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Epidemiology and clinical features of *Clostridioides difficile*-associated diarrhoea and intestinal parasitic infections in Mali

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Abbreviations

CDC	Centers for Disease Control and Prevention
CDI	*Clostridioides (syn.: Clostridium) difficile* infection
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme-Linked Immunosorbent Assay
EPG	Eggs per Gram
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ELISA	Enzyme-linked immunosorbent assay
GDH	Glutamate dehydrogenase
HAI	Healthcare-associated infections
HIV	Human immunodeficiency virus
i.e.	Id est
INRSP	Institut National de Recherche en Santé Publique
MIC	Minimum inhibitory concentration
NAAT	Nucleic acid amplification test
NIDIAG	Acronym of a study consortium entitled “Syndromic approach to Neglected Infectious Diseases (NID) at primary health care level: an international collaboration on integrated diagnostic-treatment platforms”
NSAID	Non-nonsteroidal anti-inflammatory drugs
SLS	Sample loading solution
SoC	Standard of Care
PaLoc	Pathogenicity locus
PCR	Polymerase chain reaction
TAT	Turn-around-time
TMD	transmembrane domain of the toxin
RBD	Receptor binding domain
RDT	Rapid diagnostic test
RT	Ribotyp
WHO	World Health Organization

Zusammenfassung

Hintergrund: Obwohl *Clostridioides difficile* heute weltweit als einer der wichtigsten Durchfallerreger mit einer hohen Sterblichkeit und bedeutenden Auswirkungen auf das Gesundheitssystem gilt, liegen wenig Daten zur Epidemiologie und dem klinischen Stellenwert von *C. difficile* in ärmeren Ländern des globalen Südens vor. Durchfallerkrankungen sind in vielen afrikanischen Ländern von erheblicher Relevanz, da sie häufig mit einer hohen Morbidität und Mortalität verbunden sind. Weil eine adäquate Labordiagnostik in wenig entwickelten Ländern meist fehlt, bleibt die Ätiologie von Durchfallerkrankungen häufig unklar. Aktuelle Studien aus afrikanischen Ländern zeigen, dass *C. difficile* auch dort als mögliche Differentialdiagnose für Durchfallerkrankungen in Betracht gezogen werden sollte. Zunehmend wird auch ein möglicher zoonotischer Übertragungsweg über engen Tierkontakt und tierische Produkte diskutiert.
Methoden: Zwischen Februar und April 2016 wurde eine Fall-Kontroll-Studie durchgeführt, um die Prävalenz von *C. difficile* bei Patienten mit Durchfall, asymptomatischen Kontrollen sowie Tierproben in Mali zu analysieren. In Bamako und Niono wurden insgesamt 333 Stuhlproben akquiriert, wobei 233 Proben humanen Ursprungs waren und aus Krankenhäusern und Gesundheitszentren stammten. Weitere 100 Proben wurden von Nutztieren auf lokalen Viehmärkten gesammelt. Ein diagnostischer Schnelltest (RDT), der eine *C. difficile*-spezifische Glutamatdehydrogenase (GDH) und die Toxine A und B nachweist, eine anaerobe Stuhlkultur und ein Polymerase-Kettenreaktionstest (PCR) wurden vor Ort in Bamako durchgeführt. Die Studienteilnehmer beantworteten zusätzlich einen Fragebogen, um mögliche Risikofaktoren für eine *C. difficile* Infektion zu detektieren. Alle positiven Proben wurden nach Homburg für Empfindlichkeitstests und zur weiteren Ribotypisierung transferiert. Weiterhin wurden vor Ort in Mali Stuhlproben mittels der Kato-Katz Methode und mikroskopischem Direktnachweis auf Parasiten untersucht. Kato-Katz und der mikroskopische Direktnachweis wurden bezüglich ihrer diagnostischen Übereinstimmung beurteilt.
Ergebnisse: Unter Verwendung des Schnelltests zum Nachweis der pathogen-spezifischen GDH betrug die Prävalenz von *C. difficile* beim Menschen 7,2 % in Bamako (13/180 Proben) und 0 % in Niono (0/53 Proben). Unter den 100 Proben tierischen Ursprungs war nur eine einzige von einem Huhn stammende Probe positiv. Bei den positiven Proben war der Schnelltest für *C. difficile*-spezifische Toxine in allen humanen Proben negativ, in der tierischen Probe jedoch positiv. Die weitere PCR-Untersuchung der positiven Proben in Mali zeigte in der Tierprobe sowie in drei weiteren humanen Proben die Toxine A und B. In Mali konnte in keiner Probe ein binäres Toxin nachgewiesen werden. Im Gegensatz dazu ergab die PCR in Homburg identische Ergebnisse für die positive Tierprobe, fand aber nur eine toxigene Humanprobe, die positiv für die Toxine A und B sowie das binäre Toxin war. Zuvor in der Literatur beschriebene Ribotypen konnten in 9 Fällen identifiziert werden, wobei RT084 am häufigsten nachgewiesen wurde. RT078 und RT012 wurden ebenfalls gefunden, vier Proben gehörten zu bisher unbeschriebenen Ribotypen. Antibiotika-Resistenzen fanden sich nur gegenüber Clarithomycin (9/13, 69%). Es bestand kein signifikanter Zusammenhang zwischen allgemeinen Risikofaktoren wie Alter, Antibiotikatherapie und Krankenhausaufenthalt und einer *C. difficile*-Infektion. Ein Zusammenhang zwischen dem Vorliegen einer Diarrhoe und dem Nachweis von *C. difficile* konnte gezeigt werden (n= 2, 16,6%, p=0,007). Die häufigsten Helminthen waren *Dicrocoelium dendriticum* in Bamako (9/180, 5,0%) und *Schistosoma mansoni* in Niono (34/53; 64,2%)
Schlussfolgerung *C. difficile* konnte in Mali sowohl in humanen Proben als auch in einer Tierprobe nachgewiesen werden. Einfache und günstige Screeningtests, wie beispielsweise der GDH-Test, können zu Detektion einer *C. difficile* Infektion auch in ressourcenlimitierten Settings beitragen. Als wichtige Differentialdiagnose des Durchfalls sollten in Mali parasitäre Erkrankungen in Betracht gezogen werden. Um den tatsächlichen Stellenwert von *C. difficile* als krankheitserregendes Pathogen in Mali bewerten zu können, bedarf es weiterer Studien.

Summary

Background: Although *Clostridioides difficile* is nowadays considered one of the most important diarrheal pathogens worldwide, with a high mortality and significant impact on the health care system, few data are available on the epidemiology and clinical burden of *C. difficile* in lower-income countries of the global South. Diarrheal diseases are of considerable relevance in many African countries, as they give rise to high morbidity and mortality. Because adequate laboratory diagnostics are often unavailable in less developed countries, the aetiology of diarrheal diseases remains frequently unclear. Recent studies from African countries indicate that *C. difficile* should also be considered as a possible differential diagnosis for diarrheal diseases. There is also an ongoing debate on a possible zoonotic route of transmission via close animal contact and animal products.

Methode: A case-control study was conducted between February and April 2016 to analyse the prevalence of *C. difficile* in patients with diarrhea, asymptomatic controls, and animal samples in Mali. A total of 333 stool samples were acquired in Bamako and Niono, with 233 samples of human origin from hospitals and healthcare centers. Another 100 samples were collected from animals at local livestock markets. A rapid diagnostic test (RDT) detecting the *C. difficile*-specific glutamic dehydrogenase (GDH) and toxins A and B, an anaerobic stool culture, and a polymerase chain reaction (PCR) test were performed on-site in Bamako. Study participants also answered a questionnaire to detect possible risk factors for *C. difficile* infection. All positive samples were transferred to Homburg for susceptibility testing and further ribotyping. Additionally, stool samples were examined on site for parasites using the Kato-Katz method and microscopic direct detection. Kato-Katz and microscopic direct detection were assessed for diagnostic agreement.

Results: Using the rapid test for detection of pathogen-specific GDH, the prevalence of *C. difficile* in humans was 7.2% in Bamako (13/180 samples) and 0% in Niono (0/53 samples). Among the 100 samples of animal origin, only one sample originating from a chicken was positive. Among the positive samples, the rapid test for *C. difficile*-specific toxins was negative in all human samples but positive in the animal sample. Further PCR testing of the positive samples in Mali showed toxins A and B in the animal sample and in three other human samples. In Mali, no binary toxin could be detected in any sample. The PCR in Homburg, in contrast, showed identical results for the positive animal sample, but found only one toxigenic human sample positive for toxins A and B and the binary toxin. Ribotypes previously described in the literature were identified in 9 cases, with RT084 being the most frequently detected. RT078 and RT012 were also found, and four samples belonged to previously undescribed ribotypes. Antibiotic resistance was found only against clarithomycin (9/13, 69%). There was no significant association between general risk factors such as age, antibiotic therapy, and hospitalization, with *C. difficile* infection. However, a correlation between the occurrence of diarrhea and the detection of *C. difficile* was found (n= 2, 16.6% p=0.007). The most common helminths were *Dicrocoelium dendriticum* in Bamako (9/180, 5.0%) and *Schistosoma mansoni* in Niono (34/53, 64.2%)

Conclusion: *C. difficile* was detected in both human and animal samples from Mali. Simple and inexpensive screening tests, such as the GDH test, can contribute to detection of *C. difficile* infection even in resource-limited settings. Parasitic diseases should be considered as an important differential diagnosis of diarrhea in the tropics. Further studies are needed to assess the true importance of *C. difficile* as a disease-causing pathogen in Mali.

1. Introduction

1.1. Characteristics of *Clostridioides difficile*

Clostridioides difficile (until 2016 named *Clostridium difficile*) is a Gram-positive rod-shaped, anaerobic bacterium [71]. Through its ability to form aerotolerant spores, it is resistant towards heat and extreme dehydration as well as towards chemical substances such as disinfection agents and sanitisers. Due to these characteristics, *C. difficile* can be detected ubiquitously in the environment [4]. In 1935, Hall and O'Tool were the first researchers to successfully detect *C. difficile* in stool samples from healthy infants, which was considered part of the physiological intestinal flora [50]. During the 1970s, *C. difficile* infection (CDI) was identified as a pathogen giving rise to nosocomial intestinal infections with diarrhoea and as a cause of pseudomembranous colitis, which occurred more frequently in connection with a previous antibiotic therapy [13].

Until 2003, *C. difficile* outbreaks were only sporadically documented. Yet, more than 200 individuals died in Quebec in 2003 and 2004 from CDI, which spurred further investigation as to how the bacterium could cause such a severe disease. A specific, so-called hypervirulent ribotype (RT), which was named RT027, could be identified, and was linked to the enormous fatality rate of almost 30%. Besides specific virulence characteristics, this strain showed a reduced susceptibility to vancomycin and metronidazole – the antibiotics most commonly used to treat CDI – and an acquired resistance to fluoroquinolone antibiotics such as ciprofloxacin, which facilitated this strain to spread worldwide [83].

Nowadays, *C. difficile* is considered as one of the most important pathogens of nosocomial infections, with a considerable fatality rate and a large impact on health systems. The reported number of CDIs has increased over the past 15 years. Accordingly, outbreaks of *C. difficile* were frequently observed in hospitals and other healthcare facilities. According to data put forth by the European Centre for Disease Prevention and Control (ECDC), up to 189.526 deaths related to CDI occur across Europe every year, and *C. difficile* may be responsible for over 50% of gastro-intestinal, hospital-acquired infections (HAIs) [116]. Additionally, it has also been acknowledged recently that community-acquired CDI are gaining more and more importance [68]. Indeed, 10-27 % of all CDIs are estimated to be acquired in non-healthcare settings [67,25]. More recently, *C. difficile* was also detected in food and samples of animal origin. This may on the one hand offer a new potential reservoir for the bacterium, while it may on the other hand also create another source of human infection. Indeed, toxigenic *C. difficile* strains were detected in various specimens such as cooked pork and beef meat from different countries [66,41]. Likewise, there is a controversial debate pertaining to the bacterium's potential as a zoonotic pathogen, as in particular its RT078, which also infects humans, has been repeatedly isolated from animals, e.g. on a Dutch pig farm [63].

C. difficile is transmitted by oral ingestion of the vegetative form of the bacterium or the spores, which are excreted in large quantities in the liquid stool of an infected person [64]. The vegetative form of the bacterium is extremely labile, but the spore can persist in the environment over a long period of time. The spores can be transmitted directly, i.e. through direct person-to-person contact or indirectly, through contaminated surfaces of the patient's environment [23,60]. Due to the ability to form environmentally resistant spores that cannot be removed with common disinfectants, outbreaks can occur in hospitals. An important preventative measure is therefore the washing of hands to remove the spores mechanically, in addition to hand disinfection.

- Stable in the environment for prolonged periods of time (spore-forming bacillus)
- Low inoculating dose (based on animal studies)
- Relative resistance to germicides (antiseptics and commonly used disinfectants)
- Fecal-oral transmission

Table 1. Microbiological features of *C. difficile* that may favour environmental transmission [125].

1.2. Epidemiology

C. difficile does not only occur almost ubiquitously in the environment, but it can also be regularly detected in the intestinal tract of humans and animals [4], where it has no clinical implications unless toxin-producing strains are present. In infants and toddlers, *C. difficile* can be detected in up to 80% of the samples. In up to 5 percent of the adult population, non-toxigenic *C. difficile* occur as part of the physiological intestinal flora [69]. Following hospitalisation, there is a relatively rapid increase in colonization to approximately 20-40%, with most patients remaining asymptomatic [14]. *C. difficile* causes about 15-20% of antibiotic-associated diarrheal diseases and more than 95% of cases of pseudomembranous colitis cases [15]. The increasing incidence of CDIs was first observed in the United States of America (USA) and in Canada. In Quebec, an incidence rate of 35 CDI cases per 100.000 inhabitants was recorded in 1991, as compared to 156 CDI cases per 100.000 inhabitants in 2003. An increase in 30-day mortality from 5% to 14% was also noted [92]. In the USA, it is estimated that the number of both nosocomial and community-acquired CDI cases has doubled between 2000 and 2005. In 2009, 1% of hospitalized patients in the US were affected by CDI [133,80,75]. The fluoroquinolone-resistant *C. difficile* strain RT027 (also known as toxinotype III, North American pulse field gel electrophoresis type 1 for short NAP 1) is primarily responsible for the rising incidence in the USA.

In Europe, data on the frequency of CDIs were collected for the first time in 2002, and this survey included 212 hospitals in 8 different countries. These data showed an incidence of 11 CDI cases per 10.000 individuals [10]. In 2004, 174 patients in a British hospital contracted a RT027-

positive *C. difficile* strain. 10 patients died as a result of this outbreak [111]. RT027 rapidly spread across Europe since 2008.

In Germany, data from Saxony from 2008 showed an incidence of 5 to 20 cases per 100,000 inhabitants [24]. In the years 2004-2006, there was a doubling of the *C. difficile*-associated diseases in Germany [122]. In 2008, 416 cases of severe forms of CDI were reported, of which 222 had a fatal course. In 2016, a total of 2.337 severe forms of a CDI were reported to the Robert Koch Institute (RKI). The National Reference Center for Surveillance of Nosocomial Infections published a report in 2016, which stated an overall CDI prevalence of 0.42 per 100 patients and an incidence density of 0.36 CDI cases per 1.000 patient days [88]. It is important to note that not only RT027 is of great epidemiological importance, but any toxin-producing strain can lead to serious infections. Examples of strains that regularly lead to outbreaks are RT001, RT 015, RT 017, RT018, RT 046, RT078, RT106, RT176 and RT244 [9,99]. Thus far, isolates of RT001 and RT027 predominate in nosocomial infections in Germany.

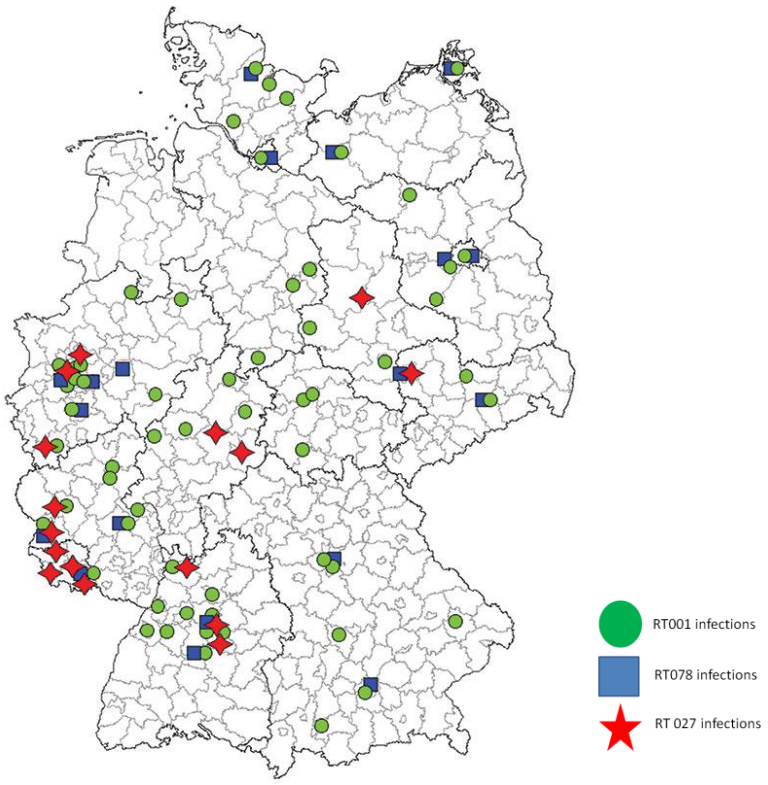

Figure 1 Approximate geographic dissemination of PCR ribotypes 001, 078, and 027 of *Clostridium difficile* in Germany in 2008 [52]

In contrast, there is comparatively little data on the prevalence of *C. difficile* in Africa. Therefore, it is difficult to evaluate the epidemiology and its clinical relevance as a pathogen giving rise to diarrhoeal diseases. Indeed, *C. difficile* is rarely considered in the clinical differential diagnostics and in microbiological laboratories. However, as many antibiotics are available in Africa without a medical prescription as 'over-the-counter' drugs, there is reason to argue that CDI might occur relatively

frequently. In 2015, a total of 298 human stool samples from the West African country Côte d'Ivoire were examined using a rapid diagnostic test for *C. difficile*. Among all samples, 16 (5%) were positive for *C. difficile* [18]. Another pilot study from Nigeria examined 140 stools of patients presenting with diarrhoea for *C. difficile*. Both inpatient and outpatient stool samples were analysed, and 97 samples stemmed from HIV-positive patients. Interestingly, toxigenic infections were observed among 10 of the 71 (14%) outpatients. It was also observed that HIV-positive outpatients with diarrhoea were more likely to be affected by a toxigenic strain than symptomatic HIV-negative outpatients [90].

In another study from South Africa, toxin-producing strains were detected in 23 (7.1%) of 322 study participants. In 20 of the toxin-positive patients, severe diarrhoea was a major clinical symptom [105]. Similar data were published in 2013 by another South African study group. They examined the incidence of CDIs in 643 patients with diarrhoea, and found CDI in 59 (9.2%) patients. Two of the infections were caused by the hypervirulent RT027 [97]. Similar results were reported from Zimbabwe [108] and Zambia [89].

1.3. Risk factors

Predisposing host factors play a major role as to whether and in what severity CDI-associated symptoms may occur. Previous antibiotic treatment is a major risk factor for CDI as it may disrupt the 'normal' composition of the intestinal flora and allow for a dysbiotic overgrowth of *C. difficile*. While the administration of clindamycin, ampicillin or cephalosporins was historically considered as a particular predisposing risk factor for CDI [22,35], it is currently believed that almost any antibiotic can trigger such an infection to a certain degree [2,110].

Additionally, proton pump inhibitors (PPI) and H2-receptor antagonists are typically seen as risk factors for CDI. While the vegetative form of *C. difficile* is not acid-stable and would not survive the gastric passage at a normal pH, the spores are acid-resistant and can thus pass on to the small intestine where they sporulate and transform into the vegetative, toxin-producing form. If the gastric pH is above 5, the vegetative forms can also enter the intestine. Bile acid promotes sporulation of *C. difficile*. In gastroesophageal reflux, bile acid is regularly detectable in the stomach and is treated with a PPI. Thus, ideal conditions (low pH for the vegetative form as well as bile acid contact) result in an increased sporulation of *C. difficile*. Co-administration of PPIs and antibiotics has an additive effect [17]. However, some researchers also argue that it may not be the intake of PPIs *per se*, but the presence of comorbidities that increases the risk for CDI [78].

Also, the regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a roughly 30% elevated risk for CDI. Other major risk factors include advanced age over 65 years, previous hospitalisation, underlying gastrointestinal diseases, renal insufficiency, neoplasia, and immunosuppression by both cytotoxic agents and diseases such as HIV and organ transplantation [16,

32,46,48,56,82,125]. In contrast to the 'common' nosocomial CDI, the recent surge of community-acquired CDI does not seem to be related to specific risk factors [82].

1.4. Clinical symptomatology

The typical clinical pattern of CDI occurs when a patient presents with an abrupt onset of watery diarrhoea with a characteristic foulish-sweet odour [46]. Blood in the stool is unusual, but may be indicative of a particularly severe course [48]. In addition to diarrhoea, *C. difficile* can cause a wide range of clinical manifestations. Nausea, vomiting and abdominal pain, often accompanied by fever, are not uncommon [32]. Severe CDI manifestations may include complications such as ileus, pseudomembranous colitis (which is a very typical *C. difficile* manifestation) and toxic megacolon with intestinal perforation and resulting sepsis [56]. However, in rare, severe cases, CDI may present without diarrhoea, for example, when there is a postoperative paralytic ileus. Important laboratory hints pointing towards CDI include a distinct leucocytosis and, in some cases, hypoalbuminemia [124]. In mild cases of CDI, systemic signs may be absent. Clinically, CDI is often indistinguishable from other causes of diarrhoea, and hence, other bacterial, viral and parasitic pathogens should also be considered [12,54].

A patient over 2 years of age must meet one or more of the points below:
1. Diarrhoea or toxic megacolon as well as positive evidence of toxin-producing *C.difficile* strains
2. Pseudomembranous colitis detected by endoscopy
3. Histopathological evidence of *C. difficile* infection (with or without diarrhea) in one Endoscopy, colectomy or autopsy.

Table 2 Definition of a *C. difficile* Infection (CDI) [81]

According to estimates, approximately 1-2% of patients with CDI have a fatal course. However, mortality may be significantly higher in risk patients with comorbidities and patients that are infected with hypervirulent strains [93]. For example, mortality rates of more than 10-30% have been reported in patients over the age of 80 in whom *C. difficile* strains of RT027 could be isolated [40,74].

1.5. Toxins and ribotypes of *C. difficile*

C. difficile-specific toxins are responsible for clinically symptomatic infections. Hence, there is an important distinction between a simple *C. difficile* colonisation (in which only a mere colonisation of the intestine is present with an apathogenic *C. difficile* strain) and an infection in which the patient is symptomatic and toxigenic strains are detectable. *C. difficile* strains without toxin genes are unable to produce toxins and are therefore considered to be non-pathogenic. The pathogenic forms can produce

the disease-relevant virulence factors enterotoxin A (toxin A) and/or cytotoxin B (toxin B). In addition, hypervirulent strains such as RT027 may produce a so-called binary toxin (CdtA).

Toxin A (*TcdA*) is an enterotoxin responsible for the development of diarrhoea and colonic inflammation. *C. difficile* toxin B (*TcdB*) is a cell membrane-damaging cytotoxin [47]. Pathogenic strains usually produce both of these synergistically acting toxins. 2-4% of strains produce only cytotoxin B. Cytotoxin B can almost always be detected in pathogenic strains. Toxigenic strains carry a so-called pathogenicity locus (PaLoc) in their DNA sequence [104,123]. This pathogenicity locus contains the genes that code for toxins. In addition, the pathogenicity locus contains additional genes (*tcdD*, *tcdE* and *tcdC*) that have regulatory functions. The genes *tcdA* and *tcdB* encode the toxin A and toxin B. The gene *tcdD* is a positive regulator gene that increases the production of toxins A and B, whereas the gene *tcdC* is considered to be a negative regulator of toxin production. It is thought that the gene *tcdE* codes for a protein which is responsible for the release of toxins from the cell. In addition to the two toxins mentioned above, some strains also form the so-called binary toxin CdtA/B. The genes for the binary toxin are situated outside the PaLoc region. These hypervirulent strains possess a mutation in the toxin repressor gene *tcdC*, thus leading to hyperproduction of toxins A and B. In the microbiology laboratory, toxin typing is based on sequence variation analysis of the toxin A and B genes and their adjacent regulatory genes [79].

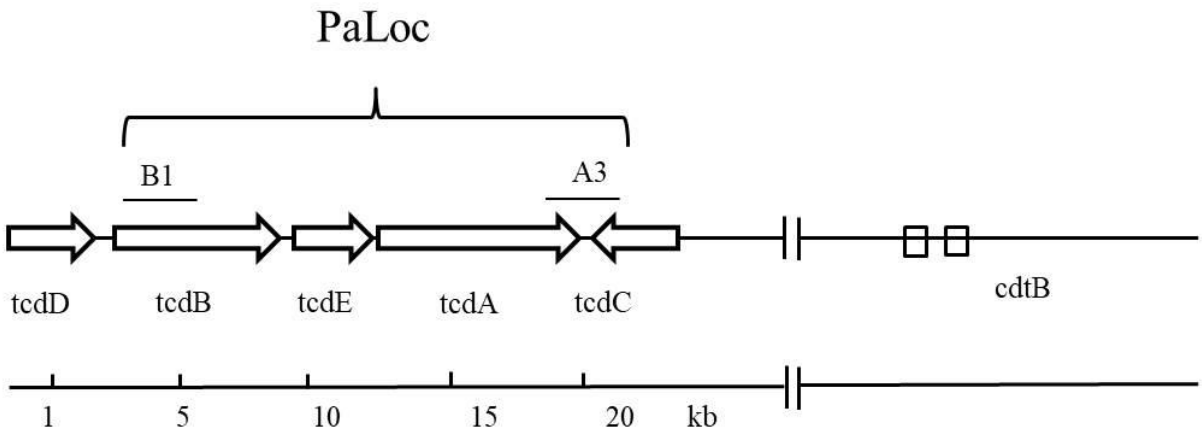

Figure 2 Gene loci for the major pathogenicity locus (PaLoc) toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin of *C. difficile* [79]

C. difficile is not an invasive microorganism: the bacterium does not penetrate into the enterocytes. The damage to the enterocytes is caused by a *C. difficile* toxin-mediated process. After the toxin has bound to the luminal membrane of the colonocytes via its receptor binding domain (RBD), the “toxin receptor complex” is endocytosed and the transmembrane domain of the toxin (TMD) is incorporated into the endosome membrane. The toxin's catalytic domain is translocated and liberated into the enterocyte's cytosol. The catalytic domain leads to monoglycosylation of Rho GTPases and Rho ATPases, thereby destroying the enterocyte actin cytoskeleton provoking the loss of cell polarity, cell retraction and finally cell death. The glycated proteins interfere with the cell migration and thus the

restoration of mucosal integrity. These inflammatory processes can lead to the pseudomembranous colitis [61].

At present, PCR ribotyping is a widely used method for typing *C. difficile* due to its high discriminatory power [115]. In 1999, a sample library of standard strains was created so that banding patterns may be compared and ribotyping is possible. Numerous strains are harmless because they do not have the ability to produce toxins. Examples of hypervirulent RTs are RT027 and RT078. Hypervirulence of ribotype 027 has been associated with increased transmissibility, increased relapse rates, and poor clinical outcomes compared with typical endemic strains [34,62].

1.6. Diagnostic techniques

It is important to note that only patients with clinical suspicion of CDI should be examined for the presence of *C. difficile*. With the exception of patients with an ileus, only liquid stool samples should be subjected to specific *C. difficile* diagnostics.

There are several detection methods for the detection of *C. difficile*, which differ greatly in their diagnostic accuracy and their time expenditure. The current European guidelines put forth by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) recommend a two-stage or three-stage diagnostic algorithm, due to the limited sensitivity and specificity of a single test procedure. This algorithm comprises a primary screening test and a second confirmatory test.

For screening, the glutamate dehydrogenase (GDH) screening test is most commonly used. If the result is positive, a toxin test must be carried out to distinguish toxigenic from non-toxigenic strains. A toxin EIA can be performed for this purpose. If GDH and toxins test positive, this is compatible with CDI. However, since direct toxin detection has a low sensitivity, GDH-positive, toxin-negative tests should be further subjected to nucleic acid amplification tests (NAAT) for toxin gene detection [33].

A further characterization of the strains (e.g. ribotyping) requires growth on anaerobic culture media, which is time-consuming and laborious, and is thus restricted to reference laboratories.

1.6.1. Diagnostic algorithm for *C. difficile* detection

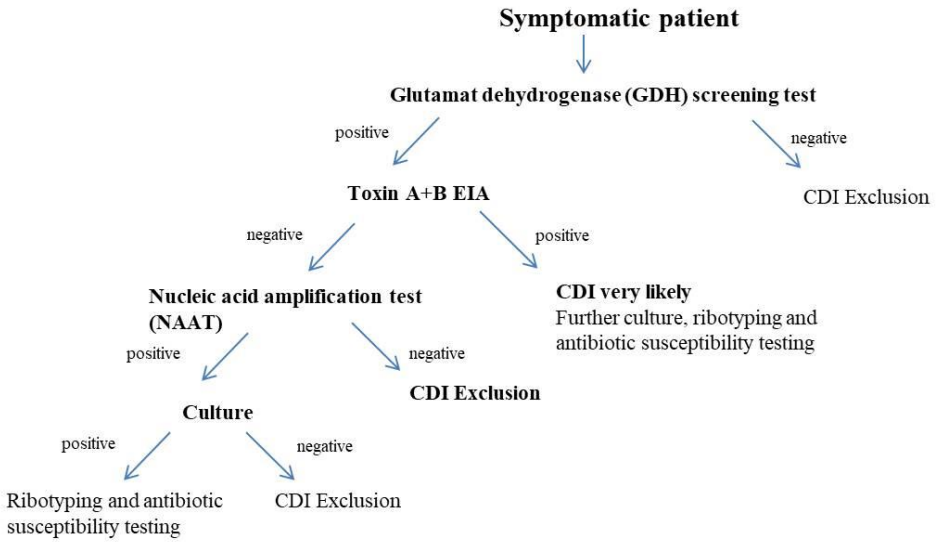

Table 3 Overview of diagnostic procedures of *C. difficile* [39]

Glutamate dehydrogenase Screnning-test TAT <2 h	Initial screening test with high sensitivity and high negative predictive value
Toxin A and B EIA TAT <2 h	Confirmatory test for the toxigenic infection in the case of a GDH positive rapid test. Good correlation with severity of infection with only limited sensitivity.
PCR TAT<4 h	confirmation test for the toxigenic infection. Not suitable as a screening test, as asymptomatic *C. difficile* colonizations are also detected
Anaerobic toxigenic culture TAT >3 d	Diagnostic gold standard as a confirmatory test for toxigenic infection. Culture is a prerequisite for ribotyping and antibiotic resistance testing

Table 4 describes the purpose and benefits of the diagnostic measures and the duration of the examination. TAT: Turn- around time; EIA: Enzyme immunoassay, PCR: Polymerase chain reaction [39]

1.7. Treatment of CDI

New international guidelines for the treatment of CDI were issued in March 2021. The guidelines distinguish between an initial form of CDI and recurrence. The severity of the disease is also included in the treatment recommendations. In mild forms, the first step should be to discontinue antibiotic treatment and monitor the patient closely. This leads in 15-23% of cases within 2-3 days to a cessation of diarrhoea with the additional benefit of low recurrence rates. The figure below shows the basic supportive steps [51,107,121].

Discontinuation of unnecessary antimicrobial therapy
Adequate replacement of fluid and electrolytes
Avoidance of anti-motility medications
Reviewing proton pump inhibitor (PPI) use.

Table 5 Basic supportive steps when treating a *C. difficile* Infection [121]

For the initial episode of CDI, fidaxomicin 200 mg given orally and twice daily for 10 days is now recommended, as opposed to the previously recommended use of oral vancomycin or metronidazole. Yet, orally administered vancomycin continues to be highly valued in the treatment of CDI in many instances. Metronidazole is no longer a recommended first-line agent for CDI, and it should be administered only when other options are not possible. A study showed that when metronidazole is given in the uncomplicated form of CDI, the rate of relapse is significantly higher than with vancomycin or fidaxomicin [6]. Also, the monoclonal antibody bezlotoxumab is now recommeded in severe cases, or cases where a high recurrence rate is expected. Bezlotoxumab is a monoclonal antibody directed against *C. difficile* toxin B. When bezlotoxumab is added to the standard of care, the risk of relapse is reduced by 10% [128]. In view of the high treatment costs as well as the cardiovascular risk profile, the administration of bezlotoxumab should be carefully discussed for each CDI case.

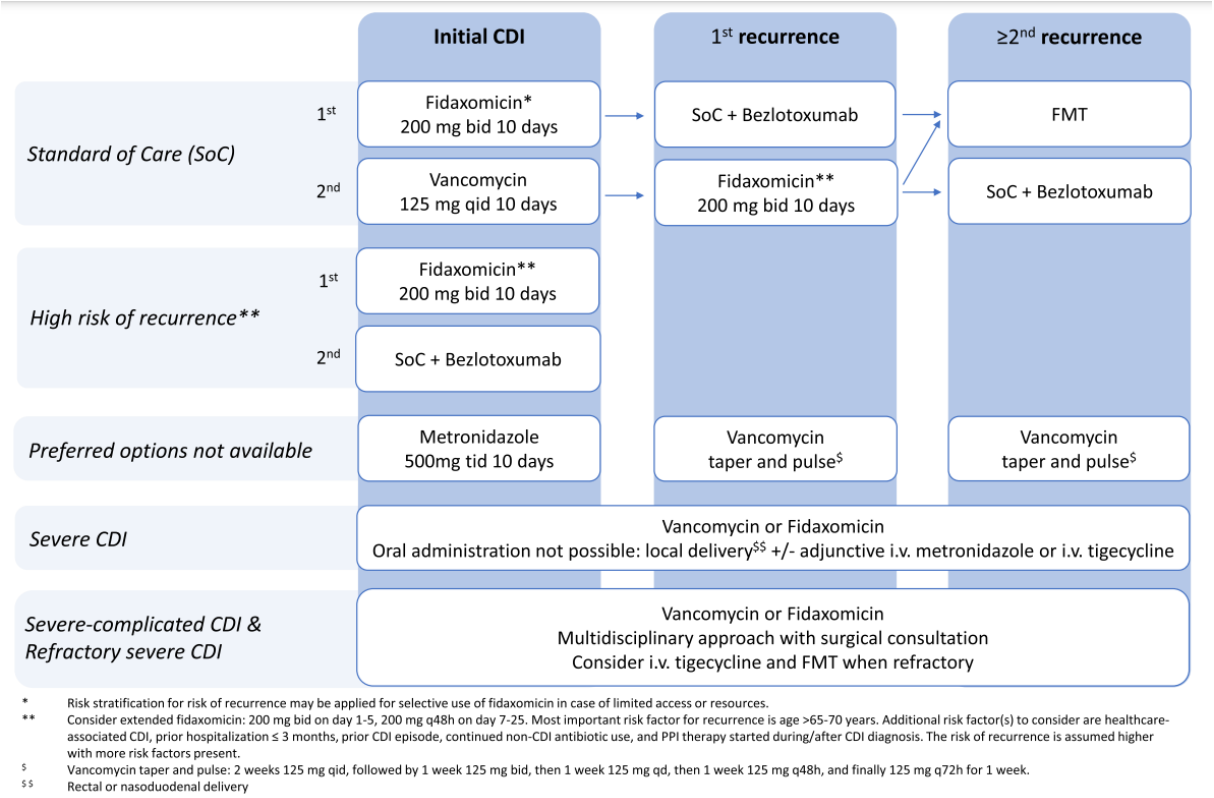

Figure 3 shows the new European guidelines for drug treatment of a *C. difficile* infection CDI [121]

The indication for surgery is rare, but may be necessary for complications such as perforation of the intestine, toxic megacolon with ileus or septic shock. In these cases, a total colectomy with a terminal ileostomy is performed. Another therapeutic approach is faecal microbiota transplantation. Treatment is considered complete when all clinical symptoms subside. Hence, no microbiological test of cure is recommended, as it may take several weeks for the tests to turn negative. The adjunctive use of probiotics is not recommended [7,129].

1.8. Important differential diagnoses

Especially in the tropics, where the role of *C. difficile* as the cause of diarrhoea is still largely unknown, further pathogens must be included in the differential diagnosis of symptomatic patients with diarrhoea. Indeed, diarrheal diseases are associated with high morbidity and mortality, and children under the age of 5 years are at highest risk [73]. It has been elucidated that diarrhoeal diseases are the second leading infectious cause of mortality among young children, second only to respiratory infections [45]. Besides bacterial and viral pathogens, parasitic infections caused by helminths (cestodes, nematodes, trematodes) or intestinal protozoa play a major role as diarrhoea-causing pathogens in the tropics. Selected parasitic pathogens of considerable importance in the study areas in Mali are thus discussed in the following chapters [19].

1.9. Intestinal protozoa infections

Giardia duodenalis, also known as *Giardia lamblia* or *Giardia intestinalis*, is a common diarrhoeagenic pathogen that can infect both humans and animals worldwide. Several species of *Giardia* are known, including *G. intestinalis*, which is the only species that can infect humans [117]. *G. intestinalis* is a single-celled flagellate that colonizes the upper small intestine. Hundreds of thousands of new cases are diagnosed each year, and it is also a common protozoan infection among returning travellers.

During its life cycle, this pathogen takes on two morphologically different forms. The trophozoites represent the vegetative form. In this form, a 10-20 µm long pear-shaped flagellate with 8 flagella can be shown in the still very fresh sample material under the light microscope. The cyst form, on the other hand, is much smaller with 8-14µm length and clear membrane with 4 nuclei. During the life cycle, this pathogen cyst represents the most environmentally resistant permanent form. The infectious cysts are excreted in the faeces and get into the environment, where they can remain vital and infectious for months even under adverse conditions.

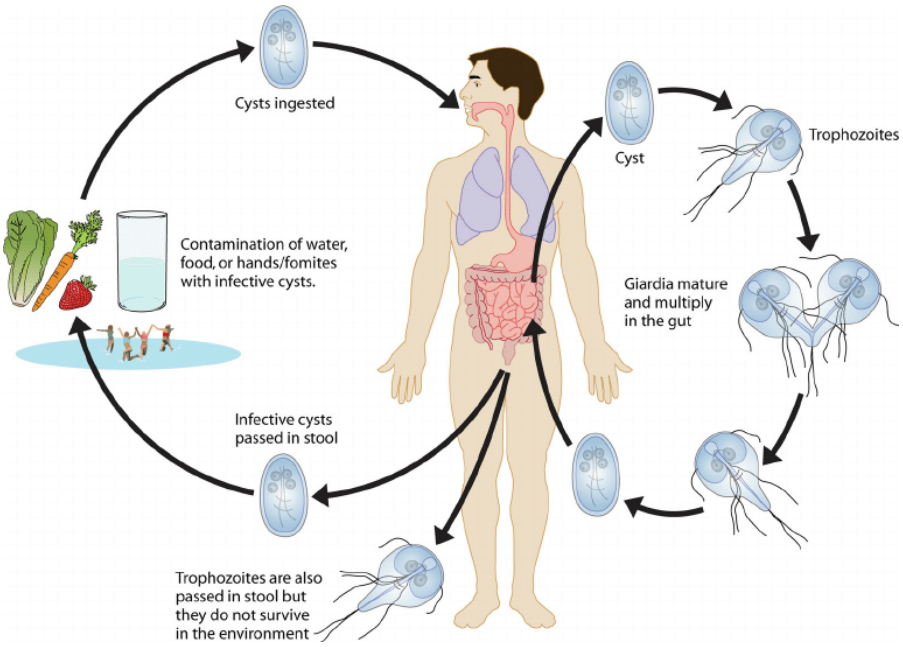

Figure 4 Life cycle of *Giardia intestinalis* [42] .

The infection is most commonly transmitted through the ingestion of contaminated water, but smear infections also provide a route of transmission [70]. Unlike other intestinal protozoa, *Giardia* is not an invasive disease, and remains thus restricted to the intestinal tract. After ingestion, the oocysts in the small intestine excise and transform into the trophozoites. These attach to the mucosa and multiply there. There is increasing damage to the mucous membrane and detachment of the epithelium. Some of the trophozoites transform into cysts and are excreted. Many cases of giardiasis are asymptomatic.

However, asymptomatic individuals may excrete infectious cysts. Infection appears clinically after an incubation period of 1-14 days. Acute giardiasis usually lasts 1-3 weeks and can be associated with diarrhoea, abdominal cramps, meteorism, nausea and epigastric pain. In severe cases and with chronic forms, malabsorption with weight loss and weakness can occur. In addition, chronic diarrhoea and secondary lactose intolerance may further complicate the clinical course of giardiasis [98] There are several methods to detect *G. intestinalis*. In many resource-constrained countries, the diagnostic gold standard is still the microscopic detection of the cysts or the trophozoites in the stool. In addition, antigen detection in stool can be carried out using ELISA or direct immunofluorescence with monoclonal antibodies. Specific PCR tests are increasingly being used for diagnostics in high-income countries. Patients with giardiasis should be treated with metronidazole or tinidazole.

1.10. Schistosomiasis

Schistosomiasis, also known as snail-fever or bilharzia is caused by a parasitic flatworm [28]. Schistosomiasis is a major tropical parasitosis, and affects at least 230 million individuals worldwide. In Mali, the two species *Schistosoma haematobium* and *Schistosoma mansoni* are the most common causes of schistosomiasis. In 2018, a Malian study team published a report indicating that the national prevalence rate of *S. haematobium* was 9% and that of *S. mansoni* 0.1% [38]. In 1981, a nationwide program for the eradication of the pathogen was established. Nevertheless, a recent publication identified a prevalence of 97% in children between 6 and 14 years living in a specific rural region of Mali [87]. Such enormous prevalence differences can be explained by the route of transmission, which is linked to freshwater contacts: An infected human excretes infective eggs through stool or urine. If freshwater bodies are infested, specific larvae, so-called miracidia, hatch from the eggs in the water. The larvae of these worms infect specific freshwater snail species, where further development occurs. They leave the snail as free-swimming cercariae. This cercaria stage is able to penetrate the intact human skin, and may cause thus infection. The cercaria is then losing its tail and becomes a schistosomulum. The schistosomulum get access to a peripheral vein of the infected host and may enter the portal vein system, where it fades and grows for several weeks. Then, the larvae migrate to the blood vessels or their target organs (most commonly the intestine or the bladder), where they become stuck and become sexually mature. The life expectancy of adult worms is 20-30 years. To lay their eggs, the females leave the male's abdominal fold and enter the end capillaries, such as the inferior mesenteric artery, which supplies sections of the colon and the bladder. The eggs get into the organs. Some of the infectious eggs are excreted in the stool or urine, causing the cycle to start over. The remaining eggs in the body can be transported via the bloodstream to other organs, where they trigger a granulomatous inflammation and can lead to fibrosis of the affected organs.

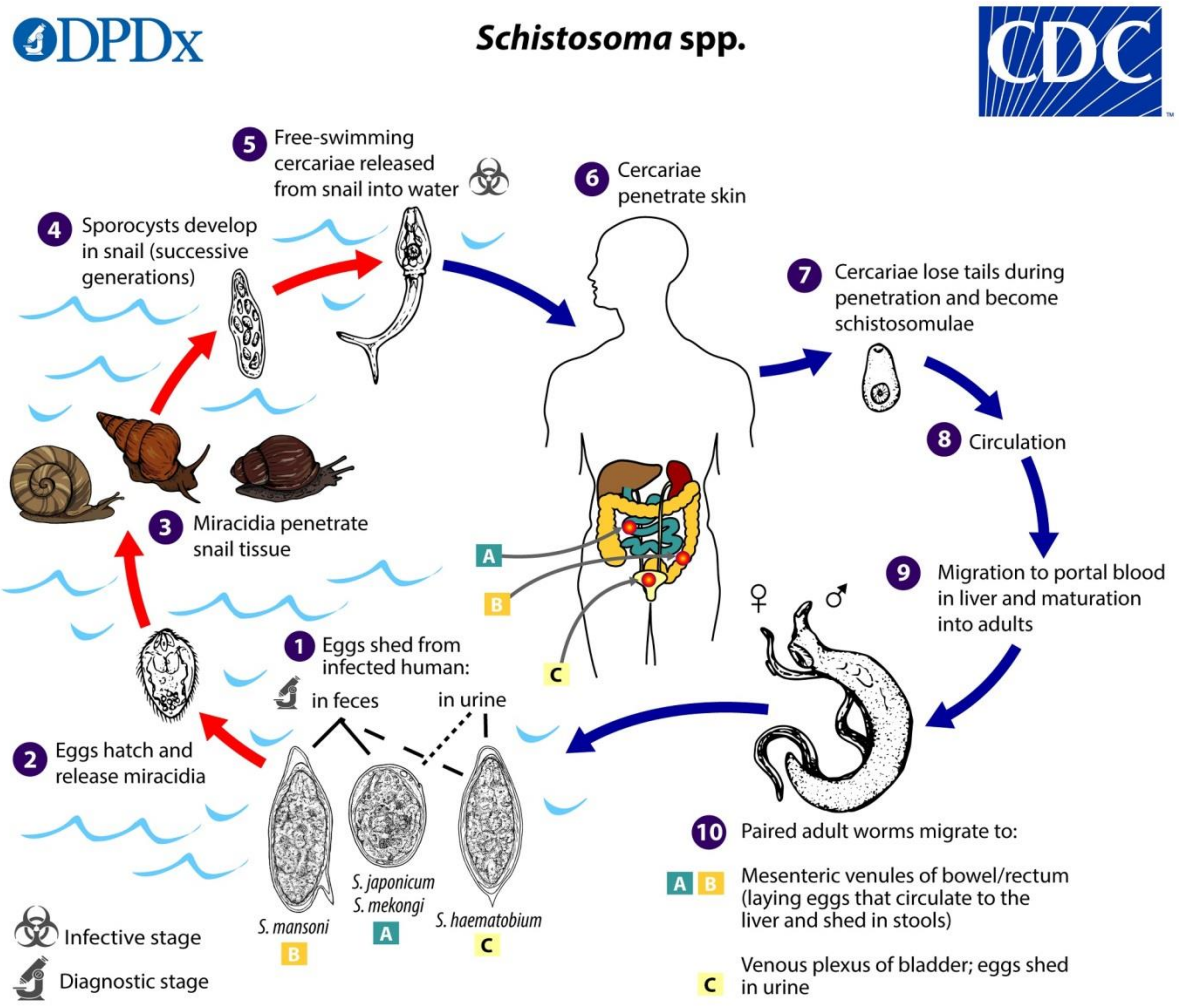

Figure 5 Life cycle of Schistosoma spp. The red arrows show the cycle outside the human. The blue arrows show the different stages inside the human body [27].

Depending on the infective species, different clinical courses are distinguishable. A major distinction is made between an acute form of schistosomiasis, also called 'Katayama syndrome', and a chronic form. The acute form usually shows a rather non-specific reaction such as fever, fatigue, myalgia, headache and pain in the upper abdomen. The chronic form is an expression of the immune reaction of the host against the worm eggs with formation of granulomas. Ultimately, it depends on the sub-form of the pathogen and the location of the egg laying, in what chronic form the disease occurs. A distinction is made between urogenital schistosomiasis, hepatosplenic schistosomiasis and the most common form of intestinal schistosomiasis. Depending on the immune status of the host and the proliferation of the eggs, rare forms such as cerebral or cardiopulmonary diseases can also occur.

The diagnostic gold standard is the microscopic detection of eggs in stool and/or urine. The eggs are excreted about 5-12 weeks after infection. Serological examinations are carried out in non-endemic countries as a screening test, but not recommended for endemic areas, since they fail to reliably distinguish between an acute and previous, already treated infection. A species determination

should be sought if the result is positive. In high-income countries, PCR tests or antigen detection tests can also be carried out. Treatment with oral praziquantel should be offered to all infected patients.

1.11. Overall goal and specific aims of this thesis

Although CDI is a well-established cause of diarrhoea in high-income countries and its transmission patterns have been studied, there are only scarce data about the epidemiology and importance of this infection in sub-Saharan Africa. Hence, CDI is not usually considered in the differential diagnosis of acute diarrhoea in most parts of sub-Saharan Africa. The goal of the current work is an in-depth epidemiological and clinical assessment in southern Mali pertaining to the prevalence of *C. difficile* in patients with diarrhoea, asymptomatic controls, as well as animal and environmental samples.

The following specific aims are related to this goal:

- To determine the prevalence of *C. difficile* in patients with diarrhoea (outpatients and inpatients) presenting to healthcare institutions in Mali and in healthy controls
- To identify the distribution of toxigenic strains and non-toxigenic *C. difficile* strains in Mali and to elucidate their antimicrobial susceptibility patterns
- To assess the predominant *C. difficile* ribotypes
- To investigate the association between specific risk factors (e.g. prior antibiotic therapy, prior hospitalisation) and frequent co-infections on CDI
- To compare the *C. difficile* strains and ribotypes obtained from humans and animals to assess the potential of *C. difficile* to be transmitted as a zoonotic pathogen

2. Materials and methods

2.1. Study area

The study was conducted in Mali, a large country in West Africa. Its capital is Bamako. Most of the population lives in the southern part of the country, which is crossed by the two rivers Niger and Senegal. The north extends deep into the Sahara and is hardly populated. The country's most important economic sectors are agriculture, fishing and, increasingly, mining. About half of the population lives below in poverty. In 2019, the country was ranked on position 184 in the United Nations' Human Development Index, and is thus one of the least developed and poorest countries in the world [57]. Likewise, Mali's health system is poorly developed and there are disparities between rural care and urban care. Overall, only 43% of the population can seek medical care when sick or injured. This is reflected in the low life expectancy at birth of only 59.3 years (in 2019), although this has considerably risen from 29.7 years in the 1950s [130]. Mali is further characterised by political instability and insecurity, especially in the central and Northern parts of the country.

This study was conducted at two different sites, i.e. Bamako and Niono. Bamako is the capital of Mali, with an estimated population of 1.8 million people. If the current population growth continues at current pace, a population of over 7.6 million people is expected by 2050 [53]. Bamako is located on the banks of the river Niger. The climate is arid and tropical, with a high annual average temperature. Rain is unusual during the dry season. From November to April, there is almost no rainfall, which is due to the Sahara anticyclone and the dry trade wind. The rainy season lasts from July to September. Bamako is divided into 6 municipalities. Each municipality has reference healthcare centres (so-called 'Centre de Reference'), the second stage of the Malian healthcare system for the care of sick patients. The foundation of the Malian healthcare system is based on a three-stage system, in which the patients usually move upwards from the bottom, depending on the severity of the disease. The primary contact is the practice where general practitioners treat. In second place is the 'Centre de Reference' of the individual municipalities. These centres are usually only visited on an outpatient basis, but in severe cases, it is possible to admit the patient. The two hospitals *Hôpital Gabriel Trouré* and *Point G* represent the highest level of care in Bamako. Due to armed conflict and civil unrest, the medical care is much worse in northern parts of the country.

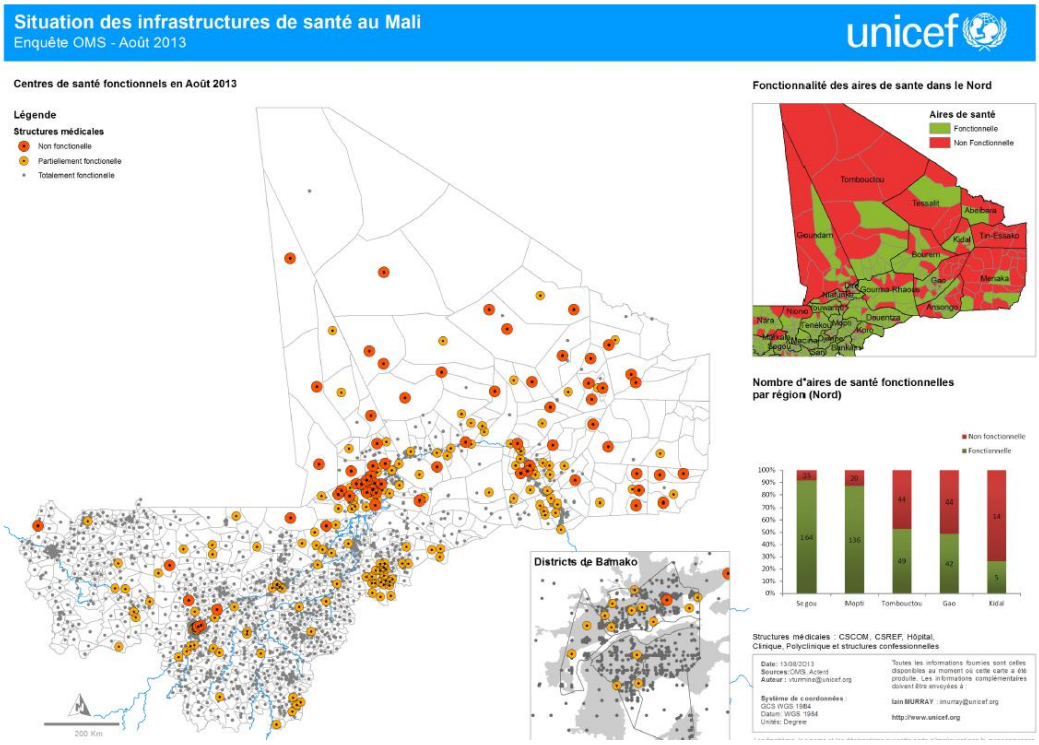

Figure 6 Malian regions with functioning medical care and regions without medically adequate infrastructure, according to UNICEF in 2013 [120]

Niono, the second study site, is a small town and municipality in the Ségou region, about 300 km northeast of Bamako. Niono is located on the northwestern edge of the interior of the Niger Delta on the main channel of the Niger River. Thanks to the wetlands of the Niger Delta, the region around Niono is one of the main rice-growing regions in Mali. In 2009, a populations of 91.554 inhabitants were counted in a census [1].

Bamako and Niono were chosen as study sites because (1) no data on the prevalence of *C. difficile* are available from these two areas; (2) they reflect an urban and a peri-urban/rural setting; and (3) because of an existing laboratory infrastructure at both sites, which was implemented during a previous multi-country study with the acronym NIDIAG, in which Saarland University participated [20]. In Mali, diarrhoea is the third leading cause of death after neonatal disorders and malaria. More people die from diarrhoea every year than from HIV and tuberculosis [26].

2.2. Study population, inclusion criteria and recruitment

Sampling was carried out from February to April 2016 during a visit of the MD thesis student and a laboratory technician from the Institute of Medical Microbiology and Hygiene, Saarland University, Homburg, Germany in Mali. The sampling consisted of a part pertaining to (1) human faecal samples, and (2) samples of animal origin.

Human samples: Samples were obtained from three different healthcare centres in the capital Bamako, namely the city's two hospitals *Point G* and *Gabriel Trouré* as well as one outpatient healthcare centre (*Commune III*). In Niono, samples were obtained from one outpatient healthcare centre providing medical care to the local population. Before inclusion, individuals were informed in detail about the purpose of the current study and written informed consent was obtained from the patients or their legal guardians before inclusion. There was no age restriction to participate in the study. Exclusion criteria applied to individuals who were unable to provide informed consent, e.g. due to an acute, severe illness. Symptomatic patients with diarrhoea were recruited using the World Health Organization (WHO) definition for diarrhoea, i.e. ≥3 unshaped stools/24 hours. For each symptomatic patient, a healthy control from the same age group and the same recruitment site was sought. All participants provided one stool sample, which was subjected to specific microbiological diagnostics for *C. difficile* and intestinal parasites, and answered a pre-tested questionnaire survey pertaining to general epidemiology, clinical symptoms and specific risk factors for CDI.

Animal samples: These samples were obtained from the four largest markets in Bamako, which are located in close proximity to the human sampling sites. Faecal samples from the most commonly traded animal species, i.e. poultry, sheep and cattle, were collected after having obtained permission from the owners of the animals. Subsequently, these specimens were subjected to microbiological diagnostics for detection of *C. difficile*.

2.3. Ethical considerations

Before the field work was carried out, ethical clearance was obtained from the 'Ethik-Kommission der Ärztekammer des Saarlandes' and the responsible Malian authority. The leading members of the cooperating medical departments of the hospitals *Point G*, *Gabriel Trouré* and the reference centres of *Commune III* were also introduced to the study plan, and the recruitment strategy and the study content were discussed. The study team then received approval.

Participation in the study was voluntary, and participation could be withdrawn at any time. All patients participating in the study were informed about the project before consenting to participate. All study procedures, including examination of stool samples, was only carried out after informed written consent had been obtained. Free treatment according to national guidelines was offered to participants found to be positive for toxigenic *C. difficile* or an intestinal parasitic infection.

2.4. Field procedures and working steps

2.4.1. Study plan

This study was designed to gain insights on the epidemiology of *C. difficile* in humans and livestock living in Bamako and Niono. According to previous sample size estimates, it was planned to acquire approximately 200 stool samples from patients with diarrhoea (as defined by WHO definition) and 200 reference stool samples from asymptomatic individuals. In addition, up to 100 samples of animal and environmental origin should be collected at local animal markets. The diagnostic tests were planned to be carried out at the Institut National de Recherche en Santé Publique (INRSP) in Bamako. In advance, a short, pre-tested questionnaire was developed in French and the local Bambara language to identify possible risk factors for CDI and/or parasitic intestinal infections.

2.4.2. Field work

During a first phase (March 8-18, 2016), study preparations including a final presentation at the Malian ethics committee, were carried out, and animal droppings were sampled at 4 different animal markets in Bamako. When collecting the droppings (e.g. from chicken), care was taken that only one sample was taken from each cage to prevent repeated analysis of samples stemming from a same animal. All animal and human samples were subjected to a diagnostic algorithm, which is presented in Figure 7.

During the second study phase (March 18 – April 15, 2016), samples from symptomatic patients and asymptomatic controls were obtained from the two hospitals and the healthcare centre in Bamako as well as from the health care centre in Niono. All participants responded to the study-specific questionnaire (Appendix). Upon receipt, all stool samples were stored in a fridge at 4°C in the local laboratories before further analysis.

2.4.3. Employed diagnostic algorithm for *C. difficile*

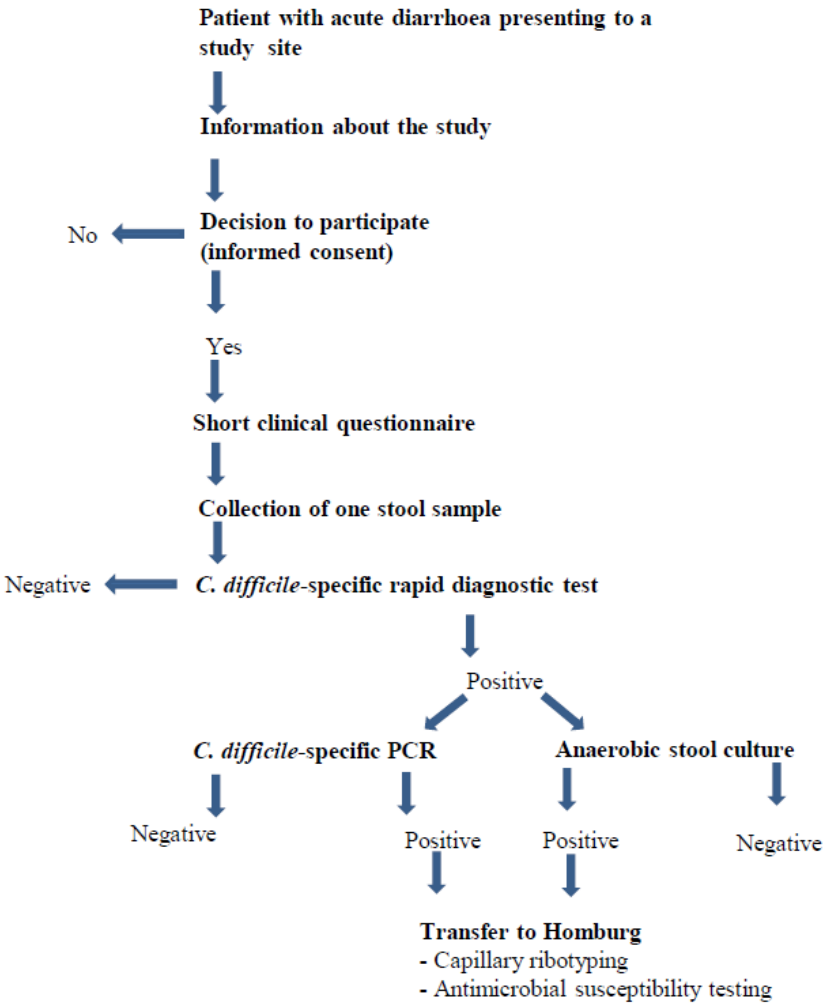

Figure 7 Diagnostic sample and analysis flowchart for the study pertaining to the occurrence of *C. difficile* in Bamako, Mali.

2.4.4. Treatment

All study participants who were diagnosed with infections were informed and, after consent, free treatment was offered by the respective treating physicians. Symptomatic CDI was treated with oral metronidazole (500 mg thrice a day for 10 days). Schistosomiasis was treated with a single dose of praziquantel (40 mg per kilogram of body weight), giardiasis and symptomatic *Entamoeba histolytica* infection were treated with metronidazole (15 mg/kg body weight for 3-5 consecutive days). Detection of *Dicrocoelium dendriticum* was not treated because the detection was considered transient colonization rather than 'true' infection.

2.4.5. Laboratory work in Mali and in Germany

The rapid diagnostic tests, specific stool cultures and PCR examinations for CDI were carried out in Mali, as well as stool microscopy for intestinal parasites. Additionally, *C. difficile*-positive stool samples from Bamako as well as all specimens from Niono were transferred to the German National Reference Centre for *C. difficile* in Homburg at Saarland University for confirmatory PCR testing and ribotyping using capillary electrophoresis. Antibiotic resistance testing was also carried out in Homburg.

Furthermore, a multiplex real-time PCR and an antigen detection rapid diagnostic test were carried out for the three intestinal protozoa species *Cryptosporidium* spp., *E. histolytica* and *G. intestinalis*, and results were compared to stool microscopy.

2.5. Diagnostic techniques

2.5.1. Kato-Katz method

The Kato-Katz method was developed for the detection of helminth eggs and is used for the diagnosis of intestinal schistosomiasis and for the detection of soil-transmitted helminthiasis such as *Ascaris lumbricoides*. The WHO still recommends the Kato-Katz technique as the method of choice in regions with a moderate to high infection rate of soil-transmitted helminths and intestinal schistosomiasis, despite its rather low sensitivity [11]. Yet, it is a major advantage that the handling is simple and without the need for sophisticated laboratory equipment.

A fresh stool sample is required as sample material. In this study, the process was started by passing a small portion of the fresh stool sample through a fine sieve to filter out coarse pieces. The stool was placed in the recessus of a spatula. The deepening of the template allowed 41.7 grams of stool to be applied in portions in order to be able to make a statement about the degree of infection. The number of eggs found was multiplied by a factor of 24 to determine the egg load, also called EPG (Eggs per gram). After the recess had been filled with the stool, the template was removed, and the remaining stool was then covered with a cellophane sheet soaked in glycerine. The glycerine cleared the faecal debits, enabling the eggs to be seen. A clean slide was then placed very carefully over the cylindrical stool covered with cellophane and pressed onto the stool until it was so thin that you could read a piece of newspaper underneath it. After approximately 30 minutes, the slide was systematically scanned under the microscope to detect any helminth eggs.

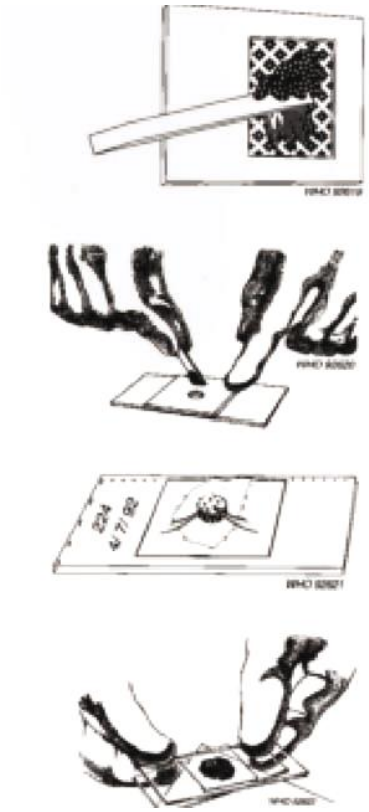

Figure 8 Shows steps of the Kato-Katz preparation. A small amount of stool is passed through a fine sieve, then the stool is placed in the deepening of the template, a cellophane soaked in glycerine is covers the stool. Subsequently a slide is pressed onto the stool [37]

The consecutive steps are illustrated in Figure 8. The first image shows the passing of the stool material through a sieve. The images 2 and 3 show how to attach the stool to the slide using the template. The last step is the careful and even pressing of the cover glass [37].

2.5.2. Direct faecal smear technique

The direct faecal smear technique continues to be an important pillar in the diagnosis of intestinal parasites. This form of diagnostics is relatively simple and can be accomplished with a laboratory that is simply equipped. The fresh stool sample is examined with a light microscope for intestinal protozoa (cysts and trophozoites) and for the presence of helminth eggs.

In this study, the slide was labelled with the patient identification number. A drop of saline solution was then placed on the slide on the left side and a drop of Lugol's solution on the right side. With the aid of a wooden stick, a small portion of stool, approximately the size of a match head, was added to the drops of saline solution and to the Lugol's solution on the slides and mixed. A cover glass was now carefully placed on each of the drops to minimize the formation of air bubbles. The slide was first examined with the light microscope under a 10x and 40 x magnifications by systematically moving the slide in a meandering pattern from right to left.

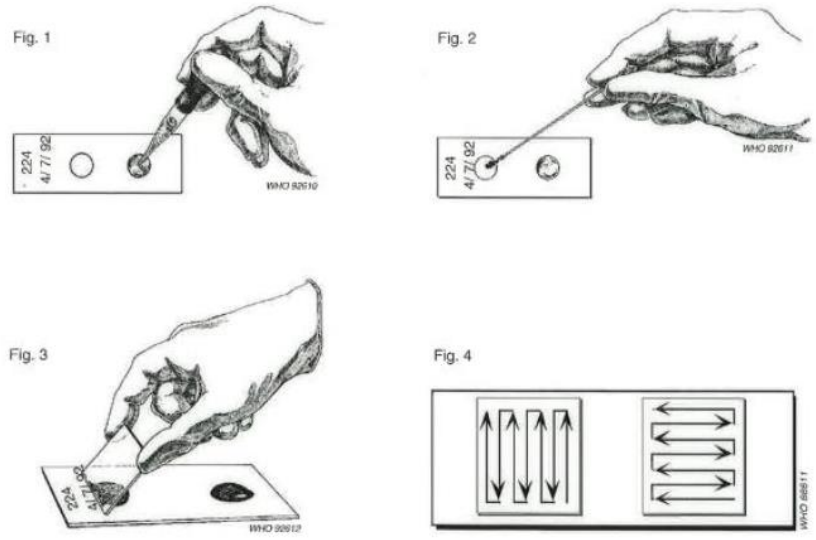

Figure 1-4. Preparation for Direct Smear Technique (adopted from WHO, 1994)

Figure 9 Preparation for direct smear technique. First scheme shows the placement of the saline and the lugol's drop into the slide. Scheme two shows the placement of the stool. Afterwards the cover glass is placed onto the slide. The fourth scheme shows the microscopy movement [127]

2.5.3. Rapid diagnostic test for *C. difficile*

The rapid test used in this study was the TECHLAB® C.DIFF QUICK CHEK COMPLETE kit from Alere Diagnostics. This test is a rapid membrane enzyme immunoassay which is used to screen patients with diarrhoea. This rapid test detects both the antigen glutamate dehydrogenase, which is characteristic of any *C. difficile* (toxigenic and non-toxigenic strains), and the toxins A and B. The test field is equipped with a reaction window with three vertical lines of immobilized antibodies. The first antigen test line contains antibodies against *C. difficile* glutamate dehydrogenase. The middle line is a control line that contains anti-horseradish peroxidase and is shown with dots if the results are valid. The last line, which is the toxin A and B test line, contains antibodies directed against toxins A and B. The conjugate consists of antibodies against glutamate dehydrogenase (GDH) and antibodies against toxins A and B bound to horseradish peroxidase [5].

In the present study, the steps were performed according to the manufacturer's instructions [5]. First, the stool sample was diluted with 750 µl dilution buffer. A drop of conjugate was added to the test tube. 500 µl of the diluted sample-conjugate mixture was added to the sample well in the upper right quadrant of the test card using a pipette. During a 15-minute incubation, glutamate dehydrogenase and toxins A and B bound to the antibody-peroxidase conjugates. The antigen-antibody-conjugate complexes passed through a filter pad to a membrane where they were captured by an immobilized glutamate dehydrogenase specific and toxin A and B specific antibodies. After incubation 300µl wash buffer was added to the reaction window. Subsequently, 2 drops of substrate

were also added to the reaction window. The test card was read after 10 min. A blue line indicated a positive test. If the antigen reaction was positive, the toxin reaction on the right side of the card had to be examined. Here too, a blue line indicated the presence of toxins. A positive Control reaction, indicated by a dotted vertical line in the centre reaction field of the Test Card, confirms that the test was working properly and that the test is valid [5].

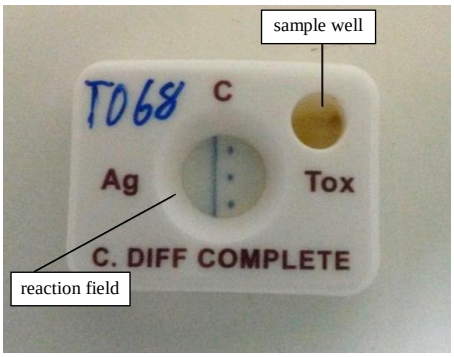

Figure 10 Shown is a typical rapid test for *C. difficile* from Alere®. In this case, the line on the left of the test indicates the presence of GDH, and the dotted line indicates the validity of the test. Toxins were not detectecd in this case.

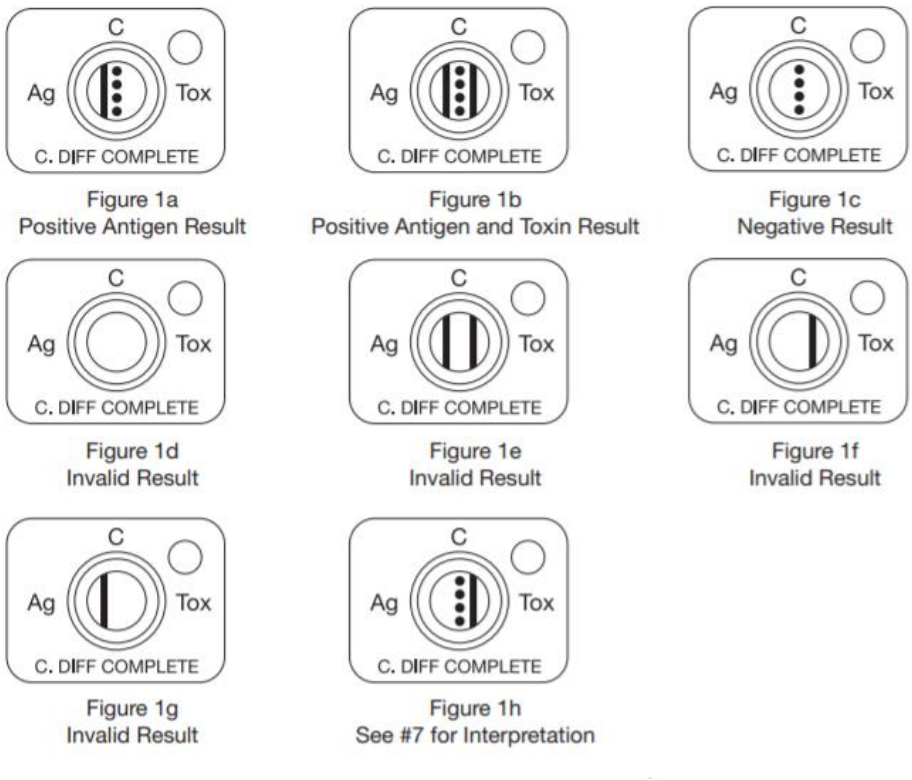

Figure 11. Rapid Test for the detection of *C. difficile*. The line on the left indicates the presence of GDH. The dotted line in the middle is the control line. The line on the right indicates the presence of toxins. For a valid result, the control line must always be visible, otherwise the test is invalid. [5] (Alere Techlab® C.DIFF QUIK CHEK COMPLETE®)

2.5.4. Microbiological *C. difficile* agar plate culture

In order to carry out more detailed investigations on microorganisms, it is usually necessary that the organism is growing in culture. A pure culture is a homogeneous culture of similar organisms of a single species. Ideally, such a culture should only have originated from a single cell. Such plating results in the formation of colonies which, when grown in isolation, can be said to have originated from a single cell or at least from a cluster of cells. Such colonies, can then be further propagated and purified by further inoculation and plating, e.g. to eliminate existing contaminants. For this purpose, selective culture media are used, which offer optimal growth conditions for the organism, while they disadvantage other, possibly competing species. Various methods are used for the purpose of separation. Usually these are plate methods, i.e. methods in which the culture is spread on an agar plate.

In the present work, no ready-to-use, pre-fabricated agar plates were brought to Mali, but a selective culture medium was freshly prepared in Petri dishes, using a commercially available agar powder for *C. difficile*-specific culture. For one litre of agar media, 54.7 g of base was dissolved in 1000 ml of H_2O. The mixture was boiled up and then cooled down. While the mixture cooled down, the supplement was prepared. The supplement was dissolved in 6 ml H_2O. When the preparation had reached 50°C, 1.2 supplement solution was added to the preparation. The agar was then poured into the petri dishes.

The stool was then applied to the agar plate using the dilution smear method (see Figure 12) and incubated anaerobically. When bacterial colonies had formed, further separation steps were performed using the cross smear technique (see Figure 12).

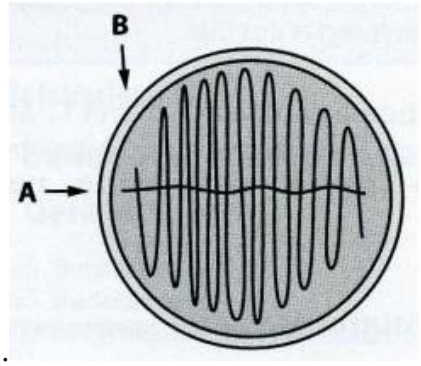

Figure 12 Dilution smear [109]: Line (A) is drawn across the plate with the inoculation loop. A new inoculation loop is placed at B and moved across the plate as shown

With the help of a loop, colonies of bacteria were spread out on a new petri dish. The Petri dish is incubated again anaerobically. Successful breeding resulted in grey colonies of 1-3 mm in size with translucent fringe and irregular borders. The characteristic odour was a further identifying feature. The grown bacterial culture from the 3rd field was swabbed with an ESwab from Bruker® and thus prepared for transport to Homburg.

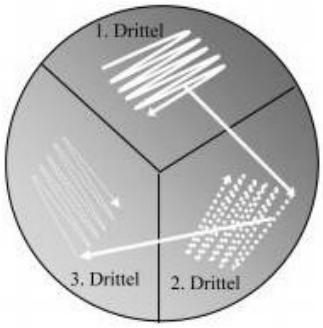

Figure 13 Cross smear for obtaining single colonies [109] The loop is moved from edge to edge. The inoculation loop is replaced, the dish is turned. Some material is taken up from the first third and drawn into a new third. The inoculation loop is replaced again, the petri dish is rotated further and a third smear is applied, following the procedure of the second smear.

2.5.5. Nucleic acid extraction and PCR tests for *C. difficile*

For subsequent PCR tests, it is necessary to extract the nucleic acids (more specifically, deoxyribonucleic acids, DNA) from a given sample. The DNA extraction in this study was performed in Mali with the QIaAMP mini DNA Kit from Qiagen®.

The DNA extraction started by pipetting 100 µl of the TE enzyme into a 1.5 ml Eppendorf reaction tube. 200 µl stool was diluted with 400 µl of water and centrifuged for 30 seconds at minimum centrifugal force. 200 µl of the diluted sample and LCR was added to each Eppendorf reagent tube and an internal control was prepared. The reagent tubes were once again vortexed and then incubated in a heating block for one hour. After the incubation period 20µl Proteinase K was added. Protein kinase K lyses the sample. A further incubation period of 30 minutes followed. In order to collect any droplets that may have formed on the lid, the test tubes were centrifuged for 1 minute at 8000 rpm. 200µl of the AL buffer was added. The tubes were briefly vortexed and then incubated for 10 minutes. In the next step 260µl 96-100% ethanol was pipetted into all reagent tubes and then vortexed for 15 seconds. Another short centrifugation followed.

The prepared sample mixture was then placed in a 2 ml QIAamp column tube. The column tube was centrifuged at 8000 rpm for one minute. 500 µl of buffer AW1 was added and centrifuge at 8000 rpm for one minute. The column in the column tube was now transferred to another new tube and washed with the second buffer AW2 and centrifuged for 3 minutes at 20 000 rpm to eliminate the remaining ethanol. The column was now pipetted into a sterile Eppendorf reagent tube and 100 µl of AE buffer was added. The purified DNA was incubated at room temperature for 5 minutes and then centrifuged again for one minute at 8000 rpm. The extracted nucleic acids were subjected to further PCR diagnostics on the same day. However, extracted DNA can be stored at –20°C for prolonged periods [96].

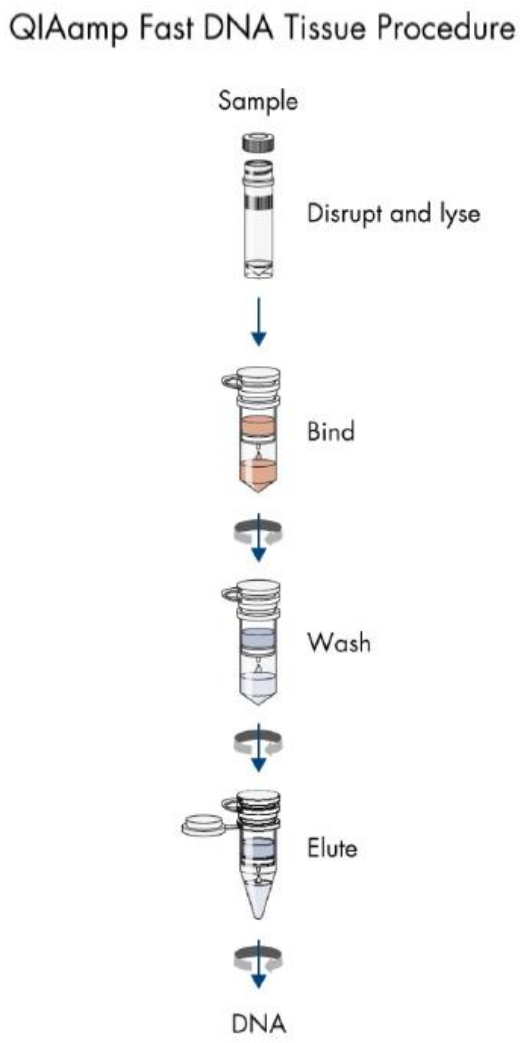

Figure 14 shows the schematic presentation of the different DNA extraction steps from QIaAMP mini DNA Kit from Qiagen® The principle of DNA extraction basically follows four steps. The lysis by the protein K, the binding of the DNA, the washing and the elution, which represents the removal of the DNA [96].

The multiplex PCR was carried out in Mali as well as in Homburg. In Mali, no PCR diagnosis for *C. difficile* had been done prior to this study, and hence, we repeated a PCR in Homburg for quality control. A real-time multiplex PCR RIDA®GENE panel from the company R-Biopharm (Darmstadt, Germany) was used.

A real-time PCR is a DNA amplification method based on the principle of the conventional PCR, but additionally provides some quantitative information pertaining to the DNA present in the analysed sample. The quantification is determined by fluorescence measurements. Amplified targets are identified with hydrolysis probes labeled at one end with a quencher and at the other end with a fluorescent reporter dye. If a target sequence is present, the probes hybridize to the amplicons. The Taq polymerase separates the quencher from the reporter during the extension. The reporter emits a fluorescent signal that is detected by the PCR device. The fluorescence signal increases with the number of formed amplicons [100].

In Mali the PCR was performed with the extracted DNA from stool samples that tested positive for *C. difficile* in the rapid diagnostic test. A positive and a negative control were included in each test run.

Steps were performed according to the PCR RIDA®GENE manual. At the beginning, a master mix was prepared. Per sample 0.1µl Taq polymerase was pipetted into 19.9µl Reaction Mix. The prepared master mix was then briefly centrifuged. To prepare a negative control 5 µl PCR water was added to the master mix. For the positive control 5 µl Positive Control was pipetted into the master mix. For each sample 20µl master mix was pipetted into the plate well and 5µl DNA extract was then added. 1 µl Internal Control was added to the Positive and Negative Control. The plate was sealed and briefly centrifuged at a few rotations per minute. The plate was transferred to the PCR device and the PCR was started according to the device settings. The software used was MXpro from Agilent. The PCR products were initially denatured for 1 minute at 95 °C at the beginning of a run. This was followed by 45 cycles, each consisting of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 30 seconds. Annealing and extension took place in the same step. When interpreting the results, both the negative and positive controls must indicate the expected results. The analytical sensitivity depends on the sample matrix, the DNA extraction and the DNA content of the sample. The detection limit was at ≤5 DNA copy/reaction [100].

2.5.6. Antibiotic susceptibility testing

The agar disc diffusion test is one of the well-established and most frequently used methods for antibiotic susceptibility testing. For this purpose, *C. difficile* isolates transferred to Homburg were grown again in culture and subjected to disc diffusion to elucidate the antimicrobial susceptibility toward the antibiotics vancomycin, metronidazole, moxifloxacin, clarithromycin and rifampicin.

To this end, a small paper discs containing the test substance in a defined concentration was used. The disk was placed on agar plates that had been previously inoculated with the *C. difficile* strain under investigation. The antibiotics dissolved in the gel culture medium and diffused radially outwards. The further the antibiotic diffused away from the platelet, the lower its concentration became. At a certain distance from the platelet, the concentration of the antibiotic was so low that the microorganism could grow despite the presence of the antibiotic. After incubation, a sufficient concentration of a growth-inhibiting substance showed a clear, circular inhibition zone, which differs from the rest of the bacterial colony of the remaining agar plate. In the next step, the minimum inhibitory concentration (MIC) of an antibiotic was estimated by measuring the diameters of the inhibition zones. The MIC is the lowest concentration of an inhibitor at which bacterial growth is inhibited.

2.5.7. Ribotyping

The determination of PCR ribotypes is a method that provides detailed information about the diversity of *C. difficile* isolates. PCR ribotyping allows for the identification of epidemiological correlations between isolates from different patients. *C. difficile* has several copies of operons in the bacterial genome that code for ribosomal DNA. The so-called intergenic space regions (ISR) lie between the individual genes of the operons. The test principle is based on the heterogeneity of sequence lengths between the operons that are compared. Using polymerase chain reaction, the different ISRs are amplified and then separated electrophoretically according to size, resulting in a band pattern typical for each ribotype.

In this study, the steps for ribotyping were performed according to the recommended instructions. First, a colony of *C. difficile* was placed with a loop in 50 0µl nuclease-free water and then purified with the Maxwell® (Promega). A minimum of 5µl DNA was required to perform the PCR. The first step took place in a clean room, to avoid exposure to contaminating DNA. To prepare the amplification mix, all reagents were brought to room temperature, vortexed and centrifuged. For one sample 12.5 µl Hot Start Mix, 0.25 µl 16S primer, 0.25 µl 23S primer and 9 µl nuclease-free water were prepared. Additional amounts had to be prepared for the positive and negative controls as well as for the pipetting loss. The DNA was added in the purification room. The DNA had to be pre-diluted 1:10 with H2O in a 96 well plate before use in the PCR. For this purpose 45 µl nuclease-free water and 5 µl DNA were mixed. Then 3µl of the 1:10 pre-diluted DNA was added to the 22µl PCR mix.
The running time of the PCR was 2 hours.

The next step was capillary electrophoresis. The gel syringe, the sample loading solution (SLS) and the separation buffer were at room temperature. Two plates in 96 well formats were prepared for capillary electrophoresis. The amplicon was diluted by adding 200 µl nuclease-free water to 5 µl amplicon. For the wells of the sample plate, a mix of 250 µl SLS and 0.5 µl standard was prepared. 30 µl was added to each well and then 2.5 µl of the amplicon was pipetted into it. Each well was sealed with a drop of mineral oil. The same rows that were filled in the "Sample Plate" were filled in the "Buffer Plate" to ¾ with Separation Buffer. The sequencer was then switched on, the computer was started and the gel syringe was installed. The Buffer and the Sample Plate were installed. Darkening was used to prevent the buffer from evaporating. The positions of the samples were entered in the system before the run start. This ensured the identity of the sample over the entire period of sample processing. Then, the run was started. After the run, the data was exported and evaluated with the software BioNumerics. For the determination of the ribotype, each sample was compared with known reference strains. For each sample, an internal marker (standard 600) was carried along to determine fragment sizes. Furthermore, the fragment pattern of each individual strain was compared with known reference strains using a database in BioNumerics.

2.6. Data management and statistical analysis

All data obtained during microbiological diagnostics and the clinical-epidemiological questionnaire survey were double-entered into a Windows Excel table (version 2007) and cross-checked. The statistical analysis was performed using SPSS from IBM. A P value of ≤ 0.05 was considered as statistically significant.

3. Results

3.1. Study population and characteristics

In total, 333 biological samples were collected during this study at the sites in Bamako and Niono. 233 specimens were of human origin, with 180 samples being collected in Bamako and 53 in Niono. In Bamako, an additional 100 animal samples were collected from different animal markets, from poultry (n=59), sheep (n=26), and cattle (n=15). The detailed origin of the samples obtained in Bamako is provided in Figure 15. In Niono, all 53 samples were collected at the same healthcare centre.

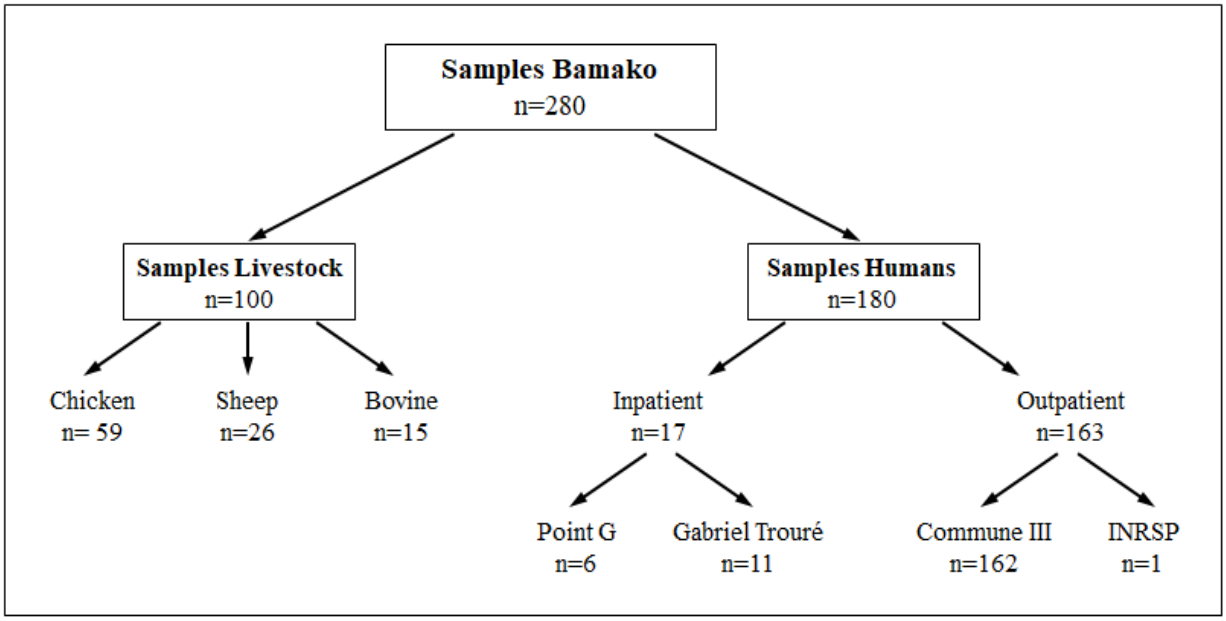

Figure 15 Origin of specimens collected in Bamako. The left side shows the number of origins of the animal samples. The distribution of human samples from the different hospitals (Point G and Gabriel Trouré) and the outpatient healthcare centres (Commune III and INRSP) are shown on the right.

In Bamako, only 9.4% (17/180) of all human samples were obtained from hospital inpatients. Diarrhoea was reported by 46.1% (83/180) of all individuals, whereas the others were healthy controls (n=97; Table 6). The median age of the study participants in Bamako was 12 years, with an age range from 10 days to 75 years. Overall, more female individuals (n=103; 57.2%) than male participants (n=77; 42.8%) participated in the study.

		Diarrhoea (Case)	No Diarrhoea (Control)	
Inpatient	Hôpital Point G	3	1	
	Hôpital Gabriel Touré	10	1	
Outpatient	Commune III	70	94	
	INRSP	0	1	
Total		83	97	180

Table 6. Absolute numbers of patients with diarrhoea and asymptomatic controls in this study on the occurrence of *C. difficile* in Bamako, Mali.

In Niono, all 53 human samples stemmed from outpatients. The sex distribution was similar as in Bamako, with more participating females (57%) than males (43%). The average age of the individuals from Niono was 9 years. The youngest patient was 2 years, the oldest patient was 23 years old.

3.2. Occurrence of *C. difficile* in the Malian study sites

Using the rapid diagnostic test (RDT) for detection of the pathogen-specific GDH, the prevalence of human *C. difficile* was 7.2% in Bamako (13/180 samples; 95% confidence interval (CI: 3.5-11.5%) and 0% in Niono (0/53 samples). Among the 100 samples of animal origin, only a single specimen obtained from a chicken was GDH-positive, owing to a prevalence of 1%. Details on the different diagnostic tests are displayed in Table 7.

Test	**Bamako**		**Niono**
	Human samples	Animal samples	Human samples
Rapid diagnostic test - Positive for *C. difficile* - Positive for toxins	 13/180 0/180	 1/100 1/100	 0/53 0/53
C. difficile-specific agar plate culture	12/13	1/1	–
PCR in Mali - Positive for *C. difficile* - Positive for toxins	 12/13 3/13	 1/1 1/1	–
PCR in Homburg - Positive for *C. difficile* - Positive for toxins	 12/13 1/13	 1/1 1/1	–
Ribotyping results	RT084 (n=7), RT078 (n=1), Unidentified/previously unknown RT (n=4)	RT012	–

Table 7 Overview of different diagnostic tests employed for the diagnosis of *C. difficile* in human and animal samples in Bamako and Niono, Mali.

In GDH-positive samples, the RDT was negative for *C. difficile*-specific toxins in all human samples, but was positive in the animal specimen. The further PCR testing of GDH-positive samples in Mali confirmed the toxigenic animal strain (toxins A and B), and identified toxins A and B in 3 human samples, whereas no binary toxin was detected in any sample. In contrast, the confirmatory PCR testing in Homburg revealed identical results for the RDT-positive animal sample, but found only one toxigenic human sample, which was positive for toxins A and B as well as the binary toxin.

All except one RDT-positive samples also grew *C. difficile* in culture (13/14). Previously described RTs could be identified in 9 cases, with RT084 being most frequently detected. RT078 and RT012 were also found. Four samples belonged to previously undescribed RTs.

With regard to the antimicrobial susceptibility of *C. difficile* isolates, all isolates were fully susceptible to the antibiotics vancomycin, metronidazole, moxifloxacin and rifampicin. Seventy percent (9/13) were, however, resistant to clarithromycin. Details are displayed in Table 8.

Substance	Susceptible isolates	Resistant isolates	Lowest MIC (µg/ml)	Highest MIC (µg/ml)	Median (µg/ml)
Vancomycin	n=13 (100%)	n=0	0.25	1	0.47
Metronidazole	n=13 (100%)	n=0	0.13	0.5	0.32
Moxifloxacin	n=13 (100%)	n=0	2.0	3.0	2.3
Rifampicin	n=13 (100%)	n=0			
Clarithromycin	n=4 (30.8%)	n=9 (69.2%)			

Table 8 shows the number of sensitive and resistant isolates for each antibiotic used. The lowest MIC, the highest MIC and the median are also given. Susceptibility toward rifampicin and clarithromycin was determined using agar disc diffusion, and hence, no MIC values can be provided.

3.3. Occurrence of intestinal parasites in the Malian study sites

3.3.1. Parasitological examinations in Bamako

178 of the 180 human samples were available for parasitological diagnostics. Following examination using the direct faecal smear and the Kato-Katz technique, a total of seven different intestinal parasite species were identified, with *Dicrocoelium dendriticum* being the most frequent helminth (n=9, prevalence: 5.1%), and *Entamoeba histolytica/Entamoeba coli* (n=16; prevalence: 9%) and *Giardia intestinalis* (n=15; prevalence: 8%) constituting the most commonly encountered intestinal protozoa species. Helminths were only detected by Kato-Katz thick smear, whereas all intestinal protozoa were found by direct faecal smear. Details and differences between symptomatic cases and asymptomatic controls as well as the correlation with sex and age groups are given in Table 9.

Parasite	N	Diarrhea			Sex			Age Group (years)				
	Prevalence 95% CI	Case	Control	P	Male	Female	P	0-1	2-5	6-18	>19	P
G. intestinalis	15	9	6	0.894	6	9	1	4	9	1	1	**0.018**
	8.3	60	40		40	60		26.67	60	6.667	6.667	
	4.3-12.4											
E. histolytica/ E.coli	16	7	9	0.719	6	10	0.898	1	3	8	4	0.129
	8.9	43,8	56,3		37.5	62.5		6.3	18.8	50	25	
	4.7-13.1											
E. hartmanni	2	2	0	0.750	1	1	1	1	1	0	0	0.837
	1.1	100	0		50	50		50	50	0	0	
	0-2.6											
D. dentriticum	9	4	5	0.867	2	7	0.43	2	2	4	1	0.75
	5.0	44.4	55.6		22.2	77.8		22.2	22.2	44.4	11.1	
	3.8-11.6											
A.lumbricoides	1	1	0	1.0	0	1	1	0	1	0	0	0.925
	0.6	100	0		0	100		0	100	0	0	
	0-1.8											

Table 9 Prevalence [%] of different intestinal parasites diagnosed in 178 stool samples from Bamako, Mali using the direct faecal smear. Data are stratified by sex, Age groupe and the occurrence of diarrhea. The Fisher's exact test was used to asses for statistical significance.

Polyparasitim, i.e. concurrent infection with more than one parasite species, was rare in Bamako, and only found in three individuals.

3.3.2. Parasitological examinations in Niono

In Niono, 51 samples could be subjected to both the Kato-Katz method and the direct faecal smear. Two samples were too loose/liquid to be examined with the Kato-Katz method, whereas all 53 samples could be examined with the direct faecal smear method. The helminth *Schistosoma mansoni* was by far the most common parasite, with a prevalence of 67% (n=34; 95% CI 53.4%-79.6%). The intensity of infection, i.e. the eggs per gram of stool (EPG) was determined according to thresholds put forth by WHO [126]. The intensity of infection was mild in the majority of analysed individuals, with an average of 14.5 EPG (range: 1 to 122 EPGs; Table 10). Infection with *S. mansoni* was most common in the age group of 6-18 years.

No infection		Light 1-99 EPG		Moderate 100-399 EPG		Heavy >400 EPG	
N	%	N	%	N	%	N	%
17	33,3	33	64,7	1	2,0	0	0

Table 10 Distribution of no, light, moderate and heavy *S. mansoni* infection intensities among 51 faecal human samples collected in Niono, Mali.

The second most common helminth was *Hymenolepis nana* (n=3; prevalence: 6%, 95 CI 0-12.4%). Both helminths occurred mainly in school-aged children aged 6-18 years. Using microscopic tests, *G. intestinalis* was the most frequent intestinal protozoon, with a prevalence of 26% (n=14; 95% CI: 14.5-38.3%). Furthermore, *Trichuris trichiura* and *Chilomasitx mesnili* were found in one sample each (n=1, 2% CI 0-5.9). Details on the different encountered parasites are displayed in Table 11.

	Prevalence [%] 95% CI	liquide	smooth	hard	p	Male	Female	p	2-5	6-18	>19	p
S. mansoni	34 **66.7** 54.9-80.4	2 4.7	26 60.5	2 4.7	0.651	13 30.3	17 40	0.526	3 7	26 60.5	1 2.3	0.174
H. nana	3 **5.9** 0-13.7	1 2.2	2 4.7	0 0	0.311	2 4.7	1 2.2	0.900	0 0	3 7	0 0	0.653
G. intestinalis	13 **23.5** 11.8-35.3	1 2.2	10 23.3	0 0	0.570	5 11.6	6 14	0.935	3 7	8 18.6	0 0	0.395
Entaboeba spp.	11 **21.6** 9.6-33	0 0	6 14	3 7	0.020	2 4.7	7 16.3	0.100	0 0	8 18.6	1 2.2	0.222

Table 11 Of the 53 samples, 51 could be assessed with both the Kato-Katz test and the direct Faecal smear. Complete questionnaires were available from 43 study participants. The left column shows the prevalence in relation to all 51 samples. However, 43 is used as the denominator in the left columns. Data are stratified by sex, age group and incidence of diarrhoea. Shown are all detected parasites above a prevalence of 5%.

3.3.2.1. Diagnostic test comparison

The diagnostic agreement between Kato-Katz and direct faecal smear technique were assessed for the diagnosis of *S. mansoni* and *H. nana* (Table 12 and
Table 13). Using Cohen's Kappa test, both methods showed an excellent agreement, i.e. κ=0.914 for *S. mansoni* and κ=1.00 for *H. nana*.

S. mansoni	Direct faecal smear		
Kato-Katz	Negative	Positive	Total
Negative	17	0	17
Positive	2	32	34
Total	19	32	51

κ=0,914

Table 12 Comparison of Kato-Katz and direct faecal smear for the diagnosis of *S. mansoni*.

H. nana	Direct faecal smear		
Kato-Katz	Negative	Positive	Total
Negative	48	0	48
Positive	0	3	3
Total	48	3	51

κ=1,00

Table 13 Comparison of Kato-Katz and direct faecal smear for the diagnosis of *H. nana*.

3.3.2.2. Polyparasitism in Niono

Polyparasitism was common in Niono, with 26% (n=14) of the patients having a double infection was detected. Two study participants were tested positive for at least 3 and 4 different parasites, respectively.

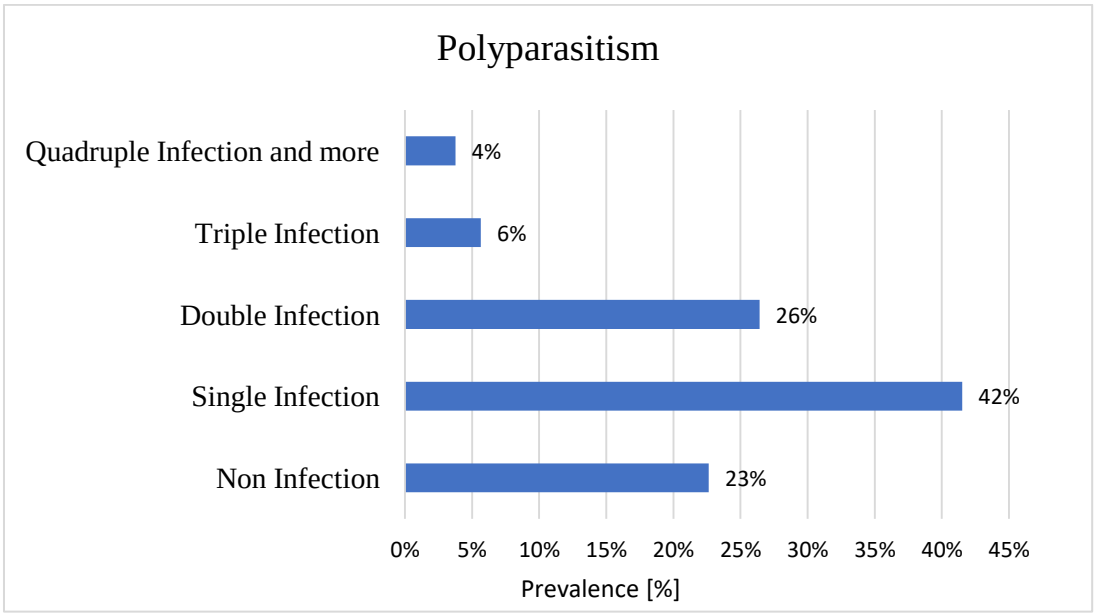

Figure 16 Polyparasitism in 53 study participants in Niono, Mali. Including both protozoa as well as helminth infections. (*S. mansoni, H.nana, T. trichura, G. intestinals, E. histolytica/dispar/coli, T. intestinalis, C.mesnili*)

3.4. Specific questionnaire survey

Of 180 study participants in Bamako, complete questionnaire data were available from 171 participants.

3.4.1. Age and sex of study participants

C. difficile

The medical history and questionnaire showed that 58% (n=7) of the *C. difficile*-positive study participants were under one year old. In both age groups 2-5 and 6-18, two positive *C. difficile* cases (17%) were detected. In the group over 19 years of age, 1 case was detected. Overall, more female patients (n=8) than male patients (n=4) were found to have *C. difficile* infection or colonisation.

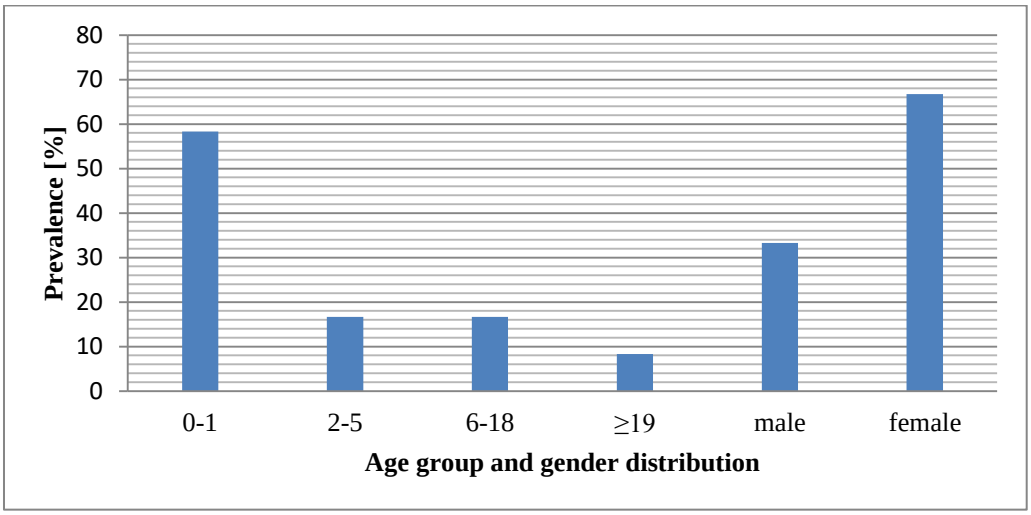

Figure 17 Age and sex distribution of detected *C. difficile* infections or colonisations.

Parasites

In the group of patients with at least one parasite infection, more female patients (n=28, 61%) were tested positive than male patients (n=18, 39%). The age distribution demonstrates that the most frequent parasite infections were found in the group of 2-5 year old children (n=17, 37%), followed by the 6-18 year olds (n=14, 30%).

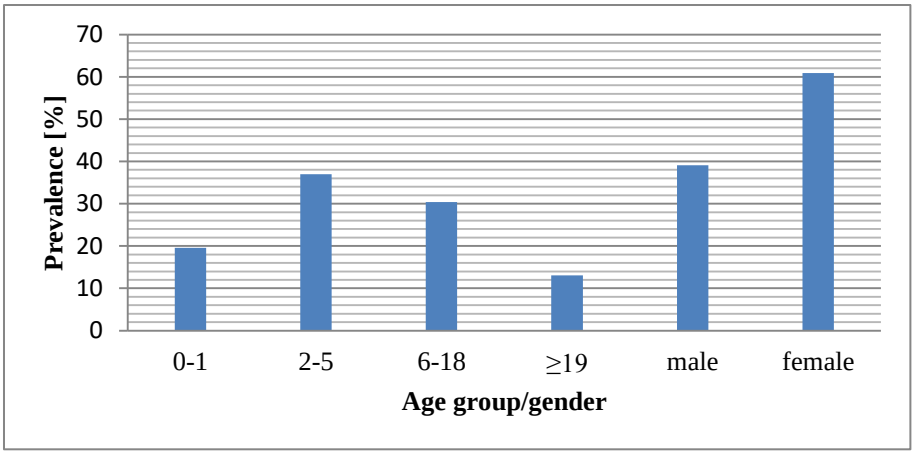

Figure 18 Prevalence of parasitic colonization or infection in relation to age distribution and sex.

3.4.2. Medical history

	Total number	Prevalence	*C. difficile*			All parasites			*G.intestinalis*		
	n	%	n	%	p		%	p	n	%	p
Self-reported diarrhea	83/180	46.1	0/13	16.6	**0.007**	25/46	54.3	0.984	9/15	60	0.88
Current hospitalization	18/180	10	0/13	0	0.232	2/46	4.4	0.132	1/15	6.7	0.802
Prior Hospitalization	3/180	1.7	0/13	0	0.641	1/46	2.2	0.764	1/15	6.7	0.284
Immunosuppression/HIV	11/180	6.1	0/13	0	0.360	2/46	4.6	0.549	1/15	6.7	0.933
Regular medication	9/180	5.1	1/13	8.3	0.592	2/46	4.4	0.97	0/15	0	0.01
Weight loss	33/180	18.8	2/13	16.6	0.848	5/46	10.9	0.125	2/15	13.3	0.456
Antibiotic intake	24/180	13.3	0/13	0	0.186	6/46	13	0.901	4/15	26.6	0.244

Table 14 displays the risk factors queried in the questionnaire in relation to the absolute number and to pathogen-specific prevalences for *C. difficile*, *G. intestinalis* and any parasitic infection.

Interestingly, only the symptom diarrhoea showed a significant correlation between the detection of *C. difficile* and self-reported diarrhoea (2, 16.6% p=0.007). A toxic strain could not be detected in these 2 samples. However, no significance could be determined for the heterogeneous group of parasitic pathogens. Also for typical intestinal parasites such as *G. intestinalis or E. histolytica*, no correlation to the symptom diarrhoea could be shown. Furthermore, only one patient (n=1, 8.3%) with a *C. difficile* colonisation stated that other fellow residents in the close environment suffered from diarrhoea (p=0.725).

In Bamako, 10 recruitde patients were hospitalised at the time of stool collection. No association could be found in this small group, neither for CDI nor for parasitic colonisation or infection (p=0.232 and p=0.132, respectively).

Of all patients, 13.3% (n=24) reported having taken antibiotics in the last 3 months. Cotrimoxazole (n=7, 3.9%) was the most commonly used antibiotic of all 180 study participants. Amoxicillin was most commonly used in the 2-5 year-old age group.

None of the study participants who tested positive for *C. difficile* (n=0; 0%) reported taking antibiotics currently or in the last 3 months.

6.1% of the patients interviewed reported having HIV, although it was not stated how advanced the disease was at the time of the interview. None of the 11 patients reported being treated with antiviral drugs. One HIV-positive patient also suffered from tuberculosis, and another HIV-positive patient also had a stomach ulcer. All HIV-positive study participants were at least 19 years old. Regarding gender distribution, women were more frequently affected than men (81.1% vs 18.2%)

Use of continuous medication was reported by 5.1% (n=9) of the study participants.

One patient in the *C. difficile*-positive group was taking an iron supplement as a permanent medication. No significant correlation was found between long-term medication and CDI (p=0.592).

Medical history	N	%
Antibiotic therapy in the past 3 months	24/180	13,3
Ceftaxidim	1	
Amoxicillin	5	
Ciprofloxacin	2	
Metronidazol	3	
Cotimoxazole	7	
Ceftriaxon	4	
Erythromycin	1	
Other	1	

Table 15 shows the distribution of self-reported antibiotic intake of all study participants in Bamako, Mali

3.4.3. Sociocultural characteristics

46.1% (n=83) of the study participants stated that they had had regular contact with animals. Contact took place predominantly in the household (sheep, cattle and poultry). No correlation between the detection of *C. difficile* or a parasitic disease and animal contact was observed (p=0.749 and 0.877, respectively).

The majority of the study participants came from the Bamako catchment area. 96.6% (n=173) reported residency in an urban area. All *C. difficile*-positive patients were from the urban area in and around Bamako. 11 (6.1 %) of the study participants had been abroad within the previous 4 weeks.

88.3 % (n=159) of the respondents drank purified water (tap water or purchased bottled water). 17 (9.4%) patients reported drinking only spring water. 2.2 % of the study participants reported drinking unpurified water from the Niger river. No significant association between the quality of drinking water and the prevalence of *C. difficile* was observed (p=0.527).

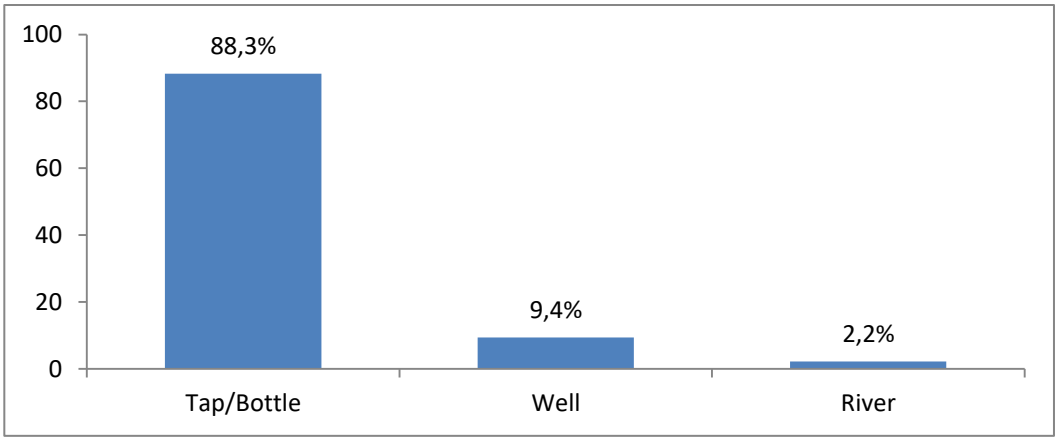

Figure 19 Main reported drinking water source of 180 participants interviewed in Bamako, Mali

A correlation between eating habits and CDI/ *C. difficile* colonisation or intestinal parasitic was not found (p=0.334 and p=0.560, respectively).

With regard to the household size, 147 of the respondents reported living with more than 8 people in the household. A smaller household size of 3 persons was indicated by only 5% (n=9) of the respondents. Household size was not associated with the presence of parasites (p=0.619) or CDI.

Sociocultural characteristcis	Total number	Prevalence	*C. difficile*			All parasites			*G. intestinalis*		
	n	%	n	%	p	n	%	p	n	%	p
Animal contact	83/180	46.1	5/13	38.5	0.749	21	45.6	0.877	8	53.3	0.363
Stays abroad	11/180	6.1	0/13	0	0.359	2	4.4	0.546	2	13.3	0.456

Table 16 shows the socio-cultural factors queried in the questionnaire in relation to the absolute number, prevalence and p-value of *C. difficile*, *G. intestinalis* and all detected parasites

4. Discussion

4.1. Epidemiology of *C. difficile* in sub-Saharan Africa

An important goal of this work was to understand the role of *C. difficile* in Sub-Saharan Africa. The results of this study indicate that *C. difficile* may play a specific, although rather minor role in the West African healthcare system and shows similarities with other African and international studies addressing *C. difficile*. In this work, *C. difficile* was isolated in 13 of 180 human samples in Bamako, while the bacterium was not found in Niono. Two *C. difficile* positive samples stemmed from patients with diarrhoea, while the other 10 samples that tested positive for *C. difficile* were from patients in the control group. Most isolates were non-toxin-producing strains. The analysis of animal samples showed a positive result in only one sample. However, toxin A, B and a binary toxin could be detected in this sample. In 9 of the 13 positive samples, specific RTs could be identified, while the others were novel, previously undescribed RTs. Hence, this study sheds additional light on the rarely assessed situation of *C. difficile* in Africa. Subsequently, the study findings will be briefly discussed in view of the international scientific literature.

4.1.1. Study design and main results

The data collected from the various African studies differ significantly in terms of their study design. A prospective cohort study, in which nearly all risk factors for CDI are accounted for, would yield different results than a case-control study. An example of the results of a cohort study is provided in a publication of Oyaro et al. from Kenya [91]. This study reported a prevalence of *C. difficile* above 93%. Of 105 patients examined, all of whom suffered from diarrhea, 98 samples tested positive for *C. difficile.* The PCR also detected the genes *tcdA*, *tcdB* or *tcdC* in 97 samples. Only in a single sample, no toxin could be detected. This study primarily examined adult, hospitalised patients. Of note, only 13.3% of the study participants were under 40 years of age. 85% of the examined in-patients reported to have taken over-the-counter antibiotics. This study underscored the importance of *C. difficile* diagnosis in adult patients with diarrhea, especially when high-risk factors for CDI are clinically or anamnestically determined. However, this result does not allow to draw conclusions on the importance of *C. difficile* in the general population. Case-control studies are better suited than cohort studies to assess the importance of *C. difficile* at the population level.

In 2016, a case-control study by Janssen et al. examined 307 patients for the presence of *C. difficile* in Ghana. Janssen found a prevalence of *C. difficile* of 4.9%. Mainly non-toxigenic strains were detected. Interestingly, 75% of the non-toxigenic strains were found in patients with diarrhoea where no other pathogen could be detected. It could be shown that in both groups, the hospitalized patients with diarrhoea and the asymptomatic study participants, there was an approximately equal distribution of the results [59].This study shows parallels to our results from Mali in many ways. Not only is the

study design similar, but the results show similarities. The most common RT found in Ghana was also RT084. Furthermore, no strain in this study showed resistance to metronidazole or vancomycin. A questionnaire was used to assess the risk profile of the study participants.

In all kinds of clinical research, the study design has a great impact on the results. In the case-control design used in the herein presented study, we were mainly able to detect asymptomatic colonisation. This is, however, important to understand the role of community-acquired CDIs, which are now increasingly being described in the western world.

4.1.2. Analysis of specific risk factors for CDI

Other African studies showed a significant correlation between antibiotic therapies, in particular ceftriaxone [59]. In Ghana, about 60% of the respondents stated that they had taken antibiotics. This result is in line with the general opinion that antibiotic therapy is a significant risk factor for a CDI. A distinction is made here between antibiotics with high and low colitis potency. Among other things, antibiotics from the group of cephalosporins and amoxicillin have been shown to have adverse effects on the intestinal tract. In our study from Bamako, 12.7 % of patients reported having taken antibiotics, but no significant association between antibiotic use and *C. difficile* infection or colonization could be shown, which can likely be explained by the low sample size.

It is noteworthy that some antibiotics are considered rather antiparasitics in Mali, and their use might thus not have been reported as ‘antibiotic use’. An important example is metronidazole, which is used in Mali primarily for the treatment of intestinal protozoan diseases and is not even primarily classified by physicians as an antibiotic.

Previous hospitalisation and healthcare system contact are also regarded as risk factors for the development of CDI. Inpatient stays in sub-Saharan African countries like Mali are fundamentally different from those in Western countries. With two state hospitals for almost 5 million people living in the urban catchment area of Bamako and an estimated 10 million inhabitants in the rest of the country with limited access to inpatient care, it is difficult to assess the risk factor of hospitalisation in such settings [76]. Data from this study show that only 1.3% of patients reported having been hospitalised in the last 3 months. If the sample of hospitalized patients with diarrhoea had been larger, the data might have been different, because with only 10% hospitalized patients and 90% outpatients, selection bias can strongly influence the results. For each inpatient case with diarrhoea, it was originally planned to examine one inpatient case in the same age group without diarrhoea. This matching was not feasible, however, because only few samples were provided from both hospitals. A meta-analysis by Curcio et al. [30] examined the prevalence of CDI in 17 studies from different low-income countries and showed a significant correlation between the symptom diarrhoea in inpatients and CDI. The authors also published data showing that up to 15% of all patients with diarrhoea in developing countries might be affected by a CDI. A case-control study from Vhembe Distict, South

Africa by Samie et al. examined 322 stool samples from inpatients using PCR. Toxigenic strains were detected in 7.1% of diarrhoea patients [105].

With regard to immunosuppression, one might wonder how CDI is influenced by HIV co-infection. A study by Samie et al. [105] showed a non-significant connection between HIV and CDI. Yet, a retrospective case control study from Italy showed a significant association between HIV and a CDI [36]. Furthermore, it was postulated that a flourishing tuberculosis, especially in connection with HIV, should be included as a separate risk factor [72]. In the herein presented study from Mali, no significant connection between immunosuppression or HIV and *C. difficile* colonisation or infection could be shown. However, this result should be viewed critically, as the study was not sufficiently conclusive to show such associations. Furthermore, the HIV status was not objectively examined using blood tests, but only asked for in the questionnaire survey. The UN AIDS Foundation estimates the HIV prevalence in Mali at around 1.4% in the 15-49 year old age group, and hence, very low compared to the HIV prevalence in other African countries. For example, the HIV infection rate in South Africa is considered to be 20.4% in the same age group [118,119].

Of note, the actual immune state may be very different in HIV-infected patients. In advanced stages, with low gamma globulin and low albumin levels, there is an increased risk of developing a CDI. Di Bella et al. discuss that the lack of a humoral immune system to play a crucial role in the development of CDI [36]. An American study group around Heines et al showed that a CD4 cell count below 50 cells/µl is a considerable risk factor for CDI [49].

An advanced age might also constitute an important risk factor for CDI, whereas children aged below 2 years are considered as frequent *C. difficile* carriers in Western countries, but only rarely develop overt disease [3]. While young asymptomatic children are thus normally not screened for *C. difficile*, we did so in the present work to obtain a complete picture of the prevalence in all age groups. Our results support previous data from other studies that symptomatic CDI is very rare in this age group, and even toxigenic *C. difficile* strains are unlikely to cause symptoms [103].

Likewise, a study group around Plants-Paris [94] from Kenya examined children with diarrhoea under 5 years of age to diagnose the cause of diarrhoea. A study population of 157 children who had presented to a hospital with community-acquired diarrhoea was included. A prevalence of *C. difficile* of 37.6% was determined, and most infections were toxigenic. The highest prevalence was found in the group of 13-18 month old children, whereas no infection or colonisation could be detected in the children over 24 months of age. In our study from Bamako, 58% of all positive *C. difficile* samples were found in infants less than one year of age. However, only one sample harboured the toxins A, B and the binary toxin; all other strains in this age group were non-toxigenic. As expected, the Plants-Paris study in Kenya found a high number of co-infections, in particular with rotavirus. Data from the USA also show frequent co-infection with rotavirus [132]. Both the Plants-Paris study and the American Zillberg study were carried out as cohort studies, i.e. only the group of

diarrhoea patients with *C. difficile* detection was examined. No healthy controls were included, and hence, an apathogenic colonisation could not be detected. Although there is little data from control groups, it is estimated that the colonisation rate in 6-12-month old children in the USA is around 14%. In 3 year olds, on the other hand, prevalences of *C. difficile* colonisation are described as similar to those of a non-hospitalized adult [58]. Studies postulate that newborns and infants may lack the cellular machinery to process the toxins from *Clostridium* species, and remain therefore asymptomatic [95]. In any event, guidelines recommend that children with *C. difficile* should only be treated if there is a risk factor such as a serious intestinal disease like Hirschsprung's disease, and after exclusion of other, more likely intestinal pathogens [106].

Very few participants from Mali presented at an advanced age, which would also have constituted a risk factor for symptomatic CDI [84]. Indeed, in West Africa including Mali, there is a different age distribution than in Western countries. For example, it is estimated that only about 3% of the Malian population is over 65 years of age, compared to 22% in Germany for those aged over 65 [114].

4.2. Zoonotic transmission: a closer look at ribotypes (RTs)

In addition to its role as a pathogenic bacterium for humans, *C. difficile* is increasingly acknowledged as a cause of gastrointestinal diseases in animals. Infections have been reported in dogs, horses, sheep and poultry [8,77]. Indeed, farm animals may serve as an important source of enteropathogenic pathogens, including *C. difficile*. Meat consumption, or close contact with farm animals, can lead to the transmission of pathogenic strains. Studies have isolated *C. difficile* from poultry and sheep faeces [4]. Pigs, goats and cattle are also moving more and more into focus and are the subject of current global discussion [101,112,113] . An American study group around Muñoz-Price investigated whether the place of residence had an influence on asymptomatic colonisation or a classical CDI. The study team observed that people living near livestock farms had a significantly higher risk of infection with toxigenic strains. Possible reasons besides direct animal contact might be sewage containing antibiotic residues that influence on the composition of the intestinal flora. [85]. In view of the specific situation in Africa, however, additional studies are needed to conclude and to discuss the extent to which antibiotics are used in the dairy and meat industry and whether these can be compared with data from the ‘Western’ world.

Molecular ribotyping allows to elucidate transmission patterns, and thus to determine whether there has been any animal-human transmission. In the herein presented study, faecal samples from animals were collected at various animal markets in Bamako. Since *C. difficile* is a spore-forming environmental microorganism [131], which has also been discussed in some African studies, the situation in Bamako should be further investigated. A toxigenic R012 strain was isolated in one out of 100 animal samples. This isolate produced not only toxins A and B, but also a binary toxin. The same

strain was not detected in any of the human samples. Additional research should also employ environmental sampling to comprehensively assess potential infection transmission chains.

The RT with the highest significance as a possible zoonotic pathogen in the Northern hemisphere is RT078 [31,65]. We found this RT in one human sample in Mali. Studies from the Netherlands that published data on the transmission of *C. difficile* from farm animals to humans showed that *C. difficile* strains with RTs 014, 078 and 020 were transmitted from animals to humans The most common RT found in Bamako in patients with asymptomatic colonisation was RT084, which is in line with a study from Ghana [59].

Animals have also been described as zoonotic pathogen reservoirs, but it is assumed that most infections are passed on from person to person. [102]. In the CLOser study, a European study that examined 2830 *C. difficile* isolates in 22 European countries between 2011 and 2014, the RTs with the highest prevalence in Europe were the RTs 001, 002, 005, 014, 015 020, 027, 078 and 126. [44]. In contrast, none of the ribotypes mentioned were detected in our study in Mali, with the exception of ribotype RT078.

A transmission of *C. difficile* from farm animals to humans is also possible in Bamako. In order to get a more precise assessment of the situation on site, investigations should be carried out.

4.3. Antibiotic resistance, treatment and antibiotic stewardship

In this study, antibiotic resistance testing did not reveal resistance to agents that are used to treat CDI, but an epidemiologically interesting high resistance towards clarithromycin was observed. Likewise, a study from Kenya in 2021 showed similar results with regard to vancomycin and metronidazole, but reported a very high resistance rate to rifampicin [86].

A pan-European study published data in 2015 showing that some RT are associated with specific antibiotic resistance patterns. For example, RTs 001, 027, 017, 018 and 356, which are endemic in Europe, showed high resistance to antibiotics [44].

Metronidazole and vancomycin remain common drugs for the treatment of CDI due to their low resistance rates. They can also be recommended in Mali, mainly because of their high sensitivity rates in CDI. However, CDI relapses are expected in up to 30% of cases after taking both metronidazole and vancomycin, as both drugs also damage part of the physiological intestinal flora. Therefore, fidaxomicin is nowadays used as the antibiotic of choice in the new guidelines. However, susceptibility testing in the laboratory is cumbersome, and the high associated cost of fidaxomicin prevents its use in low-income countries. Indeed, when choosing the appropriate antibiotic therapy for CDI, it is important to take other practical considerations into account, in addition to the guidelines. Metronidazole is a common drug, especially in developing countries, and is already used regularly by a large number of patients. It is readily available and cheap, and might thus still be recommended for

mild and moderate CDI disease courses in sub-Saharan Africa, where many patients lack health insurance [43].

The Cox study group [29] emphasised that with regard to antibiotic resistance, it is also important to establish rules on the use of antibiotics in low and middle income countries. This includes the provision of diagnostic tests; the training of laboratory staff and the general public as well as the creation and strengthening of (inter) national bodies for better regulation. In addition, controlling the manufacture, distribution and dispensing of antibiotics is essential. This requires close collaboration between politicians, scientists and physicians. Another important question is also whether the introduction of antibiotic stewardship could largely counteract the development of resistance. Data on antibiotics stewardships initiatives in Africa are scarce. A worldwide study on antibiotic stewardship showed that only 14% of the African participants in a hospital-based Antibiotic Stewardship programme had access to an acceptable programme infrastructure [55]. Data from South Africa have shown that participation in these programmes may drastically reduce antibiotics revenues [21]. Important conclusions can be drawn from these data. Since CDI is classically caused by dysbiosis after previous antibiotic therapy, a more restrictive use of antibiotics could also reduce the number of CDIs. In addition, a more targeted use of antibiotics will reduce unnecessary resistance, not only in relation to *C. difficile*.

4.4. Limitations of the present study

Several limitations of the current study are offered for consideration. First, the sample size was rather small and the prevalence of CDI lower than expected. Hence, the study was underpowered to detect e.g. associations between some specific risk factors and CDI. Second, the sample collection period was limited to a couple of weeks, thereby preventing the detection of any seasonal infection transmission or prevalence patterns. Third, the number of inpatients was quite low, and clinical and diagnostic laboratory work in Mali were only possible under challenging conditions, because a rampant meningitis outbreak at the time of the study kept laboratory and clinical stuff very busy and rendered access to the laboratory's only PCR cycler more difficult. It is recommended that future studies should include a larger sample size, more and different recruitment sites, and that they should encompass a recruitment period of at least six months, to shed further light on the clinical epidemiology of *C. difficile* in Africa.

5. References

1. 4ème RECENSEMENT GENERAL DE LA POPULATION ET DE L'HABITAT DU MALI (RGPH) République de Mali: Institut National de la Statistique.http://www.instat-mali.org/contenu/rgph/tdemo09_rgph.pdf (accessed 09.01.2020)

2. Acheson DW, Luccioli S (2004) Microbiol-gut interactions in health and disease. Mucosal immune response. Best Pract Res Clin Gastroenterol 18:387–404

3. Adlerberth I, Huang H, Lindberg E, Aberg N, Hesselmar B, Saalman R, Nord CE, Wold AE, Weintraub A (2014) Toxin-producing *Clostridium difficile* strains as long term gut colonizers in healthy infants J Clin. Microbio 52:173-179

4. Al Saif N, Brazier JS (1996) The distribution of *Clostridium difficile* in the environment of South Wales. Journal of Medical Microbiology 45:133–137

5. Alere Techlab® C.DIFF QUIK CHEK COMPLETE® manual (2016) page 6

6. Allegretti JR, Marcus J, Storm M, Sitko J, Kennedy K, Gerber GK., Bry L (2019) Clinical predictors of recurrence after primary *clostridioides difficile* infection: A prospective cohort study. Digestive Diseases and Sciences, 65: 1761–1766

7. Allen SJ (2013) Lactobacilli and bifidobacteria in the prevention of antibioticassociated diarrhoea and *Clostridium difficile* diarrhoea in older inpatients (PLACIDE):a randomised, double-blind, placebo-controlled, multicentre trial. Lancet 1249-57.

8. Arroyo LG, Weese JS, Staempfli HR (2004) Experimental *Clostridium difficile* enterocolitis in foals. Journal of Veterinary Internal Medicine 18: 734–738

9. Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G (2009) Clostidium ribotypes 001, 017and 027 are associated with lethal *C. difficile* infections in Hesse, Germany. Euro Surveill 14:45

10. Barbut F (2003) A European survey of diagnostic methods and testing protocols for *Clostridium difficile*. Clin Microbiol Infect 9: 989-96.

11. Bärenbold O, Raso G, Coulibaly JT, N'Goran EK, Utzinger J, Vounatsou P (2017) Estimating sensitivity of the Kato-Katz technique for the diagnosis of *Schistosoma mansoni* and hookworm in relation to infection intensity. PLoSNegl Trop Dis 11: e0005953

12. Barral M, Boudiaf M, Dohan A, Hoefel C, Camus M, Pautrat K (2015) MDCT of acute colitis in adults: an update in current imaging features. Diagn Interv Imaging 96:133–149

13. Bartlett JG, Moon N, Chang TW, Taylor N, Onderdonk AB (1978) Role of *Clostridium difficile* in antibiotic-associated pseudomembranous colitis. Gastroenterology 75:778-782

14. Bartlett JG (2002) Clinical practice. Antibiotic-associated diarrhea. N Engl J Med; 346: 334-9

15. Bartlett JG (2006) Narrative review: the new epidemic of *Clostridium difficile*-associated

16. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van Dissel JT, Kuijper EJ, Group ES (2011) *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377:63-73

17. Bavishi C, Dupont HL (2011) Systematic review: the use of proton pump inhibitors and increased susceptibility to enteric infection. Aliment Pharmacol Ther 34:1269-81

18. Becker SL, Chatigre JK, Coulibaly JT, Mertens P, Bonfoh B (2015) Molecular and culture-based diagnosis of *Clostridium difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions. Trans R Soc Trop Med Hyg 109:660-668

19. 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 Infect 24:13-37

20. Becker SL, Yap P, Horié NS, Alirol E, Barbé B, Bhatta NK, Bhattarai NR, Bottieau E, Chatigre JK, Coulibaly JT, Fofana HK, 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 PH, 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 Negl Trop Dis. 3:10

21. Brink AJ, Messina AP, Feldman C, Richards GA, Becker PJ, Goff DA, Bergh DVD (2016). Antimicrobial stewardship across 47 South African hospitals: an implementation study. The Lancet Infectious Diseases 16:1017–1025.

22. Brown KA, Khanafer N, Daneman N, Fisman DN (2013) Meta-analysis of antibiotics and the risk of community-associated *Clostridium difficile* infection. Antimicrob Agents Chemother 57:2326-2332.

23. Buggy BP, Wilson KH, Fekety R (1983) Comparison of methods for recovery of *Clostridium difficile* from an environmental surface. J Clin Microbiol 18:348-52.

24. Burckhardt F, Friedrich A, Beier D, Eckmanns T (2008) *Clostridium difficile* surveillance trends, Saxony, Germany. Emerg Infect Dis 14: 691

25. CDC (2005) Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states MMWR Morb Mortal Wkly Rep 54:1201-5

26. CDC Mali Facesheet (2018) https://www.cdc.gov/globalhealth/countries/mali/pdf/Mali_Factsheet.pdf (accessed November 2020)

27. Centers of Disease Control and Prevention, Schistosomiasis https://www.cdc.gov/dpdx/schistosomiasis/index.html (accessed November 2020)

28. Colley DG, Bustinduy AL, Secor WE, King CH (2014) Human schistosomiasis. The Lancet 383:2253–2264

29. Cox J, Vlieghe E, Mendelson M, Wertheim H, Ndegwa L, Villegas M, Hara GL (2017). Antibiotic stewardship in low- and middle-income countries: the same but different? Clinical Microbiology and Infection 23:812–818

30. Curcio D, Cané A, Fernández F, Correa J (2019). *Clostridium difficile*-associated Diarrhea in Developing Countries: A Systematic Review and Meta-Analysis. Infectious Diseases and Therapy 8:87–103

31. Dahms C, Hübner NO, Wilke F, Kramer A (2014) Mini-review: Epidemiology and zoonotic potenctial of multiresistant bacteria and *Clostridium difficile* in livestock and food GMS Hygiene and Infection Control 9:2196-5226

32. Dallal RM, Harbrecht BG, Boujoukas AJ, Sirio CA, Farkas LM, Lee KK (2002). Fulminant *Clostridium difficile*: an underappreciated and increasing cause of death and complications. Ann Surg 235:363-72

33. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, Delmee M, Fitzpatrick F, Ivanova K, Kuijper E (2014) Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis 14: 1208-1219

34. Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH. (2016). Diversity of *Clostridium difficile* P CR ribotypes in Europe: Results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013.Eurosurveillance 21:29

35. Deshpande A, Pasupuleti V, Thota P, Pant C, Rolston DD, Sferra TJ, Hernandez AV, Donskey CJ (2013) Community-associated *Clostridium difficile* infection and antibiotics: a meta-analysis. J Antimicrob Chemother, 68:1951-1961

36. Di Bella S, Friedrich A, García-Almodóvar E., Gallone M, Taglietti F, Topino S, Petrosillo N (2015). *Clostridium difficile* infection among hospitalized HIV-infected individuals: epidemiology and risk factors: results from a case-control study (2002-2013). BMC Infectious Diseases 15:194

37. Dietrich M, Kern P (1983) Tropenlabor Diagnostik für die ärztliche Praxis mit einfacher Laborausrüstung 1983 Fischer Verlag, Stuttgart pp 12-13

38. Doumbo Safiatou N, Ongiba A, Doumatabe D, Tran Tuan M, Traoré A (2018) Prevalence of Malaria, Intestinal and Urinary parasite infections in Kalifabougou, Mali. Mali Med 33:10-15

39. Dtsch Arzteblatt Int (2014) *Clostridium difficile*-Infektion 111:723-31

40. Dubberke ER, Butler AM, Reske KA, Agniel D, Olsen MA, D'Angelo G, McDonald LC, Fraser VJ (2008), Attributable outcomes of endemic *Clostridium difficile*-associated disease in nonsurgical patients. Emerg Infect Dis 14: 1031-1038

41. Ersöz SS, Cosansu S (2018) Prevalence of *Clostridium difficile* isolated from Beef and Chicken Mea Products in Turkey. Korean JFood Sci Resour 38:759-767

42. Esch KJ, Petersen CA (2013). Transmission and Epidemiology of Zoonotic Protozoal Diseases of Companion Animals Clinical Microbiology Reviews 26:58-85

43. Fessler B, Fidoxamicin ein neues Antibiotikum gegen CDIs unter https://www.deutsche-apotheker-zeitung.de/daz-az/2013/daz-5-2013/weniger-rezidive-mit-neuem-antibiotikum (accessed 01.2020)

44. Freeman J, Vernon J, Pilling S, Morris K, Nicholson S, Shearman S, Wilcox M (2018) The ClosER study: results from a three-year pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes, 2011–2014.Clinical Microbiology and Infection 24:724–731

45. GBD (2017) Diarrhoeal Disease Collaborators. Quantifying risks and interventions that have affected the burden of diarrhoea among children younger than 5 years: an analysis of the Global Burden of Disease Study 2017 Lancet Infect Dis. 1:37-59

46. Gerding DN (1989) Disease associated with *Clostridium difficile* infection. Ann Intern Med 110:255-7

47. Giannasca PJ, Warny M (2004) Active and passive immunization against *Clostridium difficile* diarrhea and colitis. Vaccine 22:848–856.

48. Gorkiewicz G (2009) Nosocomial and antibiotic-associated diarrhoea caused by organisms other than *Clostridium difficile*. International Journal of Antimicrobial Agents 33:37-41

49. Haines Ch, Moore R, Bartlett J, Sears C, Cosgrove S, Carroll K, Gebo K (2013). *Clostridium difficile* in a HIV-Infected Cohort Aids 27:2799–2807.

50. Hall I, O'Toole E (1935) Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, bacillus difficilis. Am J Dis Chis 49:390-402

51. Heinlen L, Ballard JD (2010) *Clostridium difficile* infection. Am J Med Sci. 340: 247-52

52. Henning Zaiß N, Witte W, Nübel U (2010) Fluoroquinolone Resistance and *Clostridium difficile*, Germany, Emerging infectious Diseases 16: 4

53. Hoornweg D, Pope K (2014). Socioeconomic Pathways and Regional Distribution of the World's 101 Largest Cities

54. Horton KM, Corl FM, Fishman EK (2000) CT evaluation of the colon: infammatory disease. Radiographics 20:399–418

55. Howard P, Pulcini C, Hara GL (2015) An international cross-sectional survey of antimicrobial stewardship programmes in hospitals J Antimicrob Chemother 70:1245-1255

56. Hull M W, Beck P L (2004) *Clostridium difficile*-associated colitis. Canadian family physician Medecin de famille canadien, 50:1536-40

57. Human Development Report 2019 United Nations https://hdr.undp.org/sites/default/files/hdr_2019_overview_-_english.pdf (accessed 04/2022)

58. Jangi S, Lamont JT (2010) Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. J Pediatr Gastroenterol Nutr 51:2–7

59. Janssen I, Cooper P, Gunka K, Rupnik M, Wetzel D, Zimmermanna O, Groß U (2016) High prevalence of nontoxigenic *Clostridium difficile* isolated from hospitalized and non-hospitalized individuals in rural Ghana International Journal of Medical Microbiology 306: 652–656

60. Jump RLP, Pultz MJ, Donskey CJ (2007) Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pumps inhibitors and *C difficile*-associated diarrhea. Antimicrob Agents Chemother 51:2883-7

61. Just I, Gerhard R (2004) Large clostridial cytotoxins. Rev Physiol Biochem Pharmacol 152:23-47

62. Kamuju V, Kumar S, Khan WH, Vivekanandan P (2018) Hypervirulent *Clostridium difficile* ribotypes are CPG depleted. Virulence 9:1422–1425

63. Keessen EC, Harmanus C, Dohmen W, Kuijper EJ, Lipman LJ (2013) *Clostridium difficile* infection associated with pig farms.Emerg Infect Dis 19:1032–1034

64. Kim KH, Fekety R, Batts DH, Brown D, Cudmore M, Silva J, Waters D (1981) Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J Infect Dis 143:42-50

65. Knetsch CW, Kumar N, Forster SC, Connor TR, Browne HP, Harmanus C, Sanders IM, Harris SR, Turner L, Morris T, Perry M, Miyajima F, Roberts P, Pirmohamed M, Songer JG, Weese JS, Indra A, Corver J, Rupnik M, Wren BW, Riley TV, Kuijper EJ, Lawley TD (2018) Zoonotic transfer of *Clostridium difficile* harboring antimicrobial resistance between farm animals and humans. J Clin Microbiol 56:01384-17

66. Kouassi KA, Dadie KF, Nguessan KM, Dje YG. (2014) *Clostridium perfringens* and *Clostridium difficile* in cooked beef sold in Côte d 'Ivoire and their antimicrobial susceptibility, Anaerobe 28:90–94

67. Kuijper E, Coignard B, Tüll P (2006). Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clinical Microbiology and Infection 12:1469-0691

68. Kuijper E, van Dissel T (2008) Spectrum of *Clostridium difficile* infections outside health care facilities. Cmaj 179:747-8

69. Larson HE (1982) Epidemiology of *Clostridium difficile* in infants. J Infect Dis 146:727-33

70. Leclerc H., Schwartzbrod L, Dei-Cas E (2002) Microbial agents associated with waterborne diseases. Crit. Rev. Microbiol. 28:371–409

71. Leffler DA, Lamont JT (2015) *Clostridium difficile* infection. N Engl J Med 372: 1539-1548

72. Legenza L, Barnett S, Rose W, Bianchini M, Safdar N, Coetzee R (2018) Epidemiology and outcomes of *Clostridium difficile* infection among hospitalised patients: results of a multicentre retrospective study in South Africa. BMJ Global Health 3:e000889

73. Liu L (2015) Global, regional, and national causes of child mortality in 2000- 13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet 385: 430–40

74. Loo VG, Poirier L, M. Miller MA, Oughton M, Libman MD, Michaud S, Bougault AM, Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ, Horn R, René P, Monczak Y, Dascal A (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. N Engl J Med 353: 2442-2449

75. Lucado, J, Gould, C, Elixhauser A. (2012) *Clostridium difficile* Infections (CDI) in Hospital Stays, 2009: Statistical Brief 124. In Healthcare Cost and Utilization Project (HCUP) Statistical Briefs. Agency for Healthcare Research and Quality (US)

76. Mali Data Portal, Indicateur Mali générale. La Taille de la population (2011) https://mali.opendataforafrica.org (accessed am 25.01.2020, in French)

77. Marks SL, Kather EJ, Kass PH, Melli AC (2002) Genotypic and phenotypic characterization of Clostridium perfringens and *Clostridium difficile* in diarrheic and healthy dogs. Journal of Veterinary Internal Medicine 16:533–540

78. Martinez FJ, Leffler DA, Kelly CA (2012) *Clostridium difficile* outbreaks: prevention and treatment strategies. Risk Management and Healthcare Policy 5:55-64

79. McDonald L, Killgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, Johnson S, Gerding DN (2005) An Epidemic, Toxin Gene-Variant Strain of *Clostridium difficile* N Engl J Med 353:2433-2441

80. McDonald LC, Owings M, Jernigan DB (2006) *Clostridium difficile* infection in patients discharged from US shortstay hospitals, 1996-2003. Emerg Infect Dis 12: 409-15

81 McDonald LC (2007) Recommendations for surveillance of *Clostridium difficile*-associated disease. Infect Control Hosp Epidemiol 28:140-5

82. McFarland LV, Beneda HW, Clarridge JE, Raugi GJ (2007) Implications of the changing face of *Clostridium difficile* disease for health care practitioners. Am J Infect Control 35:237-53

83. Miller MA (2002) Morbidity, mortality, and healthcare burden of nosocomial *Clostridium difficile*-associated diarrhea in Canadian hospitals. Infect Control Hosp Epidemiol 23:137-40

84. Mock M, Halfmann A, Herrmann M, von Müller L (2013) Aktuelles zur Epidemiologie von *Clostridium difficile* – Bericht aus dem Konsiliarlabor *C. difficile*. Epidemiologisches Bulletin 26: 241–4

85. Muñoz-Price LS, Hanson R, Singh S, Nattinger AB, Penlesky A, Buchan BW, Pezzin LE (2020). Association Between Environmental Factors and Toxigenic *Clostridioides difficile* Carriage at Hospital Admission. JAMA Network Open, 3:e1919132

86. Mutai WC, Mureithi MW, Anzala O, Revathi G, Kullin B, Burugu M, Kyany'a C, Odoyo E, Otieno P, Musila L. (2021) High Prevalence of Multidrug-Resistant *Clostridioides difficile* Following Extensive Use of Antimicrobials in Hospitalized Patients in Kenya. Front Cell Infect Microbiol. 10:604986

87. Mutombo N., Landouré A, Man WY, Fenwick A, Dembélé R, Sacko M, Mclaws ML (2019). The association between child Schistosoma spp. infections and morbidity in an irrigated rice region in Mali: A localized study. Acta Tropica, 199:105-115

88. Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen, KISS Krankenhaus-Infektions-Surveillance-System Referenzdaten 1.Januar 2016-Dezember 2016. https://www.nrz-hygiene.de/fileadmin/nrz/module/cdad/CDAD_Referenzdaten_2016_DE.pdf

89. Nehanda S, Mulundu G, Kelly P (2019). Prevalence of *Clostridium difficile* and its toxins in hospital patients with diarrhoeal diseases in Lusaka, Zambia. Trans R Soc Trop Med Hyg 00:1–5

90. Onwueme K, Fadairo Y, Idoko L, Onuh J, Alao O, Agaba P, Idoko J. (2011). High prevalence of toxinogenic *Clostridium difficile* in Nigerian adult HIV patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 105: 667–669

91. Oyaro M, Plants-Paris K, Bishoff D, Malonza P, Gontier C, DuPont H, Darkoh (2018) High rate of *Clostridium difficile* among young adults presenting with diarrhea at two hospitals in Kenya International Journal of Infectious Diseases 74:24–28

92. Pepin J (2004) *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ 171: 466-72

93. Pepin J, Valiquette L, Cossette B (2005) Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. Cmaj 173:1037-42

94. Plants-Paris K, Bishoff D, Malonza P, Oyaro M, Mwinyic B,Chapella C, Kituyi A, Nyangao J, Mbathac D, Darkoh C (2019) Prevalence of *Clostridium difficile* infections among Kenyan children with diarrhea International Journal of Infectious Diseases 81: 66–72

95. Pothoulakis C, Lamont JT (2001) Microbes and microbial toxins: paradigms for microbialmucosal interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. Am J Physiol Gastrointest Liver Physiol 280:178–183

96. QIAamp® Fast DNA Tissue Kit Handbook For rapid purification of DNA from tissue samples (2015)

97. Rajabally NM, Pentecost M, Pretorius G, Whitelaw A, Mendelson M, Watermeyer G. (2013) The *Clostridium difficile* problem: A South African tertiary institutions prospective perspective. South African Medical Journal, 103:168-72

98. Rana SV, Bhasin DK, Vinayak VK (2005) Lactose hydrogen breath test in *Giardia lamblia*-positive patients. Dig Dis Sci 50:259-61

99. Ratnayake L, McEwen J, Henderson N (2011) Control of outbreaks of diarrhea in a vascular sugery untril caused by a high level clindamycin-resistant *Clostridium difficile* PCR ribotype 106 HosptInfect 79:242-247

100. RIDA®GENE *Clostridium difficile* R-Biopharm AG Handbook (2018)

101. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS (2007) *Clostridium difficile* in retail ground meat, Canada. Emerging Infectious Diseases 13:485–487.

102. Rodriguez-Palacios A, Stampfli HR, Duffield T (2006). *Clostridium difficile* PCR ribotypes in calves, Canada Emerging Infectious Diseases 12:1730–1736

103. Rousseau C, Poilane I, Pontual D, Maherault A, Monnier A, Collignon A (2012). *Clostridium difficile* Carriage in Healthy Infants in the Community: A Potential Reservoir for Pathogenic Strains. Clinical Infectious Diseases 55:1209–1215

104. Rupnik M (2005) Revised nomenclature of *Clostridium difficile* toxins and associated genes. J Med Microbiol 54:113-7

105. Samie A, Bessong PO, Obi CL, Guerrant RL, Archbald-Pannone, Alcantara-Warren C, Franasiak J (2008) PCR Detection of *Clostridium difficile* Triose Phosphate Isomerase (tpi), Toxin A (tcdA), Toxin B (tcdB), Binary Toxin (cdtA, cdtB), and tcdC Genes in Vhembe District, South Africa. The American Journal of Tropical Medicine and Hygiene 78:577–585

106. Schutze G, Willoughby R (2013) *Clostridium difficile* Infection in Infants and Children Pediatrics 131:196-200

107. Sheth SG, LaMont JT (1998) Toxic megacolon. Lancet 351:509-13

108. Simango C, Uladi S (2014) Detection of *Clostridium difficile* diarrhoea in Harare, Zimbabwe. Trans R Soc Trop Med Hyg 108:354-357

109. Skript mikrobiologisches Praktikum Landau https://www.uni-landau.de/umwelt/study/content/files/archiv/Martin/WS10/Mikrobiologisches_Praktikum/Skript_Mibipraktikum_WS10_11.pdf (accessed Dezember 2020)

110. Slimings C, Riley TV (2014) Antibiotics and hospital-acquired *Clostridium difficile* infection: update of systematic review and meta-analysis. J Antimicrob Chemother 69: 881-891

111. Smith A (2005) Outbreak of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. Euro Surveill 10: E050630

112. Songer JG, Anderson MA (2006). *Clostridium difficile*: an important pathogen of food animals. Anaerobe 12:1–4

113. Songer JG, Post KW, Larson DJ, Jost BH, Glock RD (2000). Infection of neonatal swine with *Clostridium difficile*. Swine Health and Production 8:185–189

114. Statistisches Bundesamt Bevölkerung, Ältere Menschen die Bevölkerungsgruppe der älteren Menschen über 65 (2018) unter https://www.destatis.de/DE/Themen/Querschnitt/Demografischer-Wandel/Aeltere-Menschen/bevoelkerung-ab-65-j.html (accessed 25.01.2020)

115. Stubbs SL, Brazier JS, O'Neill GL (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 37:461–463

116. Suetens C, Latour K, Kärki T, Ricchizzi E, Kinross P, Moro ML (2018) Prevalence of healthcare-associated infections, estimated incidence and composite antimicrobial resistance index in acute care hospitals and long-term care facilities: results from two European point prevalence surveys, 2016 to 2017. Euro Surveill. 23:1560-7917

117. Thompson RCA (2002). Towards a better understanding of host specificity and the transmission of Giardia: The impact of molecular epidemiology. Giardia the cosmopolitan parasite 55-69

118. UNAIDS HIV Data Mali unter https://www.unaids.org/en/regionscountries/countries/mali (accessed 28.01.2020)

119. UNAIDS HIV Data South Africa https://www.unaids.org/en/regionscountries/countries/southafrica (accessed 28.01.2020)

120. Unicef, Situation des infrastructures de la santé au Mali, (2013). https://reliefweb.int/report/mali/health-bulletin-focus-health-situation-sahelian-countries-affected-humanitarian-crisis (acessed January 2020)

121. Van Prehn J, Reigadas E, Vogelzang EH, Bouza E, Hristea A, Guery B, Krutova M, Norén T, Allerberger F, Coia JE, Goorhuis A, van Rossen, TM, Ooijevaar RE, Burns K, Scharvik Olesen, BR, Tschudin-Sutter S, Wilcox MH, Vehreschild MJGT, Fitzpatrick F; Kuijper EJ (2021). European Society of Clinical Microbiology and Infectious Diseases: 2021 update on the treatment guidance document for *Clostridioides difficile* infection in adults. Clinical Microbiology and Infection, 27:1-21

122. Vonberg RP (2008). Infection control measures to limit the spread of *Clostridium difficile* Clin Microbiol Infect 5:2-20

123. Voth DE, Ballard JD (2005) *Clostridium difficile* Toxins: Mechanism of Action and Role in Disease. Clinical microbiology reviews, 18: 247-263

124. Wanahita A, Goldsmith EA, Musher DM (2002) Conditions associated with leukocytosis in a tertiary care hospital, with particular attention to the role of infection caused by *Clostridium difficile*. Clin Infect Dis 34:1585-92

125. Weber DJ, Anderson DJ, Sexton DJ, Rutala WA. (2013). Role of the environment in the transmission of *Clostridium difficile* in health carefacilities. American Journal of Infection Control 41:5

126. WHO (2002) Prevention and control of schistosomiasis and soil-transmitted helminthiasis. Report of a WHO Expert Committee . World Health Organization Geneva: WHO Technical Report Series No 912

127. WHO 1994 Preparation for Direct Smear Technique. Practice Guideline

128. Wilcox M.H (2017). Bezlotoxumab for Prevention of Recurrent *Clostridium difficile* Infection. N Engl J Med 376:305-317

129. Wombwell E., Patterson M. E., Bransteitter B. Gillen L. R. (2020). The effect of saccharomyces boulardii primary prevention on risk of hospital-onset *clostridioides difficile* infection in hospitalized patients administered antibiotics frequently associated with *C. difficile* infection. Clinical Infectious Diseases 73:9

130. World Population Prospects 2019, Volume II: Demographic Profiles. United Nations, Department of Economic and Social Affairs, Population Division (accessed am 24. January 2021)

131. Zidaric V, Beigot S, Lapajne S, Rupnik M (2010). The occurrence and high diversity of *Clostridium difficile* genotypes in rivers Anaerobe16:371–375

132. Zilberberg MD, Tillotson G, Mcdonald L(2010). *Clostridium difficile* Infections among Hospitalized Children, United States, 1997–2006. Emerging Infectious Diseases 16:604–609

133. Zilberberg MD, Shorr AF, Kollef MH (2008) Increase in adult *Clostridium difficile*-related hospitalizations and case fatality rate, United States, 2000-2005. Emerg Infect Dis14:929-31

6. Appendices

6.1. Written informed consent

Information sheet and informed consent form for the participation in a medical study

Epidemiology, clinical features and differential diagnosis of *Clostridium difficile*-associated diarrhoea in Bamako, Mali

1. Information sheet for patients

Identification number (ID):

Introduction:
Diarrhoea is a symptom of worldwide relevance. Diarrhoea is caused by a wide range of pathogens including specific bacteria such as Clostridium difficile. Clostridium difficile is a bacterium that can cause an extent variety of symptoms from diarrhoea to death. It is even possible that one is colonized but has no symptoms at all.
The aim of this study is to learn about the distribution and importance of this bacterium in Mali. We, a researcher groupe from the Institude de Recherche en Santé Publique, Mali and a team from Europe investigate the occurrence of this bacterium in stool samples of patients with and without diarrhoea. This will contribute to a better understanding, diagnostics and treatment in the Sub- Sahara Africa.

Study procedures:
If you agree in beeing part of this study we will ask you to provide stool into a tube. We will do a rapid screening test and a reference test to verify the result of the rapid screening test. If we detect Clostridium difficile in your stool some parts of it will be transfered to Europe for further analysis.

What we need from you:
Your participation is entirely voluntary. You can leave this study at any time without explanation nor justification.
If you agree to participate we will fill in a short questionnaire with you then we would like to ask you to provide some stool samples regardless whether you suffer from diarrhoea or not.

Advantage:
If you have diarrhoea and we find Clostridium difficile in your stool sample we will inform you and offer you a free treatment.
In addition the community will take great benefits from your participation as the information we get provides better understanding, diagnostics and following treatment.

Risks:
There are no primary risks in providing stool samples. However, if you suffer from a Clostridium difficile infection and you get medicated with antibiotics these drugs could lead to side effects (stomachache, nausea, itching and very seldom ototoxicity)

Protection of data privacy:
Your data will be treated confidentially. Your data will be anonymized with a unique nummber that will be used to identify the samples and data during the study.
If we publish the results of this study your name will not appear in the publication. Only the involved reaserchers will be able to access your personal information.

Contact in case of problem or question:

Information sheet and informed consent form for the participation in a medical study

Epidemiology, clinical features and differential diagnosis of *Clostridium difficile*-associated diarrhoea in Bamako, Mali

2. Consent Form

I have read the Information sheet or it has been read to me. I have understood its nature and its content. I know that I have the possibility to resign at any part of the study without any disadvantage.

Date: _____ (day/month/year) Location: _____

Name of **participant**: _____ ID- number:

Adults:
Signature of **participant**: _____

Minors:
Signature of **parent** or **legal guardian**: _____

Signature of the **interviewer**: _____

□ Please inform me if you find C. difficile in my stool sample.

I am available under (phone number):
Adress:

6.2. Questionnaire cases

Questionnaire for patients with diarrhoea

Sender	
Hospital/Ward: ☐ Centre de Santé de Reference Commune III ☐ Centre de Santé Reference Commune IV ☐ Hospital Gabriel Touré Contact person: _____ Phone number: _____	Pat. ID: Date:

Information about the patient	
Gender:	☐ male ☐ female
Age: _____	
Height: _____	
Weight: _____	

Material
Date of taking: _____ Date of transmittal: _____

Clinic
Hospitalisation beginning date: _____

When did you start having diarrhoea?	☐ <3 after hospitalisation ☐ >3 after hospitalisation ☐ not known

Does a person that lives with you suffer from diarroea at the moment?			
	☐ no	☐ yes	☐ not known

Therapy started?	☐no ☐ yes What medicament did you take?: _____

Did you take antibiotics the last 3 months?
□ no □ not known
□ yes Which one? _____
Have you been hospitalized the last 3 months?
□ no
□ yes
In which hospital have you been?
_____

Have you been abroad or out of your usual environment?
□ no □ yes where: _____

Did you have any animal contact? □ no □ yes which species _____
If yes: Where did/do you have animal contact?
□ household
□ job-related
□ other: _____

Do you suffer from immunosuppression? □ no □ yes

What do you usually eat? □ mixed diet □ no pork
□vegetarian □ other: _____

Infant: □mother's milk □ formula other: _____

Have you lost weight on purpose? □ no □ yes

Where do you get your drinking water from?
□ bought bottled water □tap water □ well □ tank □ other: _____

Do you take medicaments (e.g. Loperamid, PPI)
□ no □ yes? which one: _____

Where do you live? □ rural setting
□ urban setting

How many people live in your household? □ 0 □ 1 □ >3 □ >5 □ >8

What is your profession? □ farmer □physician /nurse □ unemployed
□ teacher □student □ retired
□ vendor □ other _____

Do you suffer from previous diseases? □ cancer □ tuberculosis □ HIV/AIDS
□bowel disease: _____

6.3. Questionnaire control

Questionnaire for patients without diarrhoea

Sender

Hospital/Ward:

Pat. ID:
Date:

☐ Centre de Santé de Reference Commune III
☐ Centre de Santé Reference Commune IV
☐ Hospital Gabriel Touré
Contact person: _____
Phone number: _____

Information about the patient

Gender: ☐ male
☐ female
Age: _____
Height: _____
Weight: _____

Material

Date of taking: _____

Clinic

Hospitalisation beginning date: _____

Do you suffer from diarrhoea? ☐ no ☐ yes

Did you suffer from diarrhoea the last 2 weeks? ☐ no ☐ yes

Did you take antibiotics the last 3 months?
☐ no ☐ not known
☐ yes Which one? _____

Have you been hospitalized the last 3 months?
☐ no
☐ yes
In which hospital have you been?
_____

Have you been abroad or out of your usual environment the last 2 weeks?
□ no □ yes where:
_____

Did you have any animal contact? □ no □ yes which species _____
If yes: Where did/do you have animal contact?
□ household
□ job-related
□ other: _____

Do you suffer from immunosuppression? □ no □ yes

What do you usually eat? □ mixed diet □ no pork
□vegetarian □ other: _____

Infant: □mother's milk □ formula other: _____

Have you lost weight? □ no □ yes

Where do you get your drinking water from?
□ bought bottled water □tap water □ well □ river □ other:
_____

Do you take medicaments
□ no □ yes? which one: _____

Where do you live? □ rural setting
□ urban setting

How many people live in your household? □ 0 □ 1 □ >3 □ >5 □ more than 8

What is your profession? □ farmer □physician /nurse □ unemployed
□ teacher □student □ retired
□ vendor □ other _____

Do you suffer from previous diseases?□ cancer □ tuberculosis □ HIV/AIDS
□bowel disease: _____

6.4. Curriculum vitae

6.5. Publication

Parts of this MD thesis were published in shared first authorship in the following journal:

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