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Pilot study on the epidemiology of Cryptosporidiosis and other pathogens causing infantile diarrhoea in Lambaréné, Gabon

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“The longest road out is the shortest road home.”

- Irish Proverb

Table of Contents

List of figures

List of tables

List of abbreviations

AIDS	Acquired ImmunoDeficiency Syndrome
API	Analytical Profile Index
CDC	Centres for Disease Control
CDHS	Clinical Dehydration Score
C.diff.	*Clostridium difficile*
Campylobacter jej.	*Campylobacter jejuni*
CERMEL	Centre de Récherches Médicales de Lambaréné
CHRGRL	Centre Hôpitalier Régional Georges Rawiri Lambaréné
CI	Confidence Interval
DNA	DeoxyriboNucleicAcid
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
GEMS	Global Enteric Multi-centre Study
Giardia int.	*Giardia intestinalis*
HAS	Hôpital Albert Schweitzer
HIV	HumanImmunodeficiencyVirus
km	Kilometre/s
OPD	Out-patient Department
ORS	Oral Rehydration Solution
OR	Odd's Ratio
PCR	Polymerase Chain Reaction
PhHV	Phocin HerpesVirus
RDT	Rapid Diagnostic Test
SDI	Socio-Demographic Index
SOP	Standard Operating Procedure
spp.	species pluralis = multiple species
Taq	*Thermus aquaticus*
WHO	World Health Organization

1. Introduction

1.1. Background

Millennium Development Goals

In 2000 world leaders established the so called Millennium Development Goals, which along with seven other goals postulated a reduction in child mortality by two-thirds to be achieved by 2015. (United Nations, 2015a)

Although this ambitious target was not achieved the estimated annual deaths of children under 5 years of age were reduced by 53% to around 6 million deaths worldwide in 2015.

Most of these under-five deaths occur in developing countries with about half of them (approx. 3 million) occurring in Sub-Saharan Africa with 83 per 1,000 children dying before their fifth birthday. (United Nations, 2015a)

The new Sustainable Development Goals, established in 2015 foresee a worldwide reduction to no more than 25 per 1,000 deaths of children under 5 by 2030. (United Nations, 2015b) To achieve this, it is imperative to take a closer look at what causes child morbidity and mortality worldwide.

Seeing as the percentage of children under five dying prematurely varies greatly throughout the world, it is only natural to conclude that the causes also vary. In a multi-centric study on “global, regional and national causes of under-five mortality rates” by L. Liu et al., 2016, the main causes of child mortality in developing countries were found to be neonatal deaths (45%), pneumonia (13%) and diarrhoea (9%).

Diarrhoeal disease causes more than half a million deaths of children under 5 years of age each year, with again most of these deaths occurring in Sub-Saharan Africa, as illustrated in Figure 1. This highlights diarrhoea’s role as one of the most important infectious diseases that needs to be addressed if child mortality is to be further reduced.

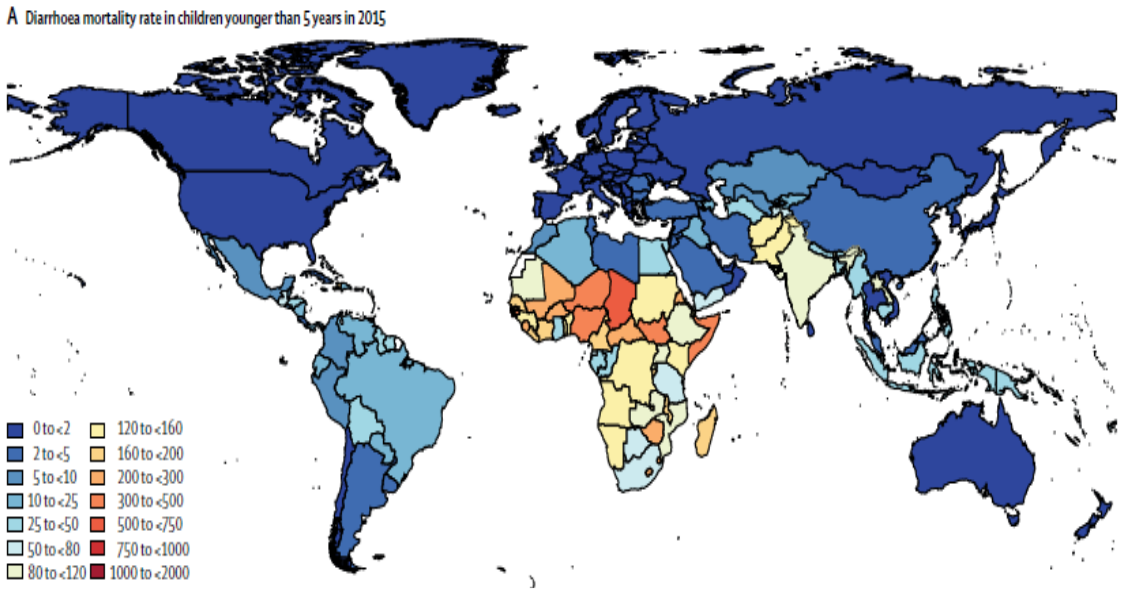

Figure 1: Diarrhoea mortality rates worldwide for children younger than 5 in 2015 (Figure by Troeger et al., 2017)

Diarrhoea is the fourth most prevalent cause of death globally in children and adolescents aged 19 years or younger in 2017, with malaria as one of the most notable tropical diseases only ranking sixth (Roba et al., 2017).

Nearly every child throughout the world suffers from diarrhoea at one point or another. It is therefore often underestimated in its potential to pose a serious threat to a child's short- and long-term health and is often seen as a passing inconvenience without further relevance, however, newer studies have shown that especially in low-resource settings, where children tend to experience more episodes of diarrhoea, the consequences for child health as well as development (Checkley et al., 2008) and survival cannot be dismissed (Troeger et al., 2017).

1.2. The physiology behind diarrhoea

Diarrhoea is defined by the WHO as the passage of three or more liquid stools during the course of 24 hours. (World Health Organization (WHO), 2013b) The emphasis lies on the consistency, allowing for individual variations in what is considered normal frequency.

Physiology of digestion

While the upper intestinal tract usually secretes large amounts of fluids, to ease digestion as well as the faeces' passage, approximately 76% of the fluid is absorbed in the ileum and another 22% (Gekle, 2018) in the colon resulting in formed stool with a consistency ranging from soft to hard, that is then excreted. Stool consists mostly of indigestible parts of foodstuffs, as well as bacterial flora that is discharged and other waste products.

While consistency and frequency can vary greatly due to nutritional factors, physical or psychological state and the bacterial flora of the individual, an increase in unformed stool secretion as observed in diarrhoeal disease is always a sign of an underlying pathology.

Pathophysiology

Diarrhoea as a symptom offers a great number of differential diagnoses, which are not limited to primary infections of the gut. Depending on the clinical presentation auto-immune diseases such as Celiac or Crohn's disease, as well as ulcerative colitis or malabsorption due to food intolerance (e.g. lactose or fructose) need to be considered. It can also be a symptom of malignant growth in the digestive tract or present as part of an infectious disease that does not primarily target the gastro-intestinal system but causes the body to react with hypersecretion, for example malaria (Ashie et al., 2017; Weinberg et al., 1997) or measles (Schuster & Kreth, 2019). In the latter case diarrhoea may even be the initial symptom of the disease, while the other more characteristic symptoms like for example fever (malaria) or fever and rash (measles) manifest themselves later.

An important aspect in the differential diagnoses of diarrhoeal disease is the patient's age. While all of these diseases can occur no matter how old the patient is, the overall likelihood varies greatly. Chronic inflammatory bowel disease or malignant growth are for example generally only found in adults.

Since acute diarrhoea (symptoms present less than two weeks) and to a lesser extent even chronic diarrhoea most often originate from infections, I decided to focus my research on infectious diarrhoea.

Acute or infectious diarrhoea, can be divided into three categories, secretory, invasive and penetrative diarrhoea, with distinct patho-mechanisms and causative agents. (Schmiedel, 2016)

Secretory Diarrhoea

Secretory diarrhoea means that the epithelial cells of the small intestine are induced to secrete more water than usual while the large intestine is not able to absorb the excess fluid sufficiently. The result is liquid to watery diarrhoea that continues even when no food is ingested. Common infectious agents causing this are *Vibrio cholerae* with the cholera toxin.

Invasive Diarrhoea

This type of diarrhoea originates in the colon. The bacteria invade the epithelial cells and destroy them, which leads to local inflammation and loss of function of the epithelial layer. The fluids secreted in the small intestine cannot be reabsorbed which combined with the inflammation causes mucoid and bloody diarrhoea. Typical pathogens in this category are *Shigella spp.*, *Entamoeba histolytica* and *EIEC* (entero-invasive *E.coli*).

Penetrative Diarrhoea

Bacteria such as *Salmonella enteritidis* and *Yersinia enterocolitica* pass through the epithelial cells of the small intestine into the submucosa and Peyer-Plaques where they cause inflammation. The exact patho-mechanism causing diarrhoea is not clear, however, generally this type of diarrhoea presents with fever as an additional symptom.

Irrespective of the underlying pathology, apart from the aforementioned change in stool consistency and frequency, children suffering from diarrhoea may present a multitude of different symptoms such as vomiting, loss of appetite or fatigue. The most feared complication of diarrhoea is severe dehydration and an

exaggeration of or subsequent malnourishment. Studies have shown that children being hospitalised for severe malnutrition accompanied by diarrhoea have a much higher mortality rate than those without diarrhoea (Talbert et al., 2012).

In cases where diarrhoea persists over weeks and months, it may lead to stunting and in extreme cases to wasting, as well as an overall higher susceptibility to other infections (Checkley et al., 2008).

1.3. Pathogens causing diarrhoea

Many studies have been conducted in the last few decades on the epidemiology of diarrhoeal disease world-wide. The spectrum of pathogens found and deemed relevant in causing diarrhoeal disease has varied greatly depending on when and where the studies were conducted, depending on which age group was examined or whether the participants were afflicted with further illnesses, and last but not least, on which diagnostic methods were employed. Since outbreaks of diarrhoea have long been linked to low sanitation standards, which then lead to contaminated food or water, it is tempting to conclude that a country's level of development, a marker of which can among others be its population's access to safe drinking water (World Health Organization (WHO) & United Nation's Children's Fund (UNICEF), 2017), may be related to the number of diarrhoeal episodes experienced per person. It is however not possible to directly link hygiene and sanitation to morbidity and mortality, because there are many different variables involved (Crocker & Bartram, 2016).

There are a number of pathogens that disrupt the digestive system and cause diarrhoea. Since the gut is host to a very complex microbiome where millions of bacteria co-exist by forming an intricate web of interdependence that generally regulates itself, every individual will react differently when exposed to a potential pathogen. Certain bacteria and viruses are obligate pathogens (e.g. *Vibrio cholerae*, Norovirus) and will always lead to a disruption in the function of the

digestive tract resulting in diarrhoea unless an immune response is quickly meditated in cases of re-exposure.

It is not clear whether the spectrum of pathogens causing diarrhoeal disease varies between developed and developing countries. A comprehensive study comparing the pathogens most often responsible for under-five deaths in different countries and between high and low SDIs found similar rankings (with rotavirus always part of and *Shigella spp.* and cholera most often represented in the top 3 and with *C. diff.* more significant in high SDI and *Cryptosporidium spp.* in low SDI countries) (Troeger et al., 2017), however, this does not necessarily mean that the spectrum of pathogens causing diarrhoea is similarly distributed, only that the same pathogens prove to be the most lethal irrespective of a country's SDI.

One of the most recent and most relevant studies in this field is by any measure the GEMS (Global Enteric Multi-centre study). This study focused mainly on children below five years of age in several countries throughout Africa and Asia and used a case-control approach to determine which pathogens were mainly responsible for episodes of diarrhoea in their cohorts. In young children, diarrhoea was mostly attributed to rotavirus, *Cryptosporidium spp.*, Enterotoxigenic *E. coli* (ETEC) and *Shigella spp.* (Kotloff et al., 2013). Upon re-evaluation with more sensitive diagnostic methods, the ranking changed to *Shigella spp*., rotavirus, adenovirus, ETEC, *Cryptosporidium spp.*, and *Campylobacter spp.* (J. Liu et al., 2016).

The inclusion of *Cryptosporidium spp.* in the top 6 pathogens causing diarrhoea in children was at the time wholly unexpected. So far, this parasite had been considered of no importance in immune-competent individuals (Striepen, 2013).

Aside from studies focusing on small children or travellers, it proves difficult to find comprehensive literature on causes of diarrhoea in the general population. Too little is known about the aetiology of diarrhoea in patients older than 5 and younger than 65, since aside from these age groups patients affected are not as likely to seek medical assistance (Wilson, 2005). Therefore, case numbers can only be assumed, and further identification of the underlying cause is seldomly undertaken unless diarrhoea persists longer than a few days.

1.4. Treatment principles

The treatment of infantile diarrhoea should follow the WHO guidelines (World Health Organization (WHO), 2005) which postulate the use of oral rehydration solution (ORS) until diarrhoea cedes or in cases with severe dehydration intravenous fluids, additionally zinc supplementation for 10-14 days and continued feeding. The WHO warns against the use of antimicrobial agents unless bloody stools are reported, cholera is suspected or if the child suffers from a severe non-intestinal infection. Anti-motility drugs should generally not be administered to children below 5 years of age.

This treatment approach is mostly symptomatic. So far when it comes to infectious diarrhoea there is hardly any direct approach to treating the underlying cause of diarrhoea.

2. Aims of the project presented

The aim of this pilot study was to determine the aetiology of childhood diarrhoea in Lambaréné and its immediate surroundings, with a special interest on the possible occurrence of *Cryptosporidium spp.*

Additionally, patient's symptoms were characterized and possible correlations between pathogenic agents identified and patient presentation were assessed.

Furthermore, as part of the project, new diagnostic methods for *Cryptosporidium spp*. were established and evaluated.

3. Cryptosporidium

3.1. Biological aspects of the parasite

Crypotsporidium is a parasite belonging to (the subkingdom of protozoa and) the phylum of apicomplexa and is thus related to *Plasmodium* and *Toxoplasma*. It has recently been classified as a gregarine instead of a coccidian parasite, which shows a change in perception of the parasite's biology. (Clode et al., 2015)

As opposed to other well-known apicomplexans *Cryptosporidium* shows a very reduced metabolism. It contains the apical complex, which is the defining feature for this phylum of parasites, but instead of some of the normally characteristic organelles for this phylum like the apicoplast, (a relict, formerly photosynthetic plastid) *Cryptosporidium spp.* has an extended pellicle, which increases the surface and the amount of micropores to access the readily available nutrients in the intestinal tract (Ryan et al., 2016).

The parasite passes through its entire life cycle in one host, multiplying both sexually and asexually in the small intestine of the host and exiting through the large intestine in the form of hardy oocysts that are then ingested either by the same or another host. While this work focuses mainly on human infection, which is notably caused mainly by *Cryptosporidium hominis* or *Cryptosporidium parvum*, the parasite was originally discovered to infect cattle and pigs. Many

other subspecies have been found to infect animals, with the parasites following the same life cycle and mostly causing similar symptoms of infection. Groups of animals discovered to be susceptible are mostly mammalians, but also birds, where infection manifests in the respiratory tract and reptiles, in which illness is generally severe and usually proves to be fatal (O'Donoghue, 1995).

Once a host has been infected and the parasite has reached a suitable mucous membrane (generally in the small intestine, although it has been shown that the parasite can also multiply in other parts of the body e.g. inside respiratory epithelial cells) the oocysts hatch and the sporozoites invade the epithelial cells where they develop into trophozoites and subsequently into meronts, which produce merozoites that then go on to infect new epithelial cells and replicate either asexually producing further merozoites or go on to reproduce sexually via differentiation into macro- and microgamonts and ending in the production of oocysts that are either excreted or reinfect the host. (Figure 2)

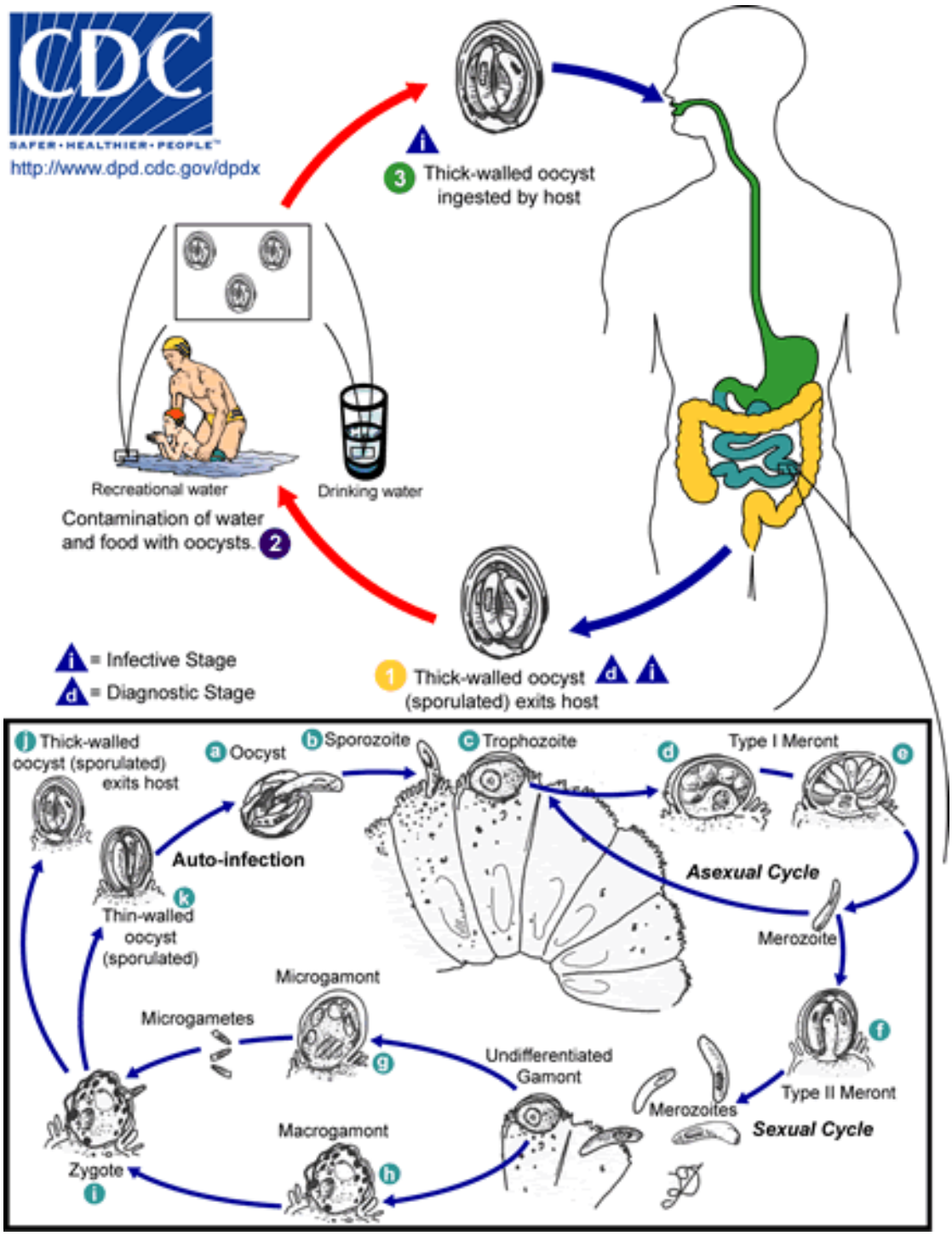

Figure 2: Cryptosporidium life cycle (Centers for Disease Control and Prevention et al., 2015b)[1]

[1] Disclaimer: The use of this figure does not constitute an endorsement or recommendation of this dissertation by the U.S. Government, Department of Health and Human Services, or Centers for Disease Control and Prevention.

3.2. Cryptosporidiosis

Cryptosporidium parvum and *hominis* are the two subtypes that are most often isolated in human cryptosporidiosis. At least 11 others have been isolated in a few cases (Squire & Ryan, 2017).

Symptoms of Cryptosporidiosis include watery diarrhoea, stomach pain, moderate to severe dehydration, occasionally fever and other complaints associated with acute diarrhoea. In case of parasite persistence over months, additional symptoms may include substantial weight loss, failure to thrive in infants, stunting and extra-intestinal manifestations in immunosuppressed patients (Cheifetz, 2011; Herold, 2016).

Cryptosporidiosis in its most extreme form is generally only found in immunocompromised individuals and has therefore until recently been viewed as a mainly opportunistic infection and is for example still listed as an AIDS-defining illness (Herold, 2016).

That immunocompetent individuals may also be susceptible to the parasite, even if this seems to mostly occur in children, is not wholly new (Casemore et al., 1985), but nevertheless the impact of this parasite on diarrhoeal disease globally had not been realized until recently (Striepen, 2013).

3.3. Current diagnostic methods

There is a variety of diagnostic tests available for the detection of *Cryptosporidium spp.* in faeces. The gold standard for diagnosis is generally stool microscopy using the Ziehl-Neelsen or modified acid-fast staining method. Oocysts are then identified under a conventional light microscope (CDC - Global Health Division of Parasitic Diseases and Malaria, 2016). This method has proven to be very specific but unfortunately necessitates a high level of training and experience in differentiating oocysts of Cryptosporidium from debris or other parasites, like *Cyclospora cayetanensis.* In addition, the preparation and subsequent microscopy are quite time consuming.

Therefore, methods such as PCR or even simpler RDTs are up and coming as with many other microbial pathogens. The main advantage for RDTs is that they are easy to use and do not require special training, however, they usually require another more sensitive method to confirm any positive or negative finding.

PCR on the other hand is extremely sensitive but requires expensive equipment and professional training.

3.4. The treatment dilemma

Effective treatment of acute Cryptosporidiosis has long proven to be elusive. Part of the problem is the relatively small number of cases and the illness being disregarded as an exclusively opportunistic infection with no impact on healthy individuals. Difficulties arise through the parasite's location intracellularly, the necessity of targeted treatment so as not to further disrupt the gut's microbiome and the parasite's possible resistance mechanisms (Mead, 2002).

Treatment regimens with different types of antibiotics have been used in the past with mostly unsatisfying outcomes. At present the only FDA approved drug for Cryptosporidiosis is Nitazoxanide, a broad-spectrum anti-infective drug belonging to the class of thiazolides (Shakya et al., 2017), which has been shown to aid recovery in immunocompetent individuals, but is not recommended for individuals <12 months of age, thereby excluding a significant number of patients. Unfortunately, it has next to no effect in immunocompromised patients, who are of course in much greater need of an effective treatment (Abubakar et al., 2012; Amadi et al., 2009).

Drug development and subsequent approval are a costly and complicated process. A study published in 2017 conducted a high-throughput screen for possible new drugs against *Cryptosporidium spp.*. This resulted in the identification of several possible drugs, clofazimine is such a drug thus far used in the treatment of leprosy (Love et al., 2017).

Despite these promising findings an effective, targeted treatment for those most affected by Cryptosporidiosis is still years away.

3.5. Possible modes of transmission

Faecal-oral transmission has long been hypothesized as the main mode of transmission in individual cases, whereas outbreaks of cryptosporidiosis have been traced back to contaminated water sources, especially swimming pools in Europe or North America (Efstratiou et al., 2017).

Seeing as newer studies show more cases outside of these documented outbreaks especially in children (Kotloff et al., 2013; Platts-Mills et al., 2015) and without the otherwise characteristic outbreak-like case accumulation, it is unclear what the main route of infection is.

Aside from faecal-oral transmission and contaminated water sources as sources of infection, recent studies have also given weight to the possibility of airborne infection. (Mor et al., 2010; Sponseller et al., 2014)

Newer insights illustrate that *Cryptosporidium spp.* are capable of extracellular replication (Aldeyarbi & Karanis, 2016) and might be able to survive and replicate in biofilms (Ryan et al., 2016). This poses problems for monitoring systems that might not register the presence of oocysts in drinking water, because they are held up in bacterial biofilms, where they accumulate and are released in bulks, additionally monitoring systems tend to focus on the oocyst stages, but seeing as replication is ongoing the contamination might be missed entirely or severely underestimated (Ryan et al., 2016).

Contrary to other microbes that can be transmitted through water, chlorination has next to no effect on the very hardy *Cryptosporidium* oocysts. They are poorly susceptible to alcohol, chlorine or iodine disinfection (hence the outbreaks in public swimming pools) and are only eliminated by reverse osmosis filters or boiling water for at least 1 minute (Centers for Disease Control and Prevention et al., 2015a). This makes them more difficult to eliminate than most other water transmittable pathogens.

Since studies concerning the parasite’s mode of transmission have been mainly conducted in developed countries following outbreaks, there is a certain bias in the knowledge regarding transmission accumulated through these investigations.

It has been assumed that outbreaks of cryptosporidiosis are simply not identified in developing countries due to lack of readily available diagnostic tools (Mmbaga & Houpt, 2017) and appropriate monitoring of disease outbreaks in general (Efstratiou et al., 2017). This lack of conclusive epidemiological data hampers researchers looking for possible modes of transmission.

A review by Aldeyarbi et al., (2016) cites that many water sources throughout Africa are contaminated, but whether it is the consumption of contaminated water, human-human or human-animal interaction that then leads to infection cannot be readily determined.

In any case further investigation is necessary, by first establishing the overall number of actual cases in developing countries and then delineating possible modes of infection.

4. Material & Methods

4.1. Material

Equipment

- Stool containers
- Gloves
- Cell culture plates
- Eppendorf tubes
- Inoculation loops
- Pipettes
- Cotton swabs
- Vortex
- Spectrometer
- Incubator
- PCR Machine ROTOR GENE Q

Rapid Diagnostic Tests

- CerTest Cryptosporidium Kit
- SD Rapid Test Rota/Adenovirus Kit

Microbiological Culture

- McConkey Agar
- Salmonella-Shigella Agar
- Hektoen Agar
- Müller-Hinton Agar
- Brain-Heart-Infusion
- Antibiotic Diffusion Discs
- Gram-staining equipment
- Oxidase test
- API 20E

Multiplex-PCR

- MO BIO PowerSoil DNA Isolation kit

- Qiagen, HotStarTaq Master Mix Kit
- Roche, MgCl2 25mM
- Primers:
    - *E.histolytica*:
      Ehd-239F: 5´-ATT GTC GTG GCA TCC TAA CTC A-3´
      Ehd-88R: 5´-GCG GAC GGC TCA TTA TAA CA-3´
      E.hist T: 5´-Joe-TCA TTG GAA TGA ATT GGC CAT TT-BHQ1-3´
    - *Giardia lamblia*:
      Giardia F: 5´-GAC GGC TCA GGA CAA CGG TT-3´
      Giardia R: 5´-TTG CCA GCG GTG TCC G-3´
      Giardia T: 5´-Fam-CCC GCG GCG GTC CCT GCT AG-TAMRA-3´
    - *Cryptosporidium parvum*:
      Crypto F: 5´-CGC TTC TCT AGC CTT TCA TGA-3´
      Crypto R: 5´-CTT CAC GTG TGT TTG CCA AT-3´
      Crypto T: 5´-Rox-CCA ATC ACA GAA TCA TCA GAA TCG ACT GGT ATC-BHQ2-3´
    - *Cyclospora cayetanensis*:
      Cyclo F: 5´-TAG TAA CCG AAC GGA TCG CAT T-3´
      Cyclo R: 5´-AAT GCC ACG GTA GGC CAA TA-3´
      Cyclo T: 5-Cy5-CCG GCG ATA GAT CAT TCA AGT TTC TGA CC-BHQ2-3´
    - Phocin Herpes Virus 1 (internal Control):
      PhHV F: 5´-GGG CGA ATC ACA GAT TGA ATC-3´
      PhHV R: 5´-GCG GTT CCA AAC GTA CCA A-3´
      PhHV T: 5´-Cy5.5-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-BBQ-3´

Software

- JMP 13
- Microsoft Office 2016
- Mendeley Desktop 2008

4.2. Methods

4.2.1. Study design

With the goal of determining possible modes of transmission for *Cryptosporidium parvum/hominis* in Sub-Saharan Africa a multi-centric, non-interventional observational study was conducted with CERMEL in Lambaréné as one of the four study sites. To investigate the circumstances surrounding a patient's infection with *Cryptosporidium spp.,* children between 0 and 5 years of age, presenting at one of the two study hospitals with diarrhoea were included and subsequently screened for the parasite. Once a positive index case was identified an out-break-like investigation into that patient's environment was conducted with the goal of collecting information about the living circumstances of the patient and including family members, friends and animals that the patient was in contact with.

To gain more insight into the general epidemiology of diarrhoeal disease in children below 5 at the study site, I devised an additional study protocol, keeping within the non-interventional observational study design, with the aim of collecting additional clinical data on patients and screening for other pathogens known to cause diarrhoea in children. The data analysed in this dissertation was mainly generated through this ancillary study with the primary focus being the pathogen distribution and clinical characteristics of the patients.

Data from the household surveys conducted for positive index cases was not used in this thesis.

The ancillary study was approved by the Comité d'Ethique Institutionel Lambaréné (CEI-CERMEL: 003/2017)[2].

[2] See appendix

4.2.2. Study site

The study sites for the primary recruitment of initial patients were the Albert Schweitzer hospital (HAS) and the regional hospital Georges Rawiri (CHRGRL) in Lambaréné. These two are the main health care centres in Lambaréné, which also provide health care for patients coming from much farther away. For the study only patients from Lambaréné and its immediate surroundings were considered, which means we included patient's residing as far as 20km from Lambaréné's centre. Lambaréné, the seventh biggest city in Gabon with a population of approximately 21 000 people (Worldometers, 2019), is situated on the Ogooué river just south of the equator. The climate is typical for the tropical rainforest with high levels of humidity and two types of seasons alternating. Rainy season from March to May and October to December, characterized by high levels of temperature and humidity, as well as heavy rains and thunderstorms as opposed to lower temperatures (20°- 27°C) and the near complete absence of rainfall during dry season from January to February and June to September (Deutscher Wetterdienst, n.d.). The change of climatic conditions as well as the accompanying change in water access, mosquito vitality lead to a certain seasonality not only in malaria incidence but also in the incidence of diarrhoeal disease as observed in the local hospital.

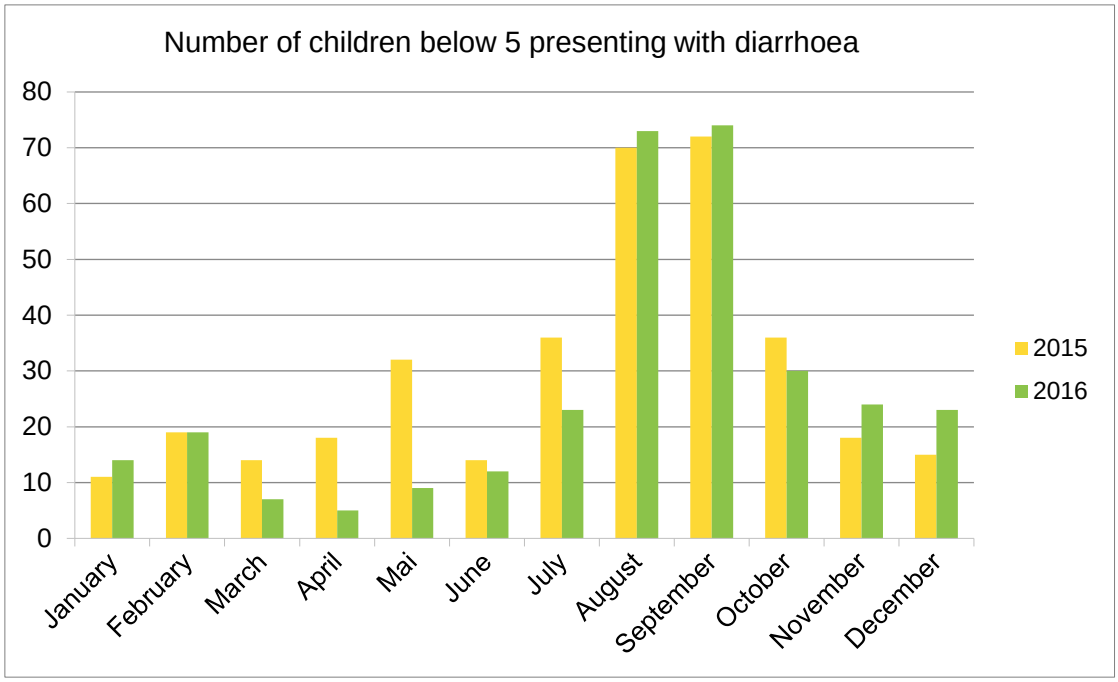

Figure 3: Data collected from the hospital register of the paediatrics' department at the Albert Schweitzer Hospital (HAS) in Lambaréné, Gabon

4.2.3. Recruitment

The recruitment process started in the respective hospitals' patient reception area. Patients below 5 years of age for whom diarrhoea was cited as the main complaint, were selected and the study procedures explained to the parent or guardian present. After obtaining written informed consent, two standardized questionnaires[3] were filled out and a basic clinical examination was conducted, assessing the status of hydration using a 4-item, 8-point Clinical Dehydration Scale (see Table 1), that groups the patients assessed into three groups. No dehydration with a score of 0, mild dehydration (score 1-4) and moderate to severe dehydration (score 5-8) (Goldman et al., 2008). Additionally, weight, further symptoms and medication intake were recorded. Afterwards a stool

[3] See appendix

collection tube was provided with instructions on sample collection to the parent or guardian.

Table 1: Clinical Dehydration Scale established by Goldman et al., 2008.

TABLE 1 CDS[10]

Characteristic	Score of 0	Score of 1	Score of 2
General appearance	Normal	Thirsty, restless, or lethargic but irritable when touched	Drowsy, limp, cold, or sweaty; comatose or not
Eyes	Normal	Slightly sunken	Very sunken
Mucous membranes (tongue)	Moist	Sticky	Dry
Tears	Tears	Decreased tears	Absent tears

Exclusion criteria were children not meeting the criteria defining diarrhoea, which in this study were the passage of three or more liquid stools in 24 hours during at least one of the last three days, as well as children living outside of the study area and children older than five years.

4.2.4. Sample processing and diagnostic methods

Stool samples from initial patients were collected during the first 48 hours after inclusion either at the hospital or at the patient's home. Samples were immediately put on ice and transported to the respective laboratories.

For the initial patients an RDT for Cryptosporidium was performed in the parasitology laboratory. Conventional microbiological culture and an RDT for Rota-/Adenovirus were performed in the microbiology lab. Conservation was done in Parasitology for PCR at -20°C. If there was a sufficient amount of sample, part of it was also conserved at -20°C in the Microbiology lab for possible further testing.

DNA was extracted from all samples using a MO BIO PowerSoil DNA Isolation kit.

For initial patients a Multiplex PCR detecting *Giardia intestinalis* and *Cyclospora cayetanensis* in addition to *Cryptosporidium spp.* was run.

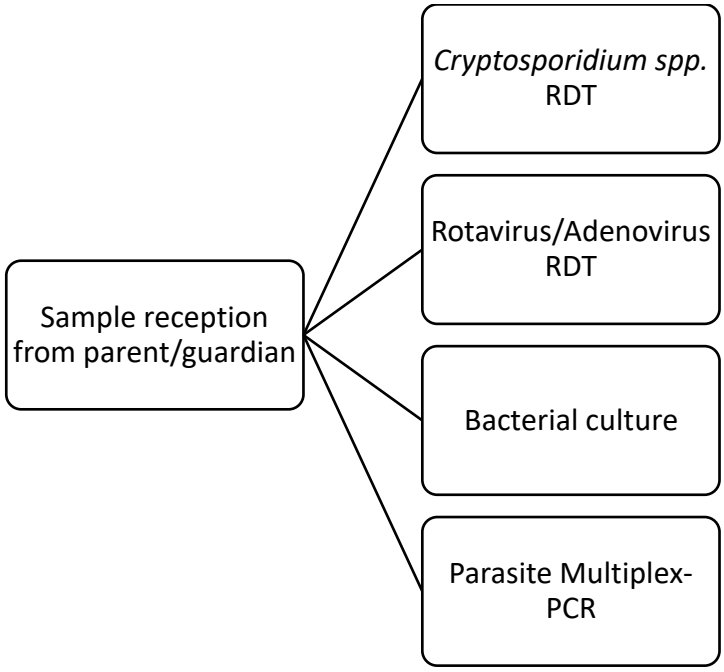

Figure 4: Testing algorithm for stool samples collected within 48 hrs of patient's inclusion into the study. If sample amount was sufficient part of the sample was conserved at -20°C.

4.2.4.1. RDTs and Microbiological testing

The RDT used for the detection of Cryptosporidium oocysts was a coloured chromatographic immunoassay using a lateral flow technique. The same principle applies to the RDT for Rota-/Adenovirus.

Testing for bacterial pathogens followed ATCC standards for microbiological testing of specimens. All tests are explained in detail in "Introduction to Microbiology" (American Type Culture Collection (ATCC), 2015). Culture plates containing agar and specific conductive and inhibiting factors for the selective growth of the most common bacterial agents (e.g. *Shigella spp.*, *Salmonella spp.*, *E. coli*) were used, MacConkey agar, Hektoen enteric agar and Salmonella-Shigella (SS) agar respectively. After incubation at 37° C for 16 – 24 hours, suspicious colonies were re-plated and further identified through colony

morphology examination, Gram-staining and Oxidase testing. Depending on the outcome of this characterization, further tests cataloguing biochemical reactions using different API kits were performed. In case a pathogen was identified, anti-microbial-susceptibility testing using Müller-Hinton Agar was added.

If no suspicious growth was observed after a further incubation period of up to 48 hours on the initial agar plates, the sample was declared negative for the bacterial pathogens sought for.

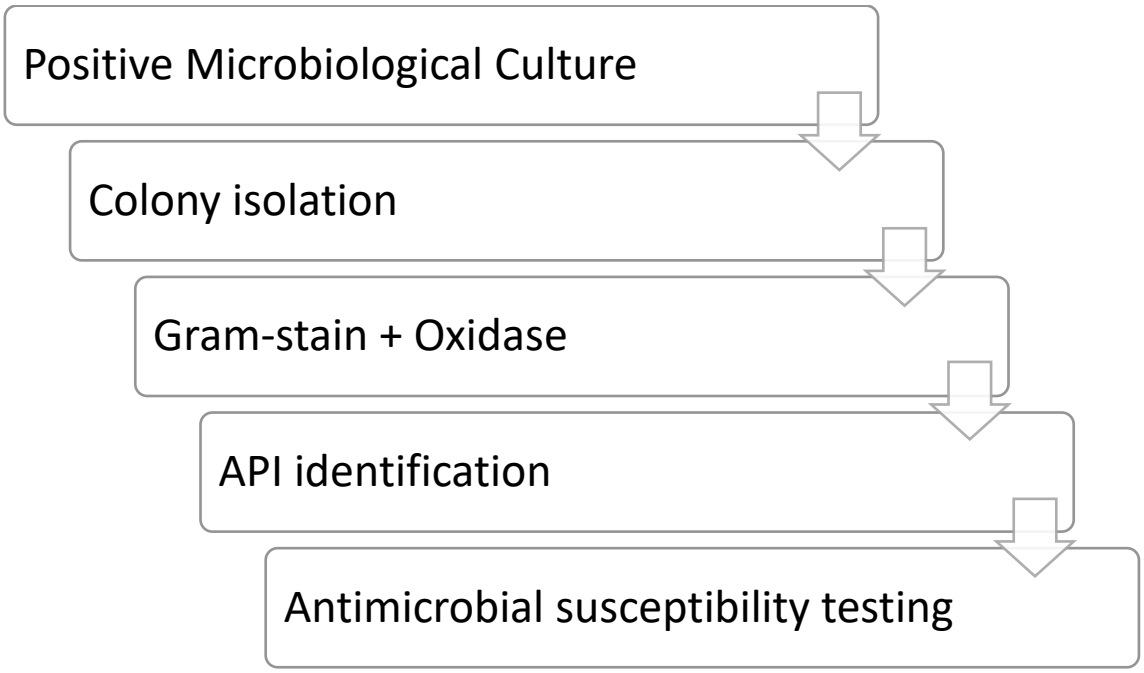

Figure 5: Simplified algorithm for microbiological testing on stool samples.

4.2.4.2. Multiplex-PCR

To confirm the results of the Cryptosporidium RDT and to identify other pathogenic parasites, a Multiplex-Realtime-Quantitative-PCR using the Qiagen Rotor Gene Q PCR instrument was performed on most samples.

The DNA needed for the following amplification was extracted from the samples using MO BIO PowerSoil DNA Isolation kit according to the protocol established for the CRYPTO multi-centre study, which was based on the manufacturer's

manual[4]. It is especially designed to remove all manner of PCR amplification inhibitors from a variety of sample types. The procedure comprises several steps using chemical and mechanical methods to achieve cell lysis and ends with the DNA being captured on a silica membrane and then washed off.

After obtaining a sufficient amount of DNA from each sample PCR was performed according to the procedure described in the SOP, which had also been established for the CRYPTO multi-centre study.

Each sample was added to a so called “reaction mix” containing HotStarTaq Master Mix, magnesium chloride, Primer Mixes for *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Giardia lamblia* and PhHV and PhHV plasmide.

PhHV plasmide was used as an inhibition control. Since the plasmide and respective primers are added to every test tube, all samples should show an amplification of PhHV plasmide, unless some type of amplification inhibition is present (e.g. bile salts or complex polysaccharides (Bessetti, 2007)).

Mixes for each sample were pipetted into Rotorgene tubes, as well as positive controls containing specified amounts of parasite DNA for each specimen and a negative control containing only the reaction mix and nuclease-free water. The tubes were loaded into the RotorGeneQ machine and a standardized programme started.

This programme begins with a 15 minutes activation phase at 95°C. The HotStar Taq-Polymerase[5] used is inactive at room temperature, making it possible to set

[4]Available at : https://www.qiagen.com/de/resources/resourcedetail?id=5c00f8e4-c9f5-4544-94fa-653a5b2a6373&lang=en (20th January 2020)

[5] Taq-Polymerase is derived from a very heat-stable bacterium, Thermus aquaticus, that can withstand very high temperatures and shows a maximum activity at 75° to 80°C and 2-4 mM $MgCl_2$ concentration. (Lawyer et al., 1993) Optimum temperature for DNA-polymerases shows great variation depending on the organism’s normal environment. For the E. coli DNA-polymerase a temperature of 37° C is optimal, since this bacterium colonizes and therefore replicates in open nature or in different eukaryotic species. This enzyme would denaturate alongside the DNA during the first part of PCR and would therefore have to be newly added after denaturation in each cycle.

the reaction mix up at ambient temperature, but necessitating this so-called activation period to ensure optimal enzyme function further on.

The actual PCR then commences with an initial **denaturation** of the double-stranded DNA at 95° C for 15 seconds, then continuing with 30 seconds of **annealing or renaturation** where primers bind to their respective target sites in the sample DNA. This leads to the polymerase binding to the 3'-hydroxyl end of the primers once the **synthesis** phase at 72° C for another 30 seconds starts. The polymerase then joins complementary bases to the existing DNA strand, resulting in a newly synthesized strand of double-stranded DNA. The synthesis is stopped by the temperature being raised to 95° C, resulting in the separation of polymerase and DNA and further denaturation of the newly synthesized strands of DNA. This leads to another round of amplification, repeating the three phases of denaturation, annealing and synthesis until after 45 cycles the amplification is complete and the mix is cooled down to 40° C for 30 seconds and can be analysed.

The mix contains specific fluorescent reporter probes, that can only bind to a specific region in the targeted DNA. These probes are marked with a fluorescent reporter and a quencher, that prevents fluorescence. Once the target DNA is denatured, the probes hybridize with the DNA. Fluorescence is however still quenched. Only once the Taq-Polymerase has connected the probe with the newly synthesized DNA strand, is the reporter released and can then emit fluorescence. The more strands are amplified, the more fluorescence is emitted, thus making it possible to measure the amount after each round of amplification. This creates a characteristic curve only when amplification occurred. Since a so called Realtime-PCR was conducted, amplification was measured after each cycle, showing how the amount of DNA contained in the mix grows exponentially. When comparing this to the curve measured in the positive controls, that contained a known quantity of DNA it becomes possible to quantify the amount contained in the original sample. This is not possible when using other PCR methods, that often use gel-electrophoresis to determine whether the sample contained DNA or not.

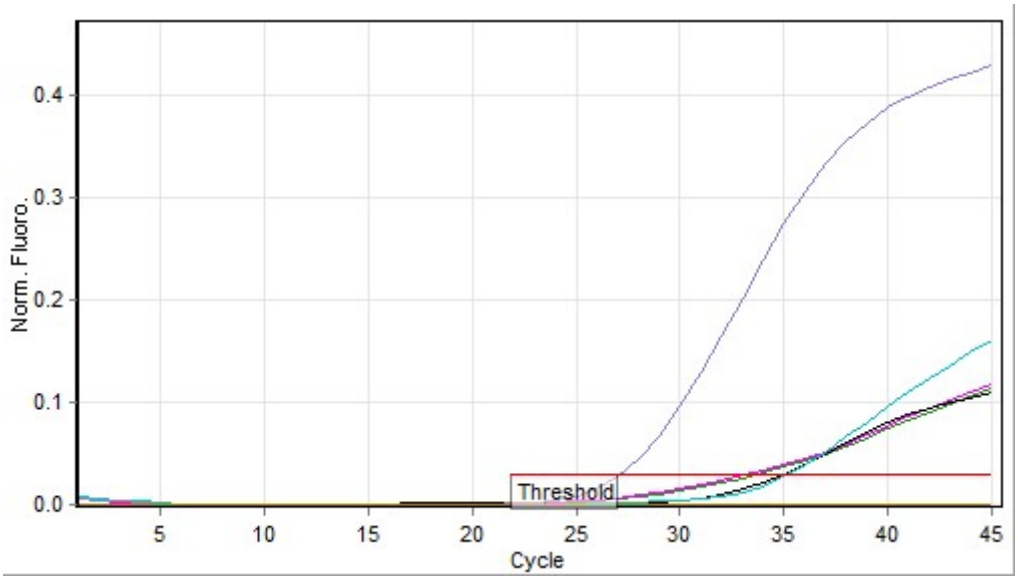

Figure 6: PCR result from a run containing two positive patients' samples. Each patient's samples were included twice in the run.

4.2.4.3. Additional diagnoses

Systemic infections, like malaria and measles, that can present diarrhoea as one of their symptoms, diarrhoea sometimes even predating other more characteristic symptoms, were also taken into consideration. Diagnosis was made by hospital staff in these cases and information either noted down directly or collected from patient's files.

Furthermore, the wet-mound technique, a standard diagnostic test performed in the microbiology laboratory, was performed on samples (American Type Culture Collection (ATCC), 2015). This method is conducted by suspending a small amount of the fresh sample in saline solution and examining a drop under a conventional (light-)microscope. This technique can be used to identify different parasites, as well as fungi or bacteria. Certain parasites, that can be thus identified, such as helminths are not generally considered to be causes of diarrhoea but can be associated with contaminated food or water and the presence of these parasites was therefore recorded as well.

4.2.4.4. Statistical analysis

Statistical analysis was performed mainly using JMP Software (Version 13) and Excel (Microsoft Office 2016) for Figure 3 and Figure 9. Likelihood-ratio X^2-test was used to test for associations between pathogens and clinical findings or seasons, as well as Odd's Ratio to measure association between pathogens and age groups, as well as pathogens and symptoms, when likelihood-ratio X^2-test had shown a significant association. To counteract the problem of multiple comparisons Bonferroni correction was utilised.

5. Results

5.1. Study cohort

From the 20th February 2017 until the 17th November 2017 100 initial patients were recruited and a stool sample analysed. 57% were recruited at the Albert Schweitzer Hospital (HAS) with 33% admitted at the regional Hospital Georges Rawiri (CHRGRL) and 10% for which the hospital of admission had not been recorded. Distribution across seasons, namely rainy and dry, was quite equal, with slightly more patients recruited during rainy season (56%).

49% of the initial patients were female, 51% were male. The mean age on admission was 14.2 months with a majority of 67% of children being between 6 and 24 months old.

Weight for age percentiles recorded were mostly below 50% (65% of patients) with 48% of patients coming in at less than 20%.

Most patients were reported to have been breast-fed (86%). Of those 6 months old or younger 47.6% were still being exclusively breast-fed and of those older than 6 months 38% had been breast-fed between 6 to 12 months. The mean duration of breast-feeding was 7.8 months.

Demographic details of patients are presented in Table 2.

Table 2: Demographic data of study cohort. Information on the duration of breast-feeding was not clear in some cases and the weight of 7 of the patients, as well as the hospital at which 10 of the patients first presented was not recorded correctly and is therefore marked as unknown.

Sex		**N**	**%**
	F	49	49%
	M	51	51%
	All	100	
Age categories			
	0 – 6 months	21	21%
	6-24 months	67	67%
	Older than 24 months	12	12%
	All	100	
Breastfed 0-6 months of age			
	Exclusively	10	48%
	Already weaned	5	24%
	Never	5	24%
	Unknown	1	5%
	All	21	
Breast-fed older than 6 months			
	Up to 6 months of age	24	30%
	Up to 12 months of age	30	38%
	More than 12 months	10	13%
	Never	8	10%
	Unknown duration	1	1%
	Unknown	6	8%
	All	79	
Weight for Age-Percentile			
	Above 50%	28	28%
	Between 20% and 50%	17	17%
	Below 20%	48	48%
	Weight unknown	7	7%
	All	100	
Season			
	Dry Season	44	44%
	Rainy Season	56	56%
	All	100	
Hospital			
	CHRGRL (Regional Hospital)	33	33%
	HAS (Albert Schweitzer Hospital)	57	57%
	Unknown	10	10%
	All	100	

Clinical presentation

Of the 100 initial patients 81 were hospitalised. Upon inclusion into the study, basic clinical characteristics were recorded (Table 3).

The median time between the onset of diarrhoea until seeking health care at one of the study hospitals was 4 days with some patients having waited up to a month until presenting at a hospital.

Upon admission 88% of patients showed signs of dehydration, with 26% being classified as moderately to severely dehydrated.

For 23% of patients the parent or guardian reported blood in the stool, and 64% had mucus in their faeces.

The most common associated symptoms were stomach pain, vomiting, loss of appetite, fatigue and fever. Additionally, 38% of patients were reported to have a cough as well.

In 23% of cases the parent or guardian reported that, aside from the initial patient, at least one other member of the child's household was or had very recently suffered from diarrhoea.

Table 3: Clinical presentation of patients 0-5 presenting at OPD with diarrhoea. Assessment of clinical symptoms was not complete for all patients. Percentages are based on results of patients assessed for the specific characteristic.

	N	%	Median	Min	Max
Out-patient	19	19.0%	.	.	.
In-patient	81	81.0%	.	.	.
Total	100	100.0%	.	.	.
Time since onset of diarrhoea (days)	98	.	4.0	1	30
Stools per day	98	.	4.0	1	9
Temperature in °C	93	.	38.7	35.8	40
Signs of dehydration (CDHS)	**91**				
No dehydration (0)	11	12.1%	.	.	.
Some dehydration (1-4)	56	61.5%	.	.	.
Moderate to severe dehydration (5-8)	24	26.4%	.	.	.
All	91		.	.	.
Stool characteristics					
Blood	23	23.2%	.	.	.
Mucus	63	63.6%	.	.	.
Blood and Mucus	15	15.2%	.	.	.
All	99				

	N	%	Median	Min	Max
Reported symptoms					
Stomach pain	49	67.1%	.	.	.
All	73		.	.	.
Vomiting	49	52.1%	.	.	.
All	94		.	.	.
Loss of appetite	65	65.0%	.	.	.
All	100		.	.	.
Fatigue	72	73.5%	.	.	.
All	98		.	.	.
Fever	51	60.7%	.	.	.
All	84		.	.	.
Cough	32	38.1%	.	.	.
All	84		.	.	.
Other symptoms	25	29.8%	.	.	.
All	84		.	.	.
Chronic disease reported	6	6.5%	.	.	.
All	93		.	.	.
Members of the same household afflicted	**95**				
None	73	76.8%	.	.	.
1 or more household members	22	23.2%	.	.	.

For 3 of the patients with a positive RDT for *Cryptosporidium spp.* an HIV test was performed, the other Crypto positive patients were not tested due to the respective parents' objection. All three patients tested were negative.

Antibiotic treatment

Most patients had already received some form of treatment when presenting at the hospitals' OPDs. ORS, antibiotics and various other medication, including anti-motility drugs, were employed.

Table 4 gives an overview of the number of patients that received any of the listed treatment options during the course of their illness.

Table 4: Treatments administered either before or during hospitalization to patients 0-5 years that presented at OPD with diarrhoea. (Total: 100)

Treatment	Antibiotics	Anti-motility drugs	Intravenous fluids	ORS	Zinc
Number of patients	67	15	26	58	1

23 patients (53% out of 43 patients for whom type of antibiotic administered had been noted) had been treated with metronidazole (in various combinations with other antibiotics), which is a widely used antibiotic, that is however not known to

affect bacteria commonly causing diarrhoea and would in this context mainly be used to eradicate *Giardia int.* or *Entamoeba hist.* (Herold, 2016). Regarding infection or carriage of *Giardia int.,* no difference could be observed between patients who had been treated with metronidazole prior to presentation at OPD or those that had not.

Regarding the prescription of antibiotics at the study sites, patients were more likely to receive antibiotic treatment when presenting bloody stool, as required by the WHO guidelines. Nevertheless, most patients, that were treated with antibiotics, had not reported bloody stools.

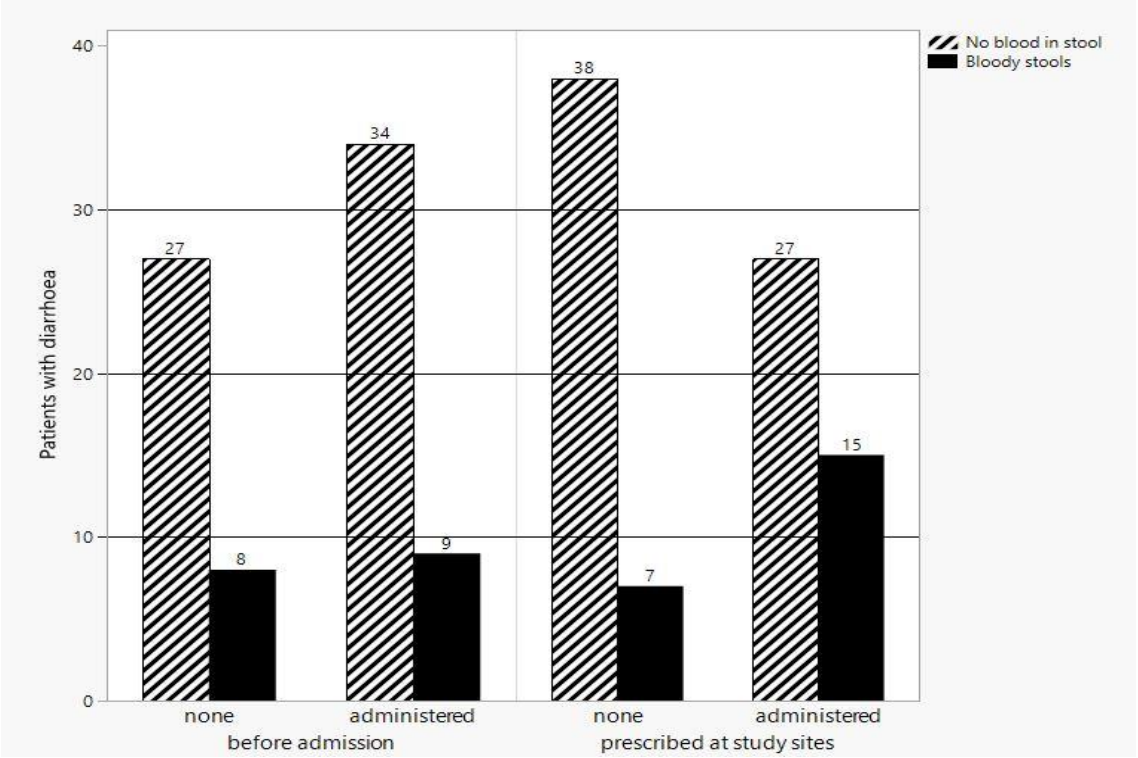

Figure 7: Administration of antibiotics to patients 0-5 years with diarrhoea before and after admission in the OPD broken down by whether bloody stools had been reported or not.

5.2. Pathogens identified

While each sample was tested for *Cryptosporidium spp.* by RDT, not all of the remaining tests could be performed. This was mainly due to an insufficient

amount of the samples provided. The following table presents an overview of the tests conducted (Table 5).

Table 5: Number of samples for which the different tests were conducted. Tests missing were not performed due to an insufficient amount of sample.

	Cryptosporidium RDT		Parasite PCR		Rota-/Adenovirus RDT		Microbiological Culture	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
missing	0	0%	6	6%	16	16%	13	13%
performed	100	100%	94	94%	84	84%	87	87%

Of the 100 samples collected in total, all tests were conducted for only 76 samples. Of the 24 samples, that had not been fully analysed as required by the study protocol, 15 were positive for one or more pathogens and three were clinically diagnosed with measles, which in this study was also viewed as a cause of diarrhoea.

The remaining 6 patients, whose samples had not been fully analysed and for whom no cause had been identified, were excluded from the further analysis in this thesis, leaving 94 patients to be analysed in total. The 18 with one or two tests missing were included in so far as the pathogenic agent identified is further analysed but were excluded when specifically analysing the impact of co-infection with multiple pathogens.

In total, an infectious agent was detected for 61 (65%) of the initial patients.

The most frequently isolated pathogen was *Giardia int.* (20%), directly followed by *Cryptosporidium spp.* in 19% of cases. The third most frequently isolated pathogen was rotavirus with a total of 14 cases (15%).

Other pathogens isolated were *Shigella spp.* (5%), *E. coli* (2%), *Cyclospora cayetanensis* (3%) and one case of adenovirus (1%). Additionally, *Taenia spp.* was identified in 2 samples (2%) and eggs of *Ascaris lumbricoides* were present in one sample (1%).

In 42 of the samples (45%), no pathogen could be identified by the tests conducted as part of this study. Of these 9 patients were however diagnosed with

either malaria or measles at the respective hospitals, which in these cases was assumed to be the cause of diarrhoea. Thus leaving 33 of the participants (35%) for whose symptoms no etiologic agent could be determined.

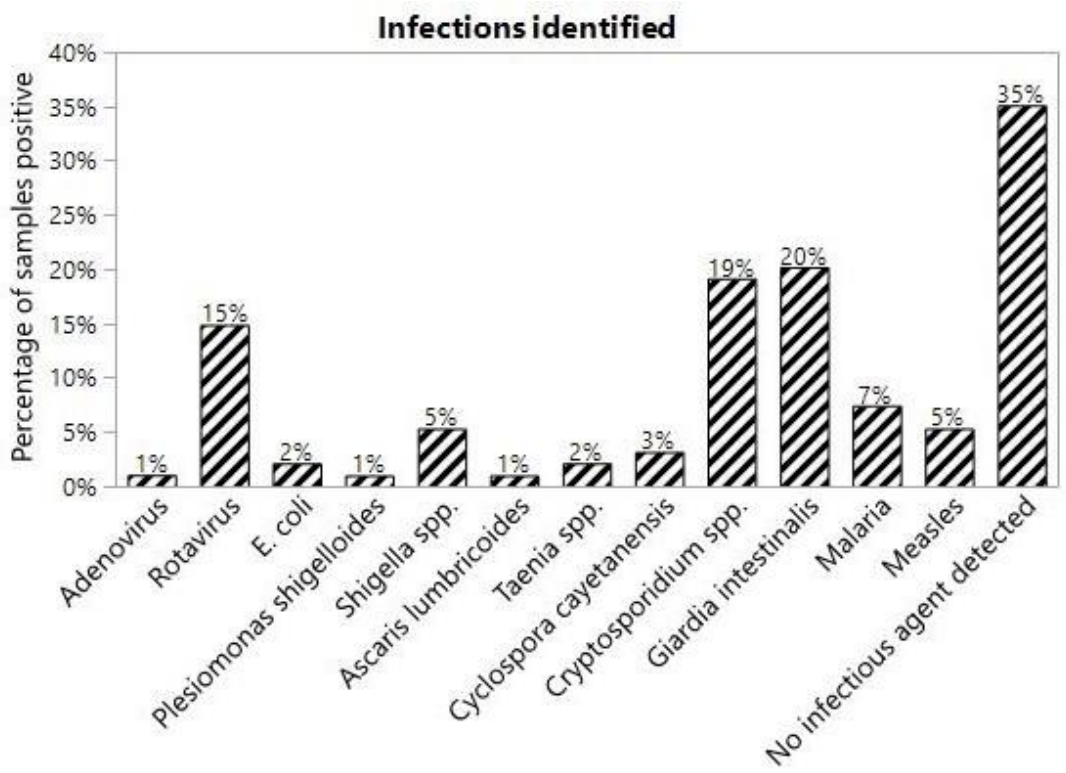

Figure 8: Infectious agents and diseases identified in children 0-5 years presenting at the hospital with diarrhoea.

Co-infections with multiple pathogens were very common as illustrated in Figure 9. 16% of patients were positive for more than one pathogen with *Crypotsporidium spp*. and *Giardia int.* most frequently encountered in the same sample (5% of all samples).

In fact, patients infected with *Cryptosporidium spp.* or *Giardia int.* were more likely to be diagnosed with an additional infectious agent present in the same sample than without (X^2=9.2; p<0.01 and X^2=23.1; p<0.0001, respectively).

Rotavirus showed no association with co-infection (X^2=2.9; p=0.09).

When utilising the Bonferroni correction however, only *Giardia int.*'s association with co-infection remains significant ($p<0.004$).

Odds of these pathogens being isolated with at least one other infectious agent present are displayed in Table 6.

Table 6: Odds of Cryptosporidium spp. and Giardia int. to be present in a case of co-infection. Only the association of Giardia int. is statistically significant when utilising Bonferroni correction for multiple comparisons.

	Co-infection +	Co-infection -	Odds Ratio	Lower 95%	Upper 95%
Cryptosporidium spp.	7/15	7/66	7.4	2	26.6
Giardia int.	10/15	5/66	24.4	6	99.8

Pathogens like *Cyclospora cayetanensis* and *Taenia spp*. were never isolated without another pathogen present.

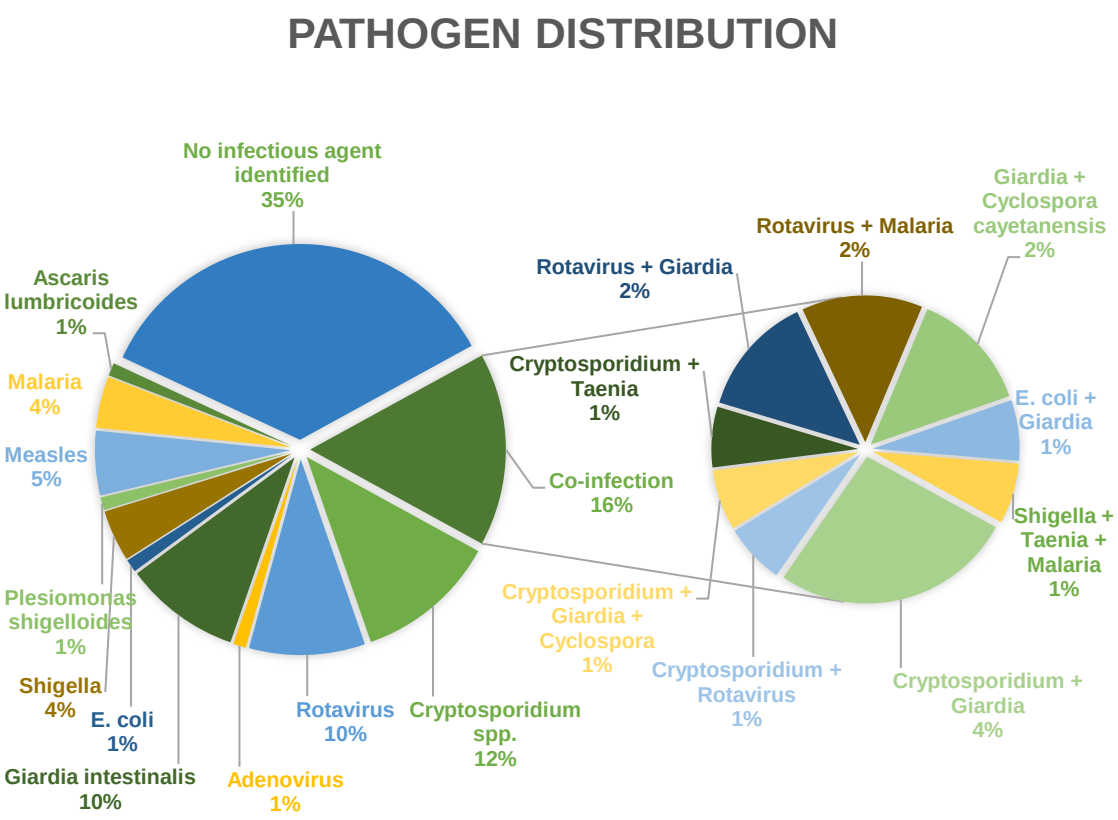

Figure 9: Distribution of mono- and coinfections with the respective infectious agents and diseases identified in children between 0-5 years presenting at the hospital with diarrhoea.

5.3. Explorative analyses

While the small sample size of my pilot study limits the statistical power of further analyses as to correlations in disease manifestations and pathogens, I performed exploratory analyses regarding the spectrum of pathogens identified in different age groups or in patients exhibiting different symptoms.

Age distribution

As illustrated in Table 7, the distribution of identified pathogens differed between age groups. Due to the three age groups not being of similar size, most of these observations are not statistically significant. Nevertheless, for *Cryptosporidium spp.* the odds of a patient being infected were 6.1 in children aged 6 to 24 months as compared to infants (0- 6 months). (CI: [1.1, 114.6]; p=0.04), likely reflecting different levels of exposure (breast feeding versus solid foods).

Table 7: Pathogen distribution by age groups

	Pathogens	N	% of Total
0-6 months (n=19)	*Cryptosporidium spp.*	1	1%
	Giardia intestinalis	3	3%
	Rotavirus	5	5%
	Adenovirus	0	0%
	E. coli	0	0%
	Shigella spp.	0	0%
	Plesiomonas shigelloides	0	0%
	Cyclospora cayetanensis	0	0%
	Taenia spp.	0	0%
	Ascaris lumbricoides	0	0%
	Malaria	0	0%
	Measles	1	1%
	No infectious agent identified	10	11%
6-24 months (n=63)	*Cryptosporidium spp.*	16	17%
	Giardia intestinalis	11	12%
	Rotavirus	9	10%
	Adenovirus	1	1%
	E. coli	1	1%
	Shigella spp.	4	4%
	Plesiomonas shigelloides	1	1%
	Cyclospora cayetanensis	2	2%
	Taenia spp.	1	1%
	Ascaris lumbricoides	1	1%
	Malaria	5	6%
	Measles	3	3%

	Pathogens	N	% of Total
	No infectious agent identified	20	21%
>24 months (n=12)	*Cryptosporidium spp.*	1	1%
	Giardia intestinalis	5	5%
	Rotavirus	0	0%
	Adenovirus	0	0%
	E. coli	1	1%
	Shigella spp.	1	1%
	Plesiomonas shigelloides	0	0%
	Cyclospora cayetanensis	1	1%
	Taenia spp.	1	1%
	Ascaris lumbricoides	0	0%
	Malaria	2	2%
	Measles	1	1%
	No infectious agent identified	3	3%

For *Giardia int.* and rotavirus no statistically significant odds could be determined but nevertheless a certain trend as illustrated in Figure 10 can be distinguished.

For infection with *Giardia int.* odds were highest for group 3 (older than 24 months) with 3.8 (CI: [0.7, 23.2]; p>0.05) compared to group 1 (0-6 months) and 3.4 (CI: [0.9, 12.7]; p>0.05) compared to group 2 (6 to 24 months).

For rotavirus the odds of infection in the first age group (0-6 months) were 2.1 (CI: [0.6, 7.3]; p>0.05) as compared to the second age group (6 to 24 months).

The odds of no infectious agent being identified were higher in the first age group (0-6 months) compared to the second (6 to 24 months) and third (>24 months) groups, with 2.4 (CI: [0.8, 6.9]; p>0.05) and 3.3 (CI: [0.7, 18.8]; p>0.05) respectively.

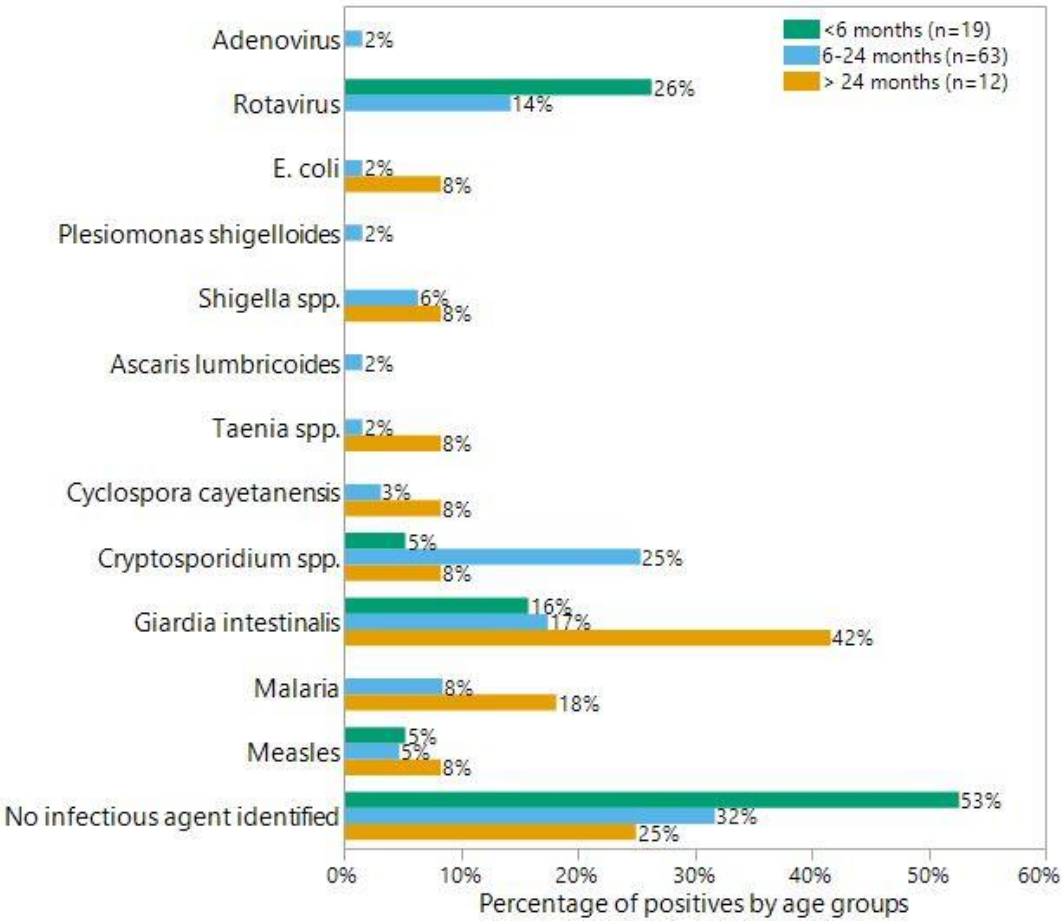

Figure 10: Distribution of pathogens in the different age groups of children between 0-5 years presenting at the hospital with diarrhoea.

Malnutrition

No significant difference in the spectrum of infectious agents could be observed between chronically malnourished patients and those with a healthy weight-for-age ratio. Children had been grouped into three groups by the weight-for-age percentiles determined (above 50%, between 50 and 20%, below 20%), but no significant correlations of infectious agents identified could be determined (X^2=4.6; p=0.2).

Symptoms

As illustrated in Figure 11 the various symptoms exhibited by patients in addition to diarrhoea varied depending on which pathogens were identified. Symptoms

mainly recorded were those generally associated with gastro-intestinal infections like stomach pain, vomiting, loss of appetite, fatigue and fever, however since a substantial number of patients (38%) had also been reported to experience an involvement of the respiratory tract (coughing) (Table 3) and infections with rotavirus are reported to involve the respiratory tract in up to 50% of cases, as well as the possibility of patients developing a form of respiratory *cryptosporidiosis* (Mor et al., 2010; Sponseller et al., 2014), I decided to include coughing as a symptom in this analysis.

Nearly all symptoms could be found with each of the respective pathogens, excepting *Shigella spp.* (which was not found in patients with cough). All patients diagnosed with measles had run a fever (X^2=5; p=0.03), which was expected since this symptom is one of the diagnostic criteria (Herold, 2016), and an infection with rotavirus was positively associated with vomiting (X^2=10.6; p=0.001).

Infection with *Giardia int.* was negatively associated with vomiting (X^2=3.9; p=0.049) and positively associated with coughing (X^2=5; p=0.02).

When using the Bonferroni correction, the required p-level for statistical significance changes to p<0.004, leaving only the positive association of rotavirus with vomiting as statistically significant.

Table 8: Odds of patients infected with rotavirus or Giardia int. reporting vomiting.

	Vomiting **+**	Vomiting **-**	**Odds Ratio**	**Lower 95%**	**Upper 95%**
Rotavirus	12/48	1/41	13.3	1.7	107.7
Giardia int.	6/48	12/41	0.3	0.1	1

Table 9: Odds of patients infected with Giardia int. reporting coughing.

	Cough **+**	Cough **-**	**Odds Ratio**	**Lower 95%**	**Upper 95%**
Giardia int.	9/30	5/50	3.9	1.1	12.9

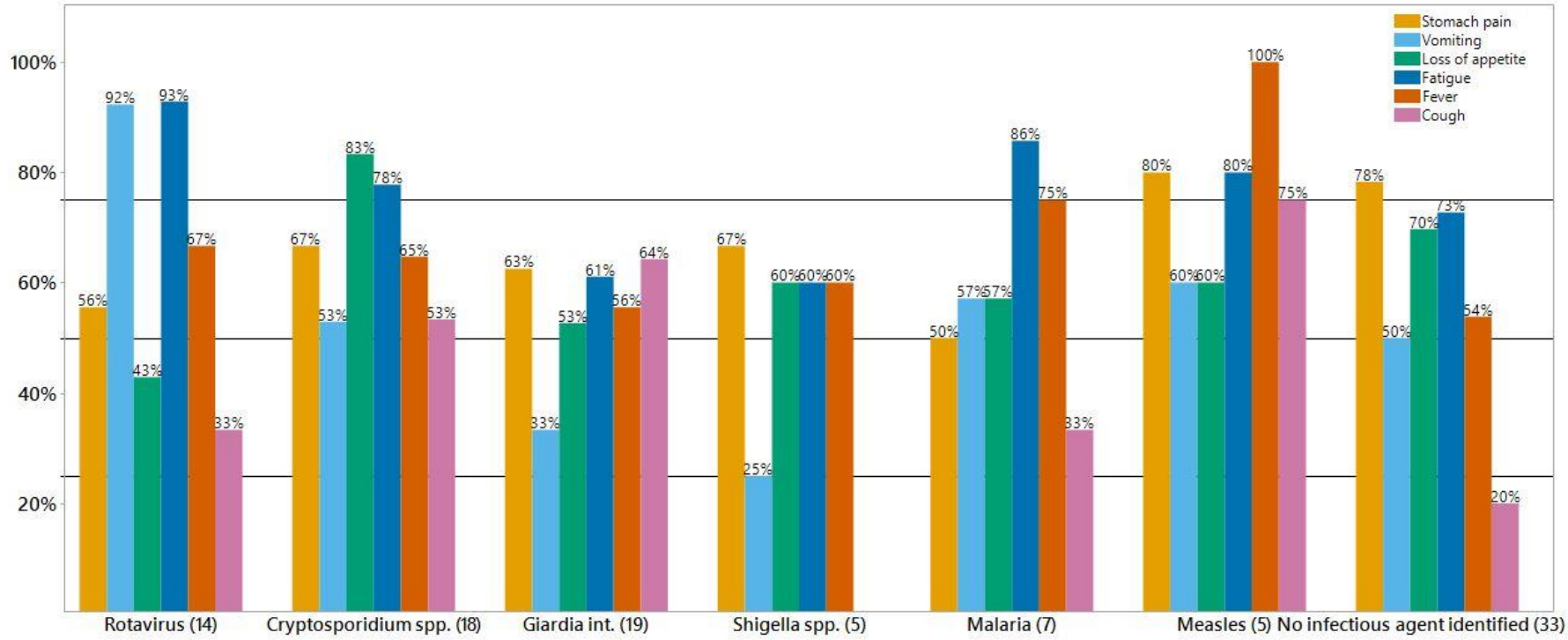

Figure 11: Distribution of symptoms exhibited by infections identified in children 0-5 years presenting at the hospital with diarrhoea. Pathogens that had been found in less than 5 cases were excluded from analysis.

When excluding cases of co-infection, the association previously shown for infection with rotavirus remains the same (X^2=10.6; p=0.001), additionally an association with fatigue can be shown (X^2=5.1; p=0.02; p<0.005 required after Bonferroni correction).

Odds ratios could not be calculated due to all nine cases of infection with rotavirus presenting with the respective symptom, leading to a cell count of zero for those not infected.

Since less than 5 cases of mono-infection with *Shigella spp.* and malaria had been recorded, these infections were not further analysed. Moreover 3 out of the 5 cases diagnosed with measles had not been fully analysed as required by the study protocol, therefore measles was also excluded from the further analysis in this case.

While not statistically significant in this population, a higher percentage of patients were reported to experience stomach pain and vomiting when only *Giardia int.* (80%) was present than in all cases of infection with this pathogen (63% and 33% respectively). The same can be observed for vomiting in cases of mono-infection with *Cryptosporidium spp.* (83%) and in combined analysis of co- and mono-infection (53%).

In reverse, mono-infection with *Giardia int.* showed a lesser percentage of patients experiencing a loss of appetite (20%) as opposed to the combined analysis (53%).

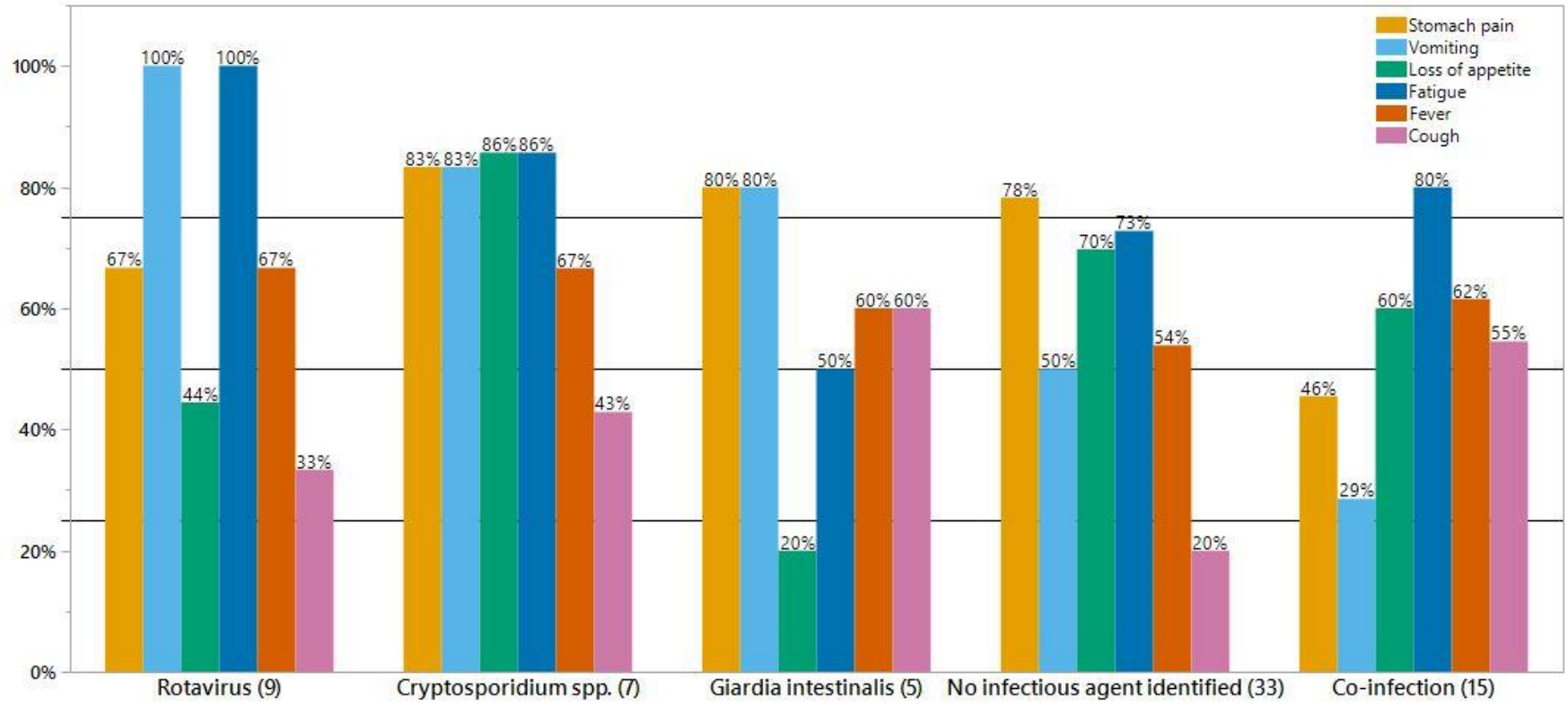

Figure 12: Symptoms by infections with a single pathogen identified and infections with multiple pathogens in children 0-5 years presenting at the hospital with diarrhoea. Only pathogens that had been identified in at least 5 cases were included. Cases where not all tests were performed were excluded from the analysis of specific pathogens. Those where more than one pathogen had been identified were however included in the group with co-infections.

Regarding the excretion of blood or mucus with the patients' stool, no clear association could be found with any specific pathogen. Infection with Cryptosporidium or rotavirus showed a negative correlation with the excretion of bloody stools (X^2=5.1; p=0.02 and X^2=8.3; p<0.01 respectively). Additionally, bloody stools were less likely to be found in cases of co-infection (X^2=9; p<0.01). Bloody stools were more likely to be reported when no infectious agent could be identified (X^2=10.5; p=0.001). After correcting with the Bonferroni correction, this leaves co-infection negatively and cases with no infectious agent identified associated with bloody stools. (Required p after correction p<0.004)

Disease severity

Patients were examined for signs of dehydration on admission to the study. This led to a classification of the severity of dehydration into the categories no dehydration, some dehydration, moderate to severe dehydration. No significant correlation between pathogens and severity of dehydration could be observed.

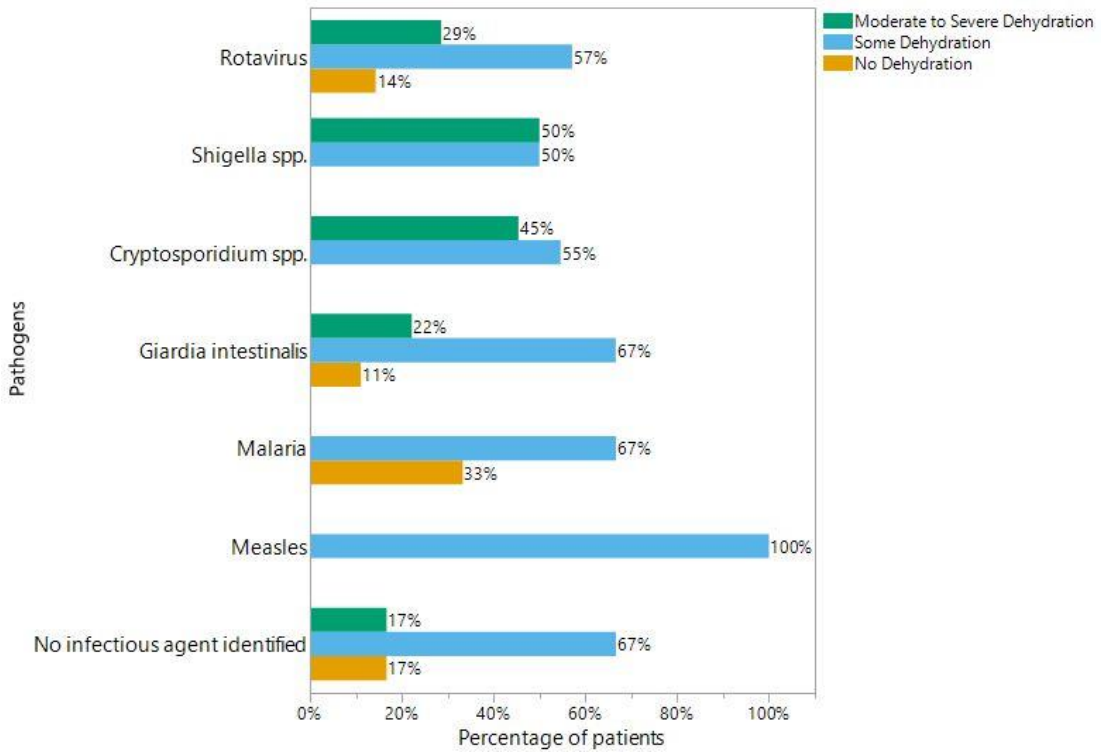

Figure 13: Severity of dehydration recorded in patients 0-5 years presenting at the hospital with diarrhoea by pathogens identified.

Seasonality

Disease variability due to seasonal differences is often reported. Overall 56 % of patients were included during rainy season as opposed to 44 % during dry season (representing 66% and 33% of the study period, respectively).

In this study the only seasonal difference could be noted for *Giardia int.* mono-infection which was more likely during the dry season (X^2=10.9; p=0.001). When including patients with an additional pathogen, the results are similar with X^2=7.1 and p<0.01, although not statistically significant due to Bonferroni correction (p<0.0038).

While the cases of malaria, which is generally agreed to be linked to mosquitoes thriving in rainy season, included in this study do not show a statistically significant difference between seasons, the fact that the majority of cases recorded in study patients (86%), were recorded during rainy season, can be seen as a positive validation of the study results.

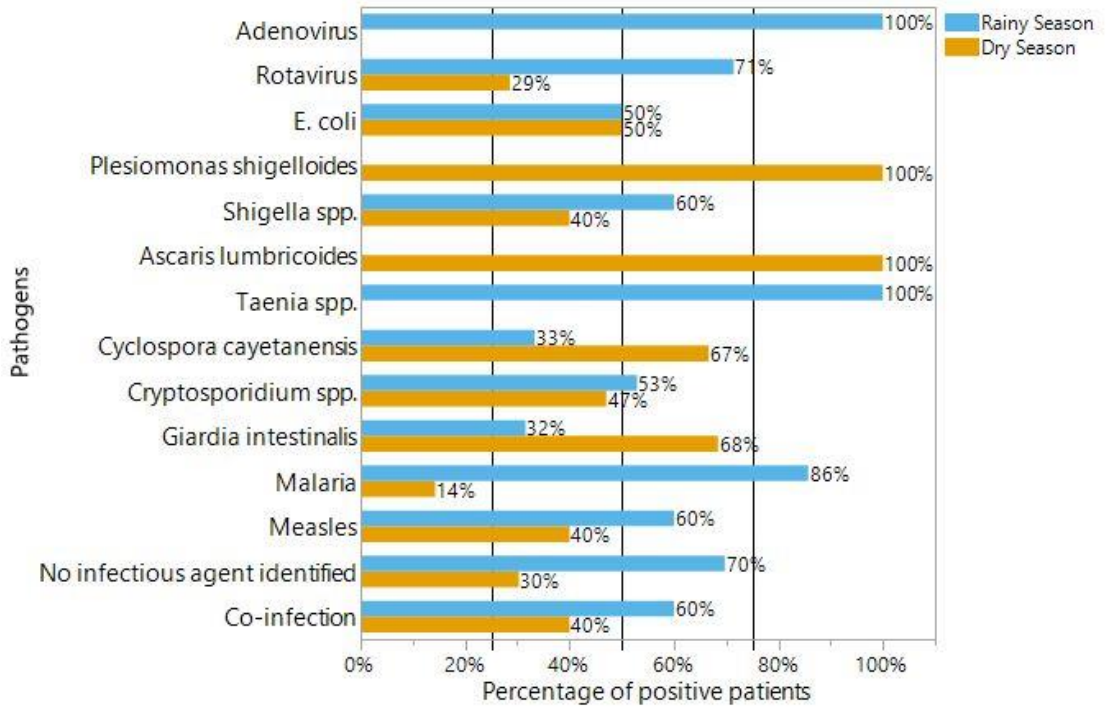

Figure 14: Distribution of pathogens identified in children 0-5 years presenting at the hospital with diarrhoea across seasons.

5.4. RDT Validation

Initially, 23 patients had a positive RDT for *Cryptosporidium spp..* Of these only 16 turned out positive on PCR. Additionally, 2 patients with a negative RDT were later found to be positive on PCR. Only the cases with a positive PCR were considered positive in my analyses. Which leaves a total of 18 cases of infection with *Cryptosporidium spp*. detected in this study.

This discrepancy between the RDT and PCR results led me to evaluate the RDT's accuracy in the detection of *Cryptosporidium spp.* oocysts as compared to PCR.

This leads to a sensitivity of 88.9% (CI: [0.7; 1]), a positive predictive value of 69.6% (CI: [0.5; 0.9]) and a specificity of 90.8% (CI: [0.8; 0.97]), with a negative predictive value of 97.2% (CI: [0.9; 1]).

Table 10: Comparison of RDT and PCR results. For 6 samples PCR could not be conducted due to insufficient quantity of the sample provided.

	Crypto PCR					
	Positive		Negative		All	
Crypto RDT	**N**	**%**	**N**	**%**	**N**	**%**
Positive	16	17%	7	7%	23	25%
Negative	2	2%	69	73%	71	76%
All	18	19%	76	81%	94	100%

The data analysed here is part of the data used by Manouana et al., (2020) to evaluate the performance of the RDT used in the CRYPTO multi-centre study.

6. Discussion

6.1. Study findings

Pathogen spectrum

This study confirms some of the conclusions drawn from recent multi-centric studies (Kotloff et al., 2013; Platts-Mills et al., 2015) highlighting that the spectrum of pathogens responsible for infantile diarrhoea is much more diverse than what had been assumed during the last few decades. The 18 cases of infection with *Cryptosporidium spp.* are the first recorded in Lambaréné, Gabon. The numbers are consistent with the estimate by Checkley et al. (2015) that around 15 - 25% of children with diarrhoea are infected by *Cryptosporidium spp.,* but are not officially diagnosed because no adequate testing is performed. This may serve to raise local physicians' awareness of this very important pathogen and in general to broaden the range of pathogens taken into consideration when deciding the further course of treatment and diagnostics applied.

Plesiomonas shigelloides was initially not included in the spectrum of pathogens sought in our study and the identification of this bacterium in one sample was therefore unexpected. There have however been several reports in the last years linking it to diarrhoeal outbreaks in Southeast Asia and Africa (Janda et al., 2016). Our finding may serve to further confirm this pathogen's role in diarrhoeal disease.

The high rate of co-infections, 16% in this study as seen in Figure 9, is consistent with other studies' findings (Kotloff et al., 2013; Stockmann et al., 2017; Zhang et al., 2016). How this affects the patients compared to mono-infection cannot be evaluated due to the small numbers in this study, nevertheless, it can be seen as a further incentive to focus more on the actual patho-mechanisms and possible pathogen interaction, which may lead to different disease outcomes.

The role of *Giardia int.* in diarrhoeal disease is not altogether clear. *Giardia int.* is often associated with traveller's diarrhoea (Valls et al., 2014), but some studies even suggest that it may be protective against diarrhoeal disease (Breurec et al., 2016; Kotloff et al., 2013; Nhampossa et al., 2015). Our study showed a high

prevalence, but since *Giardia int.* is not viewed as an obligatory pathogen, it cannot be determined whether it was a causative agent of diarrhoea or not.

Other pathogens such as *Taenia spp.* and *Ascaris lumbricoides* are not clearly associated with diarrhoea, infection does however usually occur through contaminated food. The less than ideal hygiene practices leading to this infection may have also caused infection with or heightened susceptibility to another pathogenic agent that then ultimately caused diarrhoea. In the two cases where *Taenia spp.* were present, this would be *Cryptosporidium spp.* and *Shigella spp..*

Treatment observations

The observation that many of the children were treated with antibiotics prior to hospitalisation and that many then received antibiotic treatment from the doctors at the study sites regardless of reporting blood in their stool, is worrisome.

As outlined in the WHO guidelines on treatment of acute diarrhoea (World Health Organization (WHO), 2013b), antibiotics should only be administered in cases where there is a high likelihood of infection with *Shigella spp.,* cholera or a severe systemic infection, and not generally to avoid destroying even more of the patient's healthy microbiome, which should eventually help the gut recover from the infection.

Furthermore, the very generous administration of metronidazole is problematic seeing as it is ineffective against *Shigella spp.* (World Health Organization (WHO), 2005) and would normally only be administered to clear an infection with *Giardia int.*. However, those patients treated with metronidazole were not found to be diagnosed more or less often with *Giardia int.*. Whether this is simply due to the parasite having been already cleared or the drug being administered when no infection had taken place or improper administration cannot be concluded in this setup.

Explorative analyses

The varying distribution of pathogens depending on the age of the patients has also been observed in other studies (Gasparinho et al., 2016). As demonstrated in Figure 10, most cases of *Cryptosporidium spp.* were recorded in children

between 6 and 24 months. This ties in with findings from another study conducted in Libreville where the prevalence of infection with *Cryptospordium spp.* was highest between 6 and 18 months (Duong et al., 1995, 1991). Rotavirus was more prevalent in children below 6 months and *Giardia int.* was mainly found in children older than 2 years. Since *Giardia int.* is not viewed as an obligatory pathogen, the higher prevalence in older children may be due to longer exposure to contaminated water and subsequent asymptomatic carriage.

The reason why some pathogens are more prevalent in certain age groups cannot be determined. It may be due to the youngest children still receiving a much narrower diet or not being able to move at their own volition thus limiting their exposure to certain things or people, that may be the source of infection. The fact that rates of infection drop again once children reach 2 years of age suggests a certain level of immunity is established to enteric pathogens in general throughout the first few years of life.

Direct correlations between pathogens and disease severity (measured here through the CDHS) could not be shown. Rotavirus was positively associated with vomiting, but apart from the clear association of measles with fever, which can be viewed as a positive control seeing as fever is one of the illness defining symptoms (Herold, 2016), no other statistically significant associations could be shown (Figure 11 and Figure 12).

Cryptosporidium spp. and rotavirus were negatively associated with the presentation of bloody stools. Since the total number of the other pathogens was too small, it was not possible to find any statistically significant association between them and certain symptoms. It would have for example been probable to expect a positive association between shigellosis and bloody stools (Herold, 2016), but with only 5 cases identified it was not possible to draw any conclusions.

6.2. Further studies in Gabon and Central Africa

In the early 1990s two studies were already conducted in Gabon on the epidemiology of *Cryptosporidium spp..* The studies found a rate of 3.1% in children presenting at the hospital for various reasons, not limited to diarrhoea, and 24% in children aged between 0 to 2 years whose main complaint was diarrhoea (Duong et al., 1995, 1991). Patients' immune status was only in so far evaluated as malnutrition was recorded, which was in this study correlated with higher rates of infestation with *Cryptosporidium spp.*. This finding could not be replicated with the data at hand, possibly due to the smaller sample size evaluated.

The studies by Duong et al. were conducted mainly in Libreville, making this study the first survey in Lambaréné, which presents climatic and socio-demographic differences. Surprisingly, at the time there was no direct follow-up of this study, considering that cryptosporidiosis was still considered a mainly opportunistic infection. This may be partly due to the difficulty of accurately diagnosing an infection with *Cryptosporidium spp.*. The advancement in diagnostic methods leading to the creation of simple to use RDTs, like the one employed in this study, have most certainly led to a higher feasibility of conducting studies on this parasite.

Other studies conducted in Gabon focused either on bacterial or viral causes of diarrhoea (Koko et al., 2013; Lekana-Douki et al., 2015).

As far as the general aetiology of infantile diarrhoea is concerned, there is only one study using a case-control approach from the Central African Republic available (Breurec et al., 2016). In this study rotavirus was identified as the main pathological agent, followed by *Shigella spp..* Third place was shared by *Cryptosporidium spp.,* astro- and norovirus.

This scarcity of comparable regional data further highlights the need for a concerted effort to better survey the aetiology of childhood diarrhoea in this region, for without a better knowledge of the causes any effort to better prevent and treat diarrhoea can be likened to groping in the dark.

6.3. Study limitations

Now as for limitations of this study, there is of course the relatively small sample size and descriptive nature to consider, making it difficult to draw many statistically significant conclusions on the epidemiology of infantile diarrhoea and any treatment given to the subjects at the OPD. Furthermore, the study did not include any kind of follow up, which prevents me from ascertaining any long-term effects or mortality rates and also means that any symptoms occurring later on were not taken into account.

Additionally, due to the study lacking a healthy control group it can only be surmised that pathogens identified in the participants' faeces were the causative agents of the symptoms they presented on admission. This assumption is based on the viruses, bacteria and protozoa in question having been shown to be enteropathogenic in other studies. However, newer studies in this field have shown that the simple presence of a pathogen does not necessarily lead to disease (Sultana et al., 2017). The mechanisms involved are far more complex. It is possible that the patients in this study, especially those infected with *Cryptosporidium spp.*, had an underlying cause of immunosuppression, e.g. malaria (possibly asymptomatic), HIV or any hereditary cause of immune-deficiency, that made them prone to infection with any of the infectious agents identified, which makes these so called "by-stander" infections and not the direct cause of illness.

Many recent case-control studies found a high number of pathogenic agents in the healthy control group, albeit often in lesser quantity than in the symptomatic participants (Breurec et al., 2016; Platts-Mills et al., 2014). These findings contradict "Koch's postulates" on causality, which require a pathogen to cause disease when introduced into a healthy organism (Sultana et al., 2017). These postulates however contain several deficiencies. For example, they discount asymptomatic infection, which has been shown in a number of infectious diseases, e.g. HIV and tuberculosis (Inglis, 2007). Newer approaches, e.g. the Bradford Hill criteria (Hill, 1965), have been established that consider the more

complex mechanisms involved, but nevertheless the role of certain infectious agents, particularly *Giardia int.*, in diarrhoea has not been clearly defined.

Pathogen interaction

A study conducted in China found no positive association of bacterial pathogens, except for *Shigella spp.*, with diarrhoea, while remarking that co-infection of certain bacteria and rota- or norovirus always led to symptomatic illness (Li et al., 2016). It has been suggested that an interaction of viruses and bacteria is needed to make the gut susceptible to infection, this does however require further studies for confirmation (Miura et al., 2013). The common occurrence of more than one parasite Figure 9 may also indicate a certain level of interaction between the respective pathogens, however, research in this field remains scarce.

Considering the complex interactions of the gut's microbiome, host immune system and these entero-pathogens, the pathogens isolated in this study can only be seen as possible causes of the symptoms presented.

Diagnostic methods

Another limitation involves the diagnostic methods used. While bacterial culture is a very sensitive diagnostic method and RDTs do produce relatively reliable results, molecular diagnostic methods such as PCR may lead to even more accurate diagnoses, as has been demonstrated in other studies (J. Liu et al., 2016). As can be seen in our comparison of the RDT and PCR for *Cryptosporidium spp.* (Table 10), PCR is still the more specific method and relying only on RDTs will not achieve the same measure of reliability in the data collected.

Following the old adage "seek and ye shall find", it is probable that a reanalysis of all of the patients' samples using molecular techniques would lead to even higher rates of pathogens identified, as has been demonstrated by J. Liu et al., (2016) in a re-evaluation of the GEMS multi-centre study. Especially for the bacterial pathogens, a PCR might show a completely different number of positive samples. The high number of patients taking antibiotics before we could collect a

stool sample may have also hampered the accurate diagnosis in several cases, by preventing bacterial growth in the laboratory.

The addition of further pathogens to the analysis could also lead to a more differentiated picture. For example, adding sapovirus, astrovirus and norovirus, as well as *Campylobacter jej.,* would be reasonable when considering other studies' findings (Kotloff et al., 2013; Lekana-Douki et al., 2015; J. Liu et al., 2016).

Socio-demographic determinants

A possible cause of bias in this study is that only children presenting at one of the study hospitals were considered for inclusion. While difficult to organize differently, a broader community screening could have been helpful to augment the number of inclusions and prevented the possible bias of the study towards children from somewhat wealthier families and more severe cases of diarrhoea. As in many countries not only in the developing world, a child suffering from diarrhoea would not necessarily be brought to the next healthcare facility. While having long been shown to be associated with any number of negative effects on child development and not seldomly proving to be fatal to young children, diarrhoea is still very often seen as an illness that can be very well managed without the assistance of medical personnel and children affected would therefore not be seen at a hospital, especially if the family is financially weak to begin with.

6.4. Implications and further research

This study's findings reaffirm the necessity of further diagnostic tests to distinguish the actual causes of diarrhoea, seeing as the symptoms exhibited are diverse and not usually pathogen-specific (Figure 11 and Figure 12). Since diarrhoea is usually treated symptomatically, exact diagnosis of the pathogen responsible may seem unnecessary, overly time-consuming and expensive, especially in low-resource settings like Gabon, yet a better understanding of the underlying causes may pave the way for better preventive measures and targeted treatments.

One preventive option already available is the rotavirus vaccine, which has been available since 2006 and is recommended as a routine vaccination for children from 2 to 8 months of age by the WHO (World Health Organization (WHO), 2013a). This could already be implemented following this study, seeing as rotavirus ranked third in the list of pathogens identified in this study (Figure 8) and thus implies that a significant proportion of patients would have benefitted from early vaccination. The results presented here could function as a baseline when evaluating the efficacy should a national vaccination programme be implemented in the following years.

Water and sanitation hygiene (WASH)

The high coincidence of *Giardia* and *Cryptosporidium* (Figure 9) gives rise to the suspicion of high levels of water contamination with both parasites, seeing as infection with the former has been mostly linked to contaminated water sources and the latter has been known to cause water-borne outbreaks worldwide (Efstratiou et al., 2017; Fayer, 2004).

A further step following this study would be to examine the water sources in and around Lambaréné. Especially considering the amount of co-infections with pathogens that are known or at least suspected to be sometimes transmitted through contaminated water, it could prove quite informing to see whether the respective pathogens can be found in common water sources and to see if better sanitation systems might lower the incidence of diarrhoea in the respective area.

The unsolved enigma

Diarrhoeal disease still ranks fifth in the global causes of Years of Life Lost for all age groups in 2017 (as opposed to third in 1990) (Roth et al., 2018) and fourth as the most frequent cause of death in children and adolescents globally in 2015, taking third place when only considering countries with a low socio-demographic index/SDI (Roba et al., 2017). Compared to other major causes of morbidity and mortality the future research targets are less tangible. The direct causes of pneumonia, neonatal deaths, meningitis, malaria are easier to determine and therefore easier to address. Considering the obvious need to combat diarrhoeal

disease, it is shocking that this study is one of few conducted in Gabon on this matter, and more importantly the first of its kind in Lambaréné and its surroundings. Most studies in this area are still focused on malaria and tuberculosis. Illnesses that undisputedly cause high rates of morbidity and mortality but are all the same not the sole healthcare issue to be tackled.

More comprehensive studies on the epidemiology of diarrhoea are needed, not only in children, but also in adults to determine if and why there is a different range of pathogens responsible. Rotavirus for example has long been known to mainly affect children, leaving them ostensibly immune in the aftermath. The high prevalence of infection with *Cryptosporidium spp.* in young children and the subsequent findings of many asymptomatic carriers in adults, may indicate a similar immune reaction. However, immunity towards parasites, with the partial immunity against malaria parasites in countries with high endemicity as its most prominent example, is still not well understood and leaves many questions. A better understanding of the mechanisms involved may provide new ideas on how to treat and maybe even prevent infection with *Cryptosporidium* and possibly even other parasites in the future.

7. Summary

Background

Diarrhoeal disease causes more than half a million deaths of children below five years of age each year, making it the third most frequent cause of child mortality worldwide. While much has been achieved over the last few years, there is still little known of the exact epidemiology of diarrheagenic pathogens due to mostly symptomatic treatment and subsequently infrequent testing for said pathogens. Recent studies have identified a different spectrum of pathogens than what had formerly been presumed to be responsible for most cases of diarrhoea in children (Kotloff et al., 2013).

Methods

Patients aged between 0 and 5 years of age presenting at the OPD of either one of the two hospitals in Lambaréné, Gabon were included, a clinical examination conducted, and a stool sample collected. Samples were then analysed by RDTs for Rota-/Adenovirus and *Cryptosporidium spp.*, as well as Microbiological culture, wet mound technique and Multiplex-PCR for *Cryptosporidium spp.*, *Giardia int.* and *Cyclospora cayetanensis*.

Results

Of the 94 samples included in the final analysis, *Giardia int.* was identified in 19 samples (20%), *Cryptosporidium spp.* in 18 (19%), rotavirus in 14 (15%), *Shigella spp.* in 5 (5%), *E.coli* in 4 (4%), *Cyclospora cayetanensis* in 3 (3%), *Taenia spp.* in 2 (2%) and adenovirus, *Ascaris lumbricoides* and *Plesiomonas shigelloides* in 1 sample (1%) respectively. 7 patients were found to be infected with malaria (7%) and 5 had measles (5%). 15 patients (16%) had more than one pathogen or were additionally diagnosed with either malaria or measles. No infectious agent could be identified for 33 patients (35%). No significant differences between infections identified and symptoms exhibited or incidence across seasons could be observed. But a significant difference in incidence was observed for *Cryptosporidium spp., Giardia int.* and rotavirus across different age groups.

As a side project a RDT for *Cryptosporidium spp.* was evaluated in comparison to Multiplex-PCR. This yielded a sensitivity of 88.9%, and specificity of 90.8%.

Conclusion

The findings of this study may be used as baseline data for further studies in this area, e.g., as a reference should the rotavirus vaccine be implemented to the national vaccination programme in Gabon, as recommended by the WHO. Further research needs to be conducted on transmission of different parasites especially *Cryptosporidium spp.* to implement adequate preventive measures in the future. Additionally, the role of *Giardia int.* in childhood development and childhood diarrhoea has to be further examined.

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9. German Summary

Hintergrund

Weltweit sterben jedes Jahr mehr als eine halbe Millionen Kinder unter 5 Jahren aufgrund von Durchfallerkrankungen, welche damit den dritten Platz als Grund von Kindersterblichkeit belegen. Obwohl in den letzten Jahren im Hinblick auf die Behandlung dieser Kinder viel erreicht wurde, weiß man immer noch viel zu wenig über die genaue Epidemiologie der Pathogene, die Durchfall auslösen, da Patienten meist nur symptomatisch behandelt werden und dementsprechend nicht flächendeckend auf diese getestet werden. In neueren Studien wurde ein vollkommen neues Spektrum an Pathogenen, als bisher angenommen identifiziert, das in den meisten Fällen Durchfall in Kindern auslöst (Kotloff et al., 2013).

Methoden

Es wurden Patienten zwischen 0 und 5 Jahren in die Studie eingeschlossen, die sich in der Ambulanz eines der zwei Krankenhäuser in Lambaréné, Gabun vorstellten. Es wurde eine klinische Untersuchung durchgeführt, ein Fragebogen mit den Eltern oder Sorgeberechtigten ausgefüllt und eine Stuhlprobe genommen. Die Proben wurden dann mittels Schnelltests für Rota-/Adenovirus und *Cryptosporidium spp.*, Mikrobiologischer Kultur, Nativpräparat und Multiplex-PCR auf *Cryptosporidium spp.*, *Giardia int.* und *Cyclospora cayetanensis* untersucht.

Ergebnisse

94 Proben wurden abschließend ausgewertet. Dabei wurde in 19 (20%) *Giardia int.* gefunden, *Cryptosporidium spp.* in 18 (19%), Rotavirus in 14 (15%), *Shigella spp.* in 5 (5%), *E.coli* in 4 (4%), *Cyclospora cayetanensis* in drei (3%), *Taenia spp.* in zwei (2%), sowie Adenovirus, *Ascaris lumbricoides* und *Plesiomonas shigelloides* in je einer Probe (1%). 7 Patienten waren mit Malaria (7%) infiziert und 5 hatten Masern (5%). 15 Patienten (16%) wiesen mehr als einen Krankheitserreger in der Stuhlprobe auf oder wurden zusätzlich mit Malaria oder Masern diagnostiziert. Kein Krankheitserreger oder eine spezifische Erkrankung

konnte bei 33 Patienten (35%) nachgewiesen werden. Es konnte kein signifikanter Unterschied der verschiedenen Krankheitserreger bezüglich der hervorgerufenen Symptome oder der Inzidenz nach Jahreszeiten gezeigt werden. Es konnte aber ein signifikanter Unterschied in der Inzidenz von *Cryptosporidium spp., Giardia int.* und Rotavirus je nach Altersgruppe beobachtet werden.

Als Nebenprojekt wurde ein Schnelltest für *Cryptosporidium spp.* im Vergleich zur Multiplex-PCR evaluiert, was eine Sensitivität von 88.9%, und Spezifizität von 90.8% ergab.

Schlussfolgerung

Die Ergebnisse dieser Studie können als Grundlage für weitere Studien in diesem Bereich dienen, z.B., als Referenz falls die von der WHO empfohlene Impfung gegen Rotavirus in das gabunische nationale Impfprogramm aufgenommen werden sollte. Weitere Forschung ist nötig, was die Transmission der verschiedenen Parasiten, vor allem *Cryptosporidium spp.,* angeht, damit in der Zukunft adäquate Präventionsmaßnahmen durchgeführt werden können. Außerdem muss die Rolle von *Giardia int.,* vor allem im Hinblick auf die Entwicklung im Kindesalter, sowie dessen genaue Rolle bei Durchfallerkrankungen im Kindesalter weiter erforscht werden.

10. Veröffentlichungen

Teile der vorliegenden Dissertationsschrift wurden bereits in den folgenden Publikationen veröffentlicht:

Manouana GP, Byrne N, Mbong Ngwese M, Nguema Moure A, Hofmann P, Bingoulou Matsougou G, Lotola Mougeni F, Nnoh Dansou E, Agbanrin MD, Mapikou Gouleu CS, Ategbo S, Zinsou JF, Adegbite BR, Edoa JR, Kremsner PG, Mordmüller B, Eibach D, McCall M, Abraham A, Borrmann S, Adegnika AA. Prevalence of Pathogens in Young Children Presenting to Hospital with Diarrhea from Lambaréné, Gabon. Am J Trop Med Hyg. 2021 Jul 7;105(1):254-260. doi: 10.4269/ajtmh.20-1290. PMID: 34232911; PMCID: PMC8274774.

Manouana GP, Lorenz E, Mbong Ngwese M, Nguema Moure PA, Maiga Ascofaré O, Akenten CW, Amuasi J, Rakotozandrindrainy N, Rakotozandrindrainy R, Mbwana J, Lusingu J, Byrne N, Melhem S, Zinsou JF, Adegbite RB, Hogan B, Winter D, May J, Kremsner PG, Borrmann S, Eibach D, Adegnika AA. Performance of a rapid diagnostic test for the detection of Cryptosporidium spp. in African children admitted to hospital with diarrhea. PLoS Negl Trop Dis. 2020 Jul 13;14(7):e0008448. doi: 10.1371/journal.pntd.0008448. PMID: 32658930; PMCID: PMC7377516.

Krumkamp R, Aldrich C, Maiga-Ascofare O, Mbwana J, Rakotozandrindrainy N, Borrmann S, Caccio SM, Rakotozandrindrainy R, Adegnika AA, Lusingu JPA, Amuasi J, May J, Eibach D; CRYPTO Study Group. Transmission of Cryptosporidium Species Among Human and Animal Local Contact Networks in Sub-Saharan Africa: A Multicountry Study. Clin Infect Dis. 2021 Apr 26;72(8):1358-1366. doi: 10.1093/cid/ciaa223. PMID: 32150243; PMCID: PMC8075035.

11. Erklärung zum Eigenanteil

Die Arbeit wurde am Centre de Recherches Médicales de Lambaréné (CERMEL) unter der Schirmherrschaft des Instituts für Tropenmedizin, Reisemedizin, Humanparasitologie (ITM) Tübingen, betreut von Professor Dr. Steffen Borrmann, durchgeführt.

Die Konzeption der Studie erfolgte durch mich in Zusammenarbeit mit Prof. Dr. Steffen Borrmann und Prof. Dr. Ayola Akim Adegnika in Rücksprache mit dem Studienteam.

Die Probanden wurden eigenständig durch mich oder durch Frau Danny Adèle Nguema, eine unserer Studienkrankenschwestern unter meiner Anleitung aufgeklärt und untersucht. Die Proben wurden durch mich abgeholt und versorgt. Sämtliche RDTs auf Rota-/Adenoviren wurden durch mich durchgeführt. Die bakteriellen Kulturen wurden durch mich angelegt und in Zusammenarbeit mit den Mitarbeitern des Labors für Mikrobiologie am HAS/CERMEL, Dr. Abraham Alabi und Dr. Matthew McCall ausgewertet. Die RDTs auf *Cryptosporidium spp.* wurden von den Mitarbeitern des Labors für Parasitologie durchgeführt. Die Multiplex-PCR wurde von mir nach Einarbeitung durch Herrn Mirabeau Mbong Ngwese in Zusammenarbeit mit Herrn Ngwese und Herrn Gédéon Prince Manouana aus dem Labor für Parasitologie durchgeführt. Ab 17. September 2017 übernahm Herr cand. med. Philipp Hofmann nach einer Einführung durch mich die Studienleitung und beaufsichtigte für den restlichen Zeitraum die Studie.

Die statistische Auswertung erfolgte eigenständig durch mich.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

12. Appendix

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ID : _____ Date : ____/____/_____

Formulaire de collecte de données cliniques.

Renseignements personnels

Nom: _____

Nom de parent/tuteur: _____

Sexe: M / F

Date de naissance: _____

Âge: _____ mois/ ans

Résidence/ Quartier : _____

Numéro de téléphone : _____

Données cliniques

Taille: _____ cm Poids: _____ kg

Temperature: _____ °C Pulse: _____ bpm TA: _____/_____

État général: _____

Niveau de déshydratation clinique: _____

caractéristiques	Score de 0	Score de 1	Score de 2
Etat géneral/	Normal	Ayant soif, agité ou fatigué ; irritable au toucher	Somnolent, faible, ayant froid; presque comateux
les yeux	Normal	modérément creux	profondément creux
la langue	humide	collante	sèche
les larmes	présente	diminuées	absentes

Informations médicales

Votre enfant a la diarrhée depuis combien de temps? _____ jours / mois

Combien de selles diarrhéiques fait l'enfant par jour ? _____

La consistance des selles est-elle : ○ l'eau-l'eau ○ liquide ○ molle ○ dure

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Contient-elle du/des : ○ sang ○ glaires (rhume) ○ ni l'un ni l'autre

Quelle est la couleur des selles ? ○Noire marron ○ jaune ○ verte ○ grise

Quelle odeur ont-t-elles ? ○ mauvaise ○ putride ○ nauséabonde ○ normale

Depuis quand votre enfant, est-il hospitalisé ? _____/_____/_____

Présente-t-il aussi :

Des douleurs au ventre ☐ Oui ☐ Non

Des nausées ☐ Oui ☐ Non

Des vomissement ☐ Oui ☐ Non

- Si oui, combien de fois par jours ? _____

De manque d'appétit ☐ Oui ☐ Non

De la fatigue ☐ Oui ☐ Non

De la fièvre ☐ Oui ☐ Non

- Si oui, quelle température ? _____

☐ Autres symptômes: _____

Traitement actuel : _____
_____

D'autres personnes dans la maison souffrent-elles de la diarrhée ? ☐ Oui ☐ Non

- Si oui : Âge: ___ ans /mois Sexe: M/F

  Lien avec l'enfant: _____

Antécédents médicaux

Est-ce que votre enfant souffre d'autres maladies chroniques? ☐ Oui ☐ Non

- Si oui : ☐ VIH
  ☐ d'autres : _____

- Traitement en cours : _____

Allaitement maternel: ☐ Oui ☐ Non

- Si oui, combien de temps l'aviez vous alaité? _____ mois

Comité d'Éthique Institutionnel
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AVIS DU COMITÉ D'ÉTHIQUE INSTITUTIONNEL

Dr Jeannot Zinsou
Investigateur Principal
Centre de Recherches Médicales de Lambaréné (CERMEL)

14 février 2017

Dr Zinsou,

Titre du Projet : CARACTÉRISATION DES PATHOGÈNES CAUSANT LA DIARRHÉE CHEZ LES ENFANTS DE MOINS DE CINQ ANS À LAMBARÉNÉ, GABON

Promoteur : CERMEL

Référence CEI-CERMEL : 003/2017

Nous vous remercions de votre demande d'avis éthique pour le projet de recherche susmentionné, qui a été examiné par le Comité d'Éthique Institutionnel de la recherche du CERMEL sur la base des documents fournis (voir annexe).

Date de la réunion : 02 février 2017

Confirmation de l'opinion éthique
Au nom du comité, je suis heureux de confirmer une **Opinion Favorable du Comité** sur l'éthique de la recherche sur la base décrite dans le protocole de recherche susmentionné, et les pièces justificatives, sous réserve des conditions énoncées ci-dessous.

Conditions de l'opinion favorable
Voir annexe

Après examen éthique toute modification ultérieure du protocole ou des consentements doit être soumise au Comité par une demande de soumission révisée.

Très cordialement

Ghyslain Mombo-Ngoma
Président Comité d'Éthique Institutionnel du CERMEL
ghyslain.mombongoma@cermel.org / irb@cermel.org / http://cermel.org/ethicscommittee.php

Member of MARC (Mapping African Research ethics review Capacity), http://www.researchethicsweb.org/hrweb/
Registration Office for Human Research Protections (OHRP): IORG0007336 / IRB00008812

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Référence CEI-CERMEL : 003/2017

Annexes

1. Documents soumis

- Formulaire de soumission CEI-CERMEL
- Décision du comité de revue scientifique du CERMEL (SRC2017.03)
- Protocole de l'étude en anglais, versions 01 et 02
- Formulaire de consentement versions 1 et 2
- Formulaire de collecte des données
- Formulaire de suivi version 1

2. Condition de l'avis favorable

Aucune.

3. Dates

Date de début : janvier 2017

Date de fin du projet : décembre 2020