APPLICATION OF STOOL-BASED MULTIPLEX REAL-TIME PCR FOR PERSISTENT DIGESTIVE DISORDERS IN MALI AND CÔTE D'IVOIRE

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Universitätsklinikum des Saarlandes, Homburg/Saar

Vorgelegt von: Jasmin Kaur Jasuja

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III. Acronyms

BSP	Bacterial Stool Panel
C. difficile	*Clostridioides difficile*
CDC	Centers for Disease Control and Prevention
COPD	Chronic obstructive pulmonary disease
CRF	Case report form
C_t value	Cycle threshold value
D. fragilis	*Dientamoeba fragilis*
DALYs	Disability adjusted life years
DNA	Deoxyribonucleic acid
E. histolytica	*Entamoeba histolytica*
e.g.	Exempli gratia
EAEC	*Enteroaggregative Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme immunoassay
EIEC	*Enteroinvasive Escherichia coli*
EKBB	Ethikkommission beider Basel
etc.	Et cetera
ETEC	*Enterotoxic Escherichia coli*
G. lamblia	*Giardia lamblia*
GAPPD	Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea
GEMS	Global Enteric Multicenter Study

h	Hours
HIV/AIDS	Human immune-deficiency virus/acquired immune deficiency syndrome
HUS	Haemolytic-uremic Syndrome
i.a.	Inter alia
ICD	Internal Control DNA
ICR	Internal Control RNA
IMMH	Institute of Medical Microbiology and Hygiene
INRSP	Institut National de Recherche en Santé Publique
ipaH	*Invasion plasmid antigen H gene*
IRBs	Institutional review boards
ITM	Institute of Tropical Medicine
LED	Light-emitting diode
LEV	Low Elution Volume
LT	Labile toxin
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
MAL-ED	Enteric Infections and Malnutrition and the Consequences for Child Health
max.	Maximum
NHRC	Nepal Health Research Council
NTDs	Neglected tropical diseases
NTS	Non-typhoidal salmonellosis
ORS	Oral rehydration salts
ORT	Oral rehydration therapy

PC	Positive Control
PCR	Polymerase chain reaction
RDTs	Rapid diagnostic tests
rRNA	Ribosomal ribonucleid acid
SEV	Standard Elution Volume
SOP	Standardised operating procedure
spp.	Species pluralis
ST	Stable toxin
Swiss TPH	Swiss Tropical and Public Health Institute
UN	United Nations
UNICEF	United Nations International Children's Emergency Fund
WASH	Water quality, sanitation, hygiene
WHO	World Health Organization
Y. enterocolitica	*Yersinia enterocolitica*

IV. Abstract

Background Persistent digestive disorders are common and challenging clinical entities in the tropics. While acute gastrointestinal diseases are frequently self-limiting, the clinical management of persistent diarrhoea (≥14 days) and persistent abdominal pain (≥14 days) is more complex. Currently employed diagnostic tools lack sensitivity, and hence, the infectious aetiology and appropriate treatment of persistent digestive disorders remain to be elucidated in endemic settings. The current MD thesis is part of an international research consortium (NIDIAG) and aims at contributing to an improved understanding of the aetiology of persistent digestive symptomatologies in resource-constrained settings. To this end, multiplex polymerase chain reaction (PCR) assays for intestinal pathogens were employed on stool samples stemming from Mali and Côte d'Ivoire.

Methods Stool samples from patients with persistent diarrhoea (defined as ≥3 liquid bowel movement per day lasting ≥14 days) and/or persistent abdominal pain (defined as localised or diffuse abdominal pain for ≥14 days) and matched asymptomatic controls were collected in Niono, Mali, and Dabou, Côte d'Ivoire. All specimens were fixed in ethanol and transferred to Homburg, Germany, where nucleic acid extraction was performed. Subsequently, the samples were subjected to multiplex real-time PCR for the detection of six pathogenic bacteria (*Campylobacter* spp., three *Escherichia coli* pathovars, *Salmonella* spp. and *Yersinia enterocolitica*) and four intestinal protozoa (*Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica* and *Giardia intestinalis*). Cycle threshold (C_t) values were determined as a proxy for infection intensity.

Results The study cohort in Mali comprised 553 patients and 547 asymptomatic controls. 79.7% of all cases and 75% of all asymptomatic controls were infected with at least one pathogen (p-value≥0.05). 259 symptomatic cases and 260 controls were enrolled in Côte d'Ivoire; 62.5% of the cases and 44.2% of the matched controls were infected (p-value ≤ 0.0001). Multiple infections with ≥3 pathogens were common in the entire study cohort. The most frequently detected pathogens were enteroaggregative *Escherichia coli* (EAEC) (Mali: 39.9%; Côte d'Ivoire: 21.6%), *Campylobacter* spp. (Mali: 35.3%; Côte d'Ivoire: 17.7%), and *G. intestinalis* (Mali: 20.5%; Côte d'Ivoire: 11.6%). *Campylobacter*, *G. intestinalis*, enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) were

significantly more common in cases as compared to controls. However, for most pathogens, there was no significant difference in infection intensity.

Conclusions Stool-based multiplex PCR identified a high prevalence of multiple pathogens in stool samples from two sub-Saharan African settings. However, PCR results did not allow for an accurate discrimination between patients and asymptomatic controls, and thus did not provide sufficient guidance for clinical management. Nucleic acids of infectious pathogens might be shed for prolonged periods after symptom clearance, which limits the utility of highly sensitive, molecular tests in endemic areas of the tropics. Further research is warranted to elucidate the causal role of bacteria and protozoa in the development of persistent digestive disorders.

V. Zusammenfassung

Hintergrund Persistierende Verdauungsstörungen stellen ein häufiges und zugleich herausforderndes Erkrankungsbild in den Tropen dar. Im Gegensatz zu einer akuten Durchfallepisode lässt sich die Ursache persistierender Diarrhöen (≥14 Tage) und persistierender Abdominalschmerzen (≥14 Tage) aufgrund mangelnder sensitiver Diagnostik nur unzureichend aufklären und folglich selten gezielt therapieren. Die vorliegende Dissertation ist Teil eines internationalen Forschungsprojektes (NIDIAG), welche die Ätiologie untersucht, die persistierenden Verdauungsstörungen in benachteiligten Endemiegebieten zugrunde liegt. Hierzu wurden Stuhlproben aus Mali und Côte d'Ivoire mittels multiplexer Polymerase-Kettenreaktionen (PCR) auf gastrointestinale Erreger untersucht.

Methoden Zur Analyse wurden Stuhlproben von Patienten mit persistierenden Diarrhöen (≥3 breiige bis wässrige Stuhlgänge pro Tag seit mindestens 14 Tage) und/oder persistierenden Abdominalschmerzen (lokal begrenzte oder diffuse Abdominalschmerzen seit mindestens ≥ 14 Tage) und passenden asymptomatischen Kontrollpatienten aus Niono/Mali, und Dabou/Côte d'Ivoire, in Ethanol fixiert und nach Homburg/Deutschland, transportiert. Hier erfolgte die automatisierte DNA-Extrahierung mit anschließender multiplexer Echtzeit-PCR, welche die Stuhlproben auf sechs Bakterien (*Campylobacter* spp., drei *Escherichia coli* Pathovare, *Salmonella* spp. und

Yersinia enterocolitica) und vier Parasiten (*Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica* und *Giardia intestinalis*) untersuchte.

Ergebnisse Die Studienkohorte in Mali umfasste 553 Patienten und 547 Kontrollen. 79.7% aller Fälle und 75% aller Kontrollen zeigten Infektionen mit mindestens einem Erreger auf (p-Wert ≥0,05). In Côte d'Ivoire waren 62,5% der 259 Fälle und 44,2% der 260 Kontrollen infiziert (p-Wert ≤ 0,0001). In der Gesamtkohorte waren Infektionen mit ≥3 Pathogenen häufig anzutreffen. Die meist detektierten Pathogene waren *enteroaggregative E. coli* (EAEC) (Mali: 39,9%; Côte d'Ivoire: 21,6%), *Campylobacter* spp. (Mali: 35,3%; Côte d'Ivoire: 17,7%) und *G. intestinalis* (Mali: 20,5%; Côte d'Ivoire: 11,6%). Mit Ausnahme von *Campylobacter*, *G. intestinalis,* EIEC *ipaH* und ETEC *LT*, blieb ein signifikanter Unterschied sowohl in den Prävalenzen als auch in der Infektionsintensität meist aus.

Schlussfolgerungen Die stuhlbasierte multiplexe PCR erwies sich als eine schnelle Methodik zur Identifizierung von multiplen Pathogenen in hochprävalenten Gebieten Westafrikas, konnte jedoch zwischen symptomatischen und asymptomatischen Patienten nicht differenzieren. Gründe dafür könnte u.a. der Verbleib von Rest-DNA nach ausgeheilter Infektion sein, welcher die Sensitivität der PCR negativ beeinflusste. Folglich führten PCR-Resultate nur zu einem begrenzten Zusatzgewinn für das klinische Vorgehen. Fortführende Untersuchungen werden dahingehend stark empfohlen, um die komplexe Genese, welche zu persistierenden Verdauungsstörungen führt, aufzuklären.

1. Introduction

1.1 Persistent digestive disorders in the tropics

The term ‘digestive disorders’ comprises numerous gastrointestinal symptoms such as diarrhoea, abdominal pain, constipation, flatulence, nausea and vomiting. According to the World Health Organization (WHO), diarrhoea alone is responsible for 1.7 billion cases every year, including 1.5 million deaths worldwide, particularly in young children (WHO, 2013). Consequently, this tremendous figure is reflected in the WHO’s global statistic of leading causes of death in 2015, where diarrhoeal diseases are ranked on the eighth position (Figure 1.1). Although an overall decrease in the incidence of diarrhoea was noted over the past decade, there is still a high discrepancy between low- and high-income countries. As shown in Figure 1.2a, deaths in wealthy countries are predominantly due to chronic, non-communicable illnesses like cardiovascular diseases, cancer, dementia, chronic obstructive pulmonary disease (COPD) and diabetes, while diarrhoea and most infectious diseases are of little importance. Hence, seven out of ten deaths occur in individuals aged above 70 years, whereas only one out of 100 deaths affects children under 15 years (WHO, May 2014). In contrast, low- and middle-income countries suffer from a huge burden due to infectious digestive disorders and other communicable diseases such as respiratory infections and human immune-deficiency virus / acquired immune deficiency syndrome (HIV/AIDS) (Figure 1.2b and c). Almost half of these deaths occur among children aged 15 years or below (four out of ten deaths), while only two in every ten deaths affect older adult (UNICEF). It is assumed that each year almost

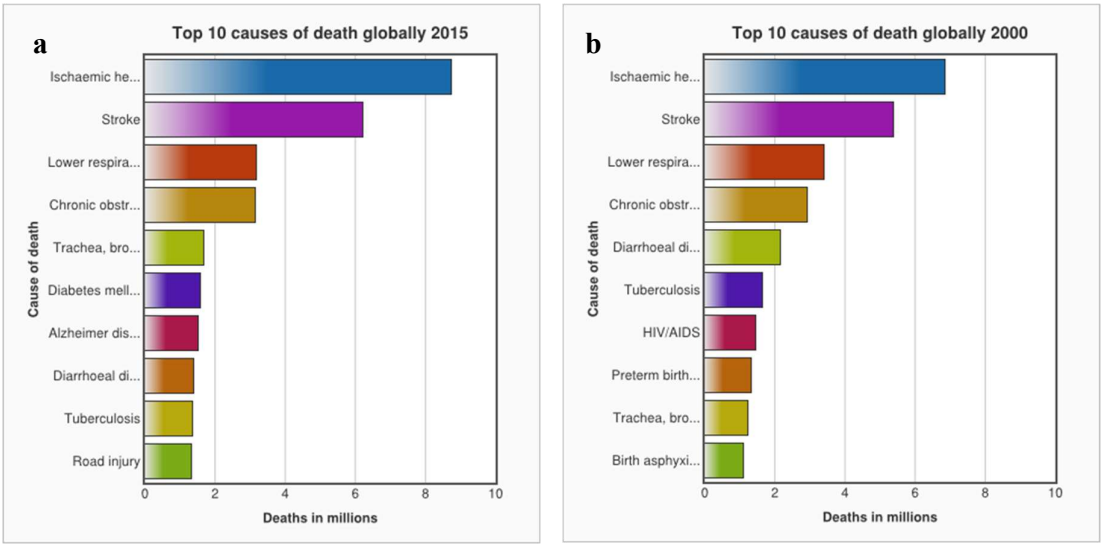


Figure 1.1 Comparison of leading causes of death over the past 15 years, (a) 2015 and (b) 2000 [published in (WHO, 2017)].

40% of global infant deaths are due to pneumonia and diarrhoea (WHO, 2017). Diarrhoea is only second to pneumonia, but remains the leading cause of death in children under five years (UNICEF, 2009; WHO, 2017). The highest burden is concentrated in tropical areas of Africa and South Asia, where 80% of the overall infant mortality is attributable to diarrhoea (UNICEF, 2009).

WHO defines diarrhoea as the passing of three or more loose or liquid stools within 24 hours (h) and differentiates between three clinical types (WHO, 1988):

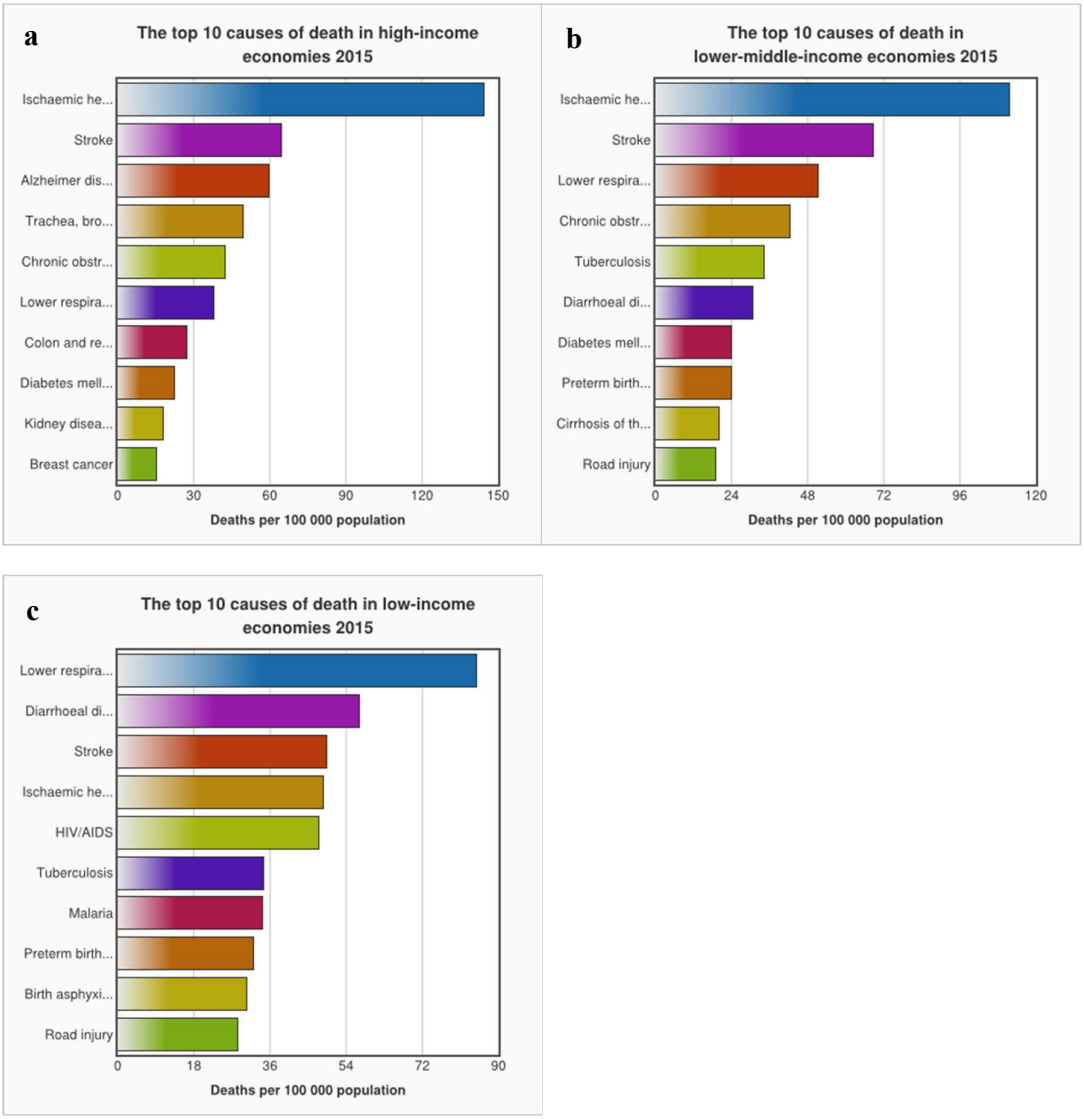


Figure 1.2 Top 10 causes of death in (a) high-income, (b) lower-middle income and (c) low-income countries in 2015 [published in (WHO, 2017)].

- Acute watery diarrhoea
- Acute bloody diarrhoea
- Persistent diarrhoea (with or without blood)

With an approximate duration of several hours or days, acute diarrhoea is time-limited, whereas persistent forms are diagnosed when symptoms last 14 days and longer (WHO, 1988). On average, an infant aged below three years experiences three episodes of diarrhoea annually, each of which might impair the nutrient supply and enhance the susceptibility to other infections at the same time (QUIGLEY et al., 2006; WHO, 1988). Especially those children and adults who also suffer from malnutrition or immunosuppression (exempli gratia (e.g.) HIV/AIDS) are more likely to develop severe and even life-threatening forms of diarrhoea. Even though multiple and simple prevention measures like clean drinking water, adequate sanitation and safe nutrition exist, these are not adequately available to individuals living in resource-constrained areas. WHO estimates that 780 million individuals still do not have access to safe drinking water and another 2.5 billion lack adequate sanitary facilities (WHO, 1988). As a consequence, poor hygienic conditions are accountable for 88% of diarrhoeal deaths worldwide (WHO, 2017). Furthermore, poor hygiene and close contact between humans and domestic animals promote dissemination of infections via faecal-oral transmission and through contaminated food and water. Hence, diarrhoea can result from multiple causative pathogens like bacteria, viruses and parasites, which either lead to an acute or a persistent disease. Further symptoms like abdominal pain, nausea and vomiting deteriorate the absorption of essential nutrients and increase fluid and electrolyte loss. Acute and particularly persistent diarrhoea may lead to reduced general wellbeing and malnourishment. If diarrhoea is not adequately treated, severe dehydration and even life-threatening events may occur. It is estimated that almost 760.000 children under five years die annually because of diarrhoea, which leads to a greater burden than that caused by AIDS, malaria and measles combined (WHO, 1988, 2017). In the 1980s, some studies reported a significant correlation between diarrhoea and death among infants, e.g. in a slum area of Lima (Peru), where 44% of deaths among children aged under five were related to diarrhoea, and it was observed that half of them suffered from persistent diarrhoea before death (QUIGLEY et al., 2006). Similar studies from other tropical settings in Bangladesh, north-eastern Brazil, northern India and Nepal also traced 36-56% of all diarrhoea-related infant deaths back to prior persistent diarrhoea (QUIGLEY et al., 2006). A study conducted in northern India even noted that the fatality rate of 14% among

persistent digestive disorders was significantly higher if compared to 0.7% in individuals with acute episodes (QUIGLEY et al., 2006). Furthermore, the incidence of persistent diarrhoea and the risk of its evolution from an acute form to a persistent presentation were investigated and showed that both peaked during the first year of life (QUIGLEY et al., 2006).

Other studies also indicated a correlation between persistent diarrhoea and persistent abdominal pain, e.g. a multinational survey displayed an overall prevalence of 10-46% for abdominal pain. Notably, the lowest scores were reported from Japan and Europe and the highest from Brazil and Mexico, where participants were also more likely to report other digestive disorders (QUIGLEY et al., 2006). However, only few studies pertaining to persistent digestive disorders were carried out thus far, despite it being a very common complaint on a global scale.

In summary, low standards of living and overall poverty substantially correlate with health problems, especially among infants, and there is ample evidence that healthcare systems in the most affected countries need to be strengthened to provide an effective treatment to their population. In order to improve this situation, in April 2013, WHO established the 'Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea' (GAPPD). It aims to put an end to preventable child deaths by 2025 through reducing the mortality and incidence of severe pneumonia and diarrhoea by at least 75% compared to the level in 2010 (QAZI et al., 2015). This concept focuses on supporting affected countries by building up co-operations between global and national policies for improved disease management, promoting collaborative research work and creating public awareness.

1.2 Clinical management of persistent diarrhoea and persistent abdominal pain

Diarrhoea may occur in an acute or long-lasting form and can be watery, mucous, and/or bloody. Watery diarrhoea can cause considerable dehydration and weight loss, while bloody diarrhoea additionally damages the intestinal mucosa and causes malnourishment. Once the symptoms last 14 days or longer, the term 'persistent diarrhoea' is used. Non-infectious digestive disorders with chronic or recurrent diarrhoea such as tropical sprue, gluten-sensitive enteropathy, other hereditary diarrhoeal disorders or blind-loop

syndrome are not regarded as persistent diarrhoea (QUIGLEY et al., 2006). The term ‘chronic diarrhoea’ is not clearly defined, but refers to diarrhoea lasting more than 4-6 weeks (POLMAN et al., 2015; QUIGLEY et al., 2006; WHO, 2005).

When suffering from diarrhoea, fluid loss through liquid stools, vomit, sweat, urine and breathing may cause an imbalance in the water and electrolyte regime and may result in severe dehydration and even death, if not treated properly (WHO, 1988; WHO, UNICEF, 2006). Hence, a precise medical history is required to classify diarrhoea correctly. Emphasis is put on questions regarding consistency, frequency, blood in stool as well as fever or other infectious symptoms. A subsequent comprehensive internal and neurological examination provides guidance on the degree of dehydration prior to therapy. Therefore, WHO recommends an assessment of the patient’s general condition and dehydration stage based on symptomatic signs, which may also guide therapeutic decision-making (Table 1.1). Key interventions for the treatment of persistent diarrhoea include inter alia (i.a.) rehydration, either orally or intravenously, in order to restore the water and electrolyte regime and to compensate ongoing fluid loss. Intravenous treatment via infusion is usually administered in severe cases or in case of inability to absorb glucose and fructose due to malabsorption, which may worsen watery diarrhoea. Oral rehydration therapy (ORT) is a simple strategy to treat dehydration properly and cost-effectively. In addition, this treatment can be performed by healthcare workers as well as by parents at home. The application starts at an early stage of dehydration as a preventive care and prior to occurrence of any symptoms. At this stage, it is sufficient to create self-made solutions containing sugar and salt (WHO, 2010b). As soon as symptoms of moderate dehydration appear, the therapy is switched to so-called oral rehydration salts (ORS) solutions. The official formula for ORS, which has been included in the WHO model list of Essential Medicines (WHO, 2010b), is illustrated in Table 1.2. It was first introduced by WHO and the United Nations International Children’s Emergency Fund (UNICEF) in 1969 and underwent several modifications since then in order to treat dehydration irrespective of the underlying aetiology or age group (WHO, 2010b). Several studies confirmed a reduction of stool output by 20% and vomit by even 30% with this formula (WHO, UNICEF, 2006; WHO, 2010b). The mixture is absorbed in the small intestine and substitutes essential nutrients and body fluids, e.g. glucose increases the efficiency of sodium absorption and consequently increases water diffusion, while sodium, potassium and trisodium citrate balance the electrolyte regime. Taking into account that many areas struggling with dehydration from diarrhoea are located in the

tropics, trisodium citrate was added to make ORS durable for 2-3 years without any further storage precautions (WHO, 2010b). The dosage for treatment with ORS is adapted to an individual's fluid deficit (Table 1.3). Hospitalisation is considered in case of severe dehydration with symptoms of shock, severe malnutrition, further weight loss or continuous diarrhoea despite therapy. The principle of hospital treatment is similar to the aforementioned aspects, but therapy is adapted more to lacking diet components and administered by infusion. If affected individuals respond satisfactorily to the treatment, they can be discharged, but should be followed up closely to ensure continued weight gain and compliance with feeding advice (WHO, UNICEF, 2006). Despite ORS being a successful treatment, only 39% of children suffering from diarrhoea in developing countries obtain proper therapy and several studies suggest that particularly endemic areas with resource-poor settings are more likely to have limited access to ORS than urban areas (WHO, 2017). As there are relatively few studies on the correct nutritional management of persistent diarrhoea, the following guidelines are mainly based on extensive experiences in the treatment of acute and chronic diarrhoea (QUIGLEY et al., 2006). It is recommended to continue with nutrient-rich food during and after a diarrhoeal episode since several studies demonstrated that despite reduced efficiency in intestinal absorption, it is still sufficient to maintain a positive balance (QUIGLEY et al., 2006; WHO, 1988; WHO, UNICEF, 2006). Moreover, increased frequency of feeding, at least six times per day, may maximise absorptive efficiency and result in less severe diarrhoea (WHO, 1988). The same applies for breastfeeding infants, particularly during the first six months of life, as breast milk provides all essential nutrients and antibodies for a protective immune system. Several studies have shown that infant feeding beyond two years of age has resulted in reduced infection rate and mild course of disease, even among infants with HIV-infected mothers (WHO, 2017). In 2010, the WHO published new guidelines on breastfeeding, after taking the risk of HIV transmission and the risk to come down with diarrhoeal diseases and pneumonia into account. Since then, infant feeding by HIV-infected mothers is not only recommended but also supported, based on the evidence that treatment with antiretroviral drugs to either the HIV-infected mother or HIV-exposed infant decreases the risk of transmitting HIV (WHO, 2012). In contrast, the sole feeding with animal milk has resulted in worsening diarrhoea, most probably because of lactose intolerance. Hence, pure yoghurt or the combination of milk and cereals have proven to be better alternatives (QUIGLEY et al., 2006; WHO, UNICEF, 2006).

Table 1.1 WHO's recommendation to estimate the degree of dehydration based on specific symptoms, rated on a scale of three [adapted from (WHO, 2005; UNCIEF, 2006)].

Degree of dehydration	Clinical signs
1. Early dehydration	• No symptoms
2. Moderate dehydration	• Thirst • Restlessness • Decreased skin elasticity • Sunken eyes
3. Severe dehydration	• Symptoms become more severe • Hypovolamic shock • Unconsciousness • Lack of urine output • Cool, moist extremities • Rapid and feeble pulse • Low or undetectable blood pressure • Pale skin and peripheral cyanosis

In general, dehydration from diarrhoea requires food mixtures of high nutrient content, low viscosity and avoiding of hyperosmolarity (QUIGLEY et al., 2006). The goal is a daily intake of at least 110 calories/kg (WHO, UNICEF, 2006). The toleration and effectiveness of the therapeutic diet is tested for seven days and reviewed afterwards. In case of no improvement, a second adjusted diet is given for another seven days. Regarding supplement of trace elements and vitamins, there is no generally acknowledged consensus, since only few studies have been conducted. However, it is known that zinc has proven beneficial on cellular level and since its deficiency is widespread in low-income countries, treatment with zinc in a dosage of 10-20 mg/day for 10-14 days to all children is recommendable (WHO, UNICEF, 2006). Several studies documented a reduction of 2-3 months in diarrhoea incidence, a decline in diarrhoea duration by 25% and reduced stool volume by 30% (WHO, 1988; WHO, UNICEF, 2006). Similar results support the substitution of vitamin A (WHO, 2005). A preventive or routine use of antimicrobials is not recommended due to the potential development of antibiotic resistance and the associated unnecessary costs (QUIGLEY et al., 2006; WHO, UNICEF, 2006).

Table 1.2 Composition of the new ORS formulation recommended by the WHO, 2006 [adapted from (UNICEF, 2006)].

New ORS	grams/litre	%	New ORS	mmol/litre
Sodium chloride	2.6	12.683	**Sodium**	75
Glucose, anhydrous	13.5	65.854	**Chloride**	65
Potassium chloride	1.5	7.317	**Glucose, anhydrous**	75
Trisodium citrate, dihydrate	2.9	14.146	**Potassium** **Citrate**	20 10
Total	**20.5**	**100**	**Total Osmolarity**	**245**

If dysentery, cholera with severe dehydration or other serious non-intestinal infections (e.g. pneumonia, sepsis, urinary tract infection and otitis media) occur, treatment starts with empirical antibiotics until microbiological results allow switching to a targeted anti-infective therapy. Other drugs like antimotilities (e.g. loperamide), antiemetics (e.g. chlorpromazine), antisecretory agents and intestinal adsorbents (e.g. kaolin) have not shown any additional effects (WHO, UNICEF, 2006). In contrast, some of these drugs may cause major side effects, particularly in children.

Whereas diarrhoea and its clinical types are clearly defined by the WHO, no common definition for persistent abdominal pain exists. Huguet *et al.* described persistent abdominal pain as a pain in the absence of anatomical abnormality, inflammation or tissue damage without defining a duration (HUGUET et al., 2017). Nevertheless, there is a definition for 'chronic abdominal pain', which is a continuous or intermittent abdominal discomfort lasting for at least six months (KEDDY, GOLDSMID, 2014). Correspondingly, persistent abdominal pain may last longer than several days but shorter than six months. Abdominal pain can be diffuse or localised which makes it difficult to determine an exact starting point of pain (WALLIS, FIKS, 2015). In addition, a lot of differential diagnoses have to be reconsidered, especially when examining children, who often indicate negative emotional symptoms with stomach pains (HUGUET et al., 2017). This underscores the importance of a thorough clinical examination by the healthcare provider.

The clinical management of digestive disorders does not only require an adequate therapy, but also a comprehensive health education. There are two preventive components:

primary and secondary prevention. The primary prevention aims at the reduction of exposure to pathogens by ensuring education on personal hygiene, supplying improved sanitary facilities and safe drinking water. Several studies have proven the benefits of these interventions, e.g. a decrease of 40% and 36% in diarrhoeal incidence by handwashing with soap and adequate sanitation, respectively (WHO, 2017). Furthermore, prevention in the form of rotavirus and measles vaccination has been introduced, since diarrhoea from rotavirus is responsible for 40% of hospital admissions among children under five years worldwide, and diarrhoea as a sequel of measles in malnourished children is one of the most common causes of death in developing countries (WHO, 2017). Secondary prevention eliminates reasons for vulnerability towards severe consequences. Therefore, exclusive breastfeeding for the first six months of life and well-balanced diet are recommended.

Table 1.3 Assessment of a child's fluid deficit recommended by the WHO. For example, a child weighing 5 kg and showing signs of 'moderate dehydration' has a fluid deficit of 250-500 ml [adapted from (WHO, 2005)].

Assessment	Fluid deficit as % of body weight	Fluid deficit in ml/kg body weight
No signs of dehydration	<5%	<50 ml/kg
Moderate dehydration	5-10%	50-100 ml/kg
Severe dehydration	>10%	>100 ml/kg

1.3 Diagnosis of persistent digestive disorders

The diagnosis of persistent diarrhoea and persistent abdominal pain includes a detailed medical history and a comprehensive physical examination. However, even a distinct symptomatology does not indicate one specific pathogen in most instances of diarrhoeal diseases. Particularly in the tropics, where inadequate hygiene and a lack of improved sanitary facilities promote the distribution of pathogens, there is a large variety of causative organisms. Many of these pathogens are zoonotic, i.e. they are transmitted horizontally from animal to humans and then spread vertically from person to person by the faecal-oral route or through contaminated food and water. In the absence of sophisticated laboratory tests, it is almost impossible to predict which pathogen is

responsible for a certain symptomatology. In the following subchapters, the main pathogens, on which particular emphasis was put in the current MD thesis, are briefly presented.

1.3.1 Bacterial infections: *Campylobacter,* EAEC, EIEC, ETEC*, Salmonella, Yersinia*

Bacterial infections pose a major health threat in the tropics, as they are capable of causing severe diarrhoea, intestinal bleeding and even intestinal perforation. Hence, an accurate diagnosis is of crucial importance and requires diagnostic tests with high sensitivity and specificity. Stool culture remains the gold standard for almost all pathogens. To supply optimal growth conditions to each microbial strain, nutrient media in the form of agar plates are equipped with inhibitory or stimulating additives (e.g. agar plates with antibiotic or nutrient addition). These selective culture media are inoculated with the patient's stool sample and are incubated for 24-72h at 35°C to allow enteric pathogens to form visible bacterial colonies (BECKER et al., 2013). The colony's phenotype and its further examination by microscope, biochemical identification panels and other phenotypic identification systems provide crucial information for the correct species identification of the culture-grown bacteria (BECKER et al., 2013). Furthermore, testing for antibiotic resistance can be performed by e.g. agar diffusion tests. However, stool culture takes at least one to three days and requires experienced laboratory personnel for a correct decision-making. Since neither experienced staff nor adequate laboratory infrastructures are available in many resource-constrained settings, rapid diagnostic tests (RDTs) have been developed for some infections, but have not proven to be sufficiently sensitive and specific (BECKER et al., 2013). Thus far, stool culture remains the standard technique, but it is rarely feasible in the tropics.

Campylobacter species (spp.) are among the most common foodborne causes of bacterial diarrhoea, with at least 400-500 million cases annually worldwide (RUIZ-PALACIOS, 2007). The highest burden remains in the tropics with 40.000-60.000 per 100.000 of infected children younger than five years of age, compared to only 300 per 100.000 infants in industrialised countries (KEDDY, GOLDSMID, 2014). Due to unhygienic conditions, the zoonosis spreads easily in low-income countries. Hence, *Campylobacter* spp. has been detected in 8-45% of symptomatic as well as asymptomatic patients (RUIZ-PALACIOS, 2007), with *Campylobacter jejuni* being the most frequently detected strain.

An infectious dose of one million pathogens is required to develop clinical symptoms (CHAMBERLAIN, 2009), and after an incubation period of two to five days, the infection leads to low-grade fever, abdominal cramps, nausea, vomiting and watery or bloody diarrhoea. The disease pattern can vary from a mild infection in healthy adults to life-threatening illnesses in elderly and immunosuppressed individuals. In rare cases, infectious complications such as Guillain-Barré and Reiter's syndrome due to cross-reactions between lipo-oligosaccharides and GM1-gangliosids have been reported (SUERBAUM et al., 2012). For detection, stool culture is set up on blood agar with antibiotic admixture (e.g. Karmali agar) and cultivated in microaerophilic atmosphere at 42°C for a minimum of 72h (BECKER et al., 2013; GILTNER et al., 2013; KEDDY, GOLDSMID, 2014). As a recent surveillance by the Centers for Disease Control and Prevention (CDC) has elucidated, a prolonged incubation plays a major role in cultivation since 66% of stool cultures showed negative results after 48h, while only 33% were negative after 72h (GILTNER et al., 2013). If suspicious colonies grew on the agar, phase contrast or darkfield microscopy is employed to look for S-shaped gram-negative rods. For a faster diagnostic approach, enzyme immunoassays (EIAs) have been developed. This technique concentrates on detecting specific antigen in stool with the help of antibodies and enzymes. One advantage is that the test results are available in less than an hour (BECKER et al., 2013). Yet, a high number of false-positive results limits the usefulness of this diagnostic tool, because positive results would still require a confirmation by stool culture (GILTNER et al., 2013).

Escherichia coli (E. coli), a facultative anaerobic, gram-negative, rod-shaped bacterium of the Enterobacterales usually colonises the intestine asymptomatically, but some strains contain plasmids, which are capable of producing virulent toxins and causing diarrhoea. Among those, there are six clinically relevant subtypes: *enteroaggregative E. coli* (EAEC), *diffusely adherent E. coli* (DAEC), *enteroinvasive E. coli* (EIEC), *enteropathogenic E. coli* (EPEC), *enterohaemorrhagic E. coli* (EHEC) and *enterotoxigenic E. coli* (ETEC). The pathogenesis differs between two types: extra- and intraintestinal infection, the latter of which is more likely to cause digestive disorders. Enterotoxins of pathogenic strains inhibit the protein biosynthesis in enterocytes, thus leading to cellular death and inflammation. Consequently, watery diarrhoea, dysentery and haemorrhagic colitis, which are frequently associated with a so-called Haemolytic-uremic Syndrome (HUS) in children, might occur (KEDDY, GOLDSMID, 2014). Since some *E. coli* strains belong to physiological flora of the intestinal tract, it is common to

grow *E. coli* on stool culture using appropriate agar media (e.g. Endo or MacConkey agar). However, further tests are required to distinguish pathogenic from apathogenic strains. Therefore, immunoassays, HEp-2 cell adherence and especially multiplex PCR can be applied to detect the specific virulence genes. PCR assays are rapid and allow a clear distinction between different strains. Despite their excellent sensitivity and specificity of over 99%, PCR assays are rarely available in endemic settings and still lack routine application (BECKER et al., 2013).

High-income as well as low-income countries struggle with a high incidence of EAEC. Several studies, particularly among children, have reported a high faecal carriage rate among different populations, with significant differences in the prevalence of EAEC between symptomatic cases and healthy control participants (WEINTRAUB, 2007). Symptoms appear after a short incubation period of 8-18 h (HUANG et al., 2006) and include watery, mucous and sometimes even bloody diarrhoea. Furthermore, abdominal pain, nausea, vomiting and a slightly elevated temperature might occur. The pathogenesis comprises three stages: First, EAEC causes increased mucus production which covers the bacteria in a biofilm. Second, a cytotoxin is released which destroys intestinal cells and thirdly causes watery diarrhoea (CHAMBERLAIN, 2009). Light microscopy may lead to detection of bacterial aggregations, but a specific diagnosis of EAEC requires identification of the virulence factor through PCR.

EIEC, resembling *Shigella* on the molecular level, are responsible for shigellosis-like diarrhoea, which is particularly occurring in developing countries. Similar to *Shigella*, plasmids of EIEC are capable of invading and destructing colonic mucosa and can cause fever, abdominal cramps, watery and even bloody diarrhoea and toxaemia (SUERBAUM et al., 2012). PCR is the only method capable of detecting the *invasion plasmid antigene H (ipaH)* gene, which allows differentiating EIEC and *Shigella* from other *E. coli* pathotypes.

Along with *Campylobacter* spp., ETEC is the most common finding in traveller's diarrhoea, being responsible for 30-60% of diarrhoea cases in travellers returning from developing countries (STEFFEN et al., 2005) (Figure 1.3). Numerically, ETEC accounts for an estimated 210 million cases of diarrhoea, thereof 380.000 deaths annually and a peak incidence within the first two years of life (STEFFEN et al., 2005). ETEC is capable of producing two toxins: *heat stable (ST)* and *heat labile (LT)* enterotoxin. The pathogenesis of *ST* induces an activation of guanylate cyclase following an increase of

guanosine monophosphate, which either stimulates the secretion of chloride or blocks NaCl absorption, both leading to watery diarrhoea (CHAMBERLAIN, 2009). *LT* is similar to *Vibrio cholerae* and capable of activating adenylate cyclase, which causes massive liquid diarrhoea after a short incubation period of one to two days. Clinical signs of infection also manifest as fever and vomiting, which can vary from mild to severe, particularly among bottle-fed infants. Duration of symptoms is usually limited to up to 14 days, but studies in Nepal have shown, that ETEC plays a major part in prolonged diarrhoea (STEFFEN et al., 2005). For detection, serological tests like latex agglutination test for *LT* and immunodiffusion for *ST* have been established, but similar to EAEC, PCR assays remain the only method for proving virulent factors.

Salmonella spp. may lead to two major clinical signs: typhoid fever (enteric fever), caused by *Salmonella enterica* serovar Typhi and Paratyphi A, B or C, and non-typhoidal salmonellosis (NTS), which is caused by *Salmonella enterica* serovar Enteritidis and >2000 other serotypes. Salmonellosis is responsible for 93.8 million diarrhoeal cases worldwide, including 155.000 deaths each year (MAJOWICZ et al., 2010). The gram-negative bacterium is very robust, since it is capable of surviving for several weeks in dry environment and even for months in water. The incidence of infection is estimated around 1.000 per 100.000 of population, particularly affecting infants and young adults aged between three and 29 years (KEDDY, GOLDSMID, 2014). The zoonosis is transmitted by faecal-oral route or contaminated food and water. For causing NTS, an infectious dose of 10^6 cells for healthy adults is necessary, whereas infants and immunosuppressed individuals require a dose of under 100 cells. Within 12-36h of incubation (WHO, 2013), *Salmonella* is ingested by macrophages in host cells, where it proliferates and then lyses the cell. Following, *Salmonella* gains access to mesenteric lymph nodes and infests the whole lymphatic and blood system. The symptoms vary in onset of fever, abdominal pain, (sometimes bloody) diarrhoea, nausea and vomiting, lasting two to seven days (WHO, 2013). Whereas typhoid fever represents high fever (39°C to 41°C), headache, muscle pain and then is followed by diarrhoea, but is self-limiting after one to three weeks. Hence, recovery without any specific treatment is possible and common in most cases. Yet, children and elderly are more vulnerable and there is a significant risk for developing bacteraemia. For diagnosis, *Salmonella* is detectable for four to six weeks after recovery and even for months in infant's faeces. Stool culture on Leifson agar is the standard method for detection of *Salmonella*, on which the bacterium grows in small, blackish colonies. After the species diagnosis has been made, additional serological testing of the

antigen composition of the respective strains is required for an exact serovar diagnosis according to the Kauffmann-White-classification. PCR assays have also been applied, but need further development and validation.

Yersinia enterocolitica (Y. enterocolitica) is one of three primarily pathogenic *Yersinia* species (besides *Y. pestis* and *Y. pseudotuberculosis*). In 2007, the European Centre for Disease Prevention and Control (ECDC) reported 8.874 cases of yersiniosis, 5.000 of which were reported from Germany (ROSNER et al., 2010). *Y. enterocolitica* belongs to the gram-negative Enterobacterales. Notably, *Y. enterocolitica* may grow at lower temperatures, which is why stool samples should also be incubated at approximately 28°C. In contrast to *Salmonella*, the infectious dose with 10^4-10^6 is very high. After oral ingestion, the bacterium invades the small intestine and reaches phagocytes of Peyer plaques, where macrophages transport it to mesenteric lymph nodes, are destroyed and allow pathogens to proliferate in the extracellular space. After an incubation period of five days, colicky abdominal pain, watery diarrhoea, tenesmae, fever, nausea and vomiting may vary and last one to two weeks or longer (ROSNER et al., 2010). Later, severe sequelae like myocarditis, arthritis and painful erythema nodosum may appear (ROSNER et al., 2010). Similar to other intestinal infections, infants are affected most frequently.

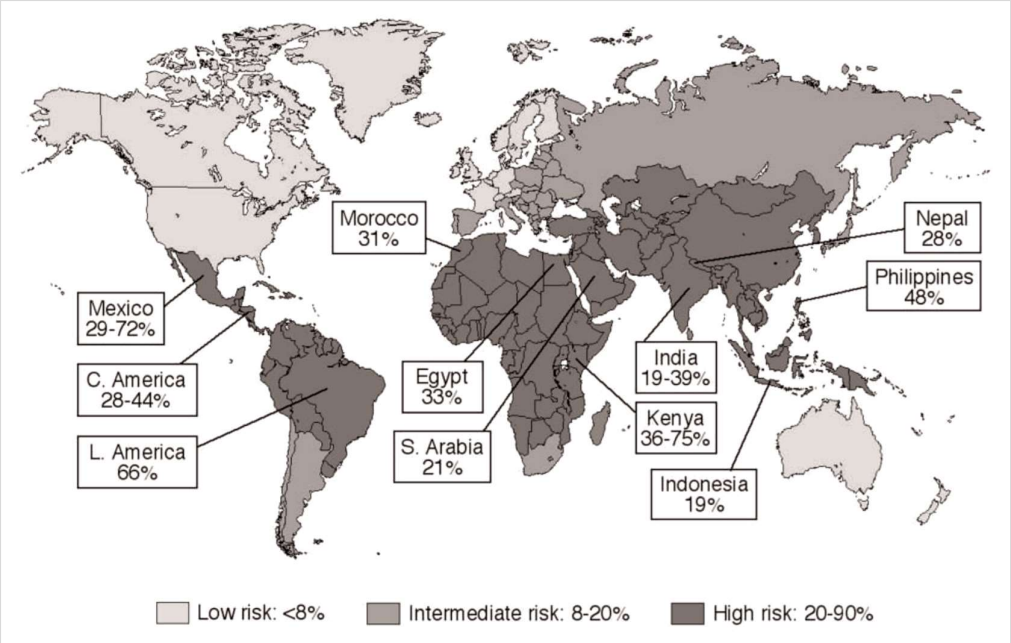


Figure 1.3 Global percentage incidence of traveller's diarrhoea caused by enterotoxigenic *Escherichia coli* [published in (STEFFEN, 2005)].

1.3.2 Parasitic infections: *Cryptosporidium* spp., *Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia*

Parasitic infections are very common in the tropics, but their 'true' medical importance has still not been comprehensively appreciated, which is the reason why several parasitic diseases are counted among the so-called neglected tropical diseases (NTDs). NTDs are communicable illnesses infecting more than one billion people who mainly live in the tropics (WHO, 2010a). Their resulting health impact is enormous, both in medical and economic dimensions. Many tropical areas struggling with poverty, lack of sanitary facilities and close interaction between humans and infectious vectors like domestic animals and livestock are most severely affected (WHO, 2010a). The diagnosis of these infections is mostly based on repeated stool examinations. For that, fresh faeces is diluted with 0.9% NaCl solution and examined with conventional light microscopy (BECKER et al., 2013). Since phenotypes of trophozoites and cysts may sometimes be difficult to differentiate visually, direct microscopy lacks high sensitivity and hence, might be improved by e.g. staining techniques, sedimentation or flotation with formalin-ether concentration technique. However, microscopy fails frequently to detect low-intensity infections, in which only small quantities of a given pathogen are shed in the stool.

Cryptosporidiosis can be detected in 1-4% of asymptomatic and 2-4% of symptomatic patients in developed countries, while the infection rate among immunosuppressive patients (e.g. HIV) is suspected to be much higher (BOUZID et al., 2013; LEITCH, HE, 2011). The oocysts of the pathogen is resistant to many environmental factors and hence, only 150 oocysts are necessary for causing diarrhoea (CHAMBERLAIN, 2009). One oocyst divides into four sporozoites, which are capable of penetrating microvilli of intestinal epithelium. There, the sporozoites proliferate asexually to merozoites, which lead to autoinfection of nearby enterocytes and where merozoites gradually proliferate asexually to gametocysts. Alternatively, merozoites can also transform into sexual cysts which then produce oocysts again (LEITCH, HE, 2011). These organisms are excreted with stool and infect other individuals through faecal-oral, sexual or postnatal route. After seven to ten days (maximum (max.) 20 days) of incubation, healthy individuals suffer from self-limiting watery diarrhoea, colicky abdominal pain, fever, nausea and vomiting, whereas immunocompromised patients undergo prolonged sickness of over two weeks with immense loss of water (≥ 50 defecations per day over months to years) (CHAMBERLAIN, 2009). Stool smear stained with Kinyoun acid-fast stain allow a rapid detection of *Cryptosporidium* cysts (CHAMBERLAIN, 2009; LEITCH, HE, 2011), but

may be false-negative in low-intensity infections. Additionally, the diagnosis is relatively time-consuming, since detection might be challenging even for experienced laboratory personnel, and several stool samples over several days are required to increase sensitivity. Antigen detection with the aid of EIA is a faster alternative, but conflicting results have been reported. Hence, the wide application of molecular methods such as PCR to differentiate species of *Cryptosporidium* is encouraged (ADEYEMO et al., 2018; VAN DEN BOSSCHE et al., 2015). Yet, PCR techniques are rarely available in the tropics.

Dientamoeba fragilis (D. fragilis) is an anaerobic intestinal protozoan. The life cycle is still underresearched, but it is a proven fact that trophozoites are capable of proliferating in colon, are excreted with faeces and then infect other individuals via faecal-oral route (Figure 1.4). Since trophozoites are very labile, a fast stool examination under microscope is required after preserving trophozoites in polyvinyl alcohol, sodium acetate-acetic, acid-formalin or Schaudinn (FOTEDAR et al., 2007; MEHLHORN, PIEKARSKI, 2002; STARK et al., 2010). In addition, several samples on alternate days are required for valid detection and higher sensitivity (six stool samples increase sensitivity to 90-95%) (STARK et al., 2010). However, the differentiation between *D. fragilis* and other amoebae species may sometimes occur. Hence, the performance of real-time PCR has proven to be the most sensitive diagnostic technique (STARK et al., 2010). Patients suffering from an infection with *D. fragilis*, often complain about abdominal pain and sometimes watery or sticky diarrhoea. Other clinical signs are nausea, vomiting, headache and fever. Usually, symptoms last one to two weeks, but several studies have shown a propensity to prolonged diarrhoea (STARK et al., 2010). The estimated prevalence in populations of developed countries varies between 0.4-71% depending on the study cohort and the employed diagnostic method (TURKELTAUB et al., 2015). However, there is also a debate whether *D. fragilis* should actually be regarded as pathogenic or non-pathogenic parasite, since the role of *D. fragilis* as a global public health pathogen is still poorly understood and has often been found in asymptomatic individuals (STARK et al., 2016; TURKELTAUB et al., 2015).

Entamoeba histolytica (E. histolytica) is the cause of amoebiasis, which is particularly prevalent in tropical and subtropical areas. According to WHO, 50 million individuals worldwide suffer from amoebiasis with 100.000 deaths annually, particularly in developing countries where a prevalence of over 10% has been reported (FOTEDAR et al., 2007). There are two pathogenic forms: trophozoite (capable of penetrating the intestinal epithelium and proliferating there) and cyst (infectious form). The oral intake of a cyst infects the colon and allows the cyst to transform into a trophozoite. The trophozoite adheres to lectins of intestinal epithelial cells and penetrates into the cell, where enzymes for lysing the enterocytes (=histolytica) are produced and cause leakage of ions. Consequently, liquid stool covered with blood and slime occurs. Histologically, flask-shaped ulcerations are visible in the cecum, appendix and ascending colon (CHAMBERLAIN, 2009). *E. histolytica* might also manifest as a severe extraintestinal form, which leads to life-threatening intestinal perforation with peritonitis and liver abscess as well as lung and brain abscess. Even asymptomatic cases have been reported and are considered as dangerous, as individuals act as vectors. The diagnosis is confirmed by microscope of diluted faeces or tissues obtained from lesions (SUERBAUM et al., 2012). The presence of intracytoplasmic erythrocytes is pathognomic for *E. histolytica*, since the pathogen is not capable to lyse red blood cells (CHAMBERLAIN, 2009; MEHLHORN, PIEKARSKI, 2002). Similar to other parasites, stool samples over several days are required for higher sensitivity, but do not promise right decision making, since *E. histolytica* resembles other subspecies (e.g. *E. dispar*). In addition, *E. histolytica* is a very environmentally labile pathogen, which makes fast microscopy within one hour after

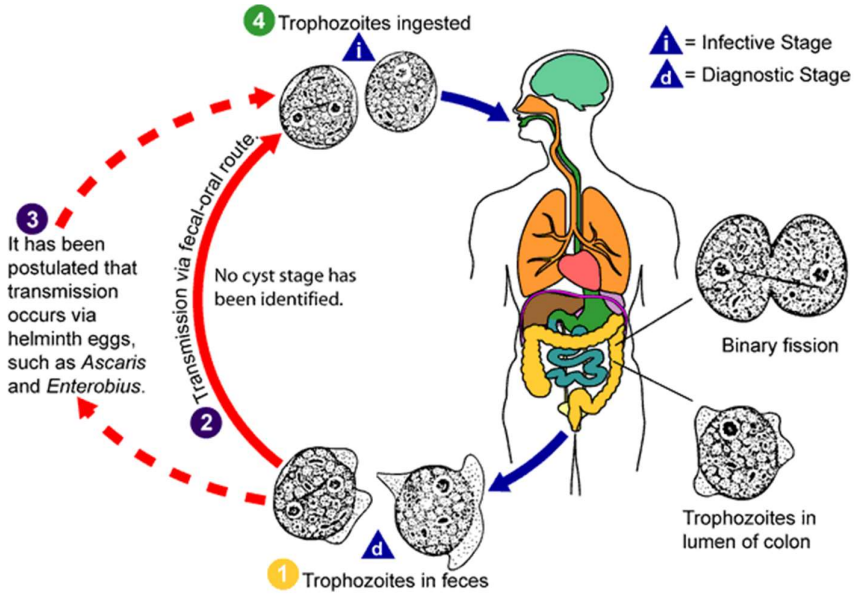


Figure 1.4 Life cycle of *D. fragilis* [published in (STARK, 2010)].

collection and fixation necessary (FOTEDAR et al., 2007). However, sensitivity for microscopy remains poor at 60% (FOTEDAR et al., 2007). Similarly, cultivation has failed as a routine diagnostic method, since it delivers false-negative results and the approach is technically difficult (FOTEDAR et al., 2007). Serologically, proof of antigen or antibodies are useable, but only sensible when liver abscess or perforation occurs. In contrast, PCR assays are capable of differentiating between species.

Giardia lamblia (G. lamblia; synonymous: *G. intestinalis)* is one of the most important causes of parasitic diarrhoea, with one third of all infected individuals living in low-income countries. In remote areas with poor sanitation and hygiene, children living in limited-resource settings, many of whom are undernourished, harbour a particularly large burden of *Giardia* infection (BARTELT, PLATTS-MILLS, 2016). In contrast, only 2% of all adults and 6-8% of all children in industrialised countries are carriers (GARDNER, HILL, 2001). In presence of gastric acid and pancreas enzymes, *G. lamblia* cysts reproduce to trophozoites in the duodenum (Figure 1.5). The trophozoites stick to enterocytes and hinder absorption of disaccharides, without infiltrating the cells. As a result, osmotic diarrhoea, malabsorption, steatorrhoea, abdominal cramps and flatulence may develop (CHAMBERLAIN, 2009). After three to four weeks, trophozoites transform into cysts, are excreted and infect other individuals. The modified metabolic status in gastrointestinal tract also allows bacterial overgrowth and deteriorates digestive complaints. Additionally, it is strongly suspected, that certain surface proteins of *G. lamblia* compete with host cells for zinc, and hence, cause malabsorption (MEHLHORN, PIEKARSKI, 2002). Clinical signs usually last for 10-14 days. The infection is easily spread through faecal-oral transmission. Diagnosis is usually made by visualisation of trophozoites and cysts upon stool microscopy. Since cysts are excreted intermittently, multiple stool collections on consecutive days increase test sensitivity. If stool microscopy is unsuccessful, duodenal secret is collected via aspiration. However, only stool microscopy is commonly available in most tropical countries. Antigen detection assays and stool-based PCR assays are also available as diagnostic tools.

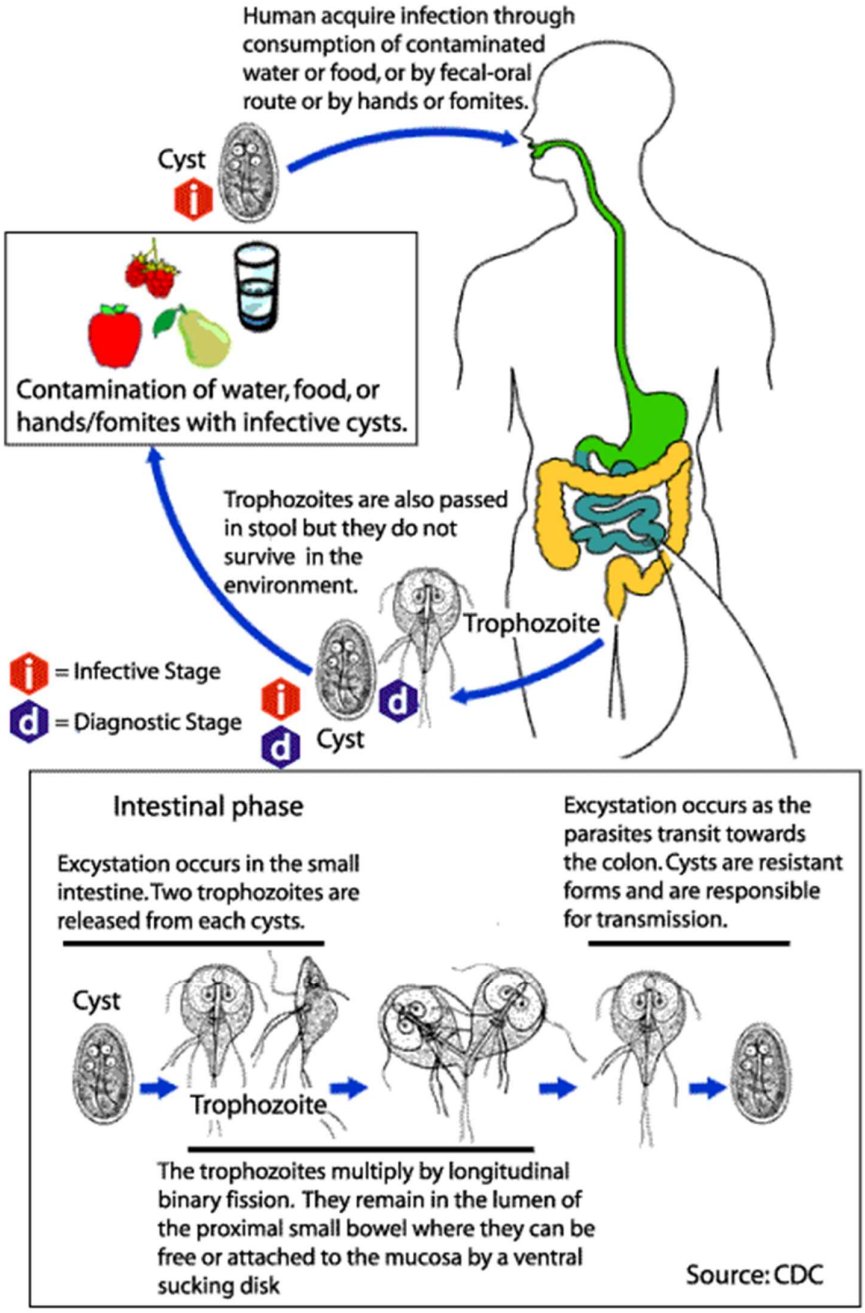


Figure 1.5 Life cycle of *G. lamblia* [published in (GARDNER, 2011)].

1.4 NIDIAG project and framework of the current MD thesis

This doctoral MD thesis was carried out as part of the NIDIAG research consortium. NIDIAG is a collaborative European research network supported by the European Commission. The consortium aims at an improved clinical management of syndromes that are frequent in low-income countries and are caused by neglected infectious diseases (NIDIAG.; WHO, 2010a). Such diseases include cryptosporidiosis, soil-transmitted helminthiasis and other intestinal infections, for which only few evidence-based recommendations with regard to diagnosis and treatment exist (NIDIAG.). The main objectives of the NIDIAG consortium are the following:

- Establishment of a syndromic diagnosis-treatment concept that primarily focuses on three NTD-related syndromes:
    - Persistent fever (≥ 14 days)
    - Neurological disorders
    - Persistent digestive disorders (≥ 14 days)
- Introduction of new evidence-based diagnosis approaches that can easily be employed in affected settings.
- Provision of guidance and recommendations pertaining to improved clinical management of these syndromes.

This MD thesis was embedded in the NIDIAG study on persistent digestive disorders. This NIDIAG study is registered on ClinicalTrials.gov (identifier: NCT02105714).

2. Goal and objectives of the study

The goal of this work is to describe the aetiological spectrum of persistent digestive disorders in Côte d'Ivoire and Mali, in an attempt to provide future guidance for an improved clinical management of digestive symptomatologies in these settings. To this end, stool samples obtained during a case-control study from symptomatic individuals and healthy controls in Côte d'Ivoire and Mali were analysed using multiplex real-time PCR assays to elucidate the presence of a host of bacterial (*Campylobacter,* EAEC, EIEC, ETEC, *Salmonella, Yersinia)* and parasitic (*Cryptosporidium, D. fragilis, E. histolytica, G. lamblia*) pathogens.

To reach this goal, the following objectives are being addressed:

1. To evaluate the use of real-time multiplex PCR as diagnostic tool for pathogens that cause gastrointestinal disorders in two different low-income countries.
2. To compare results of symptomatic cases with matched asymptomatic controls to allow for an evaluation of the clinical relevance of pathogen-specific PCR results.
3. To assess the infection intensity between different pathogens and different patient groups.
4. To elucidate the advantages and disadvantages of multiplex PCR assays in comparison to conventional diagnostic methods.
5. To contribute to the development of evidence-based recommendations for the clinical management of digestive disorders in West Africa.

3. Materials and methods

3.1 Characteristics of the NIDIAG study on persistent digestive disorders

During the NIDIAG project, a prospective study was established to investigate the two main components of digestive disorders, i.e. persistent diarrhoea (dysenteric or non-dysenteric), and persistent abdominal pain.

The study on persistent digestive disorders was designed as case-control study, in an attempt to infer the likely aetiological role of the different pathogens. It was aimed to include a total of 2.000 symptomatic patients and 2.000 matched asymptomatic controls in four different countries, namely Côte d'Ivoire, Mali, Indonesia and Nepal. Each symptomatic case and asymptomatic control provided a stool sample that was examined on site for the presence of bacteria and parasites. Subsequently, every infected participant was offered a specific treatment according to the diagnosed pathogen. Follow-up visits were also performed to evaluate and document the response to the treatment.

Within the study, the following diagnostic techniques were carried out on site:

- Microscopic examination and stool concentration techniques for the diagnosis of intestinal protozoa and helminths.
- Stool-based RDTs for the diagnosis of *Cryptosporidium* spp. and *G. intestinalis*.
- Urine-based RDTs for the detection of *Schistosoma mansoni* (only in the African study sites).
- Bacteriological stool culture for the diagnosis of selected enteric bacteria in patients presenting with persistent diarrhoea.
- PCR assays for the detection of bacterial, parasitic and viral pathogens.

For the current MD thesis, all stool samples that had been collected in Côte d'Ivoire and Mali between August 2014 and December 2015 were considered. These samples were transferred to the Institute of Medical Microbiology and Hygiene (IMMH) at Saarland University Medical Centre in Homburg/Saar, Germany, for application of molecular diagnostics as described below.

3.2 Study areas: Mali and Côte d'Ivoire

The NIDIAG study on persistent digestive disorders was conducted in four tropical countries, two in West Africa (Côte d'Ivoire and Mali) and two in South Asia (Indonesia and Nepal). The study countries were selected based on the following characteristics:

- Low- or middle-income country
- Probable presence of target pathogens (based on previously available data)
- Existence of epidemiological data on digestive disorders, particularly persistent diarrhoea and persistent abdominal pain
- Different healthcare systems

Similar to the participating Asian countries, Côte d'Ivoire and Mali have established national programmes for controlling NTDs, but lack setting-specific diagnosis-treatment concepts for the clinical management of persistent diarrhoea and persistent abdominal pain (POLMAN et al., 2015). To represent a broad range of intestinal symptoms, specific healthcare centres and related hospitals were selected as study sites after taking following criteria into account (POLMAN et al., 2015):

- Location in resource-constrained settings (remote rural or deprived urban areas)
- Good collaboration between primary healthcare centre and district hospital
- Adequate patient care in referral hospital
- Frequent occurrence of persistent diarrhoea and persistent abdominal pain in the study area
- Availability of technical equipment and expert staff to conduct standard first-line tests and to process stool samples for external work-up

For this MD thesis, the following two sites provided stool samples (Figure 3.1):

- Mali
    - Niono District Reference Healthcare Centre based in Niono
- Côte d'Ivoire
    - Hôpital Méthodiste de Dabou, regional reference hospital located in south Côte d'Ivoire

The Republic of Mali is a large country (1.240.192 km²) located in West Africa (CENTRAL INTELLIGENCE AGENCY, 2020b). The desert covers a major part of the country's territory, particularly in the north, where the climate is warm and dry, while the south is more humid, partly with rain from June to November (CENTRAL INTELLIGENCE AGENCY, 2020b). The country counts approximately 19.1 million inhabitants (status: 2018) as total population, with almost half of it being younger than 15 years and only 0.5 million belonging to an age group ≥65 years (UNITED NATIONS, 2018). The main economic activities are agriculture, fishing, livestock breeding and mining (TRAORÉ, 2009). With a gross national income of 1.965$ per head, Mali has been classified as a low-income country (UNITED NATIONS, 2018). Similar figures have been announced by the United Nations (UN), who place Mali on rank 184 out of 189 countries in the Human Development Index (status: 2018), a prosperity indicator for countries (UNITED NATIONS, 2018). Numerically, every third habitant lives below the poverty level. Hence, Mali is considered as one of the poorest countries in the world (CENTRAL INTELLIGENCE AGENCY, 2020b). In addition, high rates of corruption are reported and it is assumed that two third of children younger than 15 years of age are illiterate (UNITED NATIONS, 2018). In recent years, the government, a presidential democracy, made efforts to improve the educational system, but political turmoil has recently halted such initiatives. Furthermore, a more modern infrastructure is being developed. Only 42.4% of the population live in urban areas (UNITED NATIONS, 2018), thus large parts of the population lack access to public transportation and travelling from

Figure 3.1 World map and country-specific maps of Côte d'Ivoire, Indonesia, Mali and Nepal, indicating the sites of patient recruitment for the NIDIAG study on persistent digestive disorders [published in (POLMAN, 2015)].

rural to urban areas remains difficult, especially during the rainy season, when access to certain parts of the country is almost impossible (TRAORÉ, 2009). Regarding mortality rates, diarrhoeal diseases remain one of the leading cause, ranking only second (8%) to lower respiratory infections (WHO, 2015b).

Accordingly, there is a great shortage of clean water and acceptable sanitary facilities. As Figure 3.2a illustrates, the utilisation of proper sanitation has only marginally increased over the past two decades. Although an improvement in usage of drinking water sources has been noticed, figures still remain unsatisfactory and there is an urgent need for improvement.

The second study site providing samples for this MD thesis was the Republic of Côte d'Ivoire (Ivory Coast), which possesses 322.463 km² and counts a population of 25.1 million inhabitants (CENTRAL INTELLIGENCE AGENCY, 2020a). The climate is dry in the north, while it is more humid toward the southern coast (CENTRAL INTELLIGENCE AGENCY, 2020a). Côte d'Ivoire is relatively urbanised, with 51.7% of the population living in urban areas (CENTRAL INTELLIGENCE AGENCY, 2020a). The most common religions practiced by Ivorian people are Christianity, animism and Islam (CENTRAL INTELLIGENCE AGENCY, 2020a). Similar to Mali, Côte d'Ivoire is a young nation with 48.5% of the population belonging to an age group of under 15

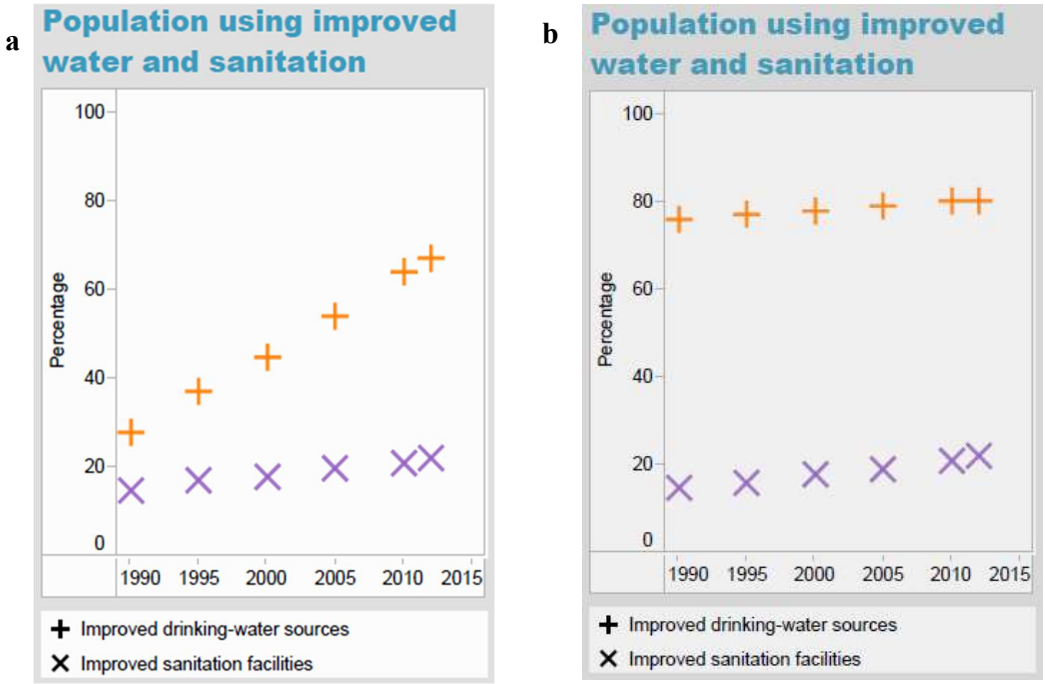


Figure 3.2 The progression to use improved water and sanitation in (a) Mali and (b) Côte d'Ivoire [published in (WHO, 2015; WHO, 2015)].

years (CENTRAL INTELLIGENCE AGENCY, 2020a; WHO, 2015a). Although Côte d'Ivoire has been classified as a lower middle-income country with a gross national income of 3.589$ per head and being ranked 165th out of 189 in the Human Development Index (status: 2018) (UNITED NATIONS DEVELOPMENT PROGRAMME), the country still struggles with a poverty rate of 46.3%, which is intensified by a high illiteracy rate despite mandatory attendance at primary school (WHO, 2015a). Moreover, illiteracy rate is assumed to be much higher in rural than in urban areas (MINISTERE DE LA SANTÉ ET DE LA LUTTE CONTRE LE SIDA, 2011). Main business sectors include agriculture, but commodity trade has also gained in importance during the past 10-15 years (CENTRAL INTELLIGENCE AGENCY, 2020a). The political system can be characterised as a presidential democracy. The public transportation is primarily via road, followed by water transportation (INSTITUT NATIONAL DE LA STATISTIQUE). According to the WHO, diarrhoea ranks fourth (5.4%) in overall causes of death, while digestive disorders ranking sixth among infant deaths (WHO, 2015a). The proportion of population using clean drinking water has remained stable at around 80% in the past two decades (Figure 3.2b). In contrast, utilisation of improved sanitary facilities still remains unsatisfactory.

3.3 General characteristics of the study population

Niono is a sub-regional administrative town, approximately 300 km northeast of Mali's capital Bamako, and covers an area of 23.063 km² with 312.123 inhabitants (status: 2010). Niono consists of twelve communes, thereof one urban and eleven rural areas, including 249 villages. Islam is the predominant religion, followed by animism and Christianity. From October to June a dry climate is expected, followed by a rainy season from July to September. Niono's landscape is divided into two different areas: an irrigated area, which is economically strong and inhabited by a large population, and a non-irrigated area, which occupies two third of the region but represents weak economy and low density of population. The economic activities are based on agriculture, fish and cattle rearing, business in trading, transporting and marketing. Pre-school education, basic and general secondary education and community school systems comprising the Koran schools represent the regional education system in Niono.

Dabou, in the south of Côte d'Ivoire, belongs to the District of Lagunes, Region Grands-Ponts, which is home to 356.495 inhabitants, half of them being males (status: 2014)

(INSTITUT NATIONAL DE LA STATISTIQUE). The department of Dabou covers 2.260 km² (ASSEMBLÉE DES RÉGIONS ET DISTRICTS DE CÔTE D'IVOIRE), with a small town and eleven villages belonging to the same health district (BECKER et al., 2015b). Despite geographical proximity to the economic capital Abidjan (approximately 30 km west), Dabou struggles with a high unemployment rate (INSTITUT NATIONAL DE LA STATISTIQUE). Agriculture is the main branch of industry.

3.4 Healthcare systems in the study countries

Ideally, the major goal of a healthcare system is to provide good healthcare to all of its citizens (WHO). Unfortunately, basic healthcare centres in low-income countries often lack funding, qualified workforce and sufficient laboratory equipment, which is a prerequisite for a higher quality of care (BECKER et al., 2013). Consequently, the clinical management of e.g. persistent digestive disorders turns out to be challenging (BECKER et al., 2013).

The healthcare system in Mali is based on primary healthcare strategies and the principles of the so-called Bamako Initiative (TRAORÉ, 2009). Main goals of the Bamako Initiative are assurance of geographical and financial availability of essential medicines and reduction of infant and maternal mortality (TRAORÉ, 2009). The health system is organised in three levels:

(i) Community Health Centre (Centre de santé communautaire)
(ii) District Reference Centre (Centre de réference)
(iii) National University Hospital Centre and the regional hospital

The District Reference Healthcare Centre in Niono, where study participants for Mali were recruited, is part of second level of healthcare. Moreover, there are seven national institutes which work for the Ministry of Health, i.a. the INRSP based in Bamako, which was also involved in the NIDIAG study project. Besides, Mali also offers governmental healthcare institutes, private hospitals, pharmacies and laboratories. However, a closer look at Table 3.1 reveals the excessive demand of clinical workforce, since one physician is responsible for close to 8.600 inhabitants. Midwives bear the highest burden, with 11.400 inhabitants per personnel.

The health system in Niono is divided into two groups: Community Health Centre and Reference Health Centre. The latter one employs doctors, medical officers, medical assistants, laboratory and health technicians, nurses, midwives and miscellaneous staff. Furthermore, the health centre includes other medical fields like dentistry, gynaecology, ophthalmology, radiology, laboratory and medical ward for consultation and two chemists.

Table 3.1 Accessibility of healthcare workers in Mali: inhabitants per physicians, midwives or nurses [adapted from (TRAORÉ, 2009)].

Health workforce	Total number of personnel	Ratio: personnel/inhabitants
Medical physician/Clinician	14.601	1/8.646
Midwives	1.106	1/11.413
Nurse/Medical Assistants	6.482	1/1.947

Table 3.2 Accessibility of healthcare workers in Côte d'Ivoire: inhabitants per physicians, midwives or nurses [adapted from (MINISTERE DE LA SANTÉ ET DE LA LUTTE CONTRE LE SIDA, 2011)].

Health workforce	Total number of personnel	Ratio: personnel/inhabitants
Medical physician/Clinician	3.220	1/5.695
Midwives	2.553	1/2.331
Nurse/Medical Assistants	7.361	1/3.717

The health system in Côte d'Ivoire consists of three administrative levels and two management sides, one for administrational work and the other for providing healthcare, comprising the following institutions (MINISTERE DE LA SANTÉ ET DE LA LUTTE CONTRE LE SIDA, 2011; USAID - FROM THE AMERICAN PEOPLE):

(i) Health Facilities of First Contact
(ii) Health recourse institutions as first reference centre
(iii) Recourse health facilities as second reference institutes

The Health Facility of First Contact is supposed to cover all villages. In 2011, Côte d'Ivoire had nine national public institutions, including four Hospital Centres and University clinics (Centres Hospitaliers et Universitaires), 17 regional hospitals (Centres Hospitaliers Régionaux) and 66 hospitals (Hôpitaux Généraux) (USAID - FROM THE AMERICAN PEOPLE). However, health-related infrastructure is still insufficient and distributed unevenly in different medical fields as well as all over the country (USAID - FROM THE AMERICAN PEOPLE). For financial reasons, technology is frequently not up to date. Moreover, rural areas often lack access to health centre due to poor infrastructure. Hospitals in resource-constrained settings even lack access to power supply system and drinking water resource (USAID - FROM THE AMERICAN PEOPLE). A private health sector is also establishing itself in the country. Although employment in health sectors has improved, Table 3.2 illustrates the persisting shortage of health workers, especially in rural areas (USAID - FROM THE AMERICAN PEOPLE).

3.5 Ethical considerations

The study protocol relevant to this MD thesis was approved by several ethics committees before the recruitment of study participants started. The NIDIAG collaboration established a study protocol, which was presented to the institutional review boards (IRBs) at the Institute of Tropical Medicine (ITM) in Antwerp (Belgium) and the Swiss Tropical and Public Health Institute (Swiss TPH) in Basel (Switzerland), where the protocol was revised, modified and permitted. The final version of study protocol was approved by the University of Antwerp in Belgium, the Ethikkommission beider Basel (EKBB) in Switzerland and the Institut National de Recherche en Santé Publiqué in Mali as well as the Ministry of Health in Côte d'Ivoire.

The NIDIAG trial on persistent digestive disorders is registered on ClinicalTrials.gov (identifier: NCT02105714) (see Appendix, p.91).

3.6 Patient recruitment and sample collection

3.6.1 Selection criteria for patient recruitment

Study participants had to fulfil several criteria to be eligible for inclusion in the study. Persistent diarrhoea and persistent abdominal pain were regarded as main clinical signs of digestive disorders. Persistent diarrhoea was judged according to the WHO definition (three or more liquid bowel movements per day lasting ≥14 days). Since abdominal pain was not defined specifically by WHO, NIDIAG set up a definition by describing persistent abdominal pain as localised or diffuse stomach pain lasting for at least 14 days with possible intermittence (POLMAN et al., 2015). Symptomatic patients for the case-control study were recruited considering following criteria:

- All individuals aged ≥ 1 year with persistent diarrhoea (≥14 days) and /or
- Children/adolescents aged 1-18 years with persistent abdominal pain (≥14 days).

While symptomatic cases were recruited irrespective of sex and residency, asymptomatic controls were eligible when they matched to symptomatic patients in the following criteria: same age group, same sex and nearby residency. Additionally, healthy controls had to be free of any gastrointestinal complaints during at least the past two months before enrolment.

Patients and controls presenting with the following symptoms were excluded from the study (POLMAN et al., 2015):

- Unwilling or unable to give declaration of consent.
- Patient or control was not fulfilling the criteria mentioned above.
- Patient or control with complaints of icterus (assessed by examining both conjunctivae).
- Patient or control was already part of another diagnostic and/or clinical study.
- Patient or control was suffering from severe disease and needed urgent intensive or surgical care.

3.6.2 Recruitment process

Prior to the start of patient recruitment, local authorities were informed in detail about the study procedures and consent was obtained. The study investigator was informed by a

health worker whenever a patient with complaints of persistent digestive disorders was admitted to the study centre. On the basis of medical history, existing laboratory tests and a short clinical examination the investigator decided whether the individual was eligible. For diagnostic purposes, the patient had to provide a stool and a urine sample in a pre-labelled container, ideally on the same day or in the next morning. Several diagnostic tests were performed on site. For this MD thesis, 500 mg of stool specimens were transferred into a 1 ml Eppendorf tube using a clean spatula. In case of liquid stool, 500 µl of stool were pipetted into a 1 ml Eppendorf tube. Afterwards, 1-2 ml of 96% of ethanol were added to the tubes containing the sample, which were gently vortexed for approximately 30 seconds and then kept in refrigerator at 4°C at the respective study site (VAN LIESHOUT L, 2010). Overall, three aliquots per study participant were collected. After three to five days, when laboratory results were available, participants were invited again. The final diagnosis of each patient was based on the combination of laboratory tests, clinical response to treatment and additional investigations requested by the treating clinician (e.g. abdominal ultrasound examination) (POLMAN et al., 2015). If the patient still suffered from persistent digestive disorders, further examinations and laboratory tests, including stool sampling, were performed. The participants for the control group were invited after ensuring exclusion of any gastrointestinal symptoms. Similar to cases, asymptomatic participants had to give their consent for taking part in the study. The clinical examination as well as the sample taking and processing was identical to the cases.

3.6.3 Storage conditions and transfer of stool aliquots to Europe

After recruitment and processing of samples, all three ethanol-preserved aliquots were stored in a refrigerator at 4°C at the respective study sites (Niono and Dabou). In Mali, the native stool aliquots were transferred from Niono District Reference Healthcare Centre to the Parasitological Department of the INRSP in Bamako once a week, where samples were stored in freezer at -20°C. From there, the aliquots were transferred in three different supplies to Europe for further molecular testing. In Côte d'Ivoire, stool samples were stored at -20°C in Dabou before being transferred to Homburg for the PCR tests carried out during this MD thesis.

3.7 Automatic nucleic acid extraction

The nucleic acid extraction of stool samples was performed in Homburg using the Maxwell® 16 MDx instrument. The machine uses the principle of sample lysis and binding to paramagnetic particles to separate DNA from body fluids like whole blood, buffy coat or – as in this case – ethanol-fixed stool (PROMEGA, 2012, 2014). The procedure was slightly modified according to recommendations put forth by the company R-Biopharm, which provided the PCR reagents and the thermocycler for the current study. With the Maxwell® system, there are two different systems for extracting DNA, the Standard Elution Volume (SEV) (for up to 400 µl,) and the Low Elution Volume (LEV) (for up to 25 µl). Here, LEV was used together with the Maxwell® 16 Blood DNA Purification Kit. The purification kit includes LEV cartridges, lysis buffer, elution buffer, elution vessels and plungers (Figure 3.3). First, the stool aliquots, the Internal Control DNA (ICD) and Internal Control RNA (ICR), which were frozen at -20°C, were thawed.

The Maxwell instrument is capable of extracting a maximum number of 16 samples in parallel. After thawing, a 200 µl stool aliquot was diluted with 400 µl of nuclease-free water and vortexed until the mixture homogenised. After dilution and mixing, the samples were centrifuged for approximately 30 seconds at 3000 rpm to obtain a supernatant for further analysis (Figure 3.4). In the next step, the sealed LEV cartridges were placed in the holder. After removing the protective film, the partition of the cartridge with seven individual wells became visible (Figure 3.5). Well 1 (facing the unnumbered side of the holder) contained lysis buffer, whereas the second well was relevant for the paramagnetic particles. The remaining wells included the wash buffer. In the first well, 300 µl of lysis buffer, 300 µl stool supernatant and 20 µl of ICD and ICR (for viral detection), respectively, were added. The ICD/ICR was used as inhibition and extraction control. Using a forceps, the plungers were placed in the last ridge and the elution tubes were filled with 100 µl of elution buffer. All samples were labelled to avoid misidentification of a sample. Next, the holder with the cartridges was placed in the Maxwell® 16 MDx instrument (Figure 3.6). Then, the purification procedure was started by selecting the DNA Blood Program which takes approximately 46 minutes. The instrument's first process is to break up every cell wall contained in the sample. To that end, a chaotropic agent, detergent and/or alcohol lyse the membrane lipid of cells in order to release DNA/RNA, which is then bound to paramagnetic particles (PROMEGA, 2014). Contaminations with stool and other cell components are washed away in the following wells. Afterwards, the DNA/RNA is resuspended in elution tubes containing the elution

buffer. If the extraction is instantly followed by PCR, the nucleic acid can shortly be kept at ambient temperature or in the refrigerator. Otherwise, the tubes were frozen in a labelled box at -20°C.

Figure 3.3 Required tools for nucleic acid purification to perform multiplex real-time PCR for a case-control study in Mali and Côte d'Ivoire. First lane from left to right: Stool aliquots with ICD and ICR, filter tips, lysis buffer, elution buffer. Second lane from left to right: Elution tubes, holder with sealed LEV cartridges and elution vessels, plungers.

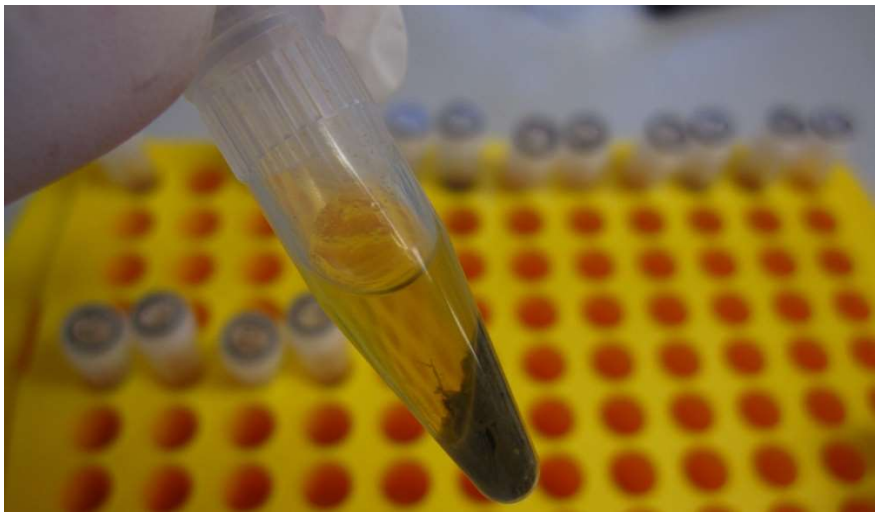


Figure 3.4 Supernatant obtained after diluting 200 µl stool sample with 400 µl of nuclease-free water and centrifuging at 3000 rpm for 30 seconds to obtain nucleic acid for further analysis by multiplex real-time PCR for a case-control study conducted in Mali and Côte d'Ivoire.

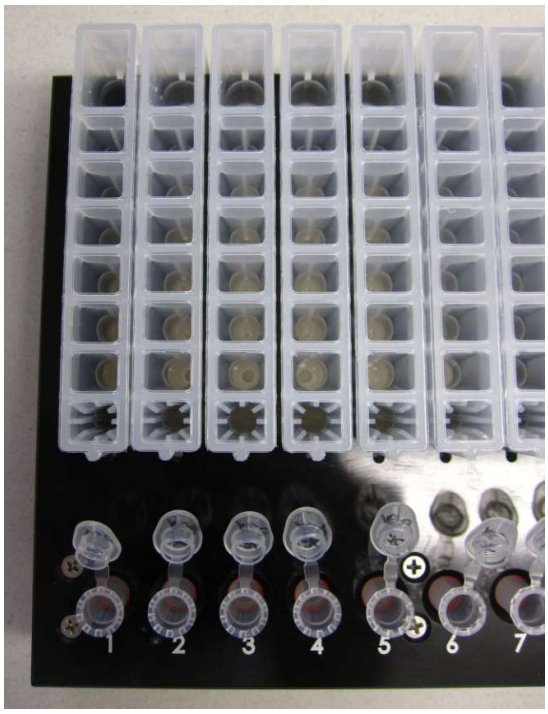


Figure 3.5 Holder with LEV cartridges and labelled elution vessels to extract nucleic acid for amplification by multiplex real-time PCR. 300 µl stool supernatant and 20 µl of ICD and ICR respectively, are added to well one. The plungers are placed in the last ridged well. The elution vessels are filled with 100 µl of elution buffer

Figure 3.6 Maxwell® 16 MDx instrument with prepared holder for nucleic acid purification utilised to conduct a case-control study in Mali and Côte d'Ivoire.

3.8 Multiplex real-time polymerase chain reaction (PCR) for multiple pathogens

3.8.1 Stratagene Mx3005P thermocycler

The Stratagene Mx3005P thermocycler was used for all analyses during this study. The aim of PCR assays is to amplify and then quantify nucleic acids of a given target pathogen. Conventional singleplex PCR targets a single pathogen and is only capable of collecting data at the end of the reaction. Due to gradual limiting reagents and accumulating inhibitors, conventional instruments fail to correctly determine the exact amount of DNA. The Stratagene Mx3005P (Figure 3.7) is an instrument for multiplex real-time PCR and as such, it differs from other conventional instruments by measuring the quantity of DNA from the very first cycle. The thermocycler collects the data after each cycle. Consequently, the real-time method is more precise since it emits a signal proportional to the amount of synthesised nucleic acid (PLATTS-MILLS et al., 2013). Furthermore, the multiplex technique allows the detection of multiple target sequences by means of synchronously running PCRs in a single reaction. This technique is based on the detection of fluorescence-marked oligonucleotides (e.g. Molecular Beacon and TaqMan probes), which are complementary to targeted DNA sequences and emit detectable fluorescence light, when the quencher (=fluorescence quenching) is distanced from the fluorophore (= fluorescence reporter) on the other end (REISCHL et al., 2012). Thus, the more replications take place, the more intense the fluorescence becomes. As a result, the amount of fluorescence at any given cycle is directly proportional to the amount of specific product present at that time (REISCHL et al., 2012). Since the Stratagene Mx3005P instrument is not only a real-time, but also a multiplex PCR instrument, a large variety of fluorescence colours allows focussing a great number of targets in a single reaction. For the current experiment four different 'stool panels' were provided, which contain all required reagents for performing PCR and differentiate between various pathogens. The 'bacterial stool panel' (BSP) allows differentiation of *Salmonella* spp*., Campylobacter* spp*.,* and *Y. enterocolitica.* The second panel detects EAEC, while the third panel traces other *E. coli* strains like EIEC *(ipaH)* and ETEC (*LT* and *ST*). Third, the 'parasitic stool panel' (PSP) was employed for detection of *Cryptosporidium, D. fragilis, E. histolytica* and *G. lamblia*. Each stool panel contains five reagents, which are sufficient for 100 determinations (Figure 3.8). The first reagent is the Reaction Mix, which includes fluorescence marked hybridisation probes, while the second reagent

contains a thermo stable Taq-Polymerase. For extraction and inhibition control ICD is provided as well. With the aid of different detection channels, the Stratagene Mx3005P is capable of targeting many pathogens in a single PCR run. Table 3.3 displays an overview of all pathogens and their detection channels, which detect at different wavelengths. The wavelength for absorption reveals the spectrum where a quencher absorbs light, while the emission spectrum demonstrates the wavelength where electromagnetic radiation is emitted by fluorophore and captured and measured by the multiplex real-time PCR instrument.

Figure 3.7 Stratagene Mx3005P multiplex real-time PCR utilised in a case-control study on persistent digestive disorders in Mali and Côte d'Ivoire.

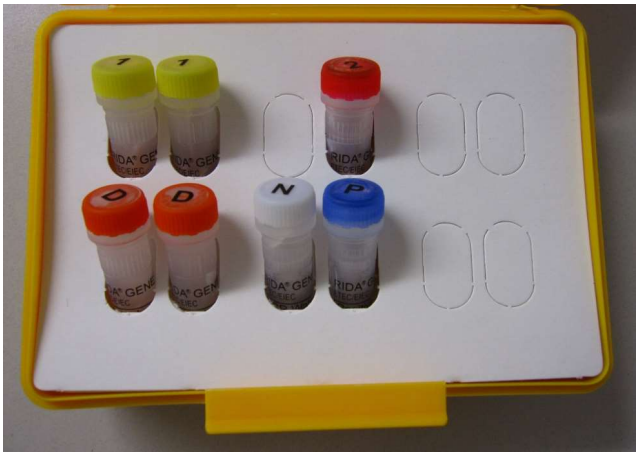


Figure 3.8 Stool panel with five reagents to perform multiplex real-time PCR with the Stratagene Mx3005P. 1: Reaction Mix. 2: Taq-Polymerase. D: Internal Control DNA. N: Negative Control. P: Positive Control.

Table 3.3 Detection channel with associated wavelength and detected pathogens for the Stratagene Mx3005P multiplex real-time PCR [adapted from (R-BIOPHARM; R-BIOPHARM; R-BIOPHARM; R-BIOPHARM)].

Detection Channel (Absorption, Emission)	Detection (Stool Panel)
ATTO (440 nm, 492 nm)	*D. fragilis* (PSP)
FAM (492 nm, 516 nm)	*Salmonella* spp. (BSP)
	EAEC (EAEC)
	LT (ETEC/EIEC)
	G. lamblia (PSP)
HEX (535 nm, 555 nm)	ICD (BSP, EAEC, ETEC/EIEC, PSP)
ROX (585 nm, 610 nm)	*Y. enterocolitica* (BSP)
	ipaH (ETEC/EIEC)
	E. histolytica (PSP)
Cy5 (635 nm, 665 nm)	*Campylobacter* spp. (BSP)
	ST (ETEC/EIEC)
	Cryptosporidium spp. (PSP)

3.8.2 Working steps

First, the Stratagene instrument is switched on in order to preheat the lamp for approximately 25 minutes. While preheating, the plate setup can be prepared on the computer (Figure 3.9). The Master-Mix is prepared in a DNA-free room to minimise contamination with nucleic acid during pipetting. Likewise, Reaction Mix and Taq-Polymerase are stored separately from the nucleic acid containing tubes (Positive and Negative Controls, ICD/ICR and sample DNA). Before creating the Master-Mix, the Reaction Mix is thawed in a dark box to prohibit influences by light. Meanwhile four Eppendorf vessels are labelled, one for each panel (Figure 3.10).

All	1	2	3	4	5	6	7	8	9	10	11	12
A	EK 62 Campy Yersi ICD Salmo	EK 63 Campy Yersi ICD Salmo	EK 65 Campy Yersi ICD Salmo	EK 66 Campy Yersi ICD Salmo	EK 67 Campy Yersi ICD Salmo	EK 68 Campy Yersi ICD Salmo	EK 72 Campy Yersi ICD Salmo	EK 74 Campy Yersi ICD Salmo	EK 77 Campy Yersi ICD Salmo	EK 78 Campy Yersi ICD Salmo	EK 64 Campy Yersi ICD Salmo	EK 69 Campy Yersi ICD Salmo
B	EK 70 Campy Yersi ICD Salmo	EK 71 Campy Yersi ICD Salmo	EK 73 Campy Yersi ICD Salmo	EK 75 Campy Yersi ICD Salmo	EK 76 Campy Yersi ICD Salmo	EK 80 Campy Yersi ICD Salmo	EK 81 Campy Yersi ICD Salmo	EK 83 Campy Yersi ICD Salmo	EK 84 Campy Yersi ICD Salmo	EK 85 Campy Yersi ICD Salmo	PK Campy Yersi ICD Salmo	NK Campy Yersi ICD Salmo
C	EK 62 ICD EAEC	EK 63 ICD EAEC	EK 65 ICD EAEC	EK 66 ICD EAEC	EK 67 ICD EAEC	EK 68 ICD EAEC	EK 72 ICD EAEC	EK 74 ICD EAEC	EK 77 ICD EAEC	EK 78 ICD EAEC	EK 64 ICD EAEC	EK 69 ICD EAEC
D	EK 70 ICD EAEC	EK 71 ICD EAEC	EK 73 ICD EAEC	EK 75 ICD EAEC	EK 76 ICD EAEC	EK 80 ICD EAEC	EK 81 ICD EAEC	EK 83 ICD EAEC	EK 84 ICD EAEC	EK 85 ICD EAEC	PK ICD EAEC	NK ICD EAEC
E	EK 62 ST IpaH ICD LT	EK 63 ST IpaH ICD LT	EK 65 ST IpaH ICD LT	EK 66 ST IpaH ICD LT	EK 67 ST IpaH ICD LT	EK 68 ST IpaH ICD LT	EK 72 ST IpaH ICD LT	EK 74 ST IpaH ICD LT	EK 77 ST IpaH ICD LT	EK 78 ST IpaH ICD LT	EK 64 ST IpaH ICD LT	EK 69 ST IpaH ICD LT
F	EK 70 ST IpaH ICD LT	EK 71 ST IpaH ICD LT	EK 73 ST IpaH ICD LT	EK 75 ST IpaH ICD LT	EK 76 ST IpaH ICD LT	EK 80 ST IpaH ICD LT	EK 81 ST IpaH ICD LT	EK 83 ST IpaH ICD LT	EK 84 ST IpaH ICD LT	EK 85 ST IpaH ICD LT	PK ST IpaH ICD LT	NK ST IpaH ICD LT
G	EK 62 Crypt D.fra E.his ICD Giard	EK 63 Crypt D.fra E.his ICD Giard	EK 65 Crypt D.fra E.his ICD Giard	EK 66 Crypt D.fra E.his ICD Giard	EK 67 Crypt D.fra E.his ICD Giard	EK 68 Crypt D.fra E.his ICD Giard	EK 72 Crypt D.fra E.his ICD Giard	EK 74 Crypt D.fra E.his ICD Giard	EK 77 Crypt D.fra E.his ICD Giard	EK 78 Crypt D.fra E.his ICD Giard	EK 64 Crypt D.fra E.his ICD Giard	EK 69 Crypt D.fra E.his ICD Giard
H	EK 70 Crypt D.fra E.his ICD Giard	EK 71 Crypt D.fra E.his ICD Giard	EK 73 Crypt D.fra E.his ICD Giard	EK 75 Crypt D.fra E.his ICD Giard	EK 76 Crypt D.fra E.his ICD Giard	EK 80 Crypt D.fra E.his ICD Giard	EK 81 Crypt D.fra E.his ICD Giard	EK 83 Crypt D.fra E.his ICD Giard	EK 84 Crypt D.fra E.his ICD Giard	EK 85 Crypt D.fra E.his ICD Giard	PK Crypt D.fra E.his ICD Giard	NK Crypt D.fra E.his ICD Giard

Figure 3.9 Plate setup on MxPro – Mx3005P Software to perform multiplex real-time PCR. E.g. case patients from Côte d'Ivoire EK 62 to EK 85 (in purple field) are investigated in A and B for BSP, C and D for EAEC, E and F for EIEC/ETEC and E and F for PSP. The different colours of pathogen indicate wavelength of detection channel.

Figure 3.10 Labelled reaction tubes for each stool panel (BSP, EAEC, ETEC/EIEC and PSP) to perform multiplex real-time PCR. The Reaction Mix (1) and Taq-Polymerase (2) are transferred into the labelled reaction tubes and are vortexed and centrifuged gently.

To prepare the Master-Mix, a Reaction Mix volume of 19.9 µl per reaction and 0.1 µl of Taq-Polymerase per reaction were required (Table 3.4). As it was advised to add an extra volume of approximately 10% to compensate pipetting errors, the total number of reactions for Master-Mix was up-scaled to 25 total reaction samples. Consequently, 498 µl of Reaction Mix (rounded up from 497.5 µl) were mixed with 2.5 µl of Taq-Polymerase in the labelled tubes (Table 3.4), were vortexed gently and centrifuged briefly. Thereafter the PCR-Mix was prepared, for which the marked PCR plate was put into an ice block to prevent any unwanted reaction between the reagents during further steps. Now 20 µl of Master-Mix were pipetted in the respective well (Figure 3.11). Further steps were continued outside the DNA-free room. Next, 5 µl of extracted DNA sample were added to the respective well, e.g. sample one was added to A1, C1, E1 and G1, sample two in A2, C2, E2 and G2 etc. The last two wells of every second row (B, D, F and H) were reserved for Positive Control in well eleven and Negative Control in well twelve. Analog to DNA extract, 5 µl of each control were added to these wells. Additionally, 1 µl of ICD was included as extraction and inhibition control. With it, the preparation of PCR-Mix was completed. The plate was sealed, spun down in a centrifuge and was ready to be placed in the Stratagene Mx3005P instrument. The plate was positioned into the thermal block and covered with a cap to start the real-time PCR (Figure 3.12). The run took approximately one hour and ten minutes.

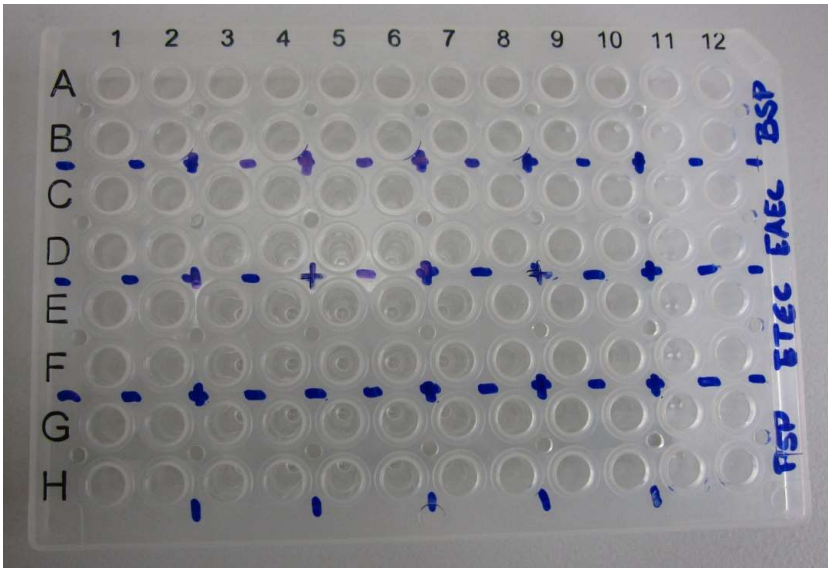


Figure 3.11 Labelled PCR plate to perform multiplex real-time PCR: A and B for BSP, C and D for EAEC, E and F for EIEC/ETEC and E and F for PSP. For better orientation during pipetting every second well is marked.

Table 3.4 Master Mix components with calculated volume per reaction and per 25 reactions.

Master Mix components	Volume per reaction	25 reactions
Reaction Mix	19.9 µl	498 µl
Taq-Polymerase	0.1 µl	2.5 µl
Total	20 µl	500.5 µl

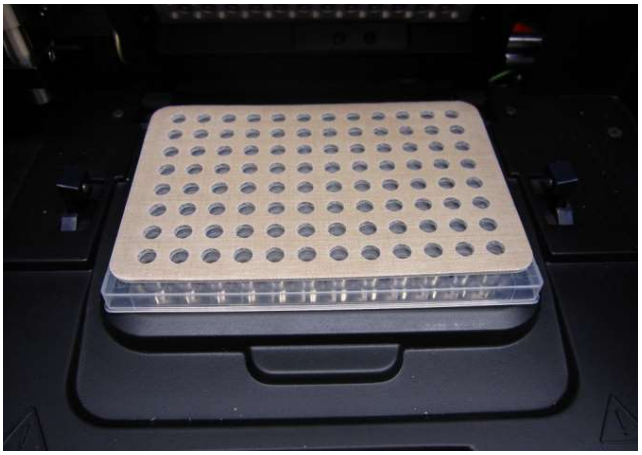


Figure 3.12 PCR plate is positioned in the Stratagene Mx3005P thermal block and covered with the brownish shade of the cap on the upper side to perform multiplex real-time PCR.

3.8.3 Evaluation methods, relevant parameters and result interpretation

The PCR run was analysed with the MxPro-Mx3005P software. Prior to sample evaluation, ICD in all stool panels was verified for extraction and inhibition control by setting up a cycle threshold value (C_t value). The C_t value shows the number of completed PCR cycles at the moment when fluorescence is detectable for the first time. It is measured by setting up a threshold and defining the value, where threshold and curve intersect (Figure 3.13). The C_t value is inversely proportional to the initial targeted DNA log, which means the more DNA template is initially present, the fewer number of cycles are required for the detection of fluorescence signal (REISCHL et al., 2012; STRATAGENE AT, 2009). Hence, the infection intensity can be deducted from the number of completed PCR cycles. Here, C_t values were classified in very high, high, low and very low pathogen quantity (Table 3.5). Amplification of ICD indicated that no inhibition during extraction and/or PCR occurred (Figure 3.14). In the next step, each

panel was interpreted by selecting all samples of one panel and reviewing the amplification of each pathogen. Similar to the evaluation procedure of ICD, every reaction was measured with the setup of a threshold (Figure 3.15). The curve for Positive Control appears in every reaction since it contains genetic information of every pathogen, while Negative Control should not show any amplification since it only contains PCR Water. As long as the Positive Control of each panel showed amplification in range (Table 3.6), there was no indication for inhibition during cycling. Results were interpreted according to Table 3.7.

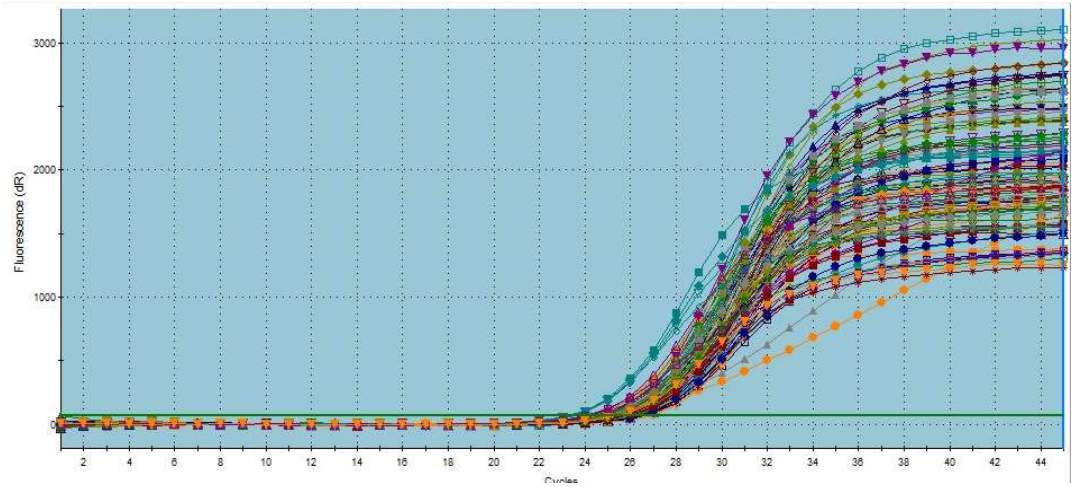


Figure 3.13 ICDs for all four stool panels. The threshold (green line) measures a C_t value between 23.0 and 27.0.

Table 3.5 Suggested classification to evaluate infection intensity on the basis of C_t value for a case-control study on persistent digestive disorders in Mali and Côte d'Ivoire.

Infection intensity	C_t value range
Very high	≤ 24.9
High	25.0 – 29.9
Low	30.0-34.9
Very Low	≥ 35.0

Well	Well Name	Well Comment	Assay	Ct (dR)	Final Call (dR)
F3	EK 182	RIDA®GENE ETEC/EIEC #	ST	15.94	+
F11	PK	RIDA®GENE ETEC/EIEC #	ST	23.00	+
F3	EK 182	RIDA®GENE ETEC/EIEC #	LT	15.13	+
F11	PK	RIDA®GENE ETEC/EIEC #	LT	24.49	+
F3	EK 182	RIDA®GENE ETEC/EIEC #	ipaH	24.31	+
F11	PK	RIDA®GENE ETEC/EIEC #	ipaH	22.75	+
B3	EK 182	RIDA®GENE BSP #	ICD	26.36	+
D3	EK 182	RIDA®GENE EAEC #	ICD	26.83	+
F3	EK 182	RIDA®GENE ETEC/EIEC #	ICD	No Ct	-
H3	EK 182	RIDA®GENE PSP I #	ICD	26.23	+
F11	PK	RIDA®GENE ETEC/EIEC #	ICD	23.65	+

Figure 3.14 Text report of case sample 182 in Côte d'Ivoire. All three pathogens (ST, LT and ipaH) have been detected and show high C_t value. ICD of ETEC/EIEC is inhibited, although ICDs in all other stool panels are available. This leads to the conclusion that ICD in *ETEC/EIEC* has been suppressed by the three detected pathogens *ST, LT* and *ipaH.* Since the Positive Control is in range as well, the reaction is valid.

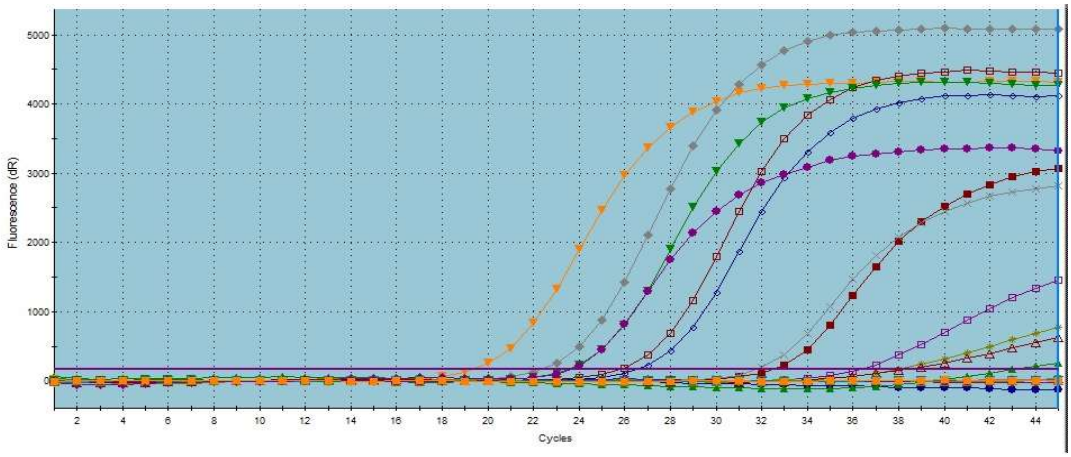


Figure 3.15 Amplification plot of *Campylobacter* spp. The purple line displays the threshold. The purple doted curve shows amplification of Positive Control, while other curves show amplification of detected pathogens in stool. Negative Control is not detectable.

Table 3.6 Suggested reference range of C_t value for Positive Control by R-Biopharm in Darmstadt, Germany.

Stool Panel	Normal range for Positive Control
BSP	22.2 – 29.1
EAEC	22.7 – 29.3
ETEC/EIEC	21.8 – 28.4
PSP	20.6 – 28.2

Table 3.7 Result interpretation of stool samples [adapted from (R-BIOPHARM)].

Target gene	Positive Control	Negative Control	ICD	Final Call
Positive	**Positive**	negative	**positive/negative**	Target gene is detectable. Reaction is **valid.**
Negative	**Positive**	negative	**positive**	Target gene is not detectable. Reaction is **valid.**
Negative	Positive	negative	**negative**	Reaction is **invalid** (PCR or extraction inhibition).
Negative/positive	**Negative**	positive/negative	positive/negative	Reaction is **invalid** (PCR inhibition).
Positive	Positive	**positive**	positive/negative	Target gene is detectable. Reaction is **valid.** Possible contamination of Negative Control.

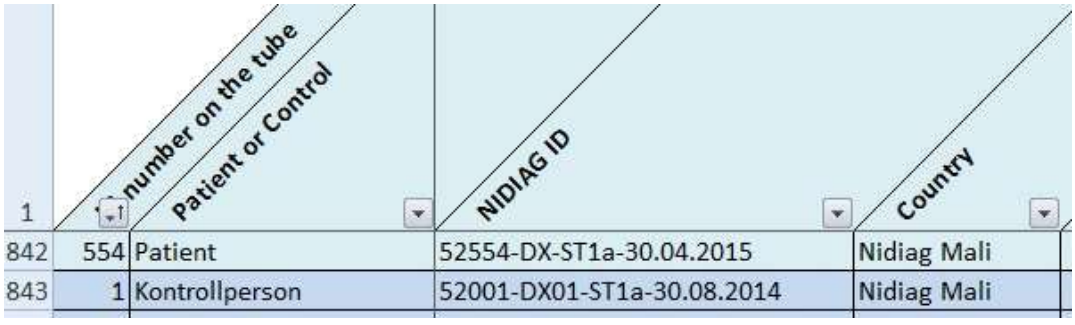
3.9 Data management and statistical analysis

3.9.1 Excel table

The results of the multiplex real-time PCR examinations for each study country were tabulated in an Excel file, which is exemplarily displayed in Figure 3.16. This Excel table comprised information on all detected pathogens and their respective C_t values as well as the relevant parameters 'Positive Control' and 'ICD'. The samples were divided into a case ('Patient') and a control ('Kontrollpatient') group (case → light blue; control → dark blue). Every sample was unambiguously labelled with a unique NIDIAG identifier.

3.9.2 Statistical methods

All data were entered in Excel version 10.0 (edition 2007, Microsoft Corporation). Prevalences and number of co-infections were calculated with Excel, while distributional differences were assessed by Pearson's chi-square. A p-value of ≤0.05 was considered statistically significant.



1	number on the tube	Patient or Control	NIDIAG ID	Country
842	554	Patient	52554-DX-ST1a-30.04.2015	Nidiag Mali
843	1	Kontrollperson	52001-DX01-ST1a-30.08.2014	Nidiag Mali

Figure 3.16 Exemplary NIDIAG ID for cases and control groups: 52=Mali, stool sample No. 554. DX=Case patient. DX01=Matched control. ST1a=First obtained stool sample. 30.04.2015=Date of sample recruitment.

4. Results

4.1 Persistent digestive disorders in Mali

In total, 1.106 individuals participated in the NIDIAG study on persistent digestive disorders in Mali. 1.100 individuals provided samples of sufficiently large quantity to constitute the final study cohort, of whom 553 were symptomatic and 547 individuals did not have any gastrointestinal complaints (asymptomatic controls). In 97.6% of the cases, 'persistent abdominal pain' was the predominant digestive symptom. PCR analyses could be run on all samples, but the automated nucleic acid extraction procedure had to be repeated in 23.9% of all samples because of initial inhibition.

4.1.1 Overall prevalence of pathogens

A considerable amount of bacterial and parasitic pathogens was found in the samples (Table 4.1). EAEC had the highest overall prevalence of 39.9%, followed by *Campylobacter* spp., which was detected in 35.3%. The intestinal protozoa species *G.lamblia* and *D. fragilis* ranked third and fourth with a prevalence of 20.5% and 16.3%, respectively. Other *E. coli* pathovars were found at moderate prevalences, while residual pathogens were rarely detected.

77.4% of all individuals were infected with at least one pathogen, and co-infections were common (Figure 4.1). In 18.4%, ≥3 co-infections were found, and 0.2% of all participants were infected with up to six pathogens concurrently.

Table 4.1 Overall prevalence and absolute numbers of investigated pathogens in a case-control study on persistent diarrhoea and persistent abdominal pain in Mali (n=1.100).

Pathogen	N	Prevalence
EAEC	439	**39.9%**
***Campylobacter* spp.**	388	**35.3%**
G. lamblia	225	**20.5%**
D. fragilis	179	**16.3%**
EIEC *ipaH*	170	**15.5%**
ETEC *LT*	130	**11.8%**
***Cryptosporidium* spp.**	46	**4.2%**
ETEC *ST*	37	**3.4%**
E. histolytica	9	**0.8%**
***Salmonella* spp.**	8	**0.7%**
***Yersinia* spp.**	1	**≤0.1%**

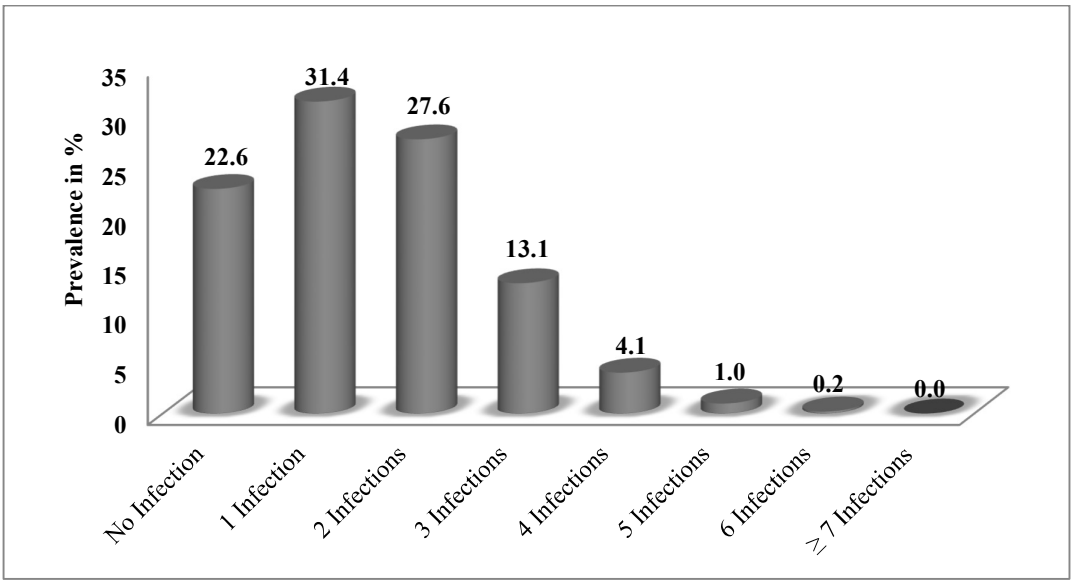


Figure 4.1 Overall co-infection rate with bacterial and parasitic pathogens in a study on persistent digestive disorders conducted in Mali (n=1.100).

4.1.2 Case-control comparison and infection intensity

EAEC had the highest prevalence among all pathogens with 40.5% in the cases and 39.3% in the controls, followed by *Campylobacter* spp., EIEC *ipah* and ETEC *LT* (Figure 4.2). Most of the aforementioned bacteria were significantly more prevalent in symptomatic patients.

Among intestinal protozoa, *G. lamblia* was more frequent among cases.

With regard to infection intensity as assessed by the C_t values, a marked difference was only found for *Salmonella* spp., with symptomatic cases having 'very high' infection intensities as compared to asymptomatic controls with 'low' infection intensities (Table 4.2, Table 4.3). The other pathogens showed similar infection intensities in both study groups.

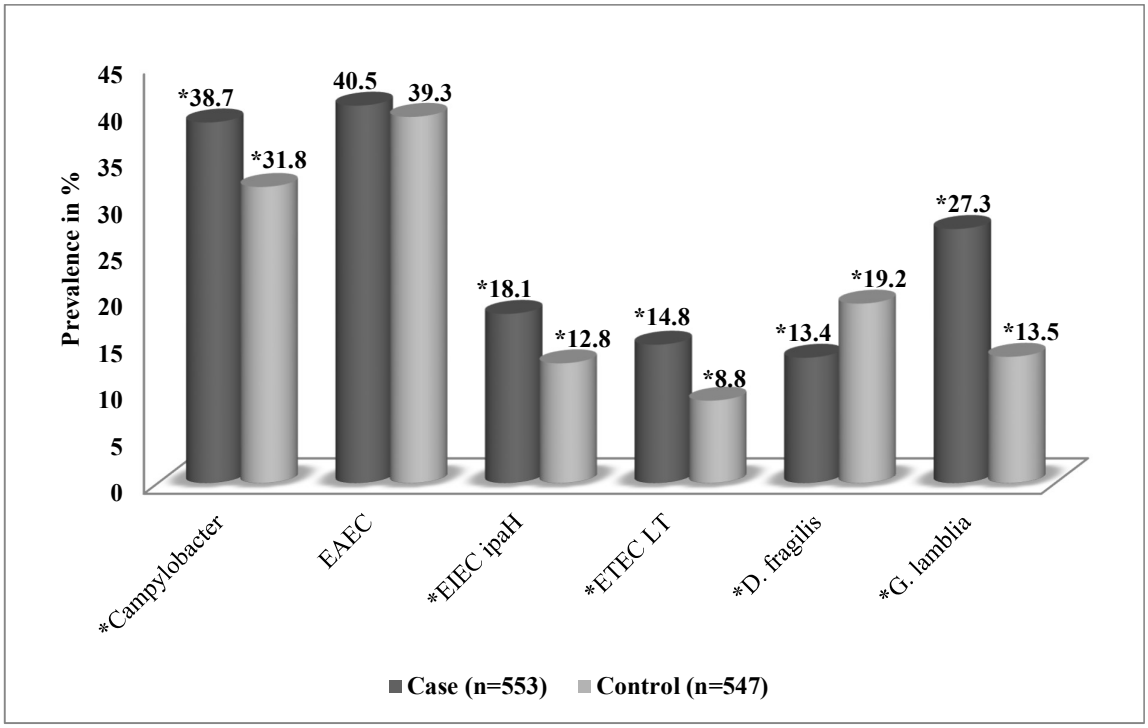


Figure 4.2 Prevalence comparison of most frequently detected pathogens in cases and controls during a study on persistent digestive disorders in Mali. *p-value ≤ 0.05

Table 4.2 Comparison of the prevalences, mean C_t values and respective standard deviation among symptomatic and asymptomatic patients in a study on persistent diarrhoea and persistent abdominal pain conducted in Mali. *p-value ≤ 0.05

Pathogen	Case (n=553)			Control (n=547)		
	N	Prevalence	Mean C_t value (standard deviation)	N	Prevalence	Mean Ct value (standard deviation)
***Campylobacter**	214	***38.7%**	32.4 (5.8)	174	***31.8%**	32.8 (6.2)
***EAEC**	224	**40.5%**	30.8 (6.4)	215	**39.3%**	29.0 (6.5)
***EIEC ipaH**	100	***18.1%**	29.3 (5.0)	70	***12.8%**	28.5 (5.3)
***ETEC LT**	82	***14.8%**	30.5 (5.0)	48	***8.8%**	30.2 (5.0)
***ETEC ST**	22	**4.0%**	29.8 (5.7)	15	**2.7%**	28.3 (4.9)
***Salmonella**	2	**0.4%**	22.8 (8.1)	6	**1.1%**	33.5 (4.6)
***Yersinia**	1	**0.2%**	29.9 (-)	0	**0.0%**	0.0 (-)
***Cryptosporidium**	20	**3.6%**	30.3 (5.8)	26	**4.8%**	32.0 (5.3)
***D. fragilis**	74	***13.4%**	35.4 (7.3)	105	***19.2%**	36.7 (7.5)
***E. histolytica**	6	**1.1%**	28.2 (2.7)	3	**0.5%**	29.2 (3.6)
***G. lamblia**	151	***27.3%**	34.2 (5.0)	74	***13.5%**	34.9 (4.9)

Table 4.3 Heat map of infection intensity, as expressed by mean C_t values, in symptomatic cases and asymptomatic controls during a study on persistent digestive disorders in Mali. The following colour code was used for infection intensity: Dark green, very low intensity (C_t ≥35.0); light green, low intensity (C_t 30.0-34.9); yellow, high intensity (C_t ≤25.0-29.9); red, very high intensity (C_t ≤24.9); white, no infection.

Pathogen	Infection Intensity Mali	
	Case	Control
Campylobacter	Low	Low
EAEC	Low	High
EIEC *ipaH*	High	High
ETEC *LT*	Low	Low
ETEC *ST*	High	High
Salmonella	Very high	Low
Yersinia	High	-
Cryptosporidium	Low	Low
D. fragilis	Very low	Very low
E. histolytica	High	High
G. lamblia	Low	Low

4.1.3 Comparison of co-infections

As shown in Figure 4.3, symptomatic patients had more co-infections than controls. Indeed, 22.8% of all cases were detected with ≥3 pathogens as compared to 13.9% in asymptomatic controls. A total of six concurrent infections were found in 0.4% of cases, but did not occur in controls. Similar trends were observed between bacterial and parasitic pathogens (Figure 4.4, Figure 4.5).

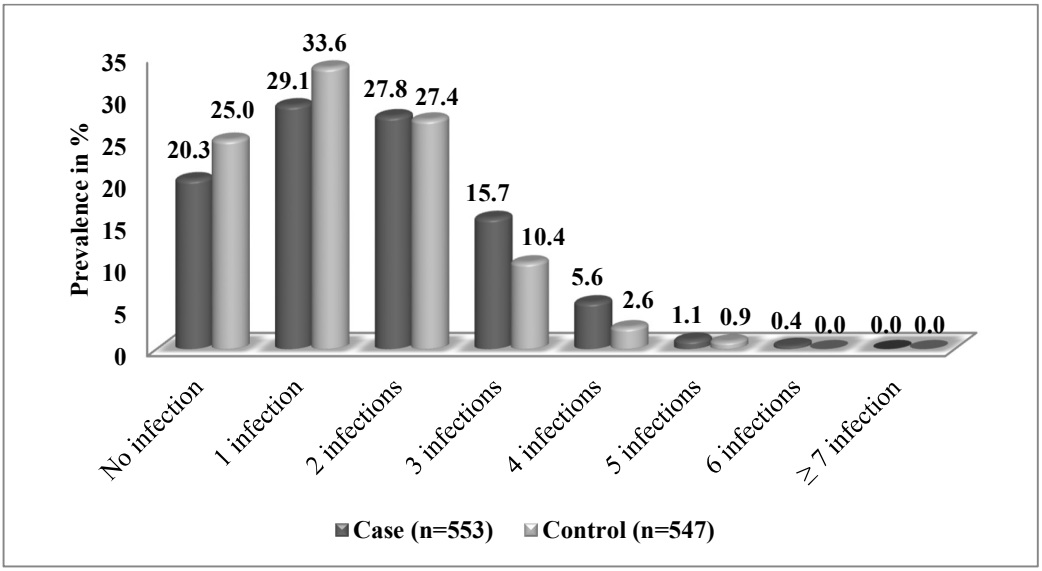


Figure 4.3 Comparison of co-infections in symptomatic patients and matched controls in a study on persistent diarrhoea and persistent abdominal pain in Mali.

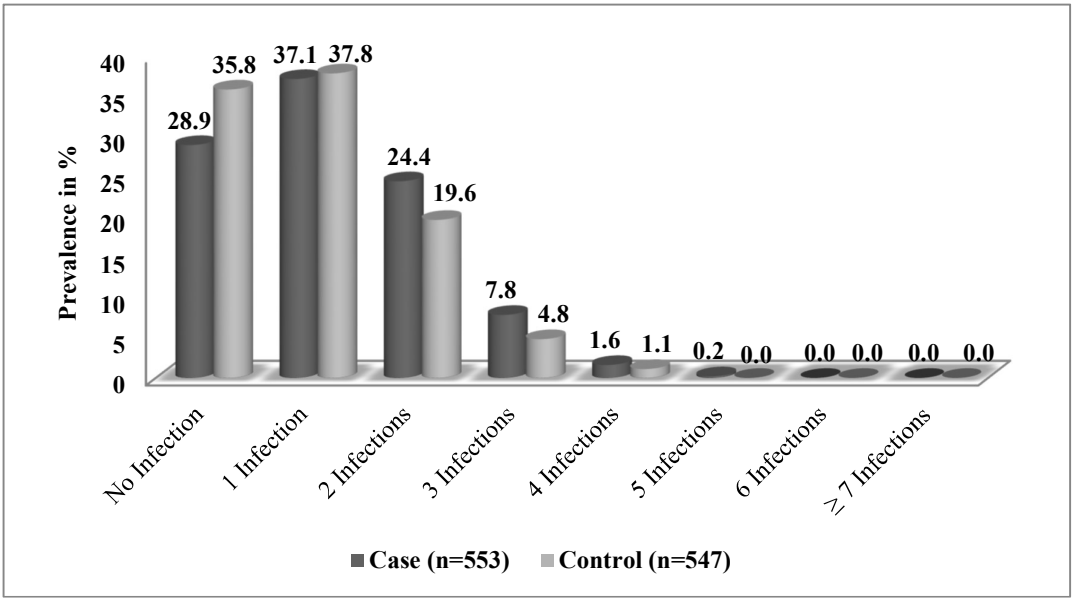


Figure 4.4 Prevalence of bacterial co-infections in symptomatic patients with complaints of persistent diarrhoea and persistent abdominal pain and matched controls in a study conducted in Mali (n=553).

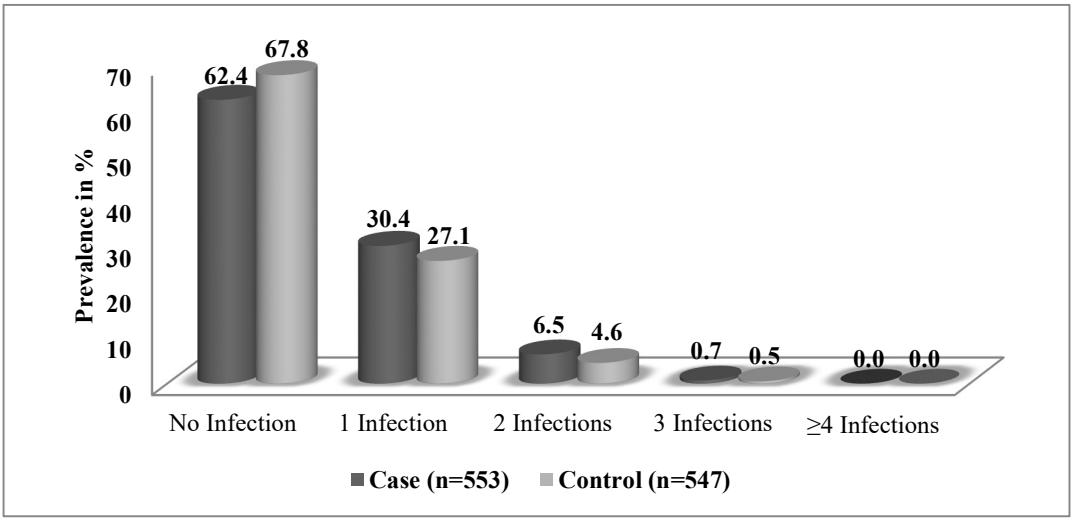


Figure 4.5 Prevalence of parasitic co-infections in cases with persistent diarrhoea and persistent abdominal pain and matched controls in study conducted in Mali (n=553).

4.2 Persistent digestive disorders in Côte d'Ivoire

In total, 520 participants were included in Côte d'Ivoire, all of which except one person provided sufficient stool for the PCR diagnostics. The control group comprised 260 asymptomatic controls, while there were 259 symptomatic patients. Approximately 85% of the cases reported 'persistent abdominal pain' as their predominant digestive symptom. 6.3% of stool samples had to undergo repeated nucleic acid extraction due to initial inhibition.

4.2.1 Overall prevalence

EAEC and *Campylobacter* spp. were the most frequently detected pathogens, with a prevalence of 21.6% and 17.7%, respectively. *G. lamblia*, EIEC *ipaH*, ETEC *LT* and *D. fragilis* were also commonly found, while the remaining pathogens were rare (Table 4.4).

53.4% of all participants were diagnosed with at least one infection, and up to ≥3 co-infections were detected in 6.4% (Figure 4.6). A maximum of six co-infections was detected in one participant.

Table 4.4 Overall prevalence of investigated pathogens in a study on persistent digestive disorders among symptomatic and asymptomatic patients in Côte d'Ivoire (n=519).

Pathogen	N	Prevalence
EAEC	112	**21.6%**
Campylobacter	92	**17.7%**
G. lamblia	60	**11.6%**
EIEC ipaH	53	**10.2%**
ETEC LT	48	**9.2%**
D. fragilis	37	**7.1%**
ETEC ST	11	**2.1%**
Cryptosporidium	11	**2.1%**
E. histolytica	5	**1.0%**
Salmonella	1	**≤ 0.0%**
Yersinia	0	**0.0%**

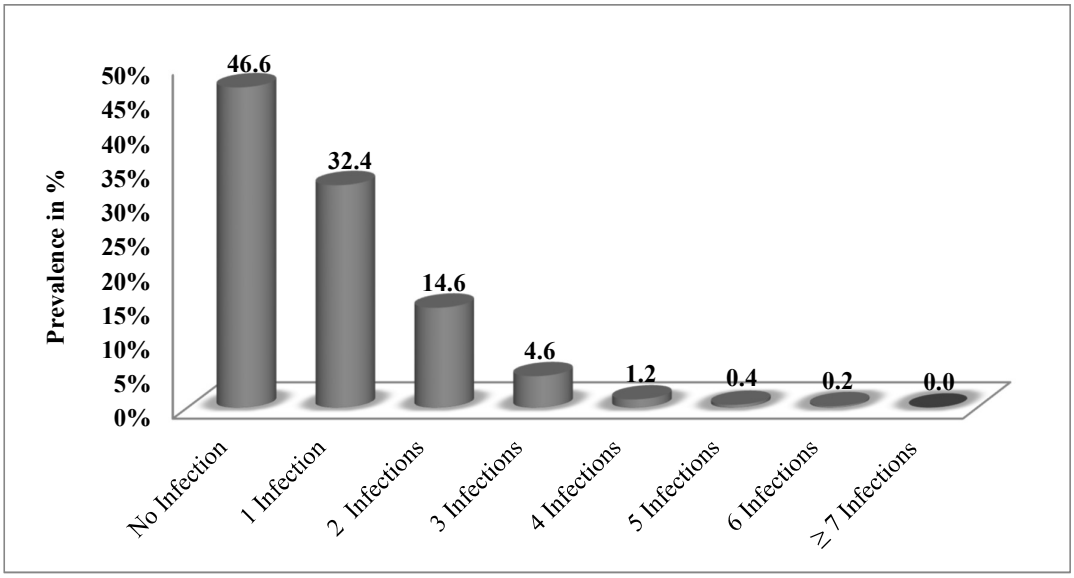


Figure 4.6 Overall co-infection rate in a case-control study on persistent diarrhoea and persistent abdominal pain in Côte d'Ivoire (n=519).

4.2.2 Case-control comparison and infection intensity

EAEC and *Campylobacter* spp. were the predominant pathogens in both symptomatic and asymptomatic individuals (Figure 4.7). Except *E. histolytica*, all pathogens were more prevalent in the cases (Table 4.5). Of note, a distinct difference between the study groups was neither determinable in C_t values nor in infection intensity (Table 4.6).

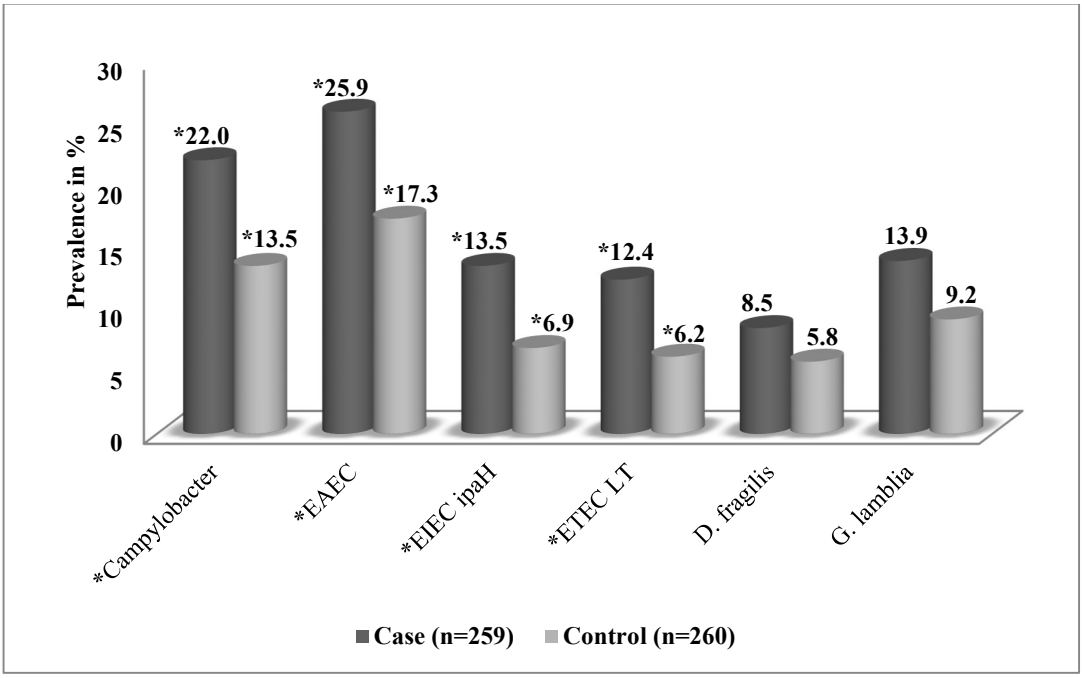


Figure 4.7 Comparison of prevalences of frequently detected pathogens in a case-control study on persistent diarrhoea and persistent abdominal pain conducted in Côte d'Ivoire. *p-value ≤ 0.05

Table 4.5 Comparison of prevalence, mean C_t value and the respective standard deviation among cases with persistent digestive disorders and matched controls in Côte d'Ivoire. *p-value ≤ 0.05

Pathogen	Case (n=259)			Control (n=260)		
	N	Prevalence	Mean C_t value (standard deviation)	N	Prevalence	Mean Ct value (standard deviation)
***Campylobacter**	57	***22.0%**	31.4 (6.3)	35	***13.5%**	33.1 (5.3)
***EAEC**	67	***25.9%**	31.7 (4.9)	45	***17.3%**	33.0 (4.4)
***EIEC ipaH**	35	***13.5%**	29.5 (6.0)	18	***6.9%**	31.3 (5.0)
***ETEC LT**	32	***12.4%**	31.6 (6.3)	16	***6.2%**	30.6 (5.2)
***ETEC ST**	8	**3.1%**	31.3 (6.5)	3	**1.2%**	31.0 (2.3)
***Salmonella**	1	**≤0.0%**	35.9 (-)	0	**0.0%**	0 (-)
***Yersinia**	0	**0.0%**	0 (-)	0	**0.0%**	0 (-)
***Cryptosporidium**	8	**3.1%**	29.7 (3.8)	3	**1.2%**	28.7 (6.0)
***D. fragilis**	22	**8.5%**	36.8 (6.9)	15	**5.8%**	33.3 (8.5)
***E. histolytica**	1	**0.4%**	30 (-)	4	**1.5%**	28.1 (4.5)
***G. lamblia**	36	**13.9%**	34.6 (5.5)	24	**9.2%**	33.7 (5.4)

Table 4.5 Heat map of infection intensity according to mean C_t values in cases with complaints of persistent digestive disorders and matched controls in Côte d'Ivoire. The following colour code was used for infection intensity: Dark green, very low intensity (C_t ≥35.0); light green, low intensity (C_t 30.0-34.9); yellow, high intensity (C_t ≤25.0-29.9); red, very high intensity (C_t ≤24.9); white, no infection.

Pathogen	Infection Intensity Côte d'Ivoire	
	Case	Control
Campylobacter	Low	Low
EAEC	Low	Low
EIEC *ipaH*	High	Low
ETEC *LT*	Low	Low
ETEC *ST*	Low	Low
Salmonella	Very low	-
Yersinia	-	-
Cryptosporidium	High	High
D. fragilis	Very low	Low
E. histolytica	Low	High
G. lamblia	Low	Low

4.2.3 Comparison of co-infections

No infections were found in 55.8% and 37.5% of all controls and symptomatic patients, respectively (Figure 4.8). More than two co-infections were rarely detected in Côte d'Ivoire (Figure 4.9, Figure 4.10).

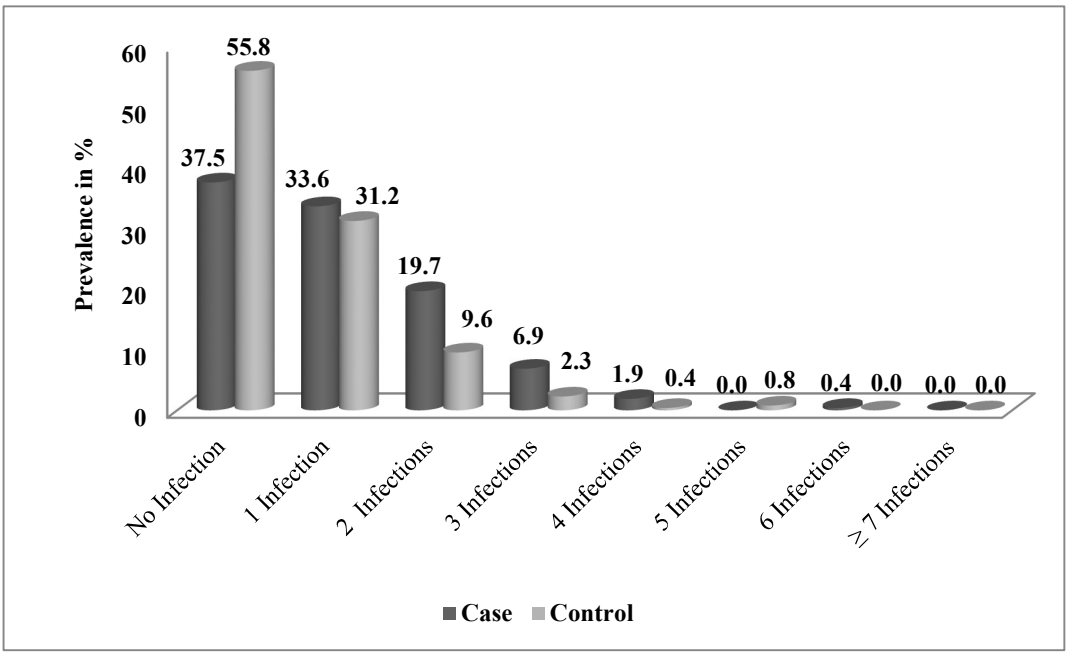


Figure 4.8 Comparison of co-infection rates in cases with symptoms of persistent diarrhoea and abdominal pain, respectively, and matched controls in a study in Côte d'Ivoire.

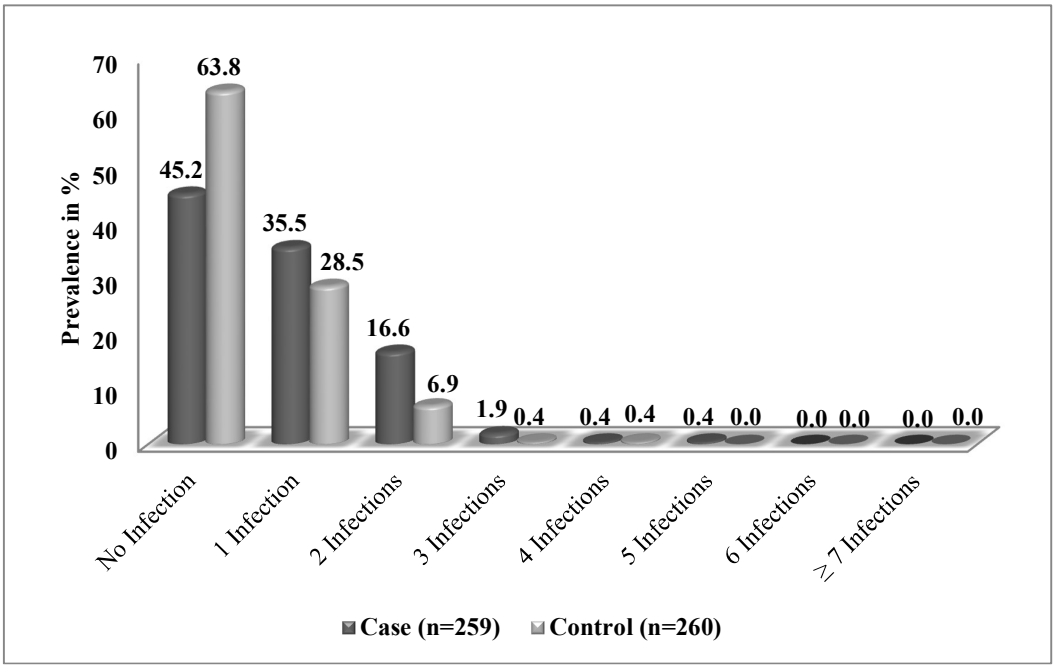


Figure 4.9 Bacterial co-infections in cases with persistent diarrhoea and persistent abdominal pain, respectively, and matched controls in a study conducted in Côte d'Ivoire.

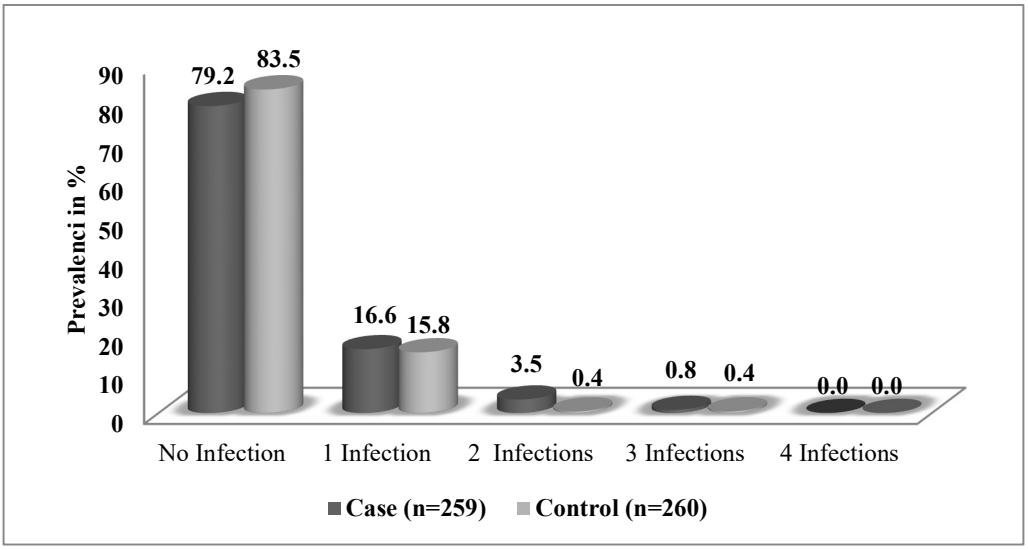


Figure 4.10 Parasitic co-infections in cases with persistent diarrhoea and persistent abdominal pain, respectively, and matched controls in a study conducted in in Côte d'Ivoire.

4.3 Comparison of findings from Mali and Côte d'Ivoire

The predominant pathogens were identical in both study countries, with EAEC, *Campylobacter* spp., EIEC and ETEC as major bacterial pathogens, and *G. lamblia* and *D. fragilis* constituting the main protozoal agents. The prevalences for most pathogens were significantly higher in Mali, both for patients and controls, as was also the number of co-infections (Figure 4.11). Infection intensity as assessed by C_t values was rather low for most pathogens in both settings (Table 4.6).

Table 4.6 Overview of obtained infection intensity in cases with symptoms of persistent diarrhoea and persistent abdominal pain and matched controls in a study in Mali and Côte d'Ivoire. The following colour code was used for infection intensity: Dark green, very low intensity (C_t ≥35.0); light green, low intensity (C_t 30.0-34.9); yellow, high intensity (C_t ≤25.0-29.9); red, very high intensity (C_t ≤24.9); white, no infection.

Pathogen	Infection Intensity Mali		Infection Intensity Côte d'Ivoire	
	Case	Control	Case	Control
Campylobacter	Low	Low	Low	Low
EAEC	Low	High	Low	Low
EIEC *ipaH*	High	High	High	Low
ETEC *LT*	Low	Low	Low	Low
ETEC *ST*	High	High	Low	Low
Salmonella	Very high	Low	Very low	-
Yersinia	High	-	-	-
Cryptosporidium	Low	Low	High	High
D. fragilis	Very low	Very low	Very low	Low
E. histolytica	High	High	Low	High
G. lamblia	Low	Low	Low	Low

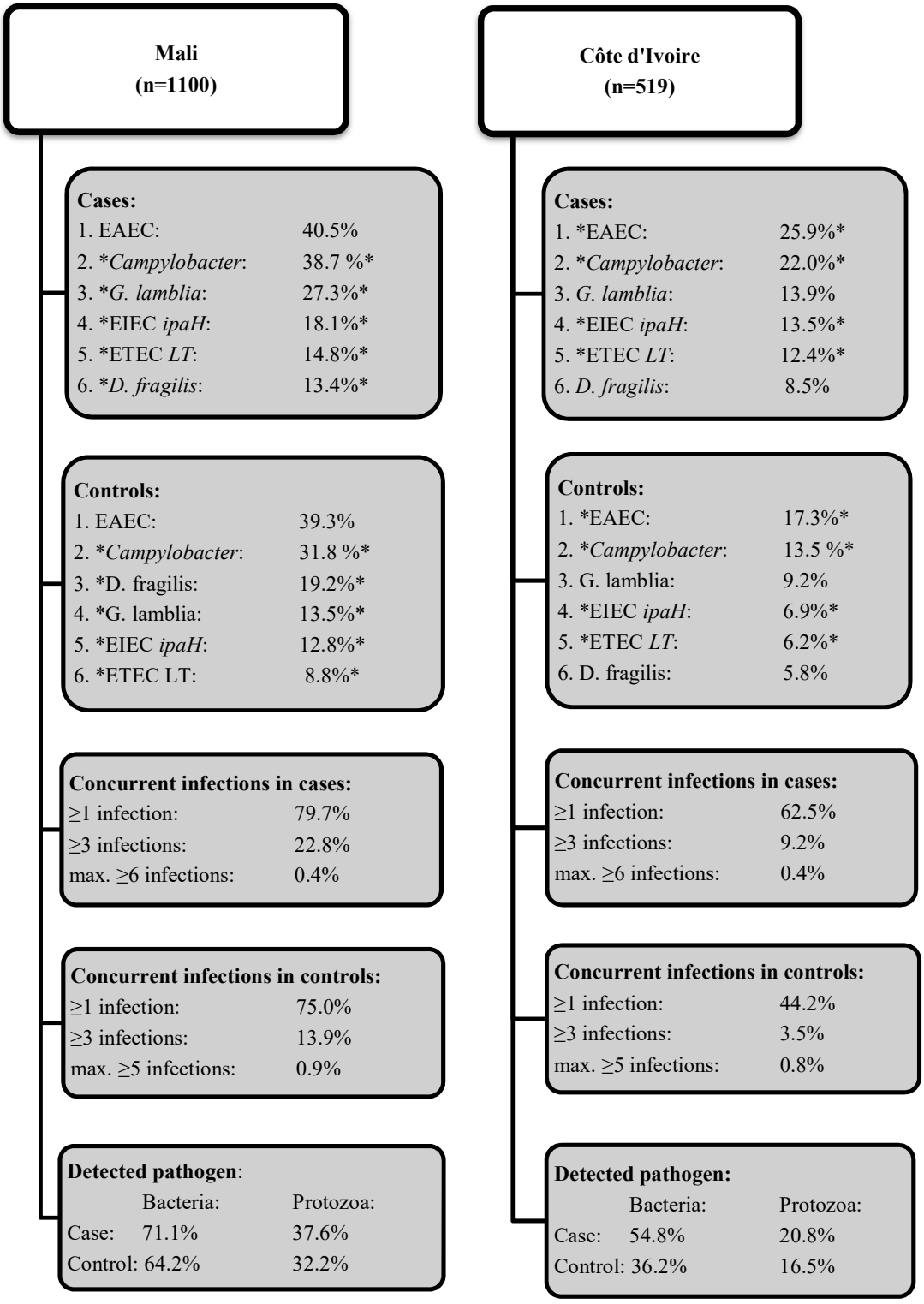


Figure 4.11 Comparison of outcomes in a case-control study on persistent digestive disorders in Mali and Côte d'Ivoire. *p-value ≤0.05

5. Discussion

The current MD thesis employed stool-based multiplex real-time PCR to provide insights into the infectious aetiology of persistent digestive disorders in Mali and Côte d'Ivoire, two West African countries with a high disease burden attributable to intestinal infections. The main findings and arising implications and challenges are discussed in this chapter.

5.1 Comparison of the current study to previously conducted studies

Mali comprised a larger study cohort (n=1.100) than Côte d'Ivoire (n=519) which may have an impact on the subsequent statistical analysis. In both countries, 'persistent abdominal pain' was the predominant symptom and less than 15% of symptomatic patients had persistent diarrhoea, which limits the significance of the study findings with regard to this clinical pattern.

In both countries, EAEC, *Campylobacter* spp., *G. lamblia, D. fragilis*, EIEC and ETEC were the most prevalent pathogens, with some country-specific characteristics. Of note, *E. histolytica, Salmonella* spp. and *Yersinia* were nearly absent in both settings.

Similar findings were observed in previously conducted studies, e.g. a study carried out in Mexico and Egypt found a prevalence of 8-45% in symptomatic and asymptomatic infants for *Campylobacter* (RUIZ-PALACIOS, 2007). In the Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) study, which was carried out in rural areas of South America, Africa and Asia, *Campylobacter* spp. was a significant and the second most detected pathogen in ≤2-year-old infants (PLATTS-MILLS et al., 2015). In the Global Enteric Multicenter Study (GEMS), a case-control study conducted in sub-Saharan Africa and South Asia, *Campylobacter* spp. was identified as one of the major causes for moderate-to-severe diarrhoea (KOTLOFF et al., 2012; KOTLOFF et al., 2013). Similar to our results, EAEC was detected in approximately 30% of cases and controls, respectively, in the MAL-ED study (PLATTS-MILLS et al., 2015). GEMS obtained a prevalence of 34% for pathogenic *E. coli* in Bamako, Mali (POP et al., 2014). *ST* and *LT* were commonly detected in children ≤2 years and significantly associated with persistent diarrhoea and severe disease, though a decrease was found in older age groups (PLATTS-MILLS et al., 2015; POP et al., 2014). Similar to *Yersinia, Salmonella* was not frequently observed in persons with persistent

digestive disorders in other tropical study areas (PLATTS-MILLS et al., 2015). A study conducted in rural areas of The Gambia that investigated the association of different EAEC strains with diarrhoea in ≤5 years-old children identified a higher prevalence for the virulence factors *aggA*, *capU* and *pet* in symptomatic children between 0-11 months (IKUMAPAYI et al., 2017). However, a significant association of several *E. coli* pathotypes with diarrhoea was not found (SARKER et al., 2017).

The prevalence of *Cryptosporidium* in our study was lower than observed elsewhere in West Africa (LEITCH, HE, 2011). Of note, the MAL-ED and GEMS studies identified *Cryptosporidium* as one of the most significant pathogens in low-income countries, particularly in children, with increased risk of death and development of moderate-to-severe diarrhoea (FOTEDAR et al., 2007; PLATTS-MILLS et al., 2015; POP et al., 2014). *E. histolytica* was rarely detected in other rural study areas, though some studies i.e. among school children conducted in an urban slum of Lagos City, Nigeria, identified *E. histolytica* and the non-pathogenic *E. dispar* in 25.3% of the children (GYANG et al., 2017). However, it must be noted that microscopy alone is not able to clearly differentiate *E. histolytica* from the non-pathogenic *E. dispar*. This was shown across multiple studies in different low-income countries (INCANI et al., 2017; KEBEDE et al., 2004; VERWEIJ et al., 2003).

Interestingly, *D. fragilis* was more common in asymptomatic individuals in this study (TURKELTAUB et al., 2015). Indeed, many studies were conducted to elucidate the pathogenic character of *D. fragilis*. In a case-control study conducted in Denmark, *D. fragilis* was frequently detected in asymptomatic controls and hence, it was concluded that this intestinal protozoa may not play a direct role in the pathogenesis of irritable bowel syndrome (KROGSGAARD et al., 2015). Similar results were found for a potential role in the pathogenesis of active ulcerative colitis and gastrointestinal infection (PETERSEN et al., 2013; ROSSEN et al., 2015; VANDENBERG et al., 2006). It is indeed still under debate whether *D. fragilis* can be attributed to persistent abdominal pain and persistent diarrhoea (JOHNSON et al., 2004; VANDENBERG et al., 2006).

In the MAL-ED study, *G. lamblia* was a common finding in diarrhoeal and non-diarrhoeal stool samples, similar to the results in the current study (PLATTS-MILLS et al., 2015). Another large study from Tanzania observed *Giardia* in all age groups, but the highest prevalence of up to 74% was seen in 2-5-year-old children (FORSELL et al., 2016). Even a study from a travel medicine center in Belgium with outpatients suspected of having

parasitic gastrointestinal infection identified *G. lamblia* (7.1%) and *D. fragilis* (6.3%) as the most frequent protozoa, while *C. parvum* and *E. histolytica* were rarely detected (VANDENBERG et al., 2006). However, a causal attribution of specific symptoms to an individual pathogen remained difficult, because multiple pathogens were concurrently detected in most samples (VANDENBERG et al., 2006). Another recent assessment from endemic settings could not unambiguously attribute digestive disorders to *Giardia* (BARTELT, PLATTS-MILLS, 2016). Indeed, the study reported even a decreased risk suffer from acute diarrhoea (BARTELT, PLATTS-MILLS, 2016).

The current case-control design revealed widespread and highly prevalent infections even among asymptomatic individuals in both study sites. Furthermore, the infectious burden was comparatively higher in Mali. Indeed, 77.4% of all Malian individuals had at least one infection, with a higher frequency of ≥3 concurrent infections, compared to only 53.4% infected individuals in Côte d'Ivoire. These findings are similar to those of the MAL-ED study, where 76.9% cases and 64.9% controls were infected (PLATTS-MILLS et al., 2015). Multiple infections with ≥2 concurrent pathogens in children of ≤2 years were also common (PLATTS-MILLS et al., 2015), similar to a Nigerian study, where polyparasitism was found in 39.1% and a study conducted in Bangladesh, where 3-5 pathogens in patients with complaints of acute diarrhoea were rather the rule than the exception (GYANG et al., 2017; SARKER et al., 2017). Underlying reasons for the comparatively higher disease burden in Mali as compared to Côte d'Ivoire might be the lower income, poor supply of clean drinking-water and inadequate sanitation, especially if considering that the study area in Niono, Mali is less urbanised than the site in Dabou, Côte d'Ivoire. Indeed, drinking unsafe water increases the risk for protozoan infections and may be linked to lower performance in educational activities and chronic symptomatic infection may prevent children from attending schools (GYANG et al., 2017), which on the other hand may impede the understanding and the importance of hygiene. To investigate the impact of unhygienic conditions, studies addressing specifically the water quality, sanitation and hygiene (WASH) behaviour were conducted in Kenya and Bangladesh (ARNOLD et al.). However, childhood diarrhoea could not be substantially reduced in the study areas, although children in Bangladesh showed a lower level of parasitic infections (ARNOLD et al.; ARNOLD et al., 2013; NULL et al., 2018).

At the country level, 'regular deworming', i.e. the regular administration of anthelminthic drugs to e.g. school-aged children, is a successful tool for control of selected NTDs.

Interestingly, there is a strong correlation between the Human Development Index, an index calculated by the World Bank that is calculated based on the average healthy life span, level of education and standard of living, and the so-called 'worm index' (KANG et al., 2018). The worm index is calculated based on the following information:

1. The number of school-aged children requiring annual deworming for their intestinal helminth infections
2. The population requiring regular preventive chemotherapy for lymphatic filariasis and
3. The number of school-aged children requiring regular preventive chemotherapy treatment for schistosomiasis (HOTEZ, HERRICKS, 2015).

There is a strong inverse correlation between both indices, which might demonstrate the long-term negative impact of worm infection on the socioeconomic situation. However, studies in India observed that improved access to sanitary facilities did not go along with change of defecation or hygienic behaviour (PRENDERGAST, KELLY, 2016). Nevertheless, Prüss-Ustün *et al.* showed that, on a global scale, approximately 502.000 deaths were associated with inadequate water and 280.000 deaths were linked to inadequate sanitation (PRÜSS-USTÜN et al., 2014). Among infants aged ≤5 years, 361.000 deaths were deemed to be preventable, which underscores the enormous potential (PRÜSS-USTÜN et al., 2014).

Besides diarrhoeal diseases and persistent abdominal pain, there are potentially further links between intestinal infections and malnutrition and even increased mortality (GYANG et al., 2017; PRÜSS-USTÜN et al., 2014; SARKER et al., 2017; THE M. A. L. E. D. NETWORK INVESTIGATORS, 2014). It is assumed that malnutrition may lead to low gastric acid secretion, which favours bacterial colonisation and may lead to a so-called gut microbiota dysbiosis, which was found in infants in Bangladesh suffering from persistent diarrhoea (PRENDERGAST, KELLY, 2016). The dysbiosis was based on a shift from *Streptococcus*- to *Escherichia*-dominated gut microbiomes and was frequently related to previous antibiotic intake, and resulted in high proportions of antibiotic-resistant *E. coli* (PRENDERGAST, KELLY, 2016; THE et al., 2017). Studies also showed that *E. coli* was predominant in patients with normal vitamin A levels while a deficiency displayed an *Enterococcus*-dominated stool, which is related to *Streptococcus* and may also explain the different gut microbiome (SARKER et al., 2017). The dysbiosis does not only influence the vulnerability to multiple infections, but also leads to a

decreased immune response to rotavirus vaccination, as suggested by a Ghanaian study (HARRIS et al., 2017). Further explorations of the potential links between diarrhoea and certain pathogens, gender and age are nowadays being carried out, as GEMS found that e.g. elderly individuals and females had a comparatively higher disease burden of diarrhoea than other patient groups (FARAG et al., 2013).

5.2 What is the clinical relevance of multiplex real-time PCR?

5.2.1 Infection intensity

As demonstrated by the study results from Mali and Côte d'Ivoire, the infection intensity was rather low in most instances, as indicated by a C_t value of 30 or higher. Similar results were found in a case-control study among infants with acute diarrhoea in Zanzibar, Tanzania, where most C_t values were above 30 (ELFVING et al., 2014). Though, in contrast to our study, the C_t values for *Campylobacter* were associated with symptomatic infections (ELFVING et al., 2014). Likewise, a study on childhood diarrhoea in Rwanda reported significantly higher C_t values for *Campylobacter* in symptomatic patients than in matched controls (KABAYIZA et al., 2014). The high rate of pathogen detection in asymptomatic children might be explained with residual nucleic acid shedding that may persist for months after previous infections (PLATTS-MILLS et al., 2015). This may also explain the low and very low infection intensity of most detected pathogens. Quantification of the pathogen load may help to distinguish clinically relevant infections, even though it was not possible to define a C_t-based threshold in the current MD thesis. Longitudinal molecular analyses and parallel bacterial cultures might help to define such clinically useful cut-off values. However, it is important to note that even subclinical PCR findings of pathogens in the stool were recently shown to be associated with considerable long-term negative effects regarding childhood growth and stunting (ROGAWSKI et al., 2018).

5.2.2 Multiple infections

Multiple co-infections were frequent in our study and in other multi-centric assessments in low-income countries (e.g. MAL-ED study (PLATTS-MILLS et al., 2018)). Symptomatic patients had more frequently ≥3 concurrent infections. Hence, it may be

hypothesised that symptoms rather develop depending on the number of co-infections than on a single causative pathogen. Indeed, an analysis of the available literature and different studies conducted in recent years displayed that there is no convincing evidence that any particular pathogen or type of pathogen is exclusively associated with persistent diarrhoea in <6-year-old infants in low- and middle-income countries (ABBA et al., 2009; BARDHAN et al., 1998). It may also be possible that a primary infection facilitates a subsequent secondary infection with another pathogen, including pathogens of lower virulence (BARDHAN et al., 1998; SARKER et al., 2017). A study carried out in South America, Africa and Asia analysed stool samples of newborns for a broad range of enteropathogens using culture, enzyme immunoassays and PCR. In the longitudinal 24-months-follow-up, it was observed that each additional pathogen increased the risk to develop diarrhoea (PLATTS-MILLS et al., 2015). In malnourished, otherwise asymptomatic children, positive signals by PCR were detected for prolonged periods, which may point toward a causal role in such detrimental long-term effects of persistent intestinal infections (PLATTS-MILLS et al., 2015).

Yet, it is also noteworthy that every fifth symptomatic patient in Mali and more than every third patient in Côte d'Ivoire with self-reported persistent abdominal pain were not found to be infected with a pathogen. The unexpected high prevalence of e.g. *D. fragilis* in asymptomatic individuals may spur the discussion whether detected microorganisms may even have a protective effect on the risk of developing intestinal disorders (BECKER et al., 2016). While the diagnostic approach in our study covered many bacterial and protozoal infections, it is likely that other infections may have gone unnoticed, in particular viruses and helminths. Additionally, it can be expected that a significant amount suffered from non-intestinal infections (e.g. HIV-associated diarrhoea) or non-infectious causes. Indeed, numerous studies reported on the occurrence of diarrhoea in up to 90% of HIV-infected patients with increased frequency and severity as immune function deteriorated (BECKER et al., 2013; BHAIJEE et al., 2011). Since the introduction of combination antiretroviral therapy, an overall decrease in infectious diarrhoeal rate was reported from many endemic areas (CLAY, CRUTCHLEY, 2014). However, HIV-infected patients frequently suffer from medication-associated diarrhoea, which increased in one study from 0% in 1995 to 45% in 1997 (CLAY, CRUTCHLEY, 2014). Other non-communicable differential diagnoses may be malignant diseases, autoimmune and chronic inflammatory diseases and food allergies.

The inclusion criteria used for this study may also be challenged because the term 'persistent abdominal pain' is relatively unspecific, and hence, various extra-abdominal causes may be considered. Especially in Mali, where the majority of patients were females, genitourinary disorders like pelvic inflammatory disease, adnexal cyst, torsion or rupture, ectopic pregnancy and uterine pain due to endomyometritis are important differential diagnosis (CARTWRIGHT SL, 2008; MACALUSO, MCNAMARA, 2012; REUST CE, 2016). In men, testicular diseases may present as abdominal pain (MACALUSO, MCNAMARA, 2012). Among participating infants and even adults, pneumonia involving the lower lobes of the lung is a rare, but important differential diagnosis to abdominal pain (CARTWRIGHT SL, 2008; MACALUSO, MCNAMARA, 2012; REUST CE, 2016). Psychosomatic reasons should also not be underestimated since it was observed that mental stress was often related to civil wars and increased the prevalence of digestive disorders (HUGUET et al., 2017; UDOH et al., 2016; WALLIS, FIKS, 2015).

The observed prevalence of multiple infections questions the frequently performed routine administration of antibiotics in patients with persistent digestive disorders, but also raises concerns with regard to the clinical relevance of such highly sensitive diagnostic assays with regard to clinical decision-making. The high number of infections found in asymptomatic individuals also underscores that these results alone should not justify routine antibiotic treatment (ABBA et al., 2009; PLATTS-MILLS et al., 2018). Indeed, unnecessary antibiotic treatment may rather lead to a gut microbiome dysbalance than improvement (BECKER, 2015; PRENDERGAST, KELLY, 2016; THE et al., 2017). Yet, the molecular approach used in this study is one among few research initiatives that targeted persistent diarrhoea and persistent digestive disorders in endemic settings, as most available data stem from single-centre studies in returning travellers or infants (DUPONT, 2016). As an advantage of such molecular diagnostics is to allow for comparability across different sites in various endemic countries.

5.2.3 Factors influencing on the accuracy of PCR

Nucleic acid extraction and amplification with multiplex real-time PCR is a rapid and sensitive method to detect pathogens but is prone to several factors that may reduce the diagnostic accuracy of this approach.

Pre-analytical considerations are of utmost importance for any molecular diagnostic test. Indeed, variable specimen quantity and quality, use of fixatives, pre-diagnostic intake of antibiotics, etc. significantly impact on the quality of subsequent PCR analyses (MCAULIFFE et al., 2013; PLATTS-MILLS et al., 2013; PLATTS-MILLS et al., 2018).

The DNA yield is conditional on the extraction method (full- or semi-automatic, vigorous shaking or vortexing), its interaction with human and non-human stool components, specimen weight and quantity (MCAULIFFE et al., 2013; MIRSEPASI-LAURIDSEN et al., 2014). Indeed, these aspects may lead to variable amounts of DNA concentration thus leading to different C_t values (WON et al., 2016).

In this study's setting, it was challenge to maintain a continuous cold chain of the stool specimens during the sample transfer. For some pathogens such as *C. difficile,* this is less important as it was shown that PCR and even culturing were feasible despite prolonged storage at disrupted cold chain conditions (BECKER et al., 2015a). Yet, even storage under appropriate conditions for more than a month may reduce the diagnostics yield of later PCR analyses (KUK, 2012).

5.3 Advantages and disadvantages of multiplex real-time PCR and conventional diagnostic techniques

In resource-constrained settings, the diagnosis of gastrointestinal infections caused by a broad range of pathogens is usually based on a combined analysis of clinical characteristics, patient history and conventional laboratory methods like microscopy or stool culture (MACFARLANE-SMITH et al., 2018). Cultural diagnostics are more affordable than PCR and well-established techniques to investigate bacterial pathogens in stool samples (Table 5.1) (MACFARLANE-SMITH et al., 2018). However, bacterial culture may show reduced sensitivity, particularly in patients with ongoing antibiotic treatment (MACFARLANE-SMITH et al., 2018; PLATTS-MILLS et al., 2013). Additionally, the unambiguous species identification of culture-grown bacterial colonies is not always accurate unless modern methods such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry are used. This is particularly true for less common agents, as was recently shown in an African setting (SAMB-BA et al., 2014). However, even MALDI-TOF mass spectrometry is unable to distinguish certain closely related species, e.g. *E. coli* and *Shigella* spp., and cannot

identify specific virulence factors, which would be required for the differentiation of the *E. coli* pathotypes.

In contrast to bacteriology, the diagnosis of intestinal parasitic infections mainly relies on microscopy of stool samples, which is inexpensive, relatively simple to perform, and which is able to detect a broad range of parasitic agents, whereas PCR using specific primers targeting a limited set of parasites is more restricted (INCANI et al., 2017).

An advantage of conventional diagnostics using microscopy and culture methods is that only actively replicating pathogens are detected – as opposed to residual nucleic acids being detected by PCR even weeks after cleared infections – and that a further characterisation is possible, e.g. antimicrobial susceptibility testing and further isolate characterisation (MACFARLANE-SMITH et al., 2018). However, utilisation of conventional culture methods has the huge disadvantage of prolonged sample processing and a longer time-to-result, which is frequently associated with a high workload (INCANI et al., 2017).

Other molecular approaches such as metagenomic whole genome sequencing allow for a direct screening of clinical faecal samples for potential pathogens, but are technically challenging, expensive, require considerable biostatistical analysis and cannot (yet) be used for individual patient diagnostics (MACFARLANE-SMITH et al., 2018). Real-time PCR itself is more expensive than standard bacteriological stool culture, but the concurrent detection of different target pathogens improves the cost-effectiveness of this method, because several agar plate cultures would be required for detection of the same set of pathogens by conventional culture (MACFARLANE-SMITH et al., 2018). If performed in daily routine diagnostics, PCR has also a shorter time-to-result period, which also enhances the infection prevention and control interventions and may thus prevent disease transmission (Table 5.1). Another significant advantage of PCR is the ability to differentially identify toxin-producing pathogens (e.g. ST and LT in the case of ETEC) and to unambiguously characterise otherwise identical agents (e.g. *Entamoeba dispar* and *Entamoeba histolytica*) (KEBEDE et al., 2004; VERWEIJ et al., 2003). However, PCR also has the disadvantage of a 'too high sensitivity' for clinical decision-making, as it is prone to detection of small amounts of e.g. environmental contamination, and does not reliably differentiate asymptomatic carriage of enteric pathogens from symptomatic disease (MACFARLANE-SMITH et al., 2018).

An analysis several years ago estimated that improved enteropathogenic diagnostics, particularly for *G. lamblia*, *Cryptosporidium* and EAEC, could save 2.8 million disability-adjusted life years (DALYs) and reduce the prevalence of underdevelopment in low-income countries by 12.5% (HOUPT, GUERRANT, 2008; RICCI et al., 2006). Hence, the further development and deployment of rapid diagnostic tools in resource-constrained areas should remain a key focus of global health initiatives in the foreseeable future.

Table 5.1 Comparison of advantages and disadvantages of multiplex real-time PCR and conventional diagnostic tools in resource-constrained tropical settings.

Diagnostic tool	Advantage	Disadvantage
PCR	- Rapid - Simultaneous detection of enteropathogens and toxins - Increased sample throughput - Decreased turnaround time - High diagnostic accuracy	- Expensive - Lack of training pertaining to molecular diagnostics outside reference laboratories - Detection of environmental contamination and asymptomatic carriage of pathogens - Prolonged nucleic acid shedding after cleared infections
Conventional methods (stool culture, microscopy)	- Affordable - Well-established routine diagnostic tools - Available in most settings - Allows for additional analyses, e.g. antimicrobial susceptibility testing	- Poor sensitivity - Time-intensive - Diagnostic accuracy heavily dependent on expertise - No detection of toxins and no differentiation of closely related species possible - Time to result for stool culture: Usually several days
Other molecular tests (RDTs, EAI)	- Rapid - Suitable in laboratories with limited technical equipment - Can be used as point-of-care tests	- Lack of routine - Restricted availability for only a few pathogens - Reduced diagnostic accuracy

6. Conclusion

6.1 Diagnostic approach to neglected digestive disorders

In an increasingly globalised and inter-connected world, a comprehensive understanding of the epidemiology of infectious diseases in different settings is of crucial importance. Thus far, relatively few data are available from low-income countries. The study reported here, which was carried out as part of the NIDIAG study on persistent digestive disorders, had the goal to achieve a better understanding of the aetiology and diagnosis of persistent diarrhoea and persistent abdominal pain in two West African low-income countries. Additional study objectives were to obtain epidemiological data and to improve a targeted clinical management of these diseases. The study was also among the first of its kind that targeted previously lesser researched groups and symptoms.

Our study in Mali and Côte d'Ivoire concluded that - despite large-scale studies like MAL-ED and GEMS - persistent diarrhoea and persistent abdominal pain are still underappreciated clinical entities that require further research. Even though the implementation of molecular diagnostics in resource-constrained settings remains challenging, PCR assays are key to identify and attribute diarrhoeal diseases to their microbiological causes, and are important prerequisites for clinical diagnosis-treatment algorithms. From an epidemiological perspective, centralised *post-hoc* molecular analyses of preserved stool samples enable objective comparisons across multiple settings, regardless of the diagnostic capacities at the respective sites.

One major result of the study was the relatively low association between PCR-positive samples and the clinical symptomatology. Despite country-specific pathogen characteristics, our findings underscore that PCR-based approaches alone without an individual clinical assessment cannot accurately differentiate symptomatic patients from asymptomatic controls. It is suggested to include multiplex real-time PCR as additional diagnostic algorithms to comprehensively improve the aetiological understanding of persistent digestive disorders in the tropics.

6.2 Recommendations and research needs

Despite the data obtained during this study, further in-depth scientific investigation of neglected digestive infections is recommended and required to understand the complex aetiology, improved clinical management and to identify prognostic factors (Figure 6.1). First, a more precise and objective definition of 'persistent diarrhoea' and 'persistent abdominal pain' is required as the enrolment in this study relied on patients' self-reported morbidity accounts of persistent digestive disorders. Hence, it was uncertain whether the symptomatology had indeed been present for at least two weeks in all patients or if they had undergone partial therapy. Second, molecular analyses were carried out several months after stool collection, and despite the best efforts to maintain a meticulous cold chain, possible nucleic acid degradation in some investigated pathogens might have led to lower positivity rates. Third, the simultaneous performance of bacterial culture is highly recommended to correlate these findings to molecular results and to confirm sensitivity and specificity of multiplex real-time PCR. Fourth, even though our PCR approach covered a wide range of pathogens, other intestinal pathogens (e.g. viruses and helminths) are missing. Fifth, individual comparison of epidemiological characteristics (age, sex and residence) of a symptomatic case to his/her matched control may reveal common features in Ct values and infection aetiology.

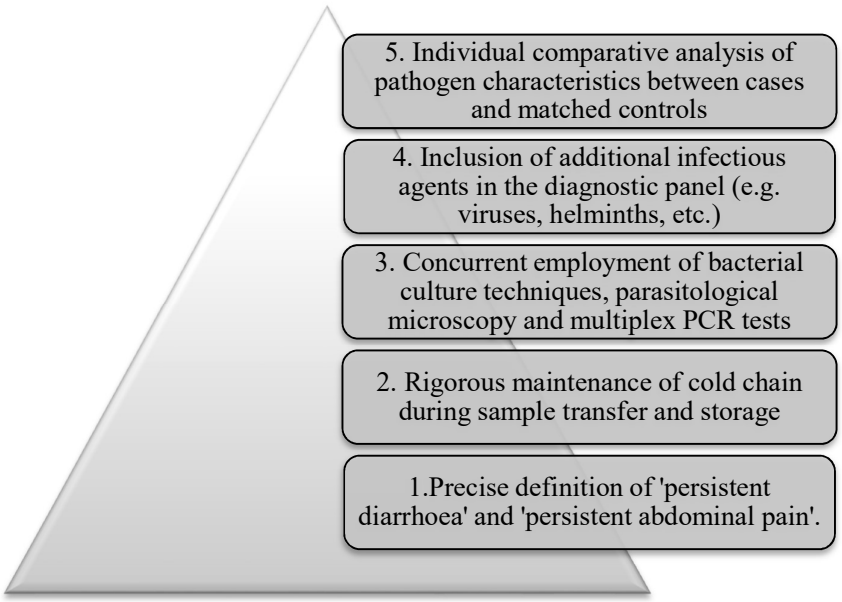


Figure 6.1 Recommendations based on experiences made during this MD thesis for future case-control studies to investigate persistent diarrhoea and persistent abdominal pain in resource-constrained settings.

7. References

1. Abba K, Sinfield R, Hart CA, Garner P (2009) Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. BMC Infectious Diseases 9:88-88

2. Adeyemo FE, Singh G, Reddy P, Stenström TA (2018) Methods for the detection of *Cryptosporidium* and *Giardia*: From microscopy to nucleic acid based tools in clinical and environmental regimes. Acta Tropica 184:15-28

3. Arnold B, Colford J, Fernald L, Kariger P, Kremer M, Lin A, et al. WASH benefits: the effects of water quality, sanitation, handwashing, and nutrition interventions on child health, growth and development in rural Kenya [Internet, cited 06 March 2018]. URL: https://www.povertyactionlab.org/evaluation/wash-benefits-effects-water-quality-sanitation-handwashing-and-nutrition-interventions

4. Arnold BF, Null C, Luby SP, Unicomb L, Stewart CP, Dewey KG, Ahmed T, Ashraf S, Christensen G, Clasen T, Dentz HN, Fernald LCH, Haque R, Hubbard AE, Kariger P, Leontsini E, Lin A, Njenga SM, Pickering AJ, Ram PK, Tofail F, Winch PJ, Colford JM, Jr. (2013) Cluster-randomised controlled trials of individual and combined water, sanitation, hygiene and nutritional interventions in rural Bangladesh and Kenya: the WASH Benefits study design and rationale. BMJ Open 3:e003476-e003476

5. Assemblée des Régions et Districts de Côte d'Ivoire. La région des Grands Ponts [Internet, cited 23 May 2016]. URL: http://www.ardci-rd.org/index.php/vie-des-regions/grands-ponts

6. Bardhan PK, Albert MJ, Alam NH, Faruque SM, Neogi PKB, Mahalanabis D (1998) Small bowel and fecal microbiology in children suffering from persistent diarrhea in Bangladesh. Journal of Pediatric Gastroenterology and Nutrition 26:9-15

7. Bartelt LA, Platts-Mills JA (2016) *Giardia*: a pathogen or commensal for children in high-prevalence settings? Current Opinion in Infectious Diseases 29:502-507

8. Becker SL, Vogt J, Knopp S, Panning M, Warhurst DC, Polman K, Marti H, von Müller L, Yansouni CP, Jacobs J, Bottieau E, Sacko M, Rijal S, Meyanti F, Miles MA, Boelaert M, Lutumba P, van Lieshout L, N'Goran EK, Chappuis F, Utzinger J (2013) Persistent digestive disorders in the tropics: causative infectious pathogens and reference diagnostic tests. BMC Infectious Diseases 13:37

9. Becker SL (2015). Syndromic approaches to approaches to persistent diegstive disorders (≥14 days) in resource-constrained settings: aetiology, clinical assessment and differential diagnostics. (Basel: Philosophisch-Naturwissenschaftliche Fakultät der Universität Basel)

10. Becker SL, Chatigre JK, Coulibaly JT, Mertens P, Bonfoh B, Herrmann M, Kuijper EJ, N'Goran EK, Utzinger J, von Müller L (2015) Molecular and culture-based diagnosis of *Clostridium difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions. Transactions of the Royal Society of Tropical Medicine and Hygiene 109:660-668

11. Becker SL, Chatigre JK, Gohou JP, Coulibaly JT, Leuppi R, Polman K, Chappuis F, Mertens P, Herrmann M, N'Goran EK, Utzinger J, von Müller L (2015) Combined stool-based multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire. Clinical Microbiology and Infection 21:591.e591-591.e510

12. Becker SL, Yap P, Horié NS, Alirol E, Barbé B, Bhatta NK, Bhattarai NR, Bottieau E, Chatigre JK, Coulibaly JT, Fofana HKM, Jacobs J, Karki P, Khanal B, Knopp S, Koirala K, Mahendradhata Y, Mertens P, Meyanti F, Murhandarwati EH, N'Goran EK, Peeling RW, Pradhan B, Ravinetto R, Rijal S, Sacko M, Saye R, Schneeberger PHH, Schurmans C, Silué KD, Steinmann P, van Loen H, Verdonck K, van Lieshout L, von Müller L, Yao JA, Boelaert M, Chappuis F, Polman K, Utzinger J (2016) Experiences and lessons from a multicountry NIDIAG study on persistent digestive disorders in the tropics. PLoS Neglected Tropical Diseases 10:e0004818-e0004818

13. Bhaijee F, Subramony C, Tang S-J, Pepper DJ (2011) Human immunodeficiency virus-associated gastrointestinal disease: common endoscopic biopsy diagnoses. Pathology Research International 2011:247923-247923

14. Bouzid M, Hunter PR, Chalmers RM, Tyler KM (2013) *Cryptosporidium* pathogenicity and virulence. Clinical Microbiology Reviews 26:115-134

15. Cartwright SL KM (2008) Evaluation of acute abdominal pain in adults. American Family Physician 77:971-978

16. Central Intelligence Agency (2020). The Worlf Factbook - Côte d'Ivoire [Internet, cited 19 September 2020]. URL: https://www.cia.gov/library/publications/the-world-factbook/geos/iv.html.

17. Central Intelligence Agency (2020). The Worlf Factbook - Mali [Internet, cited 19 September 2020]. URL: https://www.cia.gov/library/publications/the-world-factbook/geos/ml.html.

18. Chamberlain NR (2019) The Big Picture: Medical Microbiology. McGraw-Hill Education/Medical: New York

19. Clay PG, Crutchley RD (2014) Noninfectious diarrhea in HIV deropositive individuals: a review of prevalence rates, etiology, and management in the era of combination antiretroviral therapy. Infectious Diseases and Therapy 3:103-122

20. DuPont HL (2016) Persistent diarrhea: a clinical review. JAMA 315:2712-2723

21. Elfving K, Andersson M, Msellem MI, Welinder-Olsson C, Petzold M, Björkman A, Trollfors B, Mårtensson A, Lindh M (2014) Real-time PCR threshold cycle cutoffs help to identify agents causing acute childhood diarrhea in Zanzibar. Journal of Clinical Microbiology 52:916-923

22. Farag TH, Kotloff KL, Levine MM, Onwuchekwa U, Van Eijk AM, Doh S, Sow SO (2013) Seeking care for pediatric diarrheal illness from traditional healers in Bamako, Mali. The American Journal of Tropical Medicine and Hygiene 89:21-28

23. Forsell J, Granlund M, Samuelsson L, Koskiniemi S, Edebro H, Evengård B (2016) High occurrence of *Blastocystis* sp. subtypes 1-3 and *Giardia intestinalis* assemblage B among patients in Zanzibar, Tanzania. Parasites & Vectors 9:370-370

24. Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, Harkness J (2007) Laboratory diagnostic techniques for *Entamoeba* species. Clinical Microbiology Reviews 20:511-532

25. Gardner TB, Hill DR (2001) Treatment of giardiasis. Clinical Microbiology Reviews 14:114-128

26. Giltner CL, Saeki S, Bobenchik AM, Humphries RM (2013) Rapid detection of *Campylobacter* antigen by enzyme immunoassay leads to increased positivity rates. Journal of Clinical Microbiology. 51:618-620

27. Gyang V, Chuang T-W, Liao C-W, Lee Y-L, Akinwale O, Orok A, Olusola A, J. Babasola A, Cheng P-C, Chou C-M, Huang Y-C, Sonko P, Fan C-K (2019) Intestinal parasitic infections: current status and associated risk factors among school aged children in an archetypal African urban slum in Nigeria. Journal of Microbiology, Immunology and Infection 52:106-113

28. Harris VC, Armah G, Fuentes S, Korpela KE, Parashar U, Victor JC, Tate J, de Weerth C, Giaquinto C, Wiersinga WJ, Lewis KDC, de Vos WM (2017) Significant correlation between the infant gut microbiome and rotavirus vaccine response in rural Ghana. The Journal of Infectious Diseases 215:34-41

29. Hotez PJ, Herricks JR (2015) Helminth elimination in the pursuit of sustainable development goals: a "worm index" for human development. PLoS Neglected Tropical Diseases 9:e0003618

30. Houpt ER, Guerrant RL (2008) Technology in global health: the need for essential diagnostics. Lancet 372:873-874

31. Huang DB, Mohanty A, DuPont HL, Okhuysen PC, Chiang T (2006) A review of an emerging enteric pathogen: enteroaggregative *Escherichia coli*. Journal of Medical Microbiology 55:1303-1311

32. Huguet A, Olthuis J, McGrath PJ, Tougas ME, Hayden JA, Stinson JN, Chambers CT (2017) Systematic review of childhood and adolescent risk and prognostic factors for persistent abdominal pain. Acta Paediatrica 106:545-553

33. Ikumapayi UN, Boisen N, Hossain MJ, Betts M, Lamin M, Saha D, Kwambana-Adams B, Dione M, Adegbola RA, Roca A, Nataro JP, Antonio M (2017) Identification of subsets of enteroaggregative *Escherichia coli* associated with diarrheal disease among under 5 years of age children from rural Gambia. The American Journal of Tropical Medicine and Hygiene 97:997-1004

34. Incani RN, Ferrer E, Hoek D, Ramak R, Roelfsema J, Mughini-Gras L, Kortbeek T, Pinelli E (2017) Diagnosis of intestinal parasites in a rural community of Venezuela: Advantages and disadvantages of using microscopy or RT-PCR. Acta Tropica 167:64-70

35. Institut National de la Statistique. Côte d'Ivoire at-a-glance [Internet, cited 23 May 2016]. URL: http://cotedivoire.opendataforafrica.org/?lang=en.

36. Johnson EH, Windsor JJ, Clark CG (2004) Emerging from obscurity: biological, clinical, and diagnostic aspects of *Dientamoeba fragilis*. Clinical Microbiology Reviews 17:553-570

37. Kabayiza J-C, Andersson ME, Nilsson S, Bergström T, Muhirwa G, Lindh M (2014) Real-time PCR identification of agents causing diarrhea in Rwandan children less than 5 years of age. The Pediatric Infectious Disease Journal 33:1037-1042

38. Kang S, Damania A, Majid MF, Hotez PJ (2018) Extending the global worm index and its links to human development and child education. PLoS Neglected Tropical Diseases 12:e0006322

39. Kebede A, Verweij JJ, Petros B, Polderman AM (2004) Short communication: misleading microscopy in amoebiasis. Tropical Medicine & International Health 9:651-652

40. Keddy K GJ, Frean J. (2014). Tropical gastrointestinal infections. [Internet, cited 27 April 2016]. URL: http://www.tropmed.org/wp-content/uploads/2014/07/chapter06.pdf

41. Kotloff KL, Blackwelder WC, Nasrin D, Nataro JP, Farag TH, van Eijk A, Adegbola RA, Alonso PL, Breiman RF, Faruque ASG, Saha D, Sow SO, Sur D, Zaidi AKM, Biswas K, Panchalingam S, Clemens JD, Cohen D, Glass RI, Mintz ED, Sommerfelt H, Levine MM (2012) The Global Enteric Multicenter Study (GEMS) of diarrheal disease in infants and young children in developing countries: epidemiologic and clinical methods of the case/control study. Clinical Infectious Diseases 55: 232-245

42. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque ASG, Zaidi AKM, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. The Lancet 382:209-222

43. Krogsgaard LR, Engsbro AL, Stensvold CR, Nielsen HV, Bytzer P (2015) The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case-control study. Clinical Gastroenterology and Hepatology 13:507-513.e502

44. Kuk SYS, Cetinkaya U (2012) Stool sample storage conditions for the preservation of *Giardia intestinalis* DNA. Memorias do Instituto Oswaldo Cruz 107:965-968

45. Leitch GJ, He Q (2011) Cryptosporidiosis-an overview. Journal of Biomedical Research 25:1-16

46. Macaluso CR, McNamara RM (2012) Evaluation and management of acute abdominal pain in the emergency department. International Journal of General Medicine 5:789-797

47. Macfarlane-Smith LR, Ahmed S, Wilcox MH (2018) Molecular versus culture-based testing for gastrointestinal infection. Current Opinion in Gastroenterology 34:19-24

48. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM (2010) The global burden of nontyphoidal *Salmonella* Gastroenteritis. Clinical Infectious Diseases 50:882-889

49. McAuliffe GN, Anderson TP, Stevens M, Adams J, Coleman R, Mahagamasekera P, Young S, Henderson T, Hofmann M, Jennings LC, Murdoch DR (2013) Systematic application of multiplex PCR enhances the detection of bacteria, parasites, and viruses in stool samples. Journal of Infection 67:122-129

50. Mehlhorn H PG (2002) Grundriß der Parasitenkunde. Spektrum Akademischer Verlag, Heidelberg, Berlin.

51. Ministere de la santé et de la lutte contre le SIDA. Document de politique nationale de sante. Draft 1 (2011) [Internet, cited 24 May 2016]. URL: https://collectivity-prod.s3.amazonaws.com/uploads/attachment/document/731/Politique_Nationale_de_Sant%C3%A9_RCI_2011_int%C3%A9grale__1def.pdf

52. Mirsepasi-Lauridsen HC, Persson S, Struve C, Andersen LOB, Petersen A, Krogfelt KA (2014) Microbial diversity in fecal samples depends on DNA extraction method: easyMag DNA extraction compared to QIAamp DNA stool mini kit extraction, BMC Research Notes 7:50

53. NIDIAG. Nidiag. Better Diagnosis of Neglected Infectious Diseases. URL http://nidiag.eu/

54. Null C, Stewart CP, Pickering AJ, Dentz HN, Arnold BF, Arnold CD, Benjamin-Chung J, Clasen T, Dewey KG, Fernald LCH, Hubbard AE, Kariger P, Lin A, Luby SP, Mertens A, Njenga SM, Nyambane G, Ram PK, Colford JM, Jr. (2018) Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Kenya: a cluster-randomised controlled trial. The Lancet Global Health 6:e316-e329

55. Petersen AM, Stensvold CR, Mirsepasi H, Engberg J, Friis-Møller A, Porsbo LJ, Hammerum AM, Nordgaard-Lassen I, Nielsen HV, Krogfelt KA (2013) Active ulcerative colitis associated with low prevalence of *Blastocystis* and *Dientamoeba fragilis* infection. Scandinavian Journal of Gastroenterology 48:638-639

56. Platts-Mills JA, Liu J, Houpt ER (2013) New concepts in diagnostics for infectious diarrhea. Mucosal Immunology 6:876

57. Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJJ, McGrath M, Olortegui MP, Samie A, Shakoor S, Mondal D, Lima IFN, Hariraju D, Rayamajhi BB, Qureshi S, Kabir F, Yori PP, Mufamadi B, Amour C, Carreon JD, Richard SA, Lang D, Bessong P, Mduma E, Ahmed T, Lima AAAM, Mason CJ, Zaidi AKM, Bhutta ZA, Kosek M, Guerrant RL, Gottlieb M, Miller M, Kang G, Houpt ER (2015) Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). The Lancet Global Health 3:e564-e575

58. Platts-Mills JA, Liu J, Rogawski ET, Kabir F, Lertsethtakarn P, Siguas M, Khan SS, Praharaj I, Murei A, Nshama R, Mujaga B, Havt A, Maciel IA, McMurry TL, Operario DJ, Taniuchi M, Gratz J, Stroup SE, Roberts JH, Kalam A, Aziz F, Qureshi S, Islam MO, Sakpaisal P, Silapong S, Yori PP, Rajendiran R, Benny B, McGrath M, McCormick BJJ, Seidman JC, Lang D, Gottlieb M, Guerrant RL, Lima AAM, Leite JP, Samie A, Bessong PO, Page N, Bodhidatta L, Mason C, Shrestha S, Kiwelu I, Mduma ER, Iqbal NT, Bhutta ZA, Ahmed T, Haque R, Kang G, Kosek MN, Houpt ER, Acosta AM, Rios de Burga R, Chavez CB, Flores JT, Olotegui MP, Pinedo SR, Trigoso DR,

Vasquez AO, Ahmed I, Alam D, Ali A, Rasheed M, Soofi S, Turab A, Yousafzai A, Zaidi AKM, Shrestha B, Rayamajhi BB, Strand T, Ammu G, Babji S, Bose A, George AT, Hariraju D, Jennifer MS, John S, Kaki S, Karunakaran P, Koshy B, Lazarus RP, Muliyil J, Ragasudha P, Raghava MV, Raju S, Ramachandran A, Ramadas R, Ramanujam K, Rose A, Roshan R, Sharma SL, Sundaram S, Thomas RJ, Pan WK, Ambikapathi R, Carreon JD, Doan V, Hoest C, Knobler S, Miller MA, Psaki S, Rasmussen Z, Richard SA, Tountas KH, Svensen E, Amour C, Bayyo E, Mvungi R, Pascal J, Yarrot L, Barrett L, Dillingham R, Petri WA, Scharf R, Ahmed AMS, Alam MA, Haque U, Hossain MI, Islam M, Mahfuz M, Mondal D, Nahar B, Tofail F, Chandyo RK, Shrestha PS, Shrestha R, Ulak M, Bauck A, Black R, Caulfield L, Checkley W, Lee G, Schulze K, Scott S, Murray-Kolb LE, Ross AC, Schaefer B, Simons S, Pendergast L, Abreu CB, Costa H, Di Moura A, Filho JQ, Leite ÁM, Lima NL, Lima IF, Maciel BLL, Medeiros PHQS, Moraes M, Mota FS, Oriá RB, Quetz J, Soares AM, Mota RMS, Patil CL, Mahopo C, Maphula A, Nyathi E (2018) Use of quantitative molecular diagnostic methods to assess the aetiology, burden, and clinical characteristics of diarrhoea in children in low-resource settings: a reanalysis of the MAL-ED cohort study. The Lancet Global Health 6:e1309-e1318

59. Polman K, Becker SL, Alirol E, Bhatta NK, Bhattarai NR, Bottieau E, Bratschi MW, Burza S, Coulibaly JT, Doumbia MN, Horié NS, Jacobs J, Khanal B, Landouré A, Mahendradhata Y, Meheus F, Mertens P, Meyanti F, Murhandarwati EH, N'Goran EK, Peeling RW, Ravinetto R, Rijal S, Sacko M, Saye R, Schneeberger PHH, Schurmans C, Silué KD, Thobari JA, Traoré MS, van Lieshout L, van Loen H, Verdonck K, von Müller L, Yansouni CP, Yao JA, Yao PK, Yap P, Boelaert M, Chappuis F, Utzinger J (2015) Diagnosis of neglected tropical diseases among patients with persistent digestive disorders (diarrhoea and/or abdominal pain ≥14 days): a multi-country, prospective, non-experimental case–control study. BMC Infectious Diseases 15:338

60. Pop M, Walker AW, Paulson J, Lindsay B, Antonio M, Hossain MA, Oundo J, Tamboura B, Mai V, Astrovskaya I, Bravo HC, Rance R, Stares M, Levine MM, Panchalingam S, Kotloff K, Ikumapayi UN, Ebruke C, Adeyemi M, Ahmed D, Ahmed F, Alam MT, Amin R, Siddiqui S, Ochieng JB, Ouma E, Juma J, Mailu E, Omore R, Morris JG, Breiman RF, Saha D, Parkhill J, Nataro JP, Stine OC (2014) Diarrhea in young

children from low-income countries leads to large-scale alterations in intestinal microbiota composition. Genome Biology 15:R76

61. Prendergast AJ, Kelly P (2016) Interactions between intestinal pathogens, enteropathy and malnutrition in developing countries. Current Opinion in Infectious Diseases 29:229-236

62. Promega (2012) Technical Manual. Maxwell 16 DNA Purification Kits. USA

63. Promega (2014) Operating Manual. Maxwell 16 MDx Instrument. USA

64. Prüss-Ustün A, Bartram J, Clasen T, Colford JM, Jr., Cumming O, Curtis V, Bonjour S, Dangour AD, De France J, Fewtrell L, Freeman MC, Gordon B, Hunter PR, Johnston RB, Mathers C, Mäusezahl D, Medlicott K, Neira M, Stocks M, Wolf J, Cairncross S (2014) Burden of disease from inadequate water, sanitation and hygiene in low- and middle-income settings: a retrospective analysis of data from 145 countries. Tropical Medicine & International Health19:894-905

65. Qazi S, Aboubaker S, MacLean R, Fontaine O, Mantel C, Goodman T, Young M, Henderson P, Cherian T (2015) Ending preventable child deaths from pneumonia and diarrhoea by 2025. Development of the integrated Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea. Archives of Disease in Childhood 100:S23-S28

66. Quigley EM, Locke GR, Mueller-Lissner S, Paulo LG, Tytgat GN, Helfrich I, Schaefer E (2006) Prevalence and management of abdominal cramping and pain: a multinational survey. Aliment Pharmacol Ther 24:411-419

67. R-Biopharm. RIDA GENE ETEC/EIEC real-time PCR. Darmstadt: R-Biopharm AG

68. R-Biopharm. RIDA GENE Parasitic Stool Panel I real-time PCR. Darmstadt: R-Biopharm AG

69. R-Biopharm. RIDA GENE Bacterial Stool Panel real-time PCR. Darmstadt: R-Biopharm AG.

70. R-Biopharm. RIDA GENE EAEC real-time PCR. Darmstadt: R-Biopharm AG

71. Reischl U, Landt O, Rabenau HF, Geißdörfer W (2012) Nukleinsäurediagnostik im mikrobiologischen Labor: Neue Möglichkeiten des kulturunabhängigen Erregernachweises, der Speziesdifferenzierung und molekularen Resistenztestung. UNI-MED Verlag AG, Bremen

72. Reust CE, Williams A (2016) Acute abdominal pain in children. American Family Physician 93:830-837

73. Ricci KA, Girosi F, Tarr PI, Lim Y-W, Mason C, Miller M, Hughes J, von Seidlein L, Agosti JM, Guerrant RL (2006) Reducing stunting among children: the potential contribution of diagnostics. Nature 444:29

74. Rogawski ET, Liu J, Platts-Mills JA, Kabir F, Lertsethtakarn P, Siguas M, Khan SS, Praharaj I, Murei A, Nshama R, Mujaga B, Havt A, Maciel IA, Operario DJ, Taniuchi M, Gratz J, Stroup SE, Roberts JH, Kalam A, Aziz F, Qureshi S, Islam MO, Sakpaisal P, Silapong S, Yori PP, Rajendiran R, Benny B, McGrath M, Seidman JC, Lang D, Gottlieb M, Guerrant RL, Lima AAM, Leite JP, Samie A, Bessong PO, Page N, Bodhidatta L, Mason C, Shrestha S, Kiwelu I, Mduma ER, Iqbal NT, Bhutta ZA, Ahmed T, Haque R, Kang G, Kosek MN, Houpt ER, Acosta AM, Rios de Burga R, Chavez CB, Flores JT, Olotegui MP, Pinedo SR, Trigoso DR, Vasquez AO, Ahmed I, Alam D, Ali A, Rasheed M, Soofi S, Turab A, Yousafzai A, Zaidi AKM, Shrestha B, Rayamajhi BB, Strand T, Ammu G, Babji S, Bose A, George AT, Hariraju D, Jennifer MS, John S, Kaki S, Karunakaran P, Koshy B, Lazarus RP, Muliyil J, Ragasudha P, Raghava MV, Raju S, Ramachandran A, Ramadas R, Ramanujam K, Rose A, Roshan R, Sharma SL, Sundaram S, Thomas RJ, Pan WK, Ambikapathi R, Carreon JD, Doan V, Hoest C, Knobler S, Miller MA, Psaki S, Rasmussen Z, Richard SA, Tountas KH, Svensen E, Amour C, Bayyo E, Mvungi R, Pascal J, Yarrot L, Barrett L, Dillingham R, Petri WA, Scharf R, Ahmed AMS, Alam MA, Haque U, Hossain MI, Islam M, Mahfuz M, Mondal D, Nahar B, Tofail F, Chandyo RK, Shrestha PS, Shrestha R, Ulak M, Bauck A, Black R, Caulfield L, Checkley

W, Lee G, Schulze K, Scott S, Murray-Kolb LE, Ross AC, Schaefer B, Simons S, Pendergast L, Abreu CB, Costa H, Di Moura A, Filho JQ, Leite ÁM, Lima NL, Lima IF, Maciel BLL, Medeiros PHQS, Moraes M, Mota FS, Oriá RB, Quetz J, Soares AM, Mota RMS, Patil CL, Mahopo C, Maphula A, Nyathi E (2018) Use of quantitative molecular diagnostic methods to investigate the effect of enteropathogen infections on linear growth in children in low-resource settings: longitudinal analysis of results from the MAL-ED cohort study. The Lancet Global Health 6:e1319-e1328

75. Rosner BM, Stark K, Werber D (2010) Epidemiology of reported *Yersinia enterocolitica* infections in Germany, 2001-2008. BMC Public Health 10:337

76. Rossen NG, Bart A, Verhaar N, van Nood E, Kootte R, de Groot PF, D'Haens GR, Ponsioen CY, van Gool T (2015) Low prevalence of *Blastocystis* sp. in active ulcerative colitis patients. European Journal of Clinical Microbiology & Infectious Diseases 34:1039-1044

77. Ruiz-Palacios GM (2007) The health burden of *Campylobacter* infection and the impact of antimicrobial resistance: playing chicken. Clinical Infectious Diseases 44:701-703

78. Samb-Ba B, Mazenot C, Gassama-Sow A, Dubourg G, Richet H, Hugon P, Lagier J-C, Raoult D, Fenollar F (2014) MALDI-TOF identification of the human gut microbiome in people with and without diarrhea in Senegal. PloS One 9:e87419-e87419

79. Sarker SA, Ahmed T, Brüssow H (2017) Persistent diarrhea: a persistent infection with enteropathogens or a gut commensal dysbiosis? Environmental Microbiology 19:3789-3801

80. Stark D, Barratt J, Roberts T, Marriott D, Harkness J, Ellis J (2010) A review of the clinical presentation of dientamoebiasis. The American Journal of Tropical Medicine and Hygiene 82:614-619

81. Stark D, Barratt J, Chan D, Ellis JT (2016) *Dientamoeba fragilis*, the neglected Trichomonad of the human bowel. Clinical Microbiology Reviews 29:553-580

82. Steffen R CF, Nothdurft HD, Rombo L, Zuckerman JN. Vaccination against enterotoxigenic *Escherichia coli*, a cause of travelers' diarrhea. Journal of Travel Medicine 2005:102-107

83. Stratagene AT (2009). Instruction Manual. MxPro QPCR Software for Mx3000P and Mx3005P QPCR Systems. USA

84. Suerbaum S, Hahn H, Burchard GD, Kaufmann SHE, T S (2012) Medizinische Mikrobiologie und Infektiologie. Springer, Berlin Heidelberg

85. The HC, Florez de Sessions P, Jie S, Pham Thanh D, Thompson CN, Nguyen Ngoc Minh C, Chu CW, Tran T-A, Thomson NR, Thwaites GE, Rabaa MA, Hibberd M, Baker S (2017) Assessing gut microbiota perturbations during the early phase of infectious diarrhea in Vietnamese children. Gut Microbes 9:38-54

86. The MAL-ED Network Investigators (2014) The MAL-ED Study: A multinational and multidisciplinary approach to understand the relationship between enteric pathogens, malnutrition, gut physiology, physical growth, cognitive development, and immune responses in infants and children up to 2 years of age in resource-poor environments. Clinical Infectious Diseases 59:193-206

87. Traoré MS (2009). Situation Sanitaire du Mali. Journées Etudes Formation Administrative Continue., pp. 4-7

88. Turkeltaub JA, McCarty TRI, Hotez PJ (2015) The intestinal protozoa: emerging impact on global health and development. Current Opinion in Gastroenterology 31:38-44

89. Udoh E, Devanarayana NM, Rajindrajith S, Meremikwu M, Benninga MA (2016) Abdominal pain - predominant functional gastrointestinal disorders in adolescent Nigerians. Journal of Pediatric Gastroenterology and Nutrition 62:588-593

90. UNICEF, WHO (2009) Diarrhoea: Why children are still dying and what can be done

91. United Nations. Human Development Indicators 2019 - Mali (2020) [Internet, cited 17 September 2020]. URL: http://hdr.undp.org/en/countries/profiles/MLI

93. USAID - from the American people. African strategies for health - health financing profile: Côte d'Ivoire [Internet, cited 15 May 2018]. URL: http://www.africanstrategies4health.org/uploads/1/3/5/3/13538666/country_profile_-_cote_divoire_-_us_letter.pdf

94. Van den Bossche D, Cnops L, Verschueren J, Van Esbroeck M (2015) Comparison of four rapid diagnostic tests, ELISA, microscopy and PCR for the detection of *Giardia lamblia, Cryptosporidium spp.* and *Entamoeba histolytica* in feces. Journal of Microbiological Methods 110:78-84

95. Van Lieshout L, Verweij JJ (2010). Leiden University Medical Centre Sample Preservation Sheet

96. Vandenberg O, Peek R, Souayah H, Dediste A, Buset M, Scheen R, Retore P, Zissis G, Gool Tv (2006) Clinical and microbiological features of dientamoebiasis in patients suspected of suffering from a parasitic gastrointestinal illness: a comparison of *Dientamoeba fragilis* and *Giardia lamblia* infections. International Journal of Infectious Diseases 10:255-261

97. Verweij JJ, Oostvogel F, Brienen EAT, Nang-Beifubah A, Ziem J, Polderman AM (2003) Short communication: Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in northern Ghana. Tropical Medicine & International Health 8:1153-1156

98. Wallis EM, Fiks AG (2015) Nonspecific abdominal pain in pediatric primary care: evaluation and outcomes. Academic Pediatrics 15:333-339

99. Weintraub A (2007) Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. Journal of Medical Microbiology 56:4-8

100. WHO. Mali - The Health System [Internet, cited 21 April 2016]. URL: http://www.aho.afro.who.int/profiles_information/index.php/Mali:The_Health_System

101. WHO (1988) Persistent diarrhoea in children in developing countries: memorandum from a WHO meeting. Bulletin of the World Health Organisation 66:709-717

102. WHO (2005) The treatment of diarrhoea - a manual for physicians and other senior health workers

103. WHO, UNICEF (2006) Oral rehydration salts - Production of the new ORS.

104. WHO, Department of control of neglected tropical diseases. Working to overcome the global impact of neglected tropical diseases: first WHO report on neglected tropical diseases (2010). WHO Press, Geneva, Switzerland

105. WHO (2012). HIV and infant feeding 2010: an updated framework for priority action

106. WHO (2013). Diarrhoeal disease. WHO Fact sheet No. 330. [Internet]. [cited 15 February 2016]. URL: http://www.who.int/mediacentre/factsheets/fs330/en/

107. WHO (2015). Côte d'Ivoire: WHO statistical profile [Internet, cited 05 May 2016]. URL: http://www.who.int/gho/countries/civ/en/

108. WHO (2015). Mali: WHO statistical profile [Internet, cited 05 May 2016]. URL: http://www.who.int/gho/countries/mli/en/

109. WHO (2017). The Top 10 causes of death. WHO Fact sheet No. 310 [Internet, cited 13 Feburary 2018]. URL: http://www.who.int/mediacentre/factsheets/fs310/en/

110. WHO (2014). The Top 10 causes of death. WHO Fact sheet No 310 [Internet, cited 15 February 2016]. URL: http://www.who.int/mediacentre/factsheets/fs310/en/

111. WHO, UNAIDS, UNFPA, UNICEF (2010) Guidelines on HIV and infant feeding. Principles and recommendations for infant feeding in the context of HIV and a summary of evidence. WHO Document Production Services

112. Won EJ, Kim SH, Kee SJ, Shin JH, Suh SP, Chai JY, Ryang DW, Shin MG (2016) Multiplex real-time PCR assay targeting eight aarasites customized to the Korean population: potential use for detection in diarrheal stool samples from gastroenteritis patients. PLoS One 11:e0166957

8. Appendices

8.1 Research authorisation from Ethikkommission beider Basel EKBB

Ethikkommission beider Basel EKBB

Basel 28/11/2013
Org. J. Utzinger, Swiss TPH
cc M. Boelaert, ITM, Belgium
F. Chappuis, Geneva University Hospitals
K. Polman, ITM, Belgium
E.K. N'Goran, Uni FHB, Côte d'Ivoire
Y. Malandradhata, Indonesia
M. Sacko, Mali
S. Rijal, Nepal
M. Tanner, Utrecht Swiss TPH
C. Bussi / Study secretariat, Swiss TPH
J. Becker, Swiss TPH
P. Yap, Swiss TPH

Herrn
Prof. Dr. J. Utzinger
Schweizerisches Tropen- und Public Health-Institut
Socinstrasse 57
4002 Basel

Basel, 22. November 2013

217/13:
Diagnosis of neglected tropical diseases (NTDs) in patients presenting with persistent digestive disorders (≥2 weeks) in Côte d'Ivoire, Indonesia, Mali and Nepal

Sehr geehrter Herr Professor Utzinger

Die obgenannte Studie wurde am 27. August 2013 anlässlich der Sitzung der Ethikkommission beider Basel geprüft und beurteilt. Die folgenden Punkte wurden diskutiert:

- Protokoll: Gegen die Studie bestehen keine grundsätzlichen ethischen Bedenken.
  - Die EKBB geht davon aus, dass die Information der Studienteilnehmer - wie dies in früheren Studien des Swiss TPH der Fall war - mündlich in der jeweiligen Landessprache durchgeführt wird und der schriftliche Text vor allem als Leitfaden für die Information dient.
  - Die EKBB bestätigt, dass aus ihrer Sicht keine weiteren ethischen Bedenken vorliegen gegen die Durchführung der Studie.

Diese Studie wurde gemäss der ICH-GCP (International Conference on Harmonisation - Good Clinical Practice) Richtlinie evaluiert. Sie erfüllt die Voraussetzungen für die Durchführung von Forschungsstudien in der Schweiz, nämlich:

- Die wissenschaftliche Stichhaltigkeit und Relevanz des Forschungsprojekts wie auch der zu erwartenden Resultate;
- Ein günstiges Nutzen-Risiko Verhältnis;
- Das Einverständnis der Studienteilnehmer;
- Den Schutz der Privatsphäre und Vertraulichkeit;
- Die berufliche Qualifikation der in diesem Projekt involvierten Schweizer Forschenden;
- Die Festlegung der Qualifikationen, die für weitere beteiligte Forschende erforderlich sind.

Ob dieses Projekt aus ethischer Sicht gutgeheissen werden kann, hängt von lokalen Umständen ab, die hier nicht abgeschätzt werden konnten. Im Speziellen wurde im Antrag nicht auf die folgenden Punkte eingegangen:

- Vorgehen und Dokumentation der Rekrutierung von Studienteilnehmern, insbesondere die in der lokalen Sprache verfassten Informationsblätter und Einverständniserklärungen;
- die Angemessenheit der lokalen Infrastruktur (Material, Einrichtungen, Personal etc.) hinsichtlich des bestmöglichen Schutzes der Studienteilnehmer;

./.

Geschäftsführerin: Frau Irene Oberli, Hebelstrasse 53, 4056 Basel, Telefon 061 268 13 50, Fax 061 268 13 51, ekbb@bs.ch, www.ekbb.ch

- berufliche Qualifikation des Nicht-Schweizerischen Personals.

Die oben aufgelisteten Punkte müssen, je nachdem wo das Projekt durchgeführt wird, durch das für das jeweilige Land / die jeweiligen Länder zuständige(n) Ethikkomitee(s) beurteilt werden.

Zudem hat die Ethikkommission beider Basel das revidierte Studienprotokoll (Version 2.1 24/10/2013), die revidierten Informationen und Einverständniserklärungen („Patienten" & „Kontrollen" in Englisch und Französisch – jeweils Version 2.1 24/10/2013) sowie die CRFs („Patienten" & „Kontrollen" - Version 2.0 28/10/2013), zustimmend zur Kenntnis genommen.

Mit freundlichen Grüssen

Prof. A. R. Perruchoud
im Namen der Ethikkommission
beider Basel / EKBB

Prof. M. Kränzlin
Vizepräsident der Ethikkommission
beider Basel / EKBB

8.2 Research authorisation from Ethisch Comité UZA

UZA
Wilrijkstraat 10 / 2650 Edegem
Tel 03 821 30 00 / Fax 03 829 05 20
www.uza.be / BE 0874.619.603

Universiteit Antwerpen

ETHISCH COMITE

VOORZITTER
Prof. dr. Patrick Cras

SECRETARIAAT
tel: 03 821-35
fax: 03 821 42

Dr. K. POLMAN

Instituut voor Tropische Geneeskunde
Parasitologie

2000 Antwerpen

Diagnosis of neglected tropical diseases (NTDs) in patients presenting with persistent digestive disorders (>2 weeks) in Côte d'Ivoire, Indonesia, Mali and Nepal. (ITG: 872/13)
Belgisch Registratienummer: B300201318109

datum	ons kenmerk	contactpersoon
12/08/2013	13/25/258	Annelies Van Looy / Kim Vernimmen ethisch.comite@uza.be

DEFINITIEF GUNSTIG ADVIES

Geachte Collega,

Het Ethisch Comité van het Universitair Ziekenhuis Antwerpen en de Universiteit Antwerpen bevestigt dat bovenvermelde studie voldoet aan de criteria gesteld in de wet van 7 mei 2004 en geeft een gunstig advies dd. 12/08/2013.

De volgende bijlagen werden volgens de GCP-ICH richtlijnen door het Ethisch Comité goedgekeurd:

- Bewijs van "no-fault" verzekering Brief ITG versie 22/03/2013
- Protocol WP2-01-DIG, versie 2 versie 22/05/2013
- Informatie- en toestemmingsformulier ENG Patients, versie 2 (ITG) versie 22/05/2013
- Informatie- en toestemmingsformulier ENG Healthy Controls, versie 2 (ITG) versie 22/05/2013
- Informatie- en toestemmingsformulier ENG Patients, versie 1 (NIDIAG Consortium) versie 22/03/2013
- Informatie- en toestemmingsformulier ENG Healthy Controls, versie 1 (NIDIAG Consortium) versie 22/03/2013
- CV onderzoeker
  - Dr. K. Polman
  - Prof. K.E. N'Goran
  - Dr. M. Sacko
  - Dr. Y. Mahendradhata
  - Dr. S. Rijal
- Diverse - Ethics charter of the NIDIAG Consortium versie 25/04/2012

Tenslotte verzoeken wij u ons mee te delen indien een studie niet wordt aangevat, of wanneer ze wordt afgesloten of vroegtijdig onderbroken.

Met vriendelijke groeten,

Prof. dr. G. IEVEN
Ondervoorzitter Ethisch Comité

Cc: FAGG - Research & Development Department, Victor Hortaplein 40, bus 40 - 1060 Brussel
Prof. dr. M. BOELAERT, Instituut voor Tropische Geneeskunde - Volksgezondheid, Nationalestraat 155 - 2000 Antwerpen

8.3 Research authorisation from Comite d'Ethique de L'INRSP

MINISTERE DE LA SANTE
ET DE L'HYGIENE PUBLIQUE
-=-=-=-=-
COMITE D'ETHIQUE DE L'INRSP
BP 1771/ Tél : 20 21 42 31 / Fax : 20 21 43 20
Portable : 66 78 11 13 / 76 18 72 60
- Bamako
-=-=-=-=-

REPUBLIQUE DU MALI
Un Peuple - Un But - Une Foi
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DECISION N° 13/2013 /CE-INRSP

LE PRESIDENT DU COMITE D'ETHIQUE DE L'INSTITUT NATIONAL DE RECHERCHE EN SANTE PUBLIQUE (INRSP)

Vu l'arrêté n°2013-1223/MS-SG du 03 avril 2013 portant nomination des membres du Comité d'Ethique de l'Institut National de Recherche en Santé Publique (INRSP) ;

Vu les recommandations n°13/13/INRSP-CE issues de la session du 27 novembre 2013 du comité d'éthique de l'INRSP relative à l'examen de protocole de recherche intitulé ***«Le diagnostic des maladies tropicales négligées (MTN) chez les patients présentant des troubles digestifs persistants (≥ 2 semaines) en Côte d'Ivoire, en Indonésie, au Mali et au Népal» ;***

Vu les corrections apportées audit protocole conformément aux recommandations formulées par le comité d'éthique.

DECIDE

Article 1er/ : Le protocole de recherche intitulé ***«Le diagnostic des maladies tropicales négligées (MTN) chez les patients présentant des troubles digestifs persistants (≥ 2 semaines) en Côte d'Ivoire, en Indonésie, au Mali et au Népal»*** jugé conforme à l'éthique et aux droits humains, est approuvé par le comité d'éthique de l'Institut National de Recherche en Santé Publique (INRSP).

Article 2/ : Toute modification intervenant dans l'exécution dudit Protocole est portée à la connaissance du Comité d'Ethique de l'INRSP dans un délai maximum de quinze (15) jours.

Article 3/ : La présente décision valable pour toute la durée de l'étude, sera enregistrée et communiquée partout où besoin sera.

Bamako, le 28 novembre 2013
LE PRESIDENT DU COMITE D'ETHIQUE,

Ampliations :
Président /CE 1
Archives/CE 1
Institute of Tropical Medicine Antwerpen 2

INSTITUT NATIONAL DE RECHERCHE EN SANTE PUBLIQUE
(I.N.R.S.P)
Comité d'Ethique
LE PRESIDENT

Pr. Boulkassoum HAIDARA

9. Publications

1. **Jasmin K. Jasuja**, Florian Bub, Jonas Veit, Hassan K. M. Fofana, Moussa Sacko, Rénion Saye, Justin K. Chatigre, Eliézer K. N'Goran, Joel A. Yao, Basudha Khanal, Kanika Koirala, Narayan R. Bhattarai, Suman Rijal, Lutz von Müller, Emmanuel Bottieau, Marleen Boelaert, François Chappuis, Katja Polman, Jürg Utzinger, Sören L. Becker (2024). Multiplex PCR for bacterial, viral and protozoal pathogens in persistent diarrhoea or persistent abdominal pain in Côte d'Ivoire, Mali and Nepal. Sci Rep. 2024 May 13. doi: https://doi.org/10.1038/s41598-024-60491-y

2. Julia D. Michels-Zetsche, Vicky Gassmann, **Jasmin K. Jasuja**, Benjamin Neetz, Philipp Höger, Jan Meis, Simone Britsch, Urte Sommerwerck, Sebastian Fähndrich, Florian Bornitz, Michael M. Müller, Felix J.F. Herth & Franziska C. Trudzinski (2024). Role of multidrug-resistant bacteria in weaning from invasive mechanical ventilation. Respir Res 25, 69 (2024). doi: https://doi.org/10.1186/s12931-024-02694-5

3. **Jasmin K. Jasuja**, Stefan Zimmermann, Irene Burckhardt (2021). Applicability and performance of EUCAST's rapid antimicrobial susceptibility testing (RAST) on primarily sterile body fluids in blood culture bottles in laboratory routine with total lab automation. Eur J Clin Microbiol Infect Dis. 2021 Jan 12. doi: 10.1007/s10096-020-04146-6.

4. **Jasmin K. Jasuja**, Stefan Zimmermann, Irene Burckhardt (2020). Applicability and performance of EUCAST's rapid antibiotic susceptibility testing (RAST) on sterile body fluid in blood culture bottle. 6th Joint Conference of the DGHM & VAAM in Leipzig, Germany; 8-11 March 2020

5. **Jasmin K. Jasuja,** Stefan Zimmermann, Irene Burckhardt (2020). Evaluation of EUCAST rapid antimicrobial susceptibility testing (RAST) for positive blood cultures in clinical practice using a total lab automation. Eur J Clin Microbiol Infect Dis. 2020;39(7):1305-1313. doi:10.1007/s10096-020-03846-3

6. **Jasmin K. Jasuja,** Stefan Zimmermann, Irene Burckhardt (2019). Blood cultures processing using total lab automation: reduced time to report using rapid antimicrobial susceptibility testing. 29th European Congress of Clinical Microbiology and Infectious Diseases in Amsterdam, Netherlands; 13-16 September 2019

7. Stefan Zimmermann, **Jasmin K. Jasuja,** Irene Burckhardt (2019). Blood Culture Processing Using Total Lab Automation (TLA) and Rapid Antimicrobial Susceptibility Testing (RAST) Result in Reduced Time-to Report. ASM Microbe 2019 in San Francisco, USA; 20-24 June 2019

8. **Jasmin K. Jasuja**, Hassan K.M. Fofana, Jonas Veit, Rénion Saye, Mama N. Doumbia, Anna Nimmesgern, Aly Landouré, Mamadou S. Traoré, Mathias Herrmann, Peiling Yap, Katja Polman, Marleen Boelaert, François Chappuis, Jürg Utzinger, Lutz von Müller, Sören L. Becker, Moussa Sacko (2016). Multiplexed polymerase chain reaction (PCR) for the diagnosis of intestinal pathogens in patients with persistent digestive disorders: a case-control study from Mali. 68th Annual Meeting of the German Society of Hygiene and Tropical Medicine in Ulm, Germany; 12 September 2016

9. **Jasmin Jasuja**, Jonas Veit, Hassan K. M. Fofana, Anna Nimmesgern, Rénion Saye, Mama N. Doumbia, Aly Landouré, Mamadou N Traoré, Mathias Herrmann, Peiling Yap, Katja Polman, Marleen Boelaert, François Chappuis, Jürg Utzinger, Moussa Sacko, Lutz von Müller, Sören L. Becker (2015). Stool-based polymerase chain reaction for the diagnosis of multiple pathogens in Mali: a case-control study. 9th European Congress on Tropical Medicine and International Health (ECTMIH) in Basel, Switzerland; 07 September 2015

10. Acknowledgments

When I started with my MD thesis in October 2014, I would have never imagined that this time period could become one of the best and most memorable parts of my studies. The years reflect an intensive period full of hard work, fruitful cooperation, educative experience and – most of all – joy. The extensive experience I gained in these years was precious and gave me the possibility to rediscover myself and my strengths. This acknowledgement is the final touch of my MD thesis and maybe the most difficult part because words cannot describe my deep gratitude to each supportive person. This thesis would have not been possible without them.

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11. Curriculum vitae

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.

Tag der Promotion: 13. Januar 2025

Dekan: Univ.-Prof. Dr. med. dent. Matthias Hannig

Berichterstatter: Prof. Dr. med. Dr. phil. Sören Leif Becker

Prof. Dr. med. Jörn Markus Schattenberg