Cryptosporidiosis in low-resource healthcare settings

Near-patient diagnostics, risk factors, and parasite shedding dynamics in young Ethiopian children

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Scientific environment

This research project was carried out from 2016 to 2021, during my time as a PhD fellow at the Department of Clinical Science at the Medical Faculty of the University of Bergen, and while I was employed part-time by Vestfold Hospital Trust, Department of Microbiology, from 2016 to 2021, as part of a joint hospital collaboration with Tikur Anbessa Hospital and Yekatit 12 Hospital in Addis Ababa, Ethiopia. The hospital exchange programme was financed in part by FK Norway (the Norwegian Peace Corps), and in part by Vestfold Hospital Trust, followed by a PhD grant from the Norwegian Research Council GLOBVAC programme. The exchange programme and subsequent research funding allowed me the privilege to live and work in Ethiopia, with my wife, also supported by the FK Norway exchange programme, and our three young boys (born 2012, 2014 and 2017), for four wonderful years, from 2014 to 2018. The final write-up stage was completed after I started working as a Consultant microbiologist for the Southern Health and Social Care Trust in Northern Ireland from August 2021. The CRYPTO-POC project was a joint collaboration between the following institutions: University of Bergen, Jimma University, Vestfold Hospital Trust, Copenhagen University, and the Norwegian University of Life Sciences. My main PhD supervisor has been Kurt Hanevik and my co-supervisors were Nina Langeland and Lucy Robertson. Kurt Hanevik is a Professor of microbiology, and Nina Langeland is a Professor of infectious diseases, both at the Department of Clinical Science, University of Bergen. Lucy Robertson is a Professor of parasitology at the Faculty of Veterinary Medicine, Department of Paraclinical Sciences, at the Norwegian University of Life Sciences.

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Øystein Haarklau Johansen

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AP	Auramine-phenol (staining method)
ASSURED	Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Delivered (criteria)
CI	Confidence interval
CRS	Clinical composite reference standard
DALY	Disability-adjusted life-years
DFA	Direct antibody fluorescence microscopy (equivalent to IFAT)
DNA	Deoxyribonucleic acid
D-RCT	Diagnostic randomized controlled trial
DSS	Demographic surveillance site
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ETB	Ethiopian Birr
ETEC	Enterotoxigenic *Escherichia coli*
GAMM	Generalized additive mixed model
GBD	Global Burden of Disease (study)
GEMS	Global Enteric Multicentre Study
gp60	The 60-kDa glycoprotein gene
GPS	Global Positioning System
HAZ	Height-for-age Z-score
HDSS	Health and demographic surveillance site
HIV	Human immunodeficiency virus
iCCM	Integrated Community Case Management
ICLF	Immunochromatographic lateral-flow assay
IFAT	Immunofluorescent antibody testing (equivalent to DFA)
IMCI	Integrated Management of Childhood Illness
IRB	Institutional review board
JMC	Jimma Medical Centre

JUTH	Jimma University Teaching Hospital
LAMP	Loop-mediated isothermal amplification
LED	Light-emitting diode
LED-AP	Light-emitting diode auramine-phenol fluorescence microscopy
LMIC	Low and middle-income country
LNA	Locked nucleic acid
MAL-ED	Malnutrition and Enteric Disease Study
MAM	Moderate acute malnutrition
mRNA	Messenger RNA
MRS	Microbiological composite reference standard
MUAC	Mid-upper-arm circumference
mZN	Modified Ziehl-Neelsen (staining method)
NAAT	Nucleic acid amplification test
NASBA	Nucleic acid sequence-based amplification
NCrD	Non-cryptosporidiosis diarrhoea
OR	Odds ratio
PAF	Population attributable fraction
PCR	Polymerase chain reaction
POC	Point-of-care
qPCR	Quantitative PCR
RCT	Randomized controlled trial
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RPA	Recombinase polymerase amplification
rRNA	Ribosomal RNA
SAM	Severe acute malnutrition
SHINE	Sanitation, Hygiene, Infant Nutrition Efficacy (trial)
TAT	Turnaround time
TNA	Total nucleic acid (DNA and RNA)
UK	United Kingdom
UN	United Nations

USD	United States Dollars
WASH	Water, sanitation, and hygiene
WAZ	Weight-for-age Z-score
WHO	World Health Organization
WHZ	Weight-for-height/length Z-score
ZN	Ziehl-Neelsen (staining method)

Summary

Background: *Cryptosporidium* is a leading cause of diarrhoea and death in children younger than two years of age in low-and-middle income countries. Targeted treatment is needed but requires accurate and setting-appropriate diagnostic tests. Prevention should target known risk factors. Duration of parasite shedding is not well known.

Objectives: To evaluate the diagnostic accuracy of two near-patient tests for cryptosporidiosis, risk factors for disease, and parasite shedding patterns, in young children in Ethiopia.

Methods: From Dec 2016 to Jul 2018 the CRYPTO-POC study enrolled 1384 children under 5 years with diarrhoea, from a hospital and a health center, and 946 controls without diarrhoea. A diagnostic accuracy study in 912 cases and 706 controls compared results from LED-AP microscopy and a test strip, performed by local healthcare workers, against a panel of three reference tests. Risk factors in children under 2 years were examined in a case-case-control analysis of 59 cases with cryptosporidiosis, 432 diarrhoea cases without cryptosporidiosis, and 725 non-diarrhoea controls. A 60-day follow-up study was conducted in 53 cryptosporidiosis cases, supported by *Cryptosporidium* genotyping.

Results: The sensitivities of LED-AP and the test strip were 88% and 89%, and positive and negative predictive values 88% and 99%, respectively. Specificity was 99% for both tests. LED-AP was cheaper at 0.7 USD per test. Important operational issues were identified. Caregiver-related socioeconomic factors, public-tap water use, previous healthcare attendance, and acute malnutrition were important risk factors for cryptosporidiosis. Prolonged parasite shedding was common, with a median duration of 31 days, although with a 10-fold drop in quantity per week for the first four weeks.

Conclusions and consequences: LED-AP is an accurate test for cryptosporidiosis that can be integrated with existing laboratory infrastructure, near the point of care. The test strip is a good alternative. Nutritional management of moderate acute malnutrition, focused caregiver education and closer follow-up of children who present for health care can be explored as preventive interventions. Parasite shedding duration can be considered as a secondary treatment outcome in cryptosporidiosis intervention trials.

List of publications

Paper I: Øystein H. Johansen, Alemseged Abdissa, Mike Zangenberg, Zeleke Mekonnen, Beza Eshetu, Ola Bjørang, Yonas Alemu, Bizuwarek Sharew, Nina Langeland, Lucy J. Robertson, Kurt Hanevik: *Performance and operational feasibility of two diagnostic tests for cryptosporidiosis in children (CRYPTO-POC): a clinical, prospective, diagnostic accuracy study.* Lancet Infectious Diseases; Vol 21, May 2021, published 3 Dec 2020.

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Two related papers not included in this thesis:
Mike Zangenberg, Øystein H. Johansen, Alemseged Abdissa, Beza Eshetu, Jørgen A. L. Kurtzhals, Henrik Friis, Halvor Sommerfelt, Nina Langeland, Kurt Hanevik: *Prolonged and persistent diarrhoea is not restricted to children with acute malnutrition: an observational study in Ethiopia.*
Tropical Medicine & International Health; Vol 24, Issue 9, Sep 2019; published 20 Jul 2019.

Lucy J. Robertson, Øystein H. Johansen, Tsegabirhan Kifleyohannes, Akinwale Michael Efunshile, Getachew Terefe: *Cryptosporidium infections in Africa - how important is zoonotic transmission? A review of the evidence.* Frontiers in Veterinary Science; Vol 7, Oct 2020; published 8 Oct 2020.

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1. Introduction

1.1 The global challenge of diarrhoea

1.1.1 A disease of poverty

The massive reduction in diarrhoeal deaths, from 2.6 million in 1990 to 1.3 million in 2013, was a major public health success story [1]. However, in 2017, diarrhoea was still the third leading cause of mortality in children under five years old globally [2] . An estimated 540.000 children younger than 5 years died from diarrhoea that year [2]. These deaths are unequally distributed and mainly affect the global poor [3]. The strongest driver behind the reductions in diarrhoeal deaths has been socioeconomic development [4]. In Ethiopia, childhood diarrhoea is the second most important cause of life years lost, after lower respiratory tract infections [1]. On average, each Ethiopian child suffers 3-5 episodes of diarrhoea per year [5]. Although many well-known diarrhoeal diseases like cholera, amoebic dysentery, or shigellosis, are more common in warm and humid areas, diarrhoea should not be considered a "tropical disease". This is illustrated by the large variation in diarrhoeal death rates between countries within the tropical region [6]. Indeed, many of the tropical or near-tropical areas that are now considered middle-income (including, for example, most countries in meso-America), transitioned from low-income to middle-income status during the same historical time period when diarrhoeal incidence and diarrhoeal death rates dropped dramatically. Socioeconomic inequality still underpins huge internal variation in deaths from diarrhoea within countries like India, Indonesia and South Africa [7], and in underprivileged populations in high-income countries [8, 9]. Within Oslo, the capital of Norway, there was a massive drop in diarrhoeal mortality in the impoverished eastern part of the city between 1900 and 1930 following large-scale improvements in sanitation and living standards [10].

Diarrhoeal deaths are also unequally distributed at the level of individuals. For example, underlying malnutrition, and suffering from diarrhoea that last 2 weeks or longer - referred to as a persistent diarrhoeal episode - cause a disproportionate fraction of diarrhoeal deaths [11, 12]. In the Butajira community in Ethiopia, persistent

diarrhoea was estimated to cause more than 55% of the diarrhoeal deaths and almost 45% of those deaths were associated with malnutrition [13]. Short duration of exclusive breastfeeding is considered one of the most important modifiable risk factors for progression from acute to prolonged (≥7 days) and persistent (≥14 days) diarrhoea [14].

Unnecessary deaths caused by diarrhoea has understandably received most of the attention from the global health community. However, short term case fatality only represents the tip of the iceberg of the total societal cost of diarrhoeal diseases. The so-called "vicious cycle" linking repeated episodes of diarrhoea with progressive decline in length growth was first demonstrated in a landmark study undertaken in the 1960s in Mata, Guatemala, that elegantly demonstrated the progressive decline across growth centiles that occur with each successive episode of diarrhoea during early childhood [15]. Later studies have repeatedly confirmed this finding; a 2008 study estimated that each additional episode of diarrhoea increased the odds of stunting by 4% (OR 1·04) [16].

1.1.2 A handful of gastrointestinal infections cause most diarrhoeal episodes

Most paediatric diarrhoeal episodes in low-and-middle income countries are caused by enteric infections. By the early 2000s, a vast number of enteric microorganisms had been identified that were associated with diarrhoea. Many early studies focused on single microbes or single sites or did not include a control group of children without diarrhoea in the same source population, that would allow quantification of the strength of association between infection and disease [17]. Without epidemiological data from well-designed case-control or cohort studies at multiple sites the relative importance or attributable disease fractions of diarrhoeal pathogens had not been established at a large scale. This evidence gap was the key motivation behind the ground-breaking Global enteric multicentre study (GEMS) [17], a large multi-site case-control study conducted in several countries in South Asia and sub-Saharan Africa, carefully designed to provide attributable fractions of all enteric pathogens known to cause diarrhoea in children under 5 years of age in low-income countries [18].

The key finding from the GEMS study was that most cases of moderate-to-severe diarrhoea could be attributed to only four pathogens, one virus, two bacteria, and one eukaryotic parasite. Rotavirus was, by far, the top diarrhoeal pathogen [12], a finding that helped spur on the global roll-out of rotavirus vaccination. The top bacterial pathogens were *Shigella* and enterotoxigenic *Escherichia coli* expressing the human variant of the thermostable toxin (ETEC). Remarkably, the *Cryptosporidium* parasite was found to be a key diarrhoeal pathogen in all sites, and one of the four major contributors to moderate-to-severe diarrhoeal diseases, during the first 2 years of life, in all seven sites [12, 19]. The association between *Cryptosporidium* and diarrhoea was strong even in sites with low HIV prevalence, in keeping with the findings from a previous cohort study in Guinea-Bissau [20].

1.2 Cryptosporidiosis overview

1.2.1 Biology and life cycle of the *Cryptosporidium* parasite

Cryptosporidium species are unicellular parasitic protozoans in the diverse phylum Apicomplexa. The *Cryptosporidium* genus was originally assumed to be members of the coccidia but is now considered evolutionary closer to the gregarines, a large group of primitive apicomplexan parasites [21]. This "primitivity" is not developmental stagnation but can rather be thought of as an extremely successful evolutionary strategy that has been fine-tuned over millions of years of selective pressure and reductive evolution. *Cryptosporidium* can complete its whole life cycle within a single host; compare this, for example, to the complex multi-host lifecycle of the *Plasmodium* malarial apicomplexan parasites. At least 44 species of *Cryptosporidium* have been identified to date [22]. Many species are host specific, but some can infect several different host species. Infective oocysts are transmitted between hosts, usually via the faecal-oral route, and if invasion and multiplication is established in the new host, infection will either remain asymptomatic or lead to overt disease. The incubation period of the main human pathogenic species, *C. hominis,* has only been studied in a single challenge study, and only in adults, reporting a median time of four days (range 2 to 10 days) [23]. The incubation time of *C. parvum* is about the same, and has been

studied in several human challenge experiments, and is inversely related to challenge dose [24]. Pathogenicity varies by species, genotype and host factors, and severe and prolonged symptoms are common in hosts who suffer from impaired immunity or malnutrition. The most common disease manifestation is gastroenteritis with diarrhoea, but extraintestinal infections can happen, as *Cryptosporidium* is capable of invading epithelial cells of various types, for example within the respiratory tract or in the biliary system [25, 26]. Post-infection sequelae have been reported [27]; for example, there is an association between *Cryptosporidium* infection and gastrointestinal symptoms, and joint pains and fatigue several months after infection [27-29].

The *Cryptosporidium* oocyst represents a remarkable "evolutionary technology" that facilitates the environmental survival and spread of the parasite. Understanding the oocyst is therefore key to understanding the epidemiology of cryptosporidiosis. Human pathogenic species of *Cryptosporidium* produce oocysts that are a bit smaller than a human red blood cell. They are shed in massive quantities starting only days after infection, are immediately infective (i.e., no need for a maturation stage in the environment), are robust against chlorination, and can survive for months in humid environments if shaded from ultraviolet radiation [30]. After ingestion of a few oocysts – as few as nine have been demonstrated to be sufficient to establish infection [24] - four meiotic progeny harboured within the oocyst, called sporozoites, will sense the arrival of the oocyst into the environment of the stomach or small intestine. They then burst out, through a process called excystation, leaving an empty oocyst husk behind. The sporozoites manage to reach the surface of nearby epithelial cells (usually enterocytes) by a fascinating type of gliding motility. They then then proceed to invade the host cell. Invasion is a multi-step process where the apicomplexan organelle is known to play a crucial role, but where many details have yet to be resolved [31]. After invasion, the sporozoites transform into trophozoites and lodge themselves inside a parasitophorous vacuole that is located epi-cellularly, i.e., intracellular but extracytoplasmic. The vacuole protects the parasite from predation from immune cells, while allowing it to harvest essential nutrients from the host cell through a specialized feeder organelle. Parasitic feeding fuels the growth of the parasite enabling rapid asexual multiplication, leading to the production of massive amounts of merozoites.

These small invasive forms anatomically resemble sporozoites, and invade neighbouring cells, resulting in the production of new trophozoites. Sexual differentiation starts to happen about two days after infection, with the production of male and female gametes, respectively referred to as microgamonts and macrogamonts. Macrogamonts are usually fertilized by microgamonts originating from the same strain, but, as mixed infections occasionally happen, can also be fertilized by a different strain. This process contributes to genetic exchange and diversity. A fertilized macrogamont transforms to a zygote and undergoes meiosis and further development before new infective oocysts are produced and shed into host faeces, thereby reaching the environment. Some oocysts immediately re-infect the same host; such auto-infection has been hypothesized to contribute to persistence of infection in immunocompromised hosts.

Pathology is caused by indirect and direct damage to enterocytes by secretion of a range of virulence factors, e.g., proteases and haemolysins, leading to fluid loss (mainly by secretion, but also by sheer structural damage), diarrhoea, and malabsorption. Repeated infections lead to flattening of the intestinal microvilli, reduced absorptive area, and further malabsorption. These features are not unique to *Cryptosporidium* and resemble some of the characteristic gastrointestinal features of environmental enteric dysfunction (EED). This disease is thought to be caused by repeated exposure to a range of enteric pathogens, for example *Campylobacter* spp. [32]. As malabsorption leads to malnutrition, which then leads to weakened immunity against new enteric infections, the infection-diarrhoea-malnutrition process has been referred to as a "vicious cycle". Cryptosporidiosis in young children is associated with growth faltering [33] and stunting [34, 35], which again inhibits normal cognitive development in children as late as 4-7 years after infection [36-38]. Causal mechanisms have not been clearly established, but it has been proposed that host immune response or alterations to the gut microbiome could play a pathogenic role in the development of sequelae, rather than persistence of infection.

1.2.2 Recognition of *Cryptosporidium* as a human pathogen

Cryptosporidium as a genus has likely been around for most of the evolutionary history of vertebrate animals. After first being first fully described by Ernest Tyzzer in 1910 it was considered a veterinary pathogen until the 1970s, when the first case reports in humans were published [39]. It emerged into the public consciousness during the HIV pandemic when cryptosporidiosis became infamous as an opportunistic infection. In patients with AIDS, cryptosporidiosis caused protracted diarrhoea with wasting, usually resulting in a fatal outcome. Through the 1980s it became evident that the parasite was also an important and previously underrecognized cause of diarrhoea in young children in the UK [40], but that it was particularly common in poor populations, first evidenced by reports from Liberia [41, 42]. The massive water-borne cryptosporidiosis outbreak in Milwaukee in 1993 further raised the awareness and research interest in *Cryptosporidium* [43].

1.3 Cryptosporidiosis health burden

1.3.1 *Cryptosporidium* is a key diarrhoeal pathogen and is associated with excess mortality

The GEMS study found that *Cryptosporidium* was the second most important cause - after rotavirus - of moderate-to-severe childhood diarrhoea, in Africa south of Sahara, and in South Asia [12]. Furthermore, *Cryptosporidium* was the only pathogen clearly associated with mortality at 60 day follow up after acute diarrhoea [12]. Notably, the GEMS study reported that 61% of the deaths attributed to diarrhoea occurred more than 7 days after study inclusion, and 33% of the deaths occurred 21 days or later. *Cryptosporidium* infection was found to be a risk factor for such delayed mortality. The pathological mechanism is not clear but may involve prolonged duration of diarrhoea (≥7 days), more common with *Cryptosporidium* compared with other enteric infections [44], or concomitant HIV-1 infection. Unfortunately, GEMS did not test participants for HIV. A publication from the Kenyan GEMS site found that cryptosporidiosis was a risk factor for persistent diarrhoea [45], concurring with findings from the MAL-ED multi-country community cohort study [46] and earlier studies [47-49]. *Cryptosporidium* infection was associated with more than a two-fold

increase in the risk of death in children aged 12–23 months who were admitted to hospital with diarrhoea [12]. The 2016 Global Burden of Disease (GBD) study estimated that *Cryptosporidium* infections were the fifth leading diarrhoeal aetiology in children under 5 years of age, leading to 48000 deaths in that year, caused by the acute infection alone.

1.3.2 Disability-adjusted life years lost from cryptosporidiosis

A commonly used metric for disease burden is to estimate the loss of disability-adjusted life-years (DALYs). These estimates vary, based on the underlying data and modelling assumptions in large-scale epidemiological studies. The 2010 Global Burden of Disease (GBD) study provided detailed estimates allowing comparison of individual diarrhoeal pathogens. Cryptosporidiosis was estimated to have caused 8.4 million DALYs lost and 99800 deaths in 2010 [50, 51], for all ages. The 2013 GBD study estimated that 6.8% of all under-5 diarrhoeal deaths in 2013 were caused by cryptosporidiosis alone [1]. Overall mortality may be falling globally; the GBD 2016 study, the latest to provide cryptosporidiosis-specific estimates of mortality, estimated 57000 excess deaths in all age groups, of which 48000 were deaths in children younger than 5 years [35].

An acknowledged limitation of all large-scale epidemiological studies, including the GBD studies, is the variable quality of the epidemiological data underlying the analysis, and lack of high-quality data from countries with the highest childhood mortality. Notably missing were reliable data on diarrhoeal aetiology from the high-burden regions of Africa south of the Sahara and in South Asia. A main objective of the GEMS study was to fill this evidence gap. A later GBD study updated their mortality estimates in light if the GEMS findings and arrived at 59000 excess deaths caused by *Cryptosporidium* infections annually, even after limiting the analysis to the under-24-month-old group and only in the region comprising Africa South of Sahara and South Asia [19]. This was a significantly higher figure than any previous GBD estimates.

1.3.3 The added burden of cryptosporidiosis-related malnutrition

The above studies were limited by not accounting for the indirect health burden resulting from malnutrition caused by cryptosporidiosis. *Cryptosporidium* and the other so-called "neglected enteric protozoa" *Giardia duodenalis* and *Entamoeba histolytica* have long been recognized as "stunting pathogens" alongside the soil-transmitted helminths. These infections have a complex bidirectional relationship with childhood malnutrition [52, 53], and several studies have demonstrated the negative effect of *Cryptosporidium* infection on physical growth [20, 26, 54, 55]. A 2018 study attempted to address this by estimating the additional loss of DALY from cryptosporidiosis-caused malnutrition in children younger than 5 years, by combining data from the GBD 2016 study with a meta-analysis of the effect of *Cryptosporidium* infection on physical growth [35]. They first estimated what they dubbed the "acute DALYs", i.e., those DALYs that were due to diarrhoeal deaths caused by *Cryptosporidium* and arrived at an estimate of 4.2 million DALYs lost. However, when they accounted for the additional disease burden caused by the effect of cryptosporidiosis on growth faltering the total increased 2.5-fold, to 12.9 million DALYs [35]. A worrying additional finding is that early *Cryptosporidium* infections seem to correlate with reduced cognitive function in later childhood and may plausibly have a negative impact on school performance and long-term development in low-and middle-income countries [52].

1.4 Cryptosporidiosis prevention

Following the emerging evidence that a small number of enteropathogens cause most diarrhoeal episodes, major global health research funders increased their support for pathogen-specific preventive interventions. A proof-of-concept of this approach has been the development and roll-out of rotavirus vaccination. Promisingly, efforts are ongoing to develop vaccines against *Shigella* and enterotoxigenic *E. coli*, the two major bacterial diarrhoeal pathogens identified by the GEMS study.

Roll-out of vaccines may indirectly impact the clinical case management of diarrhoea, as vaccines that target specific diarrhoeal infections can alter the "case mix" of

pathogens in children who present to healthcare with diarrhoea. For example, as rotavirus vaccination leads to reduced overall numbers of children with diarrhoea that present to hospitals [56], the *relative* burden of cryptosporidiosis can plausibly be assumed to increase. Unlike rotavirus, there is no vaccine for *Cryptosporidium,* and there are no vaccine candidates in the development pipeline. Even if it becomes possible to develop a vaccine, it will likely require many years of development [57, 58].

We therefore need to identify other effective preventive interventions against cryptosporidiosis. These interventions should be based on the best available evidence on risk factors. However, there are major evidence gaps in our knowledge of cryptosporidiosis risk factors (see separate section, below). As a faecal-orally transmitted disease sharing many epidemiological and clinical features with other diarrhoeal infections, we can reasonably assume that important risk factors will overlap with risk factors for other diarrhoeal infections, e.g., poor access to safe water of sufficient quantity, lack of basic hygiene, and lack of access to improved sanitation and waste management systems. The relative importance of socioeconomic, environmental, hygiene, nutritional and other risk factors, has unfortunately not been established. Reducing the transmission of *Cryptosporidium* oocysts is particularly challenging when compared with bacterial and viral enteropathogens as the oocysts are chlorine-resistant, can persist for months in the environment, and have a low infectious dose. Prevention is further complicated by the existence of animal reservoirs for some *Cryptosporidium* species, although data on zoonotic transmission risk are patchy in countries that carry the highest burden of disease [26, 39].

Worryingly, even comprehensive interventions targeting water-sanitation-and-hygiene (WASH) related transmission pathways, e.g., the WASH Benefits and SHINE trials in Bangladesh/Kenya and Zimbabwe, respectively, failed to lead to any substantial decrease in diarrhoea [59]. Based on these sobering results, many environmental and public health scientists now advocate for an approach coined “transformative WASH”, i.e., comprehensive interventions that radically reduce faecal contamination in household environments in poor countries [59]. While agreeing, others point out that

this will require a more holistic cross-sectoral approach, and a widening of the focus from the household level to include communities, cities, and regions. This approach has been framed as a return to the strategy of "environmental sanitation", a concept pioneered by the World Health Organization as early as in the 1950s [60]. The key message still holds; that faecal contamination is closely linked to the quality of the infrastructure, service provision and regulatory systems underpinning WASH and food safety, which are, again, linked to poverty alleviation and socioeconomic development at the society level.

In summary, "quick fix" preventive interventions are unlikely to succeed against diarrhoeal pathogens like *Cryptosporidium*. Meanwhile, children will continue to seek health care for diarrhoea and cryptosporidiosis, and a significant number of these children will continue to die unless they get adequate access to medical treatment. There should be no conflict between long-term transformative change and short-term improvements in case management and health care for children with diarrhoea. Healthcare workers in low-resource countries can continue to play a key role in advocating for both approaches. As healthcare presentation is a proxy for disease severity, interventions against specific diarrhoeal pathogens, like cryptosporidiosis, may have a direct impact on case fatality. In addition to the lives saved during the acute illness, targeted diarrhoeal treatment is expected to reduce downstream morbidity by preventing malnutrition. A large modelling study in 2006 suggested that treatment against *Cryptosporidium, Giardia duodenalis* and enteroaggregative *E. coli*, all recognized as "stunting pathogens", could save an additional 19 million DALYs from the downstream positive effect on growth [34].

1.5 Cryptosporidiosis treatment

1.5.1 Therapeutics

Reducing the health burden from cryptosporidiosis through treatment will require both reliable diagnostic testing and effective therapeutics. At present, there is only one approved drug, nitazoxanide, which reduced cryptosporidiosis case fatality significantly in a randomized controlled trial in Zambia [61]. Nitazoxanide is only

available for use against cryptosporidiosis in a few low-income countries, e.g., Bangladesh, India, and Zambia, and is not currently included in the WHO list of essential medicines. The sparse use of nitazoxanide globally may be partly related to the known limitations of the drug. First, although nitazoxanide has moderate effect on diarrhoea and parasite clearance in immunocompetent children, it is not clinically effective in children with HIV infection [26, 62]. Second, there are few data on the efficacy of the drug in HIV-negative children with acute malnutrition. The key RCT from Zambia is the only trial that enrolled HIV-seronegative children with acute malnutrition, but only included 11 severely wasted and 6 moderately wasted participants, all 1-3-years old. Despite this, the trial demonstrated a significant effect of nitazoxanide on both diarrhoeal duration and case fatality [61]. Third, nitazoxanide is only approved in children aged 12 months and older, a major limitation considering that the highest prevalence of cryptosporidiosis is in the age range 6 – 24 months [63]. The drug is unfortunately not currently available in Ethiopia.

The increased awareness of the global cryptosporidiosis disease burden after the GEMS and MAL-ED studies, in light of the limitations of nitazoxanide, have led to considerable recent effort to develop better anti-cryptosporidial drugs [64, 65]. Repurposing of old drugs has shown some promise, with the antimycobacterial drug clofazimine emerging as a lead candidate. Somewhat disappointingly, when trialled in *Cryptosporidium*-infected adult Malawian HIV patients (the “CRYPTOFAZ” trial) no significant impact was reported on *Cryptosporidium* shedding or diarrhoeal symptoms. However, care should be taken to not overinterpret these negative findings. First, the case definition was based on highly sensitive qPCR detection, without applying a quantitative cutoff in order to separate incidental *Cryptosporidium* infection from clinical cryptosporidiosis. Second, there was evidence of lower plasma exposure of clofazimine in the treatment group (children with diarrhoea) than in the control group (children without diarrhoea)[65]. Although it may have been premature to rule out clofazimine for treatment of paediatric cryptosporidiosis based on the initial CRYPTOFAZ findings [66], a later pharmacodynamic study reported that therapeutic

levels of clofazimine will likely not be attainable unless safer formulations with better oral absorption are developed [67].

Complementary to these drug repurposing efforts, other groups have made promising developments with new anti-cryptosporidial candidates, and several small-molecule compounds with a favourable safety profile have been effective *in vitro* and animal models, but have not yet been trialled in humans [65]. As new therapeutics move closer to human trials there have been calls to develop an implementation strategy for new drugs, and a highlighting of the critical importance of accessible and accurate cryptosporidiosis diagnostics as part of this strategy [65, 68].

1.5.2 The role of diagnostics in treatment

Stool testing in the management of paediatric diarrhoea

The mainstay of paediatric diarrhoea case management is to replace the fluid loss caused by the diarrhoeal infection, and to give supplementation with oral zinc [69]. Empiric antimicrobial therapy is discouraged, with the exception of children with bloody diarrhoea (i.e., presumptive shigellosis), and in acute watery diarrhoea in children older than 2 years if they live in an area with a suspected or ongoing cholera outbreak. Targeted therapy is also recommended for the protozoan parasites *Entamoeba histolytica* or *Giardia duodenalis*. However, routine stool testing is not recommended for these, or any other parasites, and the issue of testing is only briefly mentioned at all within the paediatric treatment guidelines. For example, in the WHO Integrated Management of Childhood Illness (IMCI) guidelines, the basis for many standard treatment guidelines in low-income countries, stool testing is only recommended in children with persistent diarrhoea, HIV-associated diarrhoea, or dysentery; by wet microscopy, and *Shigella* bacterial culture, respectively [69]. Diagnostic stool testing for *Cryptosporidium,* which requires specific staining techniques, is only recommended for children with both HIV infection and persistent diarrhoea. *Cryptosporidium* infection is not even mentioned in the latest IMCI guidelines (2013) as a cause of acute diarrhoea, nor for persistent diarrhoea, except in HIV-infected children [69].

In Africa, no international or national guidelines provide specific guidance for cryptosporidiosis diagnosis or treatment [70]. Ethiopia was one of only 13 countries in sub-Saharan Africa that had introduced integrated community case management (iCCM) programmes for pneumonia, diarrhoea, and malaria, by 2013. An important aim of the iCCM programme was to massively expand access to life-saving diarrhoea treatment [71]. Notably missing from the iCCM algorithms, however, are any recommendations for when and how to use stool diagnostic testing for diarrhoeal infections.

Necessary features of diagnostic tests for cryptosporidiosis interventions

Diagnostic tests are necessary to enable more widespread use of nitazoxanide [72], and for trials and roll-out of new drugs that are in development. A positive test result can also lead to non-pharmaceutical interventions. For example, specific hygiene advice, targeted at the child's caregiver, could plausibly help to prevent further person-to-person spread, or recurrence of diarrhoea [73]. To enable targeted treatment, there is a need for a rapid, low-tech, reliable, and affordable diagnostic test that can be used near the point-of-care, and that is overall suitable for low-resource healthcare settings [74].

Candidate diagnostic tests need to be evaluated formally, in what is usually referred to as a diagnostic evaluation or a diagnostic accuracy study, where key test parameters are estimated. These parameters include, but are not limited to, sensitivity (the ability to pick up true positives), specificity (the ability to not pick up false positives), and, if the local prevalence of disease is known, negative and positive predictive values of testing (i.e., the ability to "rule out" or "rule in" a disease, respectively). Establishing diagnostic accuracy parameters is essential, but successful roll-out of testing also rely on data from the field on operational aspects, e.g., ease of use, turn-around-time, feasibility of staff training and supervision, and cost-per-test. To capture the importance of both diagnostic accuracy and operational performance, some authors advocate for the term "test efficacy" rather than accuracy, defined as the ability of a diagnostic test to support a clinical decision within its operational context [75]. Understood more widely, test efficacy is the ability to produce an effect or response by "administration" of a diagnostic test [75-77].

Most new diagnostics are developed by commercial companies whose main market is healthcare providers in high-income countries. It follows that most diagnostic accuracy studies are also conducted in high-income countries [78]. This is likely to skew conclusions drawn from the available evidence, as diagnostic test evaluations that are not conducted in the relevant clinical setting have been shown to overestimate diagnostic accuracy and field applicability [79]. Even in countries with considerable resources, it remains challenging to diagnose cryptosporidiosis by traditional microscopic methods. Few clinical laboratories in high-income countries receive enough samples to build up a high level of expertise in diagnostic parasitology. The oocysts are small (4-6μm) and difficult to pick out from the background without first applying particular stains. The introduction of specific microscopic detection by the application of immunofluorescent stains, and, over the last few decades, high-throughput molecular detection platforms (usually PCR) have improved diagnostic capacity in many high-income countries. However, as most of these assays are expensive or require highly specialised personnel, they can be inappropriate for low-income settings, i.e., high-prevalence settings that can benefit most from cryptosporidiosis testing.

1.6 Cryptosporidiosis diagnostics

1.6.1 Overview of tests

Detecting *Cryptosporidium* infection relies on the detection of *Cryptosporidium* oocysts in stool samples (or, in the case of a suspected respiratory infection, in sputum). Tests can visualize the oocyst (by microscopy, after applying stains of varying specificity), detect *Cryptosporidium* antigens (by plate-based assays, usually enzyme-linked immunosorbent assay, ELISA; or by paper-based immunochromatographic lateral-flow assays, ICLF), or detect specific *Cryptosporidium* nucleic acid sequences (by PCR or other nucleic acid amplification tests). The distinction between detecting the presence of oocysts, antigen or DNA in stool and diagnosing the disease of cryptosporidiosis, i.e., diarrhoea presumed to be caused by *Cryptosporidium* infection, will be further discussed in a later section.

Microscopic detection

Overview

Microscopic methods rely on the detection of *Cryptosporidium* oocysts in stool, usually in diarrhoeic stool samples. The sensitivity of all microscopic detection methods can be improved by parasite concentration techniques like formalin-ethyl-acetate concentration, or oocyst flotation using a concentrated sugar or salt solution. As these techniques involve additional hands-on steps they are not performed routinely in most resource-poor clinical laboratories. For example, in Ethiopia, where clinical laboratories are often poorly staffed, concentration methods are usually only performed as part of larger-scale surveillance surveys or research supported by universities.

Cryptosporidium oocysts are visible by conventional light microscopy at the commonly used 100x - 400x magnification. There is some variability in size and morphology between *Cryptosporidium* species, but not sufficient to reliably discern species by microscopy [80]. As the oocysts are highly light-refractory, internal structures cannot be discerned, and it is difficult to differentiate *Cryptosporidium* oocysts from other small semi-spherical objects that are commonly seen in faeces, e.g., yeast cells, white blood cells, and pollen. For this reason, even highly specialized parasitology laboratories opt for special oocyst staining methods instead of conventional wet microscopy. A variety of staining methods were tested in the 1980s and 1990s [81], including different types of tinctorial stains and negative staining of background stool material, but various acid-fast staining techniques became the predominantly used method.

Tinctorial acid-fast stains

Due to their acid-fast properties, oocysts can be semi-selectively visualized with various acid-fast stains. The same stains can visualize the coccidian parasites *Cyclospora cayetanensis* and *Cystoisospora belli.* These parasites are not commonly detected in children but can cause diarrhoea, and *C. belli* is associated with dysentery in immunocompetent children and with prolonged diarrhoea in HIV patients. The most commonly used acid-fast stains for *Cryptosporidium* are tinctorial stains, e.g., Kinyoun

staining or the more commonly used modified Ziehl-Neelsen staining method (mZN) [82].

Advantages of these methods are that the staining reagents are available at low-to-moderate cost, and that conventional light microscopes can be used. However, mZN has a sensitivity less than 70% and has inadequate sensitivity and specificity when compared with IFAT [26, 82, 83]. Incorrect recognition of oocysts with conventional acid-fast stains has been reported [84]. Another drawback is that these methods require oil-immersion microscopy at 1000x times magnification; screening large areas of a glass slide at this magnification is hard on the eyes and time-consuming. Few studies have formally evaluated microscopic detection methods in low-or-middle-income settings; we identified a Turkish study that evaluated mZN [85], reporting poor accuracy, concurring with previous reports from high-income settings [26, 83].

Auramine based fluorescent stains (AP)

Auramine-phenol (AP) is an acid-fast staining method that uses a fluorescent stain instead of a tinctorial stain. Although AP is commonly referred to as an acid-fast stain, internal structures within the oocyst can also give off fluorescence. This is likely due to some binding of AP to the nucleic acids within the four sporozoites that are wrapped up inside the oocyst [86, 87]. The reagents are inexpensive and is readily available in some facilities as the AP-method is endorsed by the WHO as the preferred primary diagnostic method for tuberculosis mycobacteria. A fluorescent oocyst stands out from the background more easily compared with the pinkish-red oocysts after tinctorial staining. This allows the microscopist to scan the whole glass slide at lower magnification than conventional microscopy, usually at 200x-400x magnification, and is therefore a less tedious and faster method.

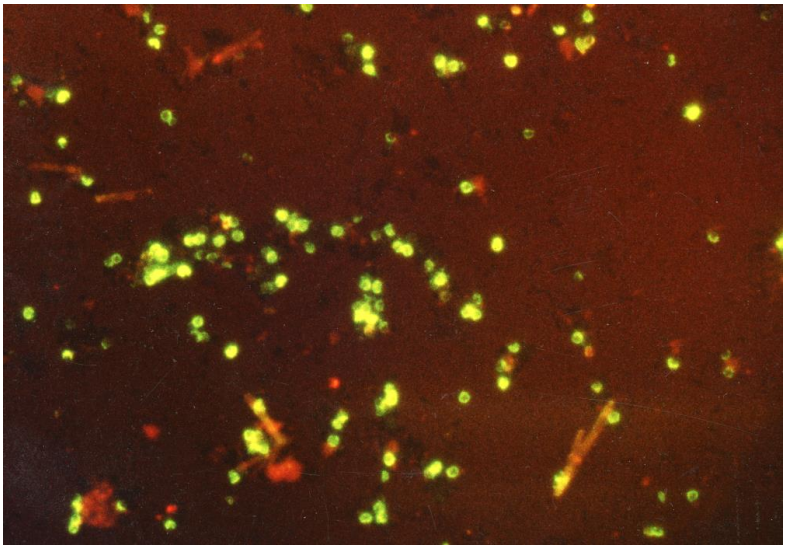


Figure 1 Auramine-phenol-stained Cryptosporidium oocysts (diameter 4-6 µm), displaying characteristic "erythrocyte pattern" of staining (section of 400x magnification field; Lucy J Robertson, personal photo)

The main drawback of AP staining has been the need for fluorescence microscope, and, until light-emitting-diode microscopes were developed, the price and cost of maintenance prohibited widespread adoption by low-resource laboratories. AP-microscopy with traditional halogen bulb fluorescence microscopes has been a well-established *Cryptosporidium* test since the 1980s [83, 86], particularly in the United Kingdom. The method has better sensitivity (92%) than mZN and comparable specificity to immunofluorescent antibody testing [83].

Immunofluorescent antibody testing (IFAT)

These methods rely on fluorescein isothiocyanate-conjugated anti-*Cryptosporidium* IgM or IgG antibodies [82]. The method is usually referred to as immunofluorescent antibody testing (IFAT) or direct antibody fluorescence microscopy (DFA) and is used for staining thin stool smears, faecal concentrates, or occasionally other samples like sputum or bile, followed by examination of the slide under a fluorescence microscope equipped with appropriate filters. IFAT is a widely adopted reference method for *Cryptosporidium* oocyst detection and enumeration. The method is mainly used in

specialised parasitology and public health laboratories, for testing of faecal material, or, following pre-staining concentration methods like salt-flotation or immunomagnetic separation, for *Cryptosporidium* testing of environmental samples and foodstuffs. Similar to AP microscopy, IFAT allows screening of glass slides at lower magnification and is quicker and less tedious than light microscopy.

IFAT is considered to have superior sensitivity and specificity compared to other available microscopic tests [82, 88]. This is a qualified statement, as we lack a clearly superior "gold standard test" that IFAT can be compared with. The superior analytical accuracy of IFAT is largely supported by early studies that compared IFAT to a broad panel of other microscopy tests, where suspensions or faeces spiked with known concentrations of oocysts were examined [82, 88]. The limit of detection obtained from these early studies was reported to be at least as low as 1000 oocysts per gram [89, 90], without preceding concentration methods [89]. Although some well-resourced laboratories use IFAT as a clinical diagnostic test, the reagents require refrigerated storage and are too expensive for routine use in low-resource clinical laboratories.

Antigen detection

Antigen detection is a different test modality than oocyst detection (microscopy) or nucleic acid detection (see below). Specific antigens are bound to the surface of oocysts or other *Cryptosporidium* parasite life stages but can also be detected as free antigen.

Multi-well plate ELISA

Cryptosporidium antigen detection with multi-well plate-based enzyme immunoassays (ELISAs) has shown good sensitivity and specificity as compared with IFAT and PCR [83]. Plate-based ELISA requires specialized equipment and expertise that is not commonly available in clinical laboratories in low-resource settings, and samples are usually processed in batch. Plate washers and plate readers are required for optimal performance and but are usually not available in low-resource laboratories. However, compared with conventional PCR platforms, plate-based ELISAs are easier to implement and may require shorter training time than IFAT, depending on the microscopy skills of the lab technicians. ELISA can therefore be a suitable option for

research studies [12] or epidemiological surveillance. Species determination and subtyping will however require supplementation with PCR-based methods.

ELISA has not been evaluated as a near-patient test in low-resource setting as part of a diagnostic accuracy study. The GEMS "qPCR reanalysis" study provided valuable data on the difference in case and control *Cryptosporidium* detection rates between ELISA and quantitative PCR [91]. However, GEMS was neither conducted nor reported [79] as a diagnostic accuracy study for cryptosporidiosis, e.g., there were no cross-tabulation of index test to reference test results, and precision of key accuracy parameters were not provided [91]. Instead, the publication focused on the change in pathogen-specific attributable incidence from using qPCR instead of the original workup. Notably, estimates for *Cryptosporidium* incidence remained similar when comparing the qPCR-based estimates to the original estimates based on ELISA.

Immunochromatographic lateral flow tests (ICLF)

Various lateral flow or immunochromatographic lateral flow tests (ICLFs) have been developed for *Cryptosporidium* antigen detection, either in paper test strips or in a plastic cartridge format. These kits have variable sensitivity, particularly when oocyst excretion is low [92, 93], but, notably, a few kits were reported to have similar accuracy to LED-AP [94]. Limitations of ICLFs are are high cost [75], short shelf-life and that most ICLFs need to be stored in a fridge. There is some evidence that ICLF tests have poor sensitivity do detect other species than *C. parvum* or *C. hominis*, and that sensitivity can vary between these two species [95].

Most diagnostic accuracy studies on ICLFs were conducted in artificial laboratory settings, or in high-income-countries [96]; diagnostic accuracy in real-life settings has been variable [97]. A single publication reported on the diagnostic accuracy of ICLFs in sub-Saharan Africa, but that study included few children and did not use a sufficiently sensitive reference method [98]. We identified only one study that evaluated ICLFs in relevant high-prevalence settings; a study conducted in children with complicated severe acute malnutrition in East Africa, where the included ICLFs were found to have only moderate sensitivity when compared with PCR [99].

Nucleic acid amplification tests (NAATs)

Tests that detect the presence of specific nucleic acid sequences are collectively referred to as nucleic acid amplification tests (NAATs). These tests usually detect DNA, but mRNA and rRNA detection assays are also possible [100].

PCR

The most commonly used NAAT is the polymerase chain reaction (PCR). PCR has comparable or better sensitivity than IFAT [89] and is the only current method that allows for *Cryptosporidium* species determination and subtyping. Real-time PCR, as opposed to traditional end-point-PCR, can be applied for quantification, and is then referred to as quantitative PCR (qPCR). Although qPCR is now a well-established technology, the technique is considered complementary rather than having superseded IFAT microscopy as a "gold standard test" for quantitative *Cryptosporidium* detection; PCR methods have not been standardized, and there are concerns that qPCR can underestimate oocyst counts compared to quantitative IFAT microscopy due to PCR inhibitors in faeces [101]. As qPCR is usually based on shorter amplicons than traditional PCR, there is a higher risk of amplifying things that are not *Cryptosporidium*. It is not clear whether IFAT or PCR most reliably detect viable (i.e., potentially infectious) parasites. In high-income countries, an important advantage of real-time PCR has been the ability to multiplex *Cryptosporidium* detection with PCRs for other parasitic, bacterial, viral and fungal targets, simplifying the laboratory workflow for detection of a broad panel of enteric pathogens [102].

Other NAATs

Constant temperature, or isothermal, nucleic acid amplification tests have been proposed as a technology that may facilitate more widespread implementation of NAAT testing in low-resource settings. The most well-known isothermal amplification technique is loop-mediated isothermal amplification (LAMP). The method has been successfully implemented for near-patient testing for human African trypanosomiasis [103], malaria, and leishmaniasis [104]. For *Cryptosporidium* testing in human faecal samples there are a few proof-of-principle studies [105, 106], but no clinical diagnostic accuracy studies. The isothermal amplification methods NASBA (nucleic acid

sequence-based amplification) and RPA (recombinase polymerase amplification) have also been developed for *Cryptosporidium* mRNA and DNA detection, respectively [107-110], but have not been evaluated beyond the initial technical development.

Issues with NAATs in low-resource settings

In many well-resourced clinical microbiology laboratories, PCR has become the first-line method for *Cryptosporidium* detection. Other NAATs can become alternatives to PCR if developed further. In most low-resource settings, PCR has not been implemented in routine clinical care due to cost and lack of access to reagents and qualified staff. In Ethiopia, there is currently no capacity for routine diagnostic PCR, even in the largest university hospital labs. The exception has been the increasing roll-out of "all-in-one" PCR systems (i.e., where extraction and detection can be performed in a small apparatus without the need for separate rooms). The most important example is the GeneXpert tuberculosis test, that has been delivered by vertical programs supported by foreign donors [111].

A caveat is that the high sensitivity of NAATs is not necessarily desirable in high-prevalence settings where low-level and often asymptomatic infections are common [91]. Overly sensitive assays may have reduced specificity for the diagnosis of cryptosporidiosis, i.e., *Cryptosporidium* infection that is causing diarrhoea currently (more on this in the section on reference tests, further down).

1.6.2 Frameworks for evaluating diagnostics in low-resource settings

The purpose of this section is to cast light on aspects of the conceptualization stage of the CRYPTO-POC study that may not be readily apparent from the published articles. The core motivation behind the project was the identified need for near-patient cryptosporidiosis tests in low-resource healthcare facilities. The next step was to identify what tests were already available, and to critically review the scientific evidence supporting their use in children with diarrhoea in low-resource settings, within existing healthcare facilities. Two conceptual tools were useful at the idea stage and during early planning. The first is to think of diagnostic research as a multi-step incremental process, similar to the way pharmaceutical intervention trials are divided into distinct "phases". The "phased approach" helpful to classify the existing diagnostic

accuracy literature. The second conceptual tool was to apply prespecified formal benchmarking criteria for prioritizing between, and to select, tests, to study further.

A phased approach to diagnostic accuracy evaluations

The first question to ask about any diagnostic test is whether it can accurately detect the condition or disease of interest. The key diagnostic accuracy parameters are sensitivity, specificity, and negative and positive predictive value. The second question is broader, but also important: whether the test has operational characteristics that are suited to the overall "diagnostic situation". This covers diverse factors that all play an important role for the usefulness or sustainability of a test; technical simplicity, need for specialized equipment, training of staff, quality control issues, laboratory support systems, sample transport, test turnaround times, cost per test, etc [112].

Before any test can be endorsed and rolled out, we should obtain up-to-date evidence of accuracy and operational performance from studies conducted in representative clinical settings, where the tests are evaluated under realistic field conditions. Simple analytical or technical performance of a test is not sufficient, but it is a necessary first step before conducting larger scale clinical evaluations.

The concept of "study phases" is well established in randomized controlled trials of therapeutic or preventive interventions (RCTs), but it is less well known that a similar framework has been proposed for diagnostic accuracy studies. In brief, diagnostic accuracy studies can be classified as follows: phase I studies are small exploratory studies, phase II studies are "challenge studies" in samples from a clinical setting, usually with a retrospective study design and often employing a so-called "multiple-gate" sampling strategy. Phase III studies are large prospective studies in a sample of patients that closely represents the target population for testing, and where the study is carefully designed to minimize important biases related to external validity, a key limitation of phase II studies [113, 114]. Table 1 summarizes the main differences between phase I, II, and III diagnostic accuracy studies, and also includes a proposed phase IV that covers "diagnostic RCTs" [115], i.e., studies that evaluate testing as an

intervention, usually combined with treatment (table adapting information from [113] and [116]).

Table 1– A phased approach to diagnostic test evaluations

Study phase	Typical design	Typical sampling plan	Diagnostic measure	Main role in test development
I	Retrospective	Artificially positive or "typical positive" cases	Crude analytic sensitivity and specificity	Proof of principle
II	Retrospective	Carefully selected spectrum of cases and controls, enrolled on basis of their status	Sensitivity and specificity - idealized	Range of sensitivities and specificities in key subgroups
III	Prospective Sample size must be large	Representative sample from the target population for testing	Sensitivity and specificity - less idealized Predictive values	Clinical validity (Can optionally also include evaluation of operational issues and field applicability)
IV	Diagnostic RCT (D-RCT)	Randomization to receive a test	Clinical outcome	Clinical usefulness of testing; operational evaluation of test-and-treatment interventions

The ASSURED criteria

In the early 2000s, a wide gap was identified between the types of tests that were needed, and the tests that were actually available, to low-resource healthcare systems. This discrepancy motivated the development of the ASSURED criteria. The acronym covers important criteria for benchmarking of diagnostic tests for use in low-resource settings: Affordable, Sensitive, Specific, User-friendly (i.e., simple to perform, uses non-invasive specimens), Rapid and Robust, Equipment-free, and Delivered (i.e., accessible to end-users). The ASSURED framework was originally proposed by the WHO for sexually transmitted diseases in 2004 [117, 118], but can be usefully applied to diagnostics for other infectious diseases that also affect poor populations globally [119].

A critical appraisal of candidate cryptosporidiosis tests

The ASSURED criteria can be thought of as a set of necessary characteristics for an "ideal" diagnostic test in a resource-constrained setting. They were helpful at the CRYPTO-POC idea stage to bring structure to our discussions of currently available

cryptosporidiosis tests. We performed a simple elimination exercise to determine which tests to not yet consider for near-patient use in Ethiopia (or similar countries) From the remainder, we then prioritised what tests to evaluate in a large diagnostic accuracy study. A full systematic review or meta-analysis of all available diagnostic tests for cryptosporidiosis was outside of the scope of the project. Informed by our general knowledge about the characteristics of various types of tests used within diagnostic clinical microbiology, and existing work that reviewed diagnostics for cryptosporidiosis [120], we were able to give a an approximate "grading" of the most important tests in current use, for each ASSURED criterion. From this high-level view, it became clear that PCR, LAMP, IFAT, and MZN all failed at least two of the ASSURED criteria (Table 2).

Table 2 – A simplified comparison of diagnostic tests for cryptosporidiosis in children, using the ASSURED criteria

	PCR	LAMP	IFAT	MZN	ELISA	ICLF	AP
Affordable							
Sensitive				Phase III study [85]		Two phase II studies [121, 122]; phase III study in SAM [99]	
Specific				Phase III study [85]		Two phase II studies [121, 122]; phase III study in SAM [99]	
User-friendly							
Robust and rapid							
Equipment-free							
Deliverable							

PCR = polymerase chain reaction; LAMP = loop mediated isothermal amplification; IFAT = immunuofluorescent antibody test; MZN = Modified Ziehl-Neelsen microscopy; ICLF = immuochromatographic lateral flow antigen tests; AP = auramine-phenol microscopy

The colours represent the degree to which each test fulfils a specific ASSURED criterion, in low-resource clinical settings, from green (criterion usually fulfilled) to red (criterion usually not fulfilled). Diagnostic accuracy studies evaluating sensitivity and specificity (i.e., phase II and III studies) in low-resource settings have been indicated in the table.

Based on this initial exercise, ICLFs and AP were the best candidates for closer scrutiny. We then conducted a semi-systematic review for studies on diagnostic accuracy for *Cryptosporidium* infection. This was done first at study conceptualization, and again while preparing paper I (the CRYPTO-POC diagnostic accuracy publication), for which the PubMed database search was last done on Jan 20, 2020; (see paper I "Research in context" section, for details). The literature search identified only two prospective studies from a consecutive series of children in LMIC. The first was a phase III study from Turkey, a middle-income country, that reported 40% sensitivity of the traditional mZN microscopy method against ELISA [85], and concluded that mZN is insufficiently accurate, a finding that concurred with previous reports [26, 83]. The second was a phase III study from Malawi and Kenya that evaluated immunochromatographic lateral flow assays (ICLFs) in hospitalised children with complicated severe acute malnutrition (SAM). They reported moderate sensitivity of all three ICLFs, against detection by PCR [99], however, their estimates may not apply to the general paediatric population. Two non-clinical phase II studies analysed birth cohort samples from children in Bangladesh and reported good accuracy of ICLFs against diarrhoea attributed to *Cryptosporidium* by qPCR [121, 122]; again, it is not known if their findings can be extrapolated to clinical settings.

The most carefully conducted diagnostic accuracy study of AP was a retrospective study conducted in the United Kingdom, where AP was found to have a sensitivity of 92% and specificity of 100% against PCR and IFAT [83]. This was a multicentre study, and it is not clear how many of the included labs performed fluorescence microscopy using traditional halogen light bulbs versus newer LED-fluorescence microscopes.

Importantly, we identified no clinical diagnostic accuracy studies on the use of AP for cryptosporidiosis in children in LMIC, using traditional fluorescence microscopes or LED-fluorescence, nor did we find any field studies that included data on operational issues within LMIC settings, such as comprehensive cost calculations or test turnaround times.

1.6.3 LED-AP fluorescence microscopy as a dual-use technology

In 2011, WHO recommended a shift in first-line tuberculosis testing, from the traditional Ziehl-Neelsen acid-fast staining method to LED-AP [123]. Large-scale roll-out of LED-microscopy is ongoing in Africa. Available at reduced price (1250 EUR per microscope, at the time) as part of the STOP-TB partnership over 1200 LED microscopes had been distributed to Ethiopia and other low-or-middle-income countries by 2013 [124]. The most widely distributed microscope is the PrimoStar iLED (ZEISS) (see photo in paper I supplementary appendix).

The increased use of LED-AP as a first-line test for tuberculosis raises the possibility for introducing near-patient *Cryptosporidium* testing into an established clinical laboratory infrastructure. AP-microscopy with traditional halogen fluorescence microscopes is a well-established and test for *Cryptosporidium* oocysts with better accuracy than mZN [83, 86]. Traditional fluorescence microscopes have mercury in their bulbs that is considered a health hazard, bringing forward their gradual replacement with LED fluorescence microscopes globally. There are additional advantages of LED-microscopes of particular relevance to low-resource settings [123]: they are cheaper, easier to operate, the LEDs last for >30000 hrs, unlike halogen bulbs, and can be run on a small battery or even solar power [125]. The auramine-phenol reagents can be stored at room temperature.

A diagnostic accuracy study conducted in a city hospital in India compared *Cryptosporidium* detection by mZN, AP, ELISA antigen detection, and PCR, in a study that enrolled 671 HIV-seropositive adults, 50 HIV-seronegative adults and 198 HIV-seronegative children [126]. They reported a sensitivity of AP of 100% and specificity 99.6%. Unfortunately, this estimate cannot be accepted as valid or even useful, as there were major flaws with the analysis and reporting. The analysis failed to distinguish between cases with and without diarrhoea, and of the nine *Cryptosporidium*-positive HIV-seronegative cases, age or presence/absence of diarrhoea was not reported. Furthermore, they failed to distinguish between index and reference testing, by defining “minimum two positive tests” of all four tests as a positive reference standard, i.e., possibly inflating sensitivity by comparing index tests with themselves.

In summary, we found no studies evaluating LED-AP for *Cryptosporidium* in Africa, and no studies comparing LED-AP with a reference standard that included IFAT or PCR in a community setting, in any LMIC. The diagnostic accuracy of LED-AP for cryptosporidiosis in children in low-income countries was the crucial evidence gap that we hoped to address with the CRYPTO-POC study. The study was designed as a phase III diagnostic accuracy study, with an additional objective of collecting operational data that may be useful for roll-out of testing.

1.6.4 A promising new rapid test strip

However, not all healthcare facilities will have access to a LED-fluorescence microscope. As an example, in Ethiopia, the smallest-level health facilities (i.e., below the village health centre level) are referred to as community health posts. These are often situated in remote rural villages with erratic or lacking power supply. Few health posts have microscopes or laboratory trained staff. Despite their possibly higher cost, ICLFs may be the only feasible option in these settings. In addition to cost, a drawback of most rapid antigen tests is a requirement for fridge storage. This precludes their use in many small health facilities. Kerosene refrigerators are sometimes available but are vulnerable to breakdowns. We were therefore encouraged to discover that a new ICLF test strip had recently been developed that can be stored in ambient temperature - the "*Cryptosporidium EZ VUE*" test (TechLab, Inc) [121]. The *EZ VUE* test strip had promising accuracy when compared with ELISA in a small phase II diagnostic accuracy study, with sensitivity 100% and specificity 89% [121]. Considering the lack of phase III evidence on the use of both LED-AP and ICLFs for cryptosporidiosis in LMIC, it is not known whether none, one or both of these tests would have adequate diagnostic performance for clinical use. If the ICLF test strip performs much better than LED-AP, even in facilities where LED-AP can be performed, it may be the preferred option for both higher- and lower-level health facilities. We decided to evaluate both tests within the same study, despite the added complexity and cost, to avoid putting all our eggs in one basket.

1.6.5 Striking the balance between sensitivity and specificity: the importance of quantification

Asymptomatic or incidental infection is common with many infectious enteric microbes, particularly in low-income countries with high infection pressure, and particularly in children [12, 46]. Although the association between *Cryptosporidium* detection and diarrhoea is strong, compared with other enteropathogens, asymptomatic infections are well described. This poses a methodological challenge for diagnostic accuracy evaluations and can a challenge when interpreting the test result for an individual patient. In a clinical setting, the usual purpose of testing is to select patients for whom intervention may be of clinical value. If another microbe is the main cause of the presenting diarrhoea, anti-cryptosporidial treatment will have little or no measurable effect on key clinical outcomes like diarrhoeal duration, dehydration, or case fatality.

To address the challenge of incidental detections, quantitative methods have been employed to allow more accurate case ascertainment. This approach is supported by findings from large-scale studies that compared quantities of enteropathogens between children with and without diarrhoea, e.g., the GEMS and MAL-ED studies [12, 46]. Non-diarrhoea controls had stool with lower-quantity detections than diarrhoea cases. If a reliable quantitative assay is used, thresholds (or "cutoffs") can be established that distinguish between incidental and symptomatic infection [91, 127, 128]. The main use of quantification is as a research tool, as accurate quantification relies on careful weighing of samples and calibration of instruments. This is currently not feasible for near-patient assays, a limitation that also applies to isothermal NAATs (e.g., LAMP) and "closed-box" PCR platforms, e.g., GeneXpert or the BioFire FilmArray. Microscopy or antigen detection has a higher limit of detection than detection of *Cryptosporidium* DNA by PCR (with the possible exception of IFAT after oocyst concentration steps). The resulting lower sensitivity can, somewhat paradoxically, be considered an advantage, as the large fraction of the "missed" infections are likely to be asymptomatic or incidental. Expressed differently, the loss of sensitivity for *any infection* may be offset by the increased specificity for *infection causing disease*.

As a side point, it follows that using an overly sensitive test as part of the case definition in a randomized controlled trial for a new cryptosporidiosis therapeutic will lead to misclassification bias. For example, if a positive *Cryptosporidium* PCR is used as the inclusion criterion, without applying a quantitative cutoff, the study may fail to detect a clinical effect and erroneously conclude that the drug lacks efficacy, when the real reason could simply be that many of the included children had diarrhoea caused by other pathogens than *Cryptosporidium* or had a non-infectious cause of diarrhoea, coupled with incidental *Cryptosporidium* detection.

1.7 The molecular epidemiology of cryptosporidiosis in Africa

1.7.1 The predominance of anthroponotic transmission in Africa

The role of animals as reservoirs of human cryptosporidiosis is not well known in Africa. *Cryptosporidium* typing data are lacking from many low-income countries, in particular from sub-Saharan Africa [129, 130]. The following summary is largely based on a literature review in June 2020 as part of a review article on this topic [39].

Although genotyping studies from human infections are few, most report a predominance of *C. hominis*, a species considered to be human-specific, although there are a few reports of detections in non-human primates. *C. parvum* is the major zoonotic species of *Cryptosporidium*. It is common in domestic animals in developed countries, where it causes serious diarrhoea in very young farm animals, particularly in even-toed ungulates (e.g., cattle, sheep, and goats). Complicating this picture is the observation that most *C. parvum* detections in humans in developing countries belong to the so-called “anthroponotic” subtypes IIc, IIe and IIm, rather than the zoonotic *C. parvum* subtypes IIa and IId, that are more common in developed countries [131, 132]. Subtype IIc has recently been proposed as a distinct subspecies, *C. parvum anthroponosum*, to separate it from the zoonotic subspecies *C. parvum parvum* (IIa and IId) [39]. There is only circumstantial evidence of transmission with the zoonotic species *C. meleagridis, C. muris, C. canis, C. suis, C. ubiquitum,* and *C. xiaoi* infections in people and associated animals in sub-Saharan Africa [39].

1.7.2 Is Ethiopia an outlier?

The only country in sub-Saharan Africa with strong evidence for zoonotic *C. parvum* transmission is Ethiopia [39]. It is not clear whether Ethiopia represents an outlier where zoonotic transmission of *Cryptosporidium* is important; the available data are limited. One study reported zoonotic *Cryptosporidium parvum* gp60 allele family IIa from two children with diarrhoea (a 4-year-old and a 3-year-old from rural Awash and Addis Ababa, respectively) [133], also, a later study found *C. parvum* IIa in patients from Wurgissa and Hawassa districts. It is however not clear how many of these infections were from young children (the study included only four children, in the range 0-9 years) [134]. Of note, *C. parvum* subtype IIa was found to be the most common genotype in stool from HIV/ADIS patients in the largest referral hospital in Addis Ababa [135]. A prospective study in 58 children (age 1-33 months) with diarrhoea attending health centres in Mekelle city found *Cryptosporidium* in three children; all were *C. hominis* [136]. One possibility is that zoonotic cryptosporidiosis in Ethiopia is restricted to adults, immunocompromised patients and/or certain geographical regions. It is too early to conclude whether Ethiopia follows the same pattern of *C. hominis* predominance as has been found in other countries in sub-Saharan Africa [39].

1.8 Risk factors for cryptosporidiosis versus other diarrhoeas – same but different?

There is an extensive body of evidence on risk factors for paediatric diarrhoea in general, both from observational studies, large-scale epidemiological studies [137], and interventional studies, e.g., as summarized in the Lancet 2012 Series on childhood pneumonia and diarrhoea [138]. For the subset of paediatric diarrhoeas caused by cryptosporidiosis, knowledge on risk factors is patchy; most studies are case-case-comparison studies, supplemented by a small number of community cohort studies.

We conducted a semi-systematic literature review of risk factors for *Cryptosporidium* infection and cryptosporidiosis in low-and-middle-income countries (LMIC) using the following MeSH search terms within PubMed (last search on 9 June 2021): (*Cryptosporidium* OR cryptosporidiosis) AND (risk factor OR case control OR cohort

OR infection OR sporadic OR prevalence), with terms limiting the search to children and without restrictions on language or publication date. This yielded a list of 263 abstracts that were reviewed for all risk factor reports concerned with children under 5 years of age in LMIC. Reference lists of all identified articles were also reviewed.

We found several puzzling contradictions between reported risk factors for cryptosporidiosis [39, 139-142] and the available knowledge on general risk factors for paediatric diarrhoea [4, 138, 143, 144]. Some examples are summarized in Table 3; a more in-depth review can be found in paper II (supplementary appendix).

Table 3- Contradictory findings on risk factors for cryptosporidiosis versus diarrhoea in general

Example risk factor	**Evidence for being a risk factor for diarrhoea in general**	**Evidence for being a risk factor for *Cryptosporium* infection and/or cryptosporidiosis**
Maternal education, household assets, income	Well established [137, 138]	Rarely reported or non-significant [139, 140]
Poor water quality	Well established [137, 138]	Not significant [139-141]
Overcrowding	Well established [138]	Contradictory findings [139-141]
Animal exposure	Depends on the pathogen (e.g., *Campylobacter,* non-typhoid *Salmonella*) [145]	Contradictory findings [39, 139-141]
Poor sanitation	Well established [137, 138]	Possibly a risk factor [139, 140]
Not breastfed exclusively first 6 months	Well established [137, 138]	Not significant [139, 140, 146]
Less severe wasting or underweight	Moderately evidence [137]	Contradictory findings [20, 33, 54, 147-156]
Acute malnutrition (MAM/SAM)	Well established [137, 143, 144, 157-159]	Not investigated as part of a comprehensive risk factor analysis [20, 33, 54, 142, 147-156, 160]

Based on the heterogeneity between the identified risk factor studies, and that few studies compared cryptosporidiosis cases with non-diarrhoea controls, we suspected that the apparent differences could be related to different methodological approaches rather than representing real differences in the underlying epidemiology. In the most comprehensive and recent literature review on cryptosporidiosis risk factors, published in 2018 [139], most of the included studies used diarrhoea cases without *Cryptosporidium* infection as comparators, i.e., they were “case-case studies” [147-154]. We identified few case-control studies on cryptosporidiosis risk factors overall, and of these, no case-control studies that distinguished between *Cryptosporidium* infection and cryptosporidiosis, i.e., diarrhoea attributed to *Cryptosporidium* infection

using quantitative methods [91, 128]. Study design and analyses rarely accounted adequately for confounding bias, and we did not identify any case-control studies that investigated hand hygiene, perinatal factors, and acute malnutrition in the same analysis [141, 142]. Furthermore, the frequent reports of animal exposure as a risk factor are puzzling in light of the currently available evidence against any significant contribution from zoonotic cryptosporidiosis in sub-Saharan Africa [39].

None of the identified cohort studies investigated moderate or severe acute malnutrition, as defined by weight-for-height z-score (WHZ) or mid-upper-arm circumference (MUAC) cutoffs (or the presence of peripheral oedema), as a risk factor for subsequent cryptosporidiosis incidence or severity. A recent GEMS post-hoc analysis on the interaction between acute malnutrition and healthcare-presenting diarrhoea assessed the influence of acute malnutrition on diarrhoeal case-fatality risk at 60-day follow up [142], finding an additional increased absolute risk of death [142]. However, their findings may not apply to a general paediatric population with diarrhoea, as they failed to use quantitative cutoffs for case ascertainment, and only included cases with moderate-to-severe diarrhoea, and of these only cases presenting with diarrhoea of shorter than 7 days duration. Furthermore, important risk factors were missing from the analysis, e.g., behavioural hygienic factors and perinatal factors, precluding comparison of the relative importance of malnutrition compared with other factors, and limiting the ability to adjust for confounding bias.

1.9 Duration of *Cryptosporidium* oocyst shedding after infection

The possible benefits of targeted treatment of cryptosporidiosis as part of paediatric diarrhoea case management has already been discussed. If prolonged shedding of infectious oocysts is common after clinical cryptosporidiosis, a possible added benefit of targeted treatment may be to reduce onwards transmission. To investigate whether this can be a feasible strategy, we need reliable estimates for the duration of oocyst shedding from low-resource clinical settings, i.e., where interventions against cryptosporidiosis are most needed.

Cryptosporidium infections that cause diarrhoea shed higher quantities of oocysts than non-diarrhoea-associated infections and symptomatic infections more often lead to secondary household transmission [161, 162]. Although it has not been formally investigated, it is plausible that secondary transmission is also affected by the intensity of infectious shedding over time.

However, few studies have investigated shedding duration after diarrhoeal infection with *Cryptosporidium*. Prior to a 2021 publication from the Malnutrition and Enteric Disease Study (MAL-ED) on post-diarrhoeal shedding of a range of enteropathogens [163], there were only a handful of studies that had investigated this question, but they relied on low-sensitivity detection methods and were conducted in heterogenous populations. A study in Finnish adults with cryptosporidiosis reported shedding up to two months after clinical recovery, and a study in Scottish children aged 9-14 years reported 19 days median shedding duration (range 9-50) from onset of symptoms [164, 165]. A community cohort study in Peruvian children (mean age 17 months) reported a mean shedding duration of 17 days [166]. All of these early studies relied on detection of oocyst by microscopy using modified Ziehl-Neelsen acid-fast staining and may therefore suffer from low sensitivity, i.e., biasing shedding estimates downwards, and/or low specificity, i.e., biasing shedding estimates upwards.

By contrast, the MAL-ED study had several important characteristics that made it a landmark study of post-diarrhoeal shedding of enteric pathogens: they used sensitive real-time PCR detection, the study population comprised a high-risk group for all-cause diarrhoea (and cryptosporidiosis), namely children younger than 2 years old, and was conducted in diverse low-resource settings [163]. They reported a median duration of *Cryptosporidium* shedding of five weeks after onset of the diarrhoeal episode [163]. This estimate was based on analysis of 108 etiologic episodes, and was, at the time of publication, the largest and most comprehensive study of shedding duration after *Cryptosporidium* infection. However, their shedding duration estimate is not necessarily valid for the population of children who are diagnosed in healthcare settings. The study population was children with cryptosporidiosis identified by community surveillance visits, rather than children who present for health care with

diarrhoea. Furthermore, as genotyping of follow-up samples was not done, later detections during the surveillance period may have represented new infections with different *Cryptosporidium* species or genotypes. If this kind of "genotype switch" happens often, and is not accounted for, estimates of shedding duration will be biased upwards.

2. Study objectives

The core idea that sparked this thesis research project was the intriguing possibility of establishing *Cryptosporidium* testing "on the back of" tuberculosis testing by using the same microscopes and staining reagents. The possibility of large-scale roll-out of cryptosporidiosis testing using an intervention that mainly involves training of staff, and the establishment of simple quality systems, without the need for any new or expensive equipment, seemed promising.

However, the feasibility of this approach depended on unknown factors. As summarized earlier, a crucial evidence gap was the unknown diagnostic accuracy of LED-AP for cryptosporidiosis in real-life low-resource healthcare settings. Secondly, although the operational feasibility of LED-AP had been established for tuberculosis testing in low-resource laboratories, the method had not yet been evaluated as part of a large-scale diagnostic test evaluation for cryptosporidiosis. Similar data were missing for the ICLF test strip, which we considered a possible alternative to LED-AP.

The CRYPTO-POC study was conceived and designed to fill these evidence gaps. We planned for it to become a phase III diagnostic accuracy study, i.e., a large-scale prospective clinical diagnostic accuracy study, conducted in a setting typical of where the test would be implemented, that also included assessment of key operational aspects of test implementation.

The underlying hypothesis was that it is possible to achieve acceptable diagnostic accuracy and operational feasibility of LED-AP in a clinical laboratory in a health centre and hospital in a low-income country. Defining what should be considered "acceptable accuracy" was not clear-cut. We made a qualified assumption that the sensitivity might be somewhat lower than that reported from studies conducted in well-resourced labs, e.g., in the UK. Although we had no clinical outcome data based on diagnostic sensitivity, a well-conducted modelling study had estimated 70% as the minimum sensitivity for a cryptosporidiosis test to be cost-effective [34]. This was based on short-term clinical outcomes combined with long-term growth impairment. We therefore decided to set the minimum acceptable sensitivity to 70%. A similar study

hypothesis of minimum 70% sensitivity was set for the antigen test strip. Although we also wanted to estimate test specificity, the limiting factor for sample size purposes is usually sensitivity rather than specificity, so we did not hypothesize a minimum level of specificity.

The CRYPTO-POC study would require considerable cost, effort, and time, and since there are many unresolved important questions in cryptosporidiosis research, we wanted to maximize the scientific output. We therefore planned to obtain data on other key questions within the field of paediatric cryptosporidiosis field and diarrhoea in general [167]. This includes (but is not limited to) the other two studies included in this PhD project; a cryptosporidiosis risk factor analysis (paper II) and a follow-up study of *Cryptosporidium* shedding duration (paper III).

2.1 Main goals

1. Estimate diagnostic accuracy, limit of detection, and quantitative cut-off for disease (by an embedded case-control study), of light-emitting diode fluorescence microscopy using auramine staining (LED-AP), and a novel room-temperature-stable antigen test strip, for the diagnosis of cryptosporidiosis in young children, near the point of care, in a primary level health centre and hospital in a resource-limited setting.
2. Determine the prevalence of *Cryptosporidium* infection in the combined study population, to allow us to calculate positive and negative predictive values of both LED-AP and the antigen test strip.
3. Evaluate key operational performance characteristics of LED-AP and the antigen test strip, including but not limited to turnaround times (for LED-AP only) and cost-per-test analysis (for both LED-AP and the test strip)

2.2 Secondary goals

1. Describe the clinical, environmental, and socio-economic characteristics of cryptosporidiosis and non-cryptosporidiosis diarrhoea, compared with

healthy controls, in order to identify risk factors for healthcare-presenting cryptosporidiosis.

2. Assess the duration and quantitative dynamics of *Cryptosporidium* parasite shedding in children with cryptosporidiosis and its association with symptom severity and malnutrition.

3. Methods

3.1 Overview of methods used in paper I, II and II

Table 4 - Overview of study methods used in papers I-III

	Paper I	Paper II	Paper III
Aims	Evaluate diagnostic accuracy, predictive values, and operational performance of two near-patient cryptosporidiosis tests	Evaluate risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea and their population attributable fractions	Evaluate duration of and quantitative dynamics of oocyst shedding in children with cryptosporidiosis
Design	Prospective clinical phase III diagnostic accuracy study; frequency matched case-control substudy	Case-case-control study using a hierarchical causal conceptual framework	Prospective clinical case series
Study population	912 children under 5 years of age with diarrhoea; substudy in 749 cases with 706 non-diarrhoea controls	59 cases with cryptosporidiosis, 432 cases with non-cryptosporidiosis diarrhoea, 725 non-diarrhoea controls; all under 2 years of age	53 cases with cryptosporidiosis, under 5 years of age (follow-up study in a subset of children enrolled in CRYPTO-POC)
Exposure variables	LED-AP microscopy for *Cryptosporidium* oocysts in faeces, test trip for *Cryptosporidium* antigen in faeces	Socioeconomic, environmental, hygiene, perinatal, nutritional, and previous illness factors	Cryptosporidiosis
Outcome variables	Cryptosporidiosis	Cryptosporidiosis; non-cryptosporidiosis diarrhoea	Median duration of *Cryptosporidium* oocyst shedding after onset of diarrhoea; quantity of *Cryptosporidium* oocyst shedding over time after onset of diarrhoea
Covariates	Age, geographical area, seasonal variation	Age, sex, geographical area, seasonal variation	Age, sex, within-subject clustering
Laboratory methods	Index tests: LED-AP, antigen test strip Reference tests: antigen ELISA, qIFAT, qPCR	Reference tests: antigen ELISA, qIFAT, qPCR	LED-AP, qIFAT, qPCR, *Cryptosporidium* species determination, *Cryptosporidium* subtyping
Statistical methods	Estimation of diagnostic accuracy parameters and predictive values, Spearman's rank correlation test; receiver operating characteristic curve analysis; odds ratios, cost analysis	Multiple imputation by chained random forests, logistic regression mixed models, hierarchical analysis, estimation of population attributable fractions	Time-to-event estimation for interval-censored data (non-parametric and parametric models); generalized additive mixed models

3.2 Study setting

This PhD project both enabled and builds on the CRYPTO-POC study. The clinical field work in CRYPTO-POC was conducted in Jimma Medical Centre (JMC), and Serbo Health Centre (SHC), in southwest Ethiopia, from December 2016 to August 2018. About 350 km from Ethiopia's capital Addis Ababa, within the large Oromia region of Ethiopia, Jimma is a small city with a population of about 200.000 people. It is the former capital of the regionally powerful Kingdom of Jimma, an autonomous hold-out that was only fully incorporated into the Ethiopian empire in 1932. At an altitude of ca 1800m, the Jimma area has a relatively cool, for its latitude, tropical monsoon climate, with an annual wet season that stretches from March to October. The wild coffee plant is native to the forests of Jimma and the neighbouring Kaffa area, and coffee cultivation is a key economic activity.

Figure 2 Drying of coffee crops outside Jimma (ØHJ, personal photo)

The Jimma zone has a population of about 2.5 million people, of which 95% are rural residents. Within the main university campus in Jimma city is Jimma Medical Centre (JMC), called Jimma University Teaching Hospital (JUTH) at the time of the study, a tertiary referral hospital with a largely urban catchment area. Serbo Health Centre

(SHC) covers a rural area around the smaller town of Serbo, approximately 16 km from Jimma. Both study sites represent typical Ethiopian healthcare settings that would be relevant for near-patient testing and treatment for cryptosporidiosis. We initially planned to use a health centre situated within a demographic surveillance site (DSS), Asendabo Health Centre, 52 km from Jimma, but with increasing political unrest in the country in 2016, this far-off site was discarded due to risk of road closures and study interruptions (see Discussion).

3.3 Study design

Details on the methods of the CRYPTO-POC study can be found in the methods sections and supplementary appendices of paper I, II and III.

3.3.1 Cryptosporidiosis diagnostic accuracy study

In brief, CRYPTO-POC was designed as a phase III prospective clinical diagnostic accuracy study [114, 168], in a consecutive series of children <5 years with diarrhoea (three or more loose stools within the previous 24 h) and/or dysentery (at least one loose stool with stains of blood within the previous 24 h). Children with diarrhoea were enrolled, whether or not diarrhoea was the primary complaint leading their carers to seek health care. Cases were enrolled from 22 December 2016 until July 2018, from morning until evening, seven days a week at JMC, and during working hours on weekdays at SHC.

Figure 3 Jimma Medical Centre, the new University hospital buildings constructed in 2016-2017 (ØHJ, personal photo)

Stool samples were tested by LED-AP and the lateral-flow test strip. Test accuracy was estimated by independent and blind comparison to a composite reference standard comprising qIFAT, ELISA, and qPCR.

Asymptomatic infection is common; hence the use of qIFAT and qPCR would be more reliable if quantitative cutoffs for diarrhoea-associated infection could be established. In previous studies, quantitative testing has been successfully applied to discriminate between asymptomatic and symptomatic infection in studies that recruited both cases and community controls [91, 127, 128]. We therefore chose a panel of reference tests that included two different quantitative assays. However, the cutoffs used in these assays needed to be valid for our study population and we could not simply apply quantitative thresholds estimated from case-control-studies conducted in other areas, e.g., from the more severely ill case populations studied in the GEMS study. To be able to obtain valid quantitative cutoffs for our reference assays, we realized during the planning stage that we needed to embed a complete case-control study within CRYPTO-POC.

3.3.2 Diarrhoea case-control sub-study

As JMC covers a large area as part of its tertiary referral function, the case-control sub-study involved only a geographical subset of cases, i.e., covering an area that we were logistically able to reach for control recruitment. We expected a large majority of cases to fall within this area, based on logbooks and data obtained from JMC and SHC. Weekly plans were made for recruitment of non-diarrhoea controls from a predefined catchment area around both study sites. The following cases were excluded from the case-control sub-study: children residing outside the 15 districts defining Jimma town and its catchment area or the 8 districts defining the SHC catchment area; enrolment as a case within the last 60 days and admission as an inpatient for longer than 24 hours prior to enrolment; or enrolment before control recruitment started on 27 Feb 2017.

The prospective recruitment of a representative control group was a complex undertaking involving large-scale week-by-week enrolment of non-diarrhoea controls from both vaccination rooms and by randomized house visits in the community. We used a procedure where controls were frequency matched to cases by enrolment week, geographical area of residence and age group. Frequency matching means that controls are matched to cases in strata, rather than by individual matching of cases to controls in case-control pairs. This study design is considered to be more flexible and less vulnerable to overadjustment bias than studies that use individual matching, assuming modern statistical software and epidemiological tools are used in the analysis [169].

The control recruitment procedure can be summarized as follows. The key criteria distinguishing a control from a case was that they reported no diarrhoea in the previous 48 hours. Every week, a plan was made for the target group of controls to enrol in the following week. The plan accounted for frequency matching of controls to cases by geography, age group and time. The age groups were 0-5 months, 6-11 months, 12-23 months, and 24-59 months. In JMC, controls would be recruited from any of the 15 districts defined as the JMC catchment area and in SHC from a random sample of the specific districts that cases had been enrolled from during the preceding week. Controls in the age groups 0-5 months and 6-11 months were recruited from vaccination rooms at the two sites. A control was eligible if the child came from one of the 15 districts in

JMC catchment area, or from one of randomly selected districts in the SHC catchment area, based on that week's control plan, and if their parent/caregiver consented to participation in the study. If it was not possible to enrol a control in the 0-5 or 6-11-month age categories from the vaccination room within one week of frequency matching, we attempted to enrol a control belonging to the same age/geography stratum from the community instead, in the following week.

Controls in the age groups 12-23 and 24-59 months were recruited by home visits in the community. We identified eligible community controls by randomly selecting a GPS point within the JMC catchment area or within the randomly selected district in the SHC catchment area, by using QGIS software v2.18 [170] and district borders from ArcGIS [171]. The GPS point was plotted on a Google Earth map [172] and selected if there was a road within 300 meters of the point accessible by a motorbike (defined as any visible path minimum 2 m wide). Using a printed map and a handheld GPS receiver, the study nurse travelled to the specific GPS location, or as close as possible, based on the road conditions, then stopped, and then faced in a pre-specified randomly selected compass direction.

Figure 4 Example of a map used by field workers for control recruitment, based on the age, included in a control recruitment plan accounting for the exact number of cases enrolled within age strata and specific community areas in the preceding week

The house nearest to this direct line of sight was selected and approached. If no child of the required age lived in the first house, or if the caregiver refused participation, the steps above were repeated, but this time with that house as the new starting point. If an eligible child resided in the house but was not home for interview and examination after two visiting attempts, the procedure was repeated as above. If the required control had not been enrolled within two weeks after frequency matching, that control was dropped, except for a few circumstances, where we accepted enrolment of a planned control in the third week after the plan was made because of unexpected public and government disruptions of study activities. Initially, the case-control ratio was 10:6 (6 controls for 10 cases), but from July 2017 it was changed to 1:1 due to more cases coming from outside the catchment areas, and also lower caseload, than expected.

As we planned to exploit the case-control design to also study risk factors for cryptosporidiosis (paper II), we carefully harmonized data collection and procedures for both cases and controls, and incorporated detailed sociodemographic, household environmental and clinical information, in all case and control questionnaires.

3.3.3 Cryptosporidiosis follow-up study

To study the duration of symptoms and *Cryptosporidium* shedding (paper III), we designed a follow-up study in a subset of children with cryptosporidiosis. This entailed follow-up visits with repeat stool sampling for 60 days after enrolment (at 1, 2, 3, 4 weeks and finally 60 days). Cases were invited to participate if LED-AP indicated the presence of *Cryptosporidium* in their stool and if they resided within about 50km, i.e., within reach of our study staff for follow-up visits or home visits. The follow-up stool samples were later analysed by both LED-AP (done concurrently with LED-AP testing performed in the diagnostic accuracy study), qIFAT, qPCR, and *Cryptosporidium* species determination and subtyping.

3.4 Clinical data collection

Demographic and clinical data were collected using standardized case report forms. Before returning home, information on treatment and clinical status was collected by

the study nurse or from the hospital medical records. All cases were offered HIV testing; first-line testing was conducted with the First Response™ HIV 1-2-O Card test (Premier Medical Corporation Ltd, Daman, India); for children younger than 18 months, positive test results were confirmed by PCR and for children older than 18 months, positive results were confirmed by a second HIV test kit, Uni-Gold™ HIV (Trinity Biotech Manufacturing Ltd, Co. Wicklow, Ireland). HIV counselling and testing was done by routine clinical staff or study nurses trained in HIV counselling and testing. Information to caregivers and HIV treatment to children were offered according to routine care.

3.5 Assessment of operational issues

We attempted to implement clinically realistic near-patient testing as much as was possible within the practical and budget constraints of a research study. The aim was to maximise the operational validity of our diagnostic accuracy estimates and to gain experience that may be of value for others who would consider implementing cryptosporidiosis testing in routine clinical practice using LED-AP or the test strip. To achieve this, LED-AP testing was performed as soon as possible after receipt of the stool sample, and the result of the test was provided back to the study nurse, clinical staff (and from there, to the patient) as soon as testing had been performed. The laboratory technicians that performed LED-AP were routine clinical lab technicians with no prior experience with the method. They were provided with two one-day training sessions on recruitment. Monthly supervision visits were conducted by the study investigators. These visits included blind review of a random sample of stored microscopy slides and on-site training. We did not ourselves take part in the testing of study samples (paper I, supplementary appendix 2). In the SHC site, we recruited two resident health centre lab technicians to conduct testing on top of their routine duties. In the Jimma hospital site, due to high sample turnover, we employed a full-time lab technician, with two part-time clinical lab technicians, also trained in the method, covering testing, when the primary technician was on annual leave. The AP staining reagents and LED microscopes were the same as are currently used for tuberculosis

point-of-care testing elsewhere in Ethiopia and Sub-Saharan Africa (Zeiss PrimoStar iLED microscopes). To ensure full blinding, antigen *EZ VUE* test strip analysis was performed independently, by a different lab technician (with an additional technician to cover during leave) who was employed in a part-time capacity, with testing performed in batch every 2 or 3 days depending on sample numbers. The test strips were stored at ambient temperature until use.

Using this approach, the operational data that could be collected included cost of reagents and laboratory expendables used (for LED-AP and ICLF testing), standardised report forms from LED-AP supervision visits, and reports on LED-AP internal quality assessment, at both sites. As antigen strip testing was performed in batch, turnaround times could only be estimated for LED-AP.

3.6 Definitions

3.6.1 Clinical definitions

Diarrhoea was defined as the passage of three or more watery or loose stools within the preceding 24 hours; the presence, and duration of, diarrhoea, was assessed by caregiver recall. Dysentery was defined as at least one loose stool per day with visible blood in the previous 24 hours. Severe acute malnutrition (SAM) was defined as one or more of the following: weight-for-height z-score (WHZ) ≤-3 of the WHO standard curves [173], and/or mid-upper arm circumference (MUAC) ≤115 mm and/or presence of bilateral oedema involving at least the feet. Moderate acute malnutrition (MAM) was defined as a WHZ ≤-2 and >-3 or a MUAC ≤125 mm and > 115 mm with no oedema. Guardians/caregivers of all participants were requested for permission for their children to be tested for HIV using the current recommended HIV testing algorithm within the Ethiopian government health system; in brief, HIV status was either based on HIV testing on enrolment or by previous testing as reported by the caregiver, and children below 18 months with an HIV-positive mother were considered HIV exposed and uninfected if a PCR result for the child was not available. Stunting was defined as a length/height-for-age z-score ≤-2 of the WHO standard curves [173]. A child had moderate to severe diarrhoea if they had diarrhoea together with very sunken eyes,

slow or very slow skin pinch as assessed by the research nurse (abnormal but ≤2 s or >2 s, respectively), had dysentery, received IV fluids or was admitted for any reason [174]. Rotavirus vaccine in Ethiopia is an oral vaccine (Rotarix TM) that is given twice, usually at 6 weeks and 10 or 14 weeks of age. We defined the child as vaccinated against rotavirus if two doses had been received at least four weeks apart. More details on definitions used are available in paper I, II and III (including supplementary appendices).

3.6.2 "Point-of-care" versus "near-patient" testing: a note on terminology

There is ongoing discussion on the best definition and use of the term "point-of-care-test" (POC). We find the following working definition most useful: "a diagnostic test that is performed near the patient or treatment facility, has a fast turnaround time, and may lead to a change in patient management". This definition was suggested by Drain *et al* in 2014 [75], who thoughtfully considered the pros and cons of various idealized criteria proposed for POC tests intended for resource-limited settings, including the ASSURED criteria (discussed earlier). However, Hänscheid *et al* warned against inflationary use of the POC term based on the fairly broad definition above [175]. Of the two index tests in our study, the test strip fulfils the requirements of speed and being independent of laboratory infrastructure and could qualify as a POC test, by most proposed definitions. LED-AP-microscopy, however, does require staining in a dedicated sink and subsequent microscopy. Staining reagents and microscopes are usually located in small clinical hospital laboratories or in basic health centre laboratories, although usually within short distance of the outpatients' department or treatment room where the child is assessed and treated. The requirement for a small lab (or, at least, a dedicated area) and training and re-training of microscopists, can be a barrier to implementation. Importantly, any test may face test-specific barriers to implementation and real-life use that can be obfuscated by simple classification of a test as "POC". These considerations, and the diverging definitions in the literature, led us to stop referring to our index tests as POC tests. We opted instead for the term "near-patient testing" to distinguish between our tests and tests that would require access to more sophisticated laboratory setups, or tests that failed several ASSURED criteria.

3.7 *Cryptosporidium* stool testing

3.7.1 Index tests

Study participants were provided with a labelled screw capped container and the caretaker informed how to collect a 5 g sample using either a potty or a nappy lined with plastic film. Unconcentrated wet stool was air dried on slides and stained with auramine-phenol before microscopy for *Cryptosporidium* by light-emitting diode microscopy (LED-AP) using a PrimoStar iLED microscope.

Figure 5 Serbo health centre laboratory and PrimoStar iLED microscope

For positive samples, a simple visual scoring system for oocyst numbers were used (1+, 2+, 3+). Testing was performed according to a standard operating procedure (paper I, supplementary appendix). The presence or absence of *Cystoisospora belli* and *Cyclospora* was also noted. Conventional direct wet mount microscopy (formed stools were mixed with a drop of saline) for cysts/ova (including, but not limited to, *Entamoeba histolytica/dispar*, *Giardia duodenalis*, and soil-transmitted helminths) was also performed. All microscopy findings, positive or negative, were reported back

to the responsible medical staff. At SHC, after LED-AP, stool samples were kept in a kerosene-powered fridge (due to frequent power cuts in the area) until transport to JMC by study staff at least once per day). The specimens were kept in a mobile cool box during transport.

ICLF strip testing (*Cryptosporidium EZ VUE* test strips, TechLab Inc, Blacksburg, VA, USA) was performed minimum two times per week, by a JMC laboratory technician, who was blinded to both LED-AP and IFAT test results.

3.7.2 Reference tests

At JMC, an air-dried slide was prepared from a 10 µg loopful of stool. Blinded to the result of LED-AP, quantitative IFAT was performed by counting *Cryptosporidium* oocysts on this slide, minimum two times per week, yielding an approximate result in oocysts per gram of wet stool. The remaining stool was frozen at -80ºC. An aliquot of stool was thawed and tested in batch by *Cryptosporidium* ELISA antigen test (Cryptosporidium II, TechLab Inc, Blacksburg, VA, USA), blinded to the results by LED-AP, dipstick, and IFAT. This ELISA assay was also used in the GEMS study [176]. The remainder was shipped to Norway for total nucleic acids (TNA) extraction followed by quantitative *Cryptosporidium* PCR, also performed blinded to results any of the index tests or other reference tests. The qPCR assay was an in-house assay with generic primers for *Cryptosporidium spp.*, based on a previously published assay [177]. By quantifying the mass of stool from which TNA was extracted, and by calibrating the qPCR using standard curves derived from serial runs of *Cryptosporidium* positive controls of known concentration (in copies per µg), *Cryptosporidium* DNA quantity could be estimated in target copies/gram of wet stool.

3.8 *Cryptosporidium* genotyping

3.8.1 *Cryptosporidium* species determination

For species determination, real-time LIB13 *C. parvum* and *C. hominis* qPCR [178] was performed in a duplex real-time PCR reaction assay targeting *C. parvum* LIB13 and *C. hominis* LIB13 using primer and probe sequences from [178], but with probes adapted

for use with the LightCycler system using locked nucleic acid (LNA) probes and a quencher at the 3' end.

3.8.2 *Cryptosporidium* subtyping

Subtyping was performed by nested PCR and sequencing of the *Cryptosporidium* gp60 gene. The gp60 gene codes for a surface glycoprotein and contains a conserved region and a hypervariable region. Phylogenetic analysis of the conserved region allows for classification into gp60 allele family. The hypervariable serine repeat region allows for classification into gp60 allele family subtypes by counting distinct serine repeat types. The nomenclature used in gp60 subtyping is well established in the *Cryptosporidium* research community and is summarized in [179] and [180]. The gp60 gene was first targeted and amplified using a nested PCR, with primary and secondary primer sequences as described in [181]. Nested PCR products were then purified using the Exo-CIP™ Rapid PCR Cleanup kit (New England BioLabs), according to the manufacturer's protocol. Purified template DNA and forward or reverse primers from the secondary nested PCR were added to a LightRun 96 plate for Sanger sequencing at a commercial sequencing facility (Eurofins Genomics). Raw gp60 gene sequence data were analysed directly by the newly developed, and freely available, CryptoGenotyper software tool [182]. Raw sequence data that were either unsuccessfully typed or flagged for manual sequence analysis by the CryptoGenotyper software were manually analysed by trimming, sequence alignment, and phylogenetic tree analysis, using Geneious Prime software (version 2021.1.1). Final sequences and phylogenetic trees were compared to reference sequences of valid gp60 allele families, following the method outlined in [183], using the CryptoGenotyper curated list of gp60 reference sequences (accessed on 010721). This was supplemented by online searches using the U.S. National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST).

3.9 Statistical methods

Data was collected by paper questionnaires labelled with unique study ID numbers and, for laboratory testing, by unique stool sample ID numbers. Double data entry was done

by two separate data entry clerks using the free software EpiData 3.1 (EpiData, Odense, Denmark). Data analysis was done using the free and open statistical programming software R (2018, R Foundation for Statistical Computing, Vienna, Austria) using the RStudio Integrated Development Environment (2016, RStudio inc., Boston, MA, USA) [184].

Only key statistical methods used will be summarized here; please refer to the individual papers for more detailed descriptions, including the specifics of the statistical software used in the analysis. For sample size considerations, please refer to paper I (main paper - methods - statistical analysis – p 726) and paper II (supplementary appendix – p5) and paper III (main paper – discussion - p7)

3.9.1 Paper I

The reference standard to which our index tests would be compared was a triple composite panel comprising independent and blinded examination of stool samples by *Cryptosporidium* qIFAT, *Cryptosporidium* qPCR, and ELISA *Cryptosporidium* antigen detection. The reference panel results were classified as follows: a positive clinical composite reference standard (CRS) was defined as two or more positive reference tests (and greater than the quantitative cutoff value for association with diarrhoea) and negative CRS as two or more negative reference tests (or less than the quantitative cutoff value for association with diarrhoea). A diarrhoea case with a positive CRS was defined as having cryptosporidiosis. A diarrhoea case with a negative CRS was defined as having non-cryptosporidiosis diarrhoea. A positive microbiological composite reference standard (MRS) was defined as two or more positive reference tests and negative MRS as two or more negative reference tests, without applying quantitative cutoffs.

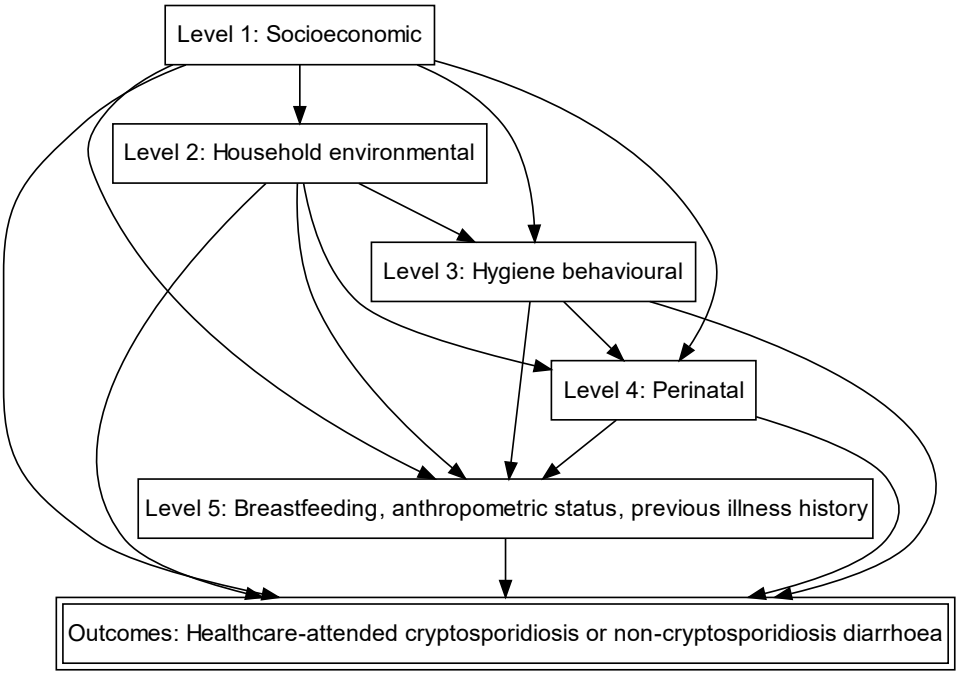
The results of the index tests under evaluation (LED-AP and the antigen test strip) were then compared, one by one, with the CRS. This enabled us to estimate diagnostic accuracy parameters for each index test, using standard statistical methods for proportions [185], with 95% confidence intervals for all accuracy estimates [186]. The strength of association between a positive *Cryptosporidium* test and diarrhoea was

approximated by calculating the odds ratio using data from the case-control sub-study [187]. Odds ratios were calculated both for the index tests and for the reference tests. A P-value lower than 0.05 was considered to represent statistical significance and 95% confidence intervals were used to represent statistical precision.

3.9.2 Paper II

This was an investigation of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea (NCrD), in healthcare-presenting young children. We analysed data collected in the CRYPTO-POC case-control substudy. From the outset we sought to adjust for possible confounding from the complex set of hierarchically related risk factors that can result in a child presenting to healthcare with cryptosporidiosis. To accomplish this a combination of epidemiological methods were applied: 1) a hierarchical conceptual framework, where risk factors are organized as a hierarchy of causal levels (Figure 6), in order to make explicit underlying assumptions about the direction of influence between various variables in the analysis, 2) case ascertainment that includes a range of tests, including two assays where quantitative cutoffs were applied, and 3) a case-case-control design, in order to distinguish between risk factors for cryptosporidiosis, and NCrD.

Figure 6



We used pragmatic "real-life" inclusion criteria in a hospital and a health centre in a low-resource population with low prevalence of HIV and high rates of malnutrition. In the case versus control comparisons, odds ratios were estimated by both univariable and multivariable mixed model logistic regression, after imputation of missing values by chained random forests.

A step-by-step approach guided the inclusion of putative risk factors thought to be progressively proximal to disease, informed by the hierarchical framework. Risk factors were first identified by univariable models, then analysed in multivariable intra-level models, then multivariable models taking all levels into account.

For risk factors found to be significant in the final hierarchical analysis, we estimated population attributable fractions, allowing us to estimate their relative contribution to cryptosporidiosis and NCrD caseload, respectively.

3.9.3 Paper III

CRYPTO-POC participants who were cases, i.e., children < 5 years presenting with diarrhoea to SHC and JMC, and who had a positive LED-AP test and resided within 50 km from JMC or SHC, respectively, were invited to participate in a separate follow-up study. This was a prospective clinical case series conducted alongside and the full 18-month period of the CRYPTO-POC field study. Stool samples were collected at 1, 2, 3, and 4 weeks, and then 60 days, after CRYPTO-POC enrolment. Samples were examined, and *Cryptosporidium* shedding quantified, using LED-AP, qIFAT and qPCR. *Cryptosporidium* species determination and subtyping were used to help distinguish between new infections and ongoing shedding.

Two complementary analytical approaches were used to estimate shedding duration. First, by time-to-event modelling (i.e., a type of survival model) where ongoing shedding was considered a binary outcome, based on microscopic detection of oocysts and/or DNA detection by PCR. Second, by quantitative modelling of the drop in DNA quantity over time, based on qPCR, using a generalized additive mixed model (GAMM) with thin plate smoothing splines. These models were adjusted for sex and age.

As a secondary analysis, we also explored how diarrhoeal severity, acute malnutrition, and *Cryptosporidium* subtypes correlated with shedding duration, and with shedding quantity over time. In the time-to-event models this was accomplished using accelerated failure time log-logistic models, adjusted for sex and age (in the time-to-event models); in the GAMM by determining regions (i.e., time windows, in days after onset of diarrhoea) of significant difference between subgroup-specific GAMM models. All models were visualized using various plotting functions in R.

3.10 Ethical issues

Ethical approval for the study had been secured by early September 2016, from both Ethiopia (Jimma University IRB, reference: RPGC/610/2016; the Ethiopian National Research Ethics Review Committee, reference: JU JURPGD/839/2017) and from

Norway (Regional Committee for Medical and Health Research Ethics of Western Norway, reference: 2016/1096). During the enrolment procedure, formal written consent was obtained from the children's parents or guardians. The purpose of the study was clearly described to the study participants and caretaker(s) including benefits and risks. Collection of stool samples and blood spots for HIV testing were considered low-risk procedures, however, should any injury occur related to participation, patients would be covered by the standard Ethiopian Ministry of Health patient insurance system. Data collected including laboratory test results were fully confidential. Children who tested positive for HIV on enrolment were counselled and considered for antiretroviral treatment and as per standard guidelines [188, 189], with free provision offered by the routine healthcare system. Costs related to allow the participants to travel to return to JMC or SHC for follow-up visits were compensated by a flat fee of 50 Ethiopian Birr (ETB) to each participant, for both cases and controls, and for cases returning for follow-up visits.

4. Main results

4.1 Paper I

The CRYPTO-POC study enrolled children between Dec 22, 2016, and July 6, 2018. The key component of the study was the diagnostic accuracy study, based on testing faecal samples from 912 children with diarrhoea or dysentery. The case-control substudy included samples from a subset of 749 cases, compared with samples from 706 non-diarrhoea controls that were frequency-matched to cases based on age group, geographical area of residence, and enrolment week.

Cryptosporidium was significantly associated with diarrhoea by all detection methods, but the strength of the association varied by test modality. Most of the qPCR-positive samples that were negative by LED-AP and the test strip had low quantities of *Cryptosporidium* DNA per gram of stool. The weakest qPCR quantity detected by LED-AP microscopy was 13836 copies/gram (a similar limit was observed for the test strip). As expected from previous case-control studies we observed a large overall difference in quantity between cases and controls, as illustrated by this figure:

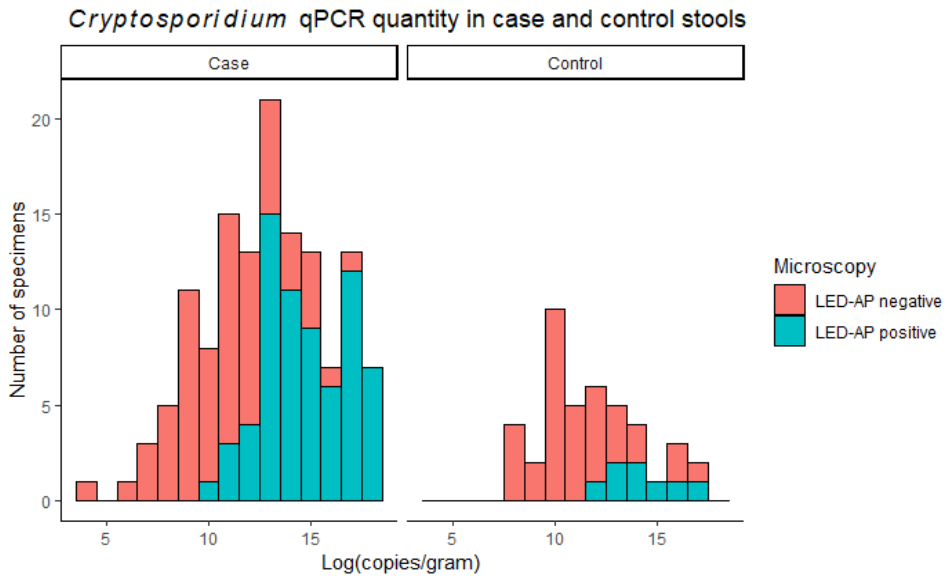


Figure 7 Cryptosporidium DNA quantity in case and control stools

Using the case-control data, we aimed to control for asymptomatic or incidental infections by including quantitative reference assays. The first step was to establish reference-test cutoff values for diarrhoea-associated infection based on receiver operating characteristic analysis. The estimated cutoff values were 2·3 × 10^5 DNA copies per g of wet stool for qPCR, and 725 oocysts per g for qIFAT. These cutoffs were integrated into the composite reference standard for cryptosporidiosis and applied to the case population (n = 912). The prevalence of cryptosporidiosis among cases was estimated as 9% (95% CI 8-12).

Comparing the result of our index tests to the reference standard, we estimated that LED-AP had a sensitivity for cryptosporidiosis of 88% (95% CI 79–94; 66 of 75 samples) and a specificity of 99% (95% CI 98–99; 717 of 726 samples); the lateral-flow test strip had accuracy similar to LED-AP, with a sensitivity of 89% (95% CI 79–94; 63 of 71 samples) and a specificity of 99% (95% CI 97–99; 626 of 635 samples). The predictive values in our population were high; a case testing positive by LED-AP had an 88% probability of having cryptosporidiosis (95% CI 79-94 for LED-AP and 78-93 for the test strip), and those testing negative had a 99% probability of not having cryptosporidiosis (95% CI 98-99 for both tests).

The cost-per-test analysis, also factoring in labour time, estimated a cost of 0.7 USD per LED-AP test, and 0.6 USD per lateral-flow strip test, excluding the cost of the test strip itself. Operational performance of LED-AP was also evaluated, and were favourable for near-patient use, but the need for supervision and quality control systems were highlighted. Turnaround times were longer than expected; but for results received the same day, test turnaround time ranged from 0.5 h to 7.8 h (median 3.5; IQR 1.7–4.9); a breakdown of turnaround times into subphases showed the preanalytical and postanalytical phases comprised the major fraction of total turnaround time.

4.2 Paper II

Acute malnutrition was strongly associated with both cryptosporidiosis and non-cryptosporidiosis diarrhoea (NCrD), and was apparently more strongly associated with cryptosporidiosis (cryptosporidiosis OR 7.2, 95% CI 2.9-17.8; NCrD OR 4.6, 95% CI 2.6-8.0). Previous healthcare attendance (OR 2.3, 95% CI 1.3-4.1) and low maternal education (OR 2.2, 95% CI 1.0-4.7) were only associated with cryptosporidiosis. Household asset ownership, use of unprotected and surface water, handwashing without soap, unsafe disposal of child stool, caesarean section, premature delivery, and early cessation of exclusive breastfeeding were significantly associated with NCrD only. Having a different primary caregiver than the child's mother was associated with both cryptosporidiosis and NCrD (cryptosporidiosis OR 5.5, 95% CI 1.7 - 17.8; NCrD OR 3.8, 95% CI 1.9-7.6), as was public tap water use (cryptosporidiosis OR 3.8, 95% CI 1.9-7.7; NCrD OR 2.5, 95% CI 1.7-3.6). By estimation of population attributable fractions (PAF) the following risk factors appeared to be more important risk contributors to cryptosporidiosis than non-cryptosporidiosis diarrhoea case load, i.e., when translated into hypothetically preventable cases: socioeconomic factors (cryptosporidiosis PAF 26%; NCrD PAF 14%), specifically low maternal education (cryptosporidiosis PAF 19%, not significantly associated with NCrD), and public tap water use (cryptosporidiosis PAF 27%; NCrD PAF 15%). For cryptosporidiosis, the strength of association was weaker for MAM than SAM (cryptosporidiosis OR 5.3 versus 16.2; NCrD OR 4.17 versus 4.1), but, considering the higher background prevalence, MAM was estimated to contribute to a higher number of cases (cryptosporidiosis PAF 10% for MAM versus 6% for SAM).

In the hierarchical mediation analysis, we found only weak evidence for mediation of distal risk factors through intermediate risk factor levels. A notable exception was the socioeconomic risk factors, in particular, for cryptosporidiosis. These factors (e.g., low maternal education) were mediated through proximal risk-factor levels to a larger extent than the NCrD risk factors (68% vs 22% of the level-1-PAF, respectively).

4.3 Paper III

From 53 confirmed cryptosporidiosis cases, a median of 4 (range 1 to 5) follow-up stool samples were collected and tested for *Cryptosporidium*. Genotyping of follow-up samples revealed a subsequent shift in the *Cryptosporidium* genotype in 5 cases (9%). These were considered likely new infections and were not counted as having "ongoing shedding" after the subtype shift had occurred. Interestingly, there were no gp60 genotype shifts at the sub-allele-family level.

From the time-to-event models, we estimated a median duration of oocyst shedding of 31 days (95% CI, 26 to 36 days) after onset of diarrhoea. Strikingly similar estimates were obtained from the quantitative GAMM models (31 days, 95% CI 27 to 37 days).

The temporal shedding dynamics were modelled by GAMM and represented in graphs. An almost linear drop in quantity was observed in the month after onset of diarrhoea, with a 10-fold drop in quantity per week, for the first 4 weeks.

When comparing key subgroups of children, only subtle or borderline significant differences were seen in the duration and quantity of shedding in the 2 months after onset of diarrhoea, although it was notable that the observed differences in shedding duration were similar when comparing the findings from the time-to-event models and the quantitative models. No window of significant difference was observed when comparing cases with and without acute malnutrition. The largest difference was seen at the *Cryptosporidium* subtype level, where *C. hominis* gp60 allele family Id was associated with a higher quantity of shedding than the other *C. hominis* allele families during the time window from 11 to 55 days after onset of diarrhoea. *C. hominis* predominated overall (46 of 53 cases), and, notably, all *C. parvum* detections (7 of 53) belonged to the anthroponotic IIc subtype.

5. Discussion

5.1 Overview

The introduction outlined the need for phase III diagnostic accuracy studies for cryptosporidiosis in children, the contradictions and evidence gaps in the available literature on risk factors, and the lack of studies of *Cryptosporidium* shedding duration supported by quantitative PCR and genotyping.

The main findings can be summarized as follows. First, LED-AP and the ICLF test strip had satisfactory accuracy for the diagnosis of cryptosporidiosis in high-burden clinical settings (paper I). Second, a select few risk factors for healthcare-presenting cryptosporidiosis were found to be more important than others, pointing to possible interventions (paper II). Third, half of all children with healthcare-presenting cryptosporidiosis will continue to shed *Cryptosporidium* one month after onset of diarrhoea, however with a 10-fold-drop-per-week in shedding intensity (paper III).

A discussion of the main results, in light of previous literature, can be found in the Discussion sections of paper I, II and III. Some key points will be summarized here.

First, our LED-AP diagnostic accuracy estimates were consistent with estimates from a previous study conducted on AP in a high-income country [83], and a small study in HIV-positive adults in India [126]. The high accuracy of the room-temperature ICLF was consistent with favourable prior evaluations of ICLFs from LMIC [99, 121, 122].

Second, the association between acute malnutrition and cryptosporidiosis was stronger than what had been found in case-case comparisons [147-154]. Contrasting with the limited previous risk factor evidence [139-141], lack of access to piped water in the household was a risk factor for cryptosporidiosis, in keeping with what has been found for all-cause diarrhoea [190].

Third, our shedding duration estimates supported the previous findings from the MAL-ED community cohort study [163]. We were unable to confirm previous reports of differences in shedding quantity between *C. parvum* and *C. hominis* [166, 191, 192].

The observed association between *C. hominis* subtype Id and prolonged shedding has not been reported before but should be interpreted with caution until confirmed by larger studies.

The external validity of our findings rests on the strengths and weaknesses of the underlying design choices and methodology in the CRYPTO-POC study. Several important limitations of the three publications included in this thesis have been included in the discussion sections and supplementary appendices of the individual papers. These limitations will only be briefly summarized here. Most of the following section will instead be devoted to a discussion of methodological issues that were omitted from, or only briefly discussed in, the published papers due to space constraints, and to discuss and expand upon some key limitations in light of the CRYPTO-POC study and of the PhD project as a whole.

5.2 Key methodological strenghts of CRYPTO-POC

The main strength of the CRYPTO-POC study was that it was the first adequately powered phase III diagnostic accuracy study for cryptosporidiosis in children with diarrhoea who present for health care in a low-income setting (Introduction, Table 2). We consider this the most important target population for therapeutic interventions against cryptosporidiosis [64, 68].

Key elements of our phase III diagnostic accuracy study design, were: blinding of all testing procedures, a realistic laboratory setup for clinical testing, and design decisions for external validity, i.e., attempting to maximise the relevance of our findings to other low-resource healthcare facilities in populations with high cryptosporidiosis burden. We used broad inclusion criteria and included all children who had diarrhoea on presentation for health care, irrespective of the duration of the diarrhoeal episode or whether the diarrhoea was the main reason for seeking care. An additional strength of our study was the inclusion of quantitative and qualitative assessment of operational issues. These are important to consider before tests are implemented in clinical practice.

Recruitment of controls not suspected to have the target condition is not usually done in diagnostic accuracy studies [116]. It is an understatement that the decision to conduct a full case-control substudy embedded within CRYPTO-POC complicated the design and execution of the field study and the subsequent statistical analysis. However, given the existing knowledge about asymptomatic or incidental *Cryptosporidium* infections and carriage [91, 127, 128] it became clear at the planning stage that reliable case ascertainment would require a reference standard that included not only the best available assays for *Cryptosporidium* detection, but also careful quantification. The quantitative cutoff values for disease ascertainment needed to be valid both for our laboratory assays and for our study population, which again would require enrolment of a representative control group without diarrhoea. Committed to this approach, epidemiological design considerations led us down the path of a frequency-matched case-control study design, in order to account for important *a priori* confounders of age, geographical area of residence, and seasonal variation.

The case-control design also enabled us to study risk factors for healthcare-presenting cryptosporidiosis (paper II). We therefore expanded the case and control questionnaires to include an extensive list of possible risk factors, including operational variables and variables that would be used mainly for confounder adjustment and bias analysis. Key strengths of the risk factor study were the combination of healthcare presentation as a proxy for severity, and concurrent recruitment of community controls without diarrhoea, using an innovative frequency matching procedure. Importantly, we carefully attempted to address important biases in case-control risk factor studies, using a hierarchical conceptual framework, case ascertainment using quantitative cutoffs, and a novel application of the "case-case-control" design, an approach originally developed to study risk factors for multidrug-resistant bacterial infections. We deliberately used pragmatic, i.e., "real-life", inclusion criteria. Our study population had low HIV prevalence in children yet high rates of malnutrition and will be representative for reasonably similar low-resource healthcare settings elsewhere.

Key strengths of the study on *Cryptosporidium* shedding dynamics (paper III) were the prospective study design, consecutive enrolment of young children who were

diagnosed with cryptosporidiosis at the point-of-care, quantitative modelling using repeated sampling with qPCR measured as *Cryptosporidium* copies per gram of stool, and the inclusion of *Cryptosporidium* species determination and subtyping to help distinguish between new infections and ongoing shedding.

5.3 Methodological limitations discussed in the papers

5.3.1 Brief summary

The most important limitations that were discussed in the diagnostic accuracy study (paper I) were the low paediatric HIV prevalence in the study area, missing data, not including comprehensive testing for co-infections with other diarrhoeal pathogens (also relevant for paper II and III), and the possibility that turnaround times may have been artificially inflated as carers had little incentive to wait for the results of testing (i.e., no available treatment).

Key limitations of the cryptosporidiosis risk factor study (paper II) were related to the inherent limitations of the observational case-control study design. These limitations could have been overcome in part by investigating risk factors using a longitudinal community cohort study design. However, as our outcome of interest was healthcare-presenting cryptosporidiosis a community cohort study would have required a larger sample size. This type of study can be done, but the required number of field visits was not feasible within the scope of the CRYPTO-POC budget or a PhD project. With necessary funding and time, such a study could be conducted as a multicentre study comprising several demographic household surveillance sites forming catchment areas around hospitals or health centres. Although CRYPTO-POC had an effective setup to make inferences about the sickest children, i.e., those children with diarrhoea who ended up presenting for health care, the case-control design limited our ability to determine the direction of causality between, e.g., malnutrition and cryptosporidiosis or the protective effect of exclusive breastfeeding. We also decided to omit interesting putative risk factors due to possible selection bias for some variables (e.g., vaccinations, diarrhoea in close contacts, handwashing). Other methodological issues that we elaborated on were differential exposure misclassification bias (found for the

sanitation variables, fully explored in the appendix for paper II), the inability to study some risk factors due to infrequent exposure in the study population (e.g., HIV or immunosuppression, measles, household water treatment), and selection bias due to enrolment location or missing outcome data (bias analyses for both types of selection bias included in the appendix for paper II). Lastly, we were not able to make strong inferences about differences in the strength of risk factor associations between cryptosporidiosis and non-cryptosporidiosis diarrhoea, due to the small sample size in the cryptosporidiosis case set.

Some limitations discussed in the cryptosporidiosis follow-up study (paper III) were the wide margins of uncertainty in the subgroup comparisons of shedding patterns, not having included contact tracing or environmental sampling within the households of the cryptosporidiosis cases, and not including the latest available laboratory methods to elucidate mixed-genotype infections.

5.3.2 Questioning the validity of the composite reference test panel

The use of a triple panel of reference tests, rather than a single reference test, was part of the protocol, and is a common way to address the problem of an imperfect or lacking "gold standard" in diagnostic accuracy studies. When combining several reference tests into a panel there should be advantages of one test that are not considered to be adequately covered by another test. For that reason, reference panels usually consist of two or more well-established tests that are based on different detection modalities. All three reference methods in our panel have strengths and weaknesses. Quantification is inherently challenging in a heterogenous and complex material like faeces, and it is not clear from the literature whether qPCR or qIFAT is more reliable for the purposes of *Cryptosporidium* quantification, as these two assays are based on different detection modalities [193]. We therefore included both. In diagnostic accuracy studies from high-income countries, ELISA testing had good sensitivity and specificity compared with both IFAT and PCR [83], and *Cryptosporidium* detection by ELISA was strongly associated with diarrhoea in both the original GEMS and MAL-ED studies [91, 128]. Antigen is a third and different detection modality [176, 194, 195] and in effect ELISA worked as a "tie breaker" when there was discrepancy between qPCR and qIFAT. We

assumed that most false-negative ELISAs (i.e., presumably due to a lower limit of detection) would be picked up by our combined reference standard anyway, as long as both of the other two reference tests were positive. We also considered it valuable to include a plate-based antigen assay in the reference panel as one of the index tests was also an antigen test (the ICLF test strip). Furthermore, the inclusion of the TechLab ELISA would allow us to compare our prevalence estimates to the findings from the original GEMS study that used the same assay [176].

5.3.3 Questioning the validity of the qPCR cutoff

By definition, applying a qPCR cutoff means that there is possibility of missing some unknown number of "real" cryptosporidiosis cases [91, 127, 128]. Although there is no other, "even better" reference test with which to compare our reference tests, we need to acknowledge that there is no gold standard to ensure external validity. However, we attempted to ensure internal validity of all assays. We also attempted to critically assess some of the underlying assumptions for the use of a quantitative diagnostic threshold.

First, we found a strong correlation between *Cryptosporidium* quantity and the presence or absence of diarrhoea. This indirectly supports the underlying logic of applying a cutoff. Statistical methods to maximise the discrimination between disease and non-disease (i.e., diarrhoea versus non-diarrhoea) was also used in the "GEMS reanalysis" study, with estimation of cutoff values [91]. It is reasonable to question our insistence on deriving our own cutoff values when other studies have applied the GEMS-derived cutoffs to other study populations and settings, and usually by "raw" real-time PCR cycle thresholds rather than values given in copies/gram (more demanding to measure as it requires careful measurement of faecal mass) [122, 196-198]. Due to the inherently variable nature of quantitative PCR across platforms (internal validity) and populations (external validity), we did not feel confident about applying the GEMS cutoffs for case ascertainment, as they were derived from a different population, enrolled by different inclusion criteria, and analysed on a different analytic platform. The case-control substudy was designed with the main purpose of deriving these cutoffs. Another important reason was to demonstrate whether *Cryptosporidium* infections were indeed associated with diarrhoea in Ethiopia, as could

reasonably be expected, based on findings from other countries in sub-Saharan Africa [12, 17, 46, 199-201]. Similar data was not available from Ethiopia. We strived to ensure consistency in our approach to quantification both during sample collection and laboratory procedures, e.g., by using plastic-covered nappies for collection (to avoid absorption of water) and by carefully weighing samples at all steps.

Interestingly, it turned out that our estimated qPCR cutoff value was reasonably close to the cutoffs estimated by GEMS. Our cutoffs were actually somewhat lower than the GEMS cutoffs, when translated to oocyst equivalents. Had we applied a higher cutoff (e.g., by using the GEMS cutoff), implying "stricter" case ascertainment, a handful of cases would have been re-classified as non-cryptosporidiosis diarrhoea, likely resulting in slightly higher estimates of sensitivity. Had we used a lower cutoff, a few cases would have moved from the "infected, non-diarrhoea associated" (i.e., MRS positive yet CRS negative) category to the cryptosporidiosis category (i.e., CRS positive). This would have result in slightly lower estimates of sensitivity, e.g., had we reclassified all "CRS negative but MRS positive" as cryptosporidiosis, the sensitivity of both LED-AP and the test strip would be 86% instead of 88% (still with 99% specificity). However, there is little evidence to support shifting the cutoffs in either direction, and such *post-hoc* changes to the analysis strategy would need to be carefully justified.

5.4 Methodological limitations that were only briefly discussed or omitted from the papers

5.4.1 Mixed infections

Misclassification from not excluding diarrhoeal episodes caused by other enteric pathogens is possible and could potentially lead to misclassification of some diarrhoeal episodes that were due to pathogens other than *Cryptosporidium* [187]. This could lead to biased risk factor estimates as well, as risk factors for infection and disease likely overlap between *Cryptosporidium* and other diarrhoeal pathogens. Previous reports indicate that rotavirus, norovirus (subtype GII), *Cryptosporidium*, and *Shigella* spp/enteroinvasive *E. coli* (EIEC) are significantly associated with diarrhoea both when present as single pathogen and when present in coinfections [202]. In the "GEMS

reanalysis" that used multi-pathogen qPCR, *Cryptosporidium* was found to be the primary or sole diarrhoea-associated pathogen in most detections, when quantitative cutoffs for diarrhoea were applied, also in mixed infections [91]. Based on this, we suspect that there would be few misclassifications due to co-infections. An important limitation of our study is that we did not test this assumption using our own data. Although we were unable to analyse for mixed infection in the current work, due to resource constraints, we plan to conduct a follow-up aetiology study using a multitarget qPCR platform in both case and control samples from CRYPTO-POC. This will enable us to re-evaluate our case ascertainment in light of co-infections and may provide much-needed case-control data on diarrhoeal aetiology in Ethiopia.

There were extremely few detections of *Cyclospora cayetanensis* and *Cystoisospora bellii* by LED-AP during the study, but we decided to not include these data in the included papers for two main reasons: first, because CRYPTO-POC was insufficiently powered to estimate diagnostic accuracy for these parasites; second, as it would distract from the main focus of the manuscript, and we were constrained by word-count limits. We hope to publish these data in a short paper or letter-format publication in the future.

Our qIFAT assay included immunofluorescent antibodies that stain *Giardia duodenalis* cysts. Not surprisingly, *Giardia* infections were very common in our study population (although with a higher median age of infection than for *Cryptosporidium*). Based on results from GEMS, *Giardia* infection is not considered to be associated with acute paediatric diarrhoea. However, we are interested in the impact *Giardia* may have on prolonged and persistent diarrhoea, and on longer-term outcomes like growth. There are few data on *Giardia* epidemiology in Ethiopia, particularly from case-control studies [136, 203]. We therefore considered *Giardia* testing and epidemiology as a separate and expanded PhD-project, supplemented by *Giardia* qPCR and genotyping.

5.4.2 Were the false negative index tests adequately scrutinized?

When compared with our reference standard, there were nine false negative LED-AP tests and eight false negative test strips. There are too few cases to be able to draw any firm conclusions on what exactly went "wrong" with the false negative index tests.

Inadequate homogenization of the stool samples with a resulting stochastic risk that oocysts did not actually end up on the slide, or errors with the staining procedure and/or microscopy, are some possibilities. These types of errors may be related to the experience of the lab technician performing the procedure. When using mean time gap between each true positive *Cryptosporidium* infection (i.e., MRS positive) detected by LED-AP as an indirect proxy for LED-AP microscopy experience, there was a negative trend between microscopy experience and false negative testing. We found out that five of the false-negative test strips were performed by the same technician, even though this staff member was only employed during the initial months of enrolment and performed only 25% of the total number of test strips during the study. It is difficult to say whether these errors were related to the test strip itself or to the testing procedure.

In retrospect, it could have been interesting to re-test index test false negatives by repeating the index tests. However, re-testing was not part of our analysis plan, and could not be reliably performed several months after completion of the field study. Furthermore, it might be overly optimistic to assume that "real-life" clinical lab performance would be less prone to error than what we accomplished with our basic setup.

5.4.3 Can false positive index tests be adressed by confirmatory testing?

Confirmatory testing of positive tests is sometimes done when there are high costs or risks associated with treatment. Confirmatory testing using the same test is theoretically possible, but would likely lead to some true positives being misclassified as negative, due to the imperfect sensitivity of the test. Even if confirmation had been implemented in our evaluation, it is worth noting that three of the eight false positive ICLF strips had confirmed *Cryptosporidium* infection, but below the quantitative cutoff (i.e., likely incidental infection) and would likely yield similar estimates for diagnostic accuracy. Finally, we consider that confirmatory testing with a separate and more specific test would, in most cases, be too impractical for low-resource settings.

5.4.4 Determining the minimum diarrhoea-free period in controls

An editorial comment questioned our choice of a short diarrhoea-free period in controls, where we defined a control as "diarrhoea-free" if they reported no diarrhoea in the 48 hours prior to enrolment, arguing that asymptomatic shedding could lead to misclassification in terms of case and control status [204]. The short diarrhoea-free period was also questioned by one of the reviewers.

First, we note that self-reported diarrhoea within the previous month were about the same among cases and controls (17% and 16%, respectively), not counting the presenting episode in cases. Some epidemiological case-control studies required a much longer diarrhoea-free for controls ; for example, a requirement of 1 month [201], 2 weeks [205] or 7 diarrhoea-free days [206], but notably without applying the same rule to cases. Putting aside the reduced power for case-control comparisons that may result from excluding controls, and the increased risk of recall bias, we only consider a 7 or 14-day diarrhoea-free period as epidemiologically sound if cases with a previous episode of diarrhoea within the same 7- or 14-day period were also excluded from the analysis. A 7-day diarrhoea-free period prior to the presenting diarrhoeal episode was enforced in GEMS [12], but for our purposes, would have artificially excluded an important, and possibly extra vulnerable, subgroup of cases from the study. Excluding cases based on a diarrhoeal episode prior to the current episode does not feel meaningful for a diagnostic test used in a healthcare setting where the primary justification for testing is to provide treatment for diarrhoea. Although the issue of case ascertainment bias from asymptomatic shedding is a crucial one, our main approach was to establish quantitative cutoffs, and by carefully distinguishing between *Cryptosporidium* infection and cryptosporidiosis in the analysis.

A sensitivity analysis applying a stricter diarrhoea-free period for controls

Simply put, a sensitivity analysis involves the experimental manipulation of the inputs (or underlying assumptions) in a model, to see what impact it may have on the outcomes. To measure the possible impact of varying the diarrhoea-free period, we re-did all statistical analyses (in paper I) after excluding all controls who reported diarrhoea within the preceding 7 days. Six controls were thereby excluded from the

qIFAT ROC-analysis for determining quantitative cutoffs; four controls were excluded from the qPCR analysis. A similar exercise was performed after imposing an even stricter 14-day exclusion criterion. The quantitative cutoffs obtained remained the same as what had been obtained with the (per protocol) 48-hour criterion, which means that the diagnostic accuracy estimates would not have been different. A caveat with this experiment is that we are unable to also exclude cases who might have had diarrhoea in the preceding 7 (or 14) days before the current episode started. A limitation of our study was the omission of questions to cases about previous diarrhoeal episodes within, e.g., 7 and 14 days prior to onset of the current episode - we only asked if they had a previous episode of diarrhoea within the last month.

5.4.5 Unknown sampling fraction for cases and controls

Case-control studies have a carefully defined source population, i.e., the "study base" from which cases and controls arise. As our cases were enrolled in health facilities, inferences about disease incidence within the source population cannot be reliably made without knowing the extent to which children with diarrhoea sought care, i.e., the "sampling fraction". For example, in the GEMS case-control study, sampling fraction was estimated by conducting a Healthcare Utilization and Attitudes Survey (HUAS) within each catchment area around the sentinel sites. Furthermore, the GEMS sites were all located within demographic surveillance sites (DSS) where detailed and up-to-date demographic data were available. We were unable to conduct our study within a DSS, furthermore, we did not have a sufficient budget or infrastructure to conduct a HUAS within our catchment areas. We accepted this limitation, as our primary focus was clinical, i.e., strengthening the evidence base for interventions targeting children who present for health care. However, it is important to not directly extrapolate from our prevalence or risk factor estimates back to the source population at large.

5.4.6 Seasonal variation as a confounding factor

As Figure 8 illustrates, there was a pattern of variation, likely seasonal in nature, for positive *Cryptosporidium* detections (using qPCR detections here for added granularity in the graph, however a similar coarse pattern was seen irrespective of test method)

Figure 8 Cryptosporidium detections over time

In the risk factor analysis, we attempted to control for residual confounding by seasonal variation, i.e., beyond what was already accomplished by the concomitant recruitment of cases and controls using frequency matching. Adjustment of all models by three-month chunks ("quarters") across the whole 18-month study period seemed a suitable compromise between adjusting for single months, yielding unnecessary granularity, and being able to capture the underlying pattern (not possible if adjusting for fewer than six quarters). By considering Feb-Apr and May-Jul in the first study year as separate quarters, we were also able to adjust for selection bias that may arise from the change in the target case:control ratio from 10:6 to 10:10 from the end of Jul 2017 (although the actual change in enrolment ratio was less dramatic). Previous reports have linked seasonal variation in cryptosporidiosis to annual variation in rainfall, humidity, and temperature, but with no simple pattern valid across countries and regions [207, 208]. We hope to investigate the underlying causes of seasonal variation in our area in a future study.

5.4.7 Confidence intervals were not provided for the PAF estimates

The width of the confidence intervals for population attributable (PAF) estimates derived from a case-control study will depend on the variability both of the estimated OR and the variability of the prevalence estimate for the risk factor in the population. It is possible to use the 95% CI of the OR to obtain a partial 95% CI around the PAF point estimate. However, this does not also take into consideration the variability around the prevalence estimate. We were unable to identify methods for estimating the CI for PAF estimates in previous hierarchical analyses similar to ours [209-211], or an applicable statistical method for PAF estimation from generalized linear mixed models after multiple chained forest imputation. PAF estimation was considered a secondary objective in the cryptosporidiosis risk factor analysis. Its main function was to avoid interpreting isolated ORs as measures of the relative "importance" of risk factors. The PAFs were intended to provide rough numerical effect estimates for what the observed risk factor associations could translate to in a population similar to ours. For this purpose, we considered it sufficient to provide point estimates.

We emphasize that there are many caveats when interpreting PAFs. We find it most useful to think of PAF estimation as a "counterfactual" or hypothetical way to assist thinking about the possible impact of a risk factors. This rests on several underlying assumptions, e.g., that the observed association represents a causal relationship, that something can be done to reduce the (effects of the) risk factor, and finally, that a significant fraction of cases would not have become cases had the exposure been removed. Ideally, interventional studies targeting putative risk factors are needed to build a stronger case for a causal relationship between the exposure and the outcome.

5.4.8 Counterintuitive reference levels in the risk factor analysis

Some referent categories used in the regression analyses may seem arbitrary or counterintuitive; for example, for household water treatment, the reference level was set to "no water treatment". As we were wanted to think of all associations as putative risk factors, and not protective factors, we would invert the categories to obtain OR point estimates that were higher than 1, if necessary. Risk factors are more meaningful than protective factors (i.e., with ORs under 1) as we would otherwise have obtained

negative PAFs. A similar approach has been taken in similar risk factor studies where PAFs were obtained from ORs [209-211]. It is considered statistically valid to "flip" referent categories as coding schemes that distinguish among the same categories yield equivalent models [212]. Other (not always fully compatible) issues needed to be considered when choosing what to assign as the referent category for a given exposure variable in the risk factor analysis: first, for continuous or integer variables, the categorical (e.g., dichotomized or trichotomized) variable levels needed to adequately reflect whether the underlying exposure-outcome relationship appeared to be linear, "hump-shaped" (e.g., quadratic), or neither. Second, if the relationship appeared to be a linear one, for either cryptosporidiosis or NCrD, but not for both, categories that allowed for both a linear and non-linear relationship needed to be used (for example, for the variable levels classifying the number of household members).

5.4.9 Determining the significance threshold for entry of variables to the intra-level models

In paper II, when deciding what risk factors from the base adjusted univariable models to include in the intra-level models, we applied a criterion that the 95% confidence intervals of the OR should not overlap with 1. This corresponds to a significance level of $P < 0.05$, and might seem like an arbitrary threshold, as some studies use a higher cutoff when performing stepwise regression [210, 213, 214]. The $P < 0.05$ was mainly chosen for simplicity of presentation, as the inclusion of a separate column of (otherwise very uninformative) P-values in addition to the 95% CIs for the ORs would complicate the already quite "busy" result tables of exposure-outcome associations. Secondly, it was simpler and more elegant to use the same criterion for deciding what variables to include in (1) the intra-level models, (2) the models used at entry into the hierarchical analysis, and (3) what was considered as a "significant" risk factor in the final hierarchical analysis. However, we did wonder if the choice of significance level could impact the results obtained in the final models. To scrutinize this further we re-ran all intra-level regression models that were included in paper II using a $P < 0.10$ (instead of $P < 0.05$) criterion for entry of a variable from the univariable models into the intra-level models. After having performed this exercise, the variables that

remained in the final intra-level models (for both cryptosporidiosis and NCrD), and therefore all risk factor associations, remained the same.

5.4.10 Inadequate power to evaluate diagnostic accuracy for asymptomatic infections

There is some evidence from community cohort studies that asymptomatic infections are associated with stunting [156]. In a recent meta-analysis of six studies, *Cryptosporidium* infection in the absence of diarrhoea was significantly associated with decreased HAZ (0.030, 95% CI 0.014 - 0.045), but infection in the absence of diarrhoea was not significantly associated with WAZ or WHZ [35]. Although the proportion of qPCR-positive detections representing "true" infections has not been clearly established, the feasibility or usefulness of screen-and-treat programs for asymptomatic infection has not been established. Evaluating tests for asymptomatic infection was not the main objective of our study, and our preliminary accuracy estimates had a wide margin of uncertainty. Furthermore, a diagnostic accuracy study designed to evaluate asymptomatic screening tests should aim to enrol a higher number of infants, e.g., prior to weaning (i.e., aged 3-6 months), as this age group was underrepresented in our study. Appropriate settings for mass screening should be used for enrolment, e.g., vaccination rooms, day-care facilities, or screening could be integrated with health promotion programmes organized at the health post level.

5.4.11 Possibility of bias from not excluding controls with *Cryptosporidium* infection

As can be seen from table 2 in paper I, 2% of the control group were infected, i.e., with positive MRS, and 1% even had a positive CRS. Of the 725 under-2-year-old controls included in the risk factor analysis (paper II), eight had a positive CRS (8/494, 1.6%, with 231 missing results, mainly because of missing stool sample). Should we have excluded the *Cryptosporidium* infected controls from the risk factor analysis? The main reason we included all controls was because we were more interested in risk factors for cryptosporidiosis than for *Cryptosporidium* infection *per se*. We could have compared *Cryptosporidium* infection rates between cases and controls with and without a given exposure. However, as CRYPTO-POC was designed as a healthcare study, our case definition had healthcare-presentation "baked into" it, meaning we were only really

seeing the tip of the iceberg of community transmission of *Cryptosporidium.* A cohort study with household visits, or a case-control study that enrolled children directly from households, would be a better study design to focus on transmission as the outcome of interest.

5.4.12 The rapid tests strips are not commercially available

Our estimates for diagnostic accuracy of the *Cryptosporidium EZ VUE* test strip should not be extrapolated to other ICLF tests, as accuracy can vary considerably between different kits and manufacturers [92, 93, 97]. We contacted TechLab after submitting the manuscript, and were informed that the company have no current plans to bring the *EZ VUE* test strip to market. The underlying reasons for this are not clear, so we can only speculate that this is based on economic considerations, e.g., the perceived size of the market in order to mass produce and sell the test. This highlights a limitation of profit-driven systems for development and distribution of diagnostics to resource-poor countries. A preliminary solution can be to "create a market" by government programmes funded by donations, for example by setting up public-private partnerships. This method was used to facilitate the roll-out of POC tests for HIV and malaria in sub-Saharan Africa [111]. A useful step in this direction would be to include cryptosporidiosis tests in the WHO model list of essential *in vitro* diagnostics [215]. A limitation to this approach is that vertical programmes supported by foreign donations can maintain and create systems of dependency for low-resource countries. This is neither sustainable, nor just, in the long term. Large-scale political reforms involving government support for research, development, production, and distribution of diagnostic tests, are likely to be needed.

5.5 Challenges and lessons learned from the field study

Several complications arose during the planning and execution of the CRYPTO-POC field study, that, in retrospect, proved to be very valuable from a learning point of view.

5.5.1 Complications during the preparation phase

From the summer of 2016 onwards, there was increasing political unrest and restrictions on communication within Ethiopia, with scattered incidents involving roadblocks and attacks on cars, particularly within the Oromia region where the study was conducted. Importing necessary laboratory equipment (e.g., ultrafreezers) was also challenging. Although we did not suffer complete disruption of study activities, all of the above impacted the execution of the study and led to delays in the initial phase of enrolment.

5.5.2 Paper questionnaires instead of electronic data capture

During the planning stage we went back and forth on whether we should use electronic data capture. The main reason we ended up using paper questionnaires - with resulting huge amount of extra work with data entry, cleaning, and validation - was lack of funding for tablet computers, lack of skilled and trained personnel to maintain a computer tablet park, limited experience with the best electronic data capture software in Jimma University at the time, and lack of reliable systems for data backup during periods of unstable internet access. Several of these issues could have been addressed with more time or better planning. For future studies we would strive to spend more time up-front to ensure a reliable infrastructure to allow electronic data capture, with gains in time after completion of the field study.

5.5.3 Change of the rural study site due to political instability

The original study site, Asendabo Health Center, was situated in Asendabo town, 52 km east from Jimma. However, by early autumn 2016, with increasing political unrest in the country, there had been a road blockade and public demonstrations within Asendabo town. We worried that transport of samples and personnel between Jimma and Asendabo could be disrupted by the unpredictable political situation. At this time both the UN and the diplomatic community within Ethiopia advised against road travel outside of major towns in the Oromia region. We therefore started looking for other health centre sites and settled on Serbo Health Centre, only 16 km east of Jimma. Protocols and ethical approvals were amended accordingly.

Figure 9 Serbo Health Centre (ØHJ, personal photo).

Due to this change in study site, in particular, additional training of local staff on how to conduct clinical field studies, we were not able to start case enrolment until 22 December 2016.

5.5.4 A modified control recruitment procedure

A drawback of changing our health centre study site was that we lost access to the resources of the Gilgel Gibe Health and Demographic Surveillance System Area (HDSS), which covers more than half of the geographical catchment area surrounding Asendabo Health Centre. We therefore lost access to the HDSS project vehicle and also the HDSS field supervisor (with motorcycle), experienced scientific staff that planned to employ part-time for supervision and home visits to conduct the follow-up study (paper III) and household recruitment of healthy controls (paper I, paper II). Crucially, it also meant that we could not use the HDSS household lists and household numbering system that would simplify selection of matched controls and follow-up visits. For these reasons, the logistics of field recruitment of controls from the catchment area around SHC would need to use a different method. On study initiation, we thought we had found a reasonable solution by recruiting controls from the busy vaccination room in SHC. However, after the initial weeks of enrolment (considered a pilot phase for control recruitment), it became apparent that most of the suitable controls from

vaccination rooms would be under the age of 12 months, as catch-up vaccinations were uncommon. This method would provide insufficient number of controls in the 12-59-month age range. We therefore decided to enrol controls aged 12-59 months by direct household visits, despite the significant added logistical challenge.

After extensive discussions within the team and with epidemiologists who had experience from large case-control studies in LMIC, we decided to replace our initial plan of individual matching of controls to cases (as was done in GEMS) with a more flexible and statistically robust system of frequency matching. This is sometimes referred to as "matching by strata". We devised a system for frequency matching by age and residential area (kebele, an administrative unit comprising around 5000 people) and accounted for seasonal variation in diarrhoeal incidence by planning target control numbers on the number and characteristics of cases enrolled in the previous week. An algorithm was devised that involved randomized satellite-map-based selection of households. To be able to reach rural households without having access to numbered households or HDSS infrastructure, we instead trained our own study nurses, who travelled by a combination of public transport and hired motorcycles.

We also attempted to limit the need for household visits in the follow-up sub-study (paper III) by providing travel cost compensation for caretakers and children returning to the JMC or SHC for follow-up visits and stool sample delivery, and by combining community follow-up visits with control recruitment visits, when feasible. Due to the change in site and in our decision to use frequency-matching, we were only able to formally start enrolling controls from 27. Feb 2017. This delay was frustrating at the time, but, in retrospect, we learned a lot from the process. The end result was, in effect, an epidemiologically sound case-control design, conducted on a shoestring budget, without access to the full resources of a HDSS.

5.5.5 Practical challenges with stool collection

During the first six months of the study, we obtained stool samples successfully from, on average, 75% of study participants. Various practical challenges in the process of stool collection became apparent during the initial phase. As most cases were not

admitted, many left the enrolment area soon after examination and interview, but before we could collect a stool sample. Many caretakers had limited time (or resources) to wait, and some caretakers struggled with the stool collection method. We encouraged all study participants to stay on-site until a sample had been collected and devised a small compensation scheme for the children's caretakers in order to facilitate stool collection. Study nurses also tried to reach caretakers by phone when available.

5.5.6 Turnaround time issues

The high test turnaround time (TAT) for some samples, mainly due to delays during the pre-analytical and post-analytical phases [216], may partly be a "study effect", because cryptosporidiosis treatment was unavailable. This would need to be addressed before implementation of LED-AP. One can speculate that the (smaller) analytical fraction of TAT can be brought down by using the test strip. Unfortunately, TAT could not be formally compared between LED-AP and the test strip, as we were unable to measure TAT for the test strip. This was because of logistical constraints of our study setup. For example, blinding between test strips and LED-AP would not have been possible in the SHC lab without more space (i.e., two separate laboratory areas) and employing additional laboratory technicians to the routine health centre staff.

6. Conclusions

This work investigated diagnostic testing, risk factors, and parasite shedding patterns in young children with cryptosporidiosis in Ethiopia. From the studies included in this thesis, the following main conclusions can be drawn:

Paper I – We evaluated two tests considered as possible candidates for near-patient cryptosporidiosis testing in low-resource healthcare settings, but where diagnostic accuracy had not yet been evaluated in large-scale prospective clinical field studies. We demonstrated that both light-emitting diode fluorescence microscopy after auramine-phenol staining (LED-AP), and a room-temperature-stable immunochromatographic lateral-flow test strip had high accuracy for the diagnosis of cryptosporidiosis. The sensitivities and specificities of the two tests were markedly similar when compared with a composite reference standard comprising quantitative immunofluorescent antibody test (qIFAT), ELISA, and quantitative PCR (qPCR). *Cryptosporidium* was significantly associated with diarrhoea by all detection methods, but the strength of the association varied by test modality. Most of the qPCR-positive samples that were negative by LED-AP and the test strip had low quantities of *Cryptosporidium* DNA. The predictive values of LED-AP and the test strip support clinical usefulness in our high-prevalence population, as children who tested positive by either test had 88% probability of having cryptosporidiosis and those testing negative had 99% probability of not having cryptosporidiosis. We described important operational issues relevant for sustainable implementation of near-patient cryptosporidiosis testing in low-resource settings, including quality control issues, turnaround times and cost-per-test estimates.

Paper II – In the final hierarchical analysis of putative risk factors, side-by-side comparisons of factors associated with cryptosporidiosis and non-cryptosporidiosis diarrhoea (NCrD) indicated that caregiver-related socioeconomic factors, public-tap water use, previous illness, and moderate acute malnutrition were more strongly associated with cryptosporidiosis than with NCrD. Unsafe child stool disposal, prematurity and early cessation of exclusive breastfeeding were significantly

associated with NCrD only, whereas previous healthcare attendance and low maternal education were only associated with cryptosporidiosis. By estimation of population attributable fractions (PAF), we estimated that some factors contributed only a small number of cases despite having a strong risk association based on odds ratios. Socioeconomic factors - particularly low maternal education - and public tap water use, were apparently more important risk factors for cryptosporidiosis than for NCrD. The most important cryptosporidiosis risk factors identified by the study, based on PAF estimation, were public tap water consumption (OR 3.8, PAF 27%), one or more visits to hospital or health centre due to illness, since birth (OR 2.3, PAF 27%), maternal education of shorter than one year (OR 2.2, PAF 19%), and acute malnutrition (OR 7.2, PAF 16%).

Paper III – The key finding from this paper was that prolonged oocyst shedding is common in a paediatric clinical population with cryptosporidiosis. Using two complementary modelling strategies, we estimated that the median duration of *Cryptosporidium* shedding was 31 days (95% CI, 26 to 36 days) after onset of the diarrhoeal episode. A striking pattern was observed with an average 10-fold drop in the quantity of shedding per week, for the first 4 weeks. No significant differences were observed in shedding patterns when we compared cases with and without acute malnutrition. There were no obvious differences by age group, sex, or diarrhoeal severity as assessed by dehydration status on enrolment. No significant differences were seen between *C. parvum* and *C. hominis*, however we observed longer and more intense shedding in *C. hominis* gp60 subtype allele family Id, as compared to other *C. hominis* subtypes.

7. Future perspectives

7.1 Implications for practice

7.1.1 Implement LED-AP testing and nitazoxanide treatment for cryptosporidiosis

CRYPTO-POC is to our knowledge the first adequately powered, prospective, diagnostic accuracy study for cryptosporidiosis in children who present for health care with diarrhoea in a low-resource setting. We have demonstrated that AP can be used for cryptosporidiosis testing using simple and robust LED fluorescence microscopes. The method can be considered a "dual-use technology" that can be easily integrated with pre-existing laboratory infrastructure for tuberculosis testing. The first steps required to establish cryptosporidiosis testing is an introductory training session and to set up simple systems for quality control. To facilitate local implementation, we have shared detailed information on operational issues, cost per test calculations, standard operating procedures, supervision, and quality control systems for LED-AP (paper I, supplementary appendix). Should the test strips become commercially available at a reasonable price, they can provide a practical alternative for facilities that cannot perform microscopy. Turnaround times should be carefully monitored. Challenges with sample transport and result communication is not isolated to stool testing and should ideally be addressed by quality improvement projects within low-resource clinical laboratory systems [216, 217].

The currently available trial evidence indicates that targeted cryptosporidiosis treatment can impact both morbidity and mortality [68]. We believe our findings support roll-out of LED-AP testing and treatment with nitazoxanide in high-prevalence settings. This will require government approval and provision of this under-utilized drug in many countries where it is currently unavailable, including Ethiopia. In Latin-America, the Middle East, India, and a handful of African countries (e.g., Egypt, South Africa, Zambia), nitazoxanide is already available as a generic drug formulation, in oral mixture form, and is registered for use in children 12 months and older. Provision of nitazoxanide will need to be supported by the incorporation of cryptosporidiosis

testing in clinical treatment guidelines. Current case management algorithms recommend stool testing only in dysentery or HIV-related persistent diarrhoea and provide no guidance on the specific choice of tests [69].

7.1.2 Antimicrobial stewardship

Watery diarrhoea is over-treated with empirical antibiotics in LMIC [218]. In GEMS, detection of *Cryptosporidium* at diarrhoea-associated quantities rarely occurred alongside other pathogens requiring antimicrobial treatment [91]. Positive *Cryptosporidium* testing may support treatment decisions of not giving antibiotics [218]. This approach could be strengthened by providing local data on the prevalence of treatable bacterial infections, e.g., *Shigella* and EIEC infections, in children who present with non-bloody diarrhoea [219].

7.1.3 Public tap water safety

The surprising finding that public tap water use was more strongly associated with cryptosporidiosis than use of water from unimproved sources provides an additional argument for improved drinking water surveillance in LMIC. Water analysis for *Cryptosporidium* is challenging and the standard methods are too expensive for low-income countries [220]. A first step can be to strengthen the routine surveillance for faecal indicator bacteria, supplemented by targeted risk assessment investigations of public tap water sources.

7.1.4 Access to treatment for both severe and moderate acute malnutrition

Our risk-factor study highlighted the key importance of acute malnutrition, including the often-neglected group with MAM, as well as caregiver-related factors, as risk factors for healthcare-presenting cryptosporidiosis. These findings may be relevant for testing provision. In settings with limited access to testing or drugs, local stakeholders can consider giving priority to children with known risk factors for cryptosporidiosis, i.e., who have a higher pre-test probability of disease, or children with an expected higher risk of morbidity or mortality from the disease, e.g.; children with MAM or SAM, severe dehydration, or prolonged or persistent diarrhoea [13, 142].

Expanding access to supplementary feeding programmes for MAM can be justified by the prevention of the high-mortality condition of SAM [221]. Supported by our findings, treatment of MAM may have the additional benefit of preventing cryptosporidiosis. Although we have focused here on its strong association with cryptosporidiosis, MAM deserves to be highlighted as an important public health issue in its own right [167]. In Ethiopia, supplementary feeding programmes for MAM are only available if an administrative region as a whole is considered food insecure. The unstated underlying assumption seems to be that MAM acquired from a large-scale special event, like a famine, warrants treatment, but not if it is suffered as the result of an endemic condition of poverty. Supplementary feeding programmes should be made available on a much larger scale, and in the very least for children who present for health care.

7.2 Relevance for future research

7.2.1 The need for further phase III diagnostic accuracy studies

As a general rule, diagnostic accuracy of new tests should be determined in a setting that is as representative as possible for where the test will be used [116]. Our study was conducted in a hospital and a health centre, where testing was conducted by clinical laboratory technicians. It might be feasible to provide testing and treatment at the village health-post level, but diagnostic accuracy may vary when testing is performed by village health workers. It would be useful to first determine diagnostic accuracy, predictive values, and operational utility in a large multi-site study involving several health posts. Community support and external validity of such a study may be improved if the study is combined with provision of nitazoxanide and supplementary food for children with MAM.

7.2.2 The need for “phase IV” diagnostic evaluations

Cryptosporidiosis testing should, ideally, be evaluated as a joint intervention of testing-and-treatment, i.e., in a diagnostic randomized controlled trial (D-RCT) [115]. For new or repurposed drugs, the first step will be to follow the conventional phased approach, culminating with an RCT of drug efficacy. Even in non-diagnostic RCTs (i.e., where

the randomization happens after testing rather than before) case ascertainment and randomization should rely on diagnostic tests of high accuracy that can be realistically implemented in care. For nitazoxanide, clinical efficacy has already been demonstrated by trials that relied on microscopic diagnosis for case ascertainment [64]. The extent to which testing itself (followed by treatment) improves outcomes has not yet been examined. A D-RCT could evaluate stool collection, turnaround times, and treatment provision, in addition to treatment efficacy. These are important operational complexities to factor in before large-scale roll-out of testing. The trial could include a separate arm to investigate nitazoxanide safety in 3-9-month-olds and should ideally include follow-up for long-term outcomes. If adequately powered, a D-RCT can also assess the impact of testing-and-treatment in specific vulnerable subgroups, e.g., children with prolonged diarrhoea or acute malnutrition [167].

7.2.3 Other treatable causes of diarrhoea

Besides *Cryptosporidium*, another key treatable cause of both acute and prolonged diarrhoea is *Shigella*. Current treatment guidelines do not recommend antibiotics or testing for *Shigella* infection unless there is dysentery [69]. However, we now have ample evidence that *Shigella* is a leading cause also of non-bloody diarrhoea and that these episodes are associated with increased mortality [219]. Near-patient tests for *Shigella* are in development [222, 223] and could be included in a D-RCT targeting both *Shigella* and cryptosporidiosis. An added benefit of multi-pathogen near-patient testing can be improved antimicrobial stewardship by limiting unnecessary empirical treatment [218].

For children presenting with prolonged diarrhoea, the full aetiological spectrum of pathogens has not been systematically studied in case-control studies using quantitative methods [167, 202, 224-226]. Baseline data on the comparative aetiology of acute, prolonged, and persistent diarrhoea will be useful for planning test-and-treat interventions for this vulnerable group of children [167]. As CRYPTO-POC enrolled children with both acute, prolonged, and persistent diarrhoea, we intend to conduct a multi-target qPCR case-control study to provide more data on the spectrum of enteropathogens associated with prolonged diarrhoea [227].

7.2.4 *Cryptosporidium* shedding duration as a secondary outcome in drug trials

Previous pharmaceutical trials for cryptosporidiosis investigated the key outcomes of diarrhoeal duration, severity, and survival in a short time period after treatment. The effect of treatment on linear and ponderal growth, cognitive development, or other long-term health sequela have not been studied [68]. It may be useful to consider *Cryptosporidium* oocyst shedding duration, and quantitative shedding patterns, as secondary outcomes in drug trials. *Cryptosporidium* oocyst shedding can be approximated using a similar approach as in paper III, e.g., by weekly follow-up stool sampling, for at least six weeks, to capture the prolonged shedders. An important caveat to this approach is that *Cryptosporidium* DNA concentration is, at best, a proxy for the total number of infective oocysts that are shed into the environment from an episode of cryptosporidiosis. A more accurate estimate would require the collection of all stools passed by the child, in order to quantify the relationship between spot measurements of DNA and the actual number of oocysts that are shed. Based on the 10-fold weekly drop in DNA concentration that we observed, we can formulate a hypothesis that an overwhelming fraction (i.e., over 90%) of the total number of oocysts are shed during the first 1-2 weeks after onset of diarrhoea. This should be testable: a small trial can perform total stool collections by frequent household follow-up visits, or in a trial restricted to inpatients. The inclusion of quantitative oocyst microscopy (by either IFAT or LED-AP) would strengthen inferences about the relationship between DNA quantification and oocysts.

7.2.5 Interventions to limit *Cryptosporidium* transmission within neighbourhoods and households

A comprehensive multi-site study in sub-Saharan Africa, supported by *Cryptosporidium* typing, found household or neighbourhood transmission clusters linked to 36% of cryptosporidiosis cases that had been diagnosed in healthcare facilities using a rapid test [161]. The study could not ascertain whether these transmission clusters represented secondary transmissions or infections arising from a common environmental source. Cryptosporidiosis drug trials can cast light on this question by including follow-up and sampling of household and neighbourhood contacts. This

would enable us to investigate whether cryptosporidiosis treatment prevents transmission, and the inclusion of a placebo group can strengthen inferences about the direction of transmission. A commentary to paper I highlighted the possibility of including close contacts in a test-and-treat strategy, even if these contacts have asymptomatic *Cryptosporidium* infections [204]. It is not clear what proportion of cryptosporidiosis episodes are caused by infections acquired from asymptomatic shedders. Drug trials can investigate this by providing prophylactic or targeted administration of anti-cryptosporidial drugs to household contacts of children diagnosed with cryptosporidiosis. All household members should be monitored for diarrhoea symptoms. The comparator should ideally be households receiving "placebo prophylaxis", and the study endpoint would be subsequent change in cryptosporidiosis incidence.

7.2.6 Interventions targeting caregivers

Children with a previous history of illness, or with current acute malnutrition, were identified as a higher-risk group for cryptosporidiosis. Low maternal education was also a risk factor for cryptosporidiosis. In addition to cryptosporidiosis testing and treatment, a bundle of secondary preventive interventions could be carefully designed to target the caregivers of children who have presented to healthcare with diarrhoea, and/or who have acute malnutrition. The bundled intervention can include training of caregivers in evidence-based diarrhoea management, with a focus on oral rehydration salts, zinc supplementation [228], breastfeeding, and supplementary feeding. Barriers to healthcare access could be specifically targeted for this higher-risk group, e.g., with monetary reimbursement of transport cost or loss of caregiver income [229] and by skipping the queue for clinical care of future diarrhoeal episodes. Although this kind of trial cannot be blinded, caregivers can be randomized to receive the intervention, with comparison of outcomes (cryptosporidiosis incidence, growth) between children in intervention and non-intervention households.

7.2.7 The importance of confounder adjustment and causal frameworks in risk factor evaluations

The importance of a causal framework in cryptosporidiosis risk factor studies was highlighted in paper II. Sociodemographic risk factors were identified and were considered as confounders of more proximal risk factor relationships. In particular, the increased risk associated with low maternal education was largely mediated through intermediate risk factor levels. Without the hierarchical framework underpinning the risk factor models, socioeconomic risk factors would have been biased downwards in importance.

7.2.8 Political interventions

In our risk factor study, we used common proxy variables to represent socioeconomic status and categorized these as "level 1" variables in our causal hierarchy. The observed level 1 associations (e.g., maternal education, and having a non-mother primary caregiver) indicate that socioeconomic inequalities are important risk factors for cryptosporidiosis. There is a strong independent association between parental education and child mortality in general, and educational reforms can be investigated as a public health intervention [230]. It is important to highlight that the most important social determinants of health, including risk factors for cryptosporidiosis, are likely to act at more distal levels than those explored by our study. These "level zero" variables include poverty and socioeconomic inequality at the village, sub-regional, regional, national, and global levels [231]. Quantifying the effect of these risk factors on cryptosporidiosis would require large-scale analyses that aggregate individual-level data from diverse countries and regions.

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Performance and operational feasibility of two diagnostic tests for cryptosporidiosis in children (CRYPTO-POC): a clinical, prospective, diagnostic accuracy study

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For the Amharic translation of the abstract see Online for appendix 1

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Summary

Background Cryptosporidiosis is a common cause of diarrhoea in young children (aged younger than 24 months) in low-resource settings but is currently challenging to diagnose. Light-emitting diode fluorescence microscopy with auramine-phenol staining (LED-AP), recommended for tuberculosis testing, can also detect *Cryptosporidium* species. A lateral-flow test not requiring refrigerator storage (by contrast with most immunochromatographic lateral-flow assays) has also recently been developed for *Cryptosporidium* spp detection. We aimed to evaluate the diagnostic accuracy and operational feasibility of LED-AP and the lateral-flow test strip for cryptosporidiosis in children.

Methods We did a prospective diagnostic accuracy study in two health-care facilities in Ethiopia, in a consecutive series of children younger than 5 years of age with diarrhoea (three or more loose stools within the previous 24 h) or dysentery (at least one loose stool with stains of blood within the previous 24 h). Stool samples were tested for *Cryptosporidium* spp by LED-AP and the lateral-flow test strip; accuracy of each test was estimated by independent and blind comparison with a composite reference standard comprising quantitative immunofluorescent antibody test (qIFAT), ELISA, and quantitative PCR (qPCR). Quantitative cutoff values for diarrhoea-associated infection were established in an embedded case-control substudy, with cases of cryptosporidiosis coming from the 15 districts in and around Jimma and the eight districts surrounding Serbo, and community controls without diarrhoea in the previous 48 h recruited by weekly frequency matching by geographical district of the household, age group, and enrolment week.

Findings Stool samples from 912 children with diarrhoea or dysentery and 706 controls from the case-control substudy were tested between Dec 22, 2016, and July 6, 2018. Estimated reference-standard cutoff values for cryptosporidiosis positivity were $2{\cdot}3\times10^5$ DNA copies per g of wet stool for qPCR, and 725 oocysts per g for qIFAT. LED-AP had a sensitivity for cryptosporidiosis of 88% (95% CI 79–94; 66 of 75 samples) and a specificity of 99% (98–99; 717 of 726 samples); the lateral-flow test strip had a sensitivity of 89% (79–94; 63 of 71 samples) and a specificity of 99% (97–99; 626 of 635 samples).

Interpretation LED-AP has high sensitivity and specificity for cryptosporidiosis and should be considered as a dual-use technology that can be easily integrated with existing laboratory infrastructures in low-resource settings. The lateral-flow test strip has similar sensitivity and specificity and provides an alternative that does not require microscopy, although purchase cost of the test strip is unknown as it is not yet available on the market.

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Introduction

Cryptosporidiosis is the fifth leading cause of diarrhoeal mortality worldwide and caused more than 48 000 deaths and led to a loss of 12·9 million disability-adjusted life-years in 2016, with the highest burden in sub-Saharan Africa.[1] The disease ranked among the top five causes of diarrhoea in the Global Enteric Multicenter Study in 2007–11,[2,3] and has probably increased in relative importance since the rotavirus vaccine was rolled out globally.[4] There is no vaccine against *Cryptosporidium* species and the only approved drug, nitazoxanide, has moderate effect on diarrhoea and parasite clearance; although the drug significantly reduced mortality in one trial, it is not effective in children with HIV.[5] Increased effort has been put into developing better drugs for cryptosporidiosis,[6] but a test-and-treat strategy will require a rapid, low-technology, reliable, and affordable diagnostic test that can be used near the point of care, and that is suitable for use in low-resource settings.[7] Diagnostic accuracy evaluations that are not done in the relevant clinical setting over-estimate diagnostic accuracy and field applicability.[8] We identified only one study from east Africa that evaluated immunochromatographic lateral-flow assays (ICLFs) in children with complicated severe acute malnutrition, reporting moderate sensitivity against PCR results,[9] and a Turkish study that evaluated modified Ziehl-Neelsen

Research in context

Evidence before this study

Cryptosporidium species are a common cause of watery childhood diarrhoea and an important cause of morbidity and mortality, particularly in sub-Saharan Africa. Current treatment for cryptosporidiosis is suboptimal, despite new drugs being in development, which emphasises the need for efficient and effective diagnostics. Most diagnostic tests in current use are either inaccurate or satisfy few of the commonly recommended criteria for an ideal diagnostic test for resource-limited settings. Targeted treatment strategies should be promoted on the basis of evidence of accuracy and operational performance from studies in representative clinical settings under field conditions.

We did a semisystematic review within PubMed using the search terms "cryptosporid*" AND ("test" OR "microscop*" OR "assay" OR "detect*") AND ("accuracy" OR "sensitivity" OR "specificity" OR "diagnos*" OR "trial*") AND ("diarrh*" OR "gastroenteritis" OR "gastrointestinal symptoms") for articles in all languages, published from database inception to Jan 20, 2020, to identify studies on diagnostic accuracy for *Cryptosporidium* spp infection that included information on diarrhoeal symptoms with a minimum of 50 positive and 50 negative samples. We initially searched for prospective studies in consecutive series of children in low-income or middle-income countries (LMICs), as this is the recommended study design for external validity. Two studies were identified: one study from a middle-income country (Turkey) that reported 40% sensitivity of the traditional modified Ziehl-Neelsen staining microscopy method against ELISA, and one study from east Africa (Kenya and Malawi) that reported moderate sensitivity (43–77%) of three immunochromatographic lateral-flow (ICLF) tests against any detection by PCR—this was, however, a study in children with complicated severe acute malnutrition, and the estimates might not apply to the general paediatric population. Two studies from Bangladesh (one retrospective) reported good accuracy of ICLFs against diarrhoea attributed to *Cryptosporidium* spp by quantitative PCR; however, they used samples from a birth cohort study, with repeated samples, and their findings might not extrapolate to clinical settings.

A retrospective multicentre study from the UK reported 92% sensitivity and 100% specificity of auramine-phenol (AP) staining against PCR and immunofluorescent antibody tests, but we identified no clinical diagnostic accuracy studies on the use of AP for cryptosporidiosis in children in LMICs. Furthermore, we did not find any field studies in LMICs that included data on operational issues, such as comprehensive cost calculations and test turnaround times.

Added value of this study

We evaluated the diagnostic accuracy of two simple tests (light-emitting diode fluorescence microscopy with AP staining [LED-AP] and a lateral-flow test strip) for cryptosporidiosis in children in a low-income country, and included cost-per-test calculations and operational assessment. Asymptomatic infections were controlled for by using quantitative reference methods. The sensitivity of LED-AP was consistent with estimates from a study in a high-income country, while maintaining high specificity. The lateral-flow test strip had similar sensitivity and specificity to LED-AP.

Implications of all the available evidence

Test-and-treat strategies for diarrhoea that include accurate detection of the aetiological agent will increase the chance of a useful clinical response. LED-AP is a reliable and affordable test for cryptosporidiosis that can be integrated with existing laboratory infrastructure, near the point of care, in LMICs. The lateral-flow test strip could be an alternative when LED-AP is unavailable, but is likely to be more expensive. Laboratories in LMICs that use modified Ziehl-Neelsen staining microscopy for *Cryptosporidium* spp testing could consider switching to LED-AP.

staining microscopy.[10] The Turkish study concluded, concurring with many previous reports, that modified Ziehl-Neelsen staining microscopy is insufficiently accurate.[5,11]

In 2011, WHO recommended a large-scale shift in first-line tuberculosis testing, to light-emitting diode fluorescence microscopy with auramine-phenol (AP) staining (LED-AP).[12] Available at a reduced price to low-income countries, thousands of LED microscopes have been distributed in Ethiopia and other low-income or middle-income countries (LMICs).[13] AP microscopy, using traditional halogen-bulb fluorescence microscopes, is well established as a diagnostic test for *Cryptosporidium* spp,[11,14] but its field performance in high-prevalence settings is largely unknown, with the exception of a small study in HIV-positive adults in India that reported high accuracy.[15] We found no studies that evaluated AP microscopy against the most accurate reference-standard tests (immunofluorescence antibody test [IFAT] microscopy or quantitative PCR [qPCR]) in children in an LMIC.

We hypothesised that LED-AP would have acceptable diagnostic accuracy (minimum sensitivity 70%, specificity 96%) and operational feasibility for cryptosporidiosis testing in children presenting to health-care facilities in a low-resource setting with high rates of malnutrition. As the most likely next-best alternative in centres without microscopy facilities, we included an ICLF in the evaluation; although ICLFs have variable performance in high-income countries,[16–18] a few have shown promising results in LMICs.[9,19,20] Most ICLFs require refrigeration; however, a lateral-flow test strip for *Cryptosporidium* spp that can be stored at room temperature has now been developed.[20] We aimed to evaluate the diagnostic accuracy and operational feasibility of LED-AP and this lateral-flow test strip for cryptosporidiosis.

Few diagnostic accuracy studies and trials distinguish between *Cryptosporidium* spp infection and cryptosporidiosis, despite asymptomatic infections being well described for *Cryptosporidium* spp and other diarrhoeal

pathogens. Simply detecting a pathogen will have reduced value when the purpose of testing is to select those patients for whom intervention might be of clinical value. Building on studies where quantitative testing in both cases of cryptosporidiosis and community controls has been applied to discriminate between asymptomatic and symptomatic infection,[2,21,22] we chose a panel of reference tests that included two different quantitative assays. We embedded a case-control study in the accuracy study to estimate quantitative cutoff values, and to allow estimation of the predictive value of LED-AP and the ICLF test strip for clinical disease in our study population.

Methods

Study design and participants

We did a prospective diagnostic accuracy study for cryptosporidiosis in children presenting to health-care facilities with diarrhoea or dysentery, in Jimma Medical Centre, Jimma, and Serbo Health Centre, Serbo, in southwest Ethiopia. Jimma Medical Centre is a tertiary referral hospital located in an urban area; Serbo Health Centre covers a rural area around the smaller town of Serbo, approximately 16 km from Jimma.

See Online for appendix 2

Study nurses screened children younger than 5 years of age at the paediatric outpatient departments and all inpatient wards at both centres for eligibility. Children were consecutively enrolled from 0800 h to 1800 h every day at Jimma Medical Centre and from 0800 h to 1700 h in Serbo Health Centre. Children were eligible if they had diarrhoea (three or more loose stools within the previous 24 h) or dysentery (at least one loose stool with stains of blood within the previous 24 h), regardless of whether these were the primary complaints leading them to seek health care. The exclusion criterion was inpatient admission for longer than 24 h before enrolment in the study. Written informed consent was obtained from the children's caregivers and we followed STARD guidelines.[8]

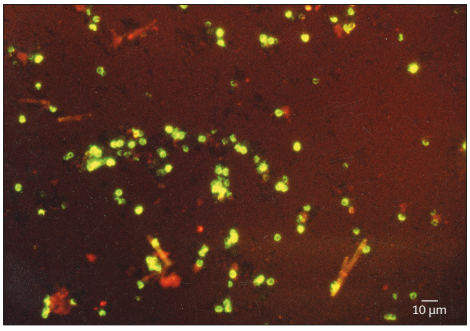

Figure 1: **Auramine-phenol stained *Cryptosporidium* spp oocysts (diameter 4–6 µm), displaying characteristic erythrocyte pattern of staining**
Section of 400 × magnification field (LJR, personal photograph).

The embedded case-control study included children with diarrhoea from the 15 districts in and around Jimma and the eight districts surrounding Serbo. Community controls without diarrhoea in the previous 48 h were recruited by weekly frequency matching by geographical district of the household, age group, and enrolment week.[23]

Jimma University Institutional Review Board (reference RPGC/610/2016), the Ethiopian National Research Ethics Review Committee (reference JU JURPGD/839/2017), and the Regional Committee for Medical and Health Research Ethics of Western Norway (reference 2016/1096) approved the study.

Procedures

Study nurses collected demographic and clinical data using standardised case-report forms. Information about treatment and clinical outcome was collected by interview with caregivers and from hospital records. All participants were tested for HIV (appendix 2 p 2), where possible, and were asked to provide a stool sample. Stool was collected using a nappy, single-use bedpan, or potty, and quickly transferred to a screw-cap plastic container. Study nurses lined nappies with plastic film to avoid water absorption from the stool into the absorbent polymer layer of the nappy. Samples were stored at 4°C until further processing.

All laboratory personnel that performed index and reference testing were fully masked to results of the other tests and to clinical information, including case or control status (ie, diarrhoea or no diarrhoea) of the participants. LED-AP testing was done by medical laboratory technicians trained in the method according to a described standardised operating procedure (appendix 2 p 16) in addition to their routine laboratory duties. AP staining was done on a manually homogenised, air-dried stool smear, without preceding sample concentration. The reagents used were the same as those used for auramine staining of sputum smears for acid-fast bacilli, but with a standard operating procedure optimised for detecting *Cryptosporidium* spp oocysts. AP-stained slides were examined using a PrimoStar iLED fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) for objects of the same size and morphology as *Cryptosporidium* spp oocysts (figure 1) and were categorised according to average number of oocysts per 200×magnification field of view: one to nine, ten to 50, or more than 50 (appendix 2 pp 2, 17).

Findings were reported on a standardised results form. Microscopy slides were kept at ambient temperature in a closed, non-transparent box. Once per month, the study investigator (ØHJ) selected ten slides for blinded review and to assess microscope settings and the quality of stains (appendix 2 pp 21–22); result discrepancies did not affect the recorded results of LED-AP testing, but were used as a basis for immediate feedback and on-site retraining for laboratory technicians.

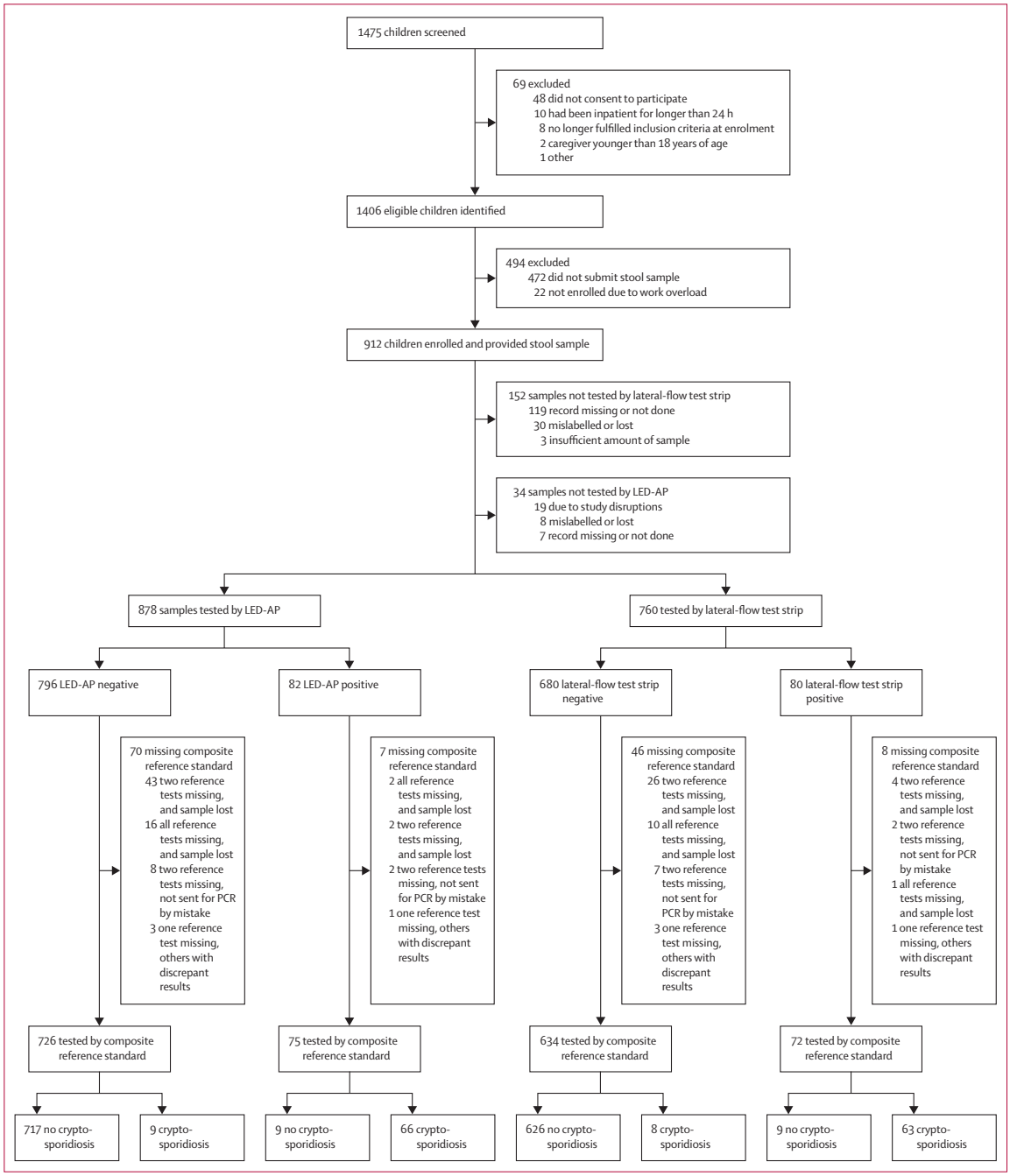

Figure 2: **Study flowchart**
LED-AP=light-emitting diode fluorescence microscopy with auramine-phenol (AP) staining.

	Participants
Age in months, median (IQR)	12 (8–22)
Female sex	379/912 (42%)
Diarrhoea episode	
Duration in days, median (IQR)	3 (2–5)*
Acute episode (1–6 days)	745/907 (82%)
Prolonged episode (7–13 days)	116/907 (13%)
Persistent episode (14 days or longer)	46/907 (5%)
Moderate-to-severe diarrhoea	190/782 (24%)
Dysentery (visible blood in stool)	
By caregiver report	78/910 (9%)
By caregiver report or by laboratory technician inspection of stool†	92/910 (10%)
Severe acute malnutrition	86/911 (9%)
Stunted growth	200/907 (22%)
HIV positive	2/682 (<1%)
HIV exposed	2/682 (<1%)
Rotavirus vaccination	853/910 (94%)
Enrolment site Jimma Medical Centre	445/912 (49%)
Enrolment site Serbo Health Centre	467/912 (51%)

Data are n/N (%) unless otherwise stated. Denominators vary because of missing data for some participants (eg, not recorded or not answered). *N=907. †By gross inspection of the sample after it arrived in the clinical laboratory (data available from 637 samples).

Table 1: **Demographic and clinical characteristics of children with diarrhoea in the diagnostic accuracy study**

Cryptosporidium EZ VUE lateral-flow test strips (TECHLAB, Blacksburg, VA, USA) were used for rapid testing for *Cryptosporidium* spp antigen (appendix 2 p 3). Lateral-flow strip testing was done by laboratory technicians other than those who did LED-AP testing and was done at the Jimma Medical Centre laboratory, twice per week.

As there is no commonly accepted reference-standard test for asymptomatic or symptomatic *Cryptosporidium* spp infections, we used a composite reference panel that included quantitative IFAT (qIFAT) for detection of *Cryptosporidium* spp oocysts, ELISA for detection of *Cryptosporidium* spp antigen, and qPCR for detection of *Cryptosporidium* spp DNA (appendix 2 p 3). Quantitative cutoff values for the association of *Cryptosporidium* spp detection with diarrhoea were defined as the qPCR and qIFAT quantity that maximally discriminated case or control status in the case-control substudy and were given as target DNA copy number (qPCR) or oocysts (qIFAT) per g of wet stool.

Positive microbiological composite reference standard (MRS) was defined as two or more positive reference tests and negative MRS as two or more negative reference tests. Positive clinical composite reference standard (CRS) was defined as two or more positive reference tests (and greater than the quantitative cutoff value for association with diarrhoea) and negative CRS as two or more negative reference tests (or less than the quantitative cutoff value for association with diarrhoea). *Cryptosporidium* spp infection was defined as a diarrhoea case or non-diarrhoea control with positive MRS. Cryptosporidiosis was defined as a diarrhoea case with positive CRS.

Operational data were also collected and these included test turnaround times, cost of reagents and laboratory expendables used for LED-AP and ICLF strip testing, standardised report forms from supervision visits to both enrolment sites, and reports on internal quality assessment at both sites.

Statistical analysis

For the diagnostic accuracy sample-size calculations, we assumed that the main limiting factor would be sensitivity, as its precision would depend on the number of children with cryptosporidiosis that we recruited into the study. With a margin of error of 10%, a power of 90%, and an assumed minimum acceptable sensitivity of 70% for a cryptosporidiosis test to be cost-effective,[24] we estimated that a minimum of 75 children with cryptosporidiosis should be enrolled to achieve the necessary precision in sensitivity. Double data entry was done with EpiData (version 3.1). All data analyses were done using R (version 3.5.2). 95% CIs were used to represent statistical precision and a p value of less than 0·05 was considered significant.

Quantitative cutoff values designed to maximise discrimination between cases and controls were established for *Cryptosporidium* spp qPCR and qIFAT by analysis of all PCR and IFAT-positive cases and controls in the substudy, using a receiver operating characteristic curve analysis with pathogen quantity in stool as the discriminating variable and case or control status as the dependent variable (appendix 2 p 7).

Difference in average *Cryptosporidium* spp quantity (DNA copies per g of stool for qPCR and oocysts per g for qIFAT) between case and control samples was assessed by comparing mean averages in an unpaired Welch two-sample *t* test; if distributions were positively skewed, we used log transformation before the *t* test to achieve normality.

95% CIs for test sensitivity, specificity, and positive and negative predictive values were calculated by the Wilson method,[25] and for positive and negative likelihood ratios with formulae from Simel and colleagues.[26] The strength of association between *Cryptosporidium* spp detection and diarrhoea was approximated by the odds ratio in the case-control substudy. Spearman's rank correlation was used to quantify the association between semiquantitative scoring of positive AP slides (ie, one to nine, ten to 50, or more than 50 oocysts per magnification field of view) with quantitative results obtained by qPCR and qIFAT.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

Results

From Dec 22, 2016, to July 6, 2018, 1475 children with diarrhoea or dysentery were screened, 1384 were enrolled and, of these, 912 provided a stool sample (figure 2; table 1). All children who fulfilled the dysentery definition also fulfilled the diarrhoea definition. The case-control substudy[23] enrolled 1134 children with diarrhoea (with 749 stool samples) and 946 controls (706 stool samples; appendix 2 pp 5–6).

Cryptosporidium spp were significantly associated with diarrhoea with all detection methods used, but the strength of the association varied by test modality (table 2). When comparing *Cryptosporidium* spp quantities in case and control samples from the substudy, the median PCR quantity was $4{\cdot}7\times10^5$ DNA copies per g of stool (IQR $0{\cdot}7–36{\cdot}8\times10^5$) in case samples and $1{\cdot}2\times10^5$ DNA copies per g ($0{\cdot}2–10{\cdot}2\times10^5$) in control samples. The PCR quantity ratio of geometric means in case stools to control stools was 3·8 (95% CI 1·5–9·8). The ratio was similar for IFAT quantification (3·3; 95% CI 1·8–6·1; appendix 2 pp 8–9). After estimating qPCR and qIFAT quantitative cutoff values (appendix 2 p 7) we applied the clinical composite reference standard to classify cases as cryptosporidiosis or non-cryptosporidiosis for the diagnostic accuracy calculations. 77 (9%) of 878 samples tested with LED-AP and 54 (7%) of 760 samples tested with the lateral-flow test strip had incomplete or missing reference-standard results and were excluded from further analysis (figure 2).

Of the 77 children with cryptosporidiosis, 25 (38%) of 66 children (clinical outcome data missing for 11) had moderate-to-severe diarrhoea (appendix 2 p 4) and of the 745 cases without cryptosporidiosis, 149 (23%) of 638 children (data missing for 107) had moderate-to-severe diarrhoea. On enrolment of children both with and without cryptosporidiosis, the median diarrhoea duration was 3 days (IQR 3–7 for children with cryptosporidiosis and 2–4 days for children without). Severe acute malnutrition (appendix 2 p 4) was diagnosed in nine (12%) of 77 children with cryptosporidiosis and 69 (9%) of 744 children without cryptosporidiosis.

In the primary analysis, LED-AP had a sensitivity for cryptosporidiosis of 88% (95% CI 79–94; 66 of 75 samples) and a specificity of 99% (98–99; 717 of 726 samples). The lateral-flow test strip had a sensitivity of 89% (79–94; 63 of 71 samples) and a specificity of 99% (97–99; 626 of 635 samples; table 3).

An exploratory comparison of LED-AP and the lateral-flow test strip showed no significant differences in sensitivity, specificity, or predictive values between the two diagnostic tests (appendix 2 p 11). We observed a higher prevalence of cryptosporidiosis at the Jimma enrolment site than at the Serbo site, but adjusting the positive and negative predictive values for local prevalence had little effect on the estimates (appendix 2 p 11).

There was a positive correlation between semiquantitative grading of LED-AP slides (ie, one to nine, ten to 50, or more than 50 oocysts per magnification field of view) and quantitative results from qIFAT (Spearman's ρ 0·32, p=0·010) and qPCR (Spearman's ρ 0·27, p=0·011). The weakest IFAT-positive sample had only three oocysts in the IFAT sample well (approximately 75 oocysts per g of stool) and this sample was also positive by both LED-AP and the lateral-flow test strip. The lowest *Cryptosporidium* spp PCR quantity that was also positive by LED-AP was 13 836 DNA copies per g of stool; this was also the lowest PCR quantity that was positive by the lateral-flow test strip. Most PCR-positive samples that were negative by LED-AP and the test strip had low quantities of *Cryptosporidium* spp DNA (figure 3). Most false-negative index test results were in the lower range of positive by PCR, and there seemed to be a negative relationship

	Positive test in cases of diarrhoea, n/N (%)	Positive test in controls, n/N (%)	Odds ratio of positive test for diarrhoea (95% CI)*
Index tests			
LED-AP	66/717 (9%)	11/689 (2%)	6·25 (3·27–11·94)
Lateral-flow test strip	67/620 (11%)	13/563 (2%)	5·13 (2·80–9·39)
Reference tests			
ELISA	69/666 (10%)	12/680 (2%)	6·43 (3·45–11·99)
PCR (any detection)	104/652 (16%)	45/668 (7%)	2·64 (1·82–3·81)
qPCR (above cutoff)	67/652 (10%)	15/670 (2%)	5·00 (2·83–8·85)
IFAT (any detection)	72/681 (11%)	25/668 (4%)	3·04 (1·90–4·86)
qIFAT (above cutoff)	48/680 (7%)	7/668 (1%)	7·17 (3·22–15·97)
Reference panels			
MRS	66/669 (10%)	14/676 (2%)	5·18 (2·88–9·31)
CRS	62/669 (9%)	8/677 (1%)	8·54 (4·06–17·98)

Denominators vary because of missing data for some participants (eg, not recorded or not answered). LED-AP=light-emitting diode fluorescence microscopy with auramine-phenol (AP) staining. qPCR=quantitative PCR. IFAT=immunofluorescent antibody test. qIFAT=quantitative IFAT. MRS=microbiological composite reference standard. CRS=clinical composite reference standard. *Wald intervals.

Table 2: **The association between various *Cryptosporidium* spp detection methods and diarrhoea in the case-control substudy**

	LED-AP	Lateral-flow test strip
Cryptosporidiosis prevalence	9% (8–12)	10% (8–13)
Sensitivity	88% (79–94)	89% (79–94)
Specificity	99% (98–99)	99% (97–99)
Positive predictive value	88% (79–94)	87·5% (78–93)
Negative predictive value	99% (98–99)	99% (98–99)
Likelihood ratio of a positive test	71·0 (36·9–136·3)	62·6 (32·6–120·4)
Likelihood ratio of a negative test	0·1 (0·1–0·2)	0·1 (0·1–0·2)

Data are point estimates with 95% CI. Cryptosporidiosis was defined as a case of diarrhoea with a positive clinical composite reference standard. Absolute numbers are shown in the appendix (p 10). LED-AP=light-emitting diode fluorescence microscopy with auramine-phenol (AP) staining.

Table 3: **Diagnostic accuracy of LED-AP and lateral-flow test strip for cryptosporidiosis**

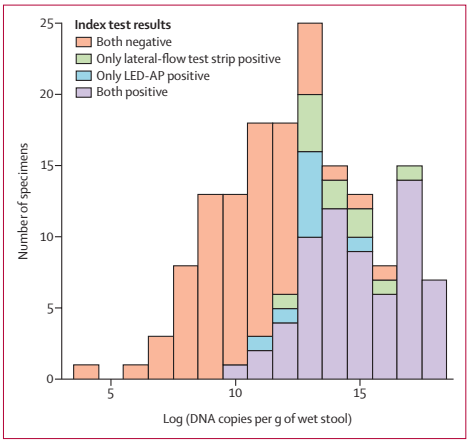

***Figure 3:* Cryptosporidium spp DNA quantity distribution**
Data from 127 diarrhoea cases and 37 diarrhoea-free controls; PCR quantity missing for two samples with positive microbiological composite reference standard. LED-AP=light-emitting diode fluorescence microscopy with auramine-phenol (AP) staining.

between laboratory technician experience and false-negative test results (data not shown).

We did an exploratory analysis of the diagnostic accuracy of the index tests for asymptomatic infection by cross-tabulating LED-AP by the microbiological composite reference standard in controls with no diarrhoea. For asymptomatic infection, LED-AP had a sensitivity of 64% and a specificity of greater than 99%, and the lateral-flow test strip had a sensitivity of 79% and a specificity of greater than 99%, but these estimates had wide margins of uncertainty (appendix 2 p 11).

For turnaround time calculations, we used available data from LED-AP tests done on both case (n=878) and control (n=689) samples (n=1567; appendix 2 p 5). LED-AP was done on the day of stool collection in 1104 (77%) of 1431 tests, the following day in 309 tests (22%), and 2 or more days later in 18 tests (1%; data missing for 136). Total test turnaround time (ie, time from when the sample was obtained until the result was received) was available from 1322 (84%) of 1567 tests; of these, 607 results (46%) came back on the day the sample was obtained, 545 (41%) the following day, and the remaining 170 (13%) came back after 2 or more days. For results received the same day, test turnaround time ranged from 0·5 h to 7·8 h (median 3·5; IQR 1·7–4·9); a breakdown of turnaround times into subphases showed that median duration of preanalytical and postanalytical phases comprised the major fraction of total turnaround time (appendix 2 p 12).

Several operational issues relevant to implementation of LED-AP testing in routine care were identified during the study (appendix 2 p 13). The overall response of the clinical laboratory technicians after commencing AP staining for *Cryptosporidium* spp was encouraging, and they reported that operation and maintenance of the LED microscope was easier than or similar to that of conventional microscopes already in use. A specific benefit of the PrimoStar iLED microscope was the ease of switching between fluorescence and conventional light microscopy operation. A cost-per-test analysis, assuming expendables are purchased in bulk for approximately 500 tests, also factoring in labour time, estimated a cost of US$0·7 per LED-AP test, and $0·6 per lateral-flow strip test (excluding the cost of the test strip itself, as it is currently not yet available for purchase; appendix 2 pp 14–15).

Discussion

We evaluated two tests suitable for near-patient use in children with community-acquired diarrhoea in a low-resource setting. In our combined population, children testing positive for *Cryptosporidium* spp by LED-AP or the lateral-flow test strip had 88% probability of having cryptosporidiosis, and those testing negative had 99% probability of not having cryptosporidiosis. Our study is the first adequately powered prospective diagnostic accuracy study for cryptosporidiosis in a consecutive series of children presenting to health-care facilities with diarrhoea in an LMIC. Testing was done by technicians who had received basic training and supervision, using the most widespread LED microscope in current use, and we found diagnostic accuracy consistent with estimates from a retrospective accuracy study in a high-income country.[11] The *Cryptosporidium* EZ VUE lateral-flow test strips were easy to use and were as accurate as LED-AP, and have the advantage, by contrast with most ICLFs, that they do not require refrigeration.

We aimed for a representative sample by using broad inclusion criteria for all children aged younger than 5 years of age who presented with diarrhoea, by prospective enrolment irrespective of presenting complaint, and without excluding important and vulnerable subgroups, such as children with severe acute malnutrition or diarrhoea of prolonged duration. Test interpretation bias was addressed by full masking of technicians, and by applying the reference standard to all patients, irrespective of index test results.

Our study was insufficiently powered to estimate diagnostic accuracy for asymptomatic infection; these estimates therefore have a wide margin of uncertainty but indicate low sensitivity. Both tests failed to detect many only qPCR-positive samples from asymptomatic children, but we consider this finding as an advantage from a clinical point of view. Many low-level *Cryptosporidium* spp detections on qPCR in children with diarrhoea can probably be explained by diarrhoea from other infectious or non-infectious causes, combined with incidental detection of *Cryptosporidium* spp DNA. These detections could be explained by various reasons other than clinical infection: asymptomatic carriage, postinfectious shedding,

or ingestion of oocysts that are no longer infectious or are from a *Cryptosporidium* species that does not infect humans. The main purpose of a test-and-treat strategy should be to detect the most likely causative agent of a diarrhoeal episode and thereby increase the chance of a useful clinical response. For the purpose of clinical drug trials for cryptosporidiosis, finding the right balance between sensitivity and specificity will increase the power to detect a clinical effect.

A range of factors should be considered when interpreting our findings. First, the study was deliberately done in an area of low HIV prevalence; however, as cryptosporidiosis is more common and severe in patients with impaired cellular immunity, the accuracy or predictive values of the tests might not be representative of settings with high prevalence of untreated HIV disease in children. Similarly, pretest and post-test probabilities for cryptosporidiosis might differ in vulnerable subgroups of children (eg, with prolonged or persistent diarrhoea, acute malnutrition, or severe diarrhoea), but we did not examine this in our study.

Second, missing composite reference-standard results for both LED-AP and the lateral-flow strip tests were somewhat higher than expected and, although the breakdown of reasons for missing tests (figure 2) indicate reasons unlikely to bias the accuracy estimates, missing-at-near-random is still an underlying assumption that warrants caution.

Third, we did not include tests for other diarrhoeal pathogens in this study because of cost constraints, which could lead to misclassification of some diarrhoeal episodes that were due to pathogens other than *Cryptosporidium* spp. Such misclassification could lead to bias and overestimation of specificity and positive predictive values. However, this bias would have a small effect, as there were few positive detections, and because previous reports indicate most high-quantity *Cryptosporidium* spp detections to be associated with diarrhoea, even when in mixed infections.[2,27] Detections of other acid-fast oocysts on LED-AP (eg, *Cystoisospora belli*, *Cyclospora cayetanensis*) occurred too infrequently to have any role (data not reported here).

Fourth, when estimating quantitative cutoff values for qIFAT and qPCR in the case-control substudy, we could not distinguish between new asymptomatic infections and shedding associated with a previous diarrhoeal episode. Although diarrhoea within the last month was reported for some children, the proportion was similar in cases and controls (appendix 2 p 6). Some epidemiological studies require a longer diarrhoea-free period for controls, but, in our opinion, the same criteria should apply to cases and controls. By using a short diarrhoea-free period (48 h), we increased the clinical validity of the quantitative cutoff values, and avoided excluding an important subgroup of cases from the study.

Fifth, the long test turnaround time for some samples could be a challenge for implementation but might, in part, be a study effect, because treatment was unavailable; ideally this should be re-evaluated as part of a test-and-treatment trial. Furthermore, test strip turnaround time was not measured, due to personnel constraints, but its hands-on time was similar to LED-AP. Considering the short hands-on time of LED-AP, the in-laboratory fraction of test turnaround time was large, but a much smaller contributor than the preanalytical and postanalytical phases. This breakdown of turnaround time is likely to reflect challenges in communication between laboratory and clinical personnel, and with sample transport. Preanalytical and postanalytical contributions to test turnaround time would need to be addressed before scaling up testing.

Lastly, as accuracies of specific ICLF kits vary considerably,[16–18] we advise caution against extrapolating the accuracy estimates for the lateral-flow test strip to other ICLFs.

The minimum essential cost of any new cryptosporidiosis drug has been suggested as $2·0 per treatment course.[28] The cost of diagnostics should be added to this, as empirical therapy would lead to unacceptably high rates of unnecessary treatment. In our setup, the added cost of testing amounted to about $0·7 per test for LED-AP, and considerably more for the rapid lateral-flow test strip when including the cost of the test strip itself. Although these cost estimates are well below the estimated cost for each cryptosporidiosis episode in LMICs,[29] our results indicate that a comprehensive cost-effectiveness evaluation and an understanding of the local so-called diagnostic ecosystem would be required before large-scale rollout of either test.[30]

In conclusion, our results show that both LED-AP and the *Cryptosporidium* EZ VUE lateral-flow test strip have high diagnostic accuracy and operational features suitable for inclusion in test-and-treat interventions for cryptosporidiosis. Where LED microscopes are already available, LED-AP testing seems the more affordable, easy-to-implement, and sustainable option.

Contributors

ØHJ had the idea for the study and ØHJ, KH, NL, LJR, and MZ designed the study and obtained funding. AA, ZM, and BE contributed to the study design. AA oversaw local data collection and data management. BE, ØHJ, and MZ supervised clinical staff. ØHJ, AA, ZM, YA, and BS supervised laboratory staff. OB optimised and did the quantitative PCR. ØHJ did the statistical analysis and wrote the first draft of the manuscript. All authors contributed to the interpretation of data and editing of the manuscript, approved the final version, and agree to be accountable for all aspects of the manuscript. ØHJ, AA, NL and KH had full access to the data and could check their validity.

Declaration of interests

We declare no competing interests.

Data sharing

Additional data (deidentified individual participant data) are available from the Norwegian Centre for Research Data, where requirements and conditions for access are also listed (under the Dokumentasjon link). Additional study documentation can be made available upon request to the corresponding author (ØHJ).

For more on **Norwegian Centre for Research Data** see http://dx.doi.org/10.18712/NSD-NSD2884-1-V1

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Supplementary appendix 1

This translation in Amharic was submitted by the authors and we reproduce it as supplied. It has not been peer reviewed. *The Lancet*'s editorial processes have only been applied to the original in English, which should serve as reference for this manuscript.

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በልጆች ላይ የሚከሰት ክሪፕቶስፖሪዲዎሲስ ህመም ለመመርመር የሚያስችሉ ሁለት የላቦራቶሪ መመርመሪያዎች የአፈፃፀም እና የአተገባበር አዋጭነት ጥናት፤

<u>በአጭሩ</u>

መግቢያ፤ ክሪፕቶስፖሪዲዎሲስ (Cryptosporidosis) በለጋ ሕፃናት ላይ (ዕድሜያቸው ከ24 ወራት በታች በሆኑ) ተቅማጥን በማስከተል ከመታወቁም በላይ፤ የኑሮ ደረጃቸው ዝቅተኛ በሆኑ አገራት ይህንን ህመም የመመርመር አቅም በጣም ውስን ነው።፡ይህንን ችግር ለመቅረፍ ለቲቢ ምርመራ እየዋለ ያለውን በብርሃን አሳላፊ ዲዮይድ ፍሎረሰንት ቀላሚ የኦራሚን-ፌኖል (Auramine-phenol) ማይክሮስኮፕ (LED-AP) በመገልገል ክሪፕቶስፖሪዲዎሲስንም መለየት ይቻላል። በቅርብ ጊዜም ማቀዝቀዣ ከምችት የማይፈለገው የላተራል-ፍሎው ምርመራ ለክሪፕቶስፖሪዲዎሲስ ልየታ ተዘጋጅቶ በአግልግሎት ላይ ውሏል። የዚህ ጥናታችንም አላማ በሕፃናት ላይ ተቅማጥ የሚያስከትለውን ክሪፕቶስፖሪዲዎሲስን ለመለየት የሚያስችሉትን የዲዮይድ ፍሎረሰንት ቀላሚ የኦራሚን-ፊኖል (Auramine-phenol) ማይክሮስኮፕ (LED-AP) እና የላተራል-ፍሎው የምርመራ (lateral-flow test) ዘዴዎችን በሽታውን በትክክለኛው የመመርመር ብቃት እና የአተገባበር አዋጭነትን መገምገም ነው ::

የጥናቱ መንገዶች፤ ይህ ጥናት የተካሄደው በኢትዮጵያ ውስጥ በጅማ እና አካባቢው ባሉ ሁለት የጤና አገልግሎት መስጫ ተቋማት ውስጥ የተቅማጥ በሽታ (ባለፉት 24 ሰዓታት ውስጥ ሶስት ወይም ከዚያ በላይ በከፊል የቀጠነ ዓይነምድር ናሙናዎች) ወይም ደም የተቀላቀበት ተቅማጥ (ባለፉት 24 ሰዓታት ውስጥ ቢያንስ አንድ በከፊል የቀጠነ እና ደም የተቀላቀለው የዓይነምድር ናሙና) ያለባቸውን፣ ዕድሜያቸው ከ 5 ዓመታት በታች የሆኑ ሕፃናት ላይ ከትትል በማድረግ ነው። የዓይነምድር ናሙናዎቹ የዲዮይድ ፍሎረሰንት ቀላሚ የኦራሚን-ፌኖል ማይክሮስኮፕ እና የላተራል-ፍሎው ምርመራ ተደርጎላቸዋል። የእያንዳንዱ ምርመራ ትክክለኝነት በተመጣጣኝ የማነፃፀሪያ መስፈርት (composite reference standard) መጠንን ባካተተ ኢሚውኖፍሎረሰንት ምርመራ (qIFAT) ፣ ኢላይዛ (ELISA) እና የፓራሳይት ዘረመል ቁጥር (qPCR) ገለልተኛ እና ስውር ንፅፅር ተገምቷል። ከተቅማጥ ጋር ተያይዞ ለሚመጣ ህመም የቁጥር ልኬት (cut off value) የተዘጋጀው በጅማ እና አካባቢው ካሉ 15 ወረዳዎች እና ከሰርቦ ዙሪያ ከሚገኙት 8 ወረዳዎች ከመጡ የክሪፕቶስፖሪዲዎሲስ ታማሚዎች ሲሆን ባለፉት 48 ሰዓታት ውስጥ ተቅማጥ የሌላቸውን እንደ ማነፃፀሪያ በመገልገል በየሳምንቱ መልካሳ ምድራዊ ክልልን ባገናዘበ የቤተሰብ፣ የእድሜ ክልል እና ሳምንታዊ የህክምና ምዝገባን መሰረት ያደረገ የማዛመድ ስራ ተከናውኖዋል።

የጥናቱ ውጤት፤ እኤአ ከዲሴምበር 22 ቀን 2016 እስከ ከጁላይ 6 ቀን 2018 ዓ.ም ባለው ግዜ ውስጥ ተቅማጥ ወይም ደም-የተቀላቀለበት ተቅማጥ ካለባቸው 912 ህጻናት እና ህመም ከሌለባቸው የማነፃፀሪያ ህፃናት ላይ ከተወሰደ 706 ናሙናዎች ላይ ምርመራ ተደርጓዋል፡፡ ለክሪፕቶስፖሪዲዎሲስ ህመም የተገመተው የማጣቀሻ መደበኛ የቁጥር ልኬቶች (reference-standard cutoff values) የነበሩት $2{\cdot}3\times10^5$ የዘረመል ቁጥር በአንድ ግራም እርጥብ ዓይነምድር በqPCR እና 725 oocysts በአንድ ግራም እርጥብ ዓይነምድር ለqIFAT ነበር። ለLED-AP ክሪፕቶስፖሪዲዎሲስ ህመም ያለባቸውን ህፃናት የመለየት አቅም 88% (95% CI 79-94፣ ከ75 ናሙናዎች ውስጥ 66) እና ህመሙ የሌላቸውን በትክክል የመለየት ሀቅም ደግሞ 99% (98 - 99፣ ከ726 ናሙናዎች ውስጥ 717)፤ የላተራል-ፍሎው ምርመራን በሚመለከት ደግሞ ህመሙ ያለባቸው በትክክል የመለየት አቅም 89% (79-94፣ ከ71 ናሙናዎች ውስጥ 63) እና ህመሙ የሌላቸውን በትክክል የመለየት አቅም ደግሞ 99% (97-99) ከ635 ናሙናዎች ውስጥ 626 የያዘ ነበር።

ትርጓሜ፤ የማይክሮስኮፕ **LED-AP** ምርመራ ህመምተኞችን በጥሩ ሁኔታ የመለየት አቅም ያለው በመሆኑ ዝቅተኛ ገቢ ባላቸው አካባቢዎች ውስጥ ከነባር የላብራቶሪ መሠረተ ልማቶች ጋር በቀላሉ በማቀናጀት ባለ ሁለትዮሽ ተግባር ቴክኖሎጂ ተደርጎ ሊወሰድ ይገባል። የላተራል-ፍሎው ምርመራም ተመሳሳይ አቅም ያለው ቢሆንም ማይክሮስኮፕ አያስፈልገውም፡፡ በእርግጥ የላተራል ፍሎው ምርመራ ገና በገበያው ላይ ስለማይገኝ ዋጋውም አይታወቅም ::

ወጪ፤ ይህንን ጥናት በገንዘብ የደገፉት የኖርዌይ ምርምር ካውንስል GLOBVAC ፈንድ፣ ቢል እና ሜሊንዳ ጌትስ ፋውንዴሽን፣ የኖርዌጂያን ሶሳይቲ ፎር ሜዲካል ማይክሮባዮሎጂ፣ የበርገን ዩኒቨርሲቲ እና ቬስትፎልድ ሆስፒታል ትረስት ናቸው።፡

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Supplementary appendix 2

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Johansen ØH, Abdissa A, Zangenberg M, et al. Performance and operational feasibility of two diagnostic tests for cryptosporidiosis in children (CRYPTO-POC): a clinical, prospective, diagnostic accuracy study. *Lancet Infect Dis* 2020; published online Dec 3. https://doi.org/10.1016/S1473-3099(20)30556-9.

Supplementary appendix for:

Performance and operational feasibility of two diagnostic tests for cryptosporidiosis in children (CRYPTO-POC): a clinical, prospective, diagnostic accuracy study

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HIV testing

Guardians/caregivers of all participants were requested for permission for their children to have recommended HIV testing. Upon consent; first-line testing with the First Response HIV 1-2-O Card™ test (Premier Medical Corporation Ltd, Daman, India); for children younger than 18 months, positive test results were confirmed by PCR; for children older than 18 months, positive results were confirmed by a second test, Uni-Gold HIV™ (Trinity Biotech Manufacturing Ltd, Co. Wicklow, Ireland). HIV counselling and testing were done by routine clinical staff or trained study nurses. Information to caregivers and HIV treatment to children were offered according to routine care procedures.

Figure S1 and S2: *Cryptosporidium* LED-AP microscopy

(Also see standard operating procedure, page 16)

Figure S1: Examining auramine-phenol stained slides on the LED fluorescence microscope (ØHJ, personal photo)

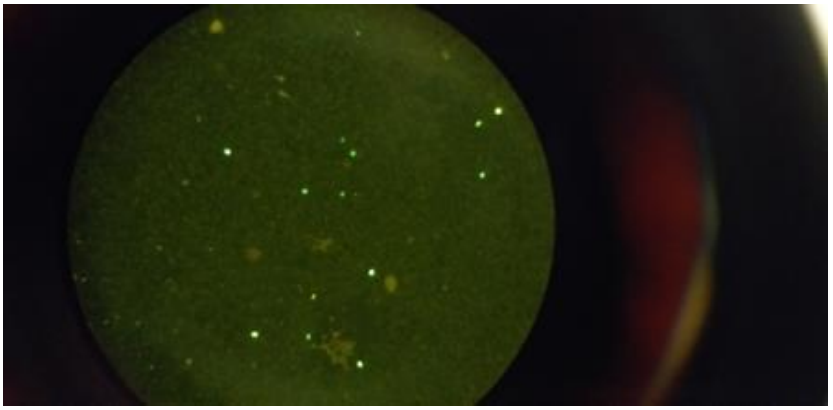


Figure S2: Brightly fluorescing circular/oval *Cryptosporidium* oocysts in an auramine-phenol stained stool smear, captured on site by smart phone camera held close to the LED microscope, through a 10x ocular and using a 40x objective; (YA, personal photo)

***Cryptosporidium EZ VUE* antigen test strip**

Stool samples were mixed well by stirring with a wooden applicator stick, and about 50 µl (or about 0.05 g) faeces were transferred by pipette to a diluent tube, into which the test end of the lateral flow test strip was inserted, then inspected after 10 minutes. One operator read each test strip. The test was considered positive if both the test line and the control line were present, without any further confirmation or repeat testing, and as negative if only the control line was visible. Lack of a control line meant an invalid result and the procedure was repeated with a new test strip (once), by the same operator. However, invalid results were not seen during this study. Fleece and colleagues[1] have further details of the procedure, including a photo of a negative and positive test strip. Note that the *Cryptosporidium EZ VUE* test strip (TECHLAB®, Blacksburg, VA, USA) is currently not commercially available, and there is currently no plan to move it forward, although this could change in the future based on the perceived need (and market) for the product (Alice Houk-Miles, TechLab, Inc., personal communication).

***Cryptosporidium* quantitative IFAT**

Immunofluorescent antibody test (IFAT) staining and microscopy were performed by a medical laboratory technician with special training in parasitology, in the JMC lab, twice weekly, on unconcentrated stool samples. Manually homogenized stool was transferred to one of three wells on a multispot microscope slide with a sterile metal wire loop calibrated to hold approximately 0.04g of stool. The samples were air-dried and then methanol fixed before staining with 5-15µl of monoclonal antibody for *Cryptosporidium* oocysts and *Giardia* cysts (Aqua-Glo G/C, Waterborne Inc., New Orleans, LA, USA) and then incubated in a humid chamber at room temperature (ranging from approx. 20 ºC to 30 ºC) for 40 minutes. The staining solution was then rinsed off with distilled water, one drop counterstain (Evans blue) added, and, after 1-minute incubation at room temperature, a drop of 1,4-diazabicyclo[2.2.2]octane anti-fade mounting medium (DABCO) and a coverslip was placed over the sample before microscopic examination. All *Cryptosporidium* oocysts in the sample were counted; if ≥ 100 oocysts were reached, the sample was diluted 1:10 in normal saline, re-stained and re-counted and this procedure was repeated until an oocyst count of <100 per well was registered. IFAT slides were kept at room temperature in a closed box and at monthly intervals the study investigator (ØHJ) selected 10 random slides for blinded review and assessed the quality of stains for the microscopy examinations; result discrepancies did not affect the recorded results of IFAT testing, but were used as the basis for feedback and on-site re-training for the lab technician.

C*ryptosporidium* ELISA

Enzyme-linked immunosorbent assay (ELISA) detection of *Cryptosporidium* antigen was performed in batches on frozen samples using a commercial assay (*Cryptosporidium II,* TECHLAB®, Blacksburg, VA, USA), with manual reading of the plates, and according to the manufacturer's protocol.

***Cryptosporidium* quantitative PCR**

After completion of the field study, sample aliquots were shipped at -80°C from Ethiopia to Norway for TNA extraction and quantitative PCR.

Pre-treatment:

After thawing, stool samples were homogenized using a lab vortex mixer. An aliquot of stool was transferred to a buffer tube that was weighed before and after transfer to calculate the exact stool mass from which total nucleic acids were subsequently extracted, thereby allowing calculation of the number of DNA target copies detected per gram of stool. After thawing, stool samples were added to pre-made 500 µl S.T.A.R. buffer (Roche) + 500 µl BLB (MagNA Pure bacterial lysis buffer, Roche), and vortexed. The stool-buffer suspension was kept in an ultrafreezer until extraction.

Extraction:

The frozen stool-buffer suspension was heated for 15 minutes at 100°C, and centrifuged for 3 minutes at 13000g, before extraction. Nucleic acids were extracted from 500 µl of the pre-treated stool-buffer suspension, with MagNA Pure 96 instrument, MagNA Pure 96 DNA, and Viral NA Large Volume Kit, and eluted in 100 µl. An internal control (DNA process control kit, Roche) was added to every sample prior to extraction. Samples yielding a negative internal-control PCR result were diluted 1:4 in stool transport and recovery buffer (S.T.A.R. buffer, Roche) and bacteria lysis buffer (BLB, Roche), and then extracted again.

Real-time quantitative PCR:

The assay target gene was *Cryptosporidium* oocyst wall protein (COWP), using forward primer CAAATTGATACCGTTTGTCCTTCTG, reverse primer GGCATGTCGATTCTAATTCAGCT and probe FAM-TGCCA(T)ACAT(T)GT(T)GTCC-BBQ, as previously described by Van Lint et al.[2] Quantitative real-time PCR was run on a Light Cycler 480II, Roche, including primers and probes for the internal control (DNA process control kit, Roche). Standard curves were established by running qPCR on tenfold dilutions of Quantitative Genomic DNA from *Cryptosporidium parvum* (ATCC® PRA-67DQ™). Quantification cycles (Cq) are the PCR cycle values at which fluorescence from amplification exceeds the background, which acts as an inverse metric of quantity of nucleic acid. All detections with a Cq greater than 38 were deemed negative.

Conversion of Cq values to copy numbers:

All positive samples with Cq below Ct 38 were considered positive. The Cq were plotted against the external standard curve, allowing calculation of target copies/µl. A positive *Cryptosporidium parvum* control of known quantity was used as a calibrator for each run, to adjust for PCR differences.

Quality assurance:

We used good laboratory practice, automated pipetting of samples (primary sample handling, PSH) and DNA to PCR setup (MagNA Pure 96) to limit the possibility of laboratory contamination. Valid results required that positive, negative, and internal controls produced the expected results.

Definitions

- **Diarrhoea**: the passage of three or more watery or loose stools (looser than normal stools) within the preceding 24 h; the presence and duration of diarrhoea were assessed by caregiver recall.
- **Dysentery:** at least one loose stool with visible blood (or stains of blood) in the previous 24 h.
- **Severe acute malnutrition (SAM):** one or more of the following: weight-for-height z-score (WHZ) ≤ -3 of the WHO standard curves, mid-upper arm circumference (MUAC) ≤ 115 mm and presence of bilateral oedema involving at least the feet.
- **HIV status:** Human immunodeficiency virus infection status; based on HIV testing on enrolment or by previous testing as reported by the caregiver. Children below 18 months with an HIV-positive mother were considered HIV-exposed and uninfected if a PCR result for the child was negative or not available.
- **Stunting:** a length/height-for-age z-score ≤ -2 of the WHO standard curves.[3]
- **Moderate to severe diarrhoea:** diarrhoea together with very sunken eyes, abdominal skin pinch that returned very slowly (>2 s) or slowly (defined as ≤ 2s but abnormal), dysentery, received IV fluids or been admitted for any reason.[4]
- **Water/sanitation, assets and maternal education (WAM) index:** calculated as in the MAL-ED study; based on access to improved or unimproved water and/or sanitation, the presence or absence of eight household assets and maternal education.[5]
- **Rotavirus vaccination:** Rotavirus vaccine in Ethiopia is an oral vaccine (Rotarix™) that is given twice, usually at 6 weeks and 10 or 14 weeks of age. We defined the child as vaccinated against rotavirus if two doses had been received at least 4 weeks apart.
- **Sensitivity, specificity, positive and negative predictive values, likelihood ratio of a positive test, likelihood ratio of a negative test:** See table S2 and S3 (page 10, appendix)

Figure S3: Flow of participants and samples in the case-control substudy

(For details on screening and eligibility, see flow diagram in previous study.[6])

Table S1: Demographic and clinical characteristics in the case-control substudy; diarrhoea cases and their frequency matched non-diarrhoea controls

	Diarrhoea cases (n = 749)[1]	**Non-diarrhoea controls** (n = 706)[1]
Age, months; median (IQR)	13 (8-24)	16 (9-25)
Age, months; mean (SD)	17 (12)	19 (13)
Female gender; n (%)	308 (41%)	360 (49%)
Exclusive breastfeeding for < 6 months; n (%)	263/700 (38%)	242/658 (37%)
WAM[2] index < 0.50; n (%)	409/685 (60%)	366/728 (50%)
WAM index; mean (SD)	0.50 (0.20); N=728	0.44 (0.15); N=685
Health facility visit in previous month; n (%)	277/748 (37%)	177/706 (25%)
Diarrhoea episode in previous month; n (%)	123/744 (17%)	111/705 (16%)
Admitted in health facility since birth; n (%)	70/746 (9.4%)	61/706 (9.6%)
Previous treatment for malnutrition; n (%)	11/746 (1.5%)	8/705 (1.1%)
MUAC[3] ≤ 125 mm (children >6 months); n (%)	68/639 (11%)	13/629 (2.1%)
Severe acute malnutrition	50/748 (7%)	8/748 (1%)
HIV[4] positive	0/554 (0%)	0/264 (0%)
HIV exposed	1/554 (0.2%)	1/264 (0.4%)
Rotavirus vaccination	706/748 (94%)	671/702 (96%)

[1]Data are n/N (%), unless otherwise specified; denominators vary slightly because of missing data for some of the participants (e.g. not recorded, or not answered). [2]Water/sanitation, assets and maternal education (definitions, appendix). [3]Mid-upper arm circumference. [4]Human immunodeficiency virus.

Figure S4: Quantitative cutoff for qPCR – ROC curve

The cutoff that maximally discriminated case/control status was obtained by finding the threshold value (number in red, on the figure) for which the point on the ROC curve has the minimum distance (indicated by the red line in the figure) to the upper left corner (where sensitivity=1 and specificity=1). By Pythagoras' theorem, this distance is the square root of $(1\text{-sensitivity})^2+(1\text{-specificity})^2$.

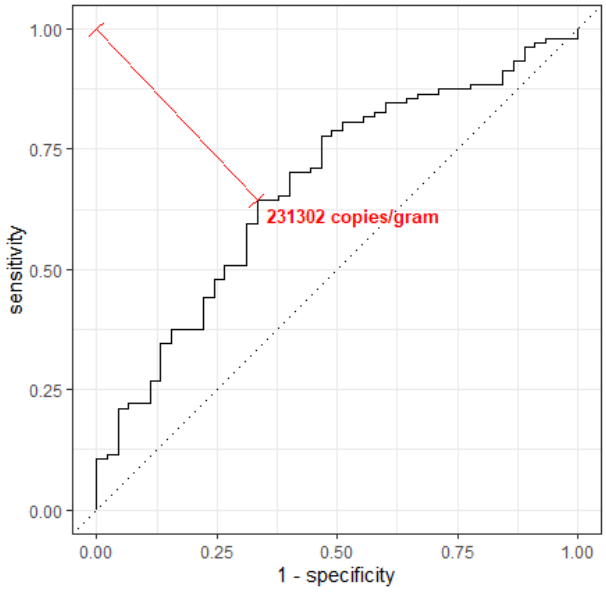
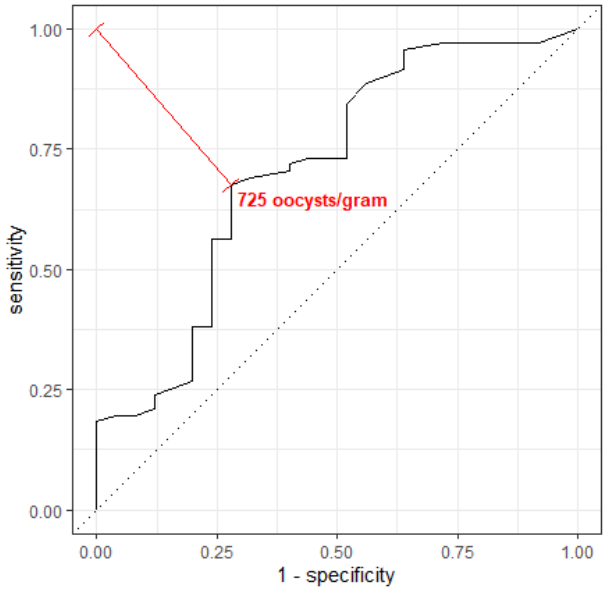


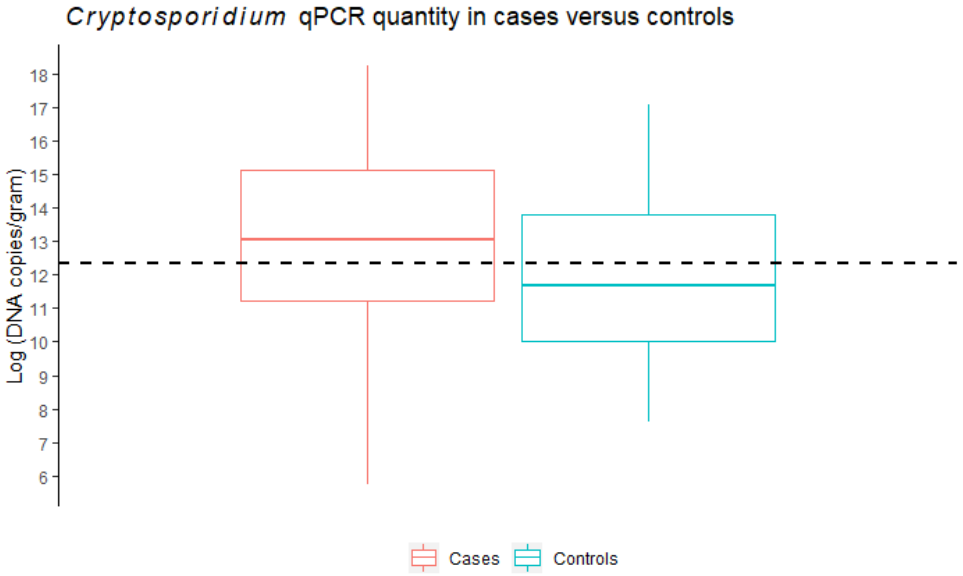
Figure S5: Quantitative cutoff for qIFAT – ROC curve

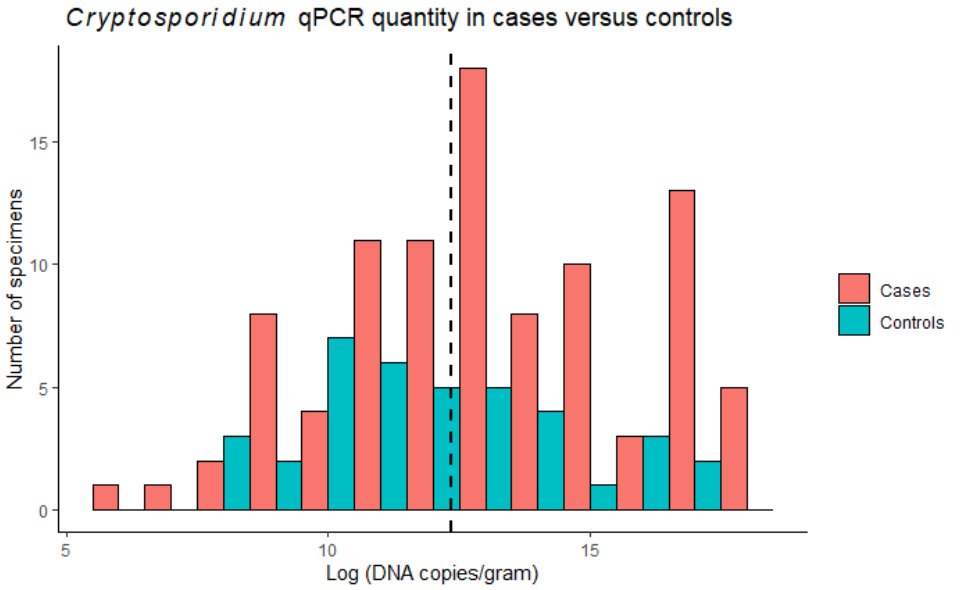


Figures S6 and S7: *Cryptosporidium* PCR quantity comparison in the case-control substudy – boxplot and histogram

The PCR quantity ratio in case to control stools was 3·8 (ratio of geometric means; 95% CI 1·5 to 9·8).

Horizontal and vertical dashed lines indicate the qPCR cutoff that maximally discriminates case/control status.

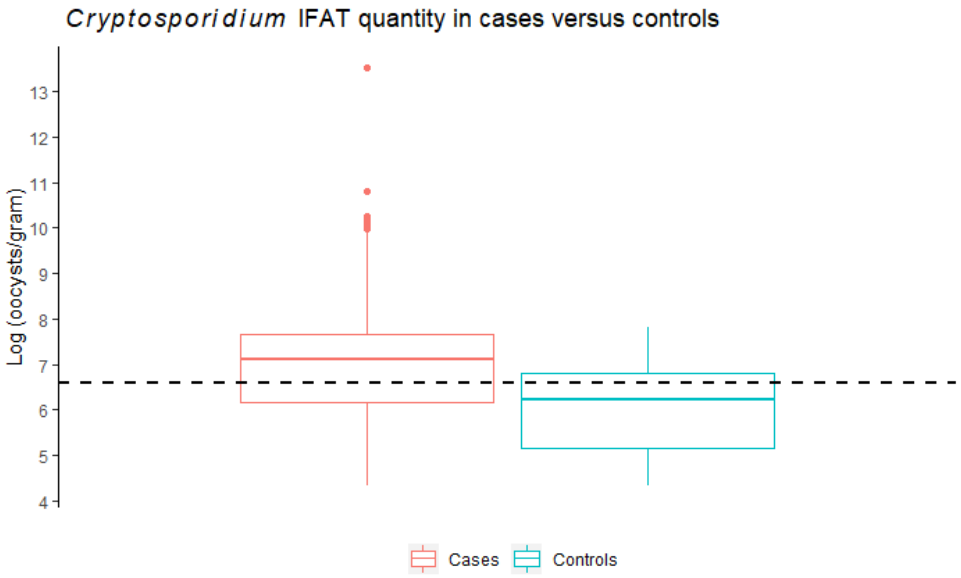




Figures S8 and S9: *Cryptosporidium* IFAT quantity comparison in the case-control substudy – boxplot and histogram

The IFAT quantity ratio in case to control stools was 3·3 (ratio of geometric means; 95% CI 1·8 to 6·1)

Horizontal and vertical dashed lines indicate the qIFAT cutoff that maximally discriminates case/control status.



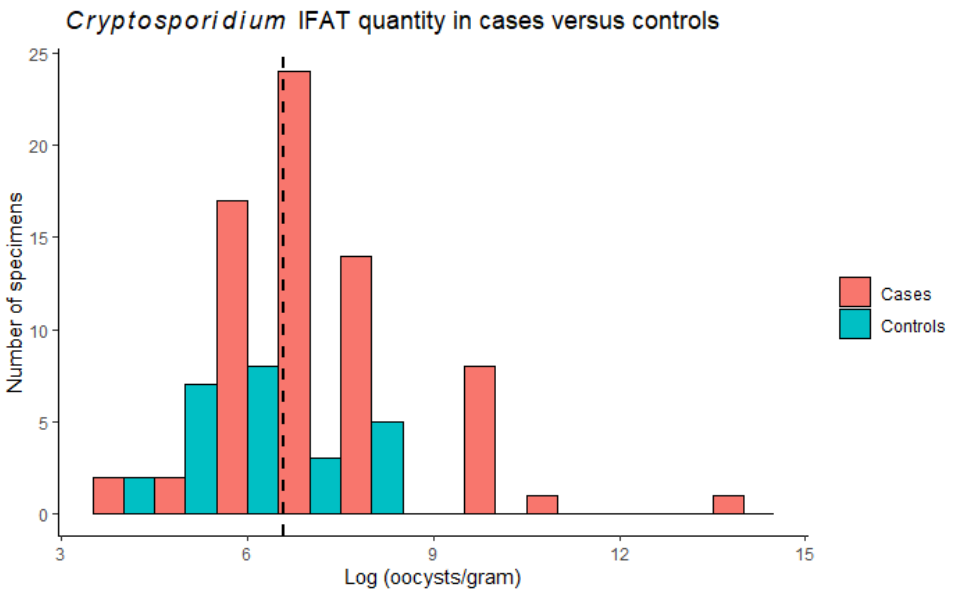


Table S2: LED-AP results in relation to cryptosporidiosis; crosstables and accuracy calculations

Cryptosporidiosis defined as a diarrhoea case with a positive clinical composite reference standard (CRS) test.

	Cryptosporidiosis		
LED-AP	Positive	Negative	Total
Positive	66 (tp)	9 (fp)	75
Negative	9 (fn)	717 (tn)	726
Total	75	726	801

The estimates in Table 3 in the main text were obtained using the following formulas:
Sensitivity = tp / (tp + fn) = 66 / 75 = 0.880
Specificity = tn / (fp + tn) = 717 / 726 = 0.988
Prevalence = (tp + fn) / (tp + fp + tn + fn) = 75 / 801 = 0.094
Positive predictive value = tp / (tp + fp) = 66 / 75 = 0.880
Negative predictive value = tn / (tn + fn) = 717 / 726 = 0.988
Likelihood ratio of a positive test = sensitivity / (1 – specificity) = 0.880 / (1 – 717 / 726) = 71.0
Likelihood ratio of a negative test = (1 – sensitivity) / specificity = (1 – 0.880) / (717 / 726) = 0.12

Table S3: Test strip results in relation to cryptosporidiosis; crosstables and accuracy calculations

Cryptosporidiosis defined as a diarrhoea case with a positive clinical composite reference standard (CRS) test.

	Cryptosporidiosis		
Test strip	Positive	Negative	Total
Positive	63 (tp)	9 (fp)	72
Negative	8 (fn)	626 (tn)	634
Total	71	635	706

The estimates in Table 3 in the main text were obtained using the following formulas:
Sensitivity = tp / (tp + fn) = 63 / 71 = 0.887
Specificity = tn / (fp + tn) = 626 / 635 = 0.986
Prevalence = (tp + fn) / (tp + fp + tn + fn) = 71 / 706 = 0.101
Positive predictive value = tp / (tp + fp) = 63 / 72 = 0.875
Negative predictive value = tn / (tn + fn) = 626 / 634 = 0.987
Likelihood ratio of a positive test = sensitivity / (1 – specificity) = (63 / 71) / (1 – (626 / 635) = 62.6
Likelihood ratio of a negative test = (1 – sensitivity) / specificity = (1 – 63 / 71) / (626 / 635) = 0.11

Table S4: Diagnostic accuracy of LED-AP versus test strip[1] for cryptosporidiosis (in reference to clinical composite reference standard)

	LED-AP	Test strip	P-value for difference	Difference
Sensitivity	90% (83 to 97)	88% (81 to 96)	1[2]	-1% (-10 to 7)
Specificity	99% (98 to 99)	99% (98 to 99)	1[2]	0% (-1 to 1)
Positive predictive value	87% (80 to 95)	87% (79 to 95)	0.96[3]	N/A
Negative predictive value	99% (99 to 100)	99% (98 to 100)	0.74[3]	N/A

[1]Note that estimates are slightly different to those in Table 3 in the main manuscript text, as this is a paired (i.e., head-to-head) analysis. Point estimates and 95% (Wald) confidence intervals[7], when applicable.
[2]Exact binominal test.[8]
[3]Generalized score statistic test.[9]

Table S5: Prevalence-adjusted predictive values

		Prevalence adjusted PPV		Prevalence adjusted NPV	
Enrolment site	**Prevalence[1]**	**LED-AP**	**Test strip**	**LED-AP**	**Test strip**
JMC	12.6% (9.7 to 16.2)	91.1% (82.5 to 95.7)	90.0% (80.9 to 95.1)	98.3% (97.0 to 99.0)	98.4% (97.1 to 99.1)
SHC	6.2% (4.3 to 9.0)	82.5% (72.4 to 89.4)	80.6% (69.9 to 88.1)	99.2% (98.2 to 99.6)	99.2% (98.2 to 99.7)

Point estimates and 95% confidence intervals. [1]Prevalence of cryptosporidiosis, defined as the proportion of diarrhoea cases with a positive CRS.

Table S6: Diagnostic accuracy of LED-AP and *Cryptosporidium EZ-VUE* test strip for asymptomatic *Cryptosporidium* infection in children < 5

	LED-AP	Test strip
Cryptosporidium infection prevalence	2.1% (1.3 to 3.5)	2.6% (1.5 to 4.2)
Sensitivity	64.3% (38.8 to 83.7)	78.6% (52.4 to 92.4)
Specificity	99.7% (98.9 to 99.9)	99.6% (98.6 to 99.9)
Positive predictive value	81.8% (52.3 to 94.9)	84.6% (57.8 to 95.7)
Negative predictive value	99.2% (98.2 to 99.7)	99.4% (98.4 to 99.8)
Likelihood ratio of a positive test	210.5 (50.0 to 886.7)	210.2 (51.3 to 861.0)
Likelihood ratio of a negative test	0.36 (0.18 to 0.72)	0.22 (0.08 to 0.59)

Point estimates and 95% confidence intervals. Asymptomatic *Cryptosporidium* infection defined as a non-diarrhoea control with a positive microbiological composite reference standard test (MRS).

Table S7: Test turnaround times with breakdown in phases, for samples where results were received by study nurse the same day

Turnaround times for samples reported the same day were subdivided into a pre-analytical phase (time from sample collection until arrival in the lab), analytical (time from sample arrival in lab until LED-AP completed) and post-analytical phase (time from LED-AP completion until result received by the study nurse).

	Both enrolment sites	JMC	SHC
Min; hrs	0.5	0.5	0.75
Max; hrs	7.8	7.8	7.3
Median; hrs (IQR)	3.5 (1.7 to 4.9)	1.8 (1.2 to 3.7)	4.6 (3.2 to 5.3)
Total TAT missing; n/N (%)	251/607 (41%)	240/415 (58%)	11/192 (6%)
Total turnaround time (TAT) by site, in hours			

Figure S10: Test turnaround times with breakdown in subphases

Breakdown of turnaround time subphases, for samples analysed on the day of sample collection:

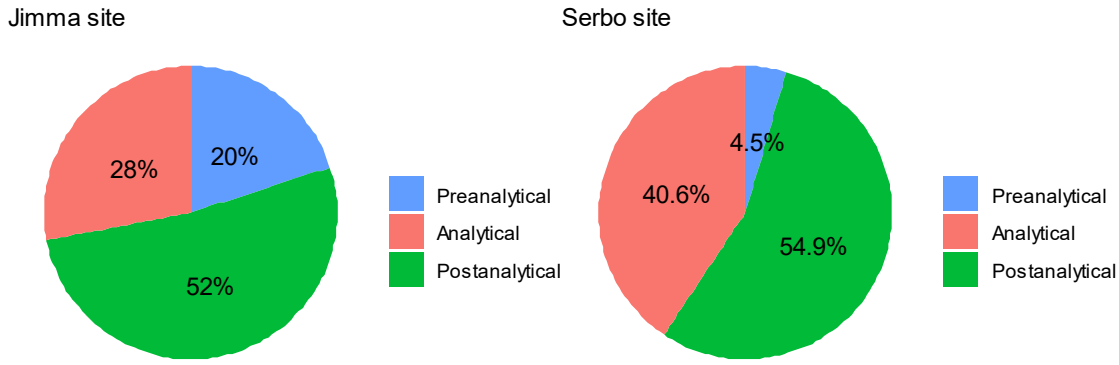


Table S8: Operational challenges with LED-AP *Cryptosporidium* staining identified during the study

Specific challenge	How the challenge was addressed during the study	Relevance to roll-out of LED-AP testing
Preanalytical issues:		
Failure to obtain stool sample because the patient left early	Information to the caretaker; transport compensation for returning with sample	Specific drug treatment, while waiting for sample provide ORS training and/or a separate waiting room for families
Long walking distance from the paediatric outpatient department and wards to the laboratory, and no clinical porter services	If the study nurse was busy, the lab tech was requested by phone to bring the sample to the lab (with ensuing delay)	Dedicated staff to transport samples, or set up small lab facility in the paediatric outpatients' department
Analytical issues – general:		
Uneven laboratory workload, with peaks just before lunch break and just before the end of the workday	Avoid sample backlog by immediate notification and transport	As above; ideally have both morning and evening shifts for lab staff
Power surge burned out the power unit in one of the LED microscopes	Voltage stabilizers installed (not routinely used in the clinical labs)	If not already in place, provide voltage stabilizers for all microscopes
Power interruptions in Serbo Health Centre as backup generator was broken and not fixed until 2 weeks later	Temporary analysis of samples in JUSTH lab instead	Solar panel and battery packs are available for most LED microscopes; general support for the lab facility (i.e., maintenance assistance)
Dwindling backup supply of AP staining reagent for several weeks; potassium permanganate difficult to import from abroad	Assistance from two regional clinical microbiology laboratories in different parts of the country	Cooperate on AP stain provision and contingency plans with the existing health infrastructure for tuberculosis diagnosis
Laboratory supervision interrupted for 2 weeks due to roadblocks	Phone communication with lab staff, supervision catch-up visit	Contingency plans for health service interruption; cell phone communication (and compensation for private use) with lab staff
Requests for follow-up training in the Serbo health centre lab	Extra training sessions organized in the JUSTH laboratory	Good support from centrally located labs to peripheral diagnostic labs
No darkroom for fluorescence microscopy	All LED microscopes equipped with plastic eye cups (eye strain low and high user satisfaction with these)	No need for darkroom, backup supply of plastic eye cups recommended
Analytical issues – related to AP staining:		
AP stain failing QC for 1 week in Serbo due to use of a plastic funnel that discoloured the stain	Staining reagent batch replaced; on-site supervision and training	Frequent and vigilant QC with blinded random review of slides recommended; on-site supervision and training recommended
Small 2-3µm circular or oval slightly fluorescent objects somewhat resembling *Cryptosporidium*	1. On-site training and SOPs in correct size measurement by micrometry; objects too small to (2-3µm vs 4-6 µm for *Cryptosporidium* spp.). 2. Specific stains for Microsporidia attempted (all negative)	Ideally equip all LED microscopes with insert eyepiece micrometer graticule, and provide SOPs and training in its use
Staining artefacts	1. Filtering of AP staining solutions, restaining of slides 2. If still staining artefacts, AP staining batch replaced 3. All AP staining bottles marked with expiry date	Written QC systems and designated QC officers for AP staining
Dust on microscope slides creating disturbing background fluorescence	1. Store all glass slides in closed box away from dust 2. Rinse slides with water before use 3. Do not use gauze or tissue paper to rinse off slides	Include similar points in SOP, provide appropriate paper for cleaning slides (i.e., lens paper or similar)
AP quantity grading discrepancies on blind review (+, ++, +++)	On-site feedback and training	Ideally include quantitative grading in blind review QC
AP slides haphazardly stored in drawers	More slide boxes provided	Ample supply of closed-lid plastic slide storage boxes for slide QC
Lab staff asking why internal oocyst contents give off fluorescence	Known phenomenon; as the AP stain likely is assumed to have nucleic acid staining properties in addition to acid-fast properties[10,11]	Laboratory bench aids with photographs of various types of appearance of *Cryptosporidium* oocysts in AP slides
Postanalytical issues:		
Nurse busy or otherwise unavailable to receive LED-AP result slip from the lab tech	Lab tech had to wait until study nurse was available as the patient's identity was unknown to the lab staff	In a non-research setting another member of the clinical staff could be approached to receive and act on the result
Nurse would sometimes call the lab tech and request result over phone	Temporary results given by phone, result form to follow	If possible, implement computer information systems to allow clinical staff to look up finished lab reports directly
The patient had already left when LED-AP result was available	Study nurse tried to reach the caretaker by phone, if possible	Possible "study effect"; if cryptosporidiosis treatment had been available, it would have been an added incentive to wait for the result

Table S9: Cost per test calculation for LED-AP

Cost item	Cost in Ethiopian Birr (ETB)	Unit	Quantity	Unit cost (ETB)	Total Cost (ETB)	Quantity used per test	Cost per test (ETB)	Average ETB to USD exchange rate during the study period	Cost per test (USD)	Notes
Staining reagents										
Auramine-O powder	55.35 for 100 mg	mg	100	0.5535	55.35	1.5	0.83	0.039763158	0.033	
Phenol crystals	147.08 for 1 kg	mg	1000	0.14708	147.08	1.5	0.22	0.039763158	0.009	(Could be slightly more expensive if < 1L purchased)
Potassium permanganate	65.0 for 100 mg	mg	100	0.65	65	1.5	0.98	0.039763158	0.039	
Methanol, absolute	240 for 1 L	ml	1000	0.24	240	2	0.48	0.039763158	0.019	(Could be slightly more expensive if < 1L purchased)
Ethanol, 96% (and HCl)	82.50 for 1 L	ml	1000	0.0825	82.5	0.5	0.04	0.039763158	0.002	(Could be slightly more expensive if < 1L purchased) Hydrochloric acid cost per test is 0.000 USD
Other laboratory expendables										
Glass microscope slides	38.50 for 50 pcs (1 pack)	pcs	50	0.77	38.5	1	0.77	0.039763158	0.031	
Applicator sticks	35.20 for 100 pcs (1 pack) =	pcs	100	0.352	35.2	1	0.35	0.039763158	0.014	
Sample collection cups	330 for 100 pcs	pcs	100	3.3	330	1	3.30	0.039763158	0.131	
Pasteur pipettes	300 for 500 pcs (1 carton)	pcs	500	0.6	300	1	0.60	0.039763158	0.024	
Gloves	118.90 for 100 (50 pairs)	pairs	50	2.378	118.9	1	2.38	0.039763158	0.095	
Tissue paper	210 for 1 big size roll	rolls	1	210	210	0.01	2.10	0.039763158	0.084	Assuming 1 roll is for 100 tests
Hands-on labour time (based on the approximate national average wage for nurses and lab technician working in the Ethiopian national health care system at the time; adjust numbers as needed.)										
Nurse (sample collection)	Weekly salary 900 ETB	min	6	0.375	2.25	1	2.25	0.039763158	0.089	
Lab tech (receipt and homogenization of sample)	Weekly salary 900 ETB	min	3	0.375	1.125	1	1.13	0.039763158	0.045	
Lab tech (staining procedure)	Weekly salary 900 ETB	min	6	0.375	2.25	0.5	1.13	0.039763158	0.045	This is the approximate hands-on time during the staining procedure, so not counting the waiting between manual steps. The "quantity used per test" is set to 0.5 as two slides would often be stained at the same time; adjust this number as needed.
Lab tech (microscopy)	Weekly salary 900 ETB	min	2	0.375	0.75	1	0.75	0.039763158	0.030	
Lab tech (reporting)	Weekly salary 900 ETB	min	4	0.375	1.5	1	1.50	0.039763158	0.060	
Total							**18.80**	0.039763158	**0.747**	

Table S10: Cost per test calculation for antigen test strip

Cost item	Cost in Ethiopian Birr (ETB)	Unit	Quantity	Unit cost (ETB)	Total cost (ETB)	Quantity used per test	Cost per test (ETB)	Average ETB to USD exchange rate during the study period	Cost per test (USD)	Notes
Laboratory expendables										
TechLab *Cryptosporidium EZ Vue* test strips	(see Notes)	1 test strip	1	25.15	25.14890801	1	25.15	0.039763158	1.000	As the test strip is not commercially available, this is a rough estimate (based on about 4 times the mean cost of malaria RDTs in health centres in Ethiopia[12]); adjust as needed
Other laboratory expendables										
Applicator sticks	35.20 for 100 pcs (1 pack)	pcs	100	0.352	35.2	1	0.35	0.039763158	0.014	
Sample collection cups	330 for 100 pcs	pcs	100	3.3	330	1	3.30	0.039763158	0.131	
Pasteur pipettes	300 for 500 pcs (1 carton)	pcs	500	0.6	300	1	0.60	0.039763158	0.024	
Gloves	118.90 for 100 (50 pairs)	pairs	50	2.378	118.9	1	2.38	0.039763158	0.095	
Tissue paper	210 for 1 big size roll	rolls	1	210	210	0.01	2.10	0.039763158	0.084	Assuming 1 roll is for 100 tests
Hands-on labour time (based on the approximate national average wage for nurses and lab technician working in the Ethiopian national health care system at the time; adjust numbers as needed.)										
Nurse (sample collection)	Weekly salary 900 ETB	min	6	0.375	2.25	1	2.25	0.039763158	0.089	
Lab tech (receipt and homogenization of sample)	Weekly salary 900 ETB	min	3	0.375	1.125	1	1.13	0.039763158	0.045	
Lab tech (test strip procedure)	Weekly salary 900 ETB	min	2	0.375	0.75	0.5	0.38	0.039763158	0.015	This is the approximate hands-on time, so not counting the waiting step. This assumes that an average of 2 samples will be received and processed at the same time
Lab tech (test strip interpretation)	Weekly salary 900 ETB	min	1	0.375	0.375	1	0.38	0.039763158	0.015	
Lab technician (reporting)	Weekly salary 900 ETB	min	4	0.375	1.5	1	1.50	0.039763158	0.060	
Total							**39.50**	0.039763158	**1.571**	

SOP: Auramine-phenol staining and light-emitting diode fluorescence microscopy (LED-AP) for *Cryptosporidium*

(Excerpt from the study Laboratory Manual; this standard operating procedure (SOP) was used for training of laboratory technicians in two half-day training sessions given one month apart; and as laboratory bench aids for the diagnostic laboratory)

Introduction

This fluorescent staining technique is used for the demonstration of *Cryptosporidium* oocysts, in faeces. Note that *Cyclospora cayetanensis* and *Cystoisospora belli* oocysts also stain by this method.

Safety considerations

Because of ocular and cutaneous toxicity, work with auramine-phenol should be performed using personal safety equipment (eye protection, gloves, clothing) in rooms with good ventilation.

Required equipment

- Microscope slides
- Methanol (absolute)
- Auramine-phenol solution (0.1% auramine-O) (ready-made commercial stain, or prepared according to the Ministry of Health protocol)
- 0.5% acid ethanol
- 0.5% potassium permanganate solution
- Pipettes
- Timer/stopwatch
- Distilled water or tap water (use a beaker, bottle with a plastic tube attached to it, or a plastic tube from the tap (gentle flow!)
- Positive control (*Cryptosporidium* oocysts, available from Waterborne Inc, mixed with faecal material)
- Negative control (faecal material known not to contain *Cryptosporidium* oocysts)

Method

a) Homogenize the stool sample as much as possible by stirring vigorously in the sample container with an applicator stick or loop
b) Prepare a smear and air dry, either in room temperature for 15-30 minutes, or in 37°C incubator (smears should be medium to thick)
c) Fix in absolute methanol for 1min, then air dry before proceeding with staining.
d) Flood the slide with Auramine-phenol solution (0.1% auramine-O) (ready-made commercial stain or prepared according to the MoH protocol) and leave the solution on the slide for 15 min. Do not heat.
e) Rinse with distilled water (or tap water if distilled water not available) from a beaker, or gently through a plastic tube attached to a bottle or a tap. Drain excess water from the slide.
f) Flood the slide with 0.5% acid ethanol and leave the destaining solution on the slide for 2min
g) Rinse with distilled water (or tap water if distilled water not available) from a beaker (not directly from the tap), or gently through a plastic tube attached to a bottle or a tap. Drain excess water from the slide.
h) Flood the slide with 0.5% potassium permanganate and leave the counterstain fluid on the slide for 2 minutes. The timing of this step is critical.
i) Rinse with distilled water (or tap water if distilled water not available) from a beaker (not directly from the tap), or gently through a plastic tube attached to a bottle or a tap. Drain excess water from the slide, and air dry. Do not blot because some blotting materials/paper may fluoresce.
j) Let the sample dry, either in room temperature (approximately 15-30 minutes) or in incubator at 37°C.
k) Examine with x 20 objective and x 10 eyepiece lens, and the PrimoStar iLED fluorescence microscope (blue light). The whole sample area should be examined for the presence of fluorescent oocysts. Suspicious objects can be re-examined with a 40x objective or with oil-immersion and the 100x objective.
l) Prepared slides should be kept in a closed slide box, at room temperature, for QC purposes.

Interpretation

Positive Result

Cryptosporidium oocysts (4-6 µm diameter) are ring or doughnut-shaped and fluoresce greeny-yellow (depending on the filter wavelengths) against a dark background. Putative oocysts may be measured by increasing the bright field light intensity and measuring the oocysts with a calibrated eye-piece graticule.

Note that *Cystoisospora belli* oocysts (about 32x16 µm, elongated oval body, tapered at both ends) and *Cyclospora cayetanensis* oocysts (8-10 m diameter, often variably stained) will also fluoresce using the auramine-phenol staining technique, due to the acid-fast properties of these organisms. Note that the oocysts of *Cystoisospora belli* and *Cyclospora cayetanensis* usually fluoresce less well than *Cryptosporidium* oocysts.

Oocyst counts (semi-quantitative method only):
Grade the samples after counting the average number of oocysts per 200x magnification field of view using the x20 objective (count the number of oocysts in minimum 10 fields, and divide by 10 to get the average number of oocysts per field)

Grade the samples after counting the number of oocysts per field of view using the x20 objective:

- 1-9 oocysts: +
- 10-50: ++
- >50: +++

Negative Result
No fluorescent objects with the correct shape or size for *Cryptosporidum, Cystoisospora belli* or *Cyclospora cayetanensis.*

Common interpretation challenges
Occasionally acid-fast objects in stool can fluoresce with AP stain, but they will not have the typical "doughnut" shape of *Cryptosporidium* spp. Yeast cells can fluoresce weakly but tend to lack the typical ring or doughnut shape of *Cryptosporidium*. In contrast to the large majority of fluorescent artefacts, the fluorescence is heterogeneously distributed in the interior of the oocysts. Refer to laboratory bench aids with photographs of the typical appearance of oocysts of *Cryptosporidium, Cystoisospora belli* and *Cyclospora cayetanensis*.

Quality control

Positive control
Cryptosporidium species oocysts; positive control material obtained from Waterborne Inc.

Negative control
A proven negative smear may be used as the negative control. Negative faecal control material can be obtained from Waterborne Inc.

Mix the positive and negative control vials before use.

Include a *Cryptosporidium* positive control and a negative control every time LED-AP microscopy is performed, minimum 1 positive and 1 negative slide per 10 slides that have been stained, or once per week, whichever comes first.

SOP: Preparation of reagents for auramine-phenol staining

The reagents used are the same as those recommended for auramine staining of sputum smears for acid-fast bacilli (e.g.,[13]).

(Excerpt from the study Laboratory manual standard operating procedure (SOP):)

Preparation of reagents – general

Batches of reagents should be prepared in adequate volumes according to need.

Equipment

- Balance, with a sensitivity of 0.1 g
- Brushes to clean bottles before reuse
- Containers for the newly prepared stains (dark amber glass bottles or plastic bottles)
- Distilled or purified water
- Flasks (conical or flat-bottomed), capacity at least 1 litre
- Filter papers, large (appropriate size for funnels)
- Funnels, large, for filling bottles
- Labels for bottles
- Stirring plate, heated, and magnetic stirrers
- Chemicals, see below

Auramine 0.1% - preparation

- Auramine 1.0 g (certified grade)
- Alcohol (denatured ethanol or methanol) 100.0 mL (technical grade)
- Phenol crystals 30.0g (analytical grade)
- Distilled water 870.0 ml

If liquefied phenol is to be used, adjust quantity as volume indicated by the manufacturer. First dissolve auramine in ethanol, then phenol crystals with water and mix both solutions. Mix only amounts that can be consumed within a few weeks, since the working solution is not stable in the long term, although the stock solution (1% auramine in alcohol) can be kept for longer (3 months). Thorough mixing for about one hour on a magnetic stirring plate is recommended, but the solution should not be heated.

Auramine stain – shelf life of working solution max 1 month

Label the bottle "0.1% auramine", add the date and sign with initials. The date the bottle is first opened must be written on the label.

Mix only amounts that can be consumed within a few weeks, since the working solution is not stable in the long term, although the stock solution (1% auramine in alcohol) can be kept for longer (3 months) "Stock and working solutions must be kept in dark bottles in the dark, and working solutions should be used within 1 month.

NB: If visible granules or precipitates form before 1 month has passed, the solution has expired, and you need to prepare a new working solution.

Auramine – safety

Wear gloves when handling patient specimens. Prepare slides from clinical specimens in safety cabinet. Use care when handling unstained slides to avoid touching infectious material.

Acid alcohol 0.5 % - preparation

- Hydrochloric acid 5 ml technical grade
- 70% ethanol 1000 ml

Use a 1-litre flask and slowly pour hydrochloric acid into alcohol.

Label the bottle "0.5% acid–alcohol", add the date and sign with initials. The date the bottle is first opened must be written on the label. This solution may be kept indefinitely.

Potassium permanganate 0.5% - preparation

- Potassium permanganate 5.0 g certified grade
- Distilled water 1000.0 ml

Label the bottle "0.5% potassium permanganate", add the date and sign with initials. The date the bottle is first opened must be written on the label. Solution should be used within 6 months.

Log-sheet for preparation of auramine, acid-alcohol, and counterstain:

Auramine method	**Quantity of reagent**	**Volume prepared**	**Date**	**Signature**
Auramine	1.0 g			
Ethanol	100 ml			
Phenol	30.0 g			
Distilled water	1000 ml			
Auramine 0.1%		1 litre		
Hydrochloric acid	5 ml			
Ethanol	1000 ml			
0.5% acid–alcohol		1 litre		
Potassium permanganate	5 g			
Distilled water	1000 ml			
Counterstaining		1 litre		

Auramine-phenol positive and negative control sheet

CRYPTO-POC study – Lab Manual Appendix – AURAMINE-PHENOL POSITIVE AND NEGATIVE CONTROL SHEET – v120417

AURAMINE-PHENOL POSITIVE AND NEGATIVE CONTROL SHEET – archive safely

NB: Mark all positive control slides with date and "AP POS CTR"; negative slides with date, and "AP NEG CTR"

Stain one *Cryptosporidium* positive and one *Cryptosporidium* negative control slide minimum 2 times per week

Completed by (study staff number) _ _ _ _ **Completed by (full name)** ______________			**JUTH** ☐ **Serbo HC** ☐
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**
Date: (DD/MM/YEAR): __ __ / __ __ / __ __ __ __	Positive control material ☐ Negative control material ☐	**Stain acceptable?** *(if no, take action!)* Yes ☐ No ☐	**Comment/actions taken:**

Auramine-phenol internal quality control sheet

INTERNAL QC ON AP SLIDES		QC microscopy performed by (name):			Date:	
ID number (on the slide)	Cryptosporidium oocysts	If Cryptosporidium oocysts are present, what is the oocyst count?	Cystoisospora belli	Cyclospora cayetanensis	Same result as on AP Lab Form?	Notes:
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	
_ _ _ _ _ - _	Present☐ Absent☐	+ ☐ ++ ☐ +++ ☐	Present☐ Absent☐	Present☐ Absent☐	Yes ☐ No ☐	

Checklist used for supervision visits to the LED-AP laboratory

(excerpt of relevant sections from the study manuals:)

LABORATORY

- Documentation
    - Laboratory manual
    - Laminated LED-AP SOP bench aid on the wall
    - Laminated size measurement bench aid
    - Laminated stool microscopy bench aids
    - AP positive and negative control sheet
    - Result slip for LED AP
    - Check that LED-AP Result Sheets have been filled in correctly
- Check the supplies and the expiry dates (if relevant)
    - Stool collection tubes
    - Nappies, potties and plastic film for stool collection
    - Stains/reagents:
        - Auramine-phenol (working solution max shelf life 1 month!)
        - Acid alcohol
        - Potassium permanganate
        - Methanol
        - Normal saline for wet microscopy
    - Positive control (*Cryptosporidium* oocysts in suspension, for staining)
    - Negative stool control, for staining
    - Glass slides
    - Pipettes
    - Cover slips
    - Applicator sticks or plastic loops
    - Lens paper, lens cleaning solution
    - Oil for immersion microscopy
- Primostar iLED microscope checklist:
    - Plastic eye cups
    - Extra eyepiece with size measurement graticule
    - Lenses and the mechanical stage clean?
    - Is the protective plastic hood present and in use?

Quality assurance and quality control of laboratory equipment

(excerpt from the Study Lab Manual:)

QA and QC of Laboratory Equipment

Routine housekeeping should be in place and adhered to in order to monitor the status of equipment, machines, and their documents. A team consisting of the PIs, the laboratory technician, and microbiologist will frequently check the housekeeping documents, availability of manuals, and maintenance of equipment, machines and its relevant records considering the following issues as QC/QA.

1. Temperature chart for the fridges should be maintained. It should be recorded twice a day, always before opening the fridge door.
2. Laboratory User manuals should be read by the lab persons and discussed among them. The person who will handle the equipment should understand every point in the manuals.
3. Expiration dates of all reagents should be checked regularly, opening date of the reagents with initials should be labelled on the containers.
4. Reagents should be available and up to date. Orders should be placed well in advance to avoid shortages. Availability of supplies should be checked in-country and import delays, customs issues, should be anticipated during shipment.
5. Backup power should be available for the fridges.
6. Ensure that the voltage stabilizers are correctly installed.

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

A comparison of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea: A case-case-control study in Ethiopian children

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Abstract

Background

Cryptosporidiosis is a major cause of diarrhoea in young children in low-and-middle-income countries. New interventions should be informed by evidence pertaining to risk factors and their relative importance. Inconsistencies in the literature may to some extent be explained by choice of methodology, furthermore, most previous risk factor studies compared cryptosporidiosis cases to diarrhoea cases of other aetiologies rather than with controls without diarrhoea.

Methodology/Principal findings

We investigated a broad set of factors in under-2-year-olds presenting with diarrhoea to a hospital and a health center in southwestern Ethiopia. We applied quantitative cut-offs to distinguish between cryptosporidiosis and incidental *Cryptosporidium* infection or carriage, a hierarchical causal framework to minimize confounding and overadjustment, and a case-case-control design, to describe risk factors for both cryptosporidiosis and non-cryptosporidiosis diarrhoea. Moderate and severe acute malnutrition were strongly associated with both cryptosporidiosis and non-cryptosporidiosis diarrhoea. Previous healthcare attendance and low maternal education were only associated with cryptosporidiosis, whereas unsafe child stool disposal, prematurity and early cessation of exclusive breastfeeding were significantly associated with non-cryptosporidiosis diarrhoea only. By estimation of population

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Competing interests: The authors have declared that no competing interests exist.

attributable fractions, socioeconomic factors—specifically low maternal education—and public tap water use, were apparently more important risk factors for cryptosporidiosis than for non-cryptosporidiosis diarrhoea.

Conclusions/Significance

Nutritional management of moderate acute malnutrition may be an effective intervention against cryptosporidiosis, particularly if combined with targeted therapy for cryptosporidiosis which, again, may mitigate nutritional insult. Focused caregiver education in healthcare settings and follow-up of children with acute malnutrition may prevent or improve outcomes of future episodes of cryptosporidiosis.

Author summary

There are puzzling contradictions between reported risk factors for cryptosporidiosis and for paediatric diarrhoea in general. We suspected that these differences are more related to different methodological approaches rather than real differences in the underlying epidemiology. To address this, we applied several epidemiological tools that, to our knowledge, have not previously been combined in a risk-factor analysis for cryptosporidiosis: 1) an underlying conceptual framework 2) the use of pragmatic "real-life" inclusion criteria, 3) quantitative cutoffs for case ascertainment, and 4) a case-case-control design, to allow side-by-side comparison of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea. Caregiver-related socioeconomic factors, public-tap water use, previous healthcare attendance, and moderate acute malnutrition were apparently more strongly associated with cryptosporidiosis than with non-cryptosporidiosis diarrhoea. Our results suggest giving priority to nutritional management of moderate acute malnutrition and exploring healthcare-initiated interventions, with focused caregiver education and closer follow-up of children who present for healthcare or have acute malnutrition, in order to prevent or ameliorate outcomes of future episodes of cryptosporidiosis.

Introduction

Infectious diarrhoea is an important cause of death in young children [1–3]. In children under 2 years of age presenting with diarrhoea, important predictors of death within 2 months include acute malnutrition [4], infection with enteropathogenic *E coli* or enterotoxigenic *E coli* expressing the human variant of the thermostable toxin, and infection with *Cryptosporidium* [2]. Excess mortality after cryptosporidiosis in infancy has also been reported [5].

Interventions that target well-established risk factors are more likely to be effective. However, there are evidence-gaps and unresolved discrepancies in the literature between risk factors for cryptosporidiosis and risk factors for pediatric diarrhoea in general [6, 7], both for environmental factors [8, 9], animal exposures [10, 11], breastfeeding and malnutrition [8, 9]. The comparators used in most risk factor studies are diarrhoea cases without *Cryptosporidium* infection, i.e., case-case comparisons [12–19]. We have not identified any case-control studies that distinguished between *Cryptosporidium* infection and cryptosporidiosis, i.e., diarrhoea attributed to *Cryptosporidium* infection [20, 21] compared with children with no diarrhoea, that investigated hand hygiene, perinatal factors, and acute malnutrition in the same analysis

[4, 11], or that adequately addressed confounding and the internal relationship between risk factors using a pre-defined conceptual causal framework.

The aim of our study was to identify and compare a broad range of risk factors for cryptosporidiosis using community controls without diarrhoea. We included several measures of socioeconomic status, drinking water source, sanitation standards, perinatal factors, factors related to caregiver hygiene, and previous illness. We also investigated the association between severe and moderate acute malnutrition (SAM or MAM) and healthcare-presenting cryptosporidiosis [4]. In order to minimize the risk of either overestimating or underestimating the relative importance of any of the above factors, we used a predefined causal conceptual framework in the analysis, primarily to guide decisions about confounder adjustment. Furthermore, in order to distinguish between those risk factors that are unique to cryptosporidiosis, and those that are common with non-cryptosporidiosis diarrhoea, comparisons of cryptosporidiosis versus non-diarrhoea controls are presented side-by-side with comparisons of non-cryptosporidiosis diarrhoea versus non-diarrhoea controls (a case-case-control design).

Methods

Ethics statement

Jimma University IRB (Reference: RPGC/610/2016), the Ethiopian National Research Ethics Review Committee (Reference: JU JURPGD/839/2017) and the Regional Committee for Medical and Health Research Ethics of Western Norway (Reference: 2016/1096) approved the study. Formal written consent was obtained from the children's parents or guardians.

Study design

Whereas community studies with longitudinal stool sampling are more appropriate for identifying risk factors for asymptomatic or less-severe infection [9, 22], we deliberately focused on risk factors in children who sought care for diarrhoea. The reason was twofold: clinical presentation is likely to be a proxy for severity [2], and low-resource healthcare centres and hospitals might represent under-utilized opportunities for simple, low-cost interventions against cryptosporidiosis that could have high impact.

To minimize the risk of confounding and overadjustment, while maintaining a pragmatic focus on cryptosporidiosis, we used three epidemiological tools that are well established, but that have not, to our knowledge, been previously combined in a risk-factor analysis for childhood diarrhoea: 1) hierarchical conceptual frameworks, where risk factors are organized in levels of a hierarchy [23, 24], 2) improved case ascertainment, using a reference standard that includes quantitative cutoffs, allowing us to distinguish between incidental infection and cryptosporidiosis [20, 21, 25, 26], and 3) a case-case-control design, an approach originally developed to study risk factors for multidrug-resistant bacterial infections [27, 28], in order to distinguish between those risk factors unique to cryptosporidiosis, and those common with non-cryptosporidiosis diarrhoea.

Selection of cases and controls

This analysis used data from a case-control study nested within the CRYPTO-POC study, a diagnostic accuracy study of light-emitting diode auramine-phenol staining microscopy and a rapid antigen test strip for near-patient diagnosis of cryptosporidiosis, conducted in southwest Ethiopia from December 2016 to July 2018 [26, 29]. In brief, the study enrolled children younger than 5 years who presented to Jimma Medical Center (JMC; formerly Jimma University Specialized Hospital) or Serbo Health Centre (SHC; approximately 16 km from JMC) with

diarrhoea (three or more loose stools within the previous 24 hours), or dysentery (at least one loose stool with stains of blood within the previous 24 h), and who lived within two nearby predefined geographical catchment areas. There was no exclusion of cases with prolonged (7–13 days) or persistent (≥14 days) diarrhoea. Community controls without diarrhoea (in the preceding 48 hours) were enrolled concurrently by weekly recruitment plans, using frequency matching to cases by age stratum (0–5 months, 6–11 months, 12–23 months) and geographical location of households (S1 Appendix). The sample size was determined by the parent study (S1 Appendix). Stool testing of controls for asymptomatic *Cryptosporidium* infection is not part of the current analysis, but was conducted as part of the CRYPTO-POC study [26].

Data collection

Study nurses obtained informed written consent from the children's caregivers, collected demographic, exposure, and clinical data using standardized questionnaires, tested the children for HIV, and asked cases to provide a stool sample [26]. Stools were tested for *Cryptosporidium* by a composite reference standard comprising antigen detection by ELISA, oocyst detection and quantification by immunofluorescent antibody test microscopy (qIFAT), and DNA quantification by qPCR. We used our previously established quantitative qIFAT and qPCR cutoffs (725 oocysts/gram and 231302 copies/gram, respectively) for diarrhoea-associated infection; these cutoffs were applied without analysis for other possible co-infecting diarrhoeal pathogens [26]. The composite reference standard was considered positive if two or more reference tests were positive (and greater than the quantitative cutoff) and negative if two or more reference tests were negative (or less than the quantitative cutoff). A diarrhoea case was defined as cryptosporidiosis if the composite reference standard was positive and as non-cryptosporidiosis diarrhoea (NCrD) if negative [26]. The included variables were obtained from key published papers on risk factors for *Cryptosporidium* infection and/or cryptosporidiosis, and other risk factors known to be important for diarrhoea in general [6]. Acute malnutrition (MAM or SAM) was defined using mid-upper arm circumference (MUAC) thresholds in 6-59-month-olds, and by WHO weight-for-height z-scores in children < 6 months [30], and/or presence of bilateral oedema involving at least the feet; see S1 Appendix for these and other variable definitions.

Statistical methods

Double data entry was done with EpiData (version 3.1). All data were analysed in R (version 4.0.3) and RStudio (version 1.3). Missing values for exposure variables were multiply imputed (100 imputations) by chained random forests (500 trees) with predictive mean matching, using the R package missRanger (v.2.1). Odds ratios (OR) and their 95% confidence intervals (CI) were estimated by unconditional mixed model logistic regression, using the R package lme4 (v.1.1). We used a case-case-control design [27, 28], where the cryptosporidiosis and NCrD case sets were compared to the same non-diarrhoea control group, with results presented side-by-side for comparison.

The multivariable analysis followed a step-by-step approach using a hierarchical conceptual framework, as first outlined by Victora *et al* [23], a method that enables adjustment for risk factors that are causally distal to disease, while, at the same time, avoiding the common mistake of underestimating distal risk factors by adjusting for more proximal ones that act as mediators of their effect [31, 32]. To accomplish this, the analysis was governed by a predefined conceptual model [23] for the causal and hierarchical relationships between the proposed risk factors (Fig 1).

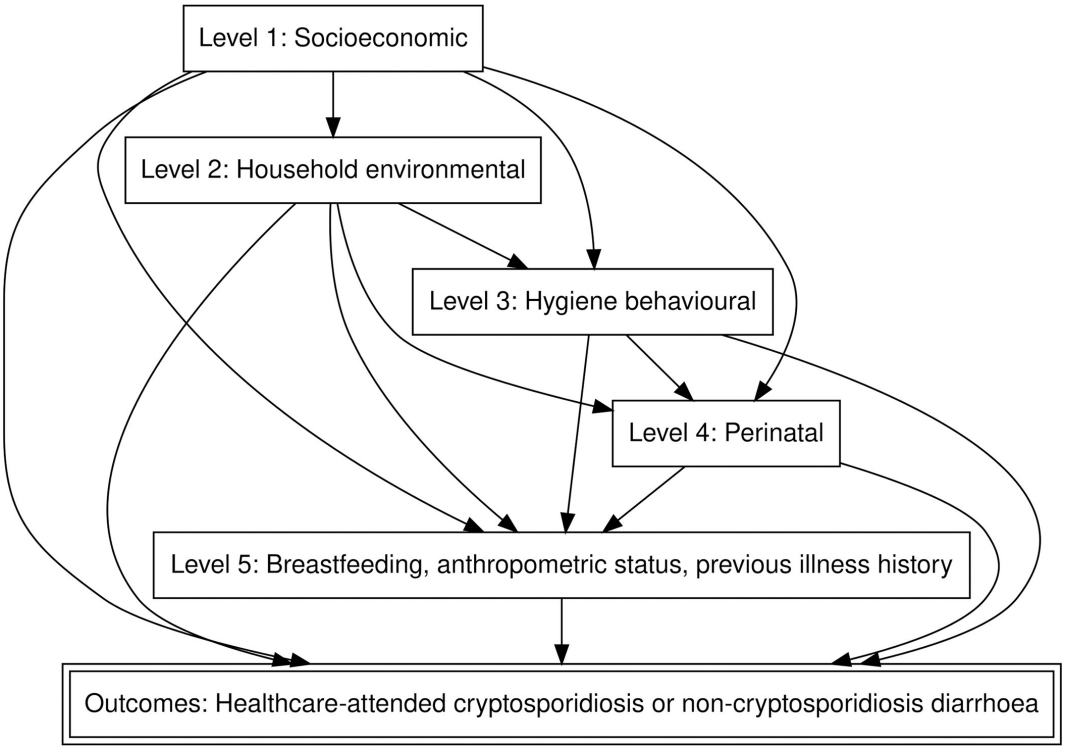

Fig 1. Hierarchical conceptual framework for the relationship between the putative risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea.

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The conceptual framework defines five hierarchical risk-factor levels [23, 24], where socioeconomic factors (level 1) are considered most distal to the outcomes of cryptosporidiosis and NCrD, and where nutritional factors and factors related to previous illness (level 5) are most proximal. Any level can be caused, in part or in full, by levels more distal to it, and the outcomes can be caused both directly and indirectly by factors at all levels. All models included adjustment for age (in months), gender, study site, and enrolment season (divided into six three-month intervals), i.e., the base adjustment set. Initial base-adjusted models were followed by intra-level models before undertaking a full hierarchical analysis. Importantly, the final estimate for the overall effect of a distal variable was the estimate derived before the introduction of more proximal-level risk factors. See S1 Appendix for details on the step-by-step modelling strategy.

Population attributable fractions (PAF) were estimated separately for cryptosporidiosis and NCrD for all risk factors that were significantly associated (i.e. with a p-value <0.05) in the hierarchical analysis, with the formula $PAF = Prevalence\ x\ \left(1 - \frac{1}{OR}\right)$, using the imputed prevalence of the risk factor in the case group and the OR estimate for the association between the risk factor and either disease [33]. Summary PAFs were calculated for each risk-factor level, derived from models that were adjusted for more distal levels, but not including more

proximal levels [24]. A summary PAF for all levels was also calculated, by taking the complement of the PAF at each level. A separate analysis was conducted to estimate the fraction of PAF that could potentially be explained by mediation through more proximal levels, using a variant of the traditional "difference method" of quantifying mediation, which is based on comparing odds ratios between two logistic regression models, where one model includes adjustment for a mediator, and the other model does not (S1 Appendix) [34].

Results

Diarrhoeal disease age distribution differed by case set; 95% (59/62) of cryptosporidiosis cases were younger than 24 months compared with 71% (432/607) of NCrD cases (Fig 2). As our priority was cryptosporidiosis risk factors, and to minimize the risk of residual confounding by age, we limited the statistical analysis to 0-23-month-olds. This included 1216 children aged 0–23 months, of whom 59 were cases with cryptosporidiosis, 432 cases with NCrD, and 725 controls. For details of screening, eligibility, and inclusion, see previous works [26, 29] and the study flowchart (Fig 2).

Table 1 shows the distribution of demographic characteristics, and distribution by enrolment period and study site, in the case sets and in the controls. Cryptosporidiosis numbers were not evenly distributed throughout the study period, with most cases during the late dry season (Feb-Apr) and early wet season months (May-Jul). A similar pattern was not seen for NCrD.

We first compared exposures between controls and each of the two case sets. There were no missing data in the base adjustment set, and no exposure variable had over 3.4% missing values (Table A in S1 Appendix). Risk factor associations are presented according to the structure of the statistical analysis; first, exposure-outcome associations estimated by univariable models (Table 2); second, risk-factor exposures, while considering other exposures at the same hierarchical level, estimated by "intra-level" multivariable models (Table 3), starting with the most distal (i.e., level 1) risk factors, and ending with the most proximal risk factors (level 5). Finally, risk-factor associations that take all hierarchical levels into account are presented, as estimated from the step-by-step hierarchical analysis (Table 4).

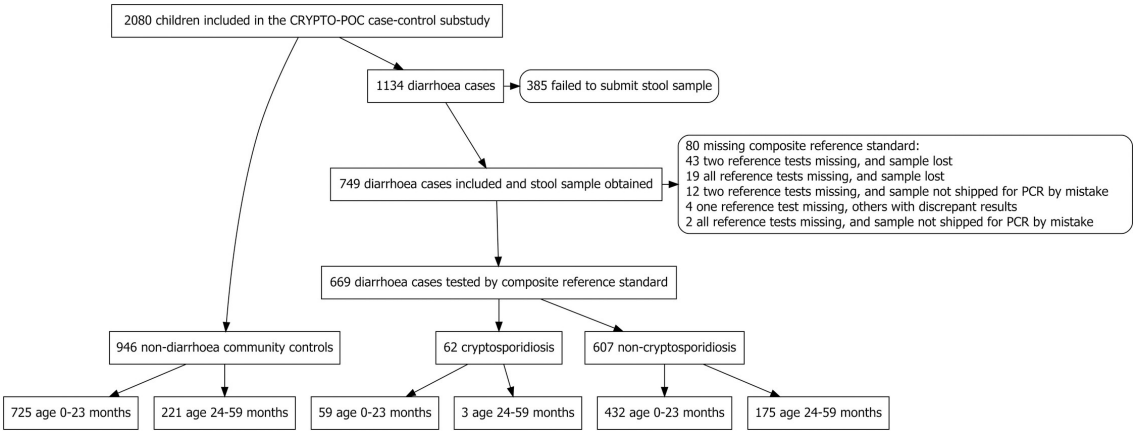

Fig 2. Study flowchart. For details on screening and eligibility, see flow diagram in previous publication [29]).

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Table 1. Distribution of diarrhoea cases with and without cryptosporidiosis and non-diarrhoea controls, according to gender and a priori defined confounding demographic variables.

		Diarrhoea cases	
Characteristic	**Controls; n (%) (N = 725)**	**Cryptosporidiosis; n (%) (N = 59)**	**Non-cryptosporidiosis; n (%) (N = 432)**
Age, in months			
< 6	92(13)	3(5)	54(12)
6–11	322(44)	34(58)	189(44)
12–23	311(43)	22(37)	189(44)
Gender[a]			
Female	331(46)	31(53)	170(39)
Male	394(54)	28(47)	262(61)
Study site			
Jimma hospital	332(46)	36(61)	186(43)
Serbo health center	393(54)	23(39)	246(57)
Season			
Feb–Apr: dry season	52(7)	18(31)	52(12)
May–Jul: wet season	120(17)	18(31)	102(24)
Aug–Oct: wet season	142(20)	4(7)	84(19)
Nov–Jan: dry season	194(27)	4(7)	101(23)
Feb–Apr: dry season (second enrolment year)	132(18)	10(17)	60(14)
May–Jul: wet season (second enrolment year)	85(12)	5(8)	33(8)

[a] Gender: evidence of selection bias due to missing outcome in some cases (S1 Appendix).

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Table 2 shows the corresponding ORs for the univariable relationships, with estimates for cryptosporidiosis and NCrD side-by-side and grouped by risk-factor level. These models contained only the base adjustment set in addition to the exposure and outcome variables, i.e., adjustment for age, gender, site, and season. Of the socioeconomic (level 1) variables, having a primary caregiver other than the mother was a strong risk factor for diarrhoea, and tended to be more strongly associated with cryptosporidiosis than with NCrD. Socioeconomic factors appeared to affect risk differently for cryptosporidiosis and NCrD; household lacking ownership of key assets was only associated with NCrD, whereas low maternal education was a risk factor specifically for cryptosporidiosis. For NCrD, there was a borderline trend of increased risk by household size. After the univariable models, the next step was adjustment for the other level-1 risk factors, but this had little impact on the observed associations (Table 3).

The following household environmental (level 2) variables were associated with NCrD in the univariable models, ordered by strength of the association from high to low: primary water source from a public piped water tap or from an unimproved source, household pre-treatment of drinking water, and cattle ownership. Of these, collecting drinking water from a public tap was also associated with cryptosporidiosis. Interestingly, however, consumption of surface water or water from unimproved sources, was not. Unimproved sanitation was neither associated with cryptosporidiosis nor NCrD, although this estimate had a wide margin of uncertainty due to evidence of differential exposure misclassification (S1 Appendix). All risk factors from the base-adjusted models remained statistically significant in the intra-level analysis (Table 3), except for the cattle ownership association with NCrD (OR 1.2, 95% CI 0.8 to 1.7).

Several hygiene-related (level 3) factors and perinatal (level 4) factors were associated with NCrD in the univariable models: handwashing without soap, unsafe disposal of child stool, premature delivery, and delivery by caesarean section (Table 2). All remained significant in the

Table 2. Risk factors for cryptosporidiosis diarrhoea and non-cryptosporidiosis diarrhoea in children under 2 years old, compared with non-diarrhoea controls; odds ratios estimated from univariable models[a].

			Diarrhoea cases		Cryptosporidiosis diarrhoea vs controls				Non-cryptosporidiosis diarrhoea vs controls			
Characteristic	**Reference level**	**Controls[b] (%) (N = 725)**	**Cryptosporidiosis[b] (%) (N = 59)**	**Non-cryptosporidiosis[b] (%) (N = 432)**	**OR**	**95% CI for OR**		**Linear trend *P***	**OR**	**95% CI for OR**		**Linear trend *P***
						from	**to**			**from**	**to**	
Level 1 – Socioeconomic factors												
Maternal education								0·02[c]				0·69[c]
< 1 year	≥ 8 years	27·4	35·6	29·9	2·4	1·1	4·9		1·1	0·78	1·4	
1–7 years	≥ 8 years	37·7	32·2	34·7	1·2[d]	0·58	2·3		0·93[d]	0·70	1·2	
Primary caregiver is not the child's mother	Mother primary caregiver	1·8	10·2	5·8	6·3	2·0	20·0		3·5	1·7	7·0	
Number of key assets owned by the household ≤ 2	3–7 key assets owned	16·0	13·6	24·1	0·93	0·42	2·1	0·10[e]	1·7	1·3	2·3	0·02[e]
Number of household members								0·27[e]				0·06[e]
4–5	< 4 members	46·6	47·5	39·6	0·72	0·38	1·4		0·88	0·65	1·2	
≥ 6	< 4 members	27·7	22·0	36·1	0·74	0·34	1·6		1·4	0·97	1·9	
Level 2 –Household environmental factors												
Persons per room ≥ 2	< 2 per room	93·5	86·4	90·7	0·52	0·22	1·2		0·72	0·46	1·1	
Animals owned by the household												
Cattle	No cattle	33·7	27·1	41·7	1·1	0·50	2·6		1·4	1·1	1·7	
Chickens	No chickens	32·1	27·1	33·6	1·1	0·55	2·0		1·0	0·81	1·4	
Dogs	No dogs	8·6	13·6	6·9	1·8	0·80	4·3		0·76	0·48	1·2	
Goats	No goats	7·4	6·8	9·5	ND	ND	ND		1·3	0·83	2·0	
Horses, donkeys, or mules	No horses, donkeys, or mules	9·1	6·8	12·5	ND	ND	ND		1·4	0·92	2·0	
Sheep	No sheep	14·8	10·2	14·1	1·2	0·45	3·1		0·99	0·70	1·4	
Other	No other animals	3·2	1·7	1·4	ND	ND	ND		0·42	0·17	1·1	
Any even-toed ungulate	No even-toed ungulates	35·9	32·2	42·6	1·6	0·69	3·6		1·3	0·99	1·6	
Any animal	No animals	49·2	45·8	50·2	1·1	0·61	2·1		0·99	0·77	1·3	
Sanitation facility[f]								0·34[c]				0·09[c]
Improved, but shared	Unimproved facility	8·6	11·9	19·0	0·63	0·15	2·7		1·3	0·68	2·3	
Improved, and not shared	Unimproved facility	8·1	37·3	28·7	1·8	0·55	5·6		1·8	0·92	3·4	
Access to "improved sanitation" (by the WHO definition)[f]	Unimproved or shared facility	8·1	37·3	28·7	2·1	0·72	5·9		1·6	0·88	2·9	
Water source for the household								0·98[c]				<0·01[c]
Public tap	Private tap	13·7	37·3	24·5	3·7[d]	1·8	7·3		2·8[d]	1·9	4·0	
Surface or rainwater, unprotected well, borehole, or protected spring	Private tap	30·6	15·3	35·9	0·99[d]	0·41	2·4		1·9[d]	1·4	2·8	

(*Continued*)

Table 2. (Continued)

			Diarrhoea cases		Cryptosporidiosis diarrhoea vs controls				Non-cryptosporidiosis diarrhoea vs controls			
Characteristic	**Reference level**	**Controls[b] (%) (N = 725)**	**Cryptosporidiosis[b] (%) (N = 59)**	**Non-cryptosporidiosis[b] (%) (N = 432)**	**OR**	**95% CI for OR**		**Linear trend *P***	**OR**	**95% CI for OR**		**Linear trend *P***
						from	**to**			**from**	**to**	
Water treated by the household (chemicals, boiling or filtering) before drinking	No water treatment	5·2	5·1	9·5	ND	ND	ND		1·9	1·2	3·1	
Level 3 –Hygiene behaviour												
Last stool disposal (from any of the caregiver's children) "unsafe" by the WHO definition	Safe disposal (i.e., in toilet/latrine, or buried)	55·7	66·1	75·7	1·50	0·77	3·0		2·4	1·7	3·3	
Caregiver will normally wash hands												
before meals	not before meals	94·6	93·2	92·1	ND	ND	ND		0·62	0·38	1·0	
before preparing food for the child	not before preparing food for the child	74·2	78·0	72·9	1·0	0·53	2·0		0·88	0·67	1·2	
after a toilet visit	not after a toilet visit	68·6	64·4	70·1	0·93	0·52	1·7		1·1	0·85	1·5	
without soap	with soap	3·3	5·1	6·9	ND	ND	ND		2·4	1·4	4·3	
Level 4 –Perinatal factors												
Mode of delivery–caesarean section	Vaginal delivery	6·5	11·9	10·9	1·6	0·66	4·0		1·6	1·1	2·5	
Child born prematurely (before week 37)	Not prematurely born	1·9	5·1	5·8	ND	ND	ND		2·9	1·5	5·6	
Level 5 – Breastfeeding, nutritional status, and previous illness history												
Early cessation of exclusive breastfeeding	No early cessation of exclusive breastfeeding	32·1	32·2	38·7	1·2	0·68	2·2		1·5	1·1	1·9	
Not breastfeeding now (or, for cases, just before the diarrhoeal episode started)	Breastfeeding now (or, for cases, just before the diarrhoeal episode started)	8·4	15·3	11·1	3·6	1·5	8·6		1·8	1·2	2·8	
Acute malnutrition								<0·01[cg]				0·02[cg]
Moderate acute malnutrition (MAM)	No acute malnutrition	2·2	11·9	10·4	5·9	2·2	15·8		5·2	2·9	9·3	
Severe acute malnutrition (SAM)	No acute malnutrition	0·6	6·8	2·1	9·3[h]	2·0	43·7		4·3	1·3	14·4	
Acute malnutrition, any (MAM or SAM)	No acute malnutrition	2·8	18·6	12·5	6·7	2·9	15·6		5·0	2·9	8·6	
One or more overnight admissions, since birth	No overnight admissions	7·3	10·2	7·9	1·0	0·40	2·6		1·0	0·64	1·6	

(*Continued*)

Table 2. (Continued)

			Diarrhoea cases		Cryptosporidiosis diarrhoea vs controls				Non-cryptosporidiosis diarrhoea vs controls			
Characteristic	**Reference level**	**Controls[b] (%) (N = 725)**	**Cryptosporidiosis[b] (%) (N = 59)**	**Non-cryptosporidiosis[b] (%) (N = 432)**	**OR**	**95% CI for OR**		**Linear trend *P***	**OR**	**95% CI for OR**		**Linear trend *P***
						from	**to**			**from**	**to**	
One or more diarrhoea episodes, during the last month	No diarrhoea episodes	15·0	27·1	16·9	2·4[d]	1·3	4·7		1·2[d]	0·83	1·6	
One or more visits to hospital or health center due to illness, since birth	No visits since birth	27·2	47·5	32·9	2·4	1·4	4·1		1·3	1·0	1·7	

OR = odds ratio. CI = confidence interval. WHO = World Health Organization. ND = Not done, due to insufficient number (n < 5) exposed for reliable estimation of OR.

[a] Logistic regression models with the addition of a "base adjustment set" with fixed effect terms for age and gender and random effect intercept terms for enrolment site and season.

[b] Prevalence, after imputing all missing values for exposure variables (see Table A in S1 Appendix for missingness breakdown)

[c] Test for linear trend (P-level), using the ordered categorical variable levels as predictor.

[d] Evidence of selection bias due to missing outcome in some cases (S1 Appendix).

[e] Test for linear trend (P-level), using the continuous variable as predictor.

[f] Models including the sanitation facility variable included a random effect intercept for nurse conducting the interview, due to evidence for differential exposure misclassification for this variable (S1 Appendix).

[g] Test for linear trend also positive (P-level < 0·01) when using MUAC as a continuous predictor variable (in ≥ 6-month-olds).

[h] Few exposed (n = 4) in the SAM subcategory.

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intra-level analysis (Table 3). None of these factors were significantly associated with cryptosporidiosis.

Of the level-5 factors, acute malnutrition was strongly associated with both cryptosporidiosis and NCrD in the univariable models, and the strength of the association varied by the degree of malnutrition. Not being breastfed immediately prior to the current diarrhoeal episode was associated with both NCrD and cryptosporidiosis, but early cessation of exclusive breastfeeding (earlier than the WHO minimum recommended age of 6 months) was only significantly associated with NCrD. Previous healthcare attendance was more strongly associated with cryptosporidiosis than with NCrD. After adjusting for other level-5 factors, diarrhoea within the last month was no longer significantly associated with cryptosporidiosis (OR 1.9, 95% CI 0.9 to 3.8; Table 3).

Results from the hierarchical analysis, i.e., taking all hierarchical levels into account, are shown in Table 4. The hierarchical framework determined the order in which variables were included from the intra-level models in the hierarchical analysis, starting with level 1 (base-adjustment-set only), then level 2 (also adjusted for level-1 risk factors), level 3 (also adjusted for level-1 and level-2 risk factors), etc. Most risk factors from the intra-level analyses (Table 3) remained significant in the hierarchical analysis, i.e., after accounting for possible confounding from more distal levels. A notable exception was not being currently breastfed, where the OR for the association with cryptosporidiosis dropped from 3.3 in the intra-level model (Table 3) to 2.0 (95% CI 0.7 to 5.6) after adjustment for more-distal risk factors.

PAF was used as an estimate for the hypothetical relative contribution of each risk factor to the number of cases (Table 4). Some factors were estimated to contribute a small number of cases despite strong risk association. For cryptosporidiosis, the most important contributors

Table 3. Risk factors for cryptosporidiosis diarrhoea and non-cryptosporidiosis diarrhoea in children under 2-years old, compared with non-diarrhoea controls; odds ratios estimated from separate intra-level multivariable regression models[a].

		Cryptosporidiosis diarrhoea vs controls			Non-cryptosporidiosis diarrhoea vs controls		
Characteristic	**Reference level**	**OR**	**95% CI for OR**		**OR**	**95% CI for OR**	
			from	**to**		**from**	**to**
Level 1 –Socioeconomic factors							
Maternal education							
< 1 year	≥ 8 years	2·2[b]	1·0	4·7			
1–7 years	≥ 8 years	1·1[b]	0·56	2·3			
Primary caregiver is not the child's mother	Mother primary caregiver	5·5	1·7	17·8	3·8	1·9	7·6
Number of key assets owned by the household ≤ 2	3–7 key assets owned				1·8[c]	1·3	2·4
Level 2 –Household environmental factors							
Water source							
Public tap	Private tap	3·7[d]	1·8	7·3	2·7[e]	1·9	3·9
Surface or rainwater, unprotected well, borehole, or protected spring	Private tap	0·99[d]	0·41	2·4	1·9[e]	1·3	2·8
Water treated by the household (chemicals, boiling, or filtering) before drinking	No water treatment				1·8	1·1	2·9
Level 3 –Hygiene behaviour							
Caregiver will normally wash hands without soap	Normally washes with soap				2·2	1·2	3·8
Last stool disposal (from any of the caregiver's children) was unsafe, by the WHO definition	In toilet/latrine, or buried (WHO "safe" stool disposal)				2·3	1·7	3·1
Level 4 –Perinatal factors							
Mode of delivery–caesarean section	Vaginal delivery				1·6	1·0	2·4
Child born prematurely (before week 37)	Not born prematurely				2·8	1·4	5·5
Level 5 –Breastfeeding, nutritional status, and previous illness							
Early cessation of exclusive breastfeeding	No early cessation of exclusive breastfeeding				1·4	1·0	1·8
Not breastfeeding now (or, for cases, just before the diarrhoeal episode started)	Breastfeeding now (or, for cases, just before the diarrhoeal episode started)	3·3	1·3	8·3	1·7	1·1	2·6
Acute malnutrition, any (MAM or SAM)	No acute malnutrition	6·1	2·6	14·6	4·8	2·8	8·3
One or more visits to hospital or health center due to illness, since birth	No visits	2·3	1·3	4·0			

Risk factor rows containing empty cells are relevant for either cryptosporidiosis or non-cryptosporidiosis diarrhoea, but not for both.

OR = odds ratio. CI = confidence interval. WHO = World Health Organization. MAM = moderate acute malnutrition. SAM = severe acute malnutrition.

[a] Presented in the table are estimates from multiple regression models with those risk factor variables that remained significant after intra-level modelling; all models were also adjusted for age and gender, with random effect intercepts for enrolment site and season.

[b] Test for linear trend P = 0·04, using the ordered categorical variable levels as predictor.

[c] Test for linear trend P = 0·01, using number of key assets owned as predictor.

[d] Test for linear trend P = 0·98, using the ordered categorical variable levels as predictor.

[e] Test for linear trend P < 0·01, using the ordered categorical variable levels as predictor.

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were, from high to low PAF values: public tap water used for drinking, previous illness leading to healthcare attendance, low maternal education, acute malnutrition, and having a primary caregiver other than the mother. By PAF contribution, socioeconomic factors and acute malnutrition were more important for cryptosporidiosis than for NCrD, whereas household environmental and hygiene factors were less important (Table 4).

In the mediation analysis, socioeconomic risk factors for cryptosporidiosis were—to a larger extent than the NCrD risk factors—mediated through proximal risk-factor levels (68% vs 22% of the level-1-PAF, respectively, Table B in S1 Appendix). With this exception, we found only weak evidence for mediation of other distal risk factors.

Discussion

A key finding from our study was the strong association between acute malnutrition and both cryptosporidiosis and NCrD. For cryptosporidiosis, the association was stronger than has previously been found in studies that compared between diarrhoea cases with and without

Table 4. Risk factors for cryptosporidiosis diarrhoea and non-cryptosporidiosis diarrhoea in children under 2-years old, compared with non-diarrhoea controls; odds ratios and population attributable fractions estimated from the hierarchical analysis[a].

		Cryptosporidiosis diarrhoea vs controls				Non-cryptosporidiosis diarrhoea vs controls			
Characteristic	**Reference level**	**OR**	**95% CI for OR**		**PAF (%)**	**OR**	**95% CI for OR**		**PAF (%)**
			from	**to**			**from**	**to**	
Level 1 –Socioeconomic factors									
Maternal education									
< 1 year	≥ 8 years	2·2[b]	1·0	4·7	19				
1–7 years	≥ 8 years	1·1[b]	0·56	2·3	NA				
Primary caregiver is not the child's mother	Mother primary caregiver	5·5	1·7	17·8	8	3·8	1·9	7·6	4
Number of key assets owned by the household ≤ 2	3–7 key assets owned					1·8[c]	1·3	2·4	11
Socioeconomic factors PAF					26				14
Level 2 –Household environmental factors									
Water source									
Public tap	Private tap	3·8[d]	1·9	7·7	27	2·5[e]	1·7	3·6	15
Surface or rainwater, unprotected well, borehole, or protected spring	Private tap	1·1[d]	0·43	2·7	NA	1·7[e]	1·2	2·5	15
Water treated by the household (chemicals, boiling or filtering) before drinking	No water treatment					1·8	1·1	3·0	4
Household environmental factors PAF					27				31
Level 3 –Hygiene behaviour									
Caregiver will normally wash hands without soap	Normally washes with soap					1·9	1·1	3·5	3
Child stool disposal unsafe, by the WHO definition	Safe child stool disposal					2·3	1·6	3·1	42
Hygiene behaviour factors PAF									44
Level 4 –Perinatal factors									
Mode of delivery–caesarean section	Vaginal delivery					1·6	1·0	2·5	4
Child born prematurely (before week 37)	Not born prematurely					3·1	1·5	6·2	4
Perinatal factors PAF									8
Level 5 –Breastfeeding, nutritional status, and previous illness									
Early cessation of exclusive breastfeeding	No early cessation of exclusive breastfeeding					1·5	1·1	2·0	13
Acute malnutrition, any (MAM or SAM)	No acute malnutrition	7·2	2·9	17·8	16[f]	4·6	2·6	8·0	10[f]
Moderate acute malnutrition (MAM)	No acute malnutrition	5·3[e]	1·8	15·5	10	4·7[g]	2·5	8·7	8
Severe acute malnutrition (SAM)	No acute malnutrition	16·2[eh]	3·1	83·4	6[h]	4·1[g]	1·2	14·6	2
One or more visits to hospital or health center due to illness, since birth	No visits	2·3	1·3	4·1	27				
Breastfeeding, nutritional status and previous illness factors PAF					39				22
Summary PAF for all levels					**67**				**76**

Risk factor rows containing empty cells are relevant for either cryptosporidiosis or non-cryptosporidiosis diarrhoea, but not for both.

OR = odds ratio. CI = confidence interval. PAF = Population attributable fraction. WHO = World Health Organization. MAM = moderate acute malnutrition.

SAM = severe acute malnutrition. NA = Not applicable, i.e., PAF not estimated as this subcategory was not a significant risk factor for cryptosporidiosis.

[a] All multiple-regression models adjusted for age and gender, with random effect intercepts for enrolment site and season.

[b] Test for linear trend P = 0·04, using the ordered categorical variable levels as predictor.

[c] Test for linear trend P = 0·01, using number of key assets owned as predictor.

[d] Test for linear trend P = 0·84, using the ordered categorical variable levels as predictor.

[e] Test for linear trend P < 0·01, using the ordered categorical variable levels as predictor.

[f] PAF estimates for acute malnutrition need to be interpreted with caution due to the cross-sectional evaluation of this exposure-outcome association (see Discussion).

[g] Test for linear trend P = 0·03, using the ordered categorical variable levels as predictor.

[h] Few exposed (n = 4) in the SAM subcategory.

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cryptosporidiosis [12–19], and may be representative of a general healthcare-presenting population with diarrhoea, i.e., not restricted to children with moderate-to-severe or acute diarrhoeal episodes. MAM was less strongly associated with cryptosporidiosis than SAM but was far more common. The WHO provides no specific guidance for the management of diarrhoea in children with MAM, besides the promotion of supplementary foods [29, 35]. Regrettably, most Ethiopian children with MAM, including in our study area, are excluded from supplementary feeding programmes, as they reside within areas that are not classified as food insecure [36]. Case-finding and proper nutritional rehabilitation for all children with acute malnutrition should be a priority. The only currently approved therapeutic for cryptosporidiosis, nitazoxanide, is still not widely used in Africa, and is not registered in Ethiopia. The drug is assumed to have a modest effect on cryptosporidiosis in HIV-negative children with acute malnutrition. However, it is worth noting that the only randomized controlled trial that enrolled HIV-seronegative children with acute malnutrition, included only 11 severely wasted and 6 moderately wasted participants, all 1-3-years old, yet demonstrated a significant effect on both diarrhoeal duration and case fatality [37]. Nitazoxanide treatment for cryptosporidiosis in children with MAM and SAM could be explored as a simple intervention, if facilitated by low-tech point-of-care tests with proven accuracy [26]. We agree with calls for further research and development of new and more effective drugs, while simultaneously recognizing the potential benefits of wider global use of nitazoxanide [38–40].

Previous healthcare visits were common in all children who presented with diarrhoea, particularly in cryptosporidiosis, where this association remained strong also after controlling for confounding from more distal levels. Previous history of illness and acute malnutrition can be considered as criteria to help prioritize which children to test for cryptosporidiosis, should testing capacity be limited. Caregivers of this higher-risk group of children might be cost-effective targets for secondary prevention of cryptosporidiosis (e.g., intervention bundles containing tools and advice on how to manage diarrhoea and malnutrition), and incentives and advice regarding when to return for review and diagnostic testing (e.g., explaining the benefits of treatment, skipping of queues for reassessment, and transport reimbursements).

Unlike previous puzzling reports [8, 9, 11], not having piped water access in the household was a risk factor for both cryptosporidiosis and NCrD, similar to what has been found for all-cause diarrhoea [41]. It is interesting that public tap water, considered an improved source, was more strongly associated with cryptosporidiosis than consumption of water from unimproved sources. This might reflect poor water quality, i.e., faecal contamination of the piped water at the point of supply or collection. Alternatively, it could be a marker for insufficient water quantity affecting household hygiene in a way that is not fully captured by the level-3-variables. The finding warrants examining the piped water at various supply points, and maybe the pump handles themselves, for both faecal indicator bacteria and *Cryptosporidium* oocysts.

Ethiopia is one of the poorest countries in the world, yet, over the last two decades, has demonstrated an impressive reduction in extreme poverty, while maintaining relative wealth equality [42]. Nevertheless, we found that socioeconomic status, particularly when assessed by maternal education, was a large contributor to healthcare-attended cryptosporidiosis in our study area. It might be possible to target caregivers with specific interventions related to hygiene behaviour and nutrition, but it seems wise to first obtain local data on the extent to which care-related risks act through behaviours and beliefs that are amenable to intervention. In our study population, the proposed effect appears to be largely mediated through intermediate risk factors (Table B in S1 Appendix). There is an important methodological lesson implicit in this finding: had we performed multivariable analysis without the application of a causal framework, socioeconomic status (specifically, low maternal education) would have appeared

to be unimportant. For NCrD, the effect of such overadjustment bias would be less dramatic, but would have resulted in lower estimates of both the strength of association and contribution to case load from level-1-factors.

Several limitations need to be considered when interpreting our findings. Defining acute malnutrition in the context of diarrhoea is challenging, as weight is affected by dehydration. To address this, MUAC was used to classify acute malnutrition in ≥ 6-month-olds, as it is less vulnerable to dehydration than weight [43, 44]. Also, the conceptual scheme used in this analysis is a simplification of the complex relationship between malnutrition and diarrhoea. It is difficult to disentangle in individual children whether malnutrition causally increases the risk of diarrhoea, whether diarrhoea inflicts a nutritional insult, or both. While most researchers accept that episodes of acute malnutrition increase the risk of all-cause diarrhoea [45], the evidence for the degree to which pre-existing malnutrition increases the risk of specific diarrhoeal syndromes, including cryptosporidiosis, is sparse (S1 Appendix) [46]. Some birth-cohort studies indicate that cryptosporidiosis can cause reduced ponderal growth in the 6-month period after an episode [47, 48] and a recent publication reporting on a large multi-country study of infants and toddlers with moderate-to-severe diarrhoea identified *Cryptosporidium* infection as a predictor of linear growth faltering [49]. In the current study, we were unable to disentangle the proportion of the observed association that was due to acute malnutrition (MAM or SAM) increasing the risk of cryptosporidiosis, and how much of the association was due to the current cryptosporidiosis episode leading to acute malnutrition. However, because most diarrhoeal episodes were short, we carefully speculate that the former link may have outweighed the latter (Table C in S1 Appendix). While we believe the OR describing the association is valid, the PAF for acute malnutrition could be an overestimate, for both cryptosporidiosis and NCrD. Appropriate analysis of case-control studies, where children with and without acute malnutrition are followed up for pathogen-attributed diarrhoeal episodes [50], will be necessary to explore this important question further.

Second, the apparent lack of association between early cessation of exclusive breastfeeding and cryptosporidiosis should be interpreted with particular care, as there were only three cases in the 0-5-month group, where introduction of foods and liquids other than breastmilk is likely to pose the highest risk (Table 1). Birth cohorts report a peak in cryptosporidiosis incidence at 6–11 months of age [9, 51], which is consistent with community-based studies that indicate a significant protective effect of breastfeeding [8, 52]. Not breastfeeding currently was significantly associated with cryptosporidiosis and NCrD in the initial models, but not in the hierarchical analysis. A possible explanation is that breastfeeding is, at least in part, a marker for one or more of the distal-level risk factors. Confounding from socioeconomic factors can bias inference about all exposure-outcome relationships at more proximal levels and is considered particularly important for breastfeeding [53].

Third, the counterintuitive finding of an association between point-of-use water treatment and NCrD should be interpreted with caution, as water treatment was uncommon overall. It is possible that the observed association is confounded by some aspect of water quality not fully captured by our water source variable.

Fourth, at least a third of the total cryptosporidiosis case load could not be attributed to any of the identified risk factors (summary PAF for all levels, 67%, Table 4). Our list of investigated risk factors was far from exhaustive. Some variables were omitted due to high risk of selection bias (e.g., vaccination status, diarrhoea in close contacts) [19], or were omitted by design (e.g., variables related to food handling, floor covering, day-care attendance, swimming).

Fifth, we did not adjust for all factors that may influence healthcare seeking, such as disease severity or dehydration, as healthcare presentation was integral to our outcome definition (Fig 1), and adjustment could therefore have induced a bias. Likewise, we are not able to quantify

how caretakers' healthcare-seeking decisions may have impacted the observed association between acute malnutrition and cryptosporidiosis.

Sixth, due to the difference in the number of children between the case sets, limited by the parent study (S1 Appendix), and because this is a case-case-control rather than a case-case study, strong inferences cannot be made based on the observed differences in OR between the cryptosporidiosis and NCrD groups. Most of the confidence intervals obtained from the analysis are sufficiently narrow to allow meaningful interpretation of their corresponding point estimates. However, we note that for some putative risk factors the confidence intervals for the OR are wide and therefore difficult to interpret, reflecting uncertainty due to the limited number of children and events for some of the comparisons, e.g., in the multivariable cryptosporidiosis vs control comparisons.

Finally, the evidence presented here can at best be used as support for a causal relationship between the identified risk factors and cryptosporidiosis, as there have been few interventional studies. For diarrhoea in general, there is now a large body of both observational and interventional evidence to support a causal role of underprivileged access to water/sanitation/hygiene, perinatal factors, lack of breastfeeding, and malnutrition [7]. There are important similarities in transmission (e.g., faecal-oral) and host susceptibility (e.g., nutrition) between various diarrhoeal infections, which likely also explain that there were many common risk factors for cryptosporidiosis and NCrD. Nevertheless, by using a hierarchical case-case-control analysis, we do observe some differences that may plausibly be related to characteristics of the *Cryptosporidium* parasite. These include stronger association with piped water from public, rather than private, taps, which could be related to the environmental robustness of *Cryptosporidium* oocysts, and higher risk associated with previous healthcare attendance and acute malnutrition, which could be related to a particular need for a healthy immune response to prevent illness and resolve symptoms. An important next step in exploring this puzzle will be obtaining more evidence from preventive interventions against diarrhoea where outcomes are considered by aetiology [54], and trials that examine the role of pharmacological treatment of cryptosporidiosis as part of the nutritional rehabilitation of malnourished children.

Supporting information

S1 Checklist. STROBE checklist.
(PDF)

S1 Appendix. Supplementary appendix. Table A in S1 Appendix. Distribution of case and control subjects according to all exposures, with counts and proportions of missing values. Table B in S1 Appendix. Hierarchical mediation analysis of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea, in children under 2 years old. Table C in S1 Appendix. Duration of diarrhoea, on enrolment, in cryptosporidiosis and non-cryptosporidiosis diarrhoea cases, in children under 2 years old.
(PDF)

S1 Dataset. Anonymized dataset.
(XLS)

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Supplementary appendix for:

A comparison of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea: a hierarchical case-case-control study in Ethiopian children

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A selective review of the risk-factor literature for cryptosporidiosis

We have not performed a comprehensive or new systematic review of the diverse risk-factor literature for *Cryptosporidium* infection and cryptosporidiosis, but have chosen to use the most recent (2018), good-quality systematic review as a starting point [1]. We then summarize some key studies conducted thereafter, with a focus on the following: 1) how methodological challenges related to case ascertainment and confounding were managed, and 2) the relevance and applicability of the findings to clinical paediatric cryptosporidiosis in LMIC. As acute malnutrition was not included in the systematic review, we give a summary of a non-systematic review of the literature on acute malnutrition as a risk factor for both cryptosporidiosis and non-cryptosporidiosis diarrhoea (NCrD).

In summary, we identified evidence-gaps and unresolved discrepancies in the literature between risk factors for cryptosporidiosis and risk factors for pediatric diarrhoea in general [2, 3]. A recent meta-analysis identified animal contact, open defecation, and diarrheic household contacts as risk factors for *Cryptosporidium* infection; surprisingly, poor quality drinking water and lack of breastfeeding were not [1]. Later, the MAL-ED community-cohort study reported a limited set of risk factors for *Cryptosporidium* infection in children below 2 years of age[4]; only overcrowding and low length-for-age in the preceding months were found to be important risk factors, and, notably, unprotected drinking water, animal exposure, and sanitation factors were not. A recent study in the GEMS site in Gambia investigated *Cryptosporidium* infection in children presenting to healthcare with diarrhoea. Household water storage and the presence of cattle and cats in the household were reported as risk factors; overcrowding was, counterintuitively, found to be protective [5]. The frequent reports of animal exposure as a risk factor are contradicted by the currently available evidence from low-income settings [6]. The comparators used in most risk factor studies are diarrhoea cases without *Cryptosporidium* infection, i.e., case-case comparisons [7-14]. We have not identified any case-control studies that distinguished between *Cryptosporidium* infection and cryptosporidiosis, i.e., diarrhoea attributed to *Cryptosporidium* infection [15, 16], or that investigated hand hygiene, perinatal factors, and acute malnutrition in the same analysis [5, 17].

When trying to identify evidence gaps in the literature, the interpretation of many reports is complicated by the distinctions between exposure, infection, and disease frequently being unclear. To classify previous studies, we found it useful to consider separately each step that leads up to a case of cryptosporidiosis that presents for healthcare. We start by defining the population, e.g., all children under 5 who live in a geographically defined area around a hospital or health center. Within this "catchment area" different subsets of children will be exposed to different enteric microorganisms, including *Cryptosporidium*, and, among these subsets, some children will become infected. A proportion of those infected will develop diarrhoeal symptoms due the infection, i.e., cryptosporidiosis, and a subset of these will seek health care.

Interventions can, and should be, targeted at each step. Study designs and methods vary widely, and it follows that there will also be large variation in the applicability of risk-factor findings to the development of new interventions.

The 2018 systematic review on risk factors for *Cryptosporidium* infection in LMIC included only 15 studies and the authors highlighted the paucity of good-quality evidence [1]. The meta-analysis identified the following risk factors for infection (note; the review did not distinguish clearly between asymptomatic *Cryptosporidium* infection and cryptosporidiosis): animal contact, open defecation, and diarrhoea in a household contact. Surprisingly, poor quality drinking water and lack of breastfeeding were not risk factors [1]. It is worth noting that perinatal and previous illness factors were not included, hand hygiene was too infrequently reported for inclusion in the meta-analysis, none of the included studies investigated unsafe disposal of children's stools, and chronic or acute malnutrition were not reviewed as risk factors. As was also highlighted by the authors, particular care should be taken when interpreting the meta-analyses, as there was substantial heterogeneity between the 15 studies included, comprising both children and adults, asymptomatic and symptomatic infections, and studies conducted both within and outside a clinical setting. The authors pooled odds ratios (OR) from univariable analyses, precluding adjustment for important confounders. Seasonal effects and demographic and socioeconomic factors were not included yet can be important confounders of any inference about risk factors related to household transmission pathways, hygiene, nutritional status, or previous illness. Adjustment for socioeconomic, maternal, and perinatal factors is considered particularly important when quantifying the risk from early cessation of breastfeeding [18]. However, the meta-analysis of the protective effect of breastfeeding (figure 7 in [1]) pooled crude OR data from four studies that reported a protective effect, with one study reporting a non-significantly increased risk from breastfeeding. This latter study was conducted in an urban slum area with notably low rates of exclusive breastfeeding (<17%) and might represent an outlier [19].

A more recent publication on *Cryptosporidium* infection in children less than 2 years of age from the MAL-ED investigators included a multivariable analysis, with some adjustment for socioeconomic factors, but the set of included risk factors was limited [4]. Overcrowding and low preceding length-for-age were found to be important risk factors, and, notably, unprotected drinking water, animal exposure, and sanitation factors were not. It is worth noting that the authors did not differentiate between asymptomatic *Cryptosporidium* infection and cryptosporidiosis, and that the findings might not be applicable to clinical cryptosporidiosis as the study was conducted in a community setting.

Another recent study, from the GEMS site in Gambia, looked at *Cryptosporidium* infection in children presenting to healthcare with diarrhoea. Overcrowding was, counterintuitively, found to be protective, and household water storage and the presence of cows and cats in the household were reported as risk factors [5]. This study did not distinguish between diarrhoea-associated infection (i.e., cryptosporidiosis) and asymptomatic infection. The GEMS and MAL-ED "re-analysis" studies performed quantitative PCR testing and highlighted the important difference between *Cryptosporidium* infection and cryptosporidiosis, i.e., diarrhoea attributed to *Cryptosporidium* infection [15, 16], but none of the above studies applied a quantitative approach to the analysis of risk factors. Finally, the frequent reports of animal exposures as a risk factor are puzzling; *Cryptosporidium* is largely considered to have an anthroponotic transmission pattern in LMIC, and, in sub-Saharan Africa, a significant contribution from zoonotic infections is contradicted by the currently available evidence [6].

Most of the reviewed studies either reported crude associations or used multivariable logistic regression with stepwise elimination starting with all variables that were significant in initial models; to our knowledge no cryptosporidiosis risk-factor studies used a multivariate modelling approach based on a pre-defined causal framework for the relationships between the included variables (for example, using a hierarchical framework to guide the analysis, by directed acyclic graphs, or by other types of causal diagrams).

Acute malnutrition is well established as a risk factor for frequency, duration, severity, and case fatality of diarrhoeal episodes [20-24], but the increase in risk can be subtle unless the malnutrition is moderate-to-severe [23, 24]. For specific diarrhoeal aetiologies, the evidence is patchy, e.g., for cryptosporidiosis, none of the risk factor studies summarized above reported on moderate or severe acute malnutrition. However, several studies have compared characteristics in diarrhoea cases with and without *Cryptosporidium* infection (i.e., case-case comparisons) and observed an association between either being underweight (as measured by weight-for-age Z score, WAZ) wasting (as measured by weight-for-height Z score, WHZ) and/or severe acute malnutrition (SAM; defined by either severe wasting or bilateral oedema) and *Cryptosporidium* infection [7-14, 25]. Protein malnutrition, thought to be a key component in acute malnutrition, has been found to be a risk factor for cryptosporidiosis in animal models [26-28], however, the evidence from prospective studies in children is limited. A community birth cohort study conducted in Guinea-Bissau from 1987-1990 found no significant difference in absolute weight, WAZ, or length in children with cryptosporidiosis, compared with non-diarrhoea controls, measured at a time point within the preceding 90 day-period, instead reporting an association with subsequent weight loss (both short- and long-term) in the 0-180-day period after the episode [29, 30]. The study design did not allow disentangling whether this pattern was shared with NCrD or specific to cryptosporidiosis. A negative impact on ponderal growth after an episode of cryptosporidiosis is also supported by animal studies [31]. A birth cohort study from Peru found an association with (short-term) weight loss in the months after *Cryptosporidium* infection in a study that mainly included asymptomatic cases [32, 33]. Although no significant difference was seen in preceding weight between children with and without cryptosporidiosis, these two cohorts included few children with acute malnutrition, moderate-to-severe wasting, or severe underweight; the Peru cohort had three children with WHZ < -2; the Guinea-Bissau study did not specifically report the numbers with acute malnutrition (or WHZ) but noted little difference in mean WAZ preceding the episode (mean WAZ -0.74 in children with cryptosporidiosis, versus -0.76 in matched controls). Although not directly comparable, as conducted in 2-to-5-year-olds, a cohort study in a Bangladeshi slum reported that children who were underweight (WAZ was < -2 in 39% of the participants) had a significantly increased risk of subsequent cryptosporidiosis [34].

None of the cohort studies summarized above investigated moderate or severe acute malnutrition, as defined by WHZ or mid-upper-arm circumference (MUAC) cutoffs or the presence of oedema, as a risk factor for subsequent cryptosporidiosis incidence or severity. However, a recent post-hoc analysis of GEMS data investigated the interaction between acute malnutrition and healthcare-presenting diarrhoea and assessed the influence of acute malnutrition on diarrhoeal case-fatality risk at 60-day follow up [17]. This is likely the most comprehensive analysis of acute malnutrition as a risk factor for healthcare-presenting diarrhoea when broken down by aetiology. Furthermore, it stands out, as it compared cases to non-diarrhoea controls, accounted for confounding by age and key socioeconomic and water/sanitation/hygiene actors, and included both the severe and moderate form of acute malnutrition. The prevalence of *Cryptosporidium* (by ELISA antigen detection) in under-2-year-old diarrhoea cases was 17% in cases with acute malnutrition (vs 9% in controls) and 9% in cases without acute malnutrition (vs 4% in controls). Furthermore, acute malnutrition plus *Cryptosporidium* infection was associated with an additional increased absolute risk of death [17]. It is worth noting that quantitative cutoffs were not applied in the case ascertainment, and that only moderate-to-severe diarrhoea and diarrhoea of shorter than 7-days duration on presentation were included. To some extent, this limits the external applicability of their findings to the general paediatric population with diarrhoea. Furthermore, the authors did not present an analysis of other risk factors for *Cryptosporidium* infection, such as household water/sanitation factors or previous illness, to allow comparison of the strength of association or attributable fraction from various exposures. Lastly, although they adjusted for confounding by socioeconomic status and water/sanitation, as is sometimes also done in case-case-comparison studies, they may not have accounted for the possibility for separate confounding from behavioural hygienic factors or perinatal factors.

Supplementary methods

Recruitment of non-diarrhoea controls

Controls without diarrhoea in the preceding 48 hours were enrolled concurrently by weekly recruitment plans in two predefined geographical catchment areas comprising 15 municipal districts (“kebeles” - the smallest administrative unit in Ethiopia) surrounding JMC (Jimma Medical Center, formerly Jimma University Specialized Hospital), and eight districts surrounding Serbo Health Center (SHC). Plans regarding which controls to recruit were made every week using a method of frequency matching (i.e., by strata) to cases by geographical location of households, week, and age stratum (0-5 months, 6-11 months, 12-23 months, 23-59 months). Due to logistical constraints, controls in the age range 0-11 months were enrolled both from vaccination rooms in JMC and SHC and by home visits, whereas all controls in the age range 12-59 months were enrolled by home visits. Although frequency matching by both age, week, and geography was applied to all controls, irrespective of control

enrolment location, a separate analysis was conducted to assess for the possibility of enrolment-location selection bias (supplementary results, below). Nurses conducted random house visits in the community by car, motorcycle, and on foot. Randomization of households for control recruitment within each district was accomplished by a procedure where the nurse would start from a randomly selected GPS point within the district specified in the control recruitment plan, and then follow a detailed standardized procedure for household selection. For further details of the design and methods used in the CRYPTO-POC case-control sub-study, see two previous publications [35, 36]

Variables

Most variables are self-explanatory, with the following exceptions:

- *Number of key assets owned by the household.* A proxy for socioeconomic status based on the presence or absence of key household assets. We collected data on the same eight assets that were part of the composite asset index used in the MAL-ED study [37], except for household members/room (instead analysed as a separate risk factor).
- *Household sanitation facility*. The following facility types were considered as improved facilities: Flush toilet (WC) or pour flush toilet that flushed to a piped sewer system or septic tank or pit latrine; ventilated improved pit (VIP) latrine; pit latrine with slab; composting toilet. The following facility types were considered as unimproved facilities: Open pit or pit latrine without slab; bush, field, or no facility. Only improved sanitation that is not shared with other households was categorized as "access to improved sanitation", according to the WHO definition.
- *Early cessation of exclusive breastfeeding*. Any cessation of exclusive breastfeeding earlier than the age of 6 months (the WHO minimum recommended age for weaning or for introducing complementary foods).
- *Acute malnutrition.* For children < 6 months, severe acute malnutrition (SAM) was defined as weight-for-height z-score (WHZ) ≤ -3 of the WHO standard curves [38] and/or presence of bilateral oedema involving at least the feet. Moderate acute malnutrition (MAM) was defined as a WHZ ≤ -2 and > -3 with no oedema. Mid-upper arm circumference (MUAC) was used instead of WHZ for 6-59-month-olds, as it was difficult to bring height measurement boards to community-control home visits (usually done by motorcycle), and because MUAC is less susceptible to dehydration than weight [39, 40]; SAM was defined as MUAC ≤ 115 mm and/or presence of bilateral oedema involving at least the feet, and MAM was defined a MUAC >115 mm and ≤ 125 mm with no oedema.

The case and control report forms also included questions about the following variables that were not included in the current analysis:

- *Household monthly income*. Intended to be used as an additional proxy for socioeconomic status, but not included in the analysis because income information was missing for >70% of the participants.
- *Handwashing hygiene*. Questions about the caregivers' normal handwashing habits also included responses for caregiver handwashing following defecation of the child, and before breastfeeding. Due to high risk of differential recall bias, we did not use these variables in case-control comparisons.
- *Rotavirus vaccine, other vaccinations, and vitamin A administration.* Not deemed appropriate for case-control comparisons, as most of the infant controls were enrolled from vaccination rooms, with resulting risk of selection bias. Note that rotavirus vaccine had been introduced in the study area at the time of the study (94% of the study diarrhoea cases had received the vaccine [35]).
- *Stunting*. Not included in the analysis as height was not measured for most controls recruited in the community, as mentioned above.
- *Antibiotics, oral rehydration salts, or zinc treatment (received last week)*. Not deemed suitable for case-control risk exposure comparison, as we were not able to ascertain whether these treatments were given before or after the diarrhoeal episode started.
- *HIV status*. Not included due to infrequent exposure; the parent case-control study found only one HIV-exposed control, one HIV exposed NCrD case, and no HIV positives [35].
- *Immunosuppressive medical conditions*. Not included due to infrequent exposure; no participants reported other immunosuppressive conditions, e.g., lymphoma, leukaemia, other cancers, chronic kidney failure, or any condition requiring regular treatment with steroids.
- *Measles episode (during the last month)*. Not included due to infrequent exposure; only reported in three controls, and in two and three cases with cryptosporidiosis and NCrD, respectively.

Note that the reference levels used when estimating ORs for exposure-outcome associations are listed as a separate column in the result tables, for each variable.

Modelling strategy and statistical methods

We did not assume that the frequency-matching procedure was sufficient to account for confounding by age, study site, and season. Instead, adjustment was forced into all models by adding a fixed effect term for age in months, and by adding random-effect intercepts for enrolment season (divided into six three-month intervals) and study site to account for epidemiologic differences by site and season. Although gender was not part of the frequency-matching procedure, it was considered a likely confounder of all level-1, -2, -3, -4 and -5 risk-factor associations, and was therefore forced into all models.

Age, gender, study site, and season were considered as the base-adjustment set. Models, both with and without base adjustment, were compared to assess the impact of adjustment, if any, on the model estimates; if deemed necessary, we performed additional adjustment for sub-site geographical area, age stratum, or by smaller enrolment-time divisions.

The modelling strategy was an interactive step-by-step process starting with base-adjusted modes, followed by intra-level models, and finally, a hierarchical analysis accounting for all levels. Importantly, the final estimate for the overall effect of a distal variable is the estimate derived before the introduction of more proximal level risk factors.

First, ORs were estimated for all putative risk factors using only base-adjusted models. Any risk factor that was statistically significant at the 0.05 significance level (i.e., by the 95% confidence interval for the OR not overlapping 1) was then entered into an intra-level multivariable model, followed by backwards stepwise elimination until only significant risk factors (at the 0.05 significance level) remained.

Finally, significant risk factors from the intra-level models were introduced into the hierarchical risk factor analysis following the predefined order in the conceptual framework: first, starting with socioeconomic (level 1) variables that were significant in the level 1 intra-level analysis, we then proceeded to add risk factors from the level 2 intra-level model, followed by a new backwards stepwise elimination procedure. To the resulting new model, we then added the significant risk factors (if any) from the next level. A similar procedure was repeated for all hierarchical levels. Importantly, the final estimate for the overall effect of distal variable is the estimate derived before the introduction of more proximal risk factors levels.

We assessed whether associations between risk factors and disease followed a dose-response relationship by both exploratory plots and by separate models with continuous variables added as linear fixed-effect terms. For categorical variables with three or more levels, we performed separate models with added ordered linear and quadratic terms and used the P value to assess the statistical significance of the linearity of the association.

Co-linearity between the variables in multivariable models was estimated by the variance inflation factor (using the R package performance, v.0.5), and for continuous variables, by exploratory plots. All putative risk factors were also assessed for interaction with age by separate models by adding an interaction term for age group (<12 versus ≥ 12-months old), and by plots.

Population attributable fractions (PAF) were estimated with the formula $PAF = Prevalence\ x\ (1 - \frac{1}{OR})$, using the imputed prevalence of the risk factor in the case group and the OR estimate for the association between the risk factor and either disease [41]. The summary PAFs for the risk factors at a given level were derived from models that were adjusted for more distal levels, but not including more proximal levels [42]. A summary PAF for all levels was also calculated, by taking the complement of the PAF at each level. We also estimated the "non-mediated portion" of PAF at each level, i.e., the attributable fraction assumed to have resulted in disease by some other route than the intermediate risk factors explored, by calculating PAF after adding to the models any significant risk factors from more proximal levels.

Sample size considerations

The sample size for the CRYPTO-POC case-control substudy was determined as one of the primary objectives of the CRYPTO-POC diagnostic accuracy study, which required determining the association between *Cryptosporidium* detection in stool and diarrhoea (i.e., case/control status). For this objective, the aim was to reach

at least 90% power to detect a difference between cases and controls, assuming a prevalence of 1.5% in the control group, and an OR between cases and controls of at least 3, with a 95% two-sided confidence level. From this, a target minimum sample size of 728 cases and 728 controls was determined. Cryptosporidiosis risk-factor assessment (the current analysis) was a predefined objective of the CRYPTO-POC-study, but as it was a secondary objective, a separate sample size calculation was not performed. We assumed that the power would be lower, due to the smaller case set with cryptosporidiosis, but that the resulting reduction in power would be somewhat counteracted by an expected much higher than 1.5% prevalence for most of the exposures.

Supplementary results

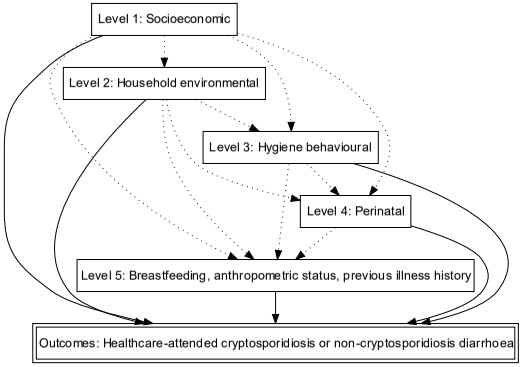
Table A: Distribution of case and control subjects according to all exposures, with counts and proportions of missing values

Of all the modelled risk-factor variables, there was at least one missing observation for 5% of the cryptosporidiosis cases, 53% of the NCrD cases, and 49% of the controls. The highest proportion of missing values for any given exposure variable was 2.6% for controls, 3.4% for cryptosporidiosis cases, and 1.9% for NCrD cases. We therefore performed multiple chained random-forest imputation before all OR and PAF calculations.

				Diarrhoea cases					
	Non-diarrhoea controls (N = 725)			Cryptosporidiosis (N = 59)			Non-cryptosporidiosis (N = 432)		
Characteristic	Exposed n	Missing n	Missing %	Exposed n	Missing n	Missing %	Exposed n	Missing n	Missing %
Level 1 – Socioeconomic factors									
Maternal education		2	0.3		0	0.0		1	0.2
< 1 year	198			21			150		
1–7 years	273			19			129		
Primary caregiver is not the child's mother	13	2	0.3	6	0	0.0	25	0	0.0
Number of key assets owned by the household ≤ 2	115	14	1.9	8	2	3.4	103	6	1.4
Number of household members		9	1.2		1	1.7		2	0.5
4-5	336			28			170		
≥ 6	199			13			156		
Level 2 – Household environmental factors									
Persons per room ≥ 2	675	3	0.4	51	0	0.0	390	2	0.5
Animals owned by the household (household ownership of ≥1 animal of the stated type)		2	0.3		0	0.0		1	0.2
Cattle	244			16			180		
Chickens	233			16			145		
Dogs	62			8			30		
Goats	54			4			41		
Horses, donkeys, or mules	66			4			54		
Sheep	107			6			61		
Other	23			1			6		
Any even-toed ungulate	260			19			184		
Any animal	357			27			217		
Sanitation facility		0	0.0		0	0.0		1	0.2
Improved, but shared	62			7			82		
Improved, and not shared	59			22			124		
Access to "improved sanitation" (by the WHO definition)	59	0	0.0	22	0	0.0	124	1	0.2
Water source for the household		2	0.3		0	0.0		1	0.2
Public tap	98			22			106		
Surface or rainwater, unprotected well, borehole, or protected spring	221			9			154		
Water treated by the household (chemicals, boiling or filtering) before drinking	38	4	0.6	3	0	0.0	41	5	1.2
Level 3 – Hygiene behaviour									
Last stool disposal (from any child of the caregiver) "unsafe" by the WHO definition	404	1	0.1	39	0	0.0	327	1	0.2
Caregiver will normally wash hands before meals	686	0	0.0	55	0	0.0	398	0	0.0

before preparing food for the child	538	0	0.0	46	0	0.0	315	0	0.0
after a toilet visit	497	0	0.0	38	0	0.0	303	0	0.0
without soap	24	0	0.0	3	0	0.0	30	0	0.0
Level 4 – Perinatal factors									
Mode of delivery – caesarean section	47	1	0.1	7	1	1	47	1	0.2
Child born prematurely (before week 37)	14	0	0.0	3	1	1.7	25	2	0.5
Level 5 – Breastfeeding, nutritional status, and previous illness history									
Early cessation of exclusive breastfeeding	231	4	0.5	19	0	0.0	164	8	1.9
Not breastfeeding now (or, for cases, just before the diarrhoeal episode started)	61	1	0.1	9	0	0.0	48	0	0.0
Acute malnutrition		19	2.6		0	0.0		5	1.2
Moderate acute malnutrition (MAM)	16			7			45		
Severe acute malnutrition (SAM)	4			4			9		
Acute malnutrition, any (MAM or SAM)	20			11			54		
One or more overnight admissions, since birth	53	1	0.1	6	0	0.0	34	2	0.5
One or more diarrhoea episodes, during the last month	109	0	0.0	16	0	0.0	73	4	0.9
Number of visits to hospital or health center due to illness, since birth		0	0.0		0	0.0		0	0.0
1-2 visits	194			21			126		
≥ 3 visits	3			7			16		

Table B: Hierarchical mediation analysis of risk factors for cryptosporidiosis and non-cryptosporidiosis diarrhoea, in children under 2 years old



The conceptual framework illustrates our underlying assumption that a risk factor can have a causal relationship with disease via several routes. In this version of the conceptual framework (figure, left), "mediated" routes are marked by dotted lines to distinguish them from the "non-mediated" routes (whole lines).

A mediation analysis is possible by deliberately adjusting for a mediator, i.e., a proximal and intermediate risk factor in the causal framework. The approach taken here is a simple variant of the traditional "difference method" of quantifying mediation [43], which is based on comparing regression coefficients (or, in logistic regression models, odds ratios) between two models, where one includes adjustment for a mediator, and the other does not include such adjustment. Odds ratios (ORs), and PAFs based on ORs, can then be estimated both with and without this adjustment. Mediator adjustment should only be done as part of a mediation analysis, as it can lead to underestimation of ORs (and population attributable fractions, PAFs) for distal risk factors (i.e., a type of overadjustment). Mediation analysis presupposes certain causal relationships, usually illustrated by directed acyclic graphs or a hierarchical framework, as above. Under the underlying assumptions implicit in the framework, a significant drop in OR, after adjusting for a proximate and intermediate step (i.e., a "mediator") provides indirect evidence for mediation of the distal risk factor through this intermediate step. As PAFs are calculated from ORs, this approach allows us to split a PAF (either a PAF from a specific risk factor or a whole-level summary PAF) into "non-mediated" and "mediated" fractions. The term "non-mediated" in this context should be cautiously interpreted, i.e., "not likely to be mediated by the other risk factors we gathered data on".

This table shows how the intra-level summary PAFs change as mediator adjustment for more proximal levels are added to the models:

	Cryptosporidiosis vs controls				Non-cryptosporidiosis diarrhoea vs controls			
Level	**Summary PAF from each level (%)[a]**	**Non-mediated PAF (%)[b]**	**Mediated PAF (%)**	**Mediated vs non-mediated fraction of PAF (%)**	**Summary PAF from each level (%)[a]**	**Non-mediated PAF (%)[b]**	**Mediated PAF (%)**	**Mediated vs non-mediated fraction of PAF (%)**
Level 1 – Socioeconomic factors	26	8	18[c]	68	14	11	3	22
Level 2 – Household environmental factors	27	27	0	0	31	30	1	1
Level 3 – Hygiene factors	0				44	40	4	8
Level 4 – Perinatal factors	0				8	8	0	0
Level 5 – Breastfeeding, nutrition, previous illness	39	39	0	0	22	22	0	0

[a] Summary PAFs were calculated for each risk factor level by taking the complement of PAFs for individual significant risk factors at that level. The summary PAFs for the risk factors a given level were derived from models that were adjusted for more distal levels, but not including more proximal levels.

[b] "Non-mediated PAF" is calculated from odds ratios obtained from models with fixed effect terms for more distal risk factors (i.e., confounder adjustment), and also terms for more proximal risk factors (i.e., mediator adjustment). This "non-mediated portion of PAF" is an estimate of the attributable fraction assumed to have resulted in disease by some other route than the intermediate risk factors explored, by calculating PAF after adding to the models any significant risk factors from more proximal levels.

[c] Maternal education <1 year becomes insignificant (with a resulting drop in PAF), after mediator adjustment for level 2, indicating mediation of this risk factor through level 2 risk factors.

Table C: Duration of diarrhoea, on enrolment, in cryptosporidiosis and non-cryptosporidiosis diarrhoea cases, in children under 2 years old

	Cryptosporidiosis (N = 59)[a]	**Non-cryptosporidiosis diarrhoea** (N = 432)[a]
Diarrhoeal duration, days; median (IQR)	3 (3-7)	3 (2-4)
Diarrhoeal duration, days; mean (SD)	4.6 (2.9)	4 (5.4)
Acute diarrhoea (2-6 days duration)	42 (71)	375 (87)
Prolonged diarrhoea (7-13 days duration)	14 (24)	43 (10)
Persistent diarrhoea (14 days duration or longer)	3 (5)	14 (3)

IQR = interquartile range. SD = standard deviation.

[a] Data are n (%), unless otherwise specified.

Bias analysis: Missing outcome for diarrhoea cases

The primary outcome of either cryptosporidiosis or non-cryptosporidiosis diarrhoea depended on successfully obtaining a stool sample for reference testing. Failure to obtain samples for all (logistically challenging as most cases were outpatients), and a few missing reference tests (see flowchart in Figure 1, main manuscript), meant that outcome status was not known for all participants. If missing outcomes are associated with risk factors under investigation, it can lead to selection bias. To detect such associations between missing outcome and exposures, all diarrhoea cases in the study were enrolled, interviewed, and examined, irrespective of the availability of a stool sample or of reference testing. We then explored differences in exposures between diarrhoea cases, with and without missing outcomes, by plots and cross tabulation, for all risk factor variables.

For most of the investigated variables, including the frequency-matching variables (age, enrolment season, study site, geographical area), there were no substantial exposure differences by missing-outcome-status, and therefore low suspicion of selection bias. However, for the following variables, we observed a small yet substantial difference: gender (higher proportion of girls among those with missing outcome), maternal education (1-7 years reported more often for cases with missing outcome), water source (unimproved water source reported more often and public tap less often among cases with missing outcome), and history of diarrhoea (reported less often by cases with missing outcome). We calculated the selection bias factor and selection bias-adjusted crude odds ratios [44], under the assumption that risk of missing outcome (i.e., probability of not being selected for analysis), was similar between cryptosporidiosis and non-cryptosporidiosis cases. See table below for crude and adjusted ORs. The results indicate that the ORs for public tap water and previous history of diarrhoea may be biased slightly upwards, due to missing-outcome selection bias.

		Cryptosporidiosis		Non-cryptosporidiosis diarrhoea	
Characteristic	**Selection bias factor[a]**	**Crude OR[b]**	**Adjusted OR[c]**	**Crude OR[b]**	**Adjusted OR[c]**
Male gender	1.13	0.76 (0.45 to 1.3)	0.67 (0.39 to 1.2)	1.3 (1.0 to 1.7)	1.1 (0.89 to 1.5)
Maternal education					
≥ 8 years		(Reference level)			
< 1 year	1.04	1.4 (0.74 to 2.7)	1.4 (0.69 to 2.6)	1.1 (0.80 to 1.5)	1.0 (0.77 to 1.4)
1–7 years	1.23	0.92 (0.48 to 1.8)	0.75 (0.37 to 1.4)	0.91 (0.69 to 1.2)	0.74 (0.55 to 0.99)
Water source for the household					
Private tap		(Reference level)			
Public tap	1.18	3.2 (1.8 to 5.9)	2.8 (1.5 to 5.1)	2.6 (1.8 to 3.6)	2.2 (1.6 to 3.0)
Surface or rainwater, unprotected well, borehole, or protected spring	0.94	0.59 (0.3 to 1.3)	0.63 (0.28 to 1.5)	1.7 (1.3 to 2.2)	1.8 (1.3 to 2.3)
One or more diarrhoea episodes, during the last month	1.22	2.1 (1.1 to 3.9)	1.7 (0.93 to 3.4)	1.2 (0.84 to 1.6)	0.95 (0.68 to 1.33)

OR = odds ratio.

[a] Selection bias factor = $S_{Aj}S_{B0} / S_{A0}S_{Bj}$, where S_{Aj} is the probability of selecting an exposed case, S_{A0} is the probability of selecting an unexposed case, S_{Bj} is the probability of selecting an exposed control, and S_{B0} is the probability of selecting an unexposed control.

[b] Point OR estimates with 95% Wald confidence intervals.

[c] Selection bias factor corrected OR, with 95% confidence intervals obtained using bootstrapping with 100 replicates, using the R package episensr (v.1.0.0).

Table D: Selection bias adjusted crude odds ratios, under 2-year-olds

Bias analysis: Enrolment of controls from vaccination rooms and by household visit

Most controls in the age group 0-11-months were enrolled from vaccination rooms in JMC and SHC, due to logistical constraints on conducting field visits. If we were unsuccessful in recruiting a control from the matching age stratum (0-5-months; 6-11 months) or geographical area, a similar control (by age stratum and geographical area) was enrolled the following week, but by the procedure for random house visits. Only 12% of the 0-11-month-old controls (50/414) were enrolled by house visits. House visit enrolment, instead of vaccination room enrolment, was mainly associated with geographical distance from the household to the healthcare facility. In

contrast, all controls ≥ 12 months old were enrolled by house visits. We therefore considered selection bias, by control recruitment location, as a real possibility, for all putative risk factors, despite geography being largely accounted for by the frequency matching procedure, and despite close harmonization of study procedures irrespective of recruitment strategy.

For this kind of selection bias to have an important effect on the exposure-outcome associations (i.e., ORs), we would expect to see substantial differences in OR by age group (below or above 12 months old; all controls ≥ 12 months were enrolled by house visits), and, also, evidence of substantial differences in exposure by control recruitment location. We also used information on the approximate distance from the household to the health facility, and the caregiver-stated preferred facility for childhood vaccinations, from both cases and controls (e.g., if the average case preferred to get vaccinated elsewhere than the average control, it was considered evidence against the assumption that they were drawn from the same underlying source population, or "study base"). Based on this, we took the following systematic approach to examine for control selection bias: for all base adjusted exposure-outcome relationships, we assessed OR differences by age group, and for substantial difference in exposure prevalence between home-recruited and vaccination-recruited controls (by inspecting the numbers, or by finding a substantial difference in exposure prevalence, as estimated by 95% Wilson confidence intervals). If present, we then proceeded to run the models both with and without adjustment and, separately, with interaction terms for vaccination location preference and by geographical distance of the household to JMC/SHC. If the OR for the exposure-outcome relationship changed substantially, it was considered evidence of control selection bias. If not, such bias was considered less likely.

Using this approach, we discovered that for the association between cessation of exclusive breastfeeding before the age of 6 months and NCrD, there was both an association with age-group (increased risk of NCrD, but not cryptosporidiosis, in the 0-11-month-olds) and a difference in exposure based on control recruitment location (22% of the vaccination room controls had stopped being exclusively breastfed earlier than 6 months versus 40% of the household controls). However, as there was minimal change in OR after adjusting for geographical distance to the health facility or when adjusting for vaccination facility preference, selection bias was not considered likely. No evidence for control selection bias was found for any of the other putative risk factors.

Bias analysis: Differential exposure misclassification for the sanitation variable

Somewhat surprisingly, the rate of access to improved sanitation was much higher in both cryptosporidiosis cases and NCrD cases than in the control group (Table 2, main manuscript). We wondered whether the complexity of how to define "improved facility", could lead to misclassification based on the individual study nurse conducting the questionnaire interview. Some misclassifications are to be expected in a large study. However, only if such misclassifications occur often, and are differential, i.e., if the misclassification operates differently for participants based on outcome (e.g., case-control status), is it likely to lead to bias. As the proportion of case to control recruitment varied to some extent by study nurse, we could not rule out differential misclassification as a possibility. In order not to single out the sanitation variable for special scrutiny, we therefore proceeded to investigate all modelled exposure variables for possible differential exposure misclassification by study nurse. The only variable with evidence of differential clustering by study nurse was the sanitation variable. To take this clustering effect into account, we therefore added a random effect intercept for study nurse to all models that included the sanitation variable.

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RESEARCH ARTICLE

Oocyst Shedding Dynamics in Children with Cryptosporidiosis: a Prospective Clinical Case Series in Ethiopia

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ABSTRACT Knowledge on the duration of *Cryptosporidium* oocyst shedding, and how shedding may be affected by subtypes and clinical parameters, is limited. Reduced transmission may be a secondary benefit of cryptosporidiosis treatment in high-prevalence areas. We conducted a prospective clinical case series in children of <5 years presenting with diarrhea to a health center and a hospital in Ethiopia over an 18-month period. Stool samples were collected repeatedly from children diagnosed with cryptosporidiosis for up to 60 days. Samples were examined, and *Cryptosporidium* shedding was quantified, using auramine phenol, immunofluorescent antibody staining, and quantitative PCR (qPCR). In addition, species determination and subtyping were used to attempt to distinguish between new infections and ongoing shedding. Duration and quantity of shedding over time were estimated by time-to-event and quantitative models (sex- and age-adjusted). We also explored how diarrheal severity, acute malnutrition, and *Cryptosporidium* subtypes correlated with temporal shedding patterns. From 53 confirmed cryptosporidiosis cases, a median of 4 (range 1 to 5) follow-up stool samples were collected and tested for *Cryptosporidium*. The median duration of oocyst shedding was 31 days (95% confidence interval [CI], 26 to 36 days) after onset of diarrhea, with similar estimates from the quantitative models (31 days, 95% CI 27 to 37 days). Genotype shift occurred in 5 cases (9%). A 10-fold drop in quantity occurred per week for the first 4 weeks. Prolonged oocyst shedding is common in a pediatric clinical population with cryptosporidiosis. We suggest that future intervention trials should evaluate both clinical efficacy and total parasite shedding duration as trial endpoints.

IMPORTANCE Cryptosporidiosis is an important cause of diarrhea, malnutrition, and deaths in young children in low-income countries. The infection spreads from person to person. After infection, prolonged release of the *Cryptosporidium* parasite in stool (shedding) may contribute to further spread of the disease. If diagnosis and treatment are made available, diarrhea will be treated and deaths will be reduced. An added benefit may be to reduce transmission to others. However, shedding duration and its characteristics in children is not well known. We therefore investigated the duration of shedding in a group of young children who sought health care for diarrhea in a hospital and health center in Ethiopia. The study followed 53 children with cryptosporidiosis for 2 months. We found that, on average, children released the parasite for 31 days after the diarrhea episode started. Point-of-care treatment of cryptosporidiosis may therefore reduce onward spread of the *Cryptosporidium* parasite within communities and households.

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Cryptosporidiosis in sub-Saharan Africa mainly affects children under the age of 2 years and is largely caused by *Cryptosporidium hominis*. *Cryptosporidium parvum* is uncommon in livestock in Sub-Saharan Africa, and human *C. parvum* infections are caused almost exclusively by anthroponotic subtypes in this region (1). The strong association between health care-presenting cryptosporidiosis and risk of death in young children was firmly established by the Global Enteric Multicenter Study (GEMS) and the GEMS-1A follow-on study (2). The global health burden has been estimated to 13 million disability-adjusted life years lost every year, of which 67% are related to decreased growth in children who suffered from cryptosporidiosis in early childhood (3). As *Cryptosporidium* oocysts are robust, enabling persistence in the environment, and the infectious dose is low, transmission may be not only person-to-person or animal-to-person but also via transmission vehicles such as water, food, or fomites. *Cryptosporidium* infections that cause diarrhea are associated with higher quantities of oocysts being shed and pose an elevated risk of household transmission (4, 5). Secondary transmission rates may also be affected by the intensity and duration of shedding after an infection. The prolonged presence of *Cryptosporidium* in the stool may also mean that other potential enteropathogens in subsequent gut infections are overlooked.

Until a 2021 landmark publication from the Malnutrition and Enteric Disease Study (MAL-ED) (6), a few studies had indicated that oocyst shedding could continue for months after a cryptosporidiosis episode, in both adults and children, and a community cohort study in children growing up in a Peruvian shantytown ($n = 25$) reported a mean shedding duration of 17 days after onset of diarrhea (7–9). These studies were conducted in heterogenous populations and relied on low-sensitivity microscopy. The MAL-ED study, by contrast, investigated postdiarrheal shedding of a broad range of enteric pathogens, used sensitive real-time PCR detection, and was conducted in under-2-year-old children in diverse low-resource settings (6). The median duration of *Cryptosporidium* shedding was estimated to be more than 5 weeks after onset of the diarrheal episode. However, genotyping of follow-up samples was not done. Thus, later detections during the surveillance period may have represented new infections with other *Cryptosporidium* species or genotypes, with a risk of overestimating the median shedding duration. Furthermore, their estimate was based on analysis of cryptosporidiosis identified by community surveillance visits and is not necessarily valid for the population of children who are diagnosed in health care settings.

Targeted treatment for cryptosporidiosis is currently limited to nitazoxanide, a drug that is not effective in immunocompromised children and regrettably is FDA-approved only for children older than 12 months, although available data indicate no adverse effects in children aged 6 to 11 months (10–13). Significant progress has been made by several research groups in developing new pharmaceutical treatments (14), and an ongoing trial in Australian Aboriginal children may provide additional safety data for use of nitazoxanide in 3- to 5-month-olds (10). If prolonged duration of shedding is confirmed in the clinical setting, tangible secondary benefits of targeted cryptosporidiosis treatment may be to reduce transmission and to enable faster recovery of intestinal function (15, 16). We therefore need reliable estimates for the duration of oocyst shedding in those settings where interventions against cryptosporidiosis are most needed, i.e., low-resource health care settings. An additional point is that it should be possible to identify these shedders using simple and affordable, yet accurate, point-of-care tests.

Our study therefore aimed to describe the temporal dynamics of *Cryptosporidium* shedding, i.e., both overall duration of shedding and quantitative shedding patterns over time, supplemented by subtyping to help distinguish between new infections and ongoing shedding. Cases were identified by near-patient testing using light-emitting diode auramine-phenol fluorescence microscopy (LED-AP), already available in many low-income countries, as the method is used for tuberculosis point-of-care testing (17). Secondary objectives were to

explore how diarrheal severity, acute malnutrition, and *Cryptosporidium* species and subtypes correlated with shedding patterns over time.

We estimated the duration of shedding by two complementary approaches. First, we defined shedding as a binary event (ongoing shedding versus no longer shedding, as confirmed by microscopic detection and/or detection by quantitative PCR [qPCR]). Second, we considered shedding as a quantitative outcome, measured as DNA copies/g of stool in follow-up samples. In addition, cases with a shift in the detected *Cryptosporidium* genotype were classified as having "ongoing shedding" only up to the last positive detection before the genotype shift happened. To our knowledge, this is the first study of temporal oocyst shedding dynamics in human cryptosporidiosis to include subtyping.

RESULTS

Cryptosporidiosis screening was performed by LED-AP microscopy on 878 diarrhea cases; 82 (9%) were positive, and, of these, 56 were eligible and consented to participate in the follow-up study. Shedding of *Cryptosporidium* DNA was confirmed in 54 of these 56 cases, as two cases had negative *Cryptosporidium* qPCR in both enrollment and follow-up stool samples (ID34 and ID40). One case that was shedding on enrollment failed to submit any follow-up samples (ID19) and was therefore excluded from the analysis. A median of 4 (range 1 to 5) follow-up stool samples were collected from each of the remaining participants. The lowest positive detection by the qPCR assay was 519 *Cryptosporidium* copies per gram of stool, corresponding to approximately 130 oocyst equivalents per gram, as there are 4 copies of the *cowp* gene per oocyst (i.e., 1 per sporozoite). See Table 1 for characteristics of the 53 participants included in the analysis of temporal shedding patterns and Fig. S1 for all microscopy, PCR, and genotyping findings for the 56 participants in the follow-up study.

Notably, two cases were still shedding oocysts at their last follow-up visits, 63 days after onset of the initial diarrheal episodes. Both were 6- to 11-month-olds, without acute malnutrition, who presented with acute diarrhea; HIV status was unknown for one and the other was HIV negative. A shift in *Cryptosporidium* genotype occurred in five cases (9%): in three cases, this was reflected by subsequent detections of a different *C. hominis gp60* allele family in samples taken 16, 24, and 25 days after onset of diarrhea, respectively (ID 9, 56, 28), in one case the shift happened after two consecutive negative samples, 34 days after onset of diarrhea (ID 48), and in one case, *C. hominis gp60* allele type Ib was detected in a sample obtained at day 37, after three consecutive samples with *C. parvum* IIc (ID 36). There were no cases where subtype shifted within the same *gp60* allele family.

Duration of shedding. As expected, the proportion of cases shedding *Cryptosporidium* declined over time. Figure 1 shows the time-to-event curves describing the decline in the proportion of cases shedding *Cryptosporidium* during the follow-up-period.

Stratification of the time-to-event curves by key subgroups revealed subtle differences by sex and age group, which might confound other comparisons. We therefore fitted a log-logistic parametric time-to-event model (Fig. S2) which allowed us to adjust for sex and age. The overall model of the decline in shedding is illustrated by Fig. 2, from which the estimated median duration of shedding was 31 days (95% confidence interval [CI], 26 to 36 days).

Shedding was protracted for several weeks in most cases; by 3 weeks after onset of diarrhea, only 21% (95% CI 11 to 32) had stopped shedding *Cryptosporidium*, rising to 75% (95% CI 62 to 87) by 6 weeks (Table 2).

Results from the parametric models, stratified by key predictors, are summarized in Table 3, where the odds ratios (OR) quantify differences in shedding duration across the follow-up period. There were no obvious differences by age group, sex, or acute malnutrition and only a trend toward shorter shedding duration for cases who were dehydrated on enrollment. No significant difference was found between *C. parvum* and *C. hominis*. However, when the shedding curves between the different *C. hominis gp60* subtype families were compared, *gp60* allele family Id, the second most common *gp60* allele family, was associated with a significantly longer duration of shedding than the other *C. hominis gp60* allele families.

Change in *Cryptosporidium* shedding quantity over time. Positive samples included in this analysis ranged in *Cryptosporidium* DNA quantity from 805 copies/g to 158 million

TABLE 1 Characteristics of cryptosporidiosis cases in the follow-up study

Characteristic	No. of cases (%)[a]
Age, in mo	
<6	1 (2)
6–11	28 (53)
12–23	22 (41)
24–59	2 (4)
Sex	
Female	25 (47)
Male	28 (53)
Study site	
Jimma hospital	41 (77)
Serbo health center	12 (23)
Acute malnutrition	
Moderate acute malnutrition (MAM)	4 (8)
Severe acute malnutrition (SAM)	7 (13)
No acute malnutrition (NAM)	42 (79)
HIV status[b]	
HIV positive	0 (0)
HIV negative	41 (100)
Diarrheal severity[c]	
No dehydration	21 (43)
Some dehydration	16 (33)
Severe dehydration	12 (24)
Diarrheal duration (on enrollment)	
Acute (<7 days duration)	37 (70)
Prolonged (7–13 days duration)	13 (24)
Persistent (≥14 days duration)	3 (6)
Cryptosporidium species and *gp60* allele family[d]	
C. hominis Ia	14 (26)
C. hominis Id	13 (25)
C. hominis Ib	10 (19)
C. hominis Ie	9 (17)
C. parvum anthroponosum IIc	6 (11)
C. parvum anthroponosum IIc + *C. hominis* (nontypeable)	1 (2)
Cryptosporidium spp. other than *C. parvum* or *C. hominis*	0 (0)

[a]*n* = 53, unless otherwise specified.
[b]*n* = 41; *n* = 12 missing HIV status.
[c]By DHAKA dehydration category; *n* = 49; *n* = 4 missing DHAKA score.
[d]Specific *gp60* subtype data can be found in Fig. S1.

copies/g. Quantities declined significantly with days since onset of diarrhea (smooth term, $P < 0.00001$) (Fig. 3).

The overall trend line was close to a quadratic relationship (effective degrees of freedom [EDF] = 1.9); however, for the first 4 weeks after onset of diarrhea, a striking pattern was seen where *Cryptosporidium* DNA shedding quantity dropped about 10-fold per week (Table S1). Thereafter, the overall trend line curved gently upwards, likely due to inclusion in the model of two cases who were still shedding after 63 days.

Although we observed some differences in shedding quantity between subgroups (Fig. 3), mirroring the subtle differences seen in the time-to-event graphs (Fig. 1), most subgroup differences were not significant at the 0.05 level, or significant only during a time window starting several weeks after onset of the diarrheal episode. The largest difference was seen at the subtype level, where *C. hominis gp60* allele family Id was associated with a higher quantity of shedding than the other *C. hominis* allele families during the time window from 11 to 55 days after onset of diarrhea (Table 3); see Fig. S3 for visualization of the models used to obtain these estimates.

We proceeded to estimate shedding duration by quantity over time by estimating the time point at which *Cryptosporidium* DNA quantity would drop below the qPCR detection

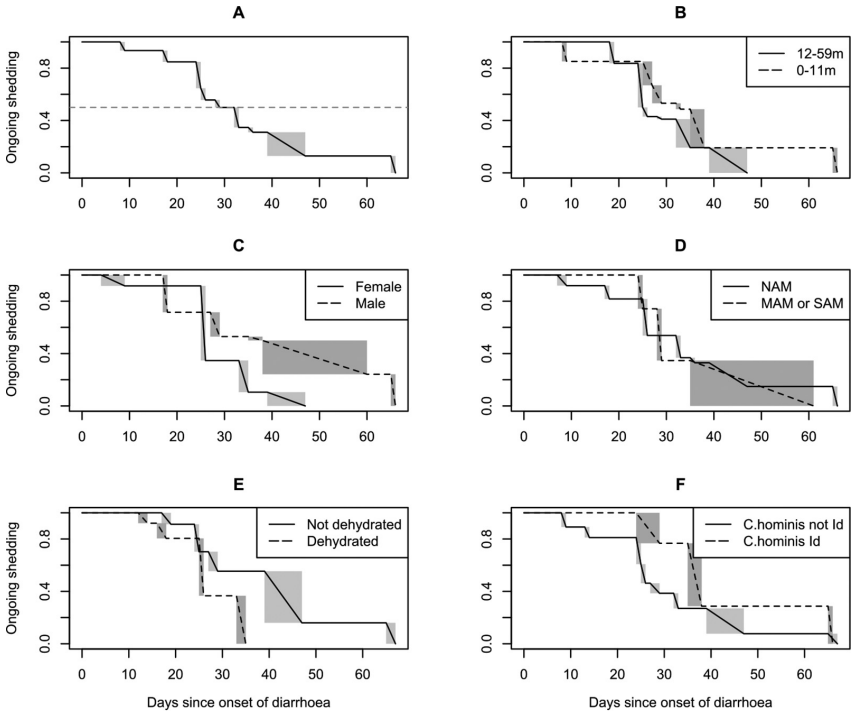

FIG 1 Duration of *Cryptosporidium* shedding nonparametric time-to-event curves. Temporal decline in ongoing shedding; (A) overall, and stratified by (B) age, (C) sex, (D) acute malnutrition, (E) dehydration, and, (F) for *C. hominis* infections, *gp60* allele family. The shaded rectangles represent ranges of indeterminate shedding status, due to the interval-censored nature of the data. MAM/SAM/NAM, moderate/severe/no acute malnutrition.

threshold (Fig. S4). This yielded an estimate for the duration of *Cryptosporidium* DNA shedding at 31 days (95% CI from 25 to 37 days).

DISCUSSION

Cryptosporidium parasite shedding was investigated by follow-up of individual children presenting to health care with cryptosporidiosis. Shedding duration was approximated using two complementary analytical approaches: first by time-to-event modeling where ongoing shedding was considered a binary outcome, based on microscopic detection of oocysts and/or DNA detection by PCR, and then by quantitative modeling of the drop in DNA quantity over time, using a highly sensitive qPCR assay. Both models yielded remarkably similar estimates for median duration of shedding, at 31 days, with 95% CIs from 26 to 36 days and 25 to 37 days, respectively. The findings demonstrate that prolonged shedding is common in a general pediatric clinical population with cryptosporidiosis. This is a slightly shorter estimate than, but still in keeping with, the 40-day estimate from the MAL-ED study, which was obtained using robust modeling and quantitative PCR for reliable case ascertainment (6). Although estimates obtained from a community study may not directly compare with our clinical population, it is worth pointing out that the MAL-ED study used a qPCR targeting the multicopy SSU rRNA gene, possibly with sensitivity better than that of our four-copy *cowp* qPCR assay at the low DNA quantities observed toward the tail end of an infection.

This is the first study of *Cryptosporidium* shedding in humans to include both species determination and subtyping. Genotype shift was seen in 5 of 53 cases. Previous estimates of shedding duration from studies where subtyping was not done may have been biased upwards. However, assuming that genotype shifts occur at a frequency similar to that which

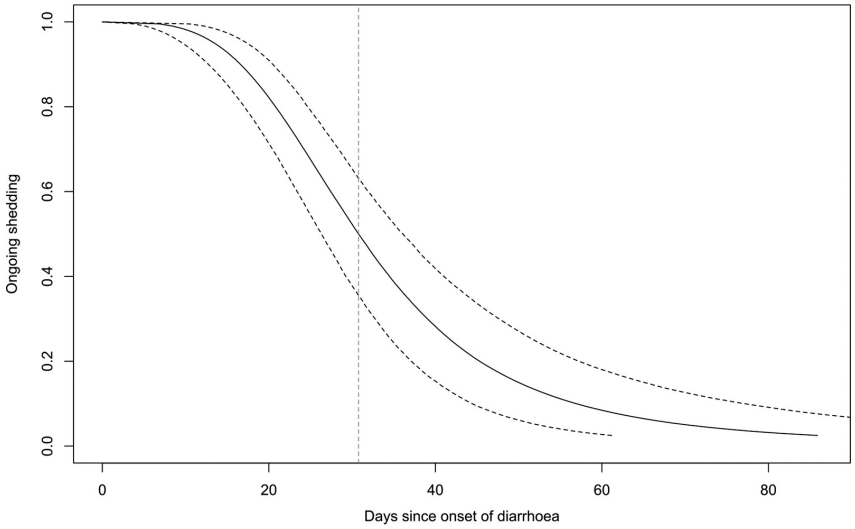

FIG 2 Duration of *Cryptosporidium* shedding parametric time-to-event curve. Log-logistic time-to-event model, adjusted for sex and age. The dashed curves indicate 95% confidence intervals around the estimated proportion with ongoing shedding at a given time point; the vertical dashed line indicates median shedding duration.

we found, the effect of such bias is likely to be small. It is interesting that there were no *gp60* genotype shifts at the sub-allele-family level. As the *gp60* gene codes for highly immunoreactive surface molecules that are involved in adhesion and invasion of host cells (18), one possible explanation could be within-*gp60*-family acquired immunity.

Although differences in oocyst shedding quantities between *Cryptosporidium* species and genotypes have been reported previously (7, 19, 20), this has not been examined by quantitative modeling of the time course of an infection using repeated follow-up samples from individual children. Our findings indicate that quantitative trends can be subtle, unless considered across the timeline of the infection; for example, the association between *C. hominis* subtype Id and prolonged shedding was significant only during the middle and tail end of the infectious episode. It is far from clear whether this is caused by real parasite-level biological differences or whether the genotype is a marker of an unmeasured prognostic or confounding factor. Even if controlled experiments were to confirm differences in shedding density that manifest late in the course of infection, it is not clear how much this would contribute to overall transmission. Supported by the observed drop in quantity by about 10-fold per week for the first 4 weeks after onset of diarrhea, we would assume that the initial weeks of an infection contribute vastly more oocysts to the immediate environment. We can speculate that the early

TABLE 2 Decline in shedding over time

Days after onset of diarrhea	Proportion no longer shedding (%)[a]	95% confidence interval (%)
7	1	0–2
14	6	2–12
21	21	11–32
28	42	29–56
35	61	48–76
42	75	62–87
49	84	72–93
56	89	79–96
63	93	84–98
70	95	88–99

[a]Estimated from the log-logistic time-to-event model, adjusted for age and sex.

TABLE 3 Duration of *Cryptosporidium* shedding compared between groups

		Odds ratio of no longer shedding, at any given time point (parametric model)			Test of difference (nonparametric model)[b]	
			95% CI for OR[a]			
Characteristic	Reference level	OR	From	To	*P* value	Window of significantly different shedding quantity[c]
Age 0–11 mo	Age 12–59 mo	1.07	0.76	1.50	0.32	22 to 52 days (higher)
Male	Female	1.15	0.83	1.59	0.12	36 to 69 days (higher)
Acute malnutrition (MAM or SAM)	No acute malnutrition (NAM)	0.97	0.68	1.38	0.70	No window of significant difference
Any dehydration	No dehydration	0.75	0.55	1.01	0.03	32 to 40 days (lower)
C. parvum	*C. hominis*	0.94	0.57	1.55	0.31	43 to 69 days (higher)
C. hominis gp60 allele family Id	*C. hominis gp60* allele family other than Id	1.51	1.01	2.23	0.04	11 to 55 days (higher)

[a]OR, odds ratios obtained from accelerated failure time log-logistic models, adjusted for sex and age (in months); age adjusted for sex only; sex adjusted for age only.
[b]Generalized log-rank test using within-subject resampling (not adjusted for sex and age).
[c]Window of significantly different shedding quantity (*Cryptosporidium* DNA copies/g, by qPCR) in days after onset of diarrhea; estimates obtained from generalized additive mixed model difference smooths, adjusted for sex and age (in months); age adjusted for sex only; sex adjusted for age only.

stage of infection is more likely to be associated with diarrhea with increased risk of onward transmission. If this is confirmed, health care-based interventions may be more effective than mass drug administration. To further investigate this question, we suggest that drug intervention studies should include repeat follow-up sampling with quantification and subtyping. Also, mathematical transmission modeling should account for the extended shedding duration of *Cryptosporidium* as well as symptoms and behavior.

Several limitations need to be kept in mind when interpreting our findings. First, our study size was limited to the number of positive cases in the CRYPTO-POC study that were within reach for follow-up. The overall sample size was decided for the primary objectives of the diagnostic accuracy study rather than for robust comparisons between groups; the observed subgroup differences have wide margins of uncertainty, should be considered trends at best, and need to be examined again in larger studies.

Second, although we attempted to account for confounding bias by adjusting the models for sex and age, this type of adjustment was not possible in the nonparametric time-to-event models. However, estimates of shedding duration from the nonparametric models were quite similar to those found in the adjusted parametric time-to-event models and to what was obtained by a generalized additive mixed model (GAMM), both of which included confounder adjustment.

Third, the observed tendency toward lower shedding in children who were dehydrated on enrollment seems counterintuitive but should at best be interpreted as a trend, as it was not a statistically significant finding in the adjusted models (Table 3).

Fourth, we interpreted repeat detection of the same *gp60* subtype as likely ongoing shedding from a single *Cryptosporidium* acquisition event. This is supported by the overall drop in quantity in repeat detections but is only a plausible assumption; another possibility is that some of the repeat detections represent new acquisitions of the same *gp60* genotype, e.g., from a still-present household source. Contact tracing has demonstrated clustering of subtypes in households (4, 5). However, determining the direction of transmission is likely to require a large-scale surveillance study with repeat stool sampling from all members of a household.

Last, although we found little evidence of mixed *gp60* sequences (data not shown), we may have missed some low-level mixed subtype infections. Elucidating this further would require the application of techniques to detect *gp60* subpopulations that are outside the scope of this analysis, e.g., deep sequencing, vector cloning, single-cell whole-genome amplification (21), or further validation of a promising new bioinformatics tool that can tease apart multistrain *C. parvum* infections by deconvoluting simple Sanger sequencing *gp60* chromatograms (22).

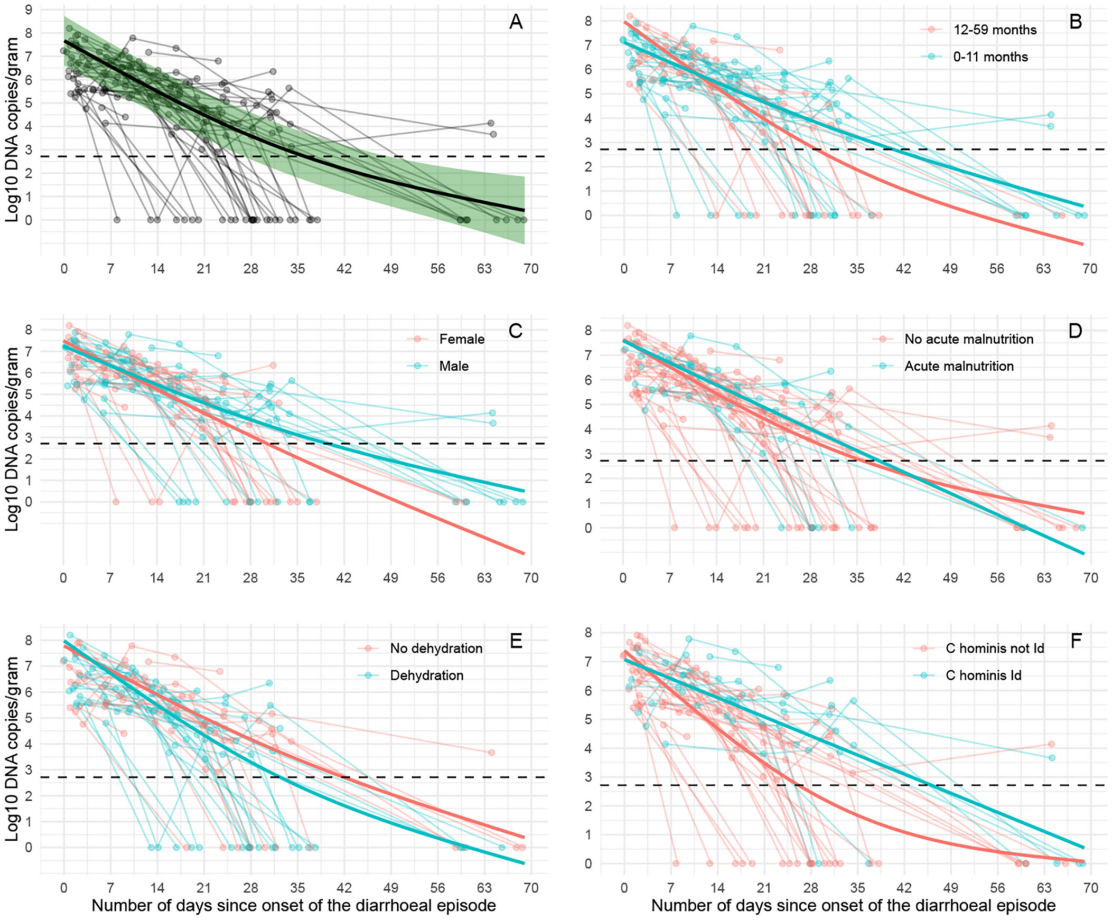

FIG 3 Temporal patterns of *Cryptosporidium* shedding. *Cryptosporidium* DNA quantity in $\log_{10}$ DNA copies/g; (A) overall, and stratified by (B) age, (C) sex, (D) acute malnutrition, (E) dehydration, and, (F) for *C. hominis* infections, *gp60* allele family. The dashed horizontal line represents the lowest reliable detection limit of the qPCR assay (519 copies/g). The shaded green area (A) represents the 95% confidence interval for the smoothed quantity estimate.

The main argument for offering testing and treatment for cryptosporidiosis in hospitals and health care centers is to reduce patient morbidity and mortality (23). In addition, targeted cryptosporidiosis treatment can be investigated as an intervention to reduce onward transmission (24) by controlling the key reservoir in children aged 6 to 24 months. Our cryptosporidiosis cases were diagnosed at the point of care, where a test-and-treat intervention might be practically feasible. Previous randomized controlled trials of nitazoxanide in children assessed parasitological clearance as a binary outcome by microscopy 7 to 10 days after initiating therapy, but total shedding duration was not a trial endpoint (25, 26). Ideally, future intervention trials of nitazoxanide or new therapeutics should include shedding duration as a separate endpoint in addition to clinical efficacy, supported by quantitative tests and subtyping. Based on the current and previous estimates of shedding duration (6), this will require minimum once-weekly follow-up of all cases for at least 6 weeks after onset of the diarrheal episode.

MATERIALS AND METHODS

Study design and participants. The study was conducted from December 2016 to August 2018 in Jimma University Hospital (now Jimma Medical Centre) and Serbo Health Center in southwest Ethiopia as a follow-up study of a subset of participants in the CRYPTO-POC diagnostic accuracy study. Details of screening

and enrollment have been published previously (17). In brief, a child under 5 years of age was eligible for inclusion if they presented for health care, they had diarrhea (3 or more loose or looser-than-normal stools within the previous 24 h), and their caregivers consented to participation. Children who had been inpatients for longer than 24 h were already excluded from the CRYPTO-POC study to avoid bias of community prevalence estimates from nosocomial cases. In contrast with many other studies, children with less-severe diarrhea, prolonged diarrhea ($\geq$7 days duration), or severe acute malnutrition (SAM) were included (17).

Data collection. Cryptosporidiosis point-of-care testing was performed by LED-AP microscopy (a semiquantitative test) at both sites, with results communicated back to the study nurse (17). Positive cases who resided within about 50 km of either site were invited to participate in a follow-up study that involved repeat clinical examination, interview, and stool samples. This started 7 days after enrollment, then continued weekly for the first 4 weeks, and ended with a final visit 60 days after enrollment. For *Cryptosporidium* oocysts, stool samples were examined by LED-AP and quantitative immunofluorescent stain antibody test (qIFAT) microscopy, and for *Cryptosporidium* DNA, stool samples were examined by a well-established quantitative PCR (qPCR) assay targeting the pan-species *Cryptosporidium cowp* gene. We generated standard curves using 10-fold dilutions of quantitative genomic *Cryptosporidium parvum* DNA, with final qPCR results expressed as DNA copies/g of wet stool, as described previously (17).

***Cryptosporidium* species determination.** On *Cryptosporidium* species qPCR-positive samples, *C. parvum* and *C. hominis* species identification was performed in a duplex real-time PCR assay targeting *C. parvum*- and *C. hominis*-specific sequences of the *lib13* gene, using primer and probe sequences from reference 27, but with probes adapted for use with the LightCycler system using locked nucleic acid probes (LC640) and a Förster resonance energy transfer quencher (BBQ-650) at the 3′ end. The PCR mixture contained LC FastStart DNA MasterPLUS HybProbe reaction mix (Roche), 625 nM each primer, 250 nM each probe, and 5 μL of DNA template, with water added to give a final reaction volume of 20 μL. This mixture was subjected to 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, with an initial denaturation at 95°C for 10 min.

***Cryptosporidium* subtyping.** We sequenced the *Cryptosporidium gp60* gene, which codes for a surface glycoprotein that contains a conserved region that allows classification of *gp60* allele families and also a hypervariable serine repeat region that allows for classification into *gp60* allele family subtypes. The *gp60* PCR is a nested PCR, with primary and secondary primer sequences as described in reference 28. The reaction mixture for both rounds of PCR contained 2$\times$ Phire Hot Start II PCR master mix (ThermoScientific), 500 nM forward and reverse primer, and 5 μL of DNA template, with water added to give a final reaction volume of 20 μL. This mixture was subjected to 35 cycles of denaturation at 98°C for 5 s, annealing at 50°C for 5 s (primary PCR) and 55°C for 5 s (secondary PCR), and extension at 72°C for 15 s, with an initial denaturation at 98°C for 30 s and a final extension at 72°C for 1 min. Nested PCR products were purified using the Exo-CIP rapid PCR cleanup kit (New England BioLabs) according to the manufacturer's protocol. A total of 5 μL of purified template DNA and 5 μL of the forward or reverse primers used in the secondary PCR (at a concentration of 5 pmol/μL) were added to a LightRun 96-well plate for Sanger sequencing (Eurofins Genomics). Raw *gp60* gene sequence data were analyzed directly using the free software CryptoGenotyper (29). Sequences that were unsuccessfully typed or flagged for manual analysis by CryptoGenotyper were trimmed and aligned using Geneious Prime software (version 2021.1.1), where final sequences and phylogenetic trees were compared to reference sequences of valid *gp60* allele families, following the method outlined in reference 30, using the CryptoGenotyper curated list of *gp60* reference sequences (29) (accessed on 1 July 2021). This was supplemented by the U.S. National Centre for Biotechnology Information Basic Local Alignment Search Tool and subtype classification by manual inspection of the serine repeat region according to the nomenclature summarized in references 31 and 32.

Dehydration score. DHAKA (dehydration: assessing kids accurately) score was calculated based on assessment of general appearance, tears, skin pinch, and respirations; a score of 4 or more was deemed severe dehydration, 2 to 3 as some dehydration, and 0 to 1 as no dehydration (33).

Acute malnutrition. For children aged $<$6 months, severe acute malnutrition (SAM) was defined as weight-for-height z-score (WHZ) of ≤ -3 of the WHO standard curves (34) and/or presence of bilateral edema involving at least the feet. Moderate acute malnutrition (MAM) was defined as a WHZ of ≤ -2 and > -3 with no edema. Midupper arm circumference (MUAC) was used instead of WHZ for 6- to 59-month-olds, as it was difficult to bring height measurement boards to home visits (by motorcycle) and because MUAC is assumed to be less susceptible to dehydration than weight (35, 36); SAM was defined as MUAC of $\leq$115 mm and/or presence of bilateral edema involving at least the feet, and MAM was defined as MUAC of $>$115 mm and $\leq$125 mm with no edema.

***Cryptosporidium* shedding.** A diarrhea case was defined as shedding *Cryptosporidium* DNA during the enrollment episode if qPCR was positive in either the enrollment sample or the 1-week follow-up sample. A case was considered no longer shedding *Cryptosporidium* from the first day, after onset of the diarrheal episode, at which both *Cryptosporidium* qPCR and oocyst microscopy were negative. Genotype shift was defined as a detection of a new *Cryptosporidium* genotype (by species, *gp60* allele family, or *gp60* subtype) in a later sample. A case was considered having ongoing shedding at the latest time point, in days after onset of diarrhea, with either a positive *Cryptosporidium* qPCR or positive *Cryptosporidium* microscopy (LED-AP and/or qIFAT) but excluding those cases where a genotype shift had occurred.

Time-to-event analysis. Time to shedding cessation was the event of interest. The nonparametric maximum likelihood estimate for the time-to-event distribution was approximated by the Turnbull estimator, a generalization of the Kaplan-Meier estimate that accounts for interval-censored data, using the R package interval (37). We fitted various parametric accelerated failure time models to be able to get 95% confidence intervals for the median shedding duration, using the R package icenReg (38). The event-variable was time to shedding cessation, in days. The model was adjusted for sex and age (in months) by separate fixed effect terms. For comparison of time-to-event curves between subgroups, fixed effect regression coefficients were

exponentiated to obtain the odds ratio of difference in ongoing shedding across the follow-up period. We therefore also performed nonparametric tests for all comparisons using a generalized log-rank test, adapted to interval-censored data using within-subject resampling (39), using the R package interval (37).

Modeling *Cryptosporidium* shedding quantity over time. *Cryptosporidium* DNA quantity (measured in $\log_{10}$ copies/g of stool) in sequential follow-up samples was plotted against time (in days since onset of diarrhea) for each cryptosporidiosis case. The visual inspection indicated that the temporal drop in quantity followed a nonlinear pattern. We therefore fitted and visualized a trend line (i.e., an overall "best fit" aggregate curve representing the drop in quantity as a function of time) using a generalized additive mixed model (GAMM), using an identity link and thin plate regression splines, with the maximum number of base functions ("knots") set to 3, using the R package mgcv (40). The overall shape ("wiggliness") of the smoothed curve was quantified by the effective degrees of freedom (EDF) of the smooth term, where an EDF of 1 would imply a linear relationship, an EDF of 2 would imply a quadratic relationship, and an EDF of 3 would imply a cubic relationship, etc., and by significance of the smooth term by the *P* value. Trend line differences between key subgroups were visualized by adding to the GAMM grouping predictors for age group (0 to 11 months versus 12 to 59 months), sex, disease severity (dehydration, acute malnutrition), and *Cryptosporidium* genotype and by separate plotting of difference smooths with 95% confidence intervals, using the R package itsadug. Overall shedding duration was estimated from DNA quantity, where shedding cessation was defined as the time point at which *Cryptosporidium* DNA quantity dropped below the reliable detection limit of the qPCR. This was estimated by a separate GAMM where days since onset of diarrhea was the outcome variable and a smoothed trend line for DNA quantity was the main predictor. To all GAMMs were added random effect intercepts for each study participant to account for within-subject clustering and also separate fixed effect linear terms to adjust for confounding by sex and age.

Ethics approval. Jimma University IRB (reference: RPGC/610/2016), the Ethiopian National Research Ethics Review Committee (reference: JU JURPGD/839/2017), and the Regional Committee for Medical and Health Research Ethics of Western Norway (reference: 2016/1096) approved the study.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 1 MB.

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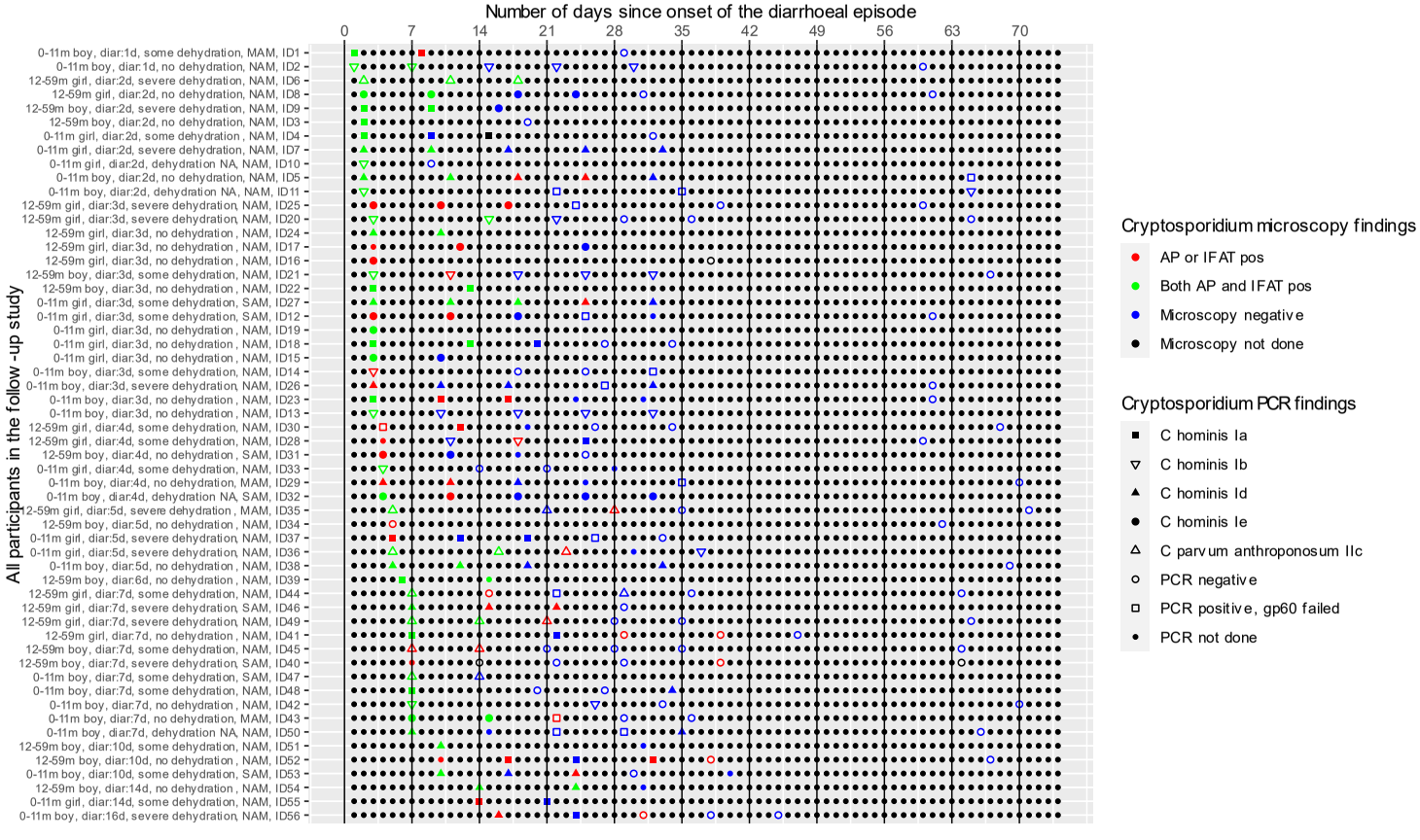
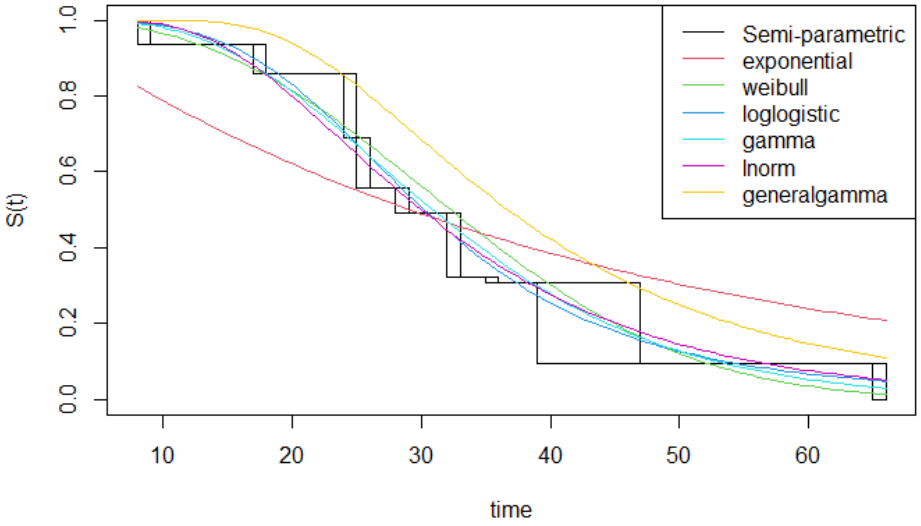
Figure S1. *Cryptosporidium* shedding over time, results for individual participants.



Note: For *Cryptosporidium* qPCR quantity and subtyping results, see **Supplementary dataset**.

Figure S2. Visual comparison and selection of time-to-event parametric models

The time-to-event graphs in Figure 1 in the main manuscript represent the aggregate interval-censored time-to-event data, overall, and stratified by subgroups. The graphs are based on the non-parametric maximum likelihood estimator (NPLME; the Turnbull estimator) and resembles a Kaplan Meier plot but is extended to allow for interval censored data. To our knowledge, there are no well-established methods to obtain confidence intervals for time-to-event duration estimates, using non-parametric or semi-parametric models. We therefore fitted several parametric models. Parametric models have the added advantage that we were able to adjust for the (a priori-assumed confounding factors of) age and sex. As there are no generally approved formal methods for choosing between time-to-event parametric models for interval censored data, we used the diag_baseline function from the icenReg R package [1] to compare various parametric models visually, with a semi-parametric Turnbull model as a baseline for assessing model fit; all models were adjusted for sex and age (in months):



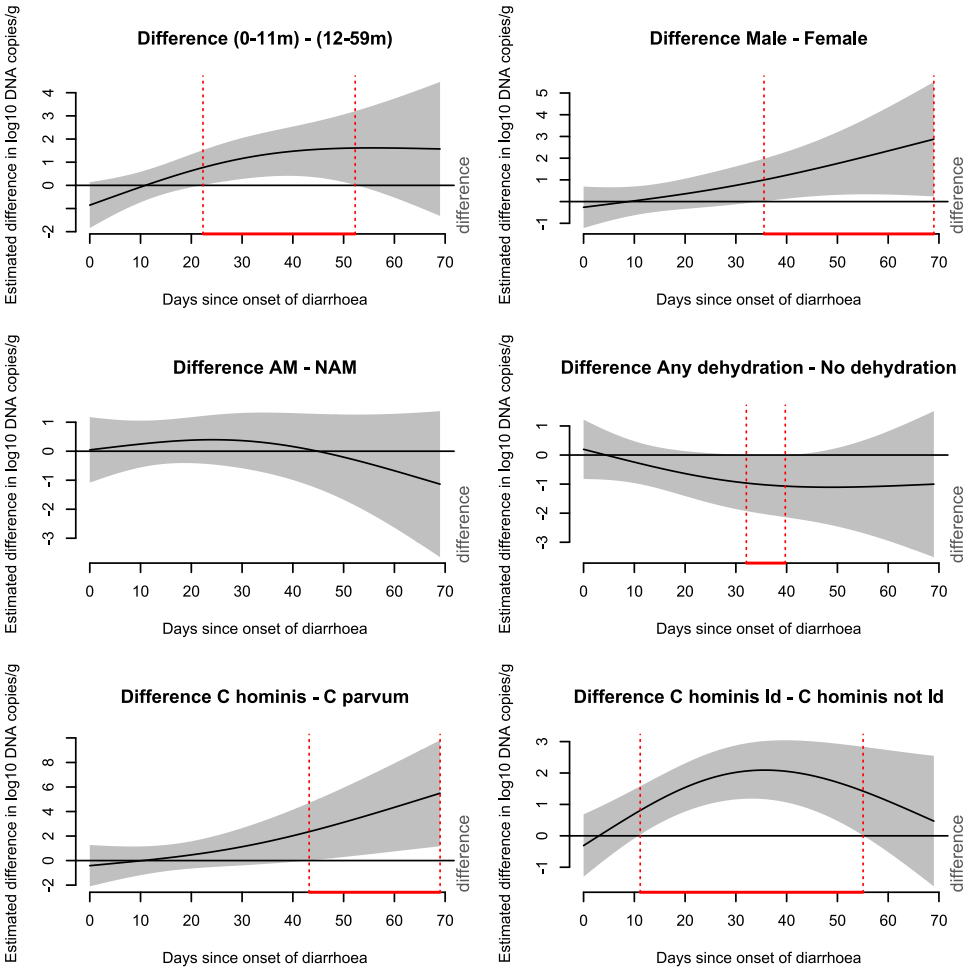
Although visual diagnostics are somewhat subjective, we see that most of the the parametric distributions show no systematic deviation from the semi-parametric baseline model, with the notable exception of the exponential and generalized gamma distributions. This implies that various parametric model familes may do a reasonable job of describing the underlying distribution. We opted for the commonly used log-logistic model, which allowed us to estimate median shedding duration with 95% confidence intervals and, also, to compare time-to-event curves between key subgroups of children by estimation of the odds ratio (also with 95% confidence intervals).

1. Anderson-Bergman C. icenReg: regression models for interval censored data in R. Journal of Statistical Software. 2017;81(1):1-23.

Supplementary table S1. Estimated *Cryptosporidium* quantity over time. Estimated by the overall generalized additive mixed model.

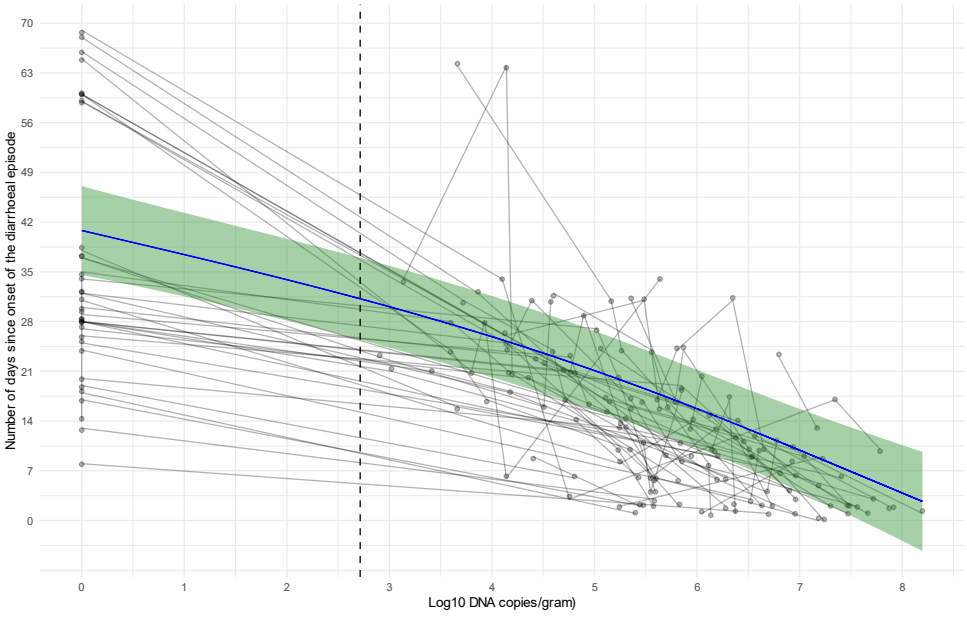
Weeks since onset of diarrhoea	*Cryptosporidium* DNA quantity (log10 copies/g)	95% confidence interval
0	7.7	6.6 to 8.7
1	6.6	5.6 to 7.6
2	5.5	4.5 to 6.5
3	4.5	3.5 to 5.5
4	3.6	2.6 to 4.6
5	2.8	1.8 to 3.8
6	2.2	1.2 to 3.2
7	1.6	0.58 to 2.7
8	1.2	0.02 to 2.3

Figure S3. Plot of difference in shedding quantity over time, compared between subgroups.



Note: These plots were made with the plot_diff function from the R package itsadug (https://CRAN.R-project.org/package=itsadug)

Figure S4. Plot of GAMM model used to predict shedding duration from overall drop in *Cryptosporidium* DNA quantity over time. Vertical dashed line represents the detection limit of the qPCR assay, and the green shaded ribbon represents the 95% confidence interval for the model predicted days since onset of diarrhoea.



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