (0.60-2.55)	0.54	59 (28.9%)	1.56 (0.79-3.09)	0.19
Open-air defecation area within 50 m of the house	8 (12.7%)	11 (7.7%)	0.57 (0.21-1.50)	0.25	22 (10.7%)	0.83 (0.35-1.97)	0.67

‡ missing data from 13 children

Table 5.3.5.(contd). Univariate logistic regression analysis for comparison of growth and nutrition related factors between cases and controls

Variable	Control (n=63)	*Cryptosporidium* associated diarrhoea			Asymptomatic infections		
		Cases (n=143)	Odds Ratio (95% CI)	*P*-value	Cases (n=204)	Odds Ratio (95% CI)	*P*-value
Nutritional Status							
Stunted at 6 months of age	12 (19%)	43 (30%)	1.82 (0.88-3.76)	0.10	53 (26%)	1.49 (0.73-3.01)	0.26
Wasted at 6 months of age	11 (17.5%)	25 (17.5%)	1.00 (0.45-2.18)	0.99	24 (11.7%)	0.63 (0.28-1.37)	0.24
Underweight at 6 months of age	13 (20.6%)	42 (29.4%)	1.59 (0.78-3.24)	0.19	65 (31.8%)	1.79 (0.91-3.54)	0.09

Table 5.3.6.: Multivariate logistic regression analysis for risk factors of *Cryptosporidium* associated diarrhoea and asymptomatic cryptosporidiosis

Variable	*Cryptosporidium* associated diarrhoea		Asymptomatic infections	
	Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Presence of older sibling	1.82 (0.91-3.66)	0.08	2.08 (1.05-4.14)	0.03
Maternal factors Mother's age <23 years	2.89 (1.40-5.95)	0.004	3.72 (1.79-7.70)	<0.001
Economic and living conditions Hut/kutcha house	-	-	4.01 (1.12-14.26)	0.03
Hygiene and sanitation Always boiling drinking water	0.33 (0.12-0.91)	0.03	-	-
Animal and environmental factors Contact with the animal ‡	2.96 (1.16-7.54)	0.02	2.08 (0.86-5.01)	0.10

‡ missing data from 13 children

5.3.3.4. Factors associated with multiple cryptosporidial infections

In the univariate analysis, when children with multiple episodes (≥3) of cryptosporidial infection (n=103) were compared with those without any infection (n=63), maternal age <23 years (OR=2.97, 95% CI=1.52-5.82), residing in a hut/kutcha house (OR=4.52, 95% CI =1.28-15.97), presence of animals in the household (OR=2.52, 95% CI=1.14-5.58) and contact with animals (OR=2.72, 95% CI=1.10-6.72) had a significantly higher odds of multiple infections. On the other hand, always boiling drinking water before consumption (OR=0.44, 95% CI=0.16-19), having a toilet in the house (OR=0.57, 95% CI=0.27-1.18) or the use of a toilet by all members of the family (OR=0.54, 95% CI=0.28-1.07) conferred some degree of protection against multiple infections, but the differences were not statistically significant (Table 5.3.7).

In the multivariate analysis, maternal age <23 years (OR=4.13, 95% CI=1.77-9.60), contact with animals (OR=2.95, 95% CI=1.06-8.26) and residing in a kutcha house (OR=4.38, 95% CI=1.17-16.4) significantly increased the odds of multiple cryptosporidial infections (Table 5.3.8).

Table 5.3.7.: Univariate logistic regression analysis for comparison of demographic and maternal factors between cases and controls

Variable	Control (n=63)	Multiple infections		
		Cases (n=103)	Odds Ratio (95% CI)	*P*-value
Male child	38 (60.3%)	56 (54.3%)	0.78 (0.41-1.48)	0.45
Low birth weight	7 (11.1%)	22 (21.3%)	2.17 (0.86-5.43)	0.09
Presence of older sibling	33 (52.4%)	59 (57.2%)	1.21 (0.64-2.28)	0.53
Exclusive breast feeding more than 6 months	8 (12.7%)	22 (21.3%)	1.86 (0.77-4.49)	0.16
Maternal factors				
Illiterate mother	24 (38.1%)	35 (34%)	0.83 (0.43-1.60)	0.59
Maternal age <23 years	18 (28.6%)	56 (54.3%)	2.97 (1.52-5.82)	0.001

Table 5.3.7.(contd) Univariate logistic regression analysis for comparison of economic, living conditions and household hygiene factors between cases and controls

Variable	Control (n=63)	Multiple infections		
		Cases (n=103)	Odds Ratio (95% CI)	*P*-value
Economic and living conditions				
Low socioeconomic status	40 (63.5%)	70 (68%)	1.21 (0.63-2.35)	0.55
Hut/kutcha house[$]	3 (4.7%)	19 (18.4%)	4.52 (1.28-15.97	0.01
Firewood as main fuel	23 (36.5%)	41 (39.8%)	1.15 (0.60-2.19)	0.67
Crowding (>5 persons per room)	15 (23.8%)	32 (31%)	1.44 (0.70-2.94)	0.31
Hygiene and sanitation				
Good hygiene	35 (55.5%)	53 (51.4%)	0.84 (0.45-1.59)	0.6
Always boiling drinking water	10 (15.8%)	8 (7.7%)	0.44 (0.16-1.19)	0.11
Presence of toilet[‡]	48 (77.4%)	67 (66.3%)	0.57 (0.27-1.18)	0.13
Use of toilet by all members[‡]	43 (69.3%)	56 (55.4%)	0.54 (0.28-1.07)	0.07

‡ missing data from 13 children

Table 5.3.7.(contd) Univariate logistic regression analysis for comparison of environmental factors between cases and controls

Variable	Control (n=63)	Multiple infections		
		Cases (n=103)	Odds Ratio (95% CI)	*P*-value
Animal and environmental factors				
Presence of cow at home‡	12 (19.3%)	28 (27.7%)	1.59 (0.74-3.43)	0.23
Presence of animal at home‡	10 (16.1%)	33 (32.6%)	2.52 (1.14-5.58)	0.02
Contact with the animal‡	7 (11.3%)	26 (25.7%)	2.72 (1.10-6.72)	0.03
Cow or other animal shed within 10 m of the house	7 (11.1%)	15 (14.5%)	1.36 (0.52-3.55)	0.52
Garbage dump within 10 m of the house	31 (49.2%)	48 (46.6%)	0.90 (0.48-1.68)	0.74
Open sewage channel within 10m of the house	13 (20.6%)	26 (25.2%)	1.29 (0.61-2.76)	0.49
Open-air defecation area within 50 m of the house	8 (12.7%)	11 (10.6%)	0.82 (0.31-2.16)	0.69

‡ missing data from 13 children

Table 5.3.7.: Univariate logistic regression analysis for comparison of growth and nutrition related factors between cases and controls

Variable	Control (n=63)	Multiple infections		
		Cases (n=103)	Odds Ratio (95% CI)	*P*-value
Nutritional Status				
Stunted at 6 months of age	12 (19%)	33 (32%)	2.00 (0.94-4.25)	0.07
Wasted at 6 months of age	11 (17.5%)	12 (11.6%)	0.62 (0.25-1.51)	0.29
Underweight at 6 months of age	13 (20.6%)	32 (31%)	1.73 (0.82-3.63)	0.14

Table 5.3.8.: Multivariate logistic regression analysis for risk factors of multiple cryptosporidiosis

Variable	Multiple infections	
	Odds Ratio (95% CI)	*P*-value
Presence of older sibling	2.48 (1.08-5.69)	0.03
Maternal factors		
Mother's age <23 years	4.13 (1.77-9.60)	0.001
Economic and living conditions		
Hut/kutcha house	4.38 (1.17-16.4)	0.03
Hygiene and sanitation		
Always boiling drinking water	0.31 (0.09-0.99)	0.04
Animal and environmental factors		
Contact with animals ‡	2.95 (1.06-8.26)	0.03

‡ missing data for 13 children

5.3.3.5. Factors associated with *C. hominis and C. parvum* species

Species data was available for 298 children, 191 children had cryptosporidiosis with only *C. hominis*, and 73 children had infection with *C. parvum*. As stated under Methods, some children infected with *C. parvum* species may also have had *C. hominis* and other zoonotic species, i.e. children with more than one species.

In the univariate analysis for *C. hominis* species, maternal age <23 years had higher odds for infection (OR=2.27, 95% CI=1.22-4.21), whereas use of a toilet by all members in the household was associated with protection (OR=0.51, 95% CI=0.27-0.94). Factors such as residing in a hut/kutcha house (OR=6.07, 95% CI=1.68-21.84), presence of animals in and around household (OR=3.30, 95% CI=1.44-7.55) and contact with any animal (OR=3.92, 95% CI=1.55-9.92) increased the odds of acquiring infection with *C. parvum* species (Table 5.3.9). Children with one or more older siblings residing in the house had a higher odds of both *C. hominis* and *C. parvum* associated cryptosporidiosis, however this difference was not significant at $P<0.2$ but this potential risk factor was retained in the multivariate analysis as it is a known risk factor and therefore of analytical importance.

In the multivariate analysis, children with an older sibling, had significantly higher odds of both *C. hominis* (OR=1.97, 95% CI=1.01-3.88) and *C. parvum* (OR=2.75, 95 CI % =1.14-6.65) respectively. Maternal age <23 years had a significantly increased odds for *C. hominis* (OR=3.05, 95% CI=1.52-6.12) but not for *C. parvum* (OR=2.36, 95% CI=0.95-5.88). Hygienic practices such as always boiling drinking water before consumption (OR=0.58, 95% CI=0.24-1.41) and use of toilet by all the household members (OR=0.55, 95% CI=0.29-1.05) were associated with protection against *C. hominis*, but the differences were not

statistically significant but for *C. parvum*, children with animal contact (OR=3.83, 95% CI=1.44-10.18) and children residing in a hut/kutcha house (OR=7.52, 95% CI=1.92-29.46) had a significantly increased odds of cryptosporidial infection (Table 5.3.10). Figure 5.3.2 shows the distribution of cryptosporidiosis by *C. hominis*, zoonotic species (*C. parvum, C. meleagridis, C. felis*) and the distribution of animals in 298 households of children with cryptosporidiosis for whom species data was available. Figure 5.3.2 (I) depicts the widespread distribution of *C. hominis* in children with cryptosporidiosis. As shown in Figure 5.3.2 (II), there were no pockets of *C. hominis* infection associated with the presence of an animal in or around the study households, while Figure 5.3.2 (III) appeared to have pockets of infection with zoonotic species around houses with any type of animals. Almost 40% of children with zoonotic species had animals in or around households, whereas 23% of children with only *C. hominis* species had animals, indicating that a possible role for animals in the vicinity of households in transmission of zoonotic species.

Table 5.3.9.: Univariate logistic regression analysis for comparison of demographic, maternal and socio-economic factors between cases and controls

Variable	Control (n=63)	Children with only *C. hominis*			Children ever infected with *C. parvum*		
		Cases (n=191)	Odds Ratio (95% CI)	*P*-value	Cases (n=73)	Odds Ratio (95% CI)	*P*-value
Male child	38 (60.3%)	106 (55.5%)	0.82 (0.46-1.46)	0.5	40 (54.8%	0.79 (0.40-1.57)	0.51
Low birth weight	7 (11.1%)	33 (17.3%)	1.67 (0.69-4.00)	0.24	12 (16.4%)	1.57 (0.57-4.27)	0.37
Presence of older sibling	33 (52.4%)	109 (57.1%)	1.20 (0.68-2.13)	0.51	47 (64.4%)	1.64 (0.82-3.27)	0.15
Breast feeding over 6months	8 (12.7%)	39 (20.4%)	1.76 (0.77-4.00)	0.17	10 (13.7%)	1.09 (0.40-2.95)	0.86
Maternal factors							
Illiterate mother	24 (38.1%)	77 (40.3%)	1.09 (0.61-1.97)	0.75	23 (31.5%)	0.74 (0.36-1.51)	0.42
Mother's age <23 years	18 (28.6%)	91 (47.6%)	2.27 (1.22-4.21)	0.009	30 (41.1%)	1.74 (0.85-3.57)	0.12
Economic and Living conditions							
Low socioeconomic status	40 (63.5%)	129 (67.5%)	1.19 (0.65-2.17)	0.55	54 (74.0%)	1.63 (0.78-3.39)	0.18
Hut/kutcha house	3 (4.7%)	21 (11.0%)	2.47 (0.71-8.58)	0.15	17 (23.3%)	6.07 (1.68-21.84)	0.006
Firewood as main fuel	23 (36.5%)	84 (44.0%)	1.36 (0.76-2.45)	0.29	33 (45.2%)	1.43 (0.71-2.85)	0.3
Crowding (>5 persons per room)	15 (23.8%)	54 (28.3%)	1.26 (0.65-2.43)	0.49	22 (30.1%)	1.38 (0.64-2.96)	0.4

Table 5.3.9.(contd) Univariate logistic regression analysis for comparison of household hygiene and environmental factors between cases and controls

Variable	Control (n=63)	Children with only *C. hominis*			Children ever infected with *C. parvum*		
		Cases (n=191)	Odds Ratio (95% CI)	*P*-value	Cases (n=73)	Odds Ratio (95% CI)	*P*-value
Hygiene and sanitation							
Good hygiene	35 (55.5%)	90 (47.1%)	0.71 (0.40-1.26)	0.24	40 (54.8%)	0.96 (0.49-1.90)	0.92
Always boiling drinking water	10 (15.8%)	24 (12.6%)	0.76 (0.34-1.69)	0.5	5 (6.8%)	0.38 (0.12-1.20)	0.1
Presence of toilet‡	48 (77.4%)	116 (64.8%)	0.53 (0.27-1.04)	0.06	55 (76.4%)	0.94 (0.42-2.11)	0.88
Use of toilet by all members‡	43 (69.3%)	96 (53.6%)	0.51 (0.27-0.94)	0.03	44 (61.1%)	0.69 (0.33-1.42)	0.32
Animal and environmental factors							
Presence of cow at home‡	12 (19.3%)	48 (26.8%)	1.52 (0.74-3.11)	0.24	17 (23.6%)	1.28 (0.56-2.96)	0.55
Presence of animals at home‡	10 (16.1%)	41 (23.0%)	1.54 (0.72-3.30)	0.26	28 (38.9%)	3.30 (1.44-7.55)	0.005
Contact with animals	7 (11.3%)	36 (20.1%)	1.97 (0.83-4.70)	0.12	24 (33.3%)	3.92 (1.55-9.92)	0.004
Garbage dump within 10 m of the house	31(49.2%)	80 (41.9%)	0.74 (0.42-1.31)	0.31	34 (46.6%)	0.89 (0.45-1.76)	0.75
Open sewage channel within 10m of the house	13 (20.6%)	41 (21.5%)	1.05 (0.52-2.12)	0.88	24 (32.8%)	1.88 (0.86-4.11)	0.11
Open-air defecation area within 50 m of the house	8 (12.7%)	20 (10.5%)	0.80 (0.33-1.92)	0.62	10 (13.7%)	1.09 (0.40-2.95)	0.86

‡ missing data from 13 children

Table 5.3.10.: Multivariate logistic regression analysis for risk factors of *C. hominis* and *C. parvum* infection

Variable	Children infected with only *C. hominis* (n=191)		Children ever infected with *C. parvum* (n=73)	
	Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Presence of older sibling	1.97 (1.01-3.88)	0.04	2.75 (1.14-6.65)	0.02
Maternal factors				
Mother's age <23 years	3.05 (1.52-6.12)	0.002	2.36 (0.95-5.88)	0.06
Economic and Living conditions				
Hut/kutcha house	2.12 (0.57-7.87)	0.25	7.52 (1.92-29.46)	0.004
Hygiene and sanitation				
Always boiling drinking water	0.58 (0.24-1.41)	0.23	-	-
Use of toilet by all members‡	0.55 (0.29-1.05)	0.07	-	-
Animal and environmental factors				
Contact with animals at home‡	-	-	3.83 (1.44-10.18)	0.007

‡ missing data for 13 children

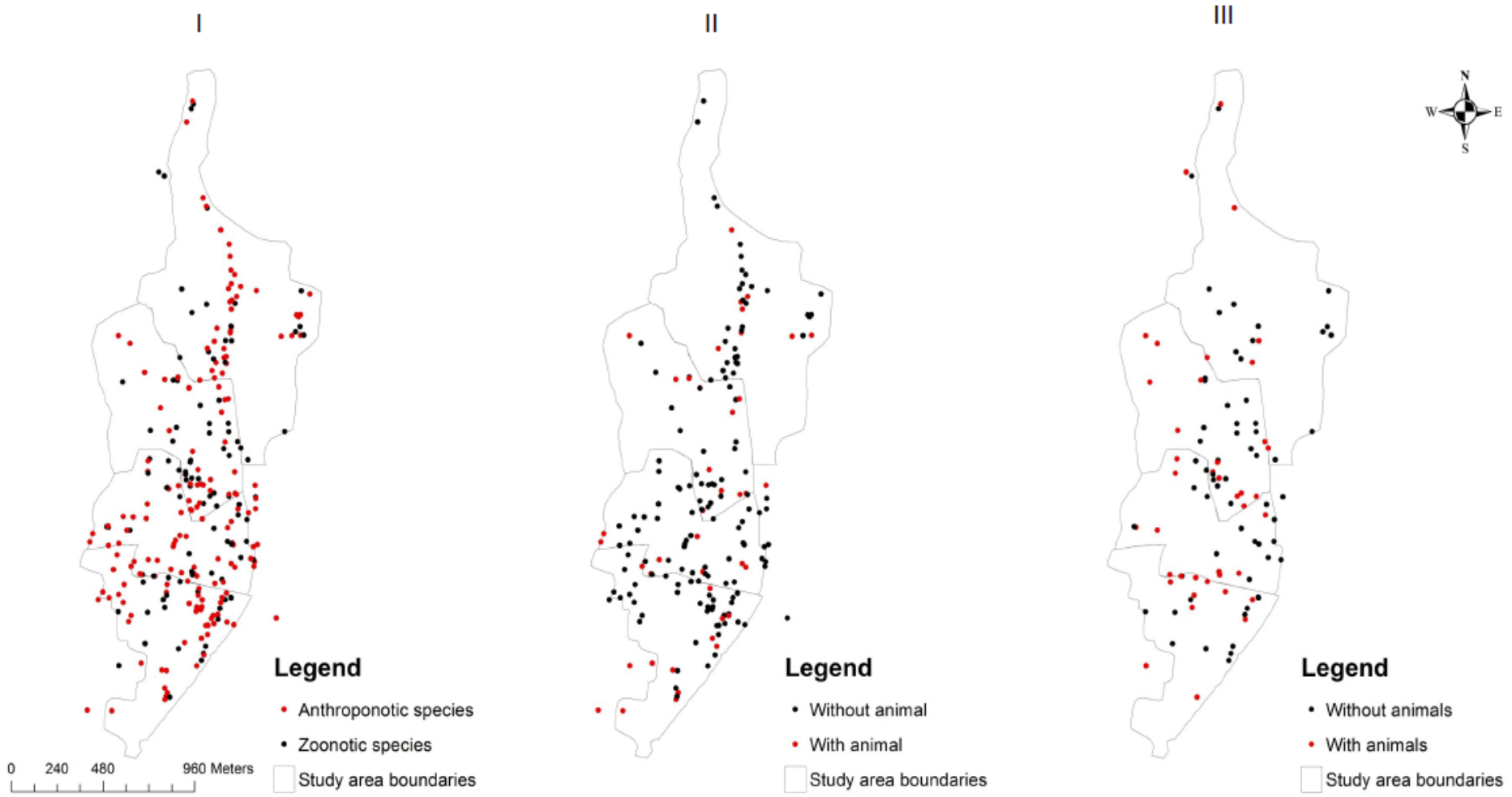


Figure 5.3.2.: Spatial distribution of cryptosporidial cases by (I) anthroponotic (*C. hominis*) and zoonotic species, (II) anthroponotic species (*C. hominis*) and presence of animals in and around household, (III) Zoonotic species and presence of animal in and around household

5.3.4. Discussion

The transmission of *Cryptosporidium* spp. is complex and may be waterborne, foodborne, zoonotic or human-to-human contact [272,117]. However, the factors that affect the acquisition of cryptosporidial infection in developing countries are poorly understood and may vary in different settings. In this study, factors known to either have a risk or confer protection were explored to evaluate their association with overall cryptosporidial infection and species-specific cryptosporidial infection in a birth cohort of a semi-urban slum community. In this study, younger age, presence of siblings in the house, maternal age, low socioeconomic status, animal contact, always boiling water before consumption and persistent stunting were associated with cryptosporidiosis among the children in the cohort. It is noteworthy that although the point estimates of the association between the risk factors and cryptosporidial infection using logistic regression analysis were large, these need to be interpreted cautiously as their confidence intervals were wide.

Cryptosporidiosis is a common enteric parasitic infection among children in the developing world. Younger children are vulnerable and are at higher risk of acquiring cryptosporidiosis because of an immature adaptive immune system. Studies from Bangladesh, South Africa, Gambia and Liberia showed a high risk for cryptosporidial infection in children less than 2 years [156, 286-288]. In this study, children in their second year of life were at higher risk of infection compared to infancy, following which there was minimal reduction in risk for the third year.

Presence of an older sibling in the house was a significant a risk factor for acquiring cryptosporidial infection. It was also a factor associated with higher odds of being infected by

C. parvum and C. hominis. This highlights the importance of close person-to-person contact, specifically with younger children in the transmission of *Cryptosporidium* spp. Studies have also reported changing diapers of children under five or helping them use the toilet [281], contact with children with diarrhoea aged >2 to 11 years [279], as risk factors for cryptosporidiosis. In a case control study, following an outbreak of cryptosporidiosis among residents of Milwaukee, Wisconsin, people living in households with young children under five years had a higher risk of endemic infection [289]. A study from England and Wales has also demonstrated an increased risk of infection in the community, where there were large proportions of children in the 0 to 4 year age group [280].

The association of infection with *C. parvum* species and the presence of an older sibling could be because older children are more likely to play with animals either within or outside the household and could play a role in transmitting zoonotic species to their younger siblings who are mostly confined to their homes.

This study has shown an inverse association between maternal age and the odds of acquiring overall cryptosporidiosis, *Cryptosporidium* associated diarrhoea, asymptomatic and *C. hominis* specific infections. Although previous studies on cryptosporidiosis have not found any association between maternal age and infection [277, 290], the protective effect of increasing maternal age on diarrhoea in general [291], and on persistent diarrhoea in particular [292], has been previously documented. A possible reason for this observed protection could be due to differences in child-rearing practices between younger and older mothers. Older mothers are known to have a more positive child rearing attitude after adjusting for various psycho-social factors such as low self-esteem, social support and cognitive abilities [293].

In this study, children residing in hut/kutcha houses had significantly higher odds of asymptomatic and multiple infections than children living in cemented houses. Children living in huts/kutcha houses could be relatively poorer and housing could be considered a proxy for low socioeconomic status. Poorer domestic environments could increase vulnerability to re-exposure and re-infections. Studies from other settings have reported low socio-economic status as either a risk [277] or a protective factor [280]. A study from Venezuela also reported that a high proportion of asymptomatic carriers were children from low socioeconomic status [20, 244]. Children from huts/kutcha houses also had a seven times higher odds for acquiring *C. parvum* infection. A possible explanation could be that floors of hut/kutcha houses are usually smeared with mud/cow dung, which creates an environment favourable for direct transmission of oocysts of *C. parvum* in children/toddlers who tend to play/crawl on the floor.

It has been well documented that animals play a vital role in transmission of cryptosporidial infections [294-295]. Contact with animals provides opportunities for direct transmission of the mature oocysts. Very often children play with animals and animals also defecate within the compound or in close proximity to where children may play, facilitating direct fecal-oral transmission. In this study children in contact with animals in and around the house had higher odds of childhood cryptosporidiosis, especially with *C. parvum* species. Studies from Brazil [296], Guatemala [245], Guinea-Bissau [274] and Indonesia [158], have also documented a higher risk of acquiring cryptosporidiosis with presence of animals within or around households. *C. hominis* has been detected in a bovine diarrhoeal sample, in India indicating the possibility of anthroponotic transmission to animals [297]. Hence in an endemic setting, transmission of *C. hominis* need not be confined to just person-to person transmission, but could include animals.

A meta-analysis has shown that drinking boiled water is associated with a 38% decrease in the risk of acquiring cryptosporidial infection [298]. In this study too, children belonging to families who always boiled their drinking water had a significantly lower risk of overall cryptosporidial infections as well as *Cryptosporidium* associated diarrhoea than those who drank water without boiling, but a significant protection against asymptomatic infections was not observed. A different study conducted in the same community had shown that provision of protected drinking water (bottled water) had not reduced the burden or delayed acquisition of cryptosporidiosis in children [22]. This suggests that prevention of cryptosporidiosis in endemic communities with poor public water and sanitation infrastructure and a high probability of recontamination during distribution, collection or storage may require point-of-use decontamination such as boiling prior to consumption.

5.3.5. Conclusion

In this study we extensively examined the host, environmental, sanitary and nutritional factors associated with cryptosporidial infection in a birth cohort in an endemic Indian slum community. The results of this study provide information on the factors associated with childhood cryptosporidiosis in an area with poor environmental and sanitary conditions and where humans and animal live in close proximity. In order to design effective control strategies for a disease with complex host-parasite interactions, a multidimensional approach has to be considered.

CHAPTER 5.4

EFFECT OF CRYPTOSPORIDIAL INFECTION AND SOCIO-DEMOGRAPHIC FACTORS ON GROWTH OF CHILDREN IN A SEMI-URBAN SLUM COMMUNITY

5.4.1. Introduction

Even in an era of rapid scientific advancement, malnutrition remains a major public health problem in the entire developing world. One of the MDGs is to decrease stunting, wasting and underweight in under five children by half, by 2015. The magnitude and distribution of nutritional deficiencies in a population depend on many factors such as socio-economic conditions, literacy levels, food production, cultural and religious customs related to nutrition, breast-feeding practices, availability of sanitation and burden caused by infectious diseases [299-300]. Infection and malnutrition are intricately linked. There is a cycle of high prevalence of infectious diseases leading to malnutrition and malnutrition increasing the susceptibility of host to infections [55, 301]. Repeated enteric infections damage the intestinal mucosa, impair the intestinal absorptive function and thereby reduce nutrient uptake and transport [302].

Cryptosporidium spp. has been recognized as an important agent causing infectious diarrhoea in children. The highest mortality and morbidity associated with children is found in third world countries [17-18]. Cryptosporidiosis appears more common in malnourished children with more severe consequences. Epidemiological studies conducted in children from Jamaica, Thailand, Peru and Israel have demonstrated an association between cryptosporidiosis and malnutrition [21, 303-305]. The direction of association is unclear, whether malnutrition predisposes to this infection, or the organism alone or along with other factors influences the development of malnutrition. This temporal ambiguity could be attributed to the cross-sectional study designs that have been employed to understand the association between cryptosporidiosis and malnutrition. There is lack of longitudinal data on cryptosporidiosis and growth in children especially in an endemic area, to understand a cause and effect

relationship. However, studies from Peru and Brazil have shown that cryptosporidial infection that occurs in early childhood is linked with long term effects on the cognitive function, growth faltering and stunting [24, 154-155].

Taking the advantage of a birth cohort with longitudinal data on growth parameters and infection, this study was undertaken to estimate the extent of malnutrition and explore the association of cryptosporidial infections and other socio-demographic factors on the growth trajectories of a birth cohort followed for three years in a semi-urban slum in southern India.

5.4.2. Methods

The description of malnutrition and effect of cryptosporidial infection on growth in the birth cohort is restricted to 410 children who completed the three-year follow up. Stool microbiology or serology results were used to identify the children with cryptosporidiosis (*Cryptosporidium* associated diarrhoea/disease and asymptomatic infection) as described in Chapter 5.2. For regression analyses, stool microbiology data was used to calculate the number of episodes of infection.

Every month, height and weight were measured for all the children (described in chapter 4). Nutritional deficiency in children was assessed by computing the weight-for-height (WHZ), height-for age (HAZ) and weight-for-age (WAZ) z-scores. The WHO child growth standards of year 2006 were used as the reference [306]. Children were classified as wasted (WHZ < -2 SD), stunted (HAZ < -2 SD), underweight (WAZ < -2 SD) or normal depending on their z-scores. Children who were stunted (HAZ < -2 SD) at 6 months, and remained so at 12, 18, 24, 30 and 36 months of age were classified as persistently stunted. Similarly, children who

showed evidence of wasting (WHZ < -2 SD) or were underweight (WAZ < -2 SD) at 6, 12, 18, 24, 30 and 36 months of age were considered to be persistently wasted or underweight, respectively. Children who were wasted, stunted or underweight at one or more, but not all time points were grouped under the intermittently malnourished category, and children who were not stunted, wasted or underweight at any time were considered never malnourished.

The effect of early childhood cryptosporidiosis on growth rate in terms of monthly height (in cm) and weight (in kg) gain was ascertained by dividing the 410 study children into exposed and unexposed groups. Children were categorised as exposed if they were infected with *Cryptosporidium* spp. in the first six months of life. The comparison/unexposed group for the analysis were children not infected within six months of age and remained infection free in the subsequent months at 12, 18, 24, 30 and 36 months of follow-up. We restricted the comparison group to children who were infection free so that the effect of cryptosporidial infections on subsequent infections in the exposure group was not diluted. Growth rates between exposed and unexposed groups were compared at age intervals of 6-12, 6-18, 6-24, 6-30 and 6-36 months.

Similarly children were categorized into exposed and unexposed, based on acquisition of infection during infancy or not (not infected by 12 months). Children from the unexposed group, who acquired infection by 24, 30 and 36 months of age, were excluded from analysis. Growth rates between exposed and unexposed groups were compared at the age intervals of 12-18, 12-24, 12-30 and 12-36 months. The association between cryptosporidial infection and proportion of stunting among exposed and unexposed groups at 12, 18, 24, 30 and 36 months of age was also explored.

The rate of growth for every month for each child was calculated using height and weight measurements at 36 months. Univariate linear regression analysis was performed at first with known predisposing socio-demographic factors, and β-coefficients with 95% confidence interval (95% CI) were calculated. The variables significant at $P \leq 0.2$ level and/or those that were known risk factors for childhood malnutrition were then included in the multivariate analysis and a final model was built using the backward stepwise method and the results have been presented as β-coefficient with 95% CI.

In order to ascertain factors associated with persistent stunting and underweight, a logistic regression was done on a subgroup of children who had been persistently stunted and underweight, comparing them separately with children who had never been stunted and had never been underweight respectively. Univariate logistic regression analysis was performed at first with potential risk factors, and crude odds ratios (OR) and 95% confidence interval (95% CI) calculated. The variables significant at $P \leq 0.2$ level and/or those that were known risk factors for childhood malnutrition were then included in the multivariate analysis and a final model built using the backward stepwise method.

5.4.3. Results

5.4.3.1. Nutritional status of the cohort

During the three-year follow up, 14433 (97%) anthropometric measurements were obtained from the 410 children. In the cohort, 280 (68.3%) children had any type (stunting/wasting/underweight) of growth faltering at the 6, 12, 18, 24, 30 or 36 month measurements. Of these 176 (43%), 139 (34%) and 143 (35%) children were intermittently

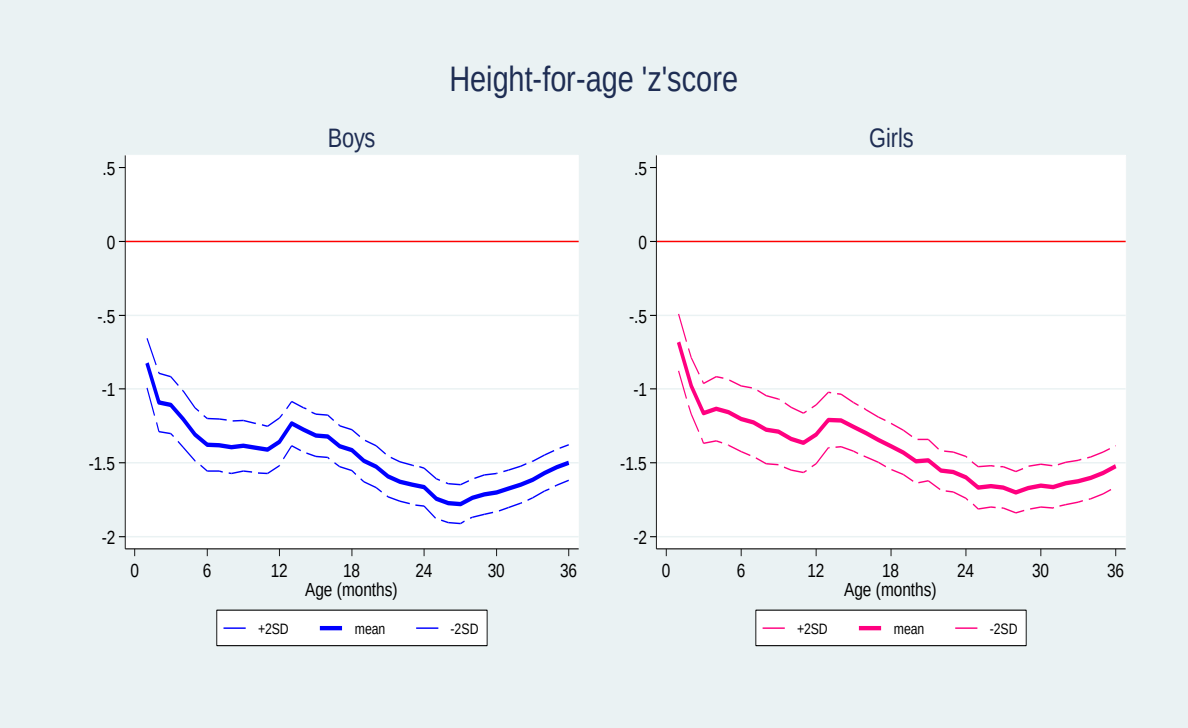
stunted, wasted and underweight, while 44 (11%), 8 (2%), 55 (13%) were persistently stunted, wasted and underweight, and 190 (46%), 263 (64%), 212 (52%) children were never stunted, wasted or underweight, respectively, as shown in Table 5.4.1. There was no association between gender and intermittent stunting (*P*=0.79), wasting (*P*=0.78) and underweight (*P*=0.41).

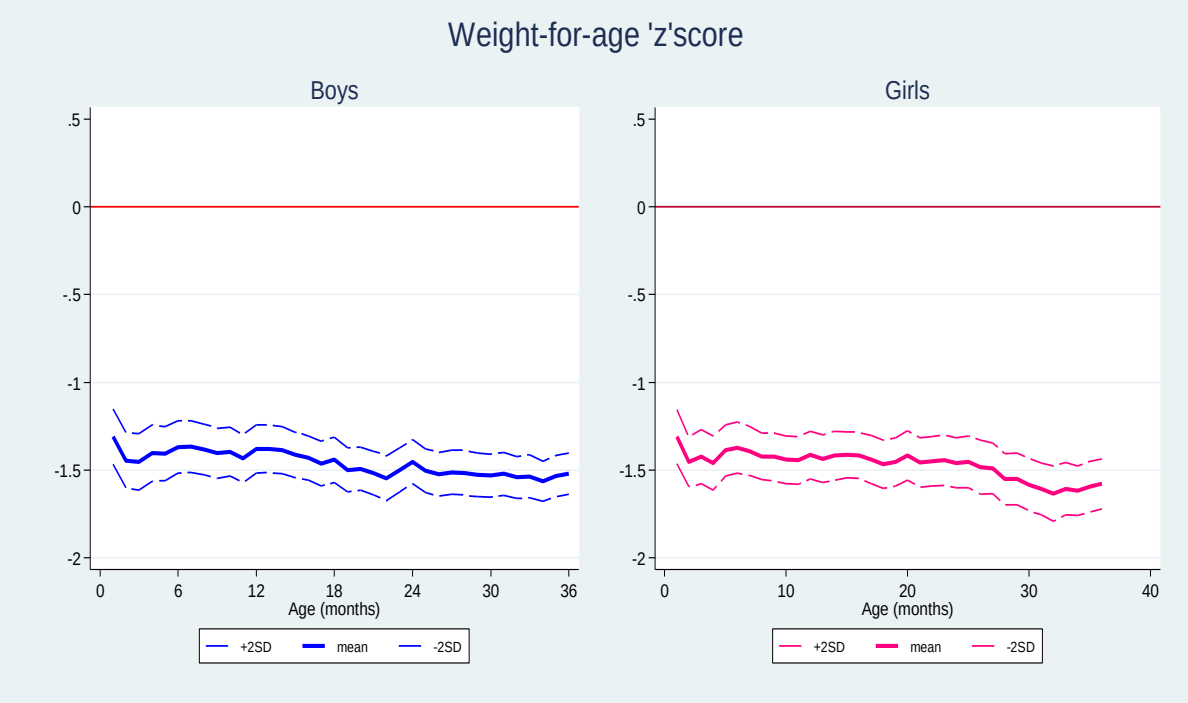
The distribution of mean (±2SD) HAZ and WAZ scores are shown in Figure 5.4.1. On average, the study children had lower HAZ and WAZ score than the WHO standard reference cut off (indicated by the red horizontal line). The elevation in the graph around 13 to 15 months was due to a discrepancy caused by change in posture while measuring length/ height from horizontal length broad method, to the vertical height scale when the child was able to stand.

Table 5.4.1.: Proportion of malnutrition among children who completed the follow-up (n=410)

Malnutrition	Never	Intermittent	Persistent
Stunting (HAZ < -2SD)	190 (46%)	176 (43%)	44 (11%)
Underweight (WAZ < -2SD)	212 (52%)	143 (35%)	55 (13%)
Wasting (WHZ < -2SD)	263 (64%)	139 (34%)	8 (2%)

Figure 5.4.1.: Growth trajectories of the birth cohort for the 36 months of follow-up





5.4.3.2. Association of cryptosporidiosis with the growth trajectory in the birth cohort

Children who acquired cryptosporidial infection during first 6 months of life tended to grow linearly at the same rate as compared to children without cryptosporidial infection. Similar results were obtained when the proportion of stunted children at each time point (12, 18, 24, 30 and 36 months) were compared by infection status at six months (Table 5.4.2). Similarly the risk of stunting was a little higher among children who acquired infection by 6 months, but this increase was not significant (Table 5.4.4). Repeating the analysis including children infected during infancy and comparing them with those not infected during infancy revealed a similar pattern, although between the 30^{th} and 36^{th} months, children with cryptosporidiosis grew linearly at a marginally lower rate compared to those without cryptosporidiosis (Table 5.4.3). There was no significant difference in the proportion of stunted children between children who acquired infection by 12 months of age and those who did not acquire infection (Table 5.4.5).

Comparing monthly weight gain (kg) between children with and without cryptosporidiosis at 6 and 12 months of age respectively, those with infection had lower weight gain compared to those without infection, but the differences were not statistically significant (Table 5.4.2 and Table5.4.3).

Table 5.4.2.: Comparison of effect of cryptosporidiosis on the growth trajectory between exposed (acquired infection by 6 months of age) and unexposed children during 36 months of follow-up

Age interval (month)	Exposed	Unexposed	Monthly Height gain (cm)		Monthly Weight gain (kg)	
			β0	β1 (95% CI)	β0	β1 (95% CI)
6-12	165	108	1.21	0.06 (-0.005, 0.14)	0.23	0.02 (-0.003, 0.04)
6-18	165	73	1.05	0.04 (-0.01, 0.10)	0.2	0.005 (-0.01, 0.02)
6-24	165	31	0.95	0.02 (-0.03, 0.08)	0.2	-0.004 (-0.02, 0.01)
6-30	165	16	0.9	0.002 (-0.06, 0.07)	0.21	-0.03 (-0.05, -0.008)
6-36	165	13	0.87	0.00006 (-0.06, 0.06)	0.18	-0.005 (-0.02, 0.01)

Table 5.4.3.: Comparison of effect of cryptosporidiosis on the growth trajectory between exposed (acquired infection by 12 months of age) and unexposed children during 36 months of follow-up

Age interval (month)	Exposed	Unexposed	Monthly Height gain (cm)		Monthly Weight gain (kg)	
			β0	β1 (95% CI)	β0	β1 (95% CI)
12-18	302	73	0.87	0.04 (-0.03, 0.12)	0.18	-0.008 (-0.03, 0.01)
12-24	302	31	0.81	0.01 (-0.06, 0.09)	0.18	-0.01 (-0.03, 0.01)
12-30	302	16	0.81	-0.02 (-0.11, 0.05)	0.19	-0.03 (-0.05, -0.01)
12-36	302	13	0.77	-0.0005 (-0.06, 0.06)	0.15	-0.002 (-0.02, 0.01)

Table 5.4.4.: Comparison of proportion of stunting in children who acquired cryptosporidial infection by 6 month and those who did not during 36 months of follow-up

Stunting by age	Exposed by 6 months	Unexposed	Relative Risk (95% CI)	*P*-value
At 12 months	n=165	n=108		
Stunted	45 (27.3%)	25 (23.1%)	1.17 (0.70-2.00)	0.51
Not stunted	120 (72.7%)	83 (76.9%)		
At 18 months	n=165	n=73		
Stunted	44 (26.7%)	19 (26.0%)	1.02 (0.58-1.85)	0.94
Not stunted	121 (73.3%)	54 (74%)		
At 24 months	n=165	n=31		
Stunted	55 (33.3%)	6 (19.3%)	1.72 (0.74-4.89)	0.19
Not stunted	110 (66.6%)	25 (80.7%)		
At 30 months	n=165	n=16		
Stunted	54 (32.7%)	3 (18.7%)	1.74 (0.56-8.72)	0.35
Not stunted	111 (67.2%)	13 (81.3%)		
At 36 months	n=165	n=13		
Stunted	39 (23.6%)	2 (15.4%)	1.53 (0.39-13.1)	0.6
Not stunted	126 (76.4%)	11 (84.6%)		

Table 5.4.5.: Comparison of proportion of stunting in children who acquired infection by 12 month and those who did not during 36 months of follow-up

Stunting by age	Exposed by 12months	Unexposed	Relative Risk (95% CI)	*P*-value
At 18 months	n=302	n=73		
Stunted	86 (28.5%)	19 (26.0%)	1.09 (0.65-1.90)	0.74
Not stunted	216 (71.5%)	54 (74%)		
At 24 months	n=302	n=31		
Stunted	113 (37.4%)	6 (19.3%)	1.93 (0.86.5.37)	0.09
Not stunted	189 (62.6%)	25 (80.7%)		
At 30 months	n=302	n=16		
Stunted	119 (39.4%)	3 (18.7%)	2.10 (0.70-10.3)	0.18
Not stunted	183 (60.6%)	13 (81.3%)		
At 36 months	n=302	n=13		
Stunted	89 (29.5%)	2 (15.4%)	1.91 (0.51-16.06)	0.37
Not stunted	213 (70.5%)	11 (84.6%)		

5.4.3.3. Association of cryptosporidial infection and socio-demographic factors on overall growth rate for 36 months of follow up

In the cohort, the average (SD) monthly height gain was 1.04 (0.10) cm and weight gain was 227 (30) gm. In the univariate analysis, presence of an older sibling (β1= -0.04, 95% CI= -0.06, -0.03), an illiterate mother (β1= -0.03, 95% CI= -0.05, -0.009), exclusive breast feeding for more than 6 months (β1= -0.03, 95% CI= -0.05, -0.0006) and use of firewood as the main fuel in the house (β1= -0.03, 95% CI= -0.05, -0.01) had a negative association with linear growth (Table 5.4.6).

In the multivariate analysis, presence of an older sibling (β1= -0.04, 95% CI= -0.06, -0.02), an illiterate mother (β1= -0.02, 95% CI= -0.04, -0.004) and use of firewood as the main fuel (β1= -0.03, 95% CI= -0.04, -0.001) resulted in significantly lower monthly height gain (Table 5.4.7).

In the univariate analysis for monthly weight gain, low birth weight (β1= -0.01, 95% CI= -0.02,-0.003), presence of an older sibling (β1= -0.01, 95% CI= -0.02, -0.004), exclusive breast feeding more than 6 months (β1= -0.01, 95% CI= -0.02, -0.003), firewood as the main fuel in the house (β1= -0.01, 95% CI= -0.02, -0.007), multiple episodes of cryptosporidial infection (β1= -0.007, 95% CI= -0.01,0.0005) and multiple GI illnesses (β1= -0.007, 95% CI= -0.01,-0.0003) were associated with decreased weight gain (Table 5.4.6).

In the multivariate analysis, low birth weight (β1= -0.01, 95% CI= -0.02, -0.002), an older sibling (β1= -0.01, 95% CI= -0.02, -0.006), use of firewood as the main fuel (β1= -0.01, 95% CI= -0.02, -0.005) and multiple GI illnesses (β1= -0.007, 95% CI= -0.04, -0.003) resulted in

significantly lower monthly weight gain. Male children had significantly higher weight gain ($\beta 1$=0.008, 95% CI=0.001, 0.01) than female children (Table 5.4.7).

Table 5.4.6.: Univariate analysis of factors associated with monthly height and weight gain during 36 months of follow-up

Variables	Monthly height gain (cm)			Monthly weight gain (kg)		
	β0	β1 (95% CI)	*P*-value	β0	β1 (95% CI)	*P*-value
Male child	1.04	0.01 (-0.006, 0.03)	0.19	0.22	0.008 (0.001, -0.01)	0.01
Low birth weight	1.04	0.007 (-0.02, 0.03)	0.6	0.22	-0.01 (-0.02, -0.003)	0.008
Presence of older sibling	1.07	-0.04 (-0.06, -0.03)	<0.0001	0.23	-0.01 (-0.02, -0.004)	0.001
Breast feeding over 6months	1.05	-0.03 (-0.05, -0.0006)	0.04	0.23	-0.01 (-0.02, -0.003)	0.008
Maternal factors						
Illiterate mother	1.06	-0.03 (-0.05, -0.009)	0.004	0.22	-0.004 (-0.01, 0.003)	0.27
Mother's age <23 years	1.05	0.006 (-0.01, 0.02)	0.5	0.22	0.0006 (-0.006, 0.007)	0.85
Economic and Living conditions						
Low socioeconomic status	1.05	-0.008 (-0.03, 0.01)	0.41	0.22	-0.0008 (-0.008, 0.006)	0.82
Hut/Kutcha house	1.05	0.02 (-0.01, 0.04)	0.25	0.22	0.006 (-0.004, 0.01)	0.26
Firewood as main fuel	1.06	-0.03 (-0.05, -0.01)	0.002	0.23	-0.01 (-0.02, -0.007)	<0.0001
Cryptosporidial infection						
Ever positive for cryptosporidiosis	1.06	-0.01 (-0.04, 0.01)	0.33	0.23	-0.009 (-0.01, 0.0003)	0.06
More than 3 episodes of infection	1.05	-0.002 (-0.02, 0.02)	0.8	0.23	-0.007 (-0.01, 0.0005)	0.06
More than 2 episodes of *Cryptosporidium* associated diarrhoea	1.05	-0.01 (-0.04, 0.02)	0.47	0.22	-0.004 (-0.01, 0.007)	0.45
Other morbidity						
Respiratory illness more the 21 episodes	1.05	-0.008 (-0.02, 0.01)	0.37	0.22	0.001 (-0.005, 0.008)	0.64
GI illness more than 5 episodes	1.05	-0.003 (-0.02, 0.01)	0.75	0.23	-0.007 (-0.01, -0.0003)	0.04

Median cut off was used to categorise maternal age, cryptosporidial infections, respiratory illness, GI illness

Table 5.4.7.: Multivariate analysis of factors associated with monthly height and weight gain during 36 months of follow-up

Variables	Monthly height gain (cm)			Monthly weight gain (kg)		
	β0	β1 (95% CI)	*P*-value	β0	β1 (95% CI)	*P*-value
Male child	1.08	0.01 (-0.004, 0.03)	0.13	0.24	0.008 (0.001, 0.01)	0.01
Low birth weight				0.24	-0.01 (-0.02, -0.002)	0.02
Presence of older sibling	1.08	-0.04 (-0.06, -0.02)	<0.0001	0.24	-0.01 (-0.02, -0.006)	<0.0001
Breast feeding over 6 months	-	-	-	0.24	-0.01 (-0.02, -0.001)	0.02
Maternal factors						
Illiterate mother	1.08	-0.02 (-0.04, -0.004)	0.01	-	-	-
Economic and Living conditions						
Firewood as main fuel	1.08	-0.03 (-0.04, -0.001)	0.003	0.24	-0.01 (-0.02, -0.005)	<0.0001
Cryptosporidial infection						
More than 3 episodes of infection	1.08	-0.003 (-0.02, 0.02)	0.77	0.24	-0.005 (-0.01, 0.002)	0.2
Other morbidity						
GI illness more than 5 episodes	-	-	-	0.24	-0.007 (-0.04, -0.0003)	0.04

5.4.3.4. Factors associated with persistent stunting in the cohort

In the univariate analysis, when children with persistent stunting (n=44) were compared with those without stunting (n=190), low birth weight (OR=8.07, 95% CI=3.63-17.92), and maternal age <23 years (OR=2.28, 95% CI =1.16-4.46) had a significantly higher odds of being persistently stunted. Although multiple episodes (≥3 episodes) of cryptosporidial infections (OR=1.97, 95% CI=0.98-3.93) and using firewood as the primary fuel (OR=1.79, 95% CI=0.92-3.49) were associated with increased odds for persistent stunting, it was insignificant (Table 5.4.8).

In the multivariate analysis, low birth weight (OR=8.45, 95% CI=3.68-19.4) significantly increased the odds of being persistently stunted. Multiple cryptosporidial infection (OR=2.04, 95% CI=0.96-4.36) and using firewood as the primary fuel (OR=1.84, 95% CI=0.89-3.82) also had higher odds of persistent stunting, and the association was very close to significance (Table 5.4.8).

Table 5.4.8.: Factors associated with persistent stunting in univariate and multivariate analyses in a birth cohort

Variables	Controls Never stunted (n=190)	Cases Persistently stunted (n=44)	Univariate Analysis		Multivariate Analysis	
			Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Male child	104 (54.7%)	24 (61.4%)	1.31 (0.67-2.56)	0.42	-	-
Low birth weight	15 (7.9%)	18 (41%)	8.07 (3.63-17.92)	<0.0001	8.45 (3.68-19.4)	<0.0001
Presence of older sibling	104 (54.7%)	25 (56.8%)	1.08 (0.56-2.10)	0.8	1.22 (0.58-2.54)	0.59
Breast feeding over 6months	24 (12.6%)	7 (16%)	1.30 (0.52-3.26)	0.56	-	-
Maternal factors						
Illiterate mother	65 (34.2%)	20 (45.5%)	1.60 (0.82-3.11)	0.16	1.43 (0.68-2.97)	0.33
Mother's age <23 years	78 (41.0%)	27 (61.4%)	2.28 (1.16-4.46)	0.01	-	-
Economic and Living conditions						
Low socioeconomic status	117 (61.6%).	29 (66%)	1.20 (0.60-2.40)	0.59	-	-
Hut/kutcha house	21 (11.0%)	7 (16%)	1.52 (0.60-3.84)	0.37	-	-
Firewood as main fuel	64 (33.7%)	21 (47.7%)	1.79 (0.92-3.49)	0.08	1.84 (0.89-3.82)	0.09
Cryptosporidial infection						
Ever positive for cryptosporidiosis	156 (82.2%)	41 (93.2%)	2.97 (0.87-10.2)	0.08	-	-
More than 3 episodes of infection	46 (24.2%)	17 (38.6%)	1.97 (0.98-3.93)	0.05	2.04 (0.96-4.36)	0.06
More than 2 episodes of *Cryptosporidium* associated diarrhoea	18 (9.5%)	6 (13.6%)	1.50 (0.56-4.05)	0.41	-	-
Other morbidity						
Respiratory illness more the 21 episodes	88 (46.3%)	19 (43.2%)	0.88 (0.45-1.70)	0.7	-	-
GI illness more than 5 more	91 (47.8%)	22 (50.0%)	1.08 (0.56-2.09)	0.8	-	-

Median cut off was used to categorise maternal age, cryptosporidial infections, respiratory illness and GI illness

5.4.3.5. Factors associated with persistent underweight in the cohort

In the univariate analysis for persistent underweight, children with low birth weight (OR=13.4, 95% CI=5.85-31) and maternal age <23 years (OR=1.84, 95% CI=1.01-3.37), had significantly higher odds of being persistently underweight (Table 5.4.9). Multiple episodes of cryptosporidial infections (OR=1.62, 95% CI=0.85-3.07) and using of firewood as the primary fuel (OR=1.74, 95% CI=0.96-3.17) were also associated with an increased odds of persistent underweight but were not significant.

In the multivariate analysis, low birth weight (OR=15.3, 95% CI=6.28-37.3), presence of an older sibling (OR=2.23, 95% CI=1.04-4.78) and maternal age <23 years (OR=2.51, 95% CI=1.19-5.30) significantly increased the odds of being persistently under weight. Multiple cryptosporidial infection (OR=1.19, 95% CI=0.55-2.53) also had higher odds of persistent underweight but the difference was not significant (Table 5.4.9).

Table 5.4.9.: Factors associated with persistent underweight in univariate and multivariate analyses in a birth cohort

Variables	Controls Never underweight (n=212)	Cases Persistently underweight (n=55)	Univariate Analysis		Multivariate Analysis	
			Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Male child	119 (56.1%)	32 (58.2%)	1.08 (0.59-1.98)	0.78	-	-
Low birth weight	10 (4.7%)	22 (40.0%)	13.4 (5.85-31)	<0.0001	15.3 (6.28-37.3)	<0.0001
Presence of older sibling	111 (52.4%)	34 (61.8%)	1.47 (0.80-2.70)	0.21	2.23 (1.04-4.78)	0.04
Breast feeding over 6months	31 (14.6%)	11 (20.0%)	1.45 (0.68-3.12)	0.33	-	-
Maternal factors						
Illiterate mother	70 (33.0%)	18 (32.7%)	0.98 (0.52-1.85)	0.96	-	-
Mother's age <23 years	91 (42.9%)	32 (58.2%)	1.84 (1.01-3.37)	0.04	2.51 (1.19-5.30)	0.01
Economic and Living conditions						
Low socioeconomic status	136 (64.1%)	34 (61.8%)	0.90 (0.49-1.67)	0.75	-	-
Hut/kutcha house	25 (11.8%)	4 (7.3%)	0.58 (0.19-1.76)	0.34	-	-
Firewood as main fuel	79 (37.3%)	28 (51.0%)	1.74 (0.96-3.17)	0.06	2.02 (1.01-4.04)	0.04
Cryptosporidial infection						
Ever positive for cryptosporidiosis	170 (80.2%)	48 (87.2%)	1.69 (0.71-4.01)	0.23	-	-
More than 3 episodes of infection	52 (24.5%)	19 (34.5%)	1.62 (0.85-3.07)	0.13	1.19 (0.55-2.53)	0.65
More than 2 episodes of *Cryptosporidium* associated diarrhoea	26 (12.6%)	8 (14.5%)	1.21 (0.51-2.86)	0.65	-	-
Other morbidity						
Respiratory illness more the 21 episodes	106 (50.0%)	23 (41.8%)	0.71 (0.39-1.31)	0.28	-	-
GI illness more than 5 more	100 (47.2%)	28 (51.0%)	1.16 (0.64-2.10)	0.62	-	-

Median cut off was used to categorise maternal age, cryptosporidial infections, respiratory illness and GI illness

5.4.4. Discussion

In this study, the magnitude of malnutrition, and the effect of cryptosporidial infections and other known socio-demographic factors associated with growth trajectories were analyzed in a birth cohort in semi-urban slum community which was intensively followed for three years.

A total of 280 (67.8%) children experienced one or more growth failures (wasted/ stunted/ underweight) during the three-year follow up period, with chronic malnutrition being the predominant type of malnutrition. The proportion of stunting (54%), wasting (36%) and underweight (48%) observed in this study exceeded the national estimates reported by NFHS-3, which were 44.9%, 22.9% and 40.4% respectively [307] and also higher compared to the Tamilnadu state urban estimates which reported 30.1% stunting, 22.3% wasting and 22.6% underweight [307]. Children in this study had an average HAZ and WAZ scores less than the standard reference value recommended by the WHO, indicating a lower growth trajectory in this community compared to the normal growth standards. The estimates of malnutrition in this study were identical to studies conducted earlier in this community by Rehman *et al.* [308] and Sarkar *et al.* [186], who reported 68% and 62.7% affected children, respectively. This reflects a continued presence of malnutrition in this community. Studies in urban slums elsewhere in India (reviewed by [309]) also reveal a high burden of malnutrition ranging from 26-94% among children from impoverished communities. Probable causes that make these children vulnerable include inadequate nutrition, lack of proper parental care, living conditions and early exposure to infectious diseases at a minimum.

Studies in Peruvian children showed cryptosporidial infection was associated with acute growth faltering in children and lack of catch up in height and weight gain [24, 154]. Molbak

et al. study reported a transient effect of cryptosporidiosis on growth among children in New Guinea; however infection in infancy had a longer negative effect on growth [155]. In our study, we have not found an association between cryptosporidial infections with monthly height gain though there was some negative relationship with weight gain, but this was mostly not significant.

In this study, children with multiple episodes of cryptosporidiosis were more associated with persistent stunting; although the difference was not significant, there was a suggestion of a relationship between multiple infections with chronic growth faltering. Checkley *et al.* showed that stunted children with cryptosporidial infection were one cm shorter than uninfected stunted children one year after infection [24]. The reason for stunting could be repeated enteric infections, since diarrhoeal diseases can damage the intestinal mucosal wall. Many of the enteric infections caused by protozoa (*Giardia, Cryptosporidium*), viruses (rotavirus, noroviruses) and bacteria severely disrupt function and cause inflammation of the absorptive villi of intestinal mucosa, there by hindering the absorption of micronutrients. Children who are marginally nourished or malnourished, have an additional problem of limited stores of vital nutrients, which may aid in repairing of mucosal damage and perpetuate the problem [302]. Studies have shown that even in the absence of overt clinical disease, asymptomatic enteric infections, especially those with *Cryptosporidium* spp. can cause damage and predispose to growth failure [24, 154].

We also examined the effect of important socio-demographic and maternal factors on growth faltering, adjusting for the cryptosporidial infection status of children in our birth cohort. In order to understand casual associations for malnutrition, it is important to examine cryptosporidial infection along with the causal constellation of factors such as birth weight,

maternal characteristics such as literacy and age and socio-economic status, duration of breastfeeding and presence of siblings in the household.

Birth weight is a very important health indicator which predicts children's vulnerability to illnesses and also their survival. Low birth weight has not only been associated with higher mortality and morbidity [212] but also with impairments in mental development [213] and malnutrition [310]. Our data showed that children with low birth weight had slower weight gain and had very high odds of being persistently stunted and underweight. Studies from Philippines, Brazil and Oman have also reported low birth weight as a predictor for persistent chronic malnutrition [311-313] and persistent underweight [314-315]. In order to reduce the proportion of children born with low birth weight, it is essential to target young women of reproductive age with macro- and micro-nutritional programmes and promote regular antenatal visits during pregnancy.

Maternal factors such as age and literacy are known to affect the child's nutritional status. In this study, children with illiterate mothers were nearly one cm shorter by three years compared to children with literate mothers. Children with mothers less than 23 years had chronic growth faltering and had two times higher odds of remaining persistently underweight. This possibly highlights the importance of better child rearing practices and knowledge of appropriate nutrition, which are acquired with age, experience and increasing education of the mother [310, 316-317].

It is interesting to note that the average monthly weight gain was inversely proportional to duration of breastfeeding. Children who had received exclusive breastfeeding for more than 6 months had lower weight gain over the three year period when compared to children who

were weaned within 6 months. Inadequate nutrient supplementation is the most likely mechanism through which prolonged breast feeding can impair growth, as breast milk alone cannot meet the nutritional needs of growing children [318-319]. Studies from Brazil and Bangladesh also showed growth pattern that is slower among children who received exclusive breastfeeding for a prolonged period of time [320-322].

Studies have demonstrated an association between low socio-economic status and malnutrition in low and middle-income countries [310, 323]. Families using firewood as the main fuel were relatively poorer within the slum community. Food insecurity and nutritional deprivation, lack of basic amenities for living are common features in these strata of society. In this study, children belonging to such poor families were shorter by 1.08 cm and weighed 360g less by three years of age. This factor also doubled the odds of being persistently stunted and underweight.

Growth rates in terms of average monthly weight gain was higher in boys in this study and is similar to a report from Brazil [320]. Social factors such as the preferential care and nutrition that boys receive compared to girls in developing countries could be a reason for this finding [324].

Studies from Brazil and Nigeria have reported that the quality of childcare in households with many children is inadequate due to the limited time available for the mother to devote herself to the care of each child [310, 316]. Another reason could also be in resource-limited settings, food in families may not meet the daily nutritional requirement of the child. Studies have also shown that, children with siblings have a higher risk of contracting infectious diseases, especially diarrhoeal diseases [325] and cryptosporidiosis [279-280], all of which are

predisposing factors for malnutrition. As seen in this study, children with siblings had growth faltering and had two times higher odds of not catching up for growth in terms of weight.

5.4.5. Conclusion

This study in a birth cohort from semi-urban slums showed significant malnutrition among children, with chronic growth faltering. Although no significant effect of cryptosporidiosis on growth could be demonstrated in this study, it can be noted that most children in the cohort were infected with cryptosporidiosis and a high proportion of them were malnourished, making it difficult to tease out the pure effect of cryptosporidial infections on the growth of the child. However socio-demographic and maternal factors, either individually, or in conjunction with cryptosporidiosis, have been shown to contribute significantly to malnutrition in the cohort. It is essential for policy makers to consider a multi-dimensional approach to address malnutrition in such communities.

CHAPTER 5.5
PROTECTION OFFERED BY NATURAL INFECTION(S) WITH CRYPTOSPORIDIUM SPP. AGAINST SUBSEQUENT INFECTION AND DISEASE

5.5.1. Introduction

Not all individuals exposed to infectious agents develop disease, but in some who are susceptible, the infectious agent multiplies and they develop disease [35]. This suggests that many individuals who come into contact with microorganisms are able to resist or eliminate them and thereby prevent the progression of an infection. Progression of infection depends on the host immune status. Both innate and adaptive immunity play a fundamental role in protecting against infectious diseases. Innate immunity offers a first-line non-specific defence mechanism immediately after exposure to the infectious agent. Phagocytic cells, such as macrophages and neutrophils, natural barriers like skin and many antimicrobial compounds formed by the host are players in innate immunity. On the other hand, adaptive immunity is antigen specific, taking time to produce an immune response which offers prolonged protection. Adaptive immunity includes "memory", which enables the host to resist repeated exposure to the same antigen. The key players of adaptive immunity are white blood cells called lymphocytes, and the antibodies. Subsequent exposure to the same antigen triggers a quicker and stronger immune response to the second challenge [326]. Individuals with immune deficiencies, either as in innate immunity (as seen in phagocytic cell dysfunction or deficiency of complement) or adaptive immunity (deficiency in antibody production or deficiency in T cell function), are known to exhibit an increased susceptibility to infections [327].

Innate and adaptive immunity are important for resistance and resolution of cryptosporidiosis [328]. The immune status of a host plays an important role in determining susceptibility, outcome and severity of this parasitic infection. In individuals who are immunocompetent, the infection is often asymptomatic and the disease self-limiting [329], but *Cryptosporidium*

spp. causes opportunistic infection in HIV/AIDS [330]. The course of the infection is severe and persistent in immunocompromised individuals [108, 329]. Studies have shown that the disease severity varies with the level of immunosuppression. HIV patients with mean CD4 counts less than 200 cells/mm^3 had *Cryptosporidium* associated diarrhoea and patients with CD4 counts more than 300 cells/mm^3 had asymptomatic infection. This highlights the important role played by CD4 T cells, which mediate resistance against the parasite [136, 145, 331-333]. In third world countries, cryptosporidial infections are more common among malnourished children with more severe consequences, possibly due to impaired T cell response [58]. Studies of Haitian children with cryptosporidial infection reported that malnourished children had increased levels of systemic and faecal proinflammatory cytokines [153].

Many seroprevalence studies have showed the presence of *Cryptosporidium* specific serum IgG, IgA and IgM antibodies following cryptosporidial exposure [132]. However, it is difficult to interpret association of naturally acquired immunity to *Cryptosporidium* with the level of specific serum antibodies. It is unclear, whether the serum antibodies have an active role in defence against the parasite, or are surrogate markers of cellular immune response [132] [250]. With a lack of effective treatment for cryptosporidiosis, there are efforts to develop effective immune-based prophylactic therapy; this may be needed in regions where the parasite is endemic [328].

Given the recently described importance of cryptosporidiosis in immunocompetent children in the multicentric GEMS study [18], is important to understand the immunity conferred by natural infection with *Cryptosporidium* spp. This would have important implications on the development of a vaccine against this infection. Many molecular studies have attempted to

understand immune response to cryptosporidiosis; however, there are no longitudinal community based studies with the specific aim of observing the protection offered by natural infection with *Cryptosporidium* spp. In this study we used epidemiological methods to ascertain if natural infection conferred any protection on subsequent cryptosporidial infections in a birth cohort in a semi-urban slum.

5.5.2. Data and analytic methods

A cohort of 410 children who had completed three years of follow-up were considered for the analysis to ascertain whether natural infection offered any evidence of protection against subsequent infection/disease. Stool microbiology and serology results were used to identify those with cryptosporidiosis (*Cryptosporidium* associated diarrhoea/disease and asymptomatic infection). All the time to event analyses, i.e. the incidence rates, were calculated using the stool microbiology data.

Analyses were conducted to determine the following:

i) whether with increase in number of infections, the child acquires protection against subsequent infection or disease.

ii) whether rates of infection/disease differ between children with early exposure to cryptosporidial infection (during the first six months of life) and children with later infection (after 6 months of age).

iii) whether early infection with *C. hominis* (during the first six months of life) offers any homotypic or heterotypic protection by comparing the rates of infections/disease with those of children who had later infection with *C. hominis* (after six months of age).

To investigate if an increase in the number of previous infections offered any protection against subsequent infection or disease, survival models accounting for multiple cryptosporidial infections within a child were fitted to obtain variance-corrected incidence rate ratios. The person time of follow-up was calculated from the first day of follow-up till the first infection for the calculation of primary infection rates and the time interval between the primary and the second infection was used for calculation of the rate of second infection and a similar approach was used for subsequent infections.

In order to study the protection offered by early natural infection, exposure to cryptosporidial infection within a specified time frame was considered. The 410 children were divided into two groups, based on whether they had acquired cryptosporidial infection (symptomatic or asymptomatic) within the first six months of life. Rates of infections were then compared in the two groups at age intervals from six months to one year, two years and three years.

Homotypic and heterotypic protection conferred by *C. hominis* species was restricted to 298 children for whom species data was available. These children were divided into those with early childhood cryptosporidiosis by *C. hominis* and those who did not have an early infection, and the risk of subsequent infections by *C. hominis* was calculated in the two groups.

In children with multiple infections, the severity of *Cryptosporidium* associated diarrhoea was evaluated by order of infections. Severity of diarrhoea was assessed using Vesikari scoring system [188]. Comparisons of Vesikari scores for the *Cryptosporidium* associated diarrhoea with order of infections were made using the Wilcoxon signed-rank test.

5.5.3. Results

5.5.3.1. Effect of prior cryptosporidial infection on subsequent infection/disease

Among 410 children, 229 children had more than one episode of cryptosporidiosis and 44 children had more than one episode of *Cryptosporidium* associated diarrhoea. Rate ratios for cryptosporidial infection and *Cryptosporidium* associated diarrhoea were calculated based on the number of earlier infections using children with no previous infection as the reference group (Table 5.5.1). Age adjusted rate ratios revealed that there was no significant protection offered by primary, second or the third cryptosporidial infection against subsequent infections.

Among children with *Cryptosporidium* associated diarrhoea, the probability of getting subsequent *Cryptosporidium* associated diarrhoea did not decrease after one or two episodes, instead the rate for subsequent *Cryptosporidium* associated diarrhoea after one episode was almost twice as high as the diarrhoeal rate for children with no previous *Cryptosporidium* associated diarrhoea. Similarly children with moderate to severe *Cryptosporidium* associated diarrhoea had a higher risk of acquiring another episode of moderate to severe diarrhoea.

Table 5.5.1.: Effect of prior cryptosporidial infection on subsequent infection/disease graded by number of previous infections – incidence rates, unadjusted and age adjusted rate ratios in the birth cohort (n=410)

Exposure as number of previous cryptosporidial infection	Person years of follow-up	No. of infections	Incidence rate (95%CI) per child year	Relative risk (95% CI) of subsequent event	
				Unadjusted	Adjusted for age
Any cryptosporidial infection					
0[$]	571.79	347	0.60 (0.54-0.67)		
1[*]	340.4	229	0.67 (0.59-0.76)	1.10 (0.95-1.30)	1.07 (0.90-1.28)
2[**]	205.62	103	0.50 (0.41-0.60)	0.82 (0.66-1.02)	0.80 (0.64-1.02)
3[**]	100.29	54	0.53 (0.42-0.68)	0.88 (0.69-1.14)	0.90 (0.68-1.20)
Cryptosporidium associated diarrhoea					
0[$]	974.52	143	0.14 (0.12-0.17)		
1[*]	182.93	44	0.24 (0.17-0.33)	1.63 (1.15-2.33)	1.80 (1.25-2.60)
2 [**]	60.65	12	0.19 (0.10-0.44)	1.34 (0.67-2.67)	1.55 (0.77-3.11)
Moderate to severe *Cryptosporidium* associated diarrhoea (score 6-20)					
0[$]	1073.41	77	0.07 (0.05-0.09)		
1[*]	114.15	22	0.19 (0.12-0.30)	2.68 (1.64-4.39)	3.20 (1.97-5.24)
2[**]	30.54	5	0.16 (0.04-1.22)	2.28 (0.70-7.41)	3.09 (0.96-9.94)

[$] the follow-up period starts from the date of birth and continues until the day a child has cryptosporidial infection (for children who experienced cryptosporidial infection) and till the third birthday (for children who did not experience cryptosporidial infection till their third birthday) (n=410) – this is the reference category for the rate ratio analysis

* the follow up period is after the day of the first cryptosporidial infection till the day of second infection (n=347)

** the follow up period starts after the day of second cryptosporidial infection till the day of third infection (n=229)

***the follow up period starts after the day of third cryptosporidial infection till the day of fourth infection (n=103)

5.5.3.2. Effect of early cryptosporidial infection on the risk of subsequent infection

There were 165 (40%) children who acquired cryptosporidial infection in the first six months of life and 245 (60%) who did not. Children, who acquired infection early (within the first six months), had an incidence rate of 0.59 (95% CI=0.44-0.82), 0.65 (95% CI=0.57-0.75) and 0.63 (95% CI=0.57-0.70) episodes per child year from six months to one, two and three years of the follow up period respectively. Incidence rates in children who did not acquire infection within the first six months of life were not significantly different across the different age intervals (Table 5.5.2), indicating a similar probability of infection in later follow-up periods irrespective of whether the child became infected early or late in infancy and early childhood.

Among 165 children with early cryptosporidiosis, 42.5%, 85.4% and 97% were re-infected from six months to one, two and three years period (Table 5.5.3). A minimal amount of protection was conferred in children with early cryptosporidiosis (RR=0.75, 95% CI=0.56-1.01) during the subsequent 6 months of follow-up. However this difference was not significant.

Table 5.5.2.: Effect of early cryptosporidial infection on the risk of subsequent infection using incidence rates

Age Interval	Overall Incidence of infection	Children infected by six months (n=165)	Children uninfected by six months (n=245)	RR (95% CI)	*P*-value
6 months - 1 year	0.63 (0.53-0.76)	0.59 (0.44-0.82)	0.66 (0.53-0.83)	0.89 (0.61-1.29)	0.56
6 months - 2 years	0.69 (0.63-0.75)	0.65 (0.57-0.75)	0.71 (0.64-0.80)	0.91 (0.74-1.11)	0.35
6 months - 3 years	0.62 (0.58-0.67)	0.63 (0.57-0.70)	0.62 (0.56-68)	1.02 (0.87-1.20)	0.74

Table 5.5.3.: Effect of early cryptosporidial infection on the risk of subsequent infection using proportions of infected children

Age Interval	Children with infection	Children infected by six months (n=165)	Children uninfected by six months (n=245)	RR (95% CI)	*P*-value
6 months - 1 year	207	70 (42.4%)	137 (55.9%)	0.75 (0.56-1.01)	0.06
6 months - 2 years	355	141 (85.4%)	214 (87.3%)	0.97 (0.78-1.21)	0.84
6 months - 3 years	392	160 (97%)	232 (94.7%)	1.02 (0.83-1.25)	0.81

5.5.3.3. Effect of early *Cryptosporidium* associated diarrhoea on the risk of subsequent *Cryptosporidium* associated diarrhoea and infection

Of the 410 children who completed three years follow up, 24 (5.8%) had *Cryptosporidium* associated diarrhoea in the first six of months of life. Incidence rates for subsequent *Cryptosporidium* associated diarrhoea among these children were 0.33 (95% CI=0.13-0.28), 0.19 (95% CI=0.10-0.39), 0.16 (95% CI=0.09-0.34) episodes per child year during six months to one, two and three years of the follow up period respectively. Children with early *Cryptosporidium* associated diarrhoea had a higher risk (RR=1.73, 95% CI=0.44-4.81) acquiring symptomatic infection in the consecutive six months (6 months-1 year) compared to the children who had not had *Cryptosporidium* associated diarrhoea within first six months. However this was not statistically significant. The relative risk for acquisition of subsequent *Cryptosporidium* associated diarrhoea during other time intervals was very similar between children with and without early *Cryptosporidium* associated diarrhoea (Table 5.5.4).

The effect of early *Cryptosporidium* associated diarrhoea on the risk of subsequent cryptosporidiosis was also explored. Children positive and symptomatic in the first six months of life had a slightly higher risk of acquiring cryptosporidiosis (RR=1.48, 95% CI=0.71-2.74) during the subsequent period from six months to one year of age, but this was not significant. The relative risk for acquisition of subsequent infections during other time intervals was very similar, as shown in Table 5.5.5.

Table 5.5.4.: Effect of early *Cryptosporidium* associated diarrhoea on the risk of subsequent *Cryptosporidium* associated diarrhoea episodes

Age Interval	Overall Incidence of infection	Children with *Cryptosporidium* associated diarrhoea by six months (n=24)	Children without *Cryptosporidium* associated diarrhoea by six months (n=386)	RR (95% CI)	*P*-value
6 months - 1year	0.20 (0.14-0.28)	0.33 (0.13-1.03)	0.19 (0.13-0.28)	1.73 (0.44-4.81)	0.31
6 months - 2 years	0.20 (0.17-0.25)	0.19 (0.10-0.39)	0.20 (0.17-0.25)	0.93 (0.36-1.97)	0.89
6 months - 3years	0.17 (0.14-0.20)	0.16 (0.09-0.34)	0.17 (0.14-0.20)	0.97 (0.46-1.83)	0.97

Table 5.5.5.: Effect of early *Cryptosporidium* associated diarrhoea on the risk of subsequent infection

Age Interval	Overall Incidence of infection	Children with *Cryptosporidium* associated diarrhoea in the first six months (n=24)	Children without *Cryptosporidium* associated diarrhoea in the first six months (n=386)	RR (95% CI)	*P*-value
6 months - 1 year	0.63 (0.53-0.76)	0.91 (0.47-2.05)	0.62 (0.51-0.74)	1.48 (0.71-2.74)	0.22
6 months - 2 years	0.69 (0.63-0.75)	0.64 (0.42-1.00)	0.69 (0.63-0.76)	0.91 (0.57-1.40)	0.71
6 months - 3 years	0.62 (0.58-0.67)	0.61 (0.47-0.81)	0.62 (0.58-0.67)	0.98 (0.68-1.37)	0.94

5.5.3.4. Diarrhoeal disease severity in consecutive cryptosporidial infections

Among the 410 children, 229 children had ≥2 episodes of cryptosporidial infection and 103 children had ≥3 episodes of infection. Among 229 children, 66 (28.8%) had *Cryptosporidium* associate diarrhoea and 163 (71.2%) had an asymptomatic infection as their first infection. Among 103 children who had ≥3 episodes of infections, 12 (11.6%) had *Cryptosporidium* associated diarrhoea as second and third infections. In children with multiple infections and *Cryptosporidium* associated diarrhoea, the severity of diarrhoea did not significantly decrease, between first and second infection (Wilcoxon signed rank=0.72) or between the second and third infection (Wilcoxon signed rank=0.40) Figure 5.5.1.1 and 5.5.1.2.

Figure 5.5.1.: Diarrhoeal disease severity in consecutive cryptosporidial infections, as estimated by the Vesikari Score

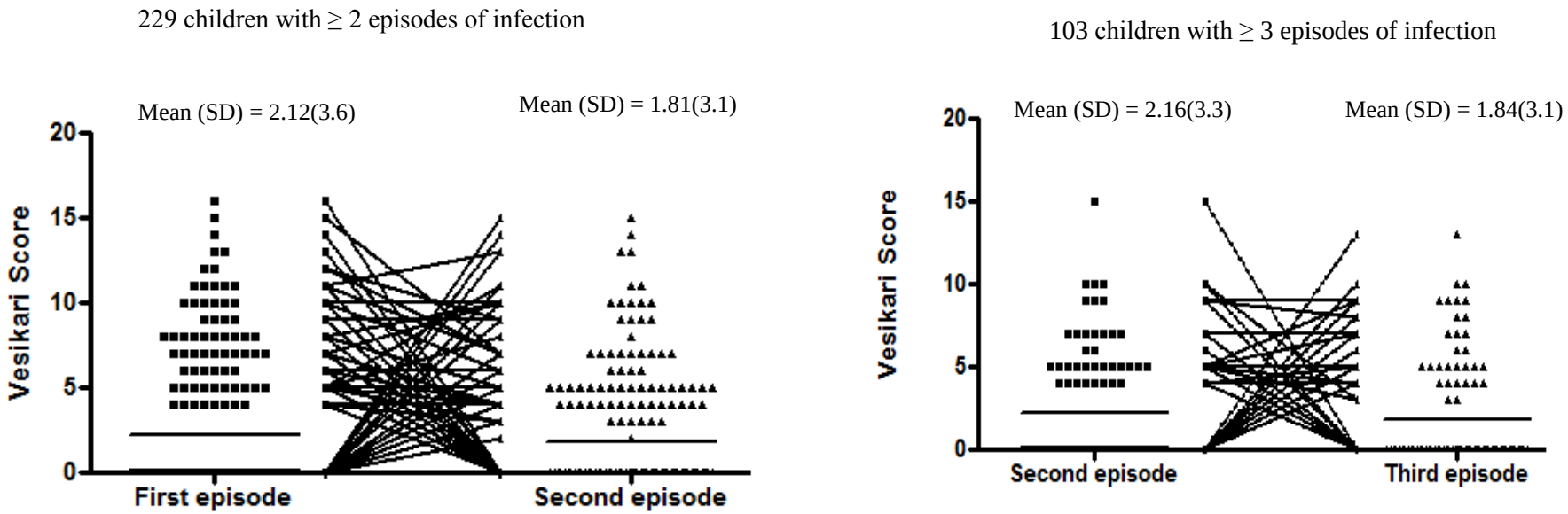


Figure 5.5.1.1. shows scores between the first and second cryptosporidial infections in 299 children with at least two infections.

Figure 5.5.1.2. shows scores between the second and third rotavirus infections in 103 children with at least three infections.

5.5.3.5. Effect of cryptosporidial infection with *C. hominis* on the risk of subsequent infection by *C. hominis* species and other zoonotic species

Among 298 children with available species data, 15 children had cryptosporidial infection with *C. hominis* species in the first six months of life. Incidence rates for subsequent infection by *C. hominis* among these children were 0.41 (95% CI=0.09-3.44), 0.50 (95% CI=0.22-1.28), 0.75 (95% CI=0.50-1.13) episodes per child year from six months to one, two, three years of the follow up period respectively. Insignificant protection against subsquent infection was observed in the group of children with early infection with *C. hominis.* A similar pattern of incidence rates for cryptospridial infection with other species were observed in children with early and late infection with *C. hominis* (Table 5.5.6 and 5.5.7).

Table 5.5.6.: Effect of early cryptosporidial infection with *C. hominis* on the risk of subsequent infection by *C. hominis*

Age Interval	Overall Incidence of infection	Children infected by six months (n=15)	Children uninfected by six months (n=283)	RR (95% CI)	*P*-value
6 months - 1 year	0.44 (0.34-0.60)	0.41 (0.09-3.44)	0.44 (0.34-0.60)	0.92 (0.10-3.48)	0.99
6 months - 2 years	0.67 (0.60-0.77)	0.50 (0.22-1.28)	0.68 (0.60-0.77)	0.73 (0.26-1.62)	0.47
6 months - 3 years	0.83 (0.76-0.91)	0.75 (0.50-1.13)	0.84 (0.76-0.91)	0.89 (0.44-1.62)	0.75

Table 5.5.7: Effect of cryptosporidial infection with *C. homnis* on the risk of subsequent infection by other zoonotic species

Age Interval	Overall Incidence of infection	Children infected by six months (n=15)	Children uninfected by six months (n=283)	RR (95% CI)	*P*-value
6 months - 1 year	0.18 (0.12-0.27)	0.20 (0.03-1.47)	0.18 (0.12-0.27)	1.12 (0.02-6.89)	0.81
6 months - 2 years	0.26 (0.21-0.32)	0.25 (0.08-0.95)	0.26 (0.21-0.32)	0.95 (0.19-2.89)	1.00
6 months - 3 years	0.31 (0.26-0.36)	0.27 (0.12-0.72)	0.30 (0.26-0.36)	0.88 (0.23-2.32)	0.86

5.5.4. Discussion

In this study, we intensively followed a cohort of 410 children from birth up to the age of three years to assess whether natural infection with *Cryptosporidium* spp. conferred protection against subsequent infections in children living in an endemic community. The results of this study suggest that previous cryptosporidial infection may not protect against subsequent infections in children. A study by Ajjampur *et al.*, showed IgG antibodies peak around 9 weeks following a *Cryptosporidium*-associated diarrhoea and levels dropped to pre-infection level by 25 weeks indicating short sustenance of IgG post-infection [334]. Studies on healthy adults have also demonstrated susceptibility to re-infection and disease following a second exposure to homologous isolates of *Cryptosporidium* spp. one year after primary exposure. These studies showed reduction in the disease severity but not the rate and duration of illness indicating protection is not conferred by a single earlier exposure [9, 335]. However Casemore cited low incidence of cryptosporidiosis among adults in the developing world and in rural communities as evidence for resistance to infection. Frequent and constant exposure to a low number of oocysts was one suggested explanation [336].

An interesting finding from this study was the fact that children with *Cryptosporidium* associated diarrhoea tended to have higher risk of a subsequent *Cryptosporidium* associated diarrhoeal episode. This possibly suggests a potential role of host genetic susceptibility in the acquisition of cryptosporidial infections. Studies by Kirkpatrick *et al.* conducted in Bangladesh and Haiti in similar impoverished endemic settings have shown children with HLA class II DQB1*0301 allele, B*15 HLA class I allele and deficiency of serum mannose binding lectin respectively were predisposed to cryptosporidial infections [251, 337]. Impaired cell mediated immunity can be another reason for susceptibility to the infection;

particularly CD4 T cells have a crucial role for protection and resolution of cryptosporidiosis [132, 338-339]. The important role of CD4 T cells is highlighted by the susceptibility of AIDS patients to cryptosporidial infection and the capacity to resolve the infection following immune reconstitution [340-341]. Severity of the disease has also been shown to vary by differences in CD4 T cell counts [136], which underscores the fact that the child's immune status likely plays a vital role in the development of disease.

In order to ascertain if early cryptosporidial infection conferred protection or differed from infection in later life, an age cut off of six months was chosen to categorise children with early cryptosporidial infection. This is because children are more susceptible to infection, as maternal antibodies start waning and children are weaned, exposing them to more environmentally transmitted agents. A study by Sarkar *et al*. from Vellore showed children were exposed for the first time to *Cryptosporidium* between the ages of three to nine months. [342]. In this study, almost 40% the children were infected by six months of age. The results show that children with early cryptosporidial infections had similar re-infection risks compared to children with late cryptosporidial infection, highlighting that early childhood cryptosporidiosis did not seem to offer any significant protection.

A previous study in Vellore suggested there is significant response to both heterotypic and homotypic cryptosporidial antigens, suggestive of cross-reactivity [334]. Demonstration of homotypic and heterotypic response against a target antigen is the initial step in vaccine development. However, it is important to determine whether immune responses to these antigens are protective against subsequent infections. With the advantage of a reasonable number of species-specific cryptosporidial infections in this birth cohort, the homotypic and heterotypic protection conferred by *C. hominis* was studied. Epidemiologically, we could not

demonstrate any evidence to support homotypic or heterotypic protection in children offered by *C. hominis.*

5.5.5. Conclusion

In this study we used epidemiological methods to examine if natural cryptosporidial infection offered any protection against subsequent infections/disease in a birth cohort of an endemic southern Indian slum community. The results of this study showed predisposition to *Cryptosporidium* associated diarrhoea in some children. However, protection by previous infections and early cryptosporidial infection against subsequent infections was not observed. Further understanding of protection will require analysis of both cell mediated and humoral immunity in conjunction with epidemiological findings.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Cryptosporidium spp. is a protozoan parasite and is a leading cause of parasitic diarrhoea in children under the age of 5 years, especially among those living in developing countries. Early exposure to cryptosporidial infections is believed to have significant ill effects on the physical and cognitive development of children. A majority of the studies on cryptosporidiosis so far have aimed at understanding the clinical course of *Cryptosporidium* associated diarrhoea and few have looked at infection in the absence of clinical symptoms. There is also a lack of longitudinal data that can help understand the natural history of cryptosporidial infection, the risk factors that influence the acquisition of infection, and the protection conferred by natural infection in children living in endemic settings.

In this birth cohort, recruited between April 2009 and May 2010, a total of 497 new born were enrolled from four adjacent semi-urban slum areas in Vellore, India, where earlier studies have shown cryptosporidiosis to be endemic. Children were then followed up twice weekly, until they attained the age of three years, and diarrhoeal and other illnesses were documented. Stool samples were collected fortnightly and also during diarrhoeal episodes. These samples were tested for the presence of *Cryptosporidium* spp. by PCR. Anthropometric measurements from children were obtained every month. Samples of blood were collected at 6-monthly intervals and were tested for the presence of *Cryptosporidium* IgG by ELISA. Baseline demographic and birth details were obtained at the time of recruitment and data on breast feeding and household hygiene were obtained at regular intervals. Of 497 children, 410 (82.5%) completed the three year follow- up period.

Morbidity in the first 1000 days of life demonstrated a high disease burden with children experiencing an average of 14.7 episodes of illnesses per year from various causes, mainly

during their infancy. Among all illnesses, the commonest were upper respiratory infections. Gastrointestinal illness accounted for around one-fifth of the total disease burden. Most morbidities (60%) resulted in contact with healthcare providers either in a clinic or a hospital setting. This was because caregivers were advised to bring their children to the study clinic whenever the child was sick. Maternal factors such as maternal haemoglobin less than 10 gm% and less than four antenatal visits were associated with an increased risk of low birth weight. Factors such as female gender and exclusive breastfeeding for six months were associated with protection against overall and GI morbidity, whereas low socio-economic status was a risk factor. This study identified antenatal factors that can potentially influence birth outcomes; and GI and respiratory illnesses continue to be the predominant morbidity in first two years of life in children living in impoverished urban slum communities with high population density and environmental exposure.

Of 410 children who completed the three-year follow-up period, 397 acquired cryptosporidiosis by three years of age. A total of 733 episodes of cryptosporidiosis were experienced by the study children, with an incidence rate of 0.60 episodes of cryptosporidiosis per child-year of observation. This high rate of infection reflects the endemicity of the disease in this community. The median (IQR) age of onset of first cryptosporidial infection was 12 (6-20) months, suggestive of early childhood exposure. A majority (534, 73%) of the infections were asymptomatic, suggesting that the circulation of cryptosporidiosis in this community was likely to be predominantly among asymptomatically infected individuals.

Cryptosporidium spp. was associated with 9.4% of all diarrhoeal episodes among the study children. Some evidence of clustering was observed in children with symptomatic

cryptosporidial infection suggesting host susceptibility to the infection. Using molecular typing, *C. hominis* was found to be the predominant *Cryptosporidium* species among the study children, followed by *C. parvum.* This again reflects the predominance of human-to-human mode of transmission of *Cryptosporidium* spp. in the study area. Some evidence of seasonal pattern of cryptosporidiosis, i.e. a preponderance of disease in the cooler months of the year (December and January) was observed in this study.

This is the first birth cohort study to combine epidemiological data with molecular typing to examine whether natural cryptosporidial infection offered any protection on subsequent infection or disease, both homotypic and heterotypic. The study found that earlier infection did not offer protection from subsequent infection. This lack of protection could be partially attributed to the quick waning of antibodies post-infection but since there is a lack of clarity on the correlate of protection, this is speculative. These data also indicate that there are likely to be significant challenges in developing effective vaccines, capable of providing long lasting immunity, for children. While it is possible that repeated exposure to the parasite over a prolonged time may lead to development of immunity against disease, like other protozoan parasites such as malaria, this will require studies with a much longer follow up.

In this study, almost 67.8% of children experienced one or more growth failures (wasted/stunted/underweight) during the three-year follow up, with chronic malnutrition (stunting) being predominant. Even though a majority of children in this cohort were infected with cryptosporidiosis and a high proportion of them were malnourished, no association between cryptosporidiosis and growth faltering could be defined. However, socio-demographic factors such as low birth weight, maternal illiteracy and age ≤23 years,

exclusively breast feeding more than six months, low socio economic status and presence of siblings were significantly associated with slower growth rates in children.

The host, environmental, sanitary and nutritional factors associated with childhood cryptosporidiosis in an endemic Indian slum community was extensively examined in this study. Factors such as the presence of an older sibling, maternal age ≤23years, low, socio-economic status and contact with animal(s) were associated with an increased likelihood of infection, while drinking boiled water was found to be protective. The results of this risk factor analysis suggests that, for a disease like cryptosporidiosis, which has a complex host parasite interaction, a multidimensional approach towards disease control has to be considered.

This study set out to fill a critical void in our understanding of the natural history of cryptosporidial infections in children in an endemic community setting. The results of this study provide valuable insights into the epidemiology of cryptosporidiosis in settings with poor environmental, sanitary conditions and close human-animal contact and can be used to design targeted disease control measures in such settings.

IMPACT OF THE STUDY

Several aspects of cryptosporidial infections, and host factors that modify the infection amongst children in this birth cohort have been explored. The impact of the findings on public health issues, particularly relevant to control of cryptosporidial infections are highlighted here.

Cryptosporidium spp. is a common cause of parasitic diarrhoea throughout the world and is one of top five pathogens causing moderate to severe diarrhoea among children in developing countries. Despite this, the epidemiology of cryptosporidiosis in endemic settings is poorly understood. This study is innovative because it is the largest birth cohort study on the natural history of childhood cryptosporidiosis. The integration of molecular and serological methods for the diagnosis of cryptosporidial infection, coupled with the intensive bi-weekly follow-up helped capture the true burden of childhood cryptosporidiosis, and found it to be higher than reported earlier.

Almost all children in the study acquired cryptosporidial infection by three years of age indicating a high rate of transmission in endemic areas, most likely through asymptomatically infected individuals. This study is the first to explore whether prior natural infection offered subsequent protection from infection or disease. The study found that an earlier infection did not offer protection from infection later in life. High rates and early infection with no subsequent protection requires public health interventions aimed at routes of transmission, rather than vaccines.

The results of the study provide important insights in understanding the natural history and epidemiology of cryptosporidiosis in slum communities in India where there is sustained transmission, through multiple routes. The risk factors identified in this study can help

develop scientifically sound, geographically relevant and socially sustainable measures that will effectively reduce the transmission of cryptosporidiosis in this and other endemic communities.

As opposed to the developed countries where water is the primary mode of transmission, in the Indian setting, the transmission of cryptosporidiosis seems to occur through multiple routes (waterborne, human-to-human and animal-to-human). Personal hygiene and safe animal handling/tethering practices hence would be the leading public health interventions in this Indian setting for short-term benefits. Social interventions such as improvement in maternal literacy, reduction of poverty and provision of basic amenities like access to safe drinking water and sanitation may also help reduce transmission in the long run, but the effect of such intervention can only be ascertained over a longer time frame. Such interventions would also involve the participation of disciplines not directly related to health, such as education, social welfare and local administration.

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APPENDICES

Appendix -1

Publications:

1. **Kattula D**, Sarkar R, Sivarathinaswamy P, Velusamy V, Venugopal S, Naumova EN, Muliyil J, Ward H, Kang G. 2014. The first 1000 days of life: prenatal and postnatal risk factors for morbidity and growth in a birth cohort in southern India. BMJ Open 4:7. pg e005404.

2. Sarkar R**, Kattula D**, Francis MR, Ajjampur SSR, Prabakaran AD, Jayavelu N, Muliyil J, Balraj V, Naumova EN, Ward H, Kang G. Risk Factors for Cryptosporidiosis among Children in a Semi Urban Slum in Southern India: A Nested Case-Control Study. AJTMH 2014 (accepted).

Appendix -2

Anti-plagiarism Report