Syndromic approaches to persistent digestive disorders (≥14 days) in resource-constrained settings: aetiology, clinical assessment and differential diagnostics

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Summary

Background: Gastrointestinal infections are among the leading causes of morbidity worldwide. In contrast to acute diarrhoea, long-lasting digestive disorders can be defined as persistent diarrhoea (≥14 days) and/or persistent abdominal pain (≥14 days). This clinical syndrome is frequently caused by intestinal infections, but its medical importance in the tropics, the range of causative pathogens and the contribution of neglected tropical diseases remain to be elucidated. Currently employed diagnostic tools for the detection of intestinal pathogens frequently lack sensitivity, and there are only few evidence-based recommendations to guide the clinical management of persistent digestive disorders in resource-constrained settings. Rapid diagnostic tests (RDTs) have become available for the diagnosis of various intestinal pathogens and hold promise to be used even in peripheral healthcare centres with only very limited laboratory infrastructure. More recently, multiplex polymerase chain reaction (PCR) assays targeting gastrointestinal pathogens have been developed, but these tests have yet to be systematically evaluated in the tropics. The current Ph.D. thesis was carried out as part of the NIDIAG project, an international research consortium that aims at developing evidence-based diagnosis-treatment algorithms for persistent digestive disorders and other common clinical syndromes in resource-constrained settings of Africa and Asia.

Methods: A systematic review was performed to elucidate the aetiological spectrum of persistent digestive disorders. A study protocol, accompanied by a set of more than 30 standard operating procedures (SOPs), was developed to conduct a multi-country, prospective case-control study to investigate persistent diarrhoea (≥14 days; all individuals aged above 1 year) and persistent abdominal pain (≥14 days; all children and adolescents aged 1-18 years) in Côte d'Ivoire, Indonesia, Mali and Nepal. In the framework of a specific site assessment, a case-control study was performed in Dabou, south Côte d'Ivoire to determine the aetiology and clinical features of persistent diarrhoea. Stool samples were subjected to a host of microscopic techniques, RDTs for *Clostridium difficile*, *Cryptosporidium* spp. and *Giardia intestinalis*, as well as the Luminex® Gastrointestinal Pathogen Panel, a stool-based multiplex PCR. A subsequent study was conducted to assess the diagnostic accuracy of real-time PCR for detection of *Strongyloides stercoralis* and to compare it to a combination of microscopic methods (Baermann funnel concentration and Koga agar plate). For the first time, a previously validated, urine-based RDT for the diagnosis of *Schistosoma mansoni* was employed for individual management of patients presenting with digestive disorders to a hospital in Europe.

Results: The systematic review identified more than 40 bacterial, parasitic (helminths and intestinal protozoa) and viral pathogens that may potentially cause persistent diarrhoea and persistent abdominal

pain. In a subsequent case-control study in southern Côte d'Ivoire, 20 different intestinal pathogens were detected and >50% of all participants had co-infections. Enterotoxigenic *Escherichia coli* (32%) and *Shigella* spp. (20%) were the most prevalent bacterial pathogens, while *G. intestinalis* (29%) and *S. stercoralis* (10%) were the predominant intestinal protozoon and helminth species, respectively. With regard to infection status, there were few differences between cases and controls. Most patients with persistent diarrhoea lived in rural areas, but clinical signs and symptoms could not distinguish between specific infections. The protocol for the multi-country NIDIAG study on persistent digestive disorders adopted a case-control approach and regular follow-up visits of symptomatic patients to monitor the clinical response to treatment.

A diagnostic study in south-central Côte d'Ivoire found that the application of a stool-based real-time PCR for *S. stercoralis* substantially improved the detection rate of this pathogen, leading to a total prevalence of 21.9%, compared to a prevalence of 10.9% according to stool microscopy. *C. difficile* could also be detected in stool samples from Côte d'Ivoire (5.4% prevalence according to RDT). Non-toxigenic *C. difficile* strains predominated and their molecular characteristics differed considerably from those observed in other settings. Prolonged storage without properly maintained cold chain only minimally affected the subsequent recovery of *C. difficile* and its toxins in stool culture.

A point-of-care (POC) test detecting a circulating cathodic antigen (CCA) in urine was successfully utilised to confirm intestinal *S. mansoni* infection in migrants from Eritrea who presented to a European hospital because of persistent abdominal pain.

Conclusions: Persistent digestive disorders are of considerable public health importance in Côte d'Ivoire and elsewhere, with the majority of cases being detected in rural areas. Many different causative agents may give rise to this syndrome and they can be accurately detected by the application of highly sensitive diagnostic techniques. The diversity of the potentially implicated pathogens underscores the need for a syndromic approach to persistent digestive disorders. RDTs are helpful tools for the detection of specific pathogens and may be implemented as part of diagnostic algorithms in endemic areas and in hospitals providing care for migrants and returning travellers. There is an urgent need to develop a stool-based RDT for *S. stercoralis*. The high asymptomatic carriage rates of intestinal pathogens call for the inclusion of healthy controls in epidemiological studies to define the specific contribution of each pathogen to the syndrome of persistent digestive disorders. Future studies employing metagenomic approaches will provide further insights into the intestinal microbiome of symptomatic patients and healthy controls.

Zusammenfassung

Hintergrund: Gastrointestinale Infektionen gehören zu den wichtigsten Infektionskrankheiten weltweit. Im Gegensatz zur akuten Diarrhoe können länger anhaltende Erkrankungen des Gastrointestinaltrakts als persistierende Diarrhoe (≥14 Tage Dauer) und/oder persistierende abdominelle Schmerzen (≥14 Tage Dauer) definiert werden. Dieses klinische Syndrom wird häufig durch Infektionserreger bedingt, aber die medizinische Bedeutung desselben in den Tropen sowie das Spektrum an verursachenden Pathogenen sind unvollständig verstanden. Die am häufigsten eingesetzten diagnostischen Verfahren zum Nachweis intestinaler Pathogene sind wenig sensitiv und es gibt nur wenige Evidenz-basierte Empfehlungen zum klinischen Management von persistierenden Erkrankungen des Intestinaltrakts. Neue Antigen-Schnelltests wurden entwickelt, die auch außerhalb von diagnostischen Laboratorien eingesetzt werden können. Darüber hinaus stehen auch neue molekulardiagnostische Verfahren (z.B. Multiplex Polymerase-Ketten-Reaktion (PCR)) zum sehr sensitiven Nachweis von Durchfallerregern zur Verfügung, aber diese Testverfahren sind bisher kaum in den Tropen eingesetzt und evaluiert worden. Dieses Dissertationsprojekt ist Teil des NIDIAG-Forschungskonsortiums, einem Zusammenschluss verschiedener Forschungsinstitutionen, welche das Ziel verfolgen, Evidenz-basierte Algorithmen für eine bessere Diagnostik und Therapie von persistierenden Durchfallerkrankungen und persistierenden abdominellen Schmerzen in ressourcenschwachen Regionen der Tropen zu entwickeln.

Methoden: Eine systematische Literaturrecherche wurde durchgeführt, um eine Übersicht über das Spektrum an infektiösen Erregern, welche persistierende Erkrankungen des Intestinaltrakts verursachen können, sowie die geeigneten diagnostischen Methoden zum Nachweis dieser Pathogene zu erarbeiten. Es wurde ein detailliertes Studienprotokoll für eine multizentrische, prospektive Fall-Kontroll-Studie zur Erforschung der persistierenden Diarrhoe und persistierender abdomineller Schmerzen in der Elfenbeinküste, Indonesien, Mali und Nepal erstellt. Im Rahmen einer ersten Untersuchung wurde das Auftreten von persistierender Diarrhoe in der Stadt Dabou im Süden der Elfenbeinküste untersucht. Menschliche Stuhlproben wurden dabei mit einer Vielzahl von konventionellen und molekularbiologischen diagnostischen Tests untersucht. Außerdem wurden Antigen-Schnelltests zum Nachweis von *Clostridium difficile*, *Cryptosporidium* spp. und *Giardia intestinalis* durchgeführt. In einer weiteren Studie wurden unterschiedliche Labormethoden hinsichtlich ihrer Sensitivität und diagnostischen Aussagekraft bei Infektionen mit dem Nematoden *Strongyloides stercoralis* verglichen. Schließlich wurde ein weiterer Antigen-Schnelltest, welcher den Saugwurm *Schistosoma mansoni* detektiert, zur Versorgung eritreischer Patienten mit persistierenden Bauchschmerzen in einem europäischen Krankenhaus evaluiert.

Ergebnisse: Die systematische Literaturrecherche ergab, dass mehr als 40 bakterielle, parasitäre (Helminthen und intestinale Protozoen) und virale Pathogene persistierende Erkrankungen des

Verdauungstraktes hervorrufen können. In der nachfolgenden Fall-Kontroll-Studie in Dabou wurden 20 unterschiedliche Pathogene detektiert, wobei mehr als >50% aller Teilnehmer mehrere Coinfektionen aufwiesen. Die am häufigsten detektierten Pathogene waren Enterotoxin-bildende *Escherichia coli* (32%), *G. intestinalis* (29%), *Shigella* spp. (20%) und *S. stercoralis* (10%). Sowohl hinsichtlich der Prävalenzen einzelner Pathogene als auch bezüglich der Rate der detektierten Coinfektionen gab es kaum Unterschiede zwischen symptomatischen Patienten und asymptomatischen Kontrollen. Die meisten Patienten mit persistierender Diarrhoe lebten in ländlichen Gegenden und die klinischen Symptome waren wenig spezifisch. Basierend auf den Daten dieser Vorstudie wurden sowohl asymptomatische Kontrollpersonen als auch standardisierte Verlaufsbeobachtungen von behandelten Patienten mit persistierender Diarrhoe in das Studienprotokoll der multizentrischen NIDIAG-Studie aufgenommen.

Eine weitere Diagnostikstudie in der Elfenbeinküste ergab, dass die Anwendung eines Stuhl-basierten PCR-Verfahrens zum Nachweis von *S. stercoralis* deutlich sensitiver ist als die alleinige Durchführung mikroskopischer Verfahren (Prävalenz 21.9% *vs.* 10.9%). Ein Schnelltest für *C. difficile* ergab außerdem ein positives Ergebnis in 5.4% der untersuchten Proben. Weitere Analysen zeigten, dass die gefundenen Stämme von *C. difficile* keine Toxine bildeten und deutlich andere Ribotyp-Muster als in anderen Regionen der Welt aufwiesen. Eine lange Stuhlproben-Transportzeit unter nicht standardisierten Bedingungen hatte keinen Einfluss auf die Sensitivität später durchgeführter bakteriologischer Stuhlkulturen zum Nachweis von *C. difficile*.

Schließlich wurde auch ein Urin-basierter Schnelltest, welcher ein zirkulierendes kathodisches Antigen von *Schistosoma mansoni* nachweist, erstmals in Europa bei Patienten aus Eritrea erfolgreich zum Nachweis einer intestinalen Schistosomiasis eingesetzt.

Schlussfolgerungen: Persistierende Durchfallerkrankungen und persistierende Bauchschmerzen sind relevante klinische Probleme in der Elfenbeinküste und anderswo in tropischen Regionen. Eine Vielzahl von Pathogenen kann diese Erkrankungen, welche besonders Menschen in armen, ländlichen Gebieten betreffen, hervorrufen. Der Einsatz hochsensitiver molekularbiologischer Diagnostikverfahren kann sehr viele dieser Krankheitserreger nachweisen. Antigen-Schnelltests können auch außerhalb von gut ausgerüsteten diagnostischen Laboratorien eingesetzt werden und sollten daher nach Möglichkeit bei der Entwicklung von klinisch-diagnostischen Algorithmen zum Management dieses Syndroms mit einbezogen werden. Der baldigen Entwicklung eines Schnelltest-Nachweisverfahrens für *S. stercoralis* sollte eine hohe Priorität zukommen. Aufgrund des sehr häufigen Nachweises von Pathogenen auch bei gesunden Individuen sollten epidemiologische Studien auch asymptomatische Kontrollpersonen untersuchen. Der akkuraten Beschreibung des intestinalen Mikrobioms wird in der Zukunft eine große Rolle zum besseren Verständnis von intestinalen Infektionen zukommen.

Résumé

Contexte: Les infections gastro-intestinales sont parmi les principales causes de morbidité dans le monde entier. Contrairement à la diarrhée aiguë, les troubles digestifs persistants sont définis comme une diarrhée persistante (≥14 jours) et/ou une douleur abdominale persistante (≥14 jours). Ce syndrome clinique est souvent causé par des infections intestinales, mais son importance médicale dans les régions tropicales, la gamme d'agents pathogènes étiologiques et la contribution des maladies tropicales négligées ne sont pas encore bien compris. Les techniques diagnostiques les plus souvent utilisées pour la détection de pathogènes intestinaux manquent souvent de sensibilité, et il n'y a actuellement que peu de recommandations factuelles pour guider la gestion clinique des troubles digestifs persistants dans les milieux aux ressources limitées. Des Tests de Diagnostic Rapides (TDR) ont été développés pour le diagnostic de certains agents pathogènes intestinaux et ils peuvent également être utilisés dans les centres de santé périphériques sans accès à l'infrastructure d'un laboratoire. Plus récemment, des tests utilisant la réaction en chaîne par polymérase (PCR) pour la détection des agents pathogènes gastro-intestinaux ont été développés, mais ces tests doivent encore être évalués systématiquement dans les zones tropicales. Cette thèse de doctorat a été réalisée dans le cadre du projet NIDIAG, un consortium de chercheurs internationaux qui vise à développer des algorithmes fondées sur des preuves de diagnostic et de traitement pour les troubles digestifs persistants et d'autres syndromes cliniques communs dans les milieux aux ressources limitées de l'Afrique et de l'Asie.

Méthodes: Une recherche systématique a été effectuée pour élucider le spectre étiologique des troubles digestifs persistants. Un protocole d'étude, accompagné d'une trentaine de Procédures Opératoires Standardisées (SOP), a été développé pour effectuer une étude prospective cas-témoins pour évaluer la diarrhée persistante (≥14 jours; tous les individus âgés de plus de 1 an) et la douleur abdominale persistante (≥14 jours; tous les enfants et adolescents âgés de 1-18 ans) en Côte d'Ivoire, en Indonésie, au Mali et au Népal. Dans le cadre de l'évaluation spécifique du site, une étude cas-témoins a été réalisée à Dabou, au sud de la Côte d'Ivoire afin de déterminer l'étiologie et les caractéristiques cliniques de la diarrhée persistante. Des échantillons de selles ont été soumis à une série de techniques microscopiques, TDR pour *Clostridium difficile*, *Cryptosporidium* spp. et *Giardia intestinalis*, ainsi que le «Gastrointestinal Pathogen Panel» de Luminex®, une PCR multiplex. Une autre

étude a été menée pour évaluer la précision diagnostique de la PCR en temps réel pour la détection de *Strongyloides stercoralis* et de la comparer à une combinaison de méthodes microscopiques (technique de Baermann et la plaque de gélose de Koga). Pour la première fois, un TDR utilisant l'urine pour le diagnostic de *Schistosoma mansoni* a été utilisée pour la gestion individuelle des patients présentant des troubles digestifs dans un hôpital européen.

Résultats: Cette recherche systématique a permis d'identifier plus de 40 bactéries, parasites (helminthes et protozoaires intestinaux) et virus qui peuvent potentiellement causer une diarrhée persistante et une douleur abdominale persistante. Dans une étude cas-témoins au sud de la Côte d'Ivoire, 20 agents pathogènes intestinaux différents ont été détectés et >50% des participants ont présenté des co-infections. *Escherichia coli* entérotoxigènes (32%) et *Shigella* spp. (20%) étaient les agents pathogènes bactériens les plus prévalents, et *G. intestinalis* (29%) et *S. stercoralis* (10%) étaient les espèces des protozoaires intestinaux et d'helminthes prédominantes, respectivement. Concernant le statut de l'infection, peu de différences ont été trouvées entre les cas et les témoins. La plupart des patients atteints de diarrhée persistante vivaient dans les zones rurales, cependant les signes et symptômes cliniques ne permettaient pas de faire la distinction entre les infections spécifiques. Le protocole de l'étude multicentrique sur les troubles digestifs persistants du projet NIDIAG a adopté une approche cas-témoins ainsi que des visites de suivi régulières des patients symptomatiques pour surveiller leur réponse clinique au traitement.

Une étude de diagnostic dans le centre-sud de la Côte d'Ivoire a montré que l'application d'une PCR à temps réel pour *S. stercoralis* permettait de détecter ce pathogène de manière beaucoup plus sensible, conduisant à une prévalence totale de 21.9%, en comparaison à une prévalence de 10.9% avec l'examen microscopique des selles. *C. difficile* pouvait également être détecté dans les échantillons de selles provenant de Côte d'Ivoire (5.4% de prévalence en fonction du TDR). Les souches non-toxigènes de *C. difficile* prédominaient et leurs caractéristiques moléculaires différaient considérablement de celles observées dans d'autres régions. Un stockage prolongé en l'absence d'une chaîne du froid appropriée n'affectait que très peu la récupération ultérieure de *C. difficile* et de ses toxines dans la culture des selles.

Un point-of-care (POC), soit un test de détection de l'antigène cathodique circulant (CCA) dans l'urine, a été utilisé avec succès pour confirmer l'infection intestinale à *S. mansoni* chez des migrants érythréens qui se sont présentés à un hôpital européen en raison de douleurs abdominales persistantes.

Conclusions: Les troubles digestifs persistants sont d'une importance considérable pour la santé publique en Côte d'Ivoire et ailleurs, avec une majorité de cas détectée en zones rurales. De nombreux agents pathogènes distincts peuvent donner lieu à ce syndrome et ils peuvent être détectés avec précision par l'application de techniques de diagnostic très sensibles. La diversité des agents pathogènes potentiellement impliqués souligne la nécessité d'une approche syndromique des troubles digestifs persistants. Les TDR sont des outils utiles pour la détection d'agents pathogènes spécifiques et peuvent être mis en œuvre dans le cadre d'algorithmes de diagnostic dans les zones endémiques et dans les hôpitaux offrant des soins pour les migrants et les voyageurs de retour. Il y a un besoin urgent de développer un TDR basé sur les échantillons de selles pour la détection de *S. stercoralis*. Du fait des taux élevés de porteurs de pathogènes intestinaux asymptomatiques, il est nécessaire d'inclure des témoins en bonne santé dans les études épidémiologiques pour définir la contribution spécifique de chaque agent pathogène au syndrome des troubles digestifs persistants. Les futures études utilisant des approches de métagénomique fourniront de nouvelles informations sur le microbiome intestinal des patients symptomatiques et des témoins asymptomatiques.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
BM	Baermann funnel technique
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CDAD	*Clostridium difficile*-associated diarrhoea
CDI	*C. difficile* infection
CMV	Cytomegalovirus
CRF	Case report form
CSRS	Centre Suisse de Recherches Scientifiques en Côte d'Ivoire
C_t	Cycle threshold
DAEC	Diffusely adherent *Escherichia coli*
DALY	Disability-adjusted life year
DNA	Deoxyribonucleic acid
EC	European Commission
EHEC	Enterohaemorrhagic *E. coli*
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *E. coli*
EKBB	Ethikkommission beider Basel
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FECT	Formalin-ether concentration technique
GBD	Global Burden of Disease
GCLP	Good Clinical Laboratory Practice
GCP	Good Clinical Practice
GDH	Glutamate dehydrogenase
GEMS	Global Enteric Multicenter Study
GPP	Gastrointestinal Pathogen Panel
HDSS	Health and demographic surveillance system
HIV	Human immunodeficiency virus
IMMH	Institute of Medical Microbiology and Hygiene
INRSP	Institut National de Recherche en Santé Publique

ISCCM	Indian Society of Critical Care Medicine
ITM	Institute of Tropical Medicine
KAP	Koga agar plate
LR	Likelihood ratio
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
M.D.	Doctorate of Medicine
MDA	Mass drug administration
MDG	Millennium Development Goal
NHRC	Nepal Health Research Council
NIDIAG	Acronym for 'Better **DIAG**nosis of **N**eglected **I**nfectious diseases'
NPV	Negative predictive value
NTD	Neglected tropical disease
OR	Odds ratio
PCR	Polymerase chain reaction
Ph.D.	Doctorate of Philosophy
PI	Principal Investigator
POC	Point-of-care
QA	Quality assurance
QALY	Quality-adjusted life year
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
RT	Ribotype
RT-PCR	Reverse transcriptase polymerase chain reaction
SOP	Standard operating procedure
STD	Sexually transmitted disease
STEC	Shiga-toxin producing *E. coli*
Swiss TPH	Swiss Tropical and Public Health Institute
TMG	Trial Management Group
WASH	Water, sanitation and hygiene
WHO	World Health Organization

1 Introduction

1.1 Global burden of disease and the role of infectious disorders

A comprehensive assessment of the prevalence, incidence and distribution of major diseases at a global scale is essential to quantify the state of health and well-being of entire populations and to identify major health problems. Initiated by the World Health Organization (WHO) and the World Bank, the Global Burden of Disease (GBD) study was designed to provide a standardised approach for the assessment of health worldwide [1]. In the 1990s, the first GBD study summarised the global state of health and provided – for the first time – data on the attribution of specific diseases and injuries to the global disease burden [2]. The introduction of the 'disability-adjusted life year' (DALY) metric was an essential development to consider not only deaths due to specific diseases, but also the associated disability that is linked to clinical conditions. DALY is defined as "the sum of life years lost due to premature mortality and years lived with disability adjusted for severity" [3]. The first GBD study identified that 43.9% of all DALYs worldwide could be attributed to communicable, maternal, perinatal and nutritional disorders, with lower respiratory infections, diarrhoeal diseases, tuberculosis and measles ranking among the most important specific causes. If considering different regions of the world, sub-Saharan Africa was found to suffer from the highest burden of infectious diseases. Indeed, infectious disorders accounted for a considerable amount of deaths and for 53% of the total DALY burden in this region [4].

A comprehensive update on the global state of health and disease was published in late 2012, when results from the GBD 2010 study were presented. A series of significant changes were observed between 1990 and 2010. While the global burden of DALYs decreased only slightly by 0.5% to a total of 2,490,385,000 DALYs, the burden due to communicable diseases could be reduced by 26.5% [5]. Major contributors to such considerable improvement were the reduction of the disease burden due to diarrhoeal diseases (–51.2%), lower respiratory infections (–44.2%), meningitis (–22.3%) and tuberculosis (–19.4%). In 2010, ischaemic heart disease replaced lower respiratory infections as the leading cause of disease burden at a global scale, but lower respiratory infections and diarrhoeal diseases still ranked second and fourth on the list of the most important causes of DALYs worldwide. Despite the global trend

to improved health reported in the GBD study 2010, the authors acknowledged enormous epidemiological heterogeneity in different geographical, social and ecological settings. If the total global burden was stratified by geographical regions, it became apparent that Eastern sub-Saharan Africa was the only African area in which a slight decrease in terms of DALYs was observed between 1990 and 2010 (–1.3%), while the total disease burden expressed in DALYs increased between 19.0% and 85.0% in Western, Central and Southern sub-Saharan Africa. Additionally, while communicable, maternal, neonatal and nutritional disorders accounted for 67-71% of the total disease burden in sub-Saharan Africa, these diseases contributed less than 10% to the disease burden of North America and Western Europe [5].

In August 2015, an analysis of the GBD study 2013 was published to provide the most recent update on the global disease burden [6]. A clear overall trend towards an improved global health was confirmed, but the global DALY burden decreased only minimally by 3.6% despite major health improvements, which can mainly be explained by a rise in life expectancy and population growth in some parts of the world. Indeed, if stratified by age, the global DALYs dropped by 27% between 1990 and 2013. The authors reported an ongoing 'epidemiological transition', i.e. an increase in DALYs due to non-communicable diseases and a further decline in many communicable diseases. Yet, the researchers acknowledged that the achieved decline, e.g. pertaining to lower respiratory infections, was not sufficient "for these conditions to be replaced as the leading causes of disease burden worldwide" [7]. Indeed, HIV/AIDS, malaria, respiratory infections, diarrhoeal diseases and tuberculosis continue to constitute the principal specific causes of disease and suffering in most countries of sub-Saharan Africa and in parts of Asia (Table 1.1). Of note, the GBD 2013 study reported also a significant increase in the burden due to a number of tropical and poverty-associated infections such as dengue fever, food-borne trematodiasis and leishmaniasis.

When analysing the global causes of child deaths in the GBD 2013 study, communicable, neonatal and nutritional deficiencies accounted for three-quarters of all deaths, even though the total number of premature child deaths could be reduced by approximately 50% since 1990 [6]. Diarrhoeal diseases, meningitis and lower respiratory infections were responsible for the majority of deaths. Eastern, central and western sub-Saharan Africa had substantially higher child mortality than all other regions of the world. The aetiological cause of diarrhoeal diseases remained unclear in at least 55.6% of all cases, which can be explained by the lack of epidemiological data and the insufficient sensitivity of many laboratory diagnostic tests.

Rotavirus played a major role in young children, but bacterial pathogens such as *Shigella* spp. and *Vibrio cholerae* and the intestinal protozoa *Cryptosporidium* spp. were also important pathogens. The bacterium *Clostridium difficile* was of considerable importance outside low-income countries and accounted for up to 95% of all diarrhoea-associated deaths in elderly people in high-income countries, particularly in North America and Western Europe [6].

Table 1.1 The three most important causes of disease (assessed by attribution of DALYs to each of the conditions) in 15 selected countries in 2013 (adapted from [7]).

	#1 cause of disease	#2 cause of disease	#3 cause of disease
Australasia			
Australia	Low back and neck pain	Ischaemic heart disease	Depression
North America			
United States of America	Ischaemic heart disease	Low back and neck pain	Chronic obstructive pulmonary disease
Western Europe			
Germany	Low back and neck pain	Ischaemic heart disease	Stroke
Italy	Low back and neck pain	Ischaemic heart disease	Alzheimer's disease
Switzerland	Low back and neck pain	Ischaemic heart disease	Falls
Latin America			
Brazil	Ischaemic heart disease	Low back and neck pain	Violence
Peru	Lower respiratory infections	Low back and neck pain	Ischaemic heart disease
Middle East			
Syria	War	Ischaemic heart disease	Stroke
South and Southeast Asia			
China	Stroke	Low back and neck pain	Ischaemic heart disease
Indonesia	Stroke	Ischaemic heart disease	Tuberculosis
Nepal	Lower respiratory infections	Ischaemic heart disease	Low back and neck pain
Sub-Saharan Africa			
Côte d'Ivoire	Lower respiratory infections	HIV/AIDS	Malaria
D.R. Congo	Diarrhoeal diseases	Lower respiratory infections	Malaria
Mali	Malaria	Diarrhoeal diseases	Lower respiratory infections
South Africa	HIV/AIDS	Tuberculosis	Lower respiratory infections

1.2 Neglected tropical diseases

In many resource-constrained countries in tropical and subtropical areas, health systems are weak and the few available resources have to be allocated to the most urgent health problems. In terms of infectious diseases affecting populations living in the tropics and subtropics, HIV/AIDS, malaria and tuberculosis constitute the 'big three' to which most attention and funding is typically addressed [8]. In September 2000, the United Nations General Assembly adopted the Millennium Declaration, in which eight Millennium Development Goals (MDGs) were specified with the overarching aim to reduce the massive burden due to poverty, diseases, weak health systems and hunger by 2015 [9]. As a direct result, funding and research on many infectious diseases, particularly the 'big three', increased considerably. However, an increasing number of researchers acknowledged that a host of infectious diseases continued to cause enormous morbidity in tropical and subtropical areas, but their health impact remained hardly ever assessed and quantified, and little funding was available to tackle these diseases. In several manuscripts published in 2005-2007, the term 'neglected tropical diseases' (NTDs) was used to summarise this group of infectious diseases [10,11].

The original list of NTDs comprised 13 major infectious diseases of bacterial, helminthic and protozoal origin [12]. Common key characteristics of these diseases are their mainly chronic character as well as their intimate connection to poverty and populations living in the most remote settings of low-income countries in the tropics and subtropics [13]. Many NTDs are clustered in similar geographical areas, thus leading to a substantial amount of co-infections with multiple pathogens in areas of endemicity [14]. Poor sanitation and hygiene, lack of adequate access to water and resources and a lack of health education are typical risk factors for acquisition of NTDs [8,15]. Children are considered to be most heavily affected by many NTDs and often suffer most from these debilitating diseases. While the long-term morbidity due to the mainly chronic NTDs is considerable, acute severe disease manifestations and subsequent deaths are rarely encountered. Hence, quantification of the disease burden due to NTDs is extremely challenging and relies on morbidity indicators such as DALYs or the more recently introduced qualiy-adjusted life years (QALYs) [16]. However, the lack of reliable epidemiological data on the occurrence of many NTDs, the poorly understood clinical symptomatology and the high rates of co-infections with multiple pathogens render pathogen- or disease-specific estimates of attributable DALYs a difficult task, e.g. in the case of intestinal parasitic infections [17]. Many helminths and intestinal protozoa may give rise to

abdominal symptoms such as pain, diarrhoea, abdominal tenderness or blood in the stool, yet few studies in endemic countries have generated epidemiological data on the role and attributable fraction of NTDs to this syndrome [18]. Additionally, some NTDs lead to enormous, frequently stigmatising long-term morbidity that persists for long time periods even after the infection is cleared. One prominent example is lymphatic filariasis, an infection that is mainly caused by the nematode *Wuchereria bancrofti*, and that is one of the leading causes of lymphoedema and hydrocoele in sub-Saharan Africa [19-21]. According to a recent analysis of the GBD 2010 study, it was estimated that the individual DALY estimates of the various NTDs may, if taken together, amount to 48 million DALYs, which is in the same range as tuberculosis (49 million DALYs in the GBD 2010) [22].

The original list of NTDs has expanded considerably over the past decade, and more recent reviews classify more than 40 diseases as NTDs [8]. Table 1.2 provides a comprehensive overview about the major diseases and the respective aetiological agents. A majority of the NTDs primarily causes gastrointestinal disorders and, hence, digestive symptomatologies are commonly seen in infected individuals. Helminths represent the largest group within the NTDs; while intestinal schistosomiasis [23,24], soil-transmitted helminthiasis [25], enterobiasis, food-borne trematodiasis [26,27] and others mainly affect the host's gastrointestinal tract, an important second group is being constituted by tissue-invading helminths with the potential to cause severe diseases, such as *Echinococcus granulosus* and *E. multilocularis*, the agents of cystic and alveolar echinococcosis, respectively, and *Taenia solium*, the cause of (neuro-)cysticercosis [28,29]. Further parasitic NTDs are caused by intestinal protozoa, which may also be categorised into intestinal protozoa (e.g. *Cryptosporidium* spp., *Entamoeba histolytica*) and tissue-invasive parasites (e.g. *Leishmania* spp.) [30-32]. Cholera, salmonellosis and shigellosis are the main bacterial NTDs that primarily lead to abdominal or intestinal symptoms. In contrast, there are also some viral (e.g. dengue virus), fungal (e.g. *Paracoccidioides brasiliensis*) and ectoparasitic pathogens (e.g. *Sarcoptes scabiei*) listed as specific causes of NTDs [33], but they are seldom associated with digestive symptoms and can usually be differentiated on clinical grounds from the pathogens that give rise to intestinal diseases.

Table 1.2 A comprehensive list of helminthic, protozoal, bacterial, fungal, viral and ectoparasitic neglected tropical diseases (adapted from [8]).

Neglected tropical disease	**Causative agent(s)**
Helminthic infections	
Cysticercosis/taeniasis	*Taenia solium*
	Taenia saginata
	Diphyllobothrium latum
Dracunculiasis	*Dracunculus medinensis*
Echinococcosis	*Echinococcus granulosus*
	Echinococcus multilocularis (less common: other *Echinococcus* spp.)
Enterobiasis	*Enterobius vermicularis*
Food-borne trematodiasis	
- Clonorchiasis	*Clonorchis sinensis*
- Fascioliasis	*Fasciola gigantica* and *F. hepatica*
- Intestinal fluke infections	*Echinostoma* spp., *Fasciolopsis buski*, *Metagonimus* spp. and *Heterophyidae*
- Opisthorchiasis	*Opisthorchis felineus*, *O. viverrini*
- Paragonimiasis	*Paragonimus* spp.
Loiasis	*Loa loa*
Lymphatic filariasis	*Wuchereria bancrofti*, *Brugia malayi* and *B. timori*
Mansonellosis	*Mansonella perstans*, *M. streptocerca* and *M. ozzardi*
Onchocerciasis	*Onchocerca volvulus*
Schistosomiasis	*Schistosoma guineensis*, *S. haematobium*, *S. intercalatum*, *S. japonicum*, *S. mansoni* and *S. mekongi*
Soil-transmitted helminthiasis	
- Ascariasis	*Ascaris lumbricoides*
- Hookworm infection	*Ancylostoma duodenale* and *Necator americanus*
- Strongyloidiasis	*Strongyloides stercoralis*
- Trichuriasis	*Trichuris trichiura*
Toxocariasis	*Toxocara canis* and *T. cati*
Trichinellosis	*Trichinella spiralis* and other *Trichinella* spp.
Protozoal infections	
Balantidiasis	*Balantidium coli*
Chagas disease	*Trypanosoma cruzi*
Human African trypanosomiasis	*Trypanosoma brucei gambiense* and *T. b. rhodesiense*
Intestinal protozoan infections	
- Amoebiasis	*Entamoeba histolytica*
- Cryptosporidiosis	*Cryptosporidium hominis* and *C. parvum*
- Giardiasis	*Giardia intestinalis* (syn.: *G. lamblia*)
Leishmaniasis	Visceral leishmaniasis: *Leishmania donovani*, *L. chagasi* and *L. infantum* (Muco-)cutaneous leishmaniasis: *L. major*, *L. tropica*, *L. braziliensis*, *L. mexicana* and other *Leishmania* spp.

Neglected tropical disease	**Causative agent(s)**
Bacterial infections	
Bartonellosis	*Bartonella henselae* and other *Bartonella* spp.
Bovine tuberculosis in humans	*Mycobacterium bovis*
Buruli ulcer	*Mycobacterium ulcerans*
Enteric bacterial infections	
- Cholera	*Vibrio cholerae*
- ETEC infection	Enterotoxigenic *Escherichia coli* (ETEC)
- Salmonellosis	Non-typhoidal salmonellosis: *Salmonella enterica* serovars *S.* Enteritidis, *S.*Typhimurium and other serovars Enteric fever: *S.enterica* serovars *S.* Typhi and *S.* Paratyphi
- Shigellosis	*Shigella dysenteriae, S. boydii, S. flexneri* and *S. sonnei*
Leprosy	*Mycobacterium leprae*
Leptospirosis	*Leptospira interrogans* group
Relapsing fever	*Borrelia recurrentis, Borrelia duttoni* and other *Borrelia* spp.
Trachoma	*Chlamydia trachomatis*
Treponematoses (bejel, pinta, syphilis, yaws)	*Treponema pallidum* (different subspecies)
Fungal infections	
Mycetoma ("Madura foot")	Various fungi (eumycetoma) and bacteria (actinomycetoma, botryomycosis)
Paracoccidioidomycosis	*Paracoccidioides brasiliensis*
Viral infections	
Dengue	Dengue fever virus (genus: *Flavivirus*)
Japanese encephalitis	Japanese encephalitis virus (genus: *Flavivirus*)
Yellow fever	Yellow fever virus (genus: *Flavivirus*)
Rabies	Rabies virus (genus: *Lyssavirus*)
Rift valley fever	Rift valley fever virus (genus: *Phlebovirus*)
Viral haemorrhagic fevers	Important *Arenaviridae*: Lassa virus, Chapare virus, Guanarito virus, Junín virus, Machupo virus and Sabiá virus *Bunyaviridae*: Crimean-Congo haemorrhagic fever virus (HFV), Hanta viruses including Puumala virus *Filoviridae*: Ebola virus and Marburg virus *Flaviviridae*: Omsk HFV and Kyasanur forest disease virus
Ectoparasitic infections	
Myiasis	Parasitic fly larvae (*Calliphoridae, Oestridae, Sarcophagidae* and others)
Scabies	*Sarcoptes scabiei*

The list of NTDs is rapidly evolving and it has recently been suggested to include also non-infectious diseases such as podoconiosis, a tropical lymphoedema of the lower limb that occurs in farmers who are walking barefoot on soil of volcanic origin [34], and venomous animal contacts (e.g. snake bites) [35]. Additionally, recent research has brought to light that

well-known pathogens such as the bacterium *Staphylococcus aureus* may present in clinically different, often more severe disease manifestations in resource-constrained settings, and hence, should also be considered as neglected pathogens to which more concerted funding and research efforts should be directed [36,37].

1.3 Syndromic management of major health problems

The adequate clinical management of infectious diseases is often challenging, particularly in the absence of accurate laboratory diagnostic techniques and if similar signs and symptoms are caused by a variety of infectious agents. Indeed, many gastrointestinal infections show substantial overlap with regard to clinical findings. In such cases, it is important to evaluate the patient's condition not only from a 'single disease' perspective, but to consider a broad range of potential pathogens that may give rise to a given clinical syndrome. In clinical research, however, efforts frequently concentrate on one specific pathogen or disease, while relatively little attention is directed to syndromic approaches that take a number of potentially implicated causative agents into consideration. One notable exception is the approach to the management of sexually transmitted diseases (STDs) in resource-constrained settings [38]. Indeed, these infections were considered as a low priority in many resource-constrained settings until clinical evidence showed that effective STD management could considerably reduce the incidence of HIV acquisition [39]. However, it proved difficult to differentiate common STDs based alone on clinical findings, because signs and symptoms are often unspecific. Hence, well-equipped laboratories would be needed to perform diagnostic testing for all major implicated pathogens. However, this rather expensive approach is rarely feasible in rural areas of resource-constrained countries. Furthermore, mixed infections are common and some laboratory tests are characterised by insufficient sensitivity. Hence, a syndromic approach has been developed for effective STD management [40].

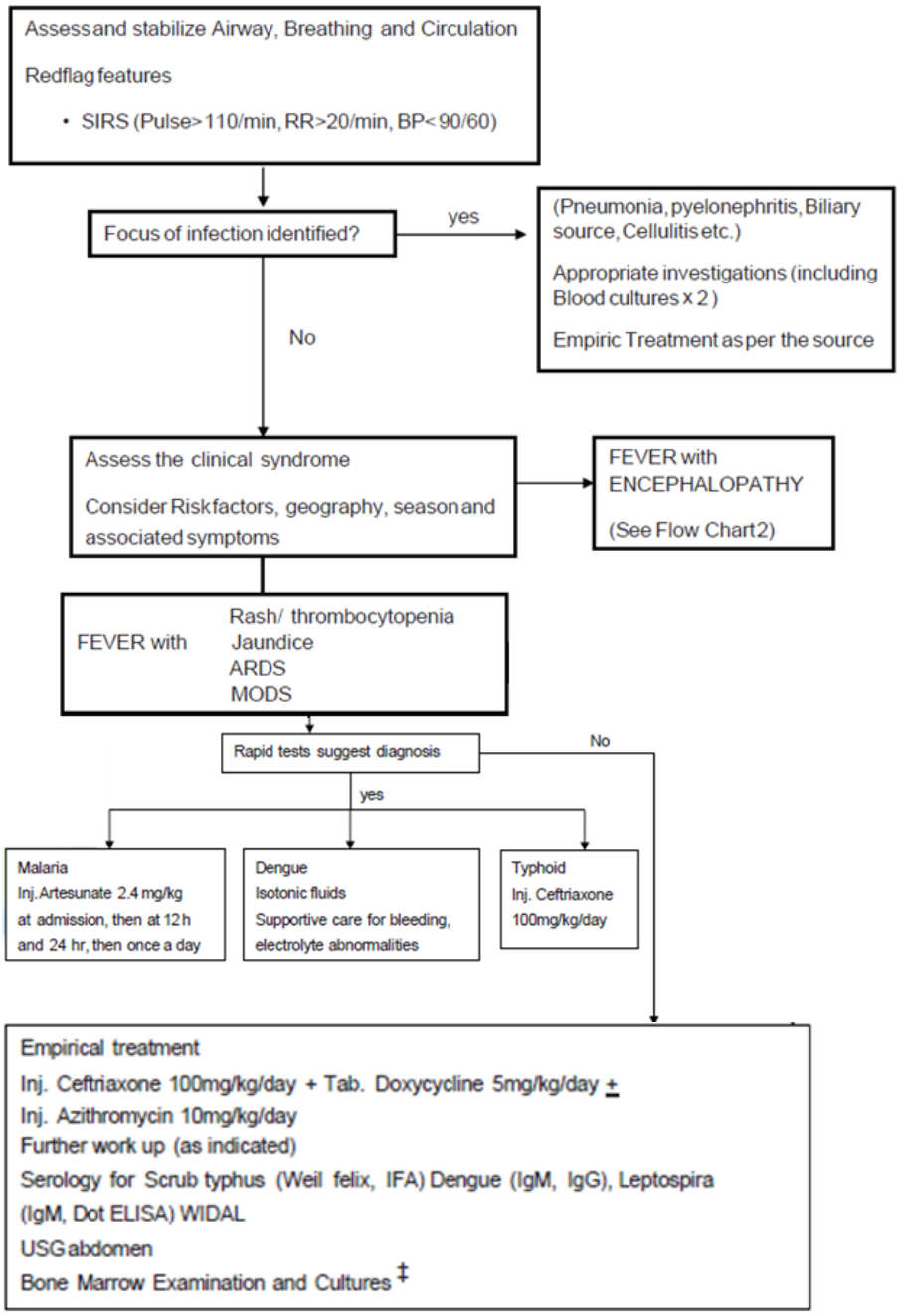
In a manuscript written by William K. Bosu in 1999, in which he argued to widely implement a syndromic STD management in resource-poor countries, the following definition was given: "Syndromic management implies an approach in which clinical algorithms such as decision trees for commonly presenting signs and symptoms (e.g. urethral discharge or genital ulcer) are used in case management. The symptoms selected are reasonably selected and easy to recognise. The algorithm provides treatment for the commonest biological causes of the syndrome. For example, for genital ulcer disease, treatment is provided concomitantly

for the two commonest causes, chancroid and syphilis, in the absence of laboratory support and in recognition of the limitations of clinical diagnosis" [41]. While the feasibility and relatively good accuracy of such a syndromic approach were confirmed in several studies [42-45], more recent research clearly expressed the urgent need to include at least some laboratory diagnostic testing parameters to establish aetiological diagnoses [46,47]. A recent study from Kenya, for example, showed that in a population in which laboratory diagnostic testing revealed a treatable STD in 32.2% of all participants, only 10.4% would have been identified by the syndromic, symptom-driven approach [48]. Hence, algorithms for syndromic management need to be continuously evaluated and adapted to the specific settings where they are employed.

The concept of syndromic approaches for an effective management of common health problems has been successfully applied to other clinical entities. In 2015, Dutch researchers analysed the travel history, clinical and diagnostic findings of 2,153 returning travellers with suspected arboviral disease [49]. Many arboviral diseases, such as dengue fever, chikungunya fever, West Nile fever and other infections may cause very similar clinical symptoms (e.g. a combination of fever, rash and arthralgia) and there is substantial geographical overlap between many arboviral infections. However, clinicians frequently request diagnostic testing for only one pathogen – typically dengue virus – despite compelling evidence that clinical judgement alone may be unable to differentiate between the different viruses. Indeed, the Dutch study revealed that diagnostic testing was only seldom requested and that only half of patients with a history suggestive of chikungunya fever were actually tested for this infection. The authors concluded that clinicians should adopt a combination of a clinical "syndromic approach and matching laboratory methods" [49].

A syndromic approach to manage common clinical problems in an effective way has gained particular interest in resource-limited areas. For some infections, rapid diagnostic tests (RDTs) are now available that can be employed even under harsh field conditions, e.g. in rural areas where no diagnostic laboratory is available [50-52]. The Indian Society of Critical Care Medicine (ISCCM), for example, has issued new guidelines for the management of tropical fevers in 2014. These guidelines include easily applicable diagnostic algorithms to aid clinical decision-making [53]. Figure 1.1 displays such an algorithm, in which it is recommended to perform RDTs for malaria, dengue fever and enteric fever (typhoid) and to administer specific treatment in case of a positive test result.

Figure 1.1 A proposed algorithm for the diagnosis and treatment of critical febrile infections in the tropics, issued by the Indian Society of Critical Care Medicine (published in [53]).



Acknowledging that many NTDs of bacterial, helminthic and protozoal origin primarily affect the human gastrointestinal tract in a chronic way and that specific treatment for the various pathogens varies considerably, it is surprising that no diagnostic algorithms for the management of long-lasting digestive symptomatologies have been developed thus far. From

a public health perspective, it may indeed be anticipated that the collective burden of diarrhoeal and other intestinal infections in resource-constrained tropical settings is considerable and the often unspecific clinical symptoms may render a syndromic approach much more appropriate than attempts that target a single pathogen. Hence, an international research consortium with the acronym NIDIAG ('Better **DIAG**nosis of **N**eglected **I**nfectious diseases') has been initiated to develop evidence-based diagnosis-treatment algorithms for an improved management of three clinical syndromes in resource-constrained settings of the tropics and subtropics (http://www.nidiag.org). These three clinical syndromes include: (i) persistent digestive disorders, defined as persistent diarrhoea (≥14 days; all individuals aged >1 year) and/or persistent abdominal pain (≥14 days; children and adolescents aged 1-18 years) [54]; (ii) neurological disorders [51]; and (iii) persistent fever [50,55]. The NIDIAG project is funded by the European Commission (EC). The current Ph.D. thesis is readily embedded in the NIDIAG consortium and aims at providing first data on the occurrence of persistent digestive disorders in resource-limited settings, associated clinical features and available diagnostic tests, which may contribute to the development of algorithms to improve the clinical management of long-lasting digestive symptomatologies in tropical and subtropical areas.

1.4 Epidemiology, clinical features, diagnosis and treatment of selected pathogens

1.4.1 *Clostridium difficile*

C. difficile is a Gram-positive, rod-shaped, anaerobic bacterium that is capable of forming endospores, which may persist for long times in the environment and are resistant to most disinfectants that are used in hospital settings [56,57]. *C. difficile* is the leading cause of nosocomial diarrhoea in high-income countries and has recently also been identified in a considerable number of patients with community-acquired diarrhoeal diseases [58]. Disease manifestations may vary from asymptomatic or mild diarrhoea to pseudomembranous colitis and potentially fatal complications, e.g. toxic megacolon [59]. According to the GBD 2013 study, *C. difficile* accounts for half of all diarrhoea-associated deaths in high-income countries and almost all deaths due to diarrhoea in elderly people [6]. While the importance of *C. difficile* infection (CDI) has been acknowledged in high-income countries and efforts

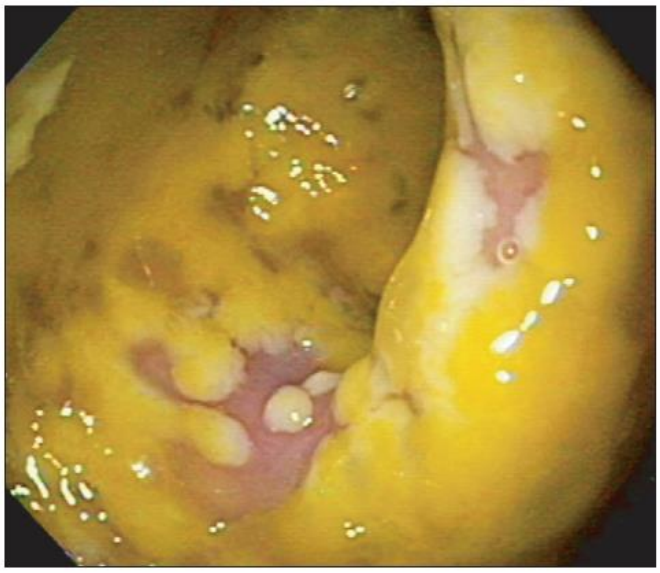
are underway to improve diagnosis and treatment, the epidemiology, burden and clinical features of this infection are poorly understood in most parts of the developing world. While some studies from large hospitals in Asia (e.g. in Shanghai) reported a low incidence of CDI [60,61], researchers from the Philippines identified *C. difficile* in 44% of patients who had been investigated for colitis [62]. In Latin America, recent data suggest that the burden due to *C. difficile* might also be considerable, and some epidemic strains are now widespread in various, geographically very diverse areas such as Costa Rica and Chile [63,64]. In contrast, almost no data are available on the occurrence of *C. difficile* and the circulating strains in sub-Saharan Africa.

C. difficile colonises the colon of humans and animals and can thus be found in the normal, healthy population. A recent study reported a carriage rate of 8% in newly admitted hospital patients in North America [65] and a meta-analysis revealed that 14.8% of all individuals living in long-term care facilities are temporarily colonised with *C. difficile* [66]. The bacterium may produce an enterotoxin (toxin A) and a cytotoxin (toxin B), which account for most of the morbidity. Strains can be differentiated by molecular methods (e.g. polymerase chain reaction (PCR) ribotyping) and some strains, such as the ribotype 027, are considered as 'hypervirulent' and may cause a more severe clinical course of disease [67]. In addition to toxins A and B, these hypervirulent strains produce a binary toxin and are characterised by typical mutations in the toxin repressor gene *tcdC*, which in turn lead to a hyperproduction of toxins A and B. There are also non-toxigenic *C. difficile* strains, but these are unable to cause disease. The amount of toxins that is produced by *C. difficile* after ingestion of either endospores or vegetative bacteria via the faecal-oral route is also influenced by the presence of physiological intestinal flora. It is a common adverse event of antibiotic treatment to disrupt this protective flora, and hence, previous antibiotic treatment is one of the most important risk factors for developing CDI [68]. Age >65 years, residency in a nursing home, medical gastric acid suppression (e.g. use of proton pump inhibitors) and immunosuppression are further risk factors [56].

Asymptomatic carriage of *C. difficile* (both toxigenic and non-toxigenic strains) is common, particularly in hospitalised patients [65]. Hence, no treatment should be initiated in the absence of clinical symptoms. If symptoms are present, these may range from watery diarrhoea, frequently accompanied by a foul odour, to severe diarrhoea with accompanying exsiccosis and pseudomembranous colitis (Figure 1.2) [61]. Some patients develop severe

and potentially life-threatening complications such as ileus, toxic megacolon, intestinal perforation and subsequent *C. difficile* bacteraemia. In these patients, a massively elevated leukocyte count and high fever may be observed and the diarrhoea may suddenly change to constipation. Rapid consultation with a visceral surgeon is important to consider life-saving surgical interventions in these patients [57].

Figure 1.2 Typical morphology of pseudomembranous colitis due to severe *C. difficile* infection, visualised during diagnostic colonoscopy (published in [61]).



The first step to establish the diagnosis of CDI in the laboratory is typically the application of a stool-based screening test, i.e. an RDT detecting a *C. difficile*-specific glutamate dehydrogenase (GDH). The test is highly sensitive and has a high negative predictive value (NPV), thus no further testing is required if the GDH RDT is negative. If the screening test is positive, this proves the presence of *C. difficile*, but a confirmation test is required to differentiate toxin-producing, pathogenic strains from non-toxigenic, apathogenic strains. Hence, either an enzyme immunoassay detecting toxins A and B or a PCR assay targeting the respective toxin genes is required to identify the toxigenic strains. The sensitivity of enzyme immunoassays for toxin detection is limited, and PCR should thus preferably be employed as confirmation test [56]. However, it is important to note that PCR is only able to detect the toxin genes, but cannot reliably predict whether these are actually transcribed and translated into toxins. Anaerobic toxigenic culture is considered the diagnostic 'gold' standard, but it is not routinely performed in most microbiology laboratories and takes at least 3 days to generate first results. In case of detection of toxigenic *C. difficile* isolates, further molecular

typing methods can be employed. PCR ribotyping is the most important method and it allows to identify strains of particular importance, e.g. the hypervirulent RT027, which is now widespread across many parts of Europe [69], and RT078, which might be transmissible between humans and animals as a zoonotic strain and is associated with community-acquired CDI [70]. An accurate diagnosis of CDI remains challenging, even in high-income countries. A recent European multi-country study estimated that approximately 40,000 hospitalised patients with symptomatic *C. difficile* infection remain untreated every year in Europe due to the use of insufficiently sensitive diagnostics [71].

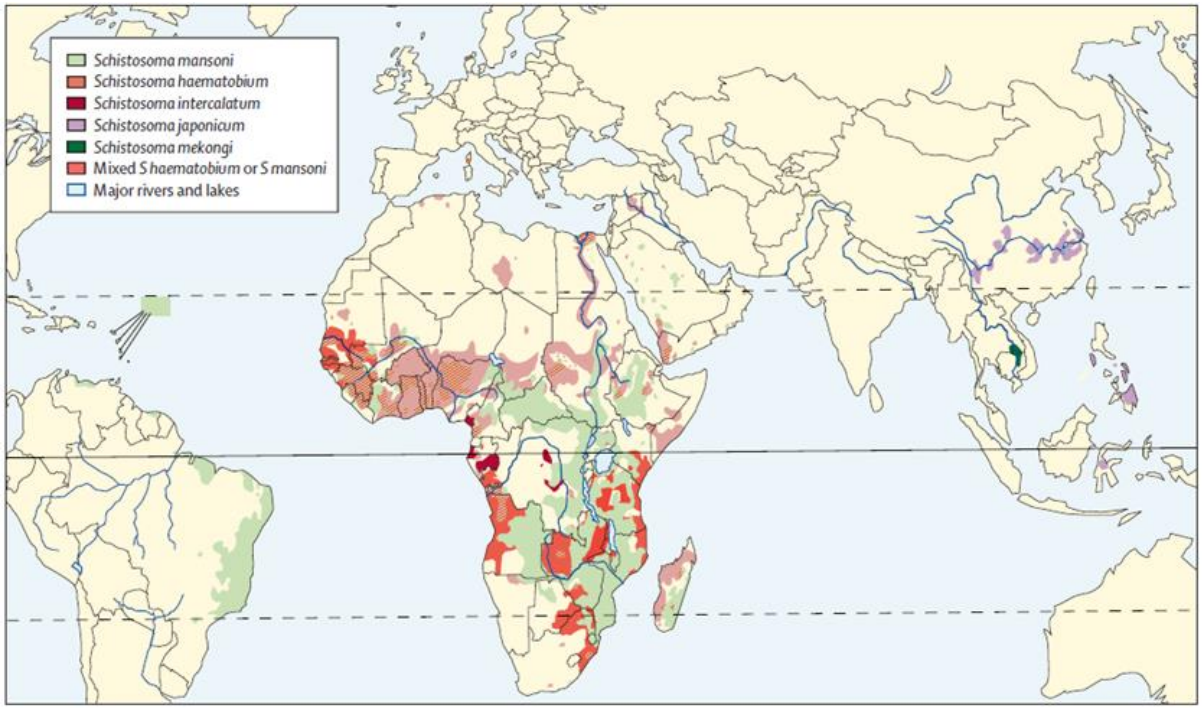
For mild *C. difficile* infections that occur during or shortly after antibiotic treatment, no specific treatment may be necessary and it can be sufficient to stop the antibiotic medication [57]. However, if a specific agent is required to treat CDI, oral metronidazole for 10 consecutive days is the preferred agent. In severe disease or for the treatment of the frequently occurring relapses, the glycopeptide vancomycin is superior to metronidazole. In case of multiple relapses and no improvement following treatment with both agents, fidaxomicin is the recommended treatment [72]. Some patients do not respond to antibiotic treatment or develop multiple relapses shortly after stopping the medication. In these patients, 'faecal microbiota transfer' or 'stool transplantation' (i.e. the transfer of the intestinal flora of a healthy donor to a symptomatic patient) has been reported to be highly efficacious. Indeed, there is growing evidence from several case series and randomised controlled trials reporting high cure rates associated with this treatment modality [73]. As clinical experience with this faecal microbiota transfer in daily clinical practice is still limited, though, it should currently only be performed in specialised centres [74].

1.4.2 *Schistosoma mansoni*

There are six different blood flukes (trematodes) of the genus *Schistosoma* that are able to cause human disease, i.e. *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *S. mekongi* and *S. guineensis* [23,75]. The latter three species are only of limited importance in some distinct foci of the world, while *S. japonicum* is of particular health relevance in the People's Republic of China and the Philippines [24]. Most of the disease burden worldwide is caused by the remaining two species, *S. haematobium* and *S. mansoni*. Both species are endemic across large parts of sub-Saharan Africa, North Africa, the Arabian peninsula and the Middle East [76]. *S. mansoni* also occurs in Brazil and some parts of Central America

(Figure 1.3). Recently, autochthonous cases of *S. haematobium* infection have been reported from Corsica and experts warn that schistosomiasis might potentially become also a European disease [77-79]. The GBD 2010 study estimated the schistosomiasis-associated disease burden to be as high as 3.3 million DALYs, with up to 250 million individuals infected. Hence, schistosomiasis is one of the most important NTDs. School-aged children and adolescents are considered at highest risk of infection and morbidity, but more recent research has also elucidated the substantial burden of schistosomiasis in infants and preschool-aged children (<5 years of age) as well as in adults [75,80-82].

Figure 1.3 The global distribution of schistosomiasis, stratified by infecting species (adapted from [24]).



Schistosomiasis is a water-based parasitic disease, which can be explained by the parasite's life cycle [23,24]. The adult female and male worms live in permanent pairs inside the human host, where they reside in the perivesical (*S. haematobium*) or portal and mesenteric blood vessels (*S. mansoni* and other species). The female worms produce up to several thousand eggs per day, which either get trapped in host tissue (e.g. liver and bladder), where they can induce considerable inflammation, or reach the bladder (*S. haematobium*) or intestine (*S. mansoni*), respectively, from where they are excreted with urine or faeces. If the eggs get

in contact with freshwater, the miracidium, a first stage larva, hatches from the eggs. For its further development into a larva that is able to infect humans, a second development step is required within specific freshwater snails that serve as intermediate hosts (e.g. *Biomphalaria* spp. in case of *S. mansoni*) [83]. Following asexual replication in the snail, so-called cercariae are released into the water, which are able to infect humans through the intact skin, e.g. during swimming or bathing activities in an infested lake. After the transcutaneous penetration, cercariae are transported hematogenously to the lungs and further onwards to the liver, where they develop into adult worms, before they migrate to their final destination where they start to produce eggs. This life cycle explains the very focal distribution of schistosomiasis, because freshwater bodies infested with suitable intermediate hosts are a necessity without which the helminth could not maintain its development cycle.

S. mansoni is the main aetiological agent of intestinal schistosomiasis, while *S. haematobium* causes genitourinary schistosomiasis. Some weeks after the first infection, an acute disease characterised by sudden headache, fever, myalgia and elevated peripheral eosinophilia, also termed as Katayama syndrome, may occur and reflects the systemic immune reaction to the infection. It is frequently seen in returning travellers, but is rare in populations living in endemic areas [75]. In general, *Schistosoma*-related morbidity is not caused by the adult worms, but rather by the produced eggs that frequently get trapped in host tissues [24]. In the case of *S. mansoni* infection, abdominal tenderness, diarrhoea, pain and abdominal discomfort and blood in the stool constitute common symptoms and clinical findings [84,85]. In some individuals, extensive periportal fibrosis may develop, with subsequent hepatosplenic disease, splenomegaly and portal hypertension [86]. Aberrant oviposition of *Schistosoma* eggs in other organs may lead to further complications, e.g. neuroschistosomiasis with subsequent paralysis if eggs are deposited into the central nervous system. It is now widely acknowledged that even individuals with light infection intensities and apparently ‘asymptomatic’ schistosomiasis suffer from long-term morbidity such as anaemia, impaired cognitive development and malnutrition [87].

The laboratory diagnosis of *S. mansoni* infection relies on the detection of helminth eggs in the faeces (or biopsies of affected tissues) [76]. Several microscopic techniques are available, such as direct faecal smear, Kato-Katz technique, FLOTAC, Mini-FLOTAC and the formalin-ether concentration technique [88]. Following WHO recommendations, the Kato-Katz technique is widely used in endemic settings [89]. However, infections of light intensity

(i.e. with few eggs shed per gram of stool in the faeces) are easily missed by all microscopic methods. More recently, a point-of-care (POC) RDT has been developed; it takes advantage of the fact that schistosomes excrete a circulating cathodic antigen (CCA) which can be detected in the serum and urine of an infected host. A urine-based POC-CCA test is now commercially available and showed a higher sensitivity than microscopy using the Kato-Katz technique in several African countries [90]. However, it remains to be elucidated how this test performs in a clinical setting and whether it can be used for individual patient management. PCR techniques have also been developed and show a much higher diagnostic accuracy than standard microscopy [91]. While molecular PCR techniques are increasingly being used in epidemiological studies and European reference laboratories, they have not yet found their way into daily clinical practice and it is unlikely that this will happen in the near future in those resource-constrained settings where the burden of schistosomiasis is highest. Serology is a helpful tool in returning travellers and can be used in epidemiological studies, e.g. to monitor populations in areas where schistosomiasis elimination is about to be achieved [76].

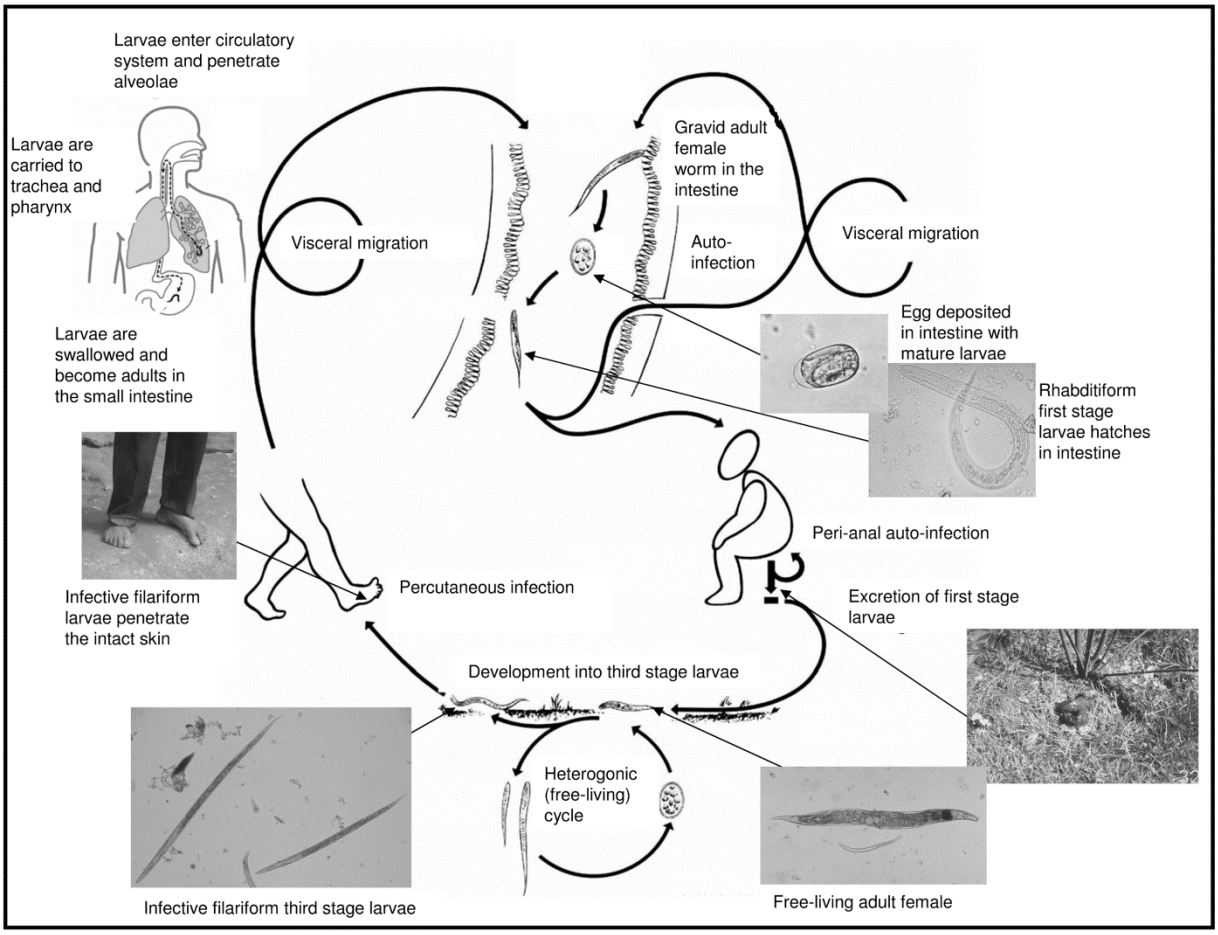
Once the diagnosis of schistosomiasis is established, oral treatment with praziquantel is highly efficacious and generally well-tolerated [92]. Besides its use for individual patient management, praziquantel is also used for 'preventive chemotherapy', i.e. a control programme recommended by WHO that consists of annual or bi-annual administration of praziquantel to all school-aged children living in highly endemic areas through teachers or community health workers [93]. While such regular mass drug administration (MDA) is not sufficient to eliminate schistosomiasis from these settings (re-infections may rapidly occur), morbidity is, however, greatly reduced through averting high-intensity infections with e.g. subsequent periportal fibrosis. In 2012, the World Health Assembley resolution 65.19 called for intensified efforts to achieve elimination of schistosomiasis in selected countries in the near future. Various activities and initiatives are underway to investigate the best approach to achieve elimination, i.e. preventive chemotherapy, improved sanitation and hygiene, behaviour change, snail control (i.e. through the use of molluscicides) and improved diagnostics [94,95].

1.4.3 *Strongyloides stercoralis*

The threadworm *Strongyloides stercoralis* is a nematode that occurs in tropical and subtropical areas [96]. Its epidemiology, distribution and clinical features are much less understood than those of the three major soil-transmitted helminths (*Ascaris lumbricoides, Trichuris trichiura* and the two hookworm species *Ancylostoma duodenale* and *Necator americanus*). The life cycle of *S. stercoralis* is complex and comprises two specific characteristics, i.e. autoinfection and hyperinfection [97,98]. The adult worms reside in the gastrointestinal tract of the human host and produce eggs, which hatch already inside the host. Hence, first-stage (L_1) larvae are passed with the faeces and may contaminate the soil in areas of poor sanitation. In case of appropriate environmental conditions (temperature, humidity), the rhabditiform L_1 larvae can develop within the soil into infective, filariform L_3 larvae that may penetrate intact human skin, e.g. when walking barefoot on contaminated soil. Following the penetration of the skin, the larvae may travel hematogenously to the lungs, where they can get into the alveolar spaces and migrate upwards to the pharyngeal area. Once arrived there, the small larvae are unconsciously swallowed to be transported into the intestine, where they develop into adult worms and start egg production [8].

The so-called autoinfection is a characteristic of *S. stercoralis*, which distinguishes it from almost all other helminths that infest humans (with the exception of *Capillaria* spp.) [99]. Indeed, some L_1 larvae that hatch in the intestine are not passed with the faeces, but may develop inside the human host into filariform L_3 larvae that can penetrate the intestinal mucosa and spread throughout the body. In immunocompetent individuals, no symptoms may be associated with such an autoinfection, but the internal replication permits the helminth to maintain infections within the same host for decades [8,98]. In case of immunosuppression (e.g. malignant diseases, corticosteroid treatment, co-infection with retroviruses and organ transplantation), the host's immune system may lose its ability to control an existing intestinal *S. stercoralis* infection. The helminth larvae may start to rapidly replicate and spread throughout the body, thereby causing a frequently fatal sepsis-like condition, the so-called hyperinfection syndrome [100].

Figure 1.4 The life cycle of *S. stercoralis* (published in [8] after being adapted from [101]).



Due to its potential to cause severe hyperinfection in patients after many years of asymptomatic infection, *S. stercoralis* should always be included in the differential diagnosis if a patient has ever travelled to an endemic area. Resource-constrained settings in tropical countries are typically considered to be endemic for *S. stercoralis* [102], but the helminth may also cause infections in high-income countries such as parts of the United States of America [103] and Italy [104]. The global burden of strongyloidiasis is ill-defined and scientific estimates range between 30 million and more than 370 million infected people worldwide [105]. The autoinfectious cycle with the potential life-long persistence of infection and the risk of developing hyperinfection make it necessary to treat every patient with a diagnosed strongyloidiasis, regardless of his/her current symptoms [8].

The clinical features of strongyloidiasis are manifold and seem to be related to the intensity of infection. While many individuals are infected asymptomatically, diarrhoea, abdominal

tenderness, weight loss and bloating are also commonly reported [18]. When the larvae migrate through the body, fast-moving cutaneous itchy eruptions called “larva currens” may sometimes be seen. The larval transition through the lungs may lead to persistent cough and/or wheezing on pulmonary auscultation. Peripheral blood eosinophilia may be present, but its absence does not rule out the possibility of strongyloidiasis, particularly in severely ill patients with a suspected hyperinfection syndrome [106].

The visualisation of *S. stercoralis* in human stool specimens is particularly challenging because no eggs are passed into the faeces and the most commonly employed microscopic techniques for intestinal parasites frequently fail to detect the L_1 larvae [107]. Hence, more laborious and time-consuming methods need to be employed, such as the Baermann funnel concentration technique or nutrient agar plate cultures (e.g. Koga agar plate) [108,109]. Ideally, two microscopic techniques should be combined and several consecutive stool specimens should be examined to achieve a high diagnostic accuracy. Stool-based PCR assays have been developed and hold promise for an improved diagnosis of *S. stercoralis*, but require further validation in areas of endemicity [110]. Outside endemic settings, serology is a very valuable tool to evaluate patients for strongyloidiasis, because anti-*Strongyloides* antibody detection is highly sensitive (with the exception of severely immunocompromised patients) and decreasing antibody titres can also be used to monitor the treatment response [111]. Ivermectin is the preferred and most efficacious treatment against strongyloidiasis, whereas albendazole and mebendazole fail to cure the disease in a considerable amount of patients [112,113].

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2 Goal and specific objectives

The overarching goal of this Ph.D. thesis was to deepen the understanding of the occurrence of persistent diarrhoea (≥14 days) and persistent abdominal pain (≥14 days) in resource-constrained settings, to elucidate the spectrum of causative infectious agents and associated clinical disease manifestations, and to contribute to the development of an evidence-based algorithm for a syndromic management of persistent digestive disorders. Five specific objectives were related to this goal.

1. To review bacterial, parasitic and viral pathogens that may give rise to persistent digestive disorders in the tropics and subtropics.

2. To develop a standardised protocol to investigate persistent digestive disorders (≥14 days) in a multi-country study, comprising a comprehensive clinical assessment and detailed laboratory investigations.

3. To investigate the prevalence of multiple intestinal pathogens in symptomatic patients with persistent diarrhoea (≥14 days) and healthy controls in south Côte d'Ivoire.

4. To evaluate multiplex PCR assays for the diagnosis of intestinal pathogens in a tropical setting and to compare the diagnostic accuracy of PCR, RDTs and standard diagnostic tools for selected pathogens, specifically *C. difficile*, *Cryptosporidium* spp., *G. intestinalis*, *S. mansoni* and *S. stercoralis*.

5. To describe the clinical signs and symptoms associated with the occurrence of specific intestinal pathogens and to synthesise clinical and microbiological data for the development of an evidence-based diagnosis-treatment algorithm pertaining to the management of persistent digestive disorders (≥14 days) in resource-limited tropical settings.

3 Persistent digestive disorders in the tropics: causative infectious pathogens and reference diagnostic tests

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REVIEW Open Access

Persistent digestive disorders in the tropics: causative infectious pathogens and reference diagnostic tests

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Abstract

Background: Persistent digestive disorders account for considerable disease burden in the tropics. Despite advances in understanding acute gastrointestinal infections, important issues concerning epidemiology, diagnosis, treatment and control of most persistent digestive symptomatologies remain to be elucidated. Helminths and intestinal protozoa are considered to play major roles, but the full extent of the aetiologic spectrum is still unclear. We provide an overview of pathogens causing digestive disorders in the tropics and evaluate available reference tests.

Methods: We searched the literature to identify pathogens that might give rise to persistent diarrhoea, chronic abdominal pain and/or blood in the stool. We reviewed existing laboratory diagnostic methods for each pathogen and stratified them by (i) microscopy; (ii) culture techniques; (iii) immunological tests; and (iv) molecular methods. Pathogen-specific reference tests providing highest diagnostic accuracy are described in greater detail.

Results: Over 30 pathogens may cause persistent digestive disorders. Bacteria, viruses and parasites are important aetiologic agents of acute and long-lasting symptomatologies. An integrated approach, consisting of stool culture, microscopy and/or specific immunological techniques for toxin, antigen and antibody detection, is required for accurate diagnosis of bacteria and parasites. Molecular techniques are essential for sensitive diagnosis of many viruses, bacteria and intestinal protozoa, and are increasingly utilised as adjuncts for helminth identification.

Conclusions: Diagnosis of the broad spectrum of intestinal pathogens is often cumbersome. There is a need for rapid diagnostic tests that are simple and affordable for resource-constrained settings, so that the management of patients suffering from persistent digestive disorders can be improved.

Keywords: Bacteria, Clinical microbiology, Diagnosis, Digestive disorders, Gastroenterology, Helminths, Intestinal protozoa, Persistent diarrhoea, Virus

Background

Diarrhoeal diseases and other digestive disorders are leading causes of morbidity and mortality worldwide, with the highest burden concentrated in tropical and subtropical areas that often lack access to clean water and adequate sanitation, and where hygienic conditions are generally poor [1]. According to the World Health Organization (WHO), diarrhoea is classified into three different categories, namely (i) acute watery diarrhoea (lasting several hours or days); (ii) acute bloody diarrhoea (synonymous: dysentery); and (iii) persistent diarrhoea (lasting 14 days or longer) [2]. 'Chronic diarrhoea' is often referred to as an individual term applicable to diarrhoea lasting more than 4–6 weeks, but it still lacks an unambiguous definition.

With an estimated burden of 89.5 million disability-adjusted life years (DALYs) caused in 2010, diarrhoeal

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diseases rank fourth in the recently published Global Burden of Disease Study [3]. Acute diarrhoeal episodes are mainly due to bacterial and viral pathogens that may cause a variety of clinical syndromes ranging from self-limiting events to life-threatening diseases. Children are most vulnerable and diarrhoeal diseases were responsible for more than 1.4 million deaths in 2010, ranking this disorder at position seven on the main causes of death [4]. In the last decades, concerted efforts have considerably improved our understanding of the epidemiology, diagnosis, treatment and control of many diarrhoeagenic pathogens globally, for instance due to the introduction of rotavirus vaccination programmes in many countries since 2006 [5]. As a result, mortality due to diarrhoeal diseases has been reduced from an estimated 2.5 million in 1990 to just under 1.5 million in 2010, a decrease of 42% [4]. However, few research activities have focused on the investigation of persistent diarrhoea and non-acute abdominal pain due to digestive disorders in the tropics. Hence, little is known regarding its aetiology, epidemiology and disease burden. It is widely acknowledged that intestinal parasites, particularly helminths and intestinal protozoa play a major role as causative agents of persistent digestive symptomatologies [6].

Infections with helminths and intestinal protozoa belong to the neglected tropical diseases, along with other diseases caused by bacterial (e.g. Buruli ulcer), viral (e.g. dengue) and fungal infections (e.g. mycetoma) [7]. More than 5 billion people are at risk of neglected tropical diseases, with the common soil-transmitted helminths (i.e. *Ascaris lumbricoides*, hookworm and *Trichuris trichiura*), exhibiting the widest geographical distribution [8]. Due to their intimate connection with poverty, the highest prevalences of neglected tropical diseases are observed in remote rural and deprived urban settings in the developing world [7,9,10]. Neglected tropical diseases drain the social and economic development in endemic countries and they negatively impact on people's quality of life and well-being at all levels [11-15].

A major challenge in the clinical management of persistent digestive disorders is the weakness of health systems in many low-income countries [16-18]. Hence, affected people might only seek care at a late stage in their therapeutic itinerary, usually at primary health care centres [19,20]. However, these primary health care centres are often under-staffed and ill-equipped, resulting in a low quality of care. The causes of persistent diarrhoea and other digestive disorders are frequently misdiagnosed due to the often unspecific clinical presentations and the absence of evidence-based algorithms for in-depth investigation [7,21]. The notorious underfinancing of health systems in many tropical and subtropical countries explains the severe neglect of laboratory networks and the only limited availability of basic tests for diagnostic services (e.g. direct faecal smears for helminth diagnosis or blood films for malaria diagnosis) [22]. Hence, in many developing countries, current diagnostic and treatment algorithms are often empirical, whereas local prevalence data and differential diagnoses are rarely taken into account at the primary care level.

Against this background, NIDIAG, an international collaboration on integrated diagnosis-treatment platforms, funded by the European Commission, sets out to develop an improved system for delivering primary health care in resource-constrained settings and proposes an integrated approach to this challenge. Emphasis is placed on a patient-centred approach starting from the presentation at the primary health care level of a clinical syndrome that might be due to 'common' pathogens. Three clinical syndromes will be investigated in the NIDIAG framework, namely (1) neurological disorders [23]; (2) persistent fever [24]; and (3) digestive disorders. Here, we focus on digestive disorders, which we define as (i) persistent (≥2 weeks) abdominal pain; (ii) persistent (≥2 weeks) diarrhoea (dysenteric or non-dysenteric); and/or (iii) blood in the stool. These digestive disorders will be investigated at different study sites in tropical areas of Africa (Côte d'Ivoire and Mali) and Asia (Indonesia and Nepal). Before clinically applicable diagnosis-treatment algorithms can be developed, the following major challenges/open issues have to be addressed. Firstly, few studies analysed the spectrum of intestinal pathogens causing persistent digestive disorders in the tropics. Therefore, epidemiological investigations targeting all potential pathogens are desirable to define the most common bacteria, parasites and viruses in the different study settings. Secondly, most diagnostic tests have only been validated in Western settings, and hence their diagnostic accuracy in the tropics remains to be determined.

In this manuscript, pursuing an extensive literature review complemented with expert opinion, we provide an overview of potential pathogens (bacterial, parasitic and viral) that might give rise to digestive disorders as defined above. Available diagnostic tests for the identified pathogens are summarised and reviewed, and we propose pathogen-specific reference tests to be utilised for an in-depth diagnostic work-up of symptomatic patients in the different study sites.

Methods

Framework

A symptomatology according to the aforementioned inclusion criteria for the syndrome of digestive disorders is likely to be caused by a large variety of infections, but also non-infectious diseases. For example, blood in the stool, accompanied by persistent abdominal pain, may be indicative of colorectal carcinoma or inflammatory disorders (e.g. Crohn's disease and ulcerative colitis), but

may also be a sign of *Schistosoma mansoni* (a helminth) or *Entamoeba histolytica* (an intestinal protozoon) infection. The aim of the NIDIAG project is to develop evidence-based diagnosis-treatment algorithms that can easily be applied in resource-constrained health care settings. As neither diagnosis nor treatment of many non-infectious diseases are currently feasible in remote rural areas of most developing countries, only infectious aetiologies of digestive disorders that may cause severe disease and that are treatable will be thoroughly assessed within the frame of the NIDIAG project and were therefore prioritised in our literature search.

Search strategy, data extraction and analysis

We performed a literature review to identify and define the bacterial, parasitic and viral pathogens that may give rise to persistent diarrhoea and chronic digestive disorders, and to obtain information on their respective diagnostic methods in order to describe appropriate reference laboratory tests. Since the role of fungi as causative pathogens of gastrointestinal infections is still under debate, fungal infections were not included in this review [25]. The available literature was reviewed by three independent groups. The results were compared, discussed and finally synthesised. Additionally, a number of experts were consulted to complement the literature review.

In a first step, we examined a series of textbooks pertaining to medical bacteriology, parasitology and virology. Moreover, we searched the electronic database MEDLINE/PubMed for infectious pathogens that may cause digestive disorders as defined in the inclusion criteria. After having identified a set of more than 30 pathogens, we searched the database with the following search term for all infectious agents: “disease name/[Mesh]” and the subheading “diagnosis” (e.g. “ascariasis/diagnosis” [Mesh]). The focus of the MEDLINE/PubMed search was on established laboratory tests as well as on newer diagnostic methods, which have been validated recently or are currently under validation (e.g. studies objectively assessing the diagnostic accuracy of different tests). Hence, we primarily focused on reviews, comparative studies and evaluation studies. Our search had no language or other restrictions and we included studies that were published until mid-April 2012.

Results

Our literature review revealed more than 30 bacterial, parasitic and viral pathogens that may cause persistent digestive disorders. Many of these infectious agents are epidemiologically well characterised in Western settings, while data regarding their occurrence in tropical and subtropical areas are scarce and often contradictory [6,26-28]. Table 1 provides a list of all selected pathogens and typical clinical characteristics that may assist clinicians to curtail their differential diagnosis. However, pathogen-specific diagnosis can rarely be done based on the clinical presentations, and hence additional diagnostic tools are needed.

The large number of available diagnostic tests for the selected pathogens is a challenge for providing the single most accurate method for a given pathogen. Hence, we classified the different methods into four diagnostic categories, namely (i) microscopy; (ii) culture; (iii) immunology (including enzyme immunoassays (EIA), serotyping of isolates and serology); and (iv) molecular biological diagnosis (e.g. polymerase chain reaction (PCR) assays and DNA sequencing). Selection of a reference test for each specific pathogen is primarily based on the sensitivity and specificity of the test as well as practical considerations (e.g. costs, ease of application, availability, etc.). The results are presented in Table 2 (bacteria), Table 3 (intestinal protozoa), Table 4 (helminths) and Table 5 (viruses). Specific issues on the diagnostic work-up are summarised in the following sub-chapters.

Bacterial pathogens

Aeromonas spp., Campylobacter jejuni, C. coli, Plesiomonas shigelloides, Salmonella enterica (non-typhoidal serovars, e.g. S. enterica ser. Enteritidis, S. enterica ser. Typhimurium), Shigella spp., Vibrio spp., Yersinia enterocolitica, Y. pseudotuberculosis

A stool culture on selective media is the test of choice to detect these diarrhoeagenic bacteria [31,48,50,52,53]. Different solid media (e.g. selective agar plates containing antibiotics and substances favouring the growth of the sought microorganism) are inoculated with a stool specimen to detect and isolate enteric pathogens. The additional use of a selective enrichment broth is helpful to identify pathogenic bacteria if their presence is quantitatively so low that they might otherwise be overlooked on solid media due to the overgrowth of non-pathogenic intestinal flora. The inoculated media are usually incubated for 24–72 hours at 35°C at ambient atmosphere to allow the bacteria to form macroscopically visible colonies. Of note, *Campylobacter* spp. are isolated using different growth conditions, i.e. incubation at a higher temperature of 42°C in microaerophilic atmosphere [53].

Following the incubation period, the agar plates are examined and morphologically suspicious colonies are identified using different biochemical identification panels or automated phenotypic identification systems (e.g. Vitek®; bioMérieux, Marcy l’Étoile, France). Recently, more rapid identification algorithms making use of mass spectrometry (MS) have successfully been implemented into clinical microbiology laboratories (e.g. matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (e.g. MicroFlex LT; Bruker Daltonics, Bremen, Germany) [98].

Stool culture remains the diagnostic ‘gold’ standard for enteric pathogenic bacteria disposing certain characteristics

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Table 1 Overview of intestinal pathogens (bacteria, intestinal protozoa, helminths and viruses) that may cause persistent digestive disorders in infected individuals

Enteric pathogen	Persistent diarrhoea	Persistent abdominal pain	Blood in the stool
Bacteria			
Aeromonas spp.	+	–	–
Campylobacter jejuni, C. coli	+	+	+
Clostridium difficile	+	+	+
Escherichia coli			
Enteroaggregative *E. coli* (EAEC)	+	+	+
Enteropathogenic *E. coli* (EPEC)	+	+	–
Enteroinvasive *E. coli* (EIEC)	+	+	+
Enterohaemorrhagic *E. coli* (STEC/EHEC)	+	+	+
Enterotoxigenic *E. coli* (ETEC)	+	+	–
Diffusely adherent *E. coli* (DAEC)	+	–	–
Mycobacterium tuberculosis and atypical mycobacteria	+	+	+
Plesiomonas shigelloides	+	–	–
Salmonella enterica (typhoidal and non-typhoidal serovars)	+	+	+
Shigella spp.	+	+	+
Tropheryma whipplei	+	–	–
Vibrio spp.	+	–	–
Yersinia enterocolitica, Y. pseudotuberculosis	+	–	–
Intestinal protozoa			
Balantidium coli	+	+	+
Blastocystis hominis[a]	(+)	(+)	–
Cryptosporidium spp.	+	+	–
Cyclospora cayetanensis	+	–	–
Dientamoeba fragilis[a]	+	+	–
Entamoeba histolytica	+	+	+
Giardia intestinalis (syn.: *G. lamblia* and *G. duodenalis*)	+	+	–
Isospora belli (syn.: *Cystoisospora belli*)	+	(+)	–
Species of microsporidia	+	+	–
Helminths			
Cestodes			
Diphyllobothrium latum	+	+	–
Hymenolepis spp.	+	–	–
Taenia spp.	+	+	–
Nematodes			
Ascaris lumbricoides	+	+	–
Capillaria philippinensis	+	+	–
Hookworm (*Ancylostoma duodenale* and *Necator americanus*)	+	+	–
Strongyloides stercoralis	+	+	(+)
Trichuris trichiura	+	+	–
Trematodes			
Intestinal flukes	+	+	–
Intestinal blood flukes: *Schistosoma mansoni, S. intercalatum, S. japonicum, S. mekongi*	+	+	+
Viruses			
Adenovirus	+	–	–
Astrovirus	(+)	–	–
Bocavirus	(+)	–	–
Coronavirus	(+)	–	–
Cytomegalovirus (CMV)	+	+	+
Enterovirus	+	–	–
Human immunodeficiency virus (HIV-1/2)	+	+	–
Norovirus	+	–	–
Parechovirus	(+)	–	–
Rotavirus	+	+	–
Sapovirus	(+)	–	–

+, existing risk; (+), low risk; –, no risk.
[a] There is an ongoing debate whether these intestinal protozoa have pathogenic potential or should rather be seen as simple commensals of the gastrointestinal tract [29,30].

which enable them to be selected out of the normal gastrointestinal flora, while other bacteria without such characteristics cannot be distinguished from apathogenic gut bacteria by culture methods alone (see below). Stool culture has important advantages, such as testing of isolated pathogens for antimicrobial susceptibility. As the successful antibiotic treatment of many bacterial intestinal infections requires knowledge of local resistance patterns (e.g. extent of fluoroquinolone-resistant *Campylobacter* strains), stool culture techniques remain mandatory to guide therapeutic interventions. However, these tests are laborious and require experienced personnel, and typically take 48–72 hours to obtain first results. Hence, other, more rapid diagnostic tests (RDTs) have been developed for some pathogens. For *Campylobacter* spp., for example, EIAs detecting a specific antigen in the stool proved to be a sensitive alternative to stool culture with results available within a few hours [32,99]. However, there is no international consensus on

immunological assays for detection of *Campylobacter* spp. and no globally validated and standardised approach, so that these tools should not replace the selective stool culture [100]. PCR assays, characterised by high sensitivity and specificity, have been developed for most of the aforementioned bacteria. Thus far, however, integration into clinical routine testing is still limited. Important drawbacks are the high cost, the need for sophisticated laboratory equipment and well-trained laboratory technicians. Moreover, PCR cannot distinguish between dead or alive bacteria and does not allow testing for antimicrobial susceptibility. However, newly developed multiplex PCR assays are increasingly being evaluated as fast screening tests for early detection of various important enteric pathogens. Besides PCR, novel molecular diagnostics are currently being developed and validated for many bacterial and viral pathogens, e.g. loop-mediated isothermal amplification (LAMP) assays. Results obtained thus far are promising [101,102], but it remains to be elucidated whether such nucleic acid amplification tests can be employed on a larger scale in resource-constrained settings in the tropics.

Salmonella enterica ser. Typhi/Paratyphi

Diagnosis of enteric fever is challenging and often delayed or not performed due to the unavailability of the most sensitive techniques in areas of high endemicity [103]. In these settings, the Widal test (measuring an increasing *S.* Typhi-specific antibody titer over the course of 10 days in patient serum samples) is often the only available test, despite its poor diagnostic performance. Contrary to non-typhoidal salmonellosis, stool culture is not sufficiently sensitive to diagnose infection due to *S.* Typhi/*S.* Paratyphi. Culturing blood and bone marrow is more sensitive, but bone marrow aspiration is only rarely performed in tropical areas due to a lack of adequately equipped hospitals and laboratories [104]. Blood cultures should be obtained during the first week of disease to achieve adequate sensitivity [45]. Serotyping of isolates (e.g. by agglutination of Vi antigen or rapid detection of various antigens or IgM antibodies by different EIA kits) is helpful for a timely diagnosis, but lacks sensitivity and specificity [46]. PCR assays have been developed for different antigens of invasive *S. enterica* serovars, but still need further development and validation before they can be more widely recommended [105].

Clostridium difficile

C. difficile can be found as part of the physiological intestinal flora, but toxin-producing strains may cause severe diarrhoea, which is most frequently seen in hospitalised patients who recently received antibiotic treatment [33]. A selective stool culture (toxigenic culture, performed on a selective agar medium or after ethanol shock pretreatment) followed by tests for toxin production remains the diagnostic 'gold' standard for *C. difficile* [33] and is particularly useful when the quantity of toxins in stool samples is small [34]. A laborious and technically difficult cell culture cytotoxicity assay is still regarded as an alternative reference standard, but is seldom performed in most microbiological laboratories. More recently developed PCR assays targeting a toxin-encoding gene are currently discussed as an alternative method for early diagnosis of *C. difficile* infection. Such molecular methods allow a more precise characterisation of isolated *C. difficile* strains, e.g. ribotype differentiation [35,106,107]. Sensitivity and specificity of PCR have been reported to vary between 85% and 100% [108]. However, various molecular assays exist which are not yet fully standardised, and the diagnostic performance of commercially available kits may differ considerably from in-house molecular testing methods in use at different laboratories. Of note, PCR can only prove the presence of the toxin-encoding gene, but cannot distinguish between asymptomatic carriage and acute infection.

In clinical practice, an easily applicable two-step approach is recommended for rapid and reasonably sensitive diagnosis of *C. difficile* [109]. Firstly, a screening test for *C. difficile*-associated glutamate dehydrogenase (GDH) should be performed to indicate the bacterium's presence in a stool sample. If positive, it should be followed by a test for toxin production (e.g. toxin A/B EIA). This procedure does not require an extensively equipped laboratory and generates accurate results within a few hours. However, the sensitivity and specificity of this two-step approach are limited, and hence toxigenic culture and PCR testing should always be performed when there is a high clinical suspicion despite negative test results [110].

Pathogenic Escherichia coli strains

Diagnosis of pathogenic *E. coli* is challenging, as these bacteria constitute an important part of the physiological intestinal flora and only some strains have diarrhoeagenic potential [41]. There are at least six groups of pathogenic *E. coli* strains, namely (i) diffusely adherent (DAEC); (ii) enteroaggregative (EAEC); (iii) enterohaemorrhagic (EHEC, including STEC = shiga toxin-producing *E. coli*); (iv) enteroinvasive (EIEC); (v) enteropathogenic (EPEC); and (vi) enterotoxigenic *E. coli* (ETEC). Pathogenic *E. coli* strains that carry simultaneously virulence factors from different pathotypes may cause severe clinical outbreaks. In mid-2011 in Germany, for example, the *E. coli* strain O104:H4 (an EAEC capable of EHEC/STEC-specific shiga toxin production) caused 2,987 cases of acute, often severe gastroenteritis and 855 cases of haemolytic-uraemic syndrome which led to 53 deaths [111].

While diagnostic procedures are poorly standardised for the pathotypes DAEC and EAEC, molecular

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Table 2 Diagnostic tests for important bacterial pathogens that may cause persistent digestive disorders

Infectious pathogen	Diagnostic method				
	Microscopy	**Stool culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Aeromonas spp.	-[a]	**Culture on cefsulodin-irgasan-novobiocin (CIN) or selective *Aeromonas* agar**	-	(Experimental, not validated)	[31]
Campylobacter jejuni, C. coli	Darkfield microscopy: motile, curved or S-shaped rods (suggestive of *Campylobacter* spp.)	**Culture on selective medium[b] (42°C, microaerophilic conditions)**	**· Faecal antigen enzyme immunoassay: *Campylobacter*-specific antigen (SA)** · Serology (important for diagnosis of postinfectious immunological diseases)	*hipO* gene (*C. jejuni*), *glyA* gene (*C. coli*)	[32]
Clostridium difficile	-[c]	**Culture on selective medium, e.g. cycloserin-cefoxitin-fructose agar (CCF) + toxigenic culture**	**· 2-step algorithm:** **1) Screening: EIA for glutamate dehydrogenase (GDH)** **2) ELISA for detection of toxin A and B** · Cell cytotoxicity assay for detection of toxin A and B	Toxin genes (increasingly being used in clinical routine)	[33-35]
Escherichia coli					
Enteroaggregative *E. coli* (EAEC)	-[a]	**HEp-2 cell adherence assay (following incubation in Luria broth)**	· Serology: antibody response against Plasmid-encoded toxin (*Pet*) · ELISA: secretory immunoglobulin A response to EAEC	*AggR, CVD432, EAST1* (most common virulence factors, not always present)	[36]
Enteropathogenic *E. coli* (EPEC)	-[a]	Culture on MacConkey (MAC) agar	-	***eae* gene**	[37]
Enteroinvasive *E. coli* (EIEC)	-[a]	Culture on MAC agar	ELISA: detection of the *ipaC* gene	***ipaH, ipaB* genes**	[38]
Enterohaemorrhagic *E. coli* (EHEC including STEC)	-[a]	Culture on sorbitol-MAC agar (most O157: H7 strains form sorbitol-negative colonies)	· O157 latex agglutination test **· Shiga toxins 1 & 2 (ELISA)**	**STEC: *stx1*, *stx2* genes** **EHEC: *stx1/stx2* + eae gene**	[39,40]
Enterotoxigenic *E. coli* (ETEC)	-[a]	Culture on MAC agar	Several immunoassays for toxin detection	***stla/stlb* and *lt* genes**	[41]
Diffusely adherent *E. coli* (DAEC)	-[a]	**HEp-2 cell adherence assay (following incubation in Luria broth)**	-	*daaD* gene	[42]
Mycobacterium tuberculosis and atypical mycobacteria	- **Histopathological examination of intestinal biopsies** - Acid-fast stain (e.g. Ziehl-Neelsen, Kinyoun, Auramin)	**Culture of biopsy material**	· Interferon-gamma-release assay (IGRA) on heparinised blood samples · Tuberculin skin test	Nucleic acid amplification tests (lacks sensitivity for diagnosis of extrapulmonary tuberculosis)	[43,44]
Plesiomonas shigelloides	-[a]	**Culture on CIN agar**	-	-	

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Table 2 Diagnostic tests for important bacterial pathogens that may cause persistent digestive disorders *(Continued)*

Infectious pathogen	Diagnostic method				
	Microscopy	**Stool culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Salmonella enterica (typhoidal and non-typhoidal serovars)	-[a]	• **Culture[d] from blood and/or bone marrow** (enteric fever) • Culture[d] from stool or duodenal aspirate (typhoidal and non-typhoidal salmonellosis)	• Serotyping of isolates (Vi antigen) • ELISA: detection of *S. typhi* antigens (blood) • Widal agglutination test (commonly used in Africa)	(Mainly for research purpose)	[45-47]
Shigella dysenteriae, S. flexneri, S. boydii, S. sonnei	-[a]	**Culture on MAC, XLD, HE or Leifson agar**	**Agglutination tests to detect serogroup and serotype**	*ipaH*, *ipl* genes	[48]
Tropheryma whipplei	**Histopathological examination of PAS-stained intestinal biopsies: sickleform particle-containing cells**	(Only in highly specialised laboratories)	Immunohistochemistry on PAS-positive biopsy material	***whip1, whip2* genes**	[49]
Vibrio spp.	Darkfield microscopy: comma-shaped, motile bacteria (highly suggestive of *Vibrio* spp.)	**Culture on TCBS agar**	-	PCR for species differentiation (*V. cholerae, V. parahaemolyticus, V. vulnificus*)	[50,51]
Yersinia enterocolitica, Y. pseudotuberculosis	-[a]	**Culture on CIN agar**	Serology (important for diagnosis of postinfectious immunological diseases)	PCR (reference laboratories and research purposes)	[52]

The laboratory techniques are divided into different categories and recommended tests for each pathogen are highlighted.
[a] Gram staining of stool samples can be useful to evaluate the presence of leucocytes, but is not helpful to differentiate between pathogenic bacteria and apathogenic microbial flora.
[b] Commonly employed selective media for detection of *Campylobacter* spp. include charcoal-cefoperazone-deoxycholate agar, *Campylobacter* blood agar plate, and cefoperazone-vancomycin-amphotericin agar [53].
[c] Detection of *C. difficile* in the Gram stain is not adequate to differentiate between clinical infection and simple colonisation with *C. difficile* [54].
[d] Commonly employed selective media for growth of *S. enterica* are MAC, XLD, HE, Leifson agar or other chromogenic media.

biological testing has revolutionized the diagnostic algorithms for the other diarrhoeagenic *E. coli*. Modern multiplex PCR assays targeting unique genes of EHEC/STEC, EIEC, EPEC and ETEC allow a rapid molecular characterisation of these pathogenic strains. Hence, multiplex PCR assays have become the test of choice with excellent sensitivity and specificity (>99%) [42]. Indeed, these tests have overcome important drawbacks of the classical stool culture, which often detects only some important strains (e.g. in the case of EHEC the O157:H7 strain on Sorbitol-MacConkey agar), but misses others that lack characteristic biochemical properties [39]. However, the integration of such multiplex PCR assays into routine testing of clinical samples remains restricted to well-equipped laboratories, and hence, these molecular techniques are only rarely available in endemic settings in the tropics.

Mycobacterium tuberculosis and atypical mycobacteria (e.g. M. avium)

Gastrointestinal tuberculosis is the sixth most common manifestation of extrapulmonary tuberculosis and causes considerable morbidity, including persistent diarrhoea and abdominal pain [112]. Atypical mycobacteria (synonymous: mycobacteria other than tuberculosis, MOTT), particularly *M. avium*, are an important cause of long-lasting diarrhoea and gastrointestinal complaints in HIV-infected individuals. Accurate diagnosis is difficult and relies on in-depth analysis of intestinal biopsy specimens by histopathological examination, microscopy after acid-fast staining (e.g. Ziehl-Neelsen, Auramin or Kinyoun techniques) and culture on selective media suitable for mycobacteria. Unless performed using oil immersion, histopathology often fails to distinguish between gastrointestinal tuberculosis and other granulomatous disorders, such as Crohn's disease [113,114]. An important drawback when culturing mycobacteria is their slow growth; it might take up to six weeks until cultures become positive. However, culture is the most sensitive technique and remains the diagnostic 'gold' standard [115]. Different molecular biological assays have been developed for various mycobacteria, but lack sensitivity for extrapulmonary tuberculosis and have not yet been validated for gastrointestinal tuberculosis [43].

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Table 3 Diagnostic tests for important intestinal protozoa that may cause persistent digestive disorders

Infectious pathogen	Diagnostic method				
	Microscopy	Stool culture	Immunology	Molecular biology (PCR)	Reference(s)
Balantidium coli	**Stool microscopy** **· Wet mount smears (unstained or iodine stain)** **· Concentration techniques (e.g. formalin-ether)** **· Permanent stains (e.g. with iron hematoxylin)**	-	-	-	[55]
Blastocystis hominis	**Stool microscopy** **· Wet mount smears (unstained or iodine stain)** **· Permanent stains (e.g. with trichrome, iron hematoxylin, Giemsa)**	Stool culture on selective liquid media (no routine procedure, but beneficial in microscopically uncertain cases)	(No routine procedure)	(PCR mainly applied in research settings)	[56-58]
Cryptosporidium spp.	**Stool microscopy** • Wet mount smears (unstained or iodine stain) **· Various staining techniques, especially acid-fast stains (e.g. Kinyoun, modified Ziehl-Neelsen)**	(No routine procedure)	**· ELISA: faecal antigen detection** • Fluorescence microscopy	**PCR** (in reference laboratories and for species differentiation)	[59-62]
Cyclospora cayetanensis	**Stool microscopy** **· Wet mount smears (light or epifluorescence microscopy)** **· Concentration techniques (e.g. formalin-ether)** • Acid-fast stains (oocysts are variably acid-fast)	(No routine procedure)	-	**PCR** (in reference laboratories)	[63]
Dientamoeba fragilis	**Stool microscopy on stained smears (e.g. iron-hematoxylin, chlorazol black dye stain)**	(No routine procedure)	**-**	**PCR** (in reference laboratories) on unpreserved stool samples	[64,65]
Entamoeba histolytica	**Stool microscopy** **· Wet mount smears (trophozoites)** **· Formalin-ether concentration (cysts)** **· Permanent stains**	(No routine procedure)	**· ELISA: faecal antigen detection able to distinguish between *E. histolytica* and *E. dispar/moshkovskii* (stool)** • Serological antibody detection tests (blood samples)	**PCR** (in reference laboratories)	[66-70]
Giardia intestinalis (syn.: *G. lamblia* and *G. duodenalis*)	**Stool microscopy** **· Wet mount smears (trophozoites)** **· Formalin-ether concentration (cysts)** **· Permanent stains**	(No routine procedure)	**· ELISA: faecal antigen detection**	**PCR** (in reference laboratories)	[60,71]

Table 3 Diagnostic tests for important intestinal protozoa that may cause persistent digestive disorders *(Continued)*

Infectious pathogen	Diagnostic method				
	Microscopy	**Stool culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Isospora belli (syn.: *Cystoisospora belli*)	**Stool microscopy** • Wet mount smears • Concentration techniques (e.g. formalin-ether) **• Acid-fast stains**	-	-	**PCR** (in reference laboratories)	[60,72,73]
Species of microsporidia (*Enterocytozoon bieneusi, Encephalitozoon* spp.)	**• Transmission electron microscopy** (gold standard, but not feasible as routine test) **- Light microscopy** (e.g. Uvitex B, Chromotrope R or Calcofluor White stain)	-	Serology: anti-microsporidial antibodies (indirect immunofluorescence assay)	**PCR** (in reference laboratories)	[60,74-76]

The laboratory techniques are divided into different categories and recommended tests for each pathogen are highlighted.

Tropheryma whipplei

Whipple's disease due to infection with *T. whipplei* is a rare disease characterised by chronic diarrhoea, wasting, abdominal pain, arthralgia and various other symptoms associated with organ involvement (e.g. encephalitis and endocarditis) [49]. The infectious agent was not identified until 1961 and many epidemiological and biological features still need to be elucidated [116]. Only highly specialised laboratories are able to grow *T. whipplei* on human fibroblast cells [117,118]. The development of a PCR assay targeting the genes *whip1* and *whip2* has been a major step forward and is nowadays the test of choice, especially in symptomatic patients without typical histopathological findings in intestinal biopsies (sickleform particle-containing cells on periodic acid-Schiff (PAS-)stained biopsy specimens) [49].

Parasitic pathogens: intestinal protozoa

Balantidium coli, Blastocystis hominis, Cryptosporidium spp., Cyclospora cayetanensis, Dientamoeba fragilis, Entamoeba histolytica, Giardia intestinalis (syn.: G. lamblia and G. duodenalis), Isospora belli (syn.: Cystoisospora belli), species of microsporidia

The three main techniques for the diagnosis of human intestinal protozoan infections include (i) light microscopy; (ii) antigen detection (EIAs); and (iii) PCR assays. Since the first description of parasitic intestinal protozoa in human stools, documented by the Dutch microscopist Antony van Leeuwenhoek in 1681 [119], microscopic detection of protozoan cysts and trophozoites has been the most widely used diagnostic approach. On fresh stool samples, direct microscopy is performed by mixing a small amount of faeces with a physiological 0.9% sodium chloride (NaCl) solution. To increase sensitivity, various stool concentration techniques have been developed, making use of either sedimentation or flotation with a formalin-ether concentration technique being the most widely used method in medical laboratories [120,121]. However, the formalin-ether concentration technique lacks sensitivity for several intestinal protozoan species as well as many helminths (described below), and hence there is a pressing need for new and more sensitive microscopic techniques (e.g. FLOTAC) [122] and non-microscopic diagnostics. Staining techniques can be helpful for microscopic parasite identification and might further improve the diagnostic accuracy. Indeed, some intestinal protozoan species require staining of the stool sample to be identified on microscopic examination. For example, acid-fast stains allow detection of *Cryptosporidium* spp., while species of microsporidia are best seen when using an Uvitex B or Calcofluor White stain. Still, correct identification of intestinal protozoan pathogens is challenging even for experienced laboratory technicians and for some species even impossible (e.g. *E. histolytica* based on cysts morphology). For *Cryptosporidium* spp., *E. histolytica* and *G. intestinalis*, sensitive EIAs detecting species-specific antigens in faecal samples have been developed, some of which are highly sensitive and complement microscopic stool examination in many clinical laboratories [123,124]. Especially for the diagnosis of *E. histolytica*, species differentiation based on alternative procedures is compulsory, since microscopy cannot readily distinguish between *E. histolytica* and the non-pathogenic *E. dispar* [66,125,126]. Of note, not all commercially available EIA antigen detection kits are *E. histolytica*-specific and some lack sensitivity, in particular if faecal samples have been stored for several days [67,127]. Over the past several years, highly sensitive PCR assays have been developed and standardised for many intestinal protozoan species.

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Table 4 Diagnostic tests for important helminths that may cause persistent digestive disorders

Infectious pathogen	Diagnostic method				
	Microscopy	**Stool culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Cestodes					
Diphyllobothrium latum	**Stool microscopy: identification of eggs or proglottids** **· Wet preparation** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques**	-	-	PCR and sequencing for species differentiation (for epidemiological purpose)	[77,78]
Hymenolepis spp.	**Stool microscopy** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques** **· FLOTAC**	-	-	PCR in research settings (for epidemiological purpose)	[79]
Taenia spp.	**Stool microscopy** • Perianal egg detection • (Graham's test applying adhesive tape) • Examination of tapeworms from purges	-	• Coproantigen EIA • Serology: detection of specific circulating antibodies against *T. solium*	PCR for species differentiation	[80]
Nematodes					
Ascaris lumbricoides	**Stool microscopy: egg detection** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques** **· FLOTAC**	-	-	PCR in research settings (for epidemiological purpose)	[81-83]
Capillaria philippinensis	**Stool microscopy: egg detection** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques** • (Kato-Katz method: great care is indicated to distinguish between *T. trichiura* and *C. philippinensis* eggs)	-	-	-	[84,85]
Hookworms (*Ancylostoma duodenale*, *Necator americanus*)	**Stool microscopy: egg detection** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques** **· FLOTAC**	Culture on Koga agar and subsequent microscopic identification of larvae		PCR mainly applied in research settings (for epidemiological purpose)	[81-83]

Table 4 Diagnostic tests for important helminths that may cause persistent digestive disorders *(Continued)*

Infectious pathogen	Diagnostic method				
	Microscopy	**Stool culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Strongyloides stercoralis	**· Stool: microscopy following Baermann funnel concentration** · Microscopy of sputum, bronchoalveolar lavage, duodenal aspirate, skin biopsy	**Culture on Koga agar and subsequent microscopic identification of larvae**	· ELISA tests detecting serum antibodies or faecal antigens · Indirect fluorescent antibody test	PCR applied in research settings (for epidemiological purpose) and increasingly used for individual patient management	[86,87]
Trichuris trichiura	**Stool microscopy: egg detection** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Sedimentation techniques** **· FLOTAC**	-	-	-	[81,82]
Trematodes					
Intestinal flukes	**Stool microscopy: egg detection** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Stoll's dilution** **· Sedimentation techniques** **· FLOTAC**	-	ELISA to detect worm-specific antibodies or antigens in serum or stool	PCR applied in research settings (for epidemiological purpose)	[88]
Intestinal blood flukes: *Schistosoma mansoni*, *S. intercalatum*, *S. japonicum*, *S. mekongi*	**Stool microscopy: egg detection** **· Kato-Katz method** **· Ethyl-acetate or formalin-ether-based concentration techniques** **· Stoll's dilution** **· Sedimentation techniques** **· FLOTAC** (first experiences for *S. mansoni*) Miracidium-hatching test from stool samples	-	- ELISA to detect serum antibodies or worm-specific antigens in serum or urine - RDT to detect CCA or CAA antigen in serum or urine (for *S. mansoni*)	PCR applied in research settings for epidemiological purpose and increasingly used for individual patient management	[89]

The laboratory techniques are divided into different categories and recommended tests for each pathogen are highlighted.

Many of these assays (e.g. *Entamoeba* spp. differentiation by PCR) are currently being integrated into parasitological reference laboratories as an additional diagnostic tool to prove diagnosis in uncertain clinical cases [59,128,129]. Such molecular biological tools are of enormous importance to improve the correct species identification of many intestinal parasites, which are difficult to diagnose using conventional techniques [60,74].

Parasitic pathogens: helminths

Ascaris lumbricoides, Capillaria philippinensis, Diphyllobothrium spp., Hymenolepis spp., hookworm (Ancylostoma duodenale and Necator americanus), Taenia spp., Trichuris trichiura, intestinal flukes

Identification of helminth eggs on microscopic stool examination is the reference test for most intestinal helminth species. In hospitals and microbiological laboratories,

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direct stool examination after prior concentration (e.g. by formalin-ether concentration technique) is most commonly employed, while the Kato-Katz thick smear technique is widely used in epidemiological studies and anthelminthic drug efficacy evaluations in endemic regions [81,130-132]. Direct microscopic examination is a cheap methodology, the microscope slides can rapidly be prepared for examination, and there is no need for sophisticated laboratory equipment. The eggs of most helminth species parasitising humans can easily be distinguished by a trained laboratory technician (see Figure 1 for eight selected helminth eggs). Hence, microscopy remains the standard reference test for *A. lumbricoides*, *T. trichiura*, hookworm, *Capillaria philippinensis*, *Diphyllobothrium* spp., *Hymenolepis* spp., *Taenia* spp. and blood flukes (*Schistosoma* spp.) [82,88,133,134]. However, microscopy is prone to a number of shortcomings. Firstly, microscopy is not very sensitive and especially infections of light intensity can be missed when only a single stool sample is analysed [131,135]. Multiple stool sampling, ideally over several consecutive days, increases the sensitivity [136], as well as the use of different concentration techniques, which are based on sedimentation (e.g. formalin-ether concentration technique), flotation or a combination of both (e.g. McMaster technique and FLOTAC) [135,137-139]. However, these techniques often require access to the power grid, a centrifuge and different chemical reagents, which are not always available in tropical settings. Moreover, the diagnostic sensitivity for different helminth species often varies considerably, and no currently available concentration technique is able to concurrently detect intestinal protozoa and helminths with the same diagnostic accuracy [122,140].

Secondly, microscopy results heavily depend on the quality of the slide preparation and on the experience of the laboratory technician reading the slides. Thirdly, the eggs of some helminth species such as the two hookworm species *A. duodenale* and *N. americanus* are virtually indistinguishable by microscopy. Fourthly, the nematode *Strongyloides stercoralis* can rarely be found when using the aforementioned microscopy techniques, because its larvae already hatch in the intestine and, hence, the eggs are not passed in the faeces [141]. Despite all these constraints, microscopy is an invaluable tool for diagnostic medical parasitology.

New molecular techniques, especially PCR assays, still need to be validated and further developed in different settings. Disadvantages of current PCR tests are their high costs, risk of contamination, the need for high-technology laboratory equipment and constant electric

Table 5 Diagnostic tests for important viral pathogens that may cause persistent digestive disorders

Infectious pathogen	Diagnostic method				
	Electron microscopy	**Cell culture**	**Immunology**	**Molecular biology (PCR)**	**Reference(s)**
Viruses					
Adenovirus	Low sensitivity (>10^6 viral particles/ml)	A549-, HEp-2-, HEK-cells	Antigen detection in faecal samples (ELISA, immunochromatography)	**PCR**	[90]
Astrovirus	Low sensitivity (>10^6 viral particles/ml)	CaCO-2-, LLC-MK2-cells	Antigen detection (ELISA)	**RT-PCR**	[91]
Bocavirus	-	-	-	**PCR**	[92]
Coronavirus	-	-	-	**RT-PCR**	[93]
Cytomegalovirus (CMV)	-	HFF-, MRC-5 cells	• pp65 antigen detection (immunofluorescence)	**PCR**	[91]
		CMV-immediate early1-pp72-antigen in HFF	• (CMV-specific antibody seroconversion)		
Enterovirus	-	MRC-5-, HEp-2-, Vero-cells	-	**RT-PCR**	[94]
Human immunodeficiency virus (HIV-1/2)	-	HUT-78-, CEM-MOLT4-cells	**• Immunoassay (e.g. 4th generation)**	**RT-PCR**	[95,96]
			• Western Blot		
Norovirus	Sensitivity 10^5-10^6 viral particles/ml	-	Antigen detection faecal samples (EIA)	**RT-PCR**	[91]
Parechovirus	-	-	-	**RT-PCR**	[97]
Rotavirus	Low sensitivity (>10^6 viral particles/ml)	MA104-, CaCO-2-cells	Antigen detection in faecal samples (ELISA), rapid tests (ELISA, immunochromatography)	**RT-PCR**	[91]
Sapovirus	-	-	-	**RT-PCR**	[91]

The laboratory techniques are divided into different categories and recommended tests for each pathogen are highlighted.
RT-PCR, reverse transcriptase-polymerase chain reaction.

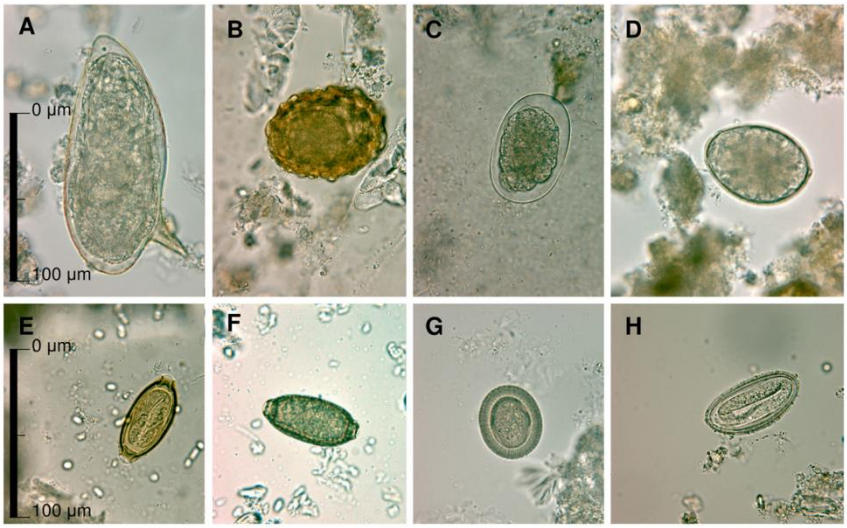

Figure 1 Morphological features of selected intestinal helminth eggs diagnosed using the formalin-ether concentration technique and standard light microscopy: A, *Schistosoma mansoni*; B, *Ascaris lumbricoides*; C, hookworm; D, *Diphyllobothrium latum*; E, *Trichuris trichiura*; F, *Capillaria* spp..; G, *Taenia* spp.; H, *Enterobius vermicularis*.

power supply which render their use for routine testing in many developing countries impossible. Indeed, PCR is seldom available in the most affected regions, and its results often do not guide clinicians' decisions, as empiric treatment with albendazole and mebendazole is commonly employed and effective against many helminth species in endemic areas [7]. Due to the variety of intestinal parasites causing digestive disorders, a multiplex real-time PCR targeting a host of various pathogens is much more desirable than individual PCR assays for each parasite, and such multiplex PCRs have been successfully developed and are increasingly used in reference laboratories in industrialised countries [59,83,142]. However, even these multiplex PCRs can only diagnose a defined host of targeted pathogens, while microscopy may sometimes detect unexpected pathogens that would have been missed by other diagnostic methods.

Strongyloides stercoralis

The diagnosis of *S. stercoralis* in human stool samples requires special, often laborious concentration techniques. Most commonly employed are the Baermann funnel and the Koga agar plate [143]. The Baermann method is a concentration technique based on the nematode's hydrophily and thermophily. It provides results within a few hours and is the technique of choice according to the World Gastroenterology Organization [144], but there is some debate whether it is as sensitive as agar plate cultures [86]. Derived from classical charcoal culture assays and its sequel, the so-called Harada-Mori culture, Koga and colleagues developed a special agar plate to detect *S. stercoralis* and hookworm larvae [145]. The agar plates are stored for 48 hours in a humid chamber and the traces of the helminths can then be seen on the agar and the larvae can easily be collected for microscopic species identification. In contrast to many other helminth infections, where exact species identification often is not necessarily required and clinical symptoms are mild, the recognition of strongyloidiasis and initiation of an effective treatment with ivermectin is essential to prevent potentially life-threatening events due to its ability to cause disseminated hyperinfection in the immunosuppressed population [141,146]. Hence, the aforementioned laborious techniques seem to be justified and a combination of the Baermann funnel and the Koga agar plate method may lead to the most accurate results.

Recently, different PCR assays targeting the helminth's 18S rRNA [87] or 28S rRNA [147] subunit have been developed. First results are promising, but still need further validation in endemic settings.

Schistosoma mansoni, S. mekongi, S. intercalatum and S. japonicum

The microscopic detection of blood fluke eggs in stool specimens still remains the cornerstone of the laboratory diagnosis of intestinal schistosomiasis, as the specificity is high and the costs of equipment are relatively low. However, the sensitivity fluctuates, depending on infection stage and intensity [148]. Hence, concentration methods like an ether-concentration, the Kato-Katz thick smear or the recently developed FLOTAC technique are important tools to increase sensitivity [89].

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Moreover, examination of multiple (preferably at least three) stool samples collected on consecutive days is recommended [136,149]. In contrast to other helminth infections, immunological RDTs have been developed for detection of intestinal (*S. mansoni*) and urogenital schistosomiasis (*S. haematobium*). Worm-gut associated glycoproteins, namely circulating cathodic antigen (CCA) and circulating anodic antigen (CAA), can be detected in the serum and the urine of *S. mansoni*-infected individuals using genus-specific monoclonal antibodies [150,151]. Immunochromatographic point-of-care (POC) dipstick or cassette tests for rapid diagnosis of *S. mansoni* via CCA detection in the urine are currently being validated in different epidemiological settings and will potentially become a valuable tool for non-microscopic diagnosis of schistosomiasis in epidemiological studies and clinical practice. Recent studies suggest that the diagnostic accuracy of a single POC-CCA test is considerably more sensitive than a single Kato-Katz thick smear and that a concurrent *S. haematobium* infection does not influence the POC-CCA test results for *S. mansoni* diagnosis, which is an important observation due to the co-endemicity of both blood fluke infections in many tropical areas [152,153]. Hence, antigen RDT assays will likely find their way into clinical practice in the foreseeable future.

PCR assays have been developed and are more sensitive than conventional parasitological and serological methods, but presently, their use is restricted to specialised reference laboratories and research institutions outside endemic areas [154,155].

Viruses

Viral infections commonly cause acute gastroenteritis with the highest burden concentrated in tropical and subtropical regions of the world. Even though these pathogens mainly lead to short-lasting and self-limiting diarrhoeal diseases, they account for considerable morbidity and even mortality, particularly in children [156]. In general, viral infections rarely cause chronic intestinal diseases, but must not be forgotten as potential pathogens that may give rise to persistent diarrhoea and chronic abdominal pain, particularly in HIV-infected individuals or otherwise immunocompromised hosts.

Traditionally, diagnosis of viral gastroenteritis is based on virus isolation by cell culture, electron microscopy and rapid antigen tests (e.g. latex agglutination or EIAs) [157]. Introduction of molecular methods led to an exponential increase in detection rates and the role of difficult-to-culture pathogens became apparent. From a technical point of view, most rapid tests can be done at the bedside, whereas cell culture, electron microscopy and molecular-based methods require laboratories with sophisticated equipment, experienced staff and appropriate biosafety procedures. This certainly limits the use of the latter methods in resource-constrained settings. Data on sensitivity and specificity of diagnostic tools for virus identification in tropical settings are currently lacking.

Adenovirus

Currently, more than 53 types of adenovirus are recognised which can cause a variety of clinical entities, but gastroenteritis is predominantly caused by types 40 and 41 [158,159]. In infected individuals, viral particles are shed in high concentrations. In general, virus isolation followed by serotyping remains the 'gold' standard for the detection of all serotypes and is possible on different cell lines (Table 5). Importantly, 293-Graham cells should be used for stool samples as adenovirus species F (adenovirus types 40 and 41) can only be cultivated on this cell line. However, virus isolation is rather laborious and time-consuming in the face of urgent requests for diagnosis. Electron microscopy is possible with high specificity, but low sensitivity. As an alternative method that is particularly useful for examination of stool samples, antigen detection assays using EIA or latex agglutination have been developed [160,161]. These assays are rapid, but displayed varying sensitivities and specificities in studies, and hence should be complemented by alternative methods. Molecular methods, in particular real-time PCR, have demonstrated superior performance over conventional methods and are now the cornerstone for diagnosis in most laboratories, but are seldom available in resource-constrained settings.

Astrovirus

Eight serotypes of astrovirus are known. In childhood, astrovirus infection with serotypes 1 and 2 predominate, whereas infection with the other serotypes occurs later in life (>4 years). Prolonged diarrhoea has been associated with astrovirus serotype 3 [162]. In the immunocompetent host, viral shedding occurs for 14–70 hours but may be prolonged in immunosuppressed patients. Virus propagation of astrovirus on CaCO-2 or LLC-MK2 cells remains restricted to expert laboratories and is not recommended for routine diagnostic use. Virus identification by electron microscopy is possible, but appearance of viral particles is not always clear. Recently developed antigen detection kits have proven their suitability and are now widely available for rapid diagnosis. However, sensitivity and specificity of rapid tests in comparison to reverse transcriptase (RT)-PCR have been reported to be comparably low [163]. Real-time RT-PCR is the most sensitive and specific method, but remains restricted to reference laboratories.

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Bocavirus

Four different species of human bocavirus (hBoV) have been described thus far [164]. The diagnosis of hBoV infection is almost exclusively based on molecular methods. hBoV has not been isolated by cell culture or in an animal model and rapid antigen tests are currently not available. Serology (e.g. using viral-like particles) has been described and can be used to complement diagnosis [165]. A variety of PCR and real-time PCR assays have been described. However, due to prolonged detection of viral DNA at low copy numbers, qualitative detection of hBoV DNA in gastrointestinal samples is not recommended. There are only few data available for hBoV species 2–4 and the relevance as a true human pathogen is still under debate [164].

Calicivirus

The family *calicivirus* comprises two human-pathogenic genera, the norovirus and sapovirus [166]. For both genera, virus isolation by cell culture is not possible. Electron microscopy is rather insensitive and rarely detects the viruses if there are fewer than 10^6 viral particles/ml of stool suspension.

Norovirus Antigen EIAs have been developed and are commercially available for rapid diagnosis. They proved to be a valuable tool especially in outbreaks, but their sensitivity is limited [167]. A recent study from Brazil reported a sensitivity of 87.9% upon use of a 3rd generation norovirus antigen detection kit [168]. More recently, real-time RT-PCR assays have been described and demonstrated excellent sensitivity and specificity [169]. In-house methods as well as commercial kits are widely available and routinely used.

Sapovirus Specific real-time RT-PCR assays have been developed, but there are no comprehensive data evaluating their diagnostic accuracy. However, there are no diagnostic alternatives because rapid antigen tests are not yet available.

Coronavirus

Five different human pathogenic coronaviruses are known which can cause respiratory and/or to a lesser extent gastrointestinal symptoms in humans. However, the relevance of coronavirus as a true human enteric pathogen is unclear [93,170]. Conventional virus isolation by cell culture can be done on human embryonal tracheal cells. Electron microscopy is possible for stool samples but displays rather low sensitivity. For coronavirus, antigen tests for stool samples are not available. Molecular methods, e.g. real-time RT-PCR assays are the method of choice for a reliable and rapid diagnosis. However, most in-house methods are restricted to reference laboratories, and hence are not commonly employed around the globe.

Cytomegalovirus

In particular immunosuppressed patients are at risk for cytomegalovirus (CMV) infection, which can affect various organ systems, including the gastrointestinal tract [158,171]. Serology represents the method of choice to differentiate primary from secondary infection. Organ-specific diagnosis (e. g. CMV-associated gastrointestinal disease) requires tissue biopsy samples. In combination with histopathology, isolation of CMV by cell culture is recommended. Detection of CMV-DNA by molecular methods alone is not sufficient.

Enterovirus

Enteroviruses belong to the family *picornaviridae* and comprise enterovirus group A to D [172]. In general, enteroviruses can cause a broad spectrum of different clinical entities. Gastroenteritis caused by coxsackievirus A is mostly seen in children. Virus isolation is possible on a range of different cell lines (Table 5). Virus typing after isolation is traditionally accomplished by virus neutralisation. Of note, enteroviruses may be shed into the stool for prolonged time after clearance of acute infection, thus limiting the significance of such a finding. RT-PCR methods are now widely available for the detection of viral genomes. However, sequence variation among the different enterovirus groups can lower the specificity and PCR-based assays should regularly be updated using latest sequence information. Serological methods for the detection of enterovirus-specific antibodies are exclusively available in reference laboratories and cannot be used for rapid diagnosis.

Parechovirus

Parechoviruses have gained recent interest, but their role in acute gastroenteritis and persistent diarrhoea has yet to be established [173,174]. At the time of writing, 16 parechoviruses types have been described. They now represent an own genus within the familiy *picornaviridae* and real-time RT-PCR is the method of choice for diagnosis in high-income settings [175].

Human rotavirus

Rotavirus infection alone is believed to account for 453,000 deaths annually in children younger than 5 years [5]. In most cases, infection causes acute diarrhoea and vomiting with viral particles being shed in high concentrations. Virus isolation is possible on MA104 or CaCO-2-cells but remains laborious and time-consuming. Antigen detection

by EIA methods is the current standard procedure for the rapid diagnosis of rotavirus infection and widely available for diagnosis as well as surveillance. These assays are able to detect virus particles even if their concentration is below 10^4 particles/ml stool suspension. Molecular methods are also available [176,177].

HIV-associated enteropathy

HIV-associated enteropathy frequently occurs in HIV-infected individuals without access to antiretroviral therapy and is characterised by persistent diarrhoea, weight loss, anorexia, abdominal pain and dysphagia. HIV-associated enteropathy should be diagnosed by obtaining intestinal biopsies via endoscopy with subsequent histological and microbiological examination [178]. Antiretroviral treatment of the HIV infection usually also cures the enteropathy.

Discussion

Persistent digestive disorders are unspecific clinical complaints which are commonly reported by many patients around the world. Gastrointestinal or systemic infections are important causes of such disorders with a broad spectrum of possible pathogens involved, including bacteria, intestinal protozoa, helminths and viruses. Due to the wide range of infectious agents which are often difficult to diagnose, great efforts have to be made to reach satisfactory detection rates and to avoid overlooking of important pathogens. Such a diagnostic work-up should include bacterial stool cultures on different selective media (including MacConkey, sorbitol-MacConkey, Leifson and other agar plate cultures), microscopic examination of unstained (e.g. direct faecal smear, Kato-Katz thick smear and formalin ether-concentration method) and stained microscope slides (acid-fast stains, e.g. Kinyoun technique) for parasite identification, and various pathogen-specific tests such as PCR for viruses and diarrhoeagenic *E. coli* pathotypes, toxin detection kits for *C. difficile* diagnosis, and stool concentration methods for *S. stercoralis* (e.g. Baermann funnel and Koga agar plate). Examination of more than one stool specimen over consecutive days is crucial, because many intestinal pathogens are irregularly shed in the faeces [149]. 'Classical' approaches to persistent diarrhoea often lead to disappointing results with up to 80% of cases in which no causative pathogen can be determined [6].

However, even exhaustive laboratory work-up is prone to a host of limitations and challenges that must be considered and addressed. Firstly, gastrointestinal complaints are often caused by non-infectious causes, and a combination of different clinical signs and symptoms as well as further tests are needed to detect and exclude such non-infectious aetiologies. Secondly, available epidemiological data regarding the sought infectious pathogens in the tropics are scarce, thus requiring broad diagnostic testing to avoid overlooking of important pathogens. Thirdly, studies should be carried out in different social-ecological settings to assess the influence of cultural, demographic, genetic, geographic, socioeconomic and health system related factors on predominating pathogens. Fourthly, such research must address all pathogen classes and should not be limited to one-dimensional approaches examining either bacteria or parasites only. Fifthly, there are certain issues unique to gastrointestinal diseases which clearly distinguish them from other organ disorders; most importantly, the finding of a given pathogen may not necessarily mean that the patient's complaints are caused by this organism [179]. Bacteria, helminths and intestinal protozoa may often be found as harmless commensals or even beneficial parts of the gastrointestinal flora, and thus such findings may represent coincidence rather than causality [180-182]. This is of particular importance when different potential pathogens are found concurrently in one faecal specimen and the causative one(s) have to be differentiated. Sixthly, even primarily non-intestinal infectious pathogens may cause gastrointestinal symptoms, as has been reported for HIV infection and even malaria in the tropics, where acute or long-lasting diarrhoea may be the only symptom in up to 20% of all observed cases [183,184]. In contrast, patients may as well start to complain about reduced well-being and develop clinical symptoms only some weeks to months after clearance of an intestinal pathogen, as is the case in postinfectious irritable bowel syndrome [185]. Finally, the variety of possible pathogens affecting the gut is so exhaustive that even very sophisticated diagnostic approaches will not be able to detect every pathogen with satisfactory sensitivity and specificity, especially when considering the cost and practical applicability of some specialised techniques that are not currently feasible in most parts of the tropics.

Conclusion

There is a pressing need for research targeting persistent digestive disorders as a coherent clinical problem rather than as a disconnected collection of pathologies. This would allow the elaboration of evidence-based diagnosis-treatment algorithms centred on patients in resource-constrained settings, where data availability is scarce and patient management often driven by experience and local beliefs. This is the overarching goal of the NIDIAG consortium, focusing on digestive disorders as discussed here, as well as on neurological disorders [23] and persistent fever [24]. Additionally, such investigations will optimise the use of existing diagnostic tests and advance the development of new methods, which are ideally able to concurrently detect a broad spectrum of intestinal pathogens with a high sensitivity and specificity, and which are

simple and affordable enough to be performed in low-income countries where prevalences of persistent digestive disorders are generally high. Moreover, the thorough evaluation of reference tests for intestinal pathogens can serve as diagnostic 'gold' standard in the standardisation and validation of easily applicable RDTs, which are highly needed tools in resource-constrained field settings. Finally, such in-depth investigations are not only important for individual patient management, but also for public health policy making (e.g. to assess the efficacy and cost-effectiveness of ongoing preventive chemotherapy control programmes targeting helminthiases). There is a need for improved diagnostics for persistent digestive disorders in the tropics. It is desirable to conduct a multicentric study to investigate the clinical presentations and respective identified pathogens of large patient cohorts presenting with non-acute gastrointestinal diseases as a first step towards more reliable and evidence-based clinical case management in the tropics.

Competing interest
The authors declare that they have no competing interests.

Authors' contributions
SLB, JV and JU took primary responsibility for the literature search. SLB, JV, SK, MP and JU drafted the manuscript. According to their areas of expertise, the authors critically revised the text chapters (bacteria: SLB, DCW, LvM, CPY and MAM; parasites: SLB, SK, DCW, KP, HM, MS, FM, MAM, LvL, EKN and JU; viruses: MP; clinical aspects: SLB, CPY, JJ, EB and SR). All authors contributed to the manuscript, read and approved the final version.

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4 Diagnosis of neglected tropical diseases among patients with persistent digestive disorders (diarrhoea and/or abdominal pain ≥14 days): a multi-country, prospective, non-experimental case-control study

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Diagnosis of neglected tropical diseases among patients with persistent digestive disorders (diarrhoea and/or abdominal pain ≥14 days): a multi-country, prospective, non-experimental case–control study

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Abstract

Background: Diarrhoea still accounts for considerable mortality and morbidity worldwide. The highest burden is concentrated in tropical areas where populations lack access to clean water, adequate sanitation and hygiene. In contrast to acute diarrhoea (<14 days), the spectrum of pathogens that may give rise to persistent diarrhoea (≥14 days) and persistent abdominal pain is poorly understood. It is conceivable that pathogens causing neglected tropical diseases play a major role, but few studies investigated this issue. Clinical management and diagnostic work-up of persistent digestive disorders in the tropics therefore remain inadequate. Hence, important aspects regarding the pathogenesis, epidemiology, clinical symptomatology and treatment options for patients presenting with persistent diarrhoea and persistent abdominal pain should be investigated in multi-centric clinical studies.

Methods/Design: This multi-country, prospective, non-experimental case–control study will assess persistent diarrhoea (≥14 days; in individuals aged ≥1 year) and persistent abdominal pain (≥14 days; in children/adolescents aged 1–18 years) in up to 2000 symptomatic patients and 2000 matched controls. Subjects from Côte d'Ivoire, Indonesia, Mali and Nepal will be clinically examined and interviewed using a detailed case report form. Additionally, each participant will provide a stool sample that will be examined using a suite of diagnostic methods (i.e., microscopic techniques, rapid diagnostic tests, stool culture and polymerase chain reaction) for the presence of bacterial and parasitic pathogens. Treatment will be offered to all infected participants and the clinical treatment response will be recorded. Data obtained will be utilised to develop patient-centred clinical algorithms that will be validated in primary health care centres in the four study countries in subsequent studies.

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Discussion: Our research will deepen the understanding of the importance of persistent diarrhoea and related digestive disorders in the tropics. A diversity of intestinal pathogens will be assessed for potential associations with persistent diarrhoea and persistent abdominal pain. Different diagnostic methods will be compared, clinical symptoms investigated and diagnosis-treatment algorithms developed for validation in selected primary health care centres. The findings from this study will improve differential diagnosis and evidence-based clinical management of digestive syndromes in the tropics.

Trial registration: ClinicalTrials.gov; identifier: NCT02105714.

Keywords: Bacteria, Diagnosis-treatment algorithm, Helminths, Intestinal protozoa, Neglected tropical diseases, Persistent diarrhoea, Côte d'Ivoire, Indonesia, Mali, Nepal

Background

Disease burden due to persistent digestive disorders in the tropics

Diarrhoeal disorders are among the major communicable diseases worldwide, with a global burden second only to lower respiratory infections and greater than the burden of HIV/AIDS, malaria and tuberculosis [1]. Lack of access to clean water, improved sanitation and hygiene puts individuals in resource-constrained settings of tropical and subtropical countries at high risk of diarrhoea and other digestive disorders [2–4]. Indeed, severe disease manifestations and associated high mortality occur in children and immunocompromised individuals, particularly in low-income countries [5]. Diarrhoea is commonly defined as three or more loose stools per day and can be classified according to the total duration of disease (e.g., acute, prolonged, persistent and chronic), the severity (e.g., light, moderate and severe) and other characteristics (e.g., watery, mucous and bloody) [6]. Yet, some of these terms lack standardisation and are often used interchangeably, which renders comparison of different studies difficult. Figure 1 depicts definitions of diarrhoea, based on recommendations put forth by the World Health Organization (WHO) and expert guidelines [7].

While the epidemiology, aetiological pathogens and clinical management of acute diarrhoea have been extensively studied in both high-income countries and resource-constrained settings [8], far less attention has been addressed to persistent diarrhoea and other non-acute digestive disorders, such as persistent abdominal pain [9]. With regard to persistent diarrhoea, for example, it is conceived that parasitic infections (e.g.,

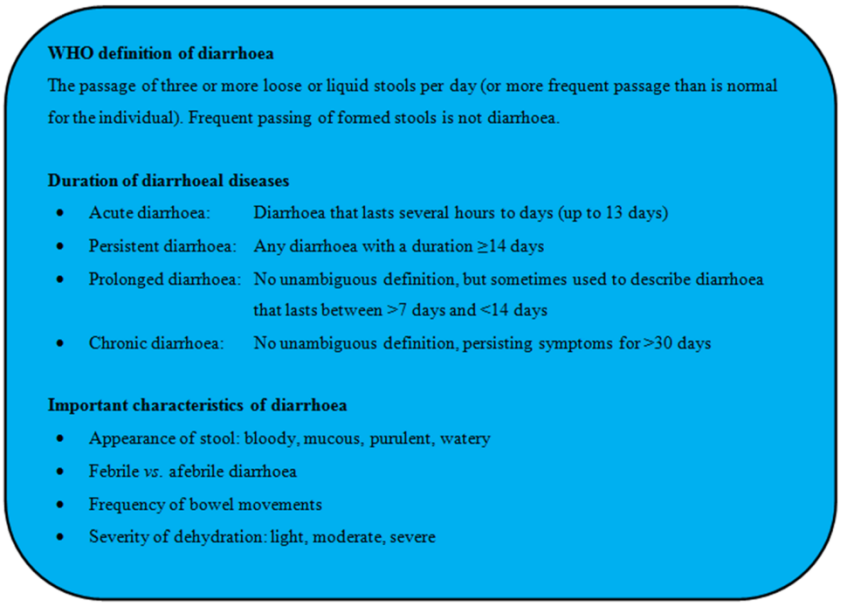

Fig. 1 Synopsis of important definitions and characteristics of diarrhoeal diseases, based on recommendations put forth by the World Health Organization (WHO) and the Infectious Diseases Society of America (IDSA)

helminths and intestinal protozoa) are major pathogens to be considered. Bacterial and viral infections are thought to be of lesser importance, although the implication of bacteria in long-lasting diarrhoea is increasingly being recognised [10]. Current knowledge on parasitic pathogens giving rise to persistent diarrhoea mainly stems from experience gained in Western travel clinics and immunocompromised individuals, while there is a paucity of data from tropical areas [11, 12]. The few published studies focussed mainly on children, sample sizes were generally small and only a limited number of studies had an appropriate design (cohort studies, case–control studies) to properly investigate the true relationship between digestive symptoms and infections caused by specific pathogens [13]. In contrast, acute diarrhoeal diseases have been studied more intensively all over the world, and hence the aetiological spectrum is well characterised [8, 11]. In addition, the recent multicountry 'Global Enteric Multicenter Study' (GEMS) thoroughly investigated the causes of acute diarrhoea in infants and young children in developing countries [14, 15]. As for persistent diarrhoea, the authors of a systematic review published in 2009 concluded that further high quality studies are required to elaborate appropriate clinical guidelines [16]. The review brought to light that diarrhoeagenic *Escherichia coli* pathotypes were found in 30–40 % of children with persistent diarrhoea and intestinal protozoa in 15–20 % of them. Thus far, the potential contribution of helminths to this syndrome has not been studied, although some of these parasitic worms are classically considered as potential causes of persistent diarrhoea and persistent abdominal pain (e.g., *Schistosoma mansoni*, *Strongyloides stercoralis* and *Trichuris trichiura*) [17].

Neglected tropical diseases and their contribution to persistent digestive disorders

The neglected tropical diseases (NTDs) comprise an evolving list of currently over 40 diseases that are caused by helminths (e.g., Schistosoma), intestinal protozoa (e.g., *Entamoeba histolytica*), bacteria (e.g., Shigella), viruses (e.g., dengue virus) and fungi (e.g., *Paracoccidioides braziliensis*) [18]. The global burden of NTDs accumulates to approximately 48 million disability-adjusted life years (DALYs) [19]. NTDs are closely linked to conditions of poverty which hinder social and economic development in endemic countries and negatively impact on people's quality of life and wellbeing at many levels [20–22].

It is poorly understood to what extent NTDs may contribute to the clinical syndrome of persistent diarrhoea and persistent abdominal pain in resource-constrained settings. Long-lasting gastrointestinal complaints are a major reason for consultation of health centres or hospitals in the tropics, but few guidelines exist regarding appropriate clinical management. The problem is exacerbated by a lack of adequate diagnostic tools to guide treatment and control [23, 24]. If diagnostic techniques are available at all in low-income countries, they are usually not sensitive enough to detect NTDs with adequate accuracy (e.g., employment of unstained direct faecal smears for the microscopic diagnosis of intestinal parasites). Additionally, individuals with persistent digestive disorders are commonly managed as outpatients in underfinanced primary health care centres in rural areas, where health system resources are even weaker than in hospital settings [25–28]. Due to the lack of adequate diagnostics and the uncertain role NTDs play as causative agents of persistent digestive disorders, clinicians may frequently assume a bacterial infection and prescribe antibiotic treatment without detailed diagnostic work-up. If symptoms persist or deteriorate despite anti-infective therapy, empirical treatment with one or several antiparasitic drugs may be added. However, it is unknown whether such an approach is medically appropriate, whether adequate clinical cure rates can be achieved and whether this strategy is cost-effective [29].

NIDIAG: developing diagnosis-treatment algorithms for neglected clinical syndromes

NIDIAG is an international collaborative research consortium that aims at improved clinical management of common clinical syndromes in the tropics (http://www.nidiag.org). This 5-year project is funded by the European Commission (EC), Framework Programme 7. NIDIAG aims to develop improved, patient-centred approaches to be applied in primary health care centres of resource-constrained settings. Three clinical syndromes are being investigated: (i) persistent digestive disorders [9]; (ii) persistent fever [30, 31]; and (iii) neurological disorders [32]. Particular emphasis is placed on the contribution of NTDs to each syndrome.

The current study protocol focuses on persistent digestive disorders, which are defined as diarrhoea (≥14 days) in individuals aged ≥1 year and/or abdominal pain (≥14 days) in children and adolescents aged 1–18 years. Adults presenting only with abdominal pain will not be studied because a considerable proportion of persistent digestive symptoms is due to non-infectious causes and beyond the scope of this study [33]. However, children presenting with persistent abdominal pain will be included, even in the absence of diarrhoea. Indeed, it is commonly assumed among tropical clinicians that long-lasting abdominal pain is associated with parasitic and other intestinal infections in this age group, but further clinical evidence is warranted [34].

Goal, aims and objectives

The overarching goal of this study is to develop clinical algorithms that provide an evidence-based syndromic

approach to NTDs at the primary health care level and shall lead to better diagnosis and management of patients with persistent digestive disorders in resource-constrained settings. To this end, we will pursue a multi-centric, prospective case–control study in Côte d'Ivoire, Indonesia, Mali and Nepal. Patients (≥1 year old) presenting with persistent diarrhoea (≥14 days) and/or children and adolescents (aged 1–18 years) with persistent abdominal pain (≥14 days) will be enrolled. Insights gained during the study will help to develop and, in a second study phase, to validate readily applicable diagnostic algorithms that shall facilitate the clinical decision-making and management of patients presenting with persistent diarrhoea and abdominal pain. The following aims and specific objectives are related to this goal.

Aims

1. To improve the quality of clinical care for persistent diarrhoea and persistent abdominal pain through the development of evidence-based diagnosis-treatment algorithms for use in primary health care centres.
2. To identify the major NTDs and other infectious agents (i.e., bacteria and parasites) that give rise to persistent digestive disorders and to assess their relative contribution to this clinical syndrome.
3. To compare different diagnostic methods and to assess their diagnostic accuracy, including clinical features, conventional laboratory techniques, rapid diagnostic tests (RDTs) and molecular assays for the diagnosis of selected pathogens.
4. To assess the clinical response to commonly employed empirical treatment options for persistent digestive disorders.

Specific objectives and activities

1. To examine stool samples from symptomatic patients presenting with persistent diarrhoea and children/adolescents with persistent abdominal pain with a range of standardised, quality-controlled laboratory techniques (e.g., microscopic techniques, RDTs and stool culture) for detection of parasites and pathogenic bacteria.
2. To examine stool samples from matched healthy controls without gastrointestinal complaints with the same suite of diagnostic tests and approaches.
3. To retrospectively analyse a (sub-)sample of stool specimens using multiplex polymerase chain reaction (PCR) assays in reference laboratories. PCR tests will be used to assess the diagnostic accuracy of conventional tests, as well as to obtain data on additional pathogens.
4. To assess the relative importance of the pathogens detected in symptomatic individuals, as compared to healthy controls.
5. To perform a standardised medical examination on all participants and to accurately document clinical signs and symptoms, risk factors, clinical management and response to treatment in a case report form (CRF).
6. To calculate the predictive values of clinical and laboratory data to provide evidence-based data for the development of a diagnostic algorithm.
7. To develop and validate one or several diagnosis-treatment algorithms for the management of persistent digestive disorders in primary health care settings of resource-limited countries.
8. To assess the cost-effectiveness of different approaches for the diagnosis of selected NTDs in patients presenting with persistent digestive disorders.

Methods/Design

Study area

For this study, four low- or middle-income countries, two in West Africa (Côte d'Ivoire and Mali) and two in Asia (Indonesia and Nepal), were selected as study sites to cover a broad geographic range, diverse pathogen profiles and different health systems. Prior to the selection of the study sites, an extensive review of existing epidemiological data on diarrhoeal diseases and the targeted NTDs in the study countries was conducted. An assessment of currently employed diagnosis-treatment practices and ongoing control activities in each country confirmed that all four countries are endemic for the main target NTDs/pathogens under investigation. In Côte d'Ivoire and Mali, currently no national guidelines or algorithms exist for the diagnosis and treatment of persistent diarrhoea or persistent abdominal pain. In Nepal, some paediatric guidelines exist, but no standardised approach towards persistent diarrhoea in adults has been developed thus far. Indonesia is the only country where, since 2011, a national programme for the management of diarrhoeal diseases is in place. All study countries have national programmes for control of NTDs, but there is considerable variation with regard to control approaches and the spectrum of NTDs covered, varying from strategies that target exclusively intestinal helminths (e.g., in Nepal) to multidimensional integrated NTD control programmes (e.g., in Mali) [35].

Our research will be conducted in peripheral health centres and referral level hospitals in order to capture a large variety of clinical presentations of the digestive syndrome. The study sites were selected on the basis of the following criteria: (i) location in remote rural areas or deprived urban settings; (ii) existing referral links between primary health care centre and district hospital (e.g., for diagnosis and treatment of complicated or severe conditions); (iii) adequate patient care in the referral hospitals; (iv) persistent digestive disorders being a relevant and frequently occurring clinical syndrome, as judged by the local physicians and nurses; (v) available

laboratory infrastructure and staff capacity to perform standard first-line tests and to process field samples for external work-up; and (vi) key staff trained in good clinical practice (GCP) and good clinical laboratory practice (GCLP).

Country-specific assessments were carried out during the site selection procedure. The following seven study centres were selected in the four study countries (Fig. 2):

- Côte d'Ivoire
  - Hôpital Méthodiste de Dabou, a regional reference hospital based in Dabou, approximately 30 km west of Abidjan, the country's economic capital.
- Indonesia
  - Tulehu Hospital, based in Tulehu in Maluku province, approximately 100 km northeast of Ambon City, the province's capital; and
  - Tulehu Health Centre, also situated in Maluku province in close proximity (approximately 1 km) to the Tulehu Hospital.
- Mali
  - Niono District Reference Health Centre, based in Niono, approximately 300 km northeast of the country's capital Bamako; and
  - Institut National de Recherche en Santé Publique (INRSP) in Bamako.
- Nepal
  - B P Koirala Institute of Health Sciences, situated in Dharan, approximately 350 km southeast of the country's capital Kathmandu; and
  - Dhankuta District Hospital in Dhankuta, approximately 50 km north of Dharan.

Study design

Our investigation is designed as a prospective, non-experimental case–control study to determine the relative importance of NTDs in patients who present with persistent digestive disorders. The case–control design was adopted because of the observation that multiple digestive pathogens may be found even in asymptomatic individuals, and hence these may rather represent 'innocent bystanders' that do not play a causal role in the aetiology of the persistent symptomatology [36, 37]. We believe that the inclusion of a control group will provide discriminative data on the distribution of pathogens and might be useful to enhance interpretation of causal associations [38, 39].

Study participants

The inclusion criteria for cases are (i) all individuals (aged ≥1 year) presenting with persistent diarrhoea (≥14 days); and/or (ii) children/adolescents (aged 1–18 years) with persistent abdominal pain (≥14 days). For persistent diarrhoea, the WHO definitions are used (see also Fig. 1). WHO defines diarrhoea as the passing of three or more loose stools within 24 h. A new episode of diarrhoea can occur after two full days without diarrhoea. Episodes of diarrhoea lasting for 14 days and longer are defined as persistent [6]. For persistent abdominal pain, no official WHO definition exists. Here, we define persistent

Fig. 2 World map and country-specific maps of Côte d'Ivoire, Indonesia, Mali and Nepal, indicating the sites of patient recruitment for the NIDIAG study on persistent digestive disorders

abdominal pain as localised or diffuse abdominal pain lasting for at least 14 days (possibly with intermittence/recurrence). To each enrolled patient, one control without any gastrointestinal complaints will be matched by age group, sex and geographical location of residence. These may be patients who present to the same hospital or outpatient facility as the case, but with non-related complaints (e.g., with ophthalmological diseases or consulting for vaccination or 'routine' check-up). If the patient was referred to the hospital by one of the peripheral health facilities in the catchment area, the control will be (actively) selected through the same or a nearby peripheral health facility.

Patients and controls presenting with the following characteristics will be excluded: (i) unwilling or unable to give informed consent; (ii) unable in the study physician's opinion to comply with the study requirements; (iii) presenting with clinical jaundice (as assessed by direct observation of the conjunctivae); (iv) already participating in other ongoing diagnostic studies and/or clinical trials; and (v) in need of immediate intensive or surgical care (including severely malnourished children).

Sample size

The lack of available data on the frequency of persistent digestive disorders in resource-constrained settings and the diversity of implicated infectious pathogens renders sample size calculations difficult [40]. A systematic review on pathogens associated with persistent diarrhoea in children in low- and middle-income countries reported prevalences of about 10 % for *Giardia intestinalis*, 5–10 % for *Campylobacter* spp. and around 5 % for *Cryptosporidium* spp. [16]. Prior data from Côte d'Ivoire [37, 41] indicate that the probability of infection with target pathogens such as *S. stercoralis* is around 10-15 % in symptomatic cases and 5–10 % in healthy controls. If the true probability of infection among cases is 10 %, 435 cases and 435 controls need to be included to be able to reject the null hypothesis that the infection rates for case and controls are identical with a probability (power) of 0.8 (2-sided alpha = 0.05). To compensate for loss to follow-up of 10 %, we aim at enrolling a total of 500 cases and 500 controls in each of the four study countries. It should be noted, however, that no robust information exist to date, and hence most data and resulting calculations remain somewhat speculative. As most reported prevalences of several important target NTDs (e.g., hookworm and *G. intestinalis*) are in the same range as described above, we adhered to these assumptions, and hence, we aim for a total of 2000 patients and 2000 matched controls.

Patient recruitment and clinical management

The recruitment strategy will be adapted to the local setting, taking into consideration the local norms, culture and health care system organisation. Before the start of the study, appropriate context-adapted mechanisms will be set up to inform the community's representatives in the catchment area of the respective study centres about the study, its objectives, the implications for the community and potential future impacts. All procedures during the study will be guided by an extensive set of specifically developed standardised operating procedures (SOPs). Figure 3 shows the patient recruitment and study flow according to the respective SOPs. Whenever a patient presents to one of the study centres with self-reported persistent digestive disorders (≥14 days), the care provider will immediately contact the study investigator. The study investigator will assess inclusion/exclusion criteria by medical history taking, review of existing medical documents (e.g., results of laboratory tests, note from referring physician) and a rapid clinical assessment. Patients who meet the inclusion criteria will be invited to participate in the trial. Once informed consent has been obtained, the selected individuals will undergo a full clinical assessment and will be asked to provide one fresh stool sample. In the two West African countries, an additional urine sample will be obtained for diagnostic work-up. Based on the initial assessment, the study physician will document a syndromic diagnosis (e.g., suspected parasitic intestinal infection) as well as any therapeutic decision (e.g., initiation of empirical treatment). Of note, all decisions will be documented, but the current practices of the clinician will not be influenced (non-interventional trial). All patients will be followed and a systematic second visit for in-depth evaluation will be conducted 3–5 days after the first visit. By the time of the second visit, all microbiological laboratory results will be available and the treatment may be adapted in case of a specific detected pathogen or an unsatisfactory clinical evolution. Patients with persistent symptoms will be asked to provide another stool sample for detailed diagnostic work-up. If need be, a patient might be assessed during a third visit that is performed one to two weeks after the second visit. In case of clinical complaints being still present, a third stool sample may be tested and treatment might be modified again. Additional visits and further laboratory work-up will be left to the discretion of the care provider. Each time, new or persistent clinical symptoms and signs as well as all treatment decisions will be recorded. The final diagnosis for each patient will be based on the combination of laboratory tests, clinical response to treatment and additional investigations requested by the treating clinician (e.g., abdominal ultrasound examination).

Individuals without any gastrointestinal complaints will be invited to participate as controls in this trial. After informed consent has been obtained, the diagnostic procedures applied to the controls are identical as for the cases.

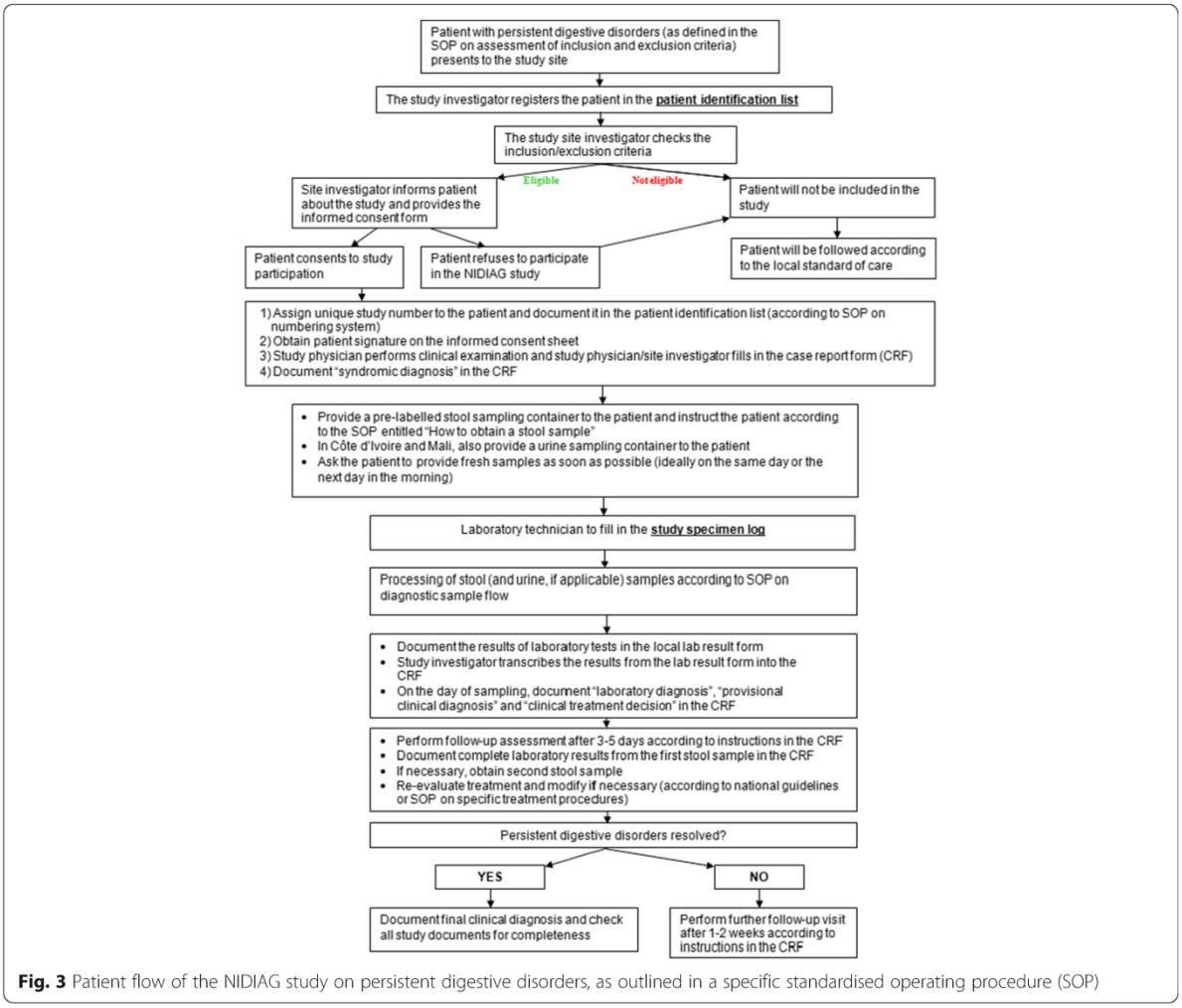

Fig. 3 Patient flow of the NIDIAG study on persistent digestive disorders, as outlined in a specific standardised operating procedure (SOP)

Diagnostic tests and laboratory procedures

All laboratory activities will be carried out according to the specific SOPs. The diagnostic tests to be employed were selected after a systematic review of the peer-reviewed literature and standard textbooks, supplemented with expert opinion [9]. Four different classes of tests will be employed during the study, namely (i) stool microscopy and stool concentration techniques for the diagnosis of intestinal protozoa and helminths (on site; all study countries); (ii) a stool-based RDT for the diagnosis of *Cryptosporidium* spp. and *G. intestinalis* (on site; all study countries) and a urine-based RDT for the detection of *S. mansoni* (on site; only in Côte d'Ivoire and Mali); (iii) bacteriological stool culture for the diagnosis of selected enteric bacteria in patients presenting with persistent diarrhoea (in national reference laboratories; all study countries except Indonesia, where the capacity and available laboratory equipment to perform bacteriological stool cultures in the selected centre did not meet the required reference standards); and (iv) PCR assays for the detection of parasitic and, possibly, additional bacterial and viral pathogens (in European reference laboratories; ethanol-fixed stool samples from all study countries). Table 1 provides an overview of the applied tests and the pathogens that can be detected by the individual techniques. Most of the test results will be available within 3 days after provision of the stool sample so that targeted treatment of the detected pathogen can be offered to the patient, based on national guidelines and evidence-based treatment recommendations. However, it is important to note that the PCR tests will only be conducted several weeks or months after stool sampling, and hence, these test results will not be available in a timely manner to guide clinical patient management.

Table 1 Diagnostic tests to be employed on stool (and urine) samples from patients with persistent digestive disorders and healthy controls in Côte d'Ivoire, Indonesia, Mali and Nepal during the NIDIAG study. Note: except for the rapid diagnostic test (RDT) for *Schistosoma mansoni* which uses urine, all tests are performed on stool samples

Diagnostic test	Targeted pathogens	Reference(s)
Microscopy		
Direct faecal smear	Helminths, intestinal protozoa	[55]
Kato-Katz thick smear	Helminths	[56]
Acid-fast staining	*Cryptosporidium* spp., *Cyclospora cayetanensis*, *Cystoisospora belli*	[57]
Baermann funnel concentration technique	*Strongyloides stercoralis*, hookworm	[41, 58]
Formalin-ether concentration technique	Helminths, intestinal protozoa	[59, 60]
Mini-FLOTAC	Helminths	[61, 62, 63]
Culture		
Bacteriological stool culture	*Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp.	[9]
Koga agar culture	*Strongyloides stercoralis*, hookworm	[64–67]
Rapid diagnostic tests (RDTs)		
Crypto/Giardia DuoStrip	*Cryptosporidium* spp., *Giardia intestinalis*	[37]
Circulating cathodic antigen (CCA)[a]	*Schistosoma mansoni*	[68–72]
Molecular post-hoc testing on ethanol-fixed stool samples		
Multiplex PCR	Helminths, intestinal protozoa (all samples); diarrhoeagenic bacteria and viruses (selected sub-sample)	[73–76]

[a]This test will only be employed in Côte d'Ivoire and Mali, because *S. mansoni* does not occur in Indonesia and Nepal

Laboratory quality control and monitoring

We will adhere to rigorous quality control procedures for all laboratory investigations specified in Table 1. A 'quality manager' will be designated in each study site to actively monitor the laboratory activities throughout the study. This person will verify the storage conditions and accountability of the reagents used for the studies. It will be carefully checked that samples are collected, transferred, processed, analysed and stored according to SOPs. Moreover, 5–10 % of all processed stool samples will be re-read under a microscope by experienced laboratory technicians. A photo of each RDT result will be taken at the time of reading, and subsequently documented. The photos and the recorded results will be checked for consistency by the quality manager. Additionally, external monitoring will be conducted at regular intervals, and according to GCLP standards. These measures will ensure quality and reproducibility of all laboratory results within and across study countries.

Economic evaluation

The cost and cost-effectiveness of the different diagnostic laboratory tests and diagnosis-treatment algorithms will be assessed and compared to existing practice. The economic evaluation will take the perspective of the public health care system, focussing on practice and implementation at the primary care level. The costing of the baseline and the diagnosis-treatment algorithm, including all diagnostic tests used in the study, will be done using a combination of top-down and bottom-up costing methodologies [42]. Information will be collected on recurrent items (e.g., staff, supplies and reagents) and capital costs (e.g., building, equipment and furniture). For the estimation of the unit costs of diagnostic tests, information on resource usage will be collected through time-and-motion studies. Resources will be valued at their opportunity cost, including the value of donated or subsidised items (i.e., economic costing). Capital items will be annualised over their expected life span.

Data collection

All clinical and laboratory data will be recorded in a specifically designed and pre-tested CRF. Two distinct CRFs were developed; one for patients and one for controls. The study investigator/clinician will complete one CRF for each study participant to document demographic data (e.g., age, sex and residency), clinical data (e.g., medical history, including data on previous exposure and risk behaviour, current symptoms, physical examination, new or persistent clinical symptoms and signs, clinical evolution, final clinical outcome), laboratory data (e.g., clinical sampling, test results for each sample,

laboratory evolution), diagnosis (e.g., syndromic diagnosis, working and final diagnosis based on available clinical and laboratory results) and treatment decisions (e.g., abstention or initiation of empirical or targeted treatment, additional treatments, treatment modifications and treatment response). All treatments are recorded in a separate document (medication form). Any additional clinical or laboratory investigations as well as unscheduled visits will be documented. The consistency and quality of data collection will be checked regularly by an internal quality control manager and during the recruitment phase via external monitor visits.

Data management and storage of data

Data management at the respective study centres will be carried out under the guidance and supervision of a central data manager. The local data manager will be responsible for supervising data entry and local data management. Completed CRFs will be submitted to the local data management, where data of the CRF will be recorded into the study database. After data cleaning and database lock, the database will be shared with the collaborating institutes. All data entry and management will be done by trained study personnel. As required by international guidelines and national regulations, the study file, signed informed consent sheets, source documents, copy of the CRF and subject identification codes will be retained for at least 2 years (or for the time period required by national legislation) at each study site (ICH-GCP 4.9.5) to allow for audits and inspections even after the study completion. The study investigators are responsible for reporting a patient's and control's personal details and identification number in a participant identification list. To ensure confidentiality, this list will be kept in a separate, locked cupboard together with the signed informed consent forms and only the study investigators and clinical study staff will have access to it.

All laboratory specimens, including preserved samples, and all subject-related reports, forms and study data will be identified by a coded number. Subject names will not be used. All local databases will be secured with password-protected access systems. A list of authorised personnel to access the databases throughout the study will be held. Regular backups of the database at the study sites will be performed and shared with the central data manager. Computers and other study hardware (e.g., external memory to store backups) will be used only for study purposes and kept in locked cupboards and/or rooms. Any personal study information will not be released to anybody outside the medical team, except to competent authorities for independent monitoring, auditing and inspection. Throughout the study, strict confidentiality, quality, and security of the subjects' data will be pursued.

Data analysis

Statistical analyses will be carried out with STATA version 12 (StataCorp LP; College Station, USA) or SPSS Statistics version 22 (IBM Corporation; Armonk, USA). The prevalence and, in case of parasitic infections, the intensity of infection with targeted NTDs will be utilised as primary outcome measure. Logistic regression models will be employed to estimate associations of putative pathogens with persistent digestive disorders. The aetiological fraction for matched case–control studies will be calculated to estimate the fraction of cases with digestive disorders due to a specific pathogen. The sensitivity of clinical and laboratory predictors and of RDTs will be calculated as the proportion of patients with a given disease ("confirmed" diagnosis only) who present with a clinical feature or show a positive test result. The specificity of clinical and laboratory predictors and of RDTs will be calculated as the proportion of individuals without the disease ("confirmed" diagnosis only) who do not present with this feature or show a negative test result. Positive and negative likelihood ratio of clinical and laboratory predictors and of RDTs will be calculated using standard formulas. The positive likelihood ratio (LR+) will be obtained by dividing the sensitivity of individual predictor by 1 minus the specificity of the predictor. The negative likelihood ratio (LR-) will be obtained by dividing 1 minus the sensitivity of the predictor by its specificity. The positive predictive value (PPV) of clinical and laboratory predictors and of RDTs as well as their combination will be calculated according to Bayes' formula as the proportion of patients with a clinical feature or a positive test result who have the disease. The negative predictive value (NPV) of clinical and laboratory predictors and of RDTs as well as their combination will be calculated as the proportion of individuals without this feature or with a negative test result who do not have the disease.

Ethical approval

The study protocol was discussed, reviewed and modified by several experts within the NIDIAG research consortium and its independent scientific and ethical board. It was approved by the institutional review boards (IRBs) at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium and the Swiss Tropical and Public Health Institute (Swiss TPH) in Basel, Switzerland prior to external review. Country-specific approvals were subsequently granted by the followings ethics committees: (i) University of Antwerp in Belgium (12 August 2013); (ii) Gadjah Mada University in Indonesia (21 November 2013); (iii) 'Ethikkommission beider Basel' (EKBB) in Switzerland (22 November 2013); (iv) 'Institut National de Recherche en Santé Publique' in Mali (28 November 2013); (v) Ministry of Health in Côte d'Ivoire (28 November 2013);

and (vi) 'Nepal Health Research Council' (NHRC) in Nepal (29 January 2014). The trial is registered on ClinicalTrials.gov (identifier: NCT02105714). Should any ethical issues arise during the conduct of the study, these will be promptly discussed within the NIDIAG ethical board and referred to the concerned IRBs.

Policy and dissemination strategy

NIDIAG aims to improve quality of clinical care for three common syndromes in the tropics and will provide specific efforts to translate evidence into policy. Hence, policy makers will be involved in the project from an early stage and will be consulted during the development of the following decision support tools: (i) development and validation of algorithms for the clinical management of persistent digestive disorders; (ii) cost estimates and cost-effectiveness analyses of integrated algorithms and diagnostic platforms compared to single-disease oriented laboratory tests; and (iii) analysis of acceptability and appropriateness of innovative diagnosis-treatment tools for application in the primary health care settings. A comprehensive translation-to-policy strategy will be put in place and insights gained through the NIDIAG study will be widely disseminated among patient groups and participating communities.

Discussion

Digestive disorders are among the major clinical syndromes that characterise a host of NTDs in Africa, Asia, Latin America and elsewhere. The morbidity due to diarrhoeal diseases in low- and middle-income countries continues to be considerable. While the aetiological spectrum of acute diarrhoea has been widely investigated, the major pathogens giving rise to persistent diarrhoea and other non-acute digestive disorders remain to be elucidated [9, 16].

There is a pressing need to assess frequently encountered clinical syndromes in resource-constrained settings from a patient-centred perspective, rather than relying on single disease-oriented approaches. Indeed, a recent study in Tanzania investigated the causes of fever in children and brought to light that viral infections predominate and are considerably more common than parasitic diseases (e.g., malaria) and bacterial infections (e.g., bacterial pneumonia) [43]. In contrast to this finding, clinicians commonly prescribe antimalarial and antibacterial medication in the absence of diagnostic testing facilities, which in turn leads to overtreatment with potential negative consequences (e.g., development of resistance and drug toxicity). It is conceivable that similar misconceptions and mismanagement apply to other clinical syndromes, including persistent diarrhoea and persistent abdominal pain, which is the focus of the current study protocol. It has been postulated that an improved syndromic approach to highly prevalent complaints such as persistent diarrhoea, persistent abdominal pain or long-lasting fever will require (i) a deeper understanding of the aetiology and epidemiology of the target diseases; (ii) the availability of readily applicable RDTs in the most affected areas; and (iii) the development of evidence-based algorithms for the management of these syndromes [30]. NIDIAG aims to address these issues for three clinical syndromes: the persistent digestive disorders discussed here, along with neurological disorders and persistent fever.

Several considerations underscore the need for a concerted, multi-centric assessment of persistent diarrhoea and persistent abdominal pain in resource-constrained settings, with particular emphasis on the primary health care level. First, digestive syndromes are associated with many different NTDs, particularly helminth and intestinal protozoa infections [44], but the magnitude of their contribution to persistent digestive disorders is largely unknown. A single disease perspective will not be able to determine the relative importance of NTDs in patients who present with persistent diarrhoea and persistent abdominal pain; hence a syndromic and patient-centred approach seems more promising. Second, for some of the NTDs that may give rise to digestive disorders, public health programmes to control these infections have been implemented [45]. For instance, the control of several helminth NTDs currently relies on preventive chemotherapy; that is the regular, large-scale administration of one or several orally available drugs with the aim of reducing the parasite load and thus preventing the development of severe disease [46, 47]. These strategies offer opportunities for a rapid impact at low cost in resource-constrained settings, especially in areas where the targeted diseases are highly prevalent. However, the long-term sustainability of these programmes has been questioned [48, 49]. Recently, public health specialists put particular emphasis on integrated control approaches that go beyond preventive chemotherapy [50, 51]. The disease-specific focus of many NTD control programmes has led to fragmentation and gaps in patient management. At the primary health care level, however, nurses and physicians do not deal with a single disease, but with patients presenting with a wide spectrum of complaints. Among these clinical syndromes, they have to recognise the specific NTDs and discriminate these from a constellation of other illnesses. Hence, there is a need for evidence-based guidelines on the management of a specific clinical syndrome and the contribution of NTDs rather than single programmes targeting one of the aetiological agents. There is growing consensus that well-functioning health systems are required to achieve sustainable NTD control and improved diagnosis-treatment algorithms are key to this [52, 53]. Third, as control of

selected NTDs is moving towards elimination in selected settings, it will be important to conduct studies in the primary health care settings to allow for an unbiased detection and surveillance of these infections. Such an assessment can only be achieved on a large scale by the first-line caregivers and available evidence-based algorithms for diagnostic work-up would provide a useful tool for patient management.

Taken together, the NIDIAG digestive study offers an integrated syndromic approach to NTD-related clinical syndromes, focussing on digestive disorders defined as persistent diarrhoea (≥14 days of diarrhoea in all individuals aged ≥1 year) and persistent abdominal pain (≥14 days in children and adolescents aged 1–18 years). The overarching goal is the elaboration of evidence-based diagnosis-treatment algorithms centred on patients in resource-constrained settings, where available data are scant and patient management is often driven by 'empirical evidence' and local beliefs. Furthermore, this study will help to assess the contribution of NTDs to persistent digestive disorders and, subsequently, develop and evaluate sustainable and cost-effective control programmes for these infections. Additionally, the NIDIAG digestive study will optimise the use of existing diagnostic tests and advance the development of new methods, which must be simple to use, affordable for application in low-income countries and able to detect a broad spectrum of intestinal pathogens with high accuracy [54]. Finally, the insights gained during this multi-country prospective study will also help to re-estimate the burden of persistent digestive disorders and may influence future public health recommendations and health policy planning.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KP, SLB, PY and JU drafted the manuscript. All authors contributed to the development of the study protocol, essential study documents and standard operating procedures to be employed during the NIDIAG study. According to their different areas of expertise, the authors critically revised specific parts of this manuscript (clinical aspects: JJ, SR, LvM, MB and FC; data management: JJ, HvL and PKY; diagnostic techniques: MWB, JJ, LvL and LvM; ethics: EA, NSH, RWP and RR; health economics: SB and FM; methodology: EA, JJ, AL, MB and FC; study country-specific issues: AL, FM, SR, KDS). All authors read and approved the final version prior to submission.

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5 Experiences and lessons from a multi-country NIDIAG study on persistent digestive disorders in the tropics

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Persistent digestive disorders can be defined as any diarrhea (i.e., three or more loose stools per day) lasting for at least 2 weeks and/or abdominal pain that persists for 2 weeks or longer [1-3]. These disorders cause considerable morbidity and human suffering, and hence, are reasons why people might seek primary health care. However, in resource-constrained settings of the tropics and subtropics, accurate point-of-care diagnostics are often lacking and treatment is empiric, particularly in remote rural areas with no laboratory infrastructure. As a result, the relative contribution of selected pathogens to the syndrome of persistent digestive disorders is poorly understood, and evidence-based guidelines for patient management in different social-ecological settings are scarce [4-6].

In order to improve the clinical management of patients with persistent digestive disorders stemming from neglected tropical diseases (NTDs), the European Commission (EC) funded a 5-year study – the NIDIAG research consortium. The overarching goal of the NIDIAG consortium is to develop and validate patient-centered diagnosis-treatment guidelines for use at the primary health care level in low- and middle-income countries (http://www.nidiag.org) [3,7-9]. Emphasis is placed on three syndromes: (i) persistent digestive disorders described here; (ii) persistent fever; and (iii) neurological disorders that latter two of which are detailed in companion pieces published in the same issue of *PLoS Neglected Tropical Diseases*.

With regard to the study on persistent digestive disorders, the main aims are (i) to identify the most important NTDs and other infectious agents that give rise to this clinical syndrome, including their relative frequency; (ii) to assess and compare the accuracy of different diagnostic methods; and (iii) to determine clinical responses to commonly employed empiric treatment options for persistent digestive disorders [9]. To this end, a case-control study has been implemented in four countries; Côte d'Ivoire and Mali in West Africa, and Indonesia and Nepal in Asia. An integral part of the NIDIAG consortium is to ensure that good clinical

practice (GCP) and good clinical laboratory practice (GCLP) are adhered to during the conduct of the studies [10,11]. A quality assurance system, which included the development and implementation of a set of standard operating procedures (SOPs), along with on-the-spot staff training and internal and external quality control activities, has been developed at project level, and introduced at each study site. The development and adherence to SOPs within harmonized study protocols were considered crucial steps for maximizing integrity of laboratory and clinical data across study settings. It also provided the basis on which quality control activities could be performed.

For Which Procedures Have SOPs Been Developed?

For the study on persistent digestive disorders, 33 specific SOPs have been developed (Supporting Information, S1-S33). As summarized in Table 1, detailed steps on clinical and laboratory procedures, data management, and quality assurance were described. With regard to clinical investigations, SOPs on history taking and clinical examination, assessing inclusion and exclusion criteria, patient recruitment, and study flow were developed (S1-S6). Detailed instructions on how to perform a set of laboratory diagnostic techniques for the detection of helminth and intestinal protozoa infections were included in the laboratory SOPs. Different conventional stool microscopy techniques were combined with more recent rapid antigen detection tests to encompass a broad spectrum of potentially implicated pathogens with high diagnostic accuracy (S7-S20). An overview of the employed diagnostic methods is provided in Table 2. Pertaining to data management, SOPs on completion of case report forms (CRFs) and on its various activities (such as data entry, data cleaning, querying, database locking, and backing up data) were also included. To ensure quality control, SOPs on internal quality control activities, external monitoring, and laboratory supervision visits were jointly developed for the three syndromes (S21-S33).

Table 1. Set of standard operating procedures (SOPs) used in the NIDIAG study on persistent digestive disorders.

Number	Type	Purpose of SOP	End user	Syndrome
1	Clinical	History taking	Site investigator	Digestive
2	Clinical	Clinical examination	Site investigator	Digestive
3	Clinical	Selection of controls without digestive syndrome	Site investigator	Digestive
4	Clinical	Specific treatment procedures (including dosage)	Site investigator	Digestive
5	Clinical	Assessing inclusion and exclusion criteria	Site investigator	Digestive
6	Clinical	Patient recruitment and patient flow	Site investigator	Digestive
7	Laboratory	Kinyoun staining technique	Laboratory technician	Digestive
8	Laboratory	Modified acid-fast staining technique	Laboratory technician	Digestive
9	Laboratory	Crypto/Giardia Duo-Strip rapid diagnostic test (RDT)	Laboratory technician	Digestive
10	Laboratory	Kato-Katz technique	Laboratory technician	Digestive
11	Laboratory	Baermann technique	Laboratory technician	Digestive
12	Laboratory	Mini-FLOTAC technique	Laboratory technician	Digestive
13	Laboratory	How to obtain a stool sample	Laboratory technician	Digestive

14	Laboratory	Formalin-ether concentration technique	Laboratory technician	Digestive
15	Laboratory	Koga agar plate culture	Laboratory technician	Digestive
16	Laboratory	Direct fecal smear technique	Laboratory technician	Digestive
17	Laboratory	Preparation of aliquots for molecular post-hoc testing	Laboratory technician	Digestive
18	Laboratory	Urine point-of-care circulating cathodic antigen (POC-CCA) RDT for the diagnosis of *Schistosoma mansoni*	Laboratory technician	Digestive
19	Laboratory	Diagnostic sample flow	Laboratory technician	Digestive
20	Laboratory	Urine sampling	Laboratory technician	Digestive
21	Quality	Obtaining informed consent	Site investigator	Common
22	Quality	Numbering system to be used in NIDIAG studies	Laboratory technician	Digestive mainly
23	Quality	Management of study documents	Principal investigator (PI)/site investigator/ nurse/laboratory technician	Common
24	Quality	SOP on SOP	SOP author	Common

25	Quality	External monitoring	PI/site investigator	Common
26	Quality	Internal quality control activities	Quality manager	Common
27	Quality	Good clinical laboratory practice (GCLP) supervision visits	Quality manager	Common
28	Quality	Min/max thermometer	Laboratory technician	Common
29	Quality	Stock management	Laboratory technician	Common
30	Quality	Handling of expired and disqualified products	Laboratory technician	Common
31	Quality	Handling and storage of rapid diagnostic tests	Laboratory technician	Digestive
32	Data	Completing case report forms (CRFs)	Site investigator	Common
33	Data	Procedure for data management	Data manager	Digestive

Table 2. Laboratory diagnostic techniques used and internally compared in the NIDIAG study on persistent digestive disorders.

Diagnostic technique	**Target pathogen(s)**			
	Soil-transmitted helminths	*Schistosoma mansoni*	*Strongyloides stercoralis*	Intestinal protozoa
Direct fecal smear	(✓)	(✓)	(✓)	(✓)
Kato-Katz technique	✓	✓	–	–
Formalin-ether concentration technique	✓	✓	(✓)	✓
Mini-FLOTAC	✓	✓	–	(✓)[a]
Baermann technique	(✓)[b]	–	✓	–
Koga agar plate culture	(✓)[b]	–	✓	–
Rapid diagnostic test for *Cryptosporidium* and *Giardia intestinalis*	–	–	–	✓[c]
Point-of-care circulating cathodic antigen (POC-CCA) urine cassette test	–	✓	–	–
Acid-fast staining procedure	–	–	–	✓[d]

The laboratory diagnostic techniques consisted of microscopic methods and rapid diagnostic tests (RDTs). They were used and compared within the NIDIAG study on persistent digestive disorders, placing particular emphasis on the suitability for the detection of helminths and

intestinal protozoa that may give rise to persistent digestive disorders (≥2 weeks). The following grading system was employed to characterize the suitability of a certain laboratory technique for the detection of specific pathogens: ✓: suitable; (✓): partially suitable; –: not suitable.

Of note, additional bacteriologic stool cultures were performed in all countries except Indonesia in case of diarrheic stool samples.

[a] Very limited published data, according to which FLOTAC techniques may detect some intestinal protozoa species (e.g., *G. intestinalis*), but further validation of the technique for this use is required.

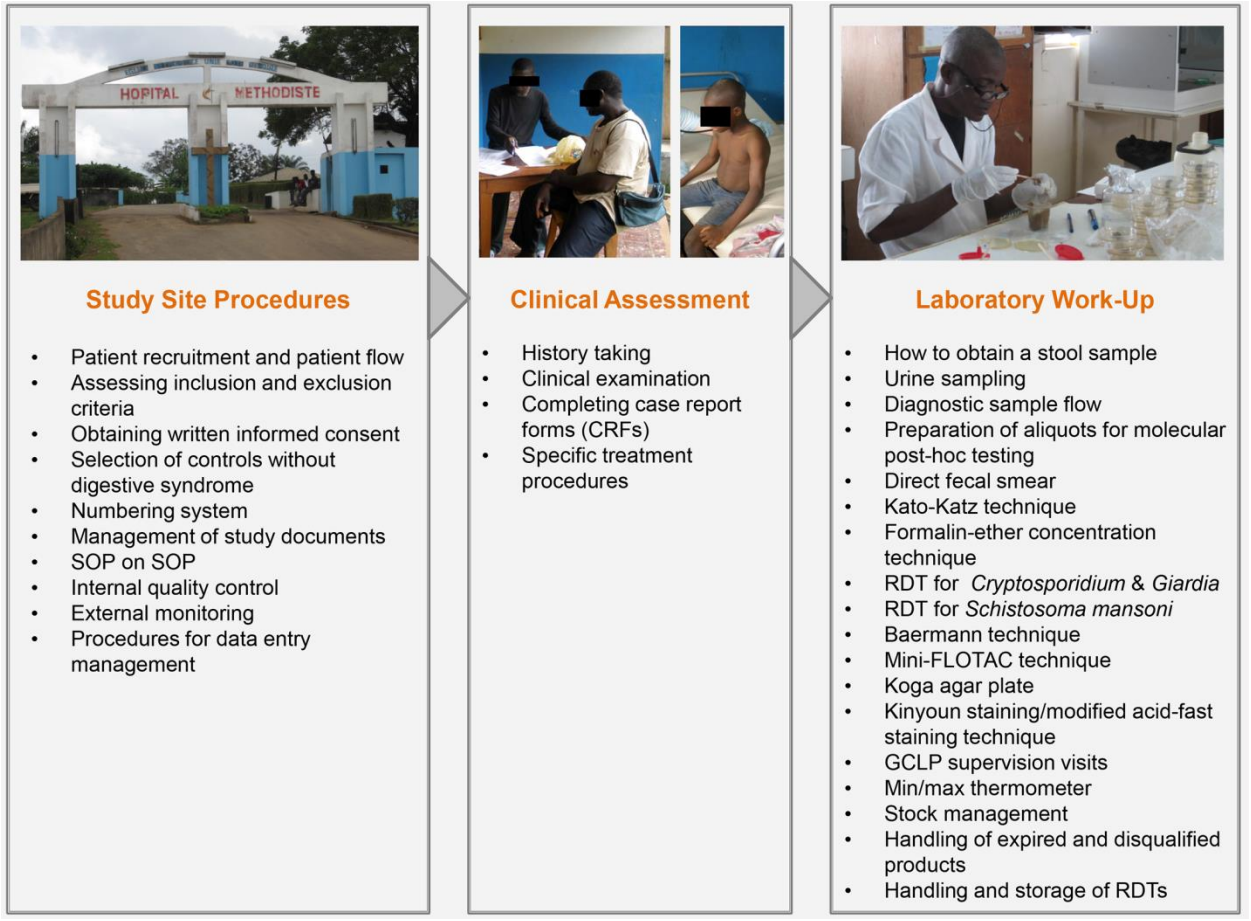
[b] Hookworm larvae can be detected, in particular by culture. Additionally, hookworm larvae can be found using the Baermann technique, if the stool sample has been kept long enough for the eggs to hatch.

[c] This RDT detects only *Cryptosporidium* spp. and *G. intestinalis*.

[d] Acid-fast staining methods (e.g., Kinyoun stain) are particularly suitable for the detection of *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Cystoisospora belli* that are easily missed by most other microscopic diagnostic techniques.

Of note, all SOPs were developed in English (for use in Nepal) and subsequently translated into French (for use in Côte d'Ivoire and Mali) and Bahasa Indonesia (for use in Indonesia). This comprehensive set of closely interconnected SOPs, which provides guidance on all essential procedures from the first presentation of an individual at a health care center until the final processing of all patient and laboratory data, is displayed in Fig 1.

Fig 1. Principal elements of the NIDIAG digestive study and the respective standard operating procedures (SOPs) used.



How Was the Development of SOPs Coordinated, and Which Quality Control Measures Were Adopted?

The development and harmonization of the various SOPs was coordinated by the quality assurance group of the NIDIAG consortium and the trial management group (TMG) of the digestive syndrome study, and followed a standard template and consortium-wide guidelines stipulated in the SOP entitled "SOP on SOP" (S24). This allowed different authors with varied background and writing styles to convey key messages and pass on their expert knowledge in a systematic, standardized manner for the benefit of the end-user of all the SOPs. In addition, it provided clear instructions on how the SOPs should be numbered, reviewed, and approved to allow for strict version control. The authors of the SOPs were chosen from within the NIDIAG consortium and allocation of topics was based on expertise and track record in the clinical, laboratory, data management, and quality assurance components of the study. Experts in the field, at the bench, and at the bedside carefully reviewed and revised the draft SOPs. Before the start of recruitment, local clinical and laboratory teams were trained on the set of SOPs through two hands-on workshops lasting three days each, conducted on site by relevant experts of the NIDIAG consortium. During these workshops, feedback from the local partners was incorporated to refine the already developed SOPs, and additional SOPs were jointly developed to meet specific demands of local clinical, epidemiologic, and laboratory conditions. For example, in Indonesia, where Kinyoun staining was not available, an SOP pertaining to a slightly modified acid-fast staining technique was developed for the local team instead. Finally, once an SOP was finalized, a member of the TMG would approve it. A quality assurance member of the NIDIAG consortium was tasked to compile and keep updated the final set of SOPs and ensure

that the latest versions were available on the NIDIAG Intranet for distribution among the different country partners.

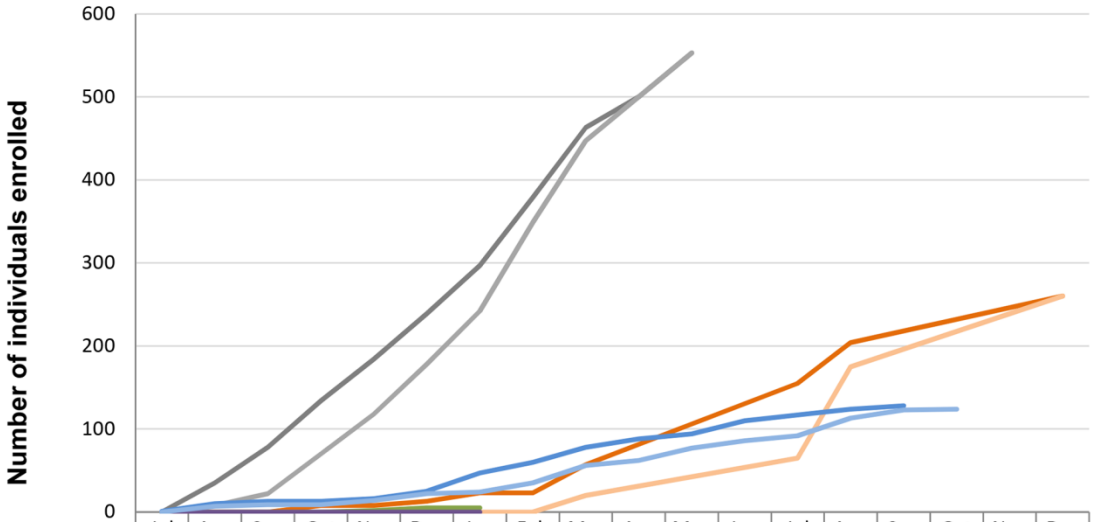
Which Patient Recruitment Patterns Have Been Observed?

In clinical trials and case-control studies, it is one of the most difficult tasks to precisely define patient inclusion and exclusion criteria, and to carry out the enrolment accordingly under 'real life' conditions, thereby minimizing selection bias [12]. Apart from a specific study protocol and a given research question, several studies have shown that many internal and external factors may considerably influence the pace of patient recruitment in clinical trials, many of which are not related to scientific issues, such as, for example, the workload for study staff who are responsible for the recruitment of patients, the distance between the study center, and the place of residence of potential participants, insufficient engagement with the community, and the complexity of consent procedures prior to the start of a trial [13,14]. In the NIDIAG study pertaining to persistent digestive disorders, several site assessments were carried out in the four countries before launching the larger multi-country study [15]. It was decided that a case-control design should be adopted [16-18] and that the ideal target sample size in each country would be in the range of 500 symptomatic patients and 500 asymptomatic controls. After obtaining all the necessary ethics approvals (August 2013 – January 2014), considerable study document and site preparations, and GCP/GCLP training of the study site staff, the first patient was recruited in Nepal on July 30, 2014.

Interestingly, the recruitment of patients and controls showed considerable heterogeneity across countries. Fig 2 displays the country-specific enrolment over time. In Mali, where almost exclusively patients with persistent abdominal pain (and very few with persistent diarrhea) were

recruited, the numbers of eligible patients and controls were high, and the recruitment went smoothly and without major obstacles. Indeed, a total of 553 patients and 553 matched controls were enrolled by May 2015, when the study deemed successfully completed in Mali.

Fig 2. Country-specific enrolment characteristics of patients and controls in the NIDIAG study on persistent digestive disorders.



	Jul 2014	Aug 2014	Sep 2014	Oct 2014	Nov 2014	Dec 2014	Jan 2015	Feb 2015	Mar 2015	Apr 2015	May 2015	Jun 2015	Jul 2015	Aug 2015	Sep 2015	Oct 2015	Nov 2015	Dec 2015
Côte d'Ivoire: Cases	0	0	0	8	8	13	23	23	57				155	204				260
Côte d'Ivoire: Controls	0	0	0	0	0	0	0	0	20				65	175				260
Indonesia: Cases	0	0	0	0	2	5	5											
Indonesia: Controls	0	0	0	0	0	0	0											
Mali: Cases	0	35	78	134	184	239	297	379	463	500	553							
Mali: Controls	0	8	22	70	118	178	242	349	447	500	553							
Nepal: Cases	1	10	13	13	16	25	47	60	78	88	94	110	117	124	128			
Nepal: Controls	0	7	9	9	14	22	24	35	56	62	77	86	92	113	123	124		

A different pattern was observed in Nepal. Patient recruitment started well in July and August 2014, but was temporarily interrupted for a period of almost 3 months due to protracted strikes and civil unrest that limited freedom of movement of the people. Later, patient enrolment

resumed at a stable recruitment pace (with some seasonality seen) until August 2015, when nation-wide strikes again affected the number of enrolled patients, so that the initially targeted sample size of 500 symptomatic patients and 500 controls could not be reached.

A slow recruitment rate was observed in Côte d'Ivoire during the first months of the study, which was mainly explained by the fact that few eligible patients presented to the study hospital, whereas higher numbers of patients with either persistent abdominal pain or persistent abdominal pain were reported from rural, remote health care posts in the surroundings of the study hospital. Hence, the recruitment was changed from a mainly passive approach, i.e., waiting for symptomatic patients to present to the study hospital, to a more pro-active enrolment strategy, which included information of health care workers in the peripheral health care institutions and villages about the purpose and scope of the study, so that they could actively refer potentially eligible patients to the study team.

A similar pro-active approach was adopted in Indonesia, but the number of enrolled patients with persistent digestive disorders remained very low. Hence, after 5 months (August 2014 – January 2015), it was decided by the study country team and the TMG to interrupt the study prematurely and to modify the design. Subsequently, a large community-based survey was carried out in the study area, which employed the same suite of diagnostic methods as foreseen in the original study protocol, to improve the diagnosis of parasitic infections, and to increase the understanding of the causes and the clinical features that are related to these infections in rural Indonesia. The respective ethics committees were readily informed about the necessary modifications and approval was obtained before the start of the survey.

What Were Key Challenges During Patient Recruitment?

During the conduct of the NIDIAG digestive study, several challenges were encountered that underscore why it remains an ambitious goal to perform studies on neglected clinical syndromes in the tropics. First, most study investigators reported difficulties in recruiting patients with persistent diarrhea as defined by the World Health Organization (WHO), i.e., any diarrhea lasting for at least 14 days [2]. Indeed, while long-lasting diarrheal diseases were perceived as a relevant clinical problem in all four study countries, many patients with persistent diarrhea had previously either taken empiric treatment (i.e., antibiotics, antiparasitics, or traditional medicines) or consulted a health care professional (e.g., village health center or local pharmacy). In this context, it is important to note that anti-diarrheal medication and anti-infective drugs are regularly available as over-the-counter drugs without prescription in many low- and middle-income countries, and are thus easily accessible [19,20]. Such medical and pharmaceutical interventions probably led to partial, transitory improvement in some patients, but the digestive symptoms returned after variable periods of intermittence in a considerable proportion of study participants. However, such patients frequently failed to meet the actual inclusion criteria because the onset of diarrheal symptoms after 2 days without diarrhea was defined as a new episode [9], and some of them could thus not be included.

Second, diarrhea and abdominal pain are relatively vague, while subjective symptoms and their reported severity depends on the perceived concepts of illness of the individual patient [21]. Previous studies have shown that the length of the recall period is inversely related to the accuracy and reliability of reported patient complaints and that less severe episodes of diarrhea can be up to 40% underreported with a recall period of one week [22]. Furthermore, there is evidence from resource-constrained settings that diarrhea is less likely to be reported as a

perceived health problem if it is common in a specific area [23,24]. For obvious reasons, 'persistent abdominal pain' is even more difficult to define and to objectively retrace. The application of a standardized medical examination as part of the routine work-up in the NIDIAG digestive study was thus considered an essential tool to complement data gathering and to counterbalance inaccuracies obtained by self-assessed morbidity reports. Third, the NIDIAG digestive study was non-interventional and study site-specific factors had thus to be considered. In Nepal, for instance, there are considerable government-run mass drug administration (MDA) campaigns that may have led to a decrease of helminthiasis-associated diarrheal diseases. Indeed, bi-annual deworming with albendazole for children aged 12-59 months has been carried out along with vitamin A supplementation since 1999, and albendazole is given twice a year to children attending grades 1-10 of all public and private schools. Albendazole has also been administered since 2005 along with diethylcarbamazine citrate (DEC) as part of the efforts to eliminate lymphatic filariasis and Nepal is expected to complete six rounds of MDA in 2018. Finally, pregnant women are also given albendazole during their second trimester. Hence, nearly all age groups in Nepal may potentially receive this anthelmintic drug through one of these MDA campaigns, and it will be interesting to see whether the NIDIAG study results confirm a beneficial effect on helminth-associated persistent digestive disorders.

Fourth, the etiology of persistent diarrhea and persistent abdominal pain is manifold and not limited to infectious causes. Autoimmune disorders, inflammatory diseases, neoplasms and specific food intolerances may, among others, also lead to persistent intestinal disorders [25]. The researchers and clinicians on site experienced that this fact needs to be explained in detail to symptomatic patients without an identified pathogen, and further investigations or referral to additional diagnostics should be initiated promptly. Of note, the detection of a certain pathogen

in a stool or urine sample does not necessarily mean that the patient's symptoms are actually linked to this finding [26]. As the proportion and quantity of encountered pathogens may vary considerably from one setting to another, the NIDIAG study employed a case-control approach to elucidate pathogen-specific attributable fractions to the syndrome of persistent digestive disorders [27,28].

Controls were recruited from the same study centers as the symptomatic patients and were further matched by age groups and sex to the symptomatic cases. It proved to be difficult to obtain consent of individuals to act as controls because they were frequently wondering why someone would be interested in analyzing stool and urine samples of healthy individuals. While careful explanation – being particularly sensitive to potential cultural barriers – helped to clarify this, some individuals still considered stool samples as very personal, intimate specimens, and refused to participate. Indeed, the Nepalese study sites observed that it was very difficult to include asymptomatic patients who were unrelated to a symptomatic patient, while it was relatively straightforward to obtain consent from friends, relatives, or neighbors of symptomatic cases with persistent diarrhea, potentially because these individuals shared similar exposure characteristics (e.g., source of drinking water) with the symptomatic patient and wanted to know whether they were found to carry the same intestinal pathogens.

Have Challenges of Specific Laboratory Techniques Been Observed, and Which Solutions Have Been Adopted?

Two main areas of laboratory-associated challenges were identified during the NIDIAG digestive study, i.e., pertaining to (i) general infrastructure and equipment; and (ii) the conduct of the diagnostic laboratory tests. First, it was noted during the pre-study field visits that the

available laboratory infrastructure in the study countries was – at most sites – not sufficient to accommodate such a broad diagnostic test panel as indicated by the NIDIAG study protocol [3]. Indeed, there was no laboratory that had routinely employed all of the methods before the onset of the study. Hence, workshops were held to familiarize study staff with the techniques and to harmonize procedures across study countries. Additionally, many efforts were required to establish an internal and external quality assurance and monitoring system, so that GCP/GCLP standards were met. It is important to note that such preparatory work is essential to ensure the proper conduct of scientific studies, but the significant human, logistic, and financial resources required are usually not considered sufficiently beforehand by both scientists and funding organizations [29]. In Indonesia, the complete laboratory infrastructure had to be set up in a remote area of an island where the study center was located, which required several weeks of construction work. A short video showing the different study procedures as well as the laboratory infrastructure before and after the onset of the NIDIAG study in Maluku Tengah can be found in the Supplementary Material of this manuscript (S34). Of note, even minor logistic issues may have a profound impact. In Côte d'Ivoire and Nepal, for example, the absence of a functioning ventilation and climate control system in the laboratories led to several short delays in recruitment and specimen processing, as stool samples are unpleasant to work with and the study was thus not initiated before the ventilation systems were fixed.

Study site investigators frequently reported that the volume of a single stool sample was smaller than the amount stipulated in the respective SOP, and was thus not sufficient to perform all required laboratory tests. Indeed, an amount of approximate 80 g of stool was required to run all tests (Table 2), as several diagnostic techniques such as the Baermann funnel concentration for the diagnosis of *Strongyloides stercoralis* require a large amount of stool. In such cases, a

second sample had to be obtained from participants, but especially children were not always able to provide the required amount and sometimes did not come back to provide the requested second sample.

Due to the host of different laboratory tests, the processing of a sample took approximately 2 to 3 hours, and it would have been ideal to collect stool specimens in the early morning and analyze them immediately in the laboratory. However, this was not always possible as the sites of patient enrolment were sometimes quite distant from the actual laboratory (e.g., the Dhankuta study site in Nepal) or the study laboratories had to send parts of the sample to reference laboratories (e.g., in Côte d'Ivoire and Mali, for stool bacteriology in case of persistent diarrhea). Such sample transfer had to be organized and carried out by couriers, so that laboratory staff sometimes worked until late in the evening when a sample arrived only in the afternoon.

With regard to difficulties associated with specific laboratory techniques, few deviations were observed and the study investigators complied very well with the various SOPs. This observation was largely attributed to the two in-depth preparatory workshops that had taken place prior to the start of the study. Some of the remaining challenges experienced and potential solutions to overcome these are presented in Table 3.

Table 3. Diagnostic challenges encountered during the NIDIAG study on persistent digestive disorders, and proposed solutions.

Diagnostic test	Problem	Possible reason	Solution
Rapid diagnostic test (RDT) for *Cryptosporidium* and *G. intestinalis*	Faintly positive test line that is hard to interpret as either positive or negative test result	An inaccurate volume of stool sample may have been used	While an exact amount of liquid stool samples could easily be taken via pipettes, this was less standardized for solid samples. All positive or faintly positive RDT results were documented by photography and results will be compared to subsequent microscopic and molecular diagnostics
RDT for *Cryptosporidium* and *G. intestinalis*	During internal quality control, a *Cryptosporidium*-positive stool sample led to inconsistent results upon RDT application	False-negative results were exclusively observed on expired or nearly expired RDTs	Strict adherence to the indicated expiration dates of RDTs in clinical studies and routine diagnostics
Formalin-ether concentration	Difficult microscopic reading of stool samples following formalin-ether concentration	Questionable quality of the locally obtained ether	Identify alternative provider for ether and other chemical products required for analysis (proved to be difficult in some study countries)
Mini-FLOTAC	Leakage of one flotation chamber	The utilized Mini-FLOTAC apparatus can be re-used after disinfection. However, the washing procedure may influence the stability of the flotation chambers	Apply vaseline on the septum/partition of the Mini-FLOTAC to prevent leakage

Conclusions

As the NIDIAG project draws to an end, we feel that experiences and lessons learnt must be shared with the broader research community, clinicians, and disease control managers in countries where digestive disorders due to NTDs remain an important public health issue (Box 1). The current set of field-tested and ready-to-use SOPs has been implemented successfully in various sites, including those located in areas with restricted resources. We hope that providing open-access to this large compilation of SOPs and highlighting key challenges met during the implementation and the conduct of the NIDIAG study on persistent digestive disorders might assist in further training and capacity building on all aspects of patient recruitment, clinical and diagnostic work-up, within GCP/GLCP standards, data management, and quality control, and thus improve the clinical-diagnostic algorithms for patients suffering from NTDs. We look forward to experiences and lessons by other groups pursuing syndromic approaches to advance the diagnosis and clinical management of NTDs among neglected populations.

Box 1. Key Learning Points from the Multi-Country NIDIAG Study on Persistent Digestive Disorders in the Tropics

- There is a need for studies investigating the etiology, diagnosis, and management of common clinical syndromes such as persistent digestive disorders in the tropics. Easily applicable, evidence-based diagnosis-treatment algorithms could substantially improve the clinical management of such syndromes at the primary health care level.
- The implementation of a quality assurance system is crucial for the conduct of multicentric clinical studies in resource-restricted settings, and it remains an ambitious goal to perform studies that fully comply with good clinical practice (GCP) and good clinical laboratory practice (GCLP).

- Study documents such as standard operating procedures (SOPs) and case report forms (CRFs) should be jointly developed and validated to harmonize procedures across study sites. Such field-tested tools are an important resource for researchers and health care staff, and should thus be made publicly available.
- In a study on persistent digestive disorders, which was carried out by the Neglected Infectious Diseases Diagnostics (NIDIAG) consortium, perceived challenges were the prolonged recall period (≥14 days) for the identification of individuals with persistent abdominal pain and persistent diarrhea, and the considerable heterogeneity seen regarding the recruitment pace of patients and controls in the four study countries.

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Supporting Information Legends

S1 Clinical SOP. History taking.

S2 Clinical SOP. Clinical examination.

S3 Clinical SOP. Selection of controls without digestive syndrome.

S4 Clinical SOP. Specific treatment procedures (including dosage).

S5 Clinical SOP. Assessing inclusion and exclusion criteria.

S6 Clinical SOP. Patient recruitment and patient flow.

S7 Laboratory SOP. Kinyoun staining technique (used in Côte d'Ivoire, Mali, and Nepal).

S8 Laboratory SOP. Modified acid-fast staining technique (used in Indonesia).

S9 Laboratory SOP. Crypto-Giardia Duo-Strip rapid diagnostic test (RDT).

S10 Laboratory SOP. Kato-Katz technique.

S11. Laboratory SOP. Baermann technique.

S12 Laboratory SOP. Mini-FLOTAC technique.

S13 Laboratory SOP. How to obtain a stool sample.

S14 Laboratory SOP. Formalin-ether concentration technique.

S15 Laboratory SOP. Koga agar plate culture.

S16 Laboratory SOP. Direct fecal smear technique.

S17 Laboratory SOP. Preparation of aliquots for molecular post-hoc testing.

S18 Laboratory SOP. Urine point-of-care circulating cathodic antigen (POC-CCA) RDT for the diagnosis of *Schistosoma mansoni*.

S19 Laboratory SOP. Diagnostic sample flow.

S20 Laboratory SOP. Urine sampling.

S21 Quality SOP. Obtaining informed consent.

S22 Quality SOP. Numbering system to be used in NIDIAG studies.

S23 Quality SOP. Management of study documents.

S24 Quality SOP. SOP on SOPs.

S25 Quality SOP. External monitoring.

S26 Quality SOP. Internal quality control activities.

S27 Quality SOP. Good clinical laboratory practice (GCLP) supervision visits.

S28 Quality SOP. Min/max thermometer.

S29 Quality SOP. Stock management.

S30 Quality SOP. Handling of expired and disqualified products.

S31 Quality SOP. Handling and storage of rapid diagnostic tests (RDTs).

S32 Data management SOP. Completing case report forms (CRFs).

S33 Data management SOP. Procedure for data management.

S34 Video. The NIDIAG study site in Maluku Tengah, Indonesia.

6 Combined stool-based multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire

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ORIGINAL ARTICLE INFECTIOUS DISEASES

Combined stool-based multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire

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Abstract

Infectious diarrhoea ranks among the leading causes of morbidity worldwide. Although most acute diarrhoeal episodes are self-limiting, the diagnosis and treatment of persistent diarrhoea (≥2 weeks) are cumbersome and require laboratory identification of the causative pathogen. Stool-based PCR assays have greatly improved the previously disappointing pathogen detection rates in high-income countries, but there is a paucity of quality data from tropical settings. We performed a case–control study to elucidate the spectrum of intestinal pathogens in patients with persistent diarrhoea and asymptomatic controls in southern Côte d'Ivoire. Stool samples from 68 patients and 68 controls were obtained and subjected to molecular multiplex testing with the Luminex® Gastrointestinal Pathogen Panel (GPP), microscopy and rapid antigen detection tests for the diagnosis of diarrhoeagenic pathogens. Overall, 20 different bacteria, parasites and viruses were detected by the suite of diagnostic methods employed. At least one pathogen was observed in 84% of the participants, and co-infections were observed in >50% of the participants. Enterotoxigenic *Escherichia coli* (32%), *Giardia intestinalis* (29%) and *Shigella* species (20%) were the predominant pathogens, and *Strongyloides stercoralis* (10%) was the most prevalent helminth. Pathogen frequencies and numbers of co-infections were similar in patients and controls. Although the Luminex® GPP detects a broad range of pathogens, microscopy for helminths and intestinal protozoa remains necessary to cover the full aetiological spectrum in tropical settings. We conclude that highly sensitive multiplex PCR assays constitute a useful screening tool, but that positive results might need to be confirmed by independent methods to discriminate active infection from asymptomatic faecal shedding of nucleic acids.

Keywords: Bacteria, Côte d'Ivoire, diagnosis, helminths, intestinal protozoa, microscopy, multiplex PCR, persistent diarrhoea, stool, viruses
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Introduction

Infectious diarrhoeal diseases constitute the fourth most common contributor to the global burden of disease, accounting for a burden of 89.5 million disability-adjusted life-years [1]. Low-income and middle-income countries are

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particularly affected, and infectious diarrhoea ranks among the three leading causes of mortality in children under the age of 5 years [2,3]. The aetiology of diarrhoea, abdominal pain and related digestive disorders may include >40 infectious pathogens, which can be grouped into bacteria, parasites (helminths and intestinal protozoa), and viruses [4]. Although many diarrhoeal episodes are self-limiting and of only light or moderate severity, a considerable proportion of patients may develop either severe acute disease or long-lasting symptoms such as persistent diarrhoea (duration: ≥2 weeks) [5]. Differentiation between several aetiological agents based on clinical presentation alone is difficult and error-prone. Hence, laboratory examinations are important to identify the causative agents of infectious diarrhoeal diseases. However, no currently available laboratory technique can diagnose the many potentially implicated pathogens with high accuracy. It follows that detection rates of intestinal pathogens are disappointing if conventional diagnostic methods are employed and only a narrow spectrum of pathogens is targeted. Prior research has shown that the causative infectious agent may remain undetected in up to 80% of stool samples obtained from patients with diarrhoea [6].

The development of molecular diagnostic methods, such as PCR assays, has considerably increased the diagnostic sensitivity for the detection of many intestinal pathogens [7]. Recently, several multiplex PCR panels that may detect multiple diarrhoeagenic pathogens concurrently in a single stool sample have become available, and they improve the diagnostic yield in symptomatic patients with digestive disorders. The Luminex® xTAG Gastrointestinal Pathogen Panel (GPP; Luminex® Corporation, Austin, TX, USA) is a technique that uses fluorescent beads to allow for the concurrent detection of 15 pathogens: nine bacteria, three viruses, and three intestinal protozoa [8]. The Luminex® GPP is licensed for use in the USA, Canada, and Europe, where it is being increasingly employed in hospitals [9,10] and travel clinics for the diagnosis of acute diarrhoea [11,12]. So far, however, experience with a multiplex Luminex® platform for the diagnosis of intestinal pathogens in the tropics is scant. Additionally, it remains to be elucidated how this multiplex assay performs if applied to stool specimens from asymptomatic individuals, especially in settings of high endemicity, where multiple pathogens are the norm rather than the exception. It is conceivable that a combination of different diagnostic tests may lead to more accurate results [13]. Most commercially available multiplex PCR assays do not cover an extensive panel of parasitic pathogens, despite the fact that these are considered to be important causative agents of diarrhoeal diseases in the tropics [4]. Thus, a combination of diagnostic methods has been proposed for the comprehensive assessment of diarrhoea and related digestive disorders in such settings.

Here, we report findings from a study that employed a suite of diagnostic methods, including the Luminex® GPP, parasitological microscopic examinations, and rapid antigen detection tests, for a detailed diagnostic work-up of patients presenting with persistent diarrhoea. The study was carried out in the southern part of Côte d'Ivoire towards the end of 2012, and also included asymptomatic controls.

Materials and methods

Ethics statement

Individuals aged ≥12 months with residency in Dabou, southern Côte d'Ivoire who had provided written informed consent (parents/guardians signed for individuals aged <18 years) were eligible to participate. Study approval was given by the institutional research commissions of the Swiss Tropical and Public Health Institute (Basel, Switzerland), the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (Abidjan, Côte d'Ivoire), and the Directorate of the Hôpital Méthodiste in Dabou. The study is registered in Current Controlled Trials (identifier: ISRCTN86951400). Within 2 days of enrolment, study participants were informed about their individual diagnostic test results based on microscopic analysis of stool samples and rapid diagnostic tests (RDTs). In the case of a parasitic infection, free treatment was offered, i.e. albendazole (400 mg) against soil-transmitted helminths, ivermectin (200 μg/kg) against *Strongyloides stercoralis*, praziquantel (40 mg/kg) against *Schistosoma mansoni*, and metronidazole against symptomatic *Giardia intestinalis* (400 mg three times daily for 5–7 days). Because the PCR tests were conducted several weeks after enrolment of study subjects in a reference laboratory abroad, these results did not guide the clinical management of infected individuals.

Study location, design, and population

Our exploratory study was part of a multi-country assessment to identify suitable settings for a subsequent prospective, non-experimental case–control study to be implemented in four countries. The purpose of the current investigation was to deepen our understanding of the aetiology and clinical presentations of persistent digestive disorders in the prospective study site in Côte d'Ivoire. The research is coordinated by the European research network with the acronym NIDIAG, which aims to develop simple and cost-effective diagnostic–treatment algorithms for three clinical syndromes (i.e. persistent digestive disorders [4], persistent fever [14] and neurological disorders [15]) in tropical settings.

The current study was carried out over a 2-week period in October 2012 in Dabou, a small town in south Côte d'Ivoire,

and 11 surrounding villages belonging to the same health district. The study area is located ~30 km west of Abidjan, the economic capital of Côte d'Ivoire. Individuals aged ≥12 months presenting with persistent diarrhoea were eligible to participate. Recommendations put forth by the World Health Organization (WHO) were used to define persistent diarrhoea, i.e. three or more loose or liquid stools per day for any period lasting for ≥2 weeks [16]. Individuals with acute diarrhoea or other abdominal symptoms with a duration of <2 weeks were excluded.

Training sessions were held with physicians from the local study hospital (Hôpital Méthodiste de Dabou) and with nurses from the surrounding village health are centres. The healthcare professionals were invited to identify eligible patients and to inform them about the purpose and procedures of the study. Additionally, medical personnel from the village healthcare centres were trained in obtaining written informed consent and filling in a short, pretested clinical questionnaire.

To allow for comparison of pathogen detection frequencies between symptomatic and asymptomatic individuals, a case–control design was adopted. Hence, individuals without any gastrointestinal complaints within the preceding 2 months were also invited to participate, and were used as controls. These controls were randomly selected from the same study area, and were matched to the cases by age group (1–5 years, 6–15 years, 16–25 years, 26–45 years, and >45 years) and village of residency (frequency matching).

Field and laboratory procedures

Stool collection containers were distributed by healthcare professionals, and participating individuals provided one fresh morning stool sample, which was transferred to the local hospital laboratory in Dabou. On the day of collection, the stool samples were examined microscopically by two senior laboratory technicians for the presence of a broad range of parasitic infections. Specifically, the following tests were employed: the Kato–Katz method (duplicate Kato–Katz thick smears for the diagnosis of *S. mansoni* and soil-transmitted helminths); the formalin–ether concentration technique (FECT) (for the diagnosis of helminths and intestinal protozoa); and Baermann funnel concentration and Koga agar plate culture (for the diagnosis of *S. stercoralis* and hookworm). Additionally, for the diagnosis of *G. intestinalis* and *Cryptosporidium* species, an antigen detection RDT was employed (Crypto/Giardia Duo-Strip; Coris BioConcept, Gembloux, Belgium). At the time of the study, the performance of bacterial stool culture was logistically not feasible at Dabou hospital.

Molecular *post-hoc* testing

From each stool sample, ~300 mg was placed in a vial and stored in a refrigerator (4°C). However, the cold chain could not be maintained consistently, owing to an irregular power supply, which is common in many parts of Côte d'Ivoire. Within 8–19 days, the samples were transferred to Homburg, Germany for further processing with the commercial Luminex® GPP. This assay concurrently detects nucleic acids from nine different bacteria (i.e. *Campylobacter* species, *Clostridium difficile*, *Escherichia coli* O107, enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli*, *Salmonella* species, *Shigella* species, *Vibrio* species, and *Yersinia enterocolitica*), three viruses (adenovirus, norovirus GI/II, and rotavirus), and three intestinal protozoa (*Cryptosporidium* species, *E. histolytica*, and *G. intestinalis*). Note that no helminths are included in the current Luminex® GPP.

Nucleic acid extraction of ~100 mg of each stool sample was performed with an automated purification system (Maxwell 16 instrument with tissue LEV DNA purification kit; Promega, Madison, WI, USA). All samples were spiked with 2 µL of internal control (MS2, a bacteriophage) before purification. Extracted nucleic acids were eluted into a final volume of 75 µL. PCR, hybridisation reactions and data analysis were carried out according to the manufacturer's instructions. In brief, 15 µL of a master mix (2.5 µL of RNAse-free water, 7.5 µL of buffer, 0.5 µL of bovine serum albumin (10 mg/mL), 2.5 µL of GPP primer mix, and 2.0 µL of enzyme mix) was mixed with 10 µL of each extracted sample and subjected to multiplex reverse transcriptase (RT-)PCR in a preheated thermal cycler (53°C). The RT-PCR programme was performed as previously described [17]. Negative and positive controls were included in each PCR run. The PCR products were mixed with the manufacturer-provided fluorescent bead mix, and a hybridisation reaction was performed in the thermal cycler (3 min at 60°C, followed by 45 min at 45°C). Samples were then transferred into the Magpix detection system. Data analysis was carried out with xTAG Data Analysis Software.

Clinical questionnaire

Study participants were interviewed with a pretested questionnaire to obtain baseline demographic information (age, sex, and residency) and to document self-reported clinical morbidity, such as the current (e.g. duration of symptoms and specific complaints) and past (e.g. antibiotic and antiparasitic treatment, and pre-existing morbidities) medical history. Questionnaires were administered by trained medical personnel.

Statistical analysis

Data were double-entered and cross-checked with Microsoft Excel version 14.0 (edition 2010; Microsoft Corporation). Statistical analysis was performed with STATA version 12.0 (StataCorp., College Station, TX, USA). In the absence of a 'true' diagnostic reference standard, a positive test result in any

TABLE 1. Epidemiological characteristics of 136 study participants in Dabou, south Côte d'Ivoire, examined in October 2012

	Total, *n*	Patients with persistent diarrhoea, *n*	Asymptomatic controls, *n*	p[a]
Sex				
Female	66	36	30	
Male	70	32	38	0.303
Age group (years)				
1–5	37	22	15	
6–15	13	5	8	
16–25	14	7	7	
26–45	43	17	26	
>45	29	17	12	0.313
Residency				
Urban (Dabou)	25	10	15	
Rural (surrounding villages)	111	58	53	0.268

[a]Derived from Pearson's χ^2-test.

of the employed diagnostic tests was considered to be a 'true positive', thus leading to a combination of all positive test results for a certain pathogen being the internal diagnostic reference standard in this study.

The prevalence of each pathogen was determined on the basis of the combination of the available diagnostic tests, and differences regarding the frequency in symptomatic vs. asymptomatic patients were assessed with Pearson's χ^2-test or Fisher's exact test, as appropriate. More than one diagnostic test was employed for the detection of *Cryptosporidium* species, *E. histolytica*, and *G. intestinalis*. Sensitivity, negative predictive value and corresponding 95% confidence intervals (CIs) were calculated for every single technique in relation to the diagnostic reference standard, and the diagnostic accuracies of the different methods were compared. A p-value of <0.05 was considered to indicate statistical significance. In the absence of a reference standard indicating the number of 'true-negative' and 'false-positive' samples, no specificity or positive predictive value could be calculated. The agreement between different diagnostic methods was evaluated with Cohen's kappa measure (κ) [18]. Univariate odds ratios (ORs) were computed to analyse significant associations between self-reported gastrointestinal symptoms and infection with a specific pathogen.

Results

Study cohort

Stool samples from 335 individuals were examined: 71 specimens from patients with persistent diarrhoea, and 264 from asymptomatic controls. The quantity of stool was sufficient for the performance of molecular *post-hoc* testing on 68 patient samples. Subsequently, 68 residency-matched and age group-matched asymptomatic controls were selected. Hence, our final study sample consisted of 136 individuals. In terms of age range, sex, and parasite spectrum (according to microscopy), the 68 stool samples selected among the 264 controls for *post-hoc* molecular analysis were comparable to those control specimens that were not further analysed (n = 196).

In the final study sample, there were slightly more males (51%, n = 70) than females (49%, n = 66), and most of the participants lived in villages surrounding the town of Dabou (82%, n = 111). The epidemiological characteristics of symptomatic patients and asymptomatic controls are shown in Table 1.

Bacterial, parasitic, and viral infections

At least one intestinal infection was diagnosed in 84% (n = 114) of all study participants. Intestinal protozoa (68%, n = 93) and bacterial (51%, n = 69) infections were far more prevalent than helminth (17%, n = 23) and viral (9%, n = 12) infections. Overall, 20 different species of intestinal pathogens were detected. The predominant infectious agents were ETEC (32%, n = 43), *Entamoeba coli* (29%, n = 40), *G. intestinalis* (29%, n = 39), and *Shigella* species (20%, n = 27). Larvae of *S. stercoralis* were found in 14 people (10%), and constituted the predominant helminth species.

Table 2 shows the prevalence of all detected bacteria, parasites and viruses, and their frequencies in patients and controls. *Shigella* species were found more often in patients than in asymptomatic controls (27% vs. 13%; p 0.053). On the other hand, *Endolimax nana* (7% vs. 32%; p 0.001) and *E. coli* O157 (0% vs. 6%; p 0.042) were significantly more common in asymptomatic controls. As shown in Fig. 1, infection rates of some intestinal pathogens were somewhat higher in rural villages located in the west of the study area (e.g. ETEC; 47% in villages located 10 km west from Dabou vs. 33% in the town of Dabou), but the difference lacked statistical significance.

Co-infections

Co-infections were common. Infections with at least two intestinal pathogen species were diagnosed in 37 patients (54%) and in 47 asymptomatic controls (69%). In two individuals with persistent diarrhoea, a concurrent infection with up to six different gastrointestinal pathogens was diagnosed. Fig. 2 summarises the occurrence of co-infections in patients and controls.

Combined diagnostic approaches

The combination of multiplex PCR, microscopy, and RDTs detected more pathogens than any of the three techniques alone. The pathogen detection rates in symptomatic patients were 69% for multiplex PCR, 56% for microscopy, and 16% for

TABLE 2. Prevalence of intestinal pathogens (helminths, intestinal protozoa, bacteria, and viruses) in 136 individuals in Dabou, south Côte d'Ivoire, in October 2012, as assessed with different diagnostic techniques

		All participants			Symptomatic patients		Asymptomatic controls		
	Diagnostic technique(s)	*N*	%	95% CI	*N*	%	*N*	%	p[a]
Helminths									
Strongyloides stercoralis	Microscopy	14	10	5–16	8	12	6	9	0.573
Hookworm	Microscopy	10	7	3–12	5	7	5	7	1.000
Ascaris lumbricoides	Microscopy	2	2	0.01–4	2	3	0	0	0.248
Intestinal protozoa									
Entamoeba coli	Microscopy	40	29	22–37	18	27	22	32	0.452
Giardia intestinalis	Microscopy, RDT, PCR	39	29	21–36	18	27	21	31	0.569
Endolimax nana	Microscopy	27	20	13–27	5	7	22	32	0.001
Blastocystis spp.	Microscopy	13	10	5–15	4	6	9	13	0.145
Chilomastix mesnili	Microscopy	13	10	5–15	9	13	4	6	0.145
Cryptosporidium spp.	RDT, PCR	12	9	4–14	3	4	9	13	0.070
Iodamoeba bütschlii	Microscopy	6	4	0.9–8	3	4	3	4	1.000
Entamoeba histolytica/E. dispar[b]	Microscopy, PCR	5	4	0.5–7	1	2	4	6	0.183
Entamoeba hartmanni	Microscopy	1	1	0.01–2.2	0	0	1	2	0.500
Bacteria[c]									
Enterotoxigenic *Escherichia coli*	PCR	43	32	24–40	23	34	20	29	0.580
Shigella spp.	PCR	27	20	13–27	18	27	9	13	0.053
Campylobacter spp.	PCR	12	9	4–14	4	6	8	12	0.227
Salmonella spp.	PCR	9	7	2–11	5	7	4	6	0.730
Escherichia coli O157	PCR	4	3	0.07–6	0	0	4	6	0.042
Viruses									
Norovirus GI/GII	PCR	9	7	2–11	6	8	3	4	0.246
Adenovirus 40/41	PCR	2	2	0.01–4	1	2	1	2	1.000
Rotavirus A	PCR	1	1	0.01–2	1	2	0	0	0.500

RDT, rapid diagnostic test.
[a]Derived from Pearson's χ^2-test or Fisher's exact test, as appropriate.
[b]Microscopy is not able to differentiate between *Entamoeba histolytica* and the apathogenic *Entamoeba dispar*. In this study, three of the five positive samples were detected by microscopy, but gave a negative PCR test result, and so most likely constituted an infection with *E. dispar*.
[c]None of the following bacterial pathogens targeted by the Luminex® GPP were detected: toxigenic *Clostridium difficile*, Shiga-toxin producing *Escherichia coli*, *Vibrio cholerae*, and *Yersinia enterocolitica*.

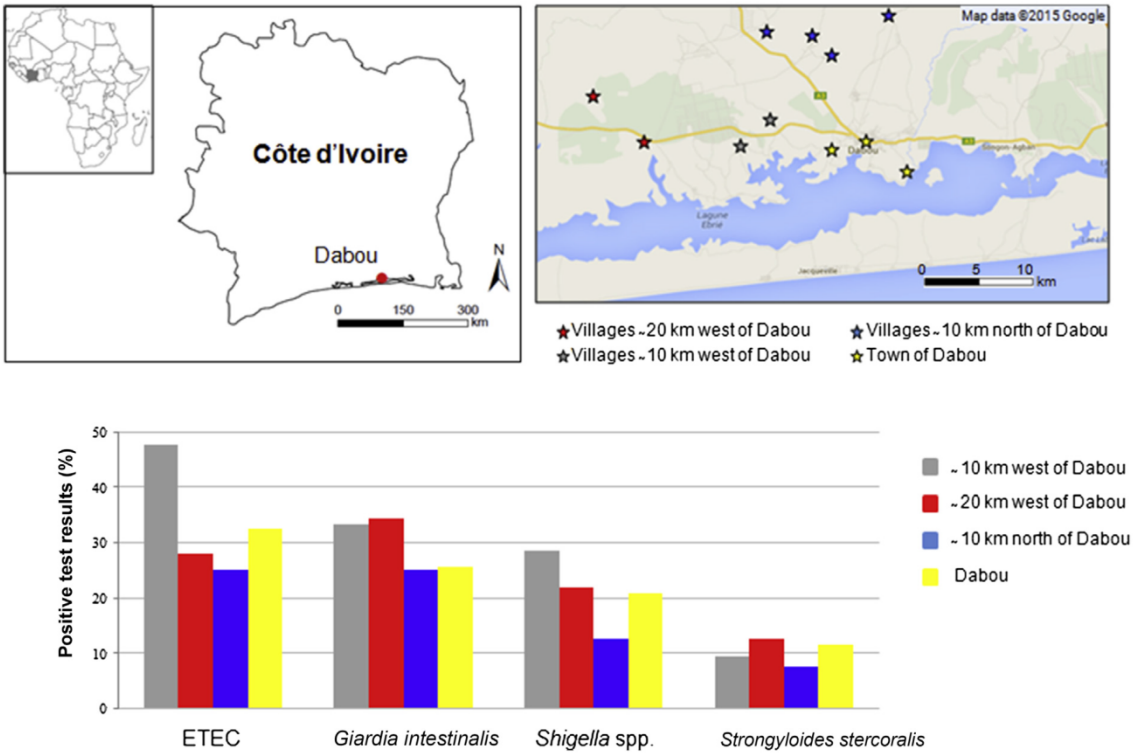

FIG. 1. Spatial distribution of selected gastrointestinal infections in 136 individuals from Dabou and surrounding villages in southern Côte d'Ivoire. ETEC, enterotoxigenic *Escherichia coli*.

RDTs. A combination of all three methods identified at least one pathogen in 84% of all stool samples (Fig. 3).

Comparison of different diagnostic methods

Among the three techniques employed for the diagnosis of *G. intestinalis* (the Luminex® GPP, microscopy with the FECT, and antigen detection with an RDT), the Luminex® PCR assay showed the highest sensitivity (84.6%; 95% CI 69.5–94.1%) and negative predictive value (94.2%; 95% CI 87.8–97.8%). Considerably lower sensitivity was observed for the FECT and the RDT, i.e. 51.3% and 40.0%, respectively. The latter two techniques, however, identified an additional six infections that remained undetected by the Luminex® PCR assay. The diagnostic agreement between microscopy and the RDT was substantial (κ = 0.64), whereas it was moderate if one of both techniques was compared with PCR (microscopy and PCR, κ = 0.47; RDT and PCR, κ = 0.46).

For the diagnosis of *Cryptosporidium* species, RDT results were available for 121 of the 136 samples, and these were compared with PCR. Only one of ten positive samples was confirmed by both methods, whereas the majority of infections were exclusively detected by one single technique (PCR, *n* = 5; RDT, *n* = 4). For *E. histolytica* detection, microscopy revealed three infections that were PCR-negative. However, care is indicated, as microscopy does not allow distinction between *E. histolytica* and the morphologically identical *E. dispar* [19]. PCR identified two *E. histolytica*-positive stool samples.

Questionnaire survey

Self-reported gastrointestinal morbidity was high among the 68 patients. Besides the main health complaint of persistent diarrhoea, abdominal pain and weight loss were commonly reported (71% and 28%, respectively). Nausea was significantly associated with hookworm (OR 4.97, 95% CI 1.11–22.25) and *S. stercoralis* (OR 5.02, 95% CI 1.31–19.25). Vomiting was significantly associated with *Salmonella* species (OR 5.85, 95% CI 1.27–27.0). Intestinal colonisation with the apathogenic intestinal protozoon *E. nana* was inversely correlated with persistent diarrhoea (OR 0.17, 95% CI 0.05–0.47) and abdominal pain (OR 0.26, 95% CI 0.08–0.79).

Self-reported treatment

Previous treatment with anti-infective drugs was reported by 25% of the symptomatic patients (*n* = 17). Albendazole (*n* = 8) was the most commonly used anthelminthic drug, followed by pyrantel pamoate (*n* = 4), and mebendazole (*n* = 2).

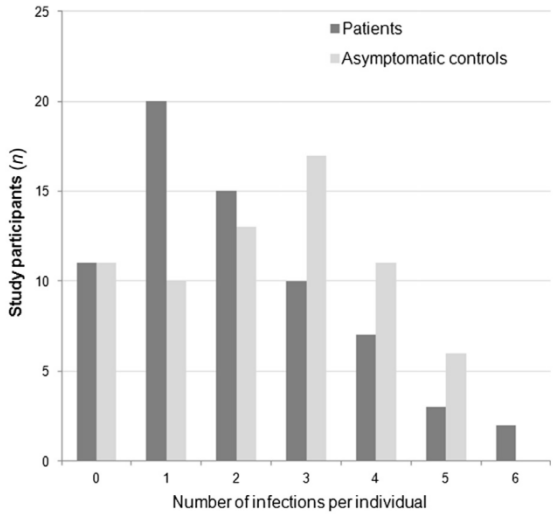

FIG. 2. Number of detected gastrointestinal infections in 136 individuals in Dabou and surrounding villages in southern Côte d'Ivoire, stratified according to symptomatic patients and asymptomatic controls.

Trimethoprim–sulphamethoxazole (n = 10) and metronidazole (n = 5) were the only antibiotics used. Combination therapy with two different drugs was reported by 12 of 17 symptomatic patients. In contrast, only two individuals without persistent diarrhoea reported any anti-infective treatment within the preceding 2 months (albendazole, n = 1; pyrantel pamoate, n = 1).

Discussion

Diarrhoea remains a major public health issue in low-income and middle-income countries, and is caused by a variety of intestinal pathogens. Previous studies have shown that the combination of several diagnostic methods for detection of pathogens in the stool generates the most accurate results [13]. Recent advances in multiplex platforms have the potential to increase sensitivity and to fast-track diagnostic testing, which in turn deepens our understanding of the causal agents giving rise to digestive disorders [20]. Here, we report the results after application of a suite of diagnostic methods, including multiplex PCR (Luminex® GPP, facilitating analysis of 15 different

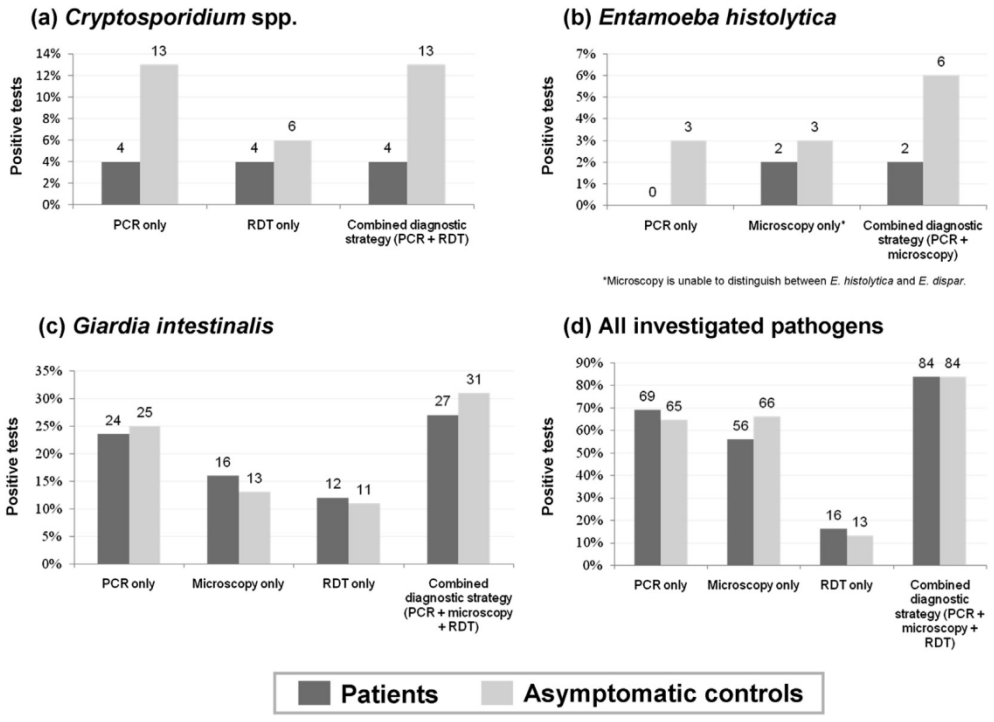

FIG. 3. Pathogen detection rates in 68 patients with persistent diarrhoea and 68 asymptomatic controls from Dabou and surrounding villages in southern Côte d'Ivoire, according to three different diagnostic methods (multiplex PCR, microscopy, and rapid diagnostic test (RDT)) and a combined diagnostic approach.

pathogens), standard microscopy, and RDTs (for *Cryptosporidium* species and *G. intestinalis*), to determine the spectrum of gastrointestinal pathogens in faecal samples of patients with persistent diarrhoea and among age group-matched and residency-matched asymptomatic controls from southern Côte d'Ivoire. As compared with each diagnostic method alone, the yield of the combined approach was considerable. Indeed, 11 of 15 pathogens targeted by our multiplex PCR were detected, and stool microscopy identified an additional nine helminth and intestinal protozoa species that are not diagnosed by the current Luminex® GPP. Hence, a total of 20 different bacteria, parasites and viruses were detected. More than 80% of the participants—regardless of whether they were symptomatic or asymptomatic—harboured at least one pathogen species, and more than half of the participants were co-infected. Self-reported gastrointestinal morbidity was high in patients with persistent diarrhoea, and >80% of them lived in rural villages surrounding the town of Dabou.

The accuracy of the Luminex® GPP for the diagnosis of acute infectious gastroenteritis has been assessed previously in various hospital settings in Europe [8,17,21,22] and the USA [23–25]. As compared with conventional techniques or real-time PCR assays, high sensitivity and specificity were reported in symptomatic patients. However, the Luminex® GPP has yet to be validated in the tropics, and no asymptomatic individuals were examined with this platform in previous studies. It is of note that a recent study from The Netherlands reported an elevated number of false-positive results for the detection of *E. histolytica* and *Salmonella* species [9]. The authors concluded that a confirmatory test might be necessary at least for these two pathogens, because PCR alone does not allow the distinction of active infection from asymptomatic shedding of nucleic acids. New research conducted in a travel clinic in Switzerland showed a pathogen detection rate of 11% in returning travellers, with ETEC being the most commonly identified organism [11]. Likewise, application of the Luminex® GPP in a Spanish travel clinic revealed at least one pathogen in 36% of stool samples obtained from symptomatic travellers returning from the tropics [12]. In the latter study, ETEC and *Shigella* species were the most prevalent bacteria, and *G. intestinalis* was the most common intestinal protozoon, which is in line with our results from a tropical setting (southern Côte d'Ivoire). In both European travel clinics, the molecular assay significantly improved the pathogen detection rate, but not all PCR-positive results could be confirmed with independent reference tests (e.g. real-time PCR and bacterial stool culture), and concerns about the suitability of nucleic acid-based tests as a single method without confirmatory tests were therefore raised. Further limitations of the Luminex® GPP for application in tropical settings include the high cost of the reagents and the need for sophisticated laboratory infrastructure.

Three issues emerge from the current study that are worth discussing. First, 58 of 68 patients with persistent diarrhoea were from villages within a 25-km radius of the town of Dabou, where the study hospital is located. If the study had not included village health centres, the true relevance of persistent diarrhoea as a health problem in this part of southern Côte d'Ivoire would have been underestimated. Hence, this finding underlines the need to conduct clinical studies in rural, remote areas to assess more accurately the true burden of infectious diseases in tropical settings. Second, co-infections were the norm rather than the exception, confirming prior research in Côte d'Ivoire [26]. The systematic application of highly sensitive diagnostic assays (i.e. multiplex PCR) has revealed that co-infections occur even in industrialised countries in a considerable proportion of patients with diarrhoea [7]. Hence, the concept of one specific pathogen causing digestive disorders is gradually being replaced by a more complex understanding of the interactions between several pathogens giving rise to diarrhoeal diseases [27]. Third, the overall detection rates for most pathogens were similar between symptomatic patients with persistent diarrhoea and asymptomatic controls. This observation may be explained either by asymptomatic colonisation or by prolonged nucleic acid shedding in the absence of viable pathogens, e.g. following a resolved infection. Whereas asymptomatic chronic infections with intestinal parasites such as *G. intestinalis* or soil-transmitted helminths commonly occur in endemic settings [28], obligate pathogens such as *Shigella* are not considered to asymptomatically colonise the gastrointestinal tract. It might be speculated that the high analytical sensitivity of molecular diagnostic assays may also result in the detection of 'environmental contamination' by inactive pathogens, thus rendering these techniques 'too sensitive' and leading to a high rate of positive results even in the absence of active infection. Hence, PCR may be used as a screening tool to sort out the negative samples, but confirmation of PCR-positive specimens by microscopy or culture-based techniques might be necessary to prove active, clinically relevant infections with vital pathogens. Further research is needed to elucidate whether the results produced by qualitative PCR assays, which merely detect the presence of pathogen nucleic acids in stool specimens, are sufficient to guide therapeutic decision-making in highly endemic areas where co-infections are common. In contrast, it may be hypothesised that quantitative PCR assays could be more useful in distinguishing ongoing infections from 'environmental contamination'.

In addition to the Luminex® GPP, a combination of several microscopic techniques revealed helminth infections in 17% of

all study participants, predominantly caused by *S. stercoralis* and hookworm. So far, however, no helminth species and only three intestinal protozoa species are targeted by the Luminex® GPP. Hence, our findings call for a combined diagnostic strategy to comprehensively assess diarrhoea in the tropics and in travel clinics, where soil-transmitted helminths and intestinal protozoa constitute important agents of digestive disorders [4,29]. Further development of the Luminex® GPP to broaden its diagnostic range, specifically detection of helminth infections, will enhance the suitability of this multiplex PCR assay for application in tropical settings and travel clinics. A recently published 'cookbook' might facilitate the development of such setting-specific multiplex assays with the Luminex® technology [30].

Our study has several limitations. First, for most pathogens that are currently targeted by the Luminex® GPP, we did not perform alternative reference diagnostic tests (e.g. bacteriological stool culture) to assess the viability of the detected pathogen. However, other studies have reported excellent sensitivity and specificity for most pathogens that are included in the current Luminex® GPP configuration [8,23,25], and our comparison of PCR, microscopy and RDTs for the detection of *G. intestinalis* confirms the superiority of the molecular assay over other diagnostic methods [31]. However, we cannot exclude the possibility that some PCR-positive results may have been false-positives. Second, the prolonged storage of the stool samples (up to 19 days) at 4°C before they were transferred to a reference laboratory in Europe for *post-hoc* molecular analysis may have negatively impacted on the PCR results, as degradation of some pathogen nucleic acids might have occurred. Third, even though we employed a suite of diagnostic tests, some pathogens that are known to potentially cause persistent diarrhoea might have been missed. The additional use of, for example, acid-fast staining for detection of *Cyclospora cayetanensis* could have detected further relevant pathogens. Fourth, examination of more than one stool sample would have been desirable for improved diagnosis of intestinal parasites that are shed in the faeces with considerable day-to-day variation [32]. Fifth, the number of examined stool samples in the current study was relatively small, thus hampering a more rigorous assessment of the association between perceived gastrointestinal morbidity and a distinct pathogen. Sixth, morbidity in this study was exclusively assessed by self-report, and no reproducible, standardised clinical examination was performed to assess an individual's health status. Some of the aforementioned shortcomings will be addressed during a larger multi-country study on persistent diarrhoea that is currently being implemented in Côte d'Ivoire, Mali, and Nepal, carried out by the NIDIAG research consortium (www.nidiag.org).

Conclusions and recommendations

A diagnostic strategy consisting of a sensitive multiplex PCR assay in combination with parasitological microscopic techniques and RDTs detected a broad range of bacteria, parasites, and viruses in >80% of patients with persistent diarrhoea in southern Côte d'Ivoire. However, correct diagnosis of the clinically most significant pathogens is hampered by co-infections and surprisingly high detection rates of facultative and obligate pathogens even in asymptomatic controls. Future studies investigating persistent diarrhoea should adopt a case–control design and attempt to quantify and compare the pathogen load between symptomatic and asymptomatic individuals.

The Luminex® GPP covers a wide range of diarrhoeagenic pathogens, and may constitute a useful screening tool to rapidly exclude common infectious agents. However, confirmatory methods may still be necessary in cases of PCR-positive results to discriminate clinically relevant infections with viable pathogens from asymptomatic carriage or nucleic acid shedding after resolved disease. In its current configuration, the Luminex® GPP does not detect helminth infections, which limits its suitability as screening tool in the tropics and subtropics, where helminthiases are still pervasive.

Transparency declaration

The reagents for the Luminex® xTAG multiplex PCR and the specific fluorescent-based detection system were provided free of charge by the Luminex® Corporation (Austin, TX, USA). P. Mertens is Director for Research and Development (R&D) at the company Coris BioConcept (Gembloux, Belgium). One of the commercially available RDTs employed in the current study, the 'Crypto/Giardia DuoStrip', is produced by Coris BioConcept.

Both companies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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7 Real-time PCR for detection of *Strongyloides stercoralis* in human stool samples from Côte d'Ivoire: diagnostic accuracy, inter-laboratory comparison and patterns of hookworm co-infection

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Real-time PCR for detection of *Strongyloides stercoralis* in human stool samples from Côte d'Ivoire: Diagnostic accuracy, inter-laboratory comparison and patterns of hookworm co-infection

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ABSTRACT

Human infections with the helminth species *Strongyloides stercoralis* encompass a wide clinical spectrum, ranging from asymptomatic carriage to life-threatening disease. The diagnosis of *S. stercoralis* is cumbersome and the sensitivity of conventional stool microscopy is low. New molecular tools have been developed to increase sensitivity. We compared the diagnostic accuracy of real-time PCR with microscopy for the detection of *S. stercoralis* and hookworm in human stool samples, and investigated the inter-laboratory agreement of *S. stercoralis*-specific real-time PCR in two European laboratories. Stool specimens from 256 randomly selected individuals in rural Côte d'Ivoire were examined using three microscopic techniques (i.e. Kato-Katz, Koga agar plate (KAP) and Baermann (BM)). Additionally, ethanol-fixed stool aliquots were subjected to molecular diagnosis. The prevalence of *S. stercoralis* and hookworm infection was 21.9% and 52.0%, respectively, whilst co-infections were detected in 35 (13.7%) participants. The diagnostic agreement between real-time PCR and microscopy was excellent when both KAP and BM tested positive for *S. stercoralis*, but was considerably lower when only one microscopic technique was positive. The sensitivity of KAP, BM and real-time PCR for detection of *S. stercoralis* as compared to a combination of all diagnostic techniques was 21.4%, 37.5% and 76.8%, respectively. The inter-laboratory agreement of *S. stercoralis*-specific PCR was substantial ($\kappa = 0.63$, $p < 0.001$). We conclude that a combination of real-time PCR and stool microscopy shows high accuracy for *S. stercoralis* diagnosis. Besides high sensitivity, PCR may also enhance specificity by reducing microscopic misdiagnosis of morphologically similar helminth larvae (i.e. hookworm and *S. stercoralis*) in settings where both helminth species co-exist.

1. Introduction

The nematode species *Strongyloides stercoralis* is known to be endemic in tropical and subtropical areas of Africa, Australia, Latin America and Southeast Asia, and has also been reported in the United States of America (e.g. Appalachian region) and Europe (e.g. along the Spanish Mediterranean Coast) (Schär et al., 2013b). At least 30 million people and perhaps up to 100 million people are affected by *S. stercoralis* worldwide (Bethony et al., 2006; Olsen et al., 2009). However, detailed analysis of available epidemiologic data suggests that the occurrence of strongyloidiasis might be more frequent than currently reported (Schär et al., 2015) with an estimated 370 million people affected (Bisoffi et al., 2013).

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S. stercoralis possesses two unique life cycle features that explain its particular medical relevance. These are, firstly, its capability of continued intestinal replication inside the host for many years (auto-infection) and, secondly, the spread of infectious third stage (L_3) larvae to extra-intestinal locations after penetration of the intestinal mucosa in immunosuppressed hosts (hyper-infection) (Utzinger et al., 2012). Such hyper-infection syndromes frequently lead to severe septicemia because intestinal bacteria residing on the helminth's surface are transported to virtually any host organ by the migrating larvae. It follows that potentially life-threatening conditions may develop up to several decades after an infection with *S. stercoralis*, for example, after starting immunosuppressive medication following organ transplantation (Keiser and Nutman, 2004). Hence, individuals at risk of *S. stercoralis* infection need to be detected and treated, even in the absence of any symptoms and without recent exposure to an endemic area.

The diagnosis of *S. stercoralis* in human fecal samples is cumbersome and the sensitivity of conventional microscopic techniques is low (Becker et al., 2013; Knopp et al., 2012; Montes et al., 2010; Siddiqui and Berk, 2001). *S. stercoralis* larvae hatch in the intestine and first stage (L_1) larvae, but no eggs, are passed with the feces. Commonly employed diagnostic methods such as direct fecal smear or the Kato-Katz technique frequently fail to identify *S. stercoralis*, and even concentration techniques based on sedimentation (e.g. formalin-ether concentration technique) or flotation (e.g. FLOTAC) are not sufficiently sensitive to detect *S. stercoralis* (Buonfrate et al., 2015a; Levenhagen and Costa-Cruz, 2014). More reliable techniques for microscopic diagnosis are the Baermann (BM) funnel (concentration method that takes advantage of the larvae's thermo- and hydrophily) (Yap et al., 2012) and a nutrient agar plate culture that was developed by Koga and colleagues (Koga agar plate; KAP) (Koga et al., 1991). However, these techniques are laborious, time-consuming and examination of several stool specimens from one patient is still required to exclude *S. stercoralis* infections (Knopp et al., 2008; Schär et al., 2015; Steinmann et al., 2007). Additionally, experienced laboratory technicians are needed to reliably differentiate *S. stercoralis* from morphologically similar larvae under a microscope, e.g. *Oesephagostomum bifurcum* and *Trichostrongylus* spp. in the BM technique and hookworm larvae in the KAP method.

In recent years, molecular tools have been developed that show a high sensitivity of stool-based diagnosis for several helminth species, including *S. stercoralis* (Janwan et al., 2011; Kramme et al., 2011; Schär et al., 2013a; Verweij et al., 2009). For example, real-time PCR targeting the helminth's 18S ribosomal ribonucleic acid (18S rRNA) gene is increasingly being employed in reference laboratories and holds promise to be used as screening tool to detect light-intensity infections in at-risk individuals (ten Hove et al., 2009; Yansouni et al., 2014). However, some researchers have challenged the sensitivity of PCR if compared to expert microscopy or a combination of different microscopic techniques (Knopp et al., 2014). Additionally, the reproducibility of PCR-based tests for *S. stercoralis* detection in different laboratories has not yet been evaluated.

We compared the diagnostic accuracy of PCR with microscopy (BM and KAP) for the diagnosis of *S. stercoralis* in stool samples from randomly selected individuals in a village in south-central Côte d'Ivoire. Moreover, we evaluated the inter-laboratory agreement of real-time PCR in two European laboratories and assessed for co-infections with hookworm.

2. Materials and methods

2.1. Ethics statement

This study was carried out as part of a cross-sectional epidemiologic baseline survey that was conducted in the Taabo health and demographic surveillance system (HDSS) in south-central Côte d'Ivoire (Becker et al., 2011a; Koné et al., 2015). The study was approved by the institutional research commissions of the Swiss Tropical and Public Health Institute (Swiss TPH; Basel, Switzerland) and the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (CSRS; Abidjan, Côte d'Ivoire). Ethical approval was granted by the ethics committee of Basel (EKBB; reference no. 316/08) and the Ministry of Higher Education and Scientific Research in Côte d'Ivoire (reference no. 124/MESRS/DGRSIT/YKS/sac). Written informed consent was obtained from all participants (parents/guardians signing for individuals aged below 18 years). Free treatment was offered to participants with a helminth infection at the end of the study (i.e. single 400 mg dose of albendazole against soil-transmitted helminths; single 200 μg/kg dose of ivermectin against *S. stercoralis*, single 40 mg/kg dose of praziquantel against *Schistosoma* spp.). The PCR results reported here were conducted independently several months after the field work in Côte d'Ivoire, thus these results did not guide the treatment decisions.

2.2. Study area, population and design

Our study was conducted in the village of Léléblé and five surrounding hamlets in south-central Côte d'Ivoire in May and June 2009. Léléblé is one of 13 main villages of the Taabo HDSS, located approximately 160 km north-west of Abidjan, the economic capital of Côte d'Ivoire (Koné et al., 2015). The design of the study was a cross-sectional survey, readily embedded in a larger epidemiologic baseline survey to assess the population prevalence of *S. stercoralis*, other helminths and *Plasmodium* infection in the Taabo HDSS. Approximately 7% of the households in Léléblé and five surrounding hamlets were randomly selected, resulting in 351 individuals to be examined as a representation for the whole village population.

2.3. Field and laboratory procedures

After written informed consent had been obtained, stool collection containers were distributed to the study participants and they were invited to collect a stool sample the next morning. The fresh stool specimens were collected at a public location and transferred to a nearby laboratory in Taabo-Cité. All samples were processed the same day and subjected to a suite of microscopic techniques for helminth diagnosis: Kato-Katz method (duplicate Kato-Katz thick smears for detection of *S. mansoni* and soil-transmitted helminth eggs) (Katz et al., 1972); BM funnel concentration and KAP culture (for detection and differentiation of *S. stercoralis* and morphologically similar hookworm larvae) (Fig. 1). Protocols for the BM and KAP techniques have been described elsewhere (Knopp et al., 2008). Of note, only one BM funnel concentration and one KAP culture were prepared from each stool sample. An apricot-sized amount of fresh stool was used for the BM technique and microscope slides were prepared after 2–3 h of incubation. For the KAP technique, approximately 2 g of fresh fecal material was placed onto the central part of a freshly prepared agar plate, and was incubated at 26–30 °C for 48 h before examination. Microscope slides were read by experienced laboratory technicians. Approximately 10% of the slides were re-examined by an independent senior laboratory technician.

In a next step, for samples with sufficient fecal material, approximately 0.5 g was put into an Eppendorf tube containing 70% ethanol. These samples were forwarded to Swiss TPH for retrospective PCR examinations. Additionally, approximately 2 g of stool was fixed in 5% formalin and was examined within 4 months for the presence of intestinal protozoa, using the formalin-ether concentration technique and the FLOTAC-400 dual technique (Cringoli et al., 2010; Utzinger et al., 2010). The comparison of these two

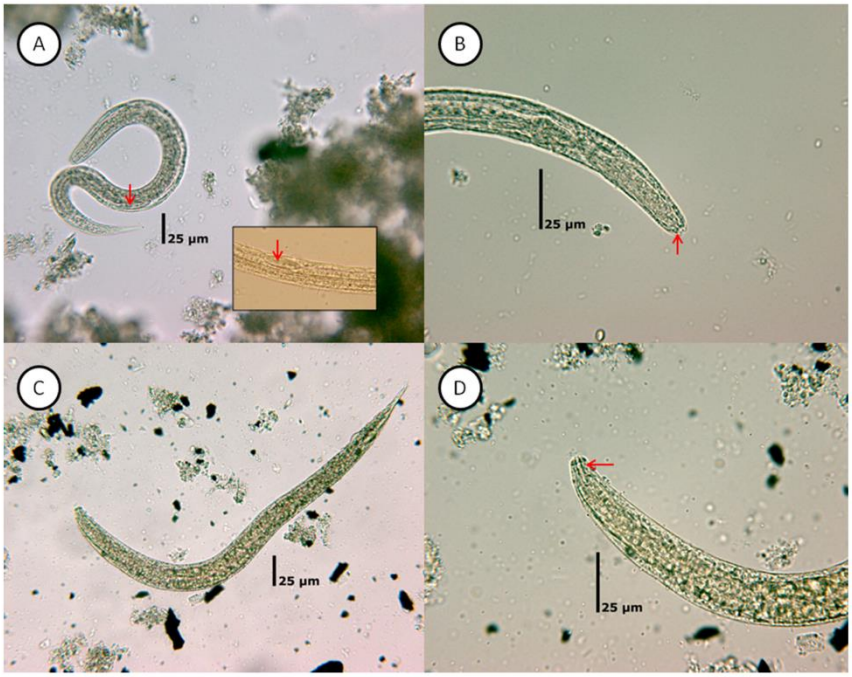

Fig. 1. Morphology of rhabditiform *Strongyloides stercoralis* L_1 larvae and hookworm L_1 larvae, as obtained using the Baermann funnel technique, examined under a microscope. Differentiating characteristics are indicated by red arrows. In *S. stercoralis*, a prominent genital primordium (A) and a short buccal cavity (B) are observed. Hookworm larvae are characterized by the absence of a visible genital primordium (C) and have a long buccal cavity (D).

diagnostic techniques for detection of intestinal protozoa infection has been presented elsewhere (Becker et al., 2011a).

2.4. Real-time PCR

Upon receipt of the ethanol-fixed stool aliquots at Swiss TPH, nucleic acid extraction was performed, using the QIAmp DNA Stool Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. A total of 200 mg of ethanol-fixed stool was used and washed three times with PBS before nucleic acid extraction. Samples were then subjected to real-time PCR for detection of the hookworm species *Ancylostoma duodenale* and *Necator americanus* (Verweij et al., 2007). The 18S *S. stercoralis*-specific real-time PCR was carried out according to a protocol published by Verweij et al. (2009) with some minor modifications. In brief, the *A. duodenale* and *N. americanus* real-time PCR assays were done as a 25 µl monoplex reaction, containing 5 µl DNA template, 12.5 µl HotStarMastermix (Qiagen), 3 mM $MgCl_2$, 400 nM of each sense and antisense primer (TIB-Molbiol; Berlin, Germany) and 200 nM of the respective probe (TIB-Molbiol). All probes were labeled with the dye FAM and BHQ as quencher. Thermal cycling on an ABI 7500 real-time PCR system (Applied Biosystems; Weiterstadt, Germany) comprised denaturation/activation of enzyme at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s (denaturation step) and 60 °C for 30 s (combined annealing/extension step). Fluorescence was measured during the 60 °C step. The *S. stercoralis* real-time PCR contained 5 µl DNA template, 12.5 µl HotStarMastermix (Qiagen), 400 nM of each sense and antisense primer (TIB-Molbiol) and 200 nM of a FAM/BHQ-labelled probe (TIB-Molbiol). Thermal cycling was carried out with the same characteristics as the hookworm PCR and fluorescence was measured during the 60 °C step.

To allow for inter-laboratory comparison of the PCR results, extracted nucleic acids were stored at –80 °C and were transferred to the Institute of Medical Microbiology and Hygiene (IMMH) in Homburg/Saar, Germany in late 2012. At IMMH, real-time PCR for *S. stercoralis* was performed, using the same protocol and identical primer and probe sequences at the same concentrations. Of note, the following minor modifications were done: for DNA amplification, 5 µl template was used in a final volume of 20 µl with 10 µl Roche FastStart Essential DNA Probes Master, which includes PCR buffer, deoxynucleotide triphosphate (dNTP) mix, $MgCl_2$ and Taq DNA polymerase (F. Hoffmann-La Roche AG; Basel, Switzerland), 3 µl water and 2 µl of primer-probe mix**.** Thermal cycling was performed on a Roche LightCycler® 96 System and comprised 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Of note, the manufacturer recommends a shorter initial denaturation/activation of 10 min, but we opted for a prolonged 15 min-period based on our own experience with this system for *S. stercoralis*-specific PCR on fecal samples. No additional real-time PCR for hookworm detection was carried out at IMMH.

2.5. Statistical analysis

Data were double entered and cross-checked in Excel, version 10.0 (2002 edition; Microsoft Corporation). For statistical analysis, STATA version 12.0 (StataCorp.; College Station, TX, USA) was utilized. In the absence of an unambiguous reference test for the diagnosis of *S. stercoralis* and hookworm, a positive test result in any of the employed microscopic and molecular diagnostic tests was considered as 'true positive', thus leading to a combination of all positive test results for a certain pathogen being the internal diagnostic 'gold' standard. The sensitivity and negative predictive value (NPV) were calculated for each method in relation to this

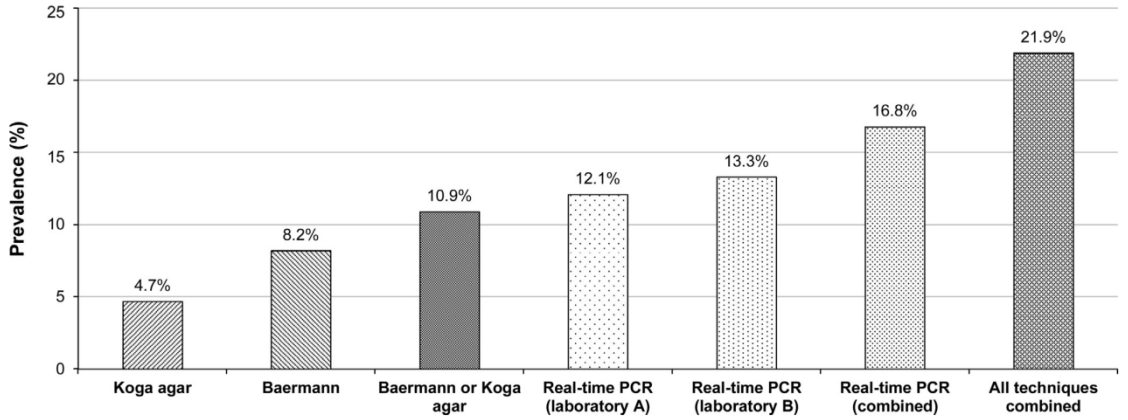

Fig. 2. Prevalence of *S. stercoralis* in 256 stool samples obtained during a cross-sectional epidemiologic survey in south-central Côte d'Ivoire in mid-2009, as determined by different diagnostic techniques.

diagnostic 'gold' standard, including 95% confidence intervals (CIs) to quantify statistical uncertainty. The specificity of the PCR assay was assessed in comparison to the results obtained by the combination of BM and KAP. The results of microscopic and molecular tests were compared and cycle threshold (C_t) values were recorded for each positive PCR result to semi-quantify the amount of target nucleic acids in a sample (i.e. the lower the C_t value, the higher the corresponding amount of target nucleic acid). A p-value <0.05 was considered to indicate statistical significance.

To assess inter-laboratory agreement of PCR for *S. stercoralis* detection, Cohen's kappa measure (κ) was used and interpreted as follows: $\kappa < 0$, no agreement; $\kappa = 0$–0.20, poor agreement; $\kappa = 0.21$–0.40, fair agreement; $\kappa = 0.41$–0.60, moderate agreement; $\kappa = 0.61$–0.80, substantial agreement; and $\kappa = 0.81$–1.0, nearly perfect agreement (Cohen, 1960).

3. Results

3.1. Study cohort

Among 351 randomly selected individuals, 256 participants provided a sufficiently large quantity of stool to perform microscopic examinations in the laboratory of the district hospital at Taabo-Cité, Côte d'Ivoire and subsequent *post-hoc* molecular testing in two laboratories in Europe. There were slightly more males than females (133 *versus* 123). Most of the participants lived in the main village of Léléblé ($n = 207$; 80.9%), while the remaining 49 subjects resided in remote rural hamlets. The median age of the final study cohort was 13.5 years with a range from 1 month to 74 years.

3.2. Prevalence of S. stercoralis and accuracy of diagnostic methods

Considering any positive test result as 'true positive', the overall prevalence of *S. stercoralis* was 21.9% ($n = 56$; 95% CI: 16.8–27.0%). Real-time PCR (combined results of both laboratories) revealed the highest prevalence (16.8%, $n = 43$; 95% CI: 12.2–21.4%), followed by BM (8.2%, $n = 21$; 95% CI: 4.8–11.6%) and KAP (4.7%, $n = 12$; 95% CI: 2.1–7.3%). About half of the *S. stercoralis* infections were detected exclusively by real-time PCR (Fig. 2).

The sensitivity of KAP, BM, the combination of KAP and BM, and real-time PCR for the diagnosis of *S. stercoralis* was 21.4% (95% CI: 11.6–34.4%), 37.5% (95% CI: 24.9–51.5%), 50.0% (95% CI: 36.3–63.7%) and 76.8% (95% CI: 63.6–87.0%), respectively. The specificity of real-time PCR as compared to the combination KAP plus BM was 89.7% (200/223 samples). The NPV was highest for real-time PCR (93.9%; 95% CI: 89.8.–96.7%) and the combination KAP plus BM (87.7%; 95% CI: 82.7–91.7%), followed by both microscopic techniques alone (BM: 85.1%; KAP: 82.0%).

All stool specimens that tested positive for *S. stercoralis* by both KAP and BM were confirmed by PCR. However, the diagnostic agreement between microscopy and PCR was considerably lower when only one microscopic technique was positive (Table 1). Half of the BM-positive and KAP-negative samples were confirmed by real-time PCR, while no *S. stercoralis*-specific DNA could be detected in 5 out of 7 specimens that were exclusively positive by KAP.

3.3. Inter-laboratory comparison of real-time PCR for S. stercoralis

The real-time PCR assay for *S. stercoralis* detection in laboratory A identified slightly less infections than in laboratory B (31 *versus* 34 infections). In contrast, there was a trend toward lower C_t values in laboratory A compared to laboratory B (median C_t 32 *versus* 37). The inter-laboratory comparison of real-time PCR in both European laboratories yielded concordant results in 235 out of 256 samples (*S. stercoralis* infection: $n = 22$; absence of *S. stercoralis* infection: $n = 213$), thus leading to a substantial diagnostic

Table 1
Comparison of the diagnostic agreement between two microscopic techniques (Baermann funnel, Koga agar plate) and real-time PCR (performed in two European laboratories) for the diagnosis of *S. stercoralis* in 256 stool samples obtained from a cross-sectional survey conducted in south-central Côte d'Ivoire in mid-2009.

N	Microscopy		Real-time PCR (laboratory A)			Real-time PCR (laboratory B)			Diagnostic agreement between PCR in both laboratories
	Baermann	Koga	Positive samples	Agreement with microscopy	Median C_t value (min–max)	Positive samples	Agreement with microscopy	Median C_t value (min–max)	
5	+	+	5	100%	32 (24–35)	5	100%	37 (28–38)	100%
16	+	–	8	50%	31 (28–36)	8	50%	34 (31–40)	100%
7	–	+	1	14.9%	32	1	14.9%	39	71.4%
228	–	–	17	92.5%	34 (28–37)	20	91.2%	38 (31–42)	91.7%

Table 2
Two-way contingency table comparing the results and respective C_t values of real-time PCR for *S. stercoralis*, independently performed in two laboratories on stool samples obtained from 256 individuals living in south-central Côte d'Ivoire in mid-2009.

Laboratory A	Laboratory B				Total
	C_t: <30	C_t: 30–35	C_t: >35	Negative	
C_t: <30	1	5	0	1	7
C_t: 30–35	0	7	7	5	19
C_t: >35	0	0	2	3	5
Negative	0	2	10	213	225
Total	1	14	19	222	256

Table 3
Two-way contingency table showing the occurrence of infections with *S. stercoralis*, hookworm and co-infection with both helminth species in 256 individuals from Léléblé, south-central Côte d'Ivoire in mid-2009.

S. stercoralis	Hookworm		
	Positive	Negative	Total
Positive	35	21	56
Negative	98	102	200
Total	133	123	256

agreement (Cohen's kappa measure, $\kappa = 0.63$, $p < 0.001$). Details on the comparative C_t values are given in Table 2. Of note, among the 21 discordant PCR results that were positive for *S. stercoralis* in only one laboratory, 13 had a C_t value above 35, while only one specimen had a C_t value <32.

3.4. Co-infection with S. stercoralis and hookworm

The prevalence of hookworm infection, as determined by BM, real-time PCR, KAP and duplicate Kato-Katz thick smears was 8.2% ($n = 21$), 14.1% ($n = 36$), 32.1% ($n = 82$) and 38.7% ($n = 99$), respectively. Considering any positive test result by any of the methods employed as 'true positive', the prevalence of hookworm infection was 52.0% ($n = 133$; 95% CI: 45.8–58.1%). 98.5% ($n = 131$) of all infections were of light intensity (<2000 eggs per gram of stool, EPG), while no heavy hookworm infections were detected. As the eggs of the two hookworm species are indistinguishable by stool microscopy, a species-specific differentiation could only be achieved by molecular tests. Real-time PCR for the two hookworm species identified *N. americanus* as the causative agent in all 36 stool samples that were PCR-positive for hookworm infection, while no case of *A. duodenale* could be detected. The sensitivity (as compared to the combination of all diagnostic tests) and specificity (as compared to the microscopic methods) of the hookworm PCR were 27.1% and 95.4%, respectively.

Co-infection with hookworm and *S. stercoralis* was observed in 35 individuals, owing to an overall co-infection prevalence of 13.7%, which was higher than what was expected by chance, considering independence of the two helminth species (11.4%; $p = 0.074$) (Table 3). The prevalence of both helminth infections was associated with age of the participants; the highest infection rates were found in individuals aged 30–49 years (Fig. 3). 63% ($n = 23$) of the co-infections were observed in males.

A hookworm co-infection was detected in 10 of 13 stool samples that were diagnosed as microscopically positive for *S. stercoralis*, but had a negative PCR result for the latter helminth. In 6 of these specimens, the hookworm co-infection was confirmed by two independent techniques ($n = 5$, Kato-Katz and KAP; $n = 1$, KAP and hookworm-specific PCR) (Table 4).

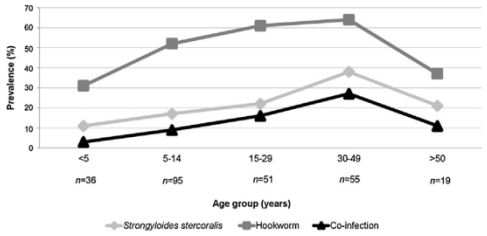

Fig. 3. Age-prevalence curves of *S. stercoralis*, hookworm and co-infection with both helminth species in 256 individuals in Léléblé, south-central Côte d'Ivoire in mid-2009.

4. Discussion

The diagnosis of *S. stercoralis* mainly relies on stool microscopy and identification of helminth larvae. No eggs are passed in the stool and the worm burden is usually low, particularly in asymptomatic individuals. Hence, commonly employed microscopic techniques frequently fail to detect *S. stercoralis* in stool samples. Microscopic concentration techniques (e.g. BM) and agar plate cultures (e.g. KAP) improve the diagnostic accuracy, but are insufficiently standardized and may still miss a considerable number of infections. Recently, different PCR protocols were developed to enhance the diagnosis of *S. stercoralis* (Verweij and Stensvold, 2014).

Here, we assessed the diagnostic accuracy of real-time PCR for the diagnosis of *S. stercoralis* in comparison to BM and KAP techniques, and we investigated the inter-laboratory agreement of real-time PCR in two European laboratories. Real-time PCR was the most sensitive technique for the diagnosis of *S. stercoralis* and half of the infections would have been missed by stool microscopy. This observation confirms previous data pertaining to the diagnostic accuracy of stool microscopy for *S. stercoralis* diagnosis (Siddiqui and Berk, 2001). According to a systematic review and meta-analysis, the sensitivity of a single KAP and a single BM is 89% and 72%, respectively (Campo Polanco et al., 2014). However, the BM technique was more sensitive than KAP in our study. Both techniques lack standardization from one laboratory to another, which may render diagnostic comparisons across different settings difficult. Even subtle modifications in the preparation of microscopic techniques for detection of *S. stercoralis* have been shown to considerably influence the test results (Anamnart et al., 2010). Hence, the sensitivity of KAP in our study setting, where mainly low-intensity infections occurred, would likely have improved if larger amounts of fecal material had been placed on the KAP or if several agar plates from each specimen had been prepared. Of note, studies employing molecular diagnostics for *S. stercoralis* were not included in the aforementioned meta-analysis. Hence, it is conceivable that the reported sensitivities overestimate the true diagnostic accuracy of these conventional parasitological techniques, particularly in light of our findings, i.e. that 50% of *S. stercoralis* cases were exclusively detected by PCR.

In the present study, the sensitivity of real-time PCR was 76.8%, which is considerably lower than in recent studies from Cambodia (Schär et al., 2013a) and Spain (Saugar et al., 2015), where a similar approach was pursued and sensitivities of 88.9% and 93.8% were reported, respectively. In contrast, a study employing PCR and the BM technique for the detection of light-intensity *S. stercoralis* infections in Tanzania observed a surprisingly low sensitivity of only 30.9% for PCR (Knopp et al., 2014). The specificity of the molecular assay in all three aforementioned studies ranged between 86.5% and 93.9%, and was thus comparable to the 89.7% observed in the current study. However, it should be noted that the specificity of real-time PCR in our study is probably underestimated because it

Table 4
Comparison of parasitological findings in 13 stool samples that were microscopically diagnosed as *S. stercoralis* infection, but gave a negative PCR result for *S. stercoralis*. The stool specimens were obtained from individuals in Léléblé, south-central Côte d'Ivoire in mid-2009.

No.	Duplicate Kato-Katz	Baermann funnel	Koga agar plate	Hookworm PCR
1	–	–	Hookworm, *S. stercoralis*	–
2	*Schistosoma mansoni*	*S. stercoralis*	–	–
3	*Ascaris lumbricoides*	*S. stercoralis*	–	–
4	Hookworm	*S. stercoralis*	–	–
5	Hookworm	*S. stercoralis*	Hookworm	–
6	–	*S. stercoralis*	Hookworm	Hookworm (*N. americanus*)
7	Hookworm	*S. stercoralis*	Hookworm	–
8	Hookworm	–	Hookworm, *S. stercoralis*	–
9	–	–	*S. stercoralis*	–
10	*Hymenolepis nana*	–	Hookworm, *S. stercoralis*	–
11	Hookworm	*S. stercoralis*	Hookworm	–
12	Hookworm	*S. stercoralis*	Hookworm	–
13	Hookworm	–	*S. stercoralis*	–

is compared to an insufficiently accurate reference standard, i.e. a combination of microscopic methods that may have misclassified some hookworm infections as *S. stercoralis* infections, thereby decreasing the specificity of the molecular assay. The substantial diagnostic agreement between real-time PCR in two independent laboratories (despite slight modifications of the PCR protocol in both laboratories) confirms the high reproducibility of standardized real-time PCR assays (Travis et al., 2011).

In chronic *S. stercoralis* infections, the number of larvae shed in the feces is generally low, which may in turn lead to false-negative PCR results (Kramme et al., 2011; Sultana et al., 2013; Verweij et al., 2009). In our work, stool samples were obtained during a cross-sectional epidemiologic survey. Even though subtle morbidity was detectable in *S. stercoralis*-positive individuals upon detailed clinical assessment (Becker et al., 2011b), it is conceivable that most individuals had chronic infections of rather light intensity. This may partially explain the relatively low sensitivity of real-time PCR in our study, in particular if compared to the aforesaid reports from a clinical center in Spain (Saugar et al., 2015), which analyzed samples stemming from symptomatic patients, and from Cambodia where high-intensity infections are frequently encountered (Khieu et al., 2013b). This hypothesis is further underscored by the previously mentioned study of Knopp et al. (2014) in Tanzania where *S. stercoralis* infections were mainly of light intensity and real-time PCR on these samples frequently gave false-negative results. Additionally, the presence of e.g. inhibitors in the stool sample may also have led to some false-negative PCR results in our study. However, genetic variability of *Strongyloides* spp. could also play an important role. Indeed, recent research has identified different *S. stercoralis* genotypes and also several microscopically indistinguishable *Strongyloides* species (Hasegawa et al., 2009; Schär et al., 2014). Hence, new research is needed to investigate this issue by using concentrated specimens, e.g. as obtained from the BM technique, rather than stool samples to perform real-time PCR and (partial) sequencing.

Co-infections with hookworm and *S. stercoralis* commonly occur and can be explained by similar transmission patterns of both helminths (Strunz et al., 2014). Hence, our findings confirm previous research from Latin America (Taranto et al., 2003) and Asia (Khieu et al., 2013a). However, further analysis of our data revealed that care is indicated when stool samples stemming from co-endemic settings for hookworm and *S. stercoralis* are analyzed microscopically. Indeed, 13 samples were reported as microscopy-positive for *S. stercoralis*, but had a negative real-time PCR result (Table 3). Ten of these 13 samples were found to be positive for hookworm, with 6 specimens being confirmed by two independent techniques. Hence, while 'true' co-infections with both helminth species frequently occur in endemic settings, we speculate that misidentification of hookworm larvae as *S. stercoralis* larvae may occasionally have occurred. Similar observations have previously been noted in the context of medical case reports (Smith et al., 1977) and diagnostic studies that compared *S. stercoralis*-specific PCR to BM and KAP techniques in Cambodia (Schär et al., 2013a) and Ghana (Verweij et al., 2007).

Our study has several limitations. First, we analyzed only one stool sample per participant, which may have led to an underestimation of the true prevalence of *S. stercoralis*, hookworm and co-infection, because helminth eggs and larvae are shed in the feces with considerable day-to-day variation (Utzinger et al., 2001). Second, sensitivity and specificity data of the different laboratory techniques need to be interpreted with caution in the absence of an independent diagnostic reference standard. Third, no (semi-) quantitative assessment of *S. stercoralis* larvae was performed upon microscopic examination, so that no comparison between C_t values and the number of microscopically detected larvae could be done. Fourth, we did not employ additional serological tests for the diagnosis of *S. stercoralis*, which could have further elucidated the local epidemiology of strongyloidiasis in our study setting. Serology is useful to evaluate an individual's exposure to *S. stercoralis* and can also be used to monitor treatment success because IgG antibody titres usually decline within 9–12 months in cured patients (Buonfrate et al., 2015b). Indeed, with the exception of recently acquired infections, serology has a high sensitivity and a NPV of nearly 100% for detection of strongyloidiasis. An excellent review on the utility of *S. stercoralis*-specific serology has been published recently (Buonfrate et al., 2015a). Fifth, this study could only compare the results obtained by *S. stercoralis*-specific PCR in two laboratories after previous nucleic acid extraction. Hence, our approach did not assess the effect of different DNA extraction procedures on the diagnostic yield of PCR tests for *S. stercoralis*. An improvement of the existing DNA isolation techniques for *S. stercoralis* has been identified as an important research need (Repetto et al., 2013) and future studies should be performed to accurately assess and compare the influence of different extraction methods on the final PCR results. Sixth, while the diagnostic accuracy of *S. stercoralis*-specific PCR was in the expected range and the inter-laboratory comparison yielded largely concordant results, the results obtained by hookworm PCR were disappointing. Almost all samples in the study area were of light infection intensity so that the number of detectable hookworm eggs in the stool samples was low, which might partially explain some false-negative PCR results. Further research is thus warranted to compare different hookworm PCR protocols and DNA extraction methods, and to investigate inter-laboratory agreement. On the other hand, we cannot exclude that some microscopy-positive, PCR-negative samples were not caused by hookworm infection. Indeed, eggs of the helminth *O. bifurcum* cannot be differentiated from hookworm eggs by light microscopy and human infections with this pathogen are known

to occur in West Africa, particularly in Ghana and Togo (Verweij et al., 2007). Integrating hookworm and *O. bifurcum* PCR examinations into epidemiologic surveys in tropical settings is of particular importance to differentiate these microscopically indistinguishable helminths that may give rise to different clinical manifestations (Jonker et al., 2012).

5. Conclusions

Real-time PCR was more sensitive than a combination of BM and KAP techniques for the diagnosis of *S. stercoralis* in a rural setting of south-central Côte d'Ivoire. We found a substantial agreement in *post-hoc* real-time PCR examinations of stool samples for *S. stercoralis* in two European laboratories. Besides the high sensitivity, PCR may also enhance specificity by preventing misdiagnosis of morphologically similar nematodes (e.g. *S. stercoralis* and hookworm larvae in BM funnel). *S. stercoralis* and hookworm co-exist in the same village, which calls for differential diagnosis with high sensitivity and specificity. A combined diagnostic algorithm that employs real-time PCR and stool microscopy using the BM and KAP techniques is recommended for detection of *S. stercoralis* with high diagnostic accuracy.

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8 Molecular and culture-based diagnosis of *Clostridium difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions

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ORIGINAL ARTICLE

Molecular and culture-based diagnosis of *Clostridium difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions

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Background: Although *Clostridium difficile* is a major cause of diarrhoea, its epidemiology in tropical settings is poorly understood. Strain characterisation requires work-up in specialised laboratories, often after prolonged storage without properly maintained cold chain.

Methods: We screened 298 human faecal samples from Côte d'Ivoire using a rapid test for *C. difficile* glutamate dehydrogenase (GDH). GDH-positive samples were aerobically stored at disrupted cold chain conditions (mean duration: 11 days) before transfer to a reference laboratory for anaerobic culture, susceptibility testing, PCR assays and ribotyping.

Results: Sixteen samples (5.4%) had a positive GDH screening test. *C. difficile* infection was confirmed in six specimens by culture and PCR, while no nucleic acids of *C. difficile* were detected in the culture-negative samples. Further analysis of stool samples harbouring toxigenic *C. difficile* strains confirmed that both GDH and toxins remained detectable for at least 28 days, regardless of storage conditions (aerobic storage at 4°C or 20°C).

Conclusions: Storage conditions only minimally affect recovery of *C. difficile* and its toxins in stool culture. A rapid GDH screening test and subsequent transfer of GDH-positive stool samples to reference laboratories for in-depth characterisation may improve our understanding of the epidemiology of *C. difficile* in the tropics.

Keywords: Côte d'Ivoire, Diagnosis, Diarrhoea, Polymerase chain reaction, Rapid diagnostic test, Storage conditions

Introduction

Clostridium difficile is an anaerobic, Gram-positive, rod-shaped and endospore-forming bacterium that may survive under extreme environmental conditions, including high temperatures, toxic chemicals and UV radiation.[1] *C. difficile* is the leading cause of nosocomial, antibiotic-associated diarrhoea worldwide and there is growing evidence that *C. difficile* is also a key pathogen of community-acquired intestinal infections.[2–5] While a steady increase of *C. difficile*-associated diarrhoea (CDAD) has been observed in Europe and North America, there is a paucity of epidemiological data from Africa, Asia[6] and South America[7] where diarrhoeal diseases remain important causes of morbidity and mortality.[8] For example, a search on PubMed/Medline on June 23, 2015 using the search strategy '*Clostridium difficile* AND Africa' yielded only 29 hits, 15 studies of which pertained to the bacterium's prevalence in humans, animals or environmental samples from Africa.[9–23]

Recent data suggest that CDAD in travellers returning from low- and middle-income countries is considerable, thus highlighting the need to deepen our understanding of the epidemiology of *C. difficile* in Africa and elsewhere in the developing world.[24] Additionally, there is ongoing debate whether *C. difficile* might be regularly transmissible as a zoonotic disease via animals or food products. Zoonotic transmission to humans has been documented for some strains (e.g., ribotype [RT] 078), but no direct food-borne outbreaks have been reported thus far.[25,26] Strains can be classified into genotypic groups by PCR ribotyping,[27]

sequence typing[28] or other genotypic methods.[29,30] Some hypervirulent RTs (e.g., RT027) are reported to be associated with a more severe clinical disease, but no data on the currently circulating RTs have been reported from Africa.[31]

The paucity of epidemiological data from resource-constrained settings is explained by the cumbersome laboratory diagnosis of *C. difficile*, which has led to its scientific neglect in many tropical areas. Comprehensive diagnostic work-up requires cultural growth of *C. difficile* on selective media under anaerobic conditions, followed by subsequent molecular typing in specialised laboratories. It follows that testing strategies for *C. difficile* vary considerably even in high-income countries. Indeed, a recent multicentre study estimated that approximately 40 000 hospitalised patients with *C. difficile* infection remain undiagnosed every year in Europe due to the use of insensitive laboratory diagnostic tests.[32] In contrast, not even the most basic diagnostic tests for *C. difficile* are available in many resource-constrained settings. One of the few recent studies pertaining to the clinical relevance of this pathogen stems from Zimbabwe; *C. difficile* was detected in 8.6% of patients with community-acquired diarrhoea,[22] which underscores the need for an improved understanding of its occurrence in Africa.

Rapid diagnostic tests (RDTs) for *C. difficile* have been developed and constitute a useful screening tool to provide point-of-care information, particularly in resource-constrained settings. In common diagnostic algorithms, a *C. difficile*-specific glutamate dehydrogenase (GDH) assay is employed as a first-line test to identify specimens that warrant further diagnostic work-up.[33,34] Though GDH is specific for *C. difficile*, it does not differentiate pathogenic toxigenic strains from apathogenic non-toxigenic strains. Some rapid membrane tests combine GDH screening with testing for toxins A and B, but the sensitivity of toxin detection is low.[35] A thorough assessment of GDH-positive samples includes an array of sophisticated tests, including anaerobic toxigenic culture, followed by genotypic and phenotypic characterisation. While the pure confirmation of *C. difficile* infection and a differentiation between toxigenic and non-toxigenic strains is possible by stool-based PCR alone, a culture isolate is required for PCR ribotyping and antimicrobial susceptibility testing.

In resource-constrained settings, the transfer of GDH-positive samples to reference laboratories within a country or abroad may take several days. Hence, it is important to understand whether and how prolonged storage, transport conditions, environmental factors and varying temperatures affect the recovery of *C. difficile* in stool samples. Thus far, it is widely believed that a sensitive diagnosis needs to be performed on fresh stool samples due to the instability of *C. difficile* antigens. Indeed, the Association for Professionals in Infection Control and Epidemiology (APIC) currently recommends that samples should be frozen at −70°C if testing cannot be performed within 24 hours after stool collection.[36] However, storage under controlled freezing conditions is limited in most developing countries.

We conducted a case-control study in south Côte d'Ivoire to investigate the epidemiology and diagnostic accuracy of different methods for detection of intestinal pathogens. In this manuscript, we report on the frequency and characterisation of *C. difficile* strains using a two-step diagnostic algorithm consisting of a GDH screening test, followed by comprehensive sample work-up in a specialised laboratory after prolonged specimen storage at disrupted cold chain conditions. Moreover, we examined the influence of a prolonged storage under standardised aerobic conditions at varying temperatures on the detection of *C. difficile* using toxigenic culture and molecular diagnostic techniques.

Materials and methods

Study area and population

The study was conducted in October 2012 in Dabou and 11 surrounding villages, located some 30 km west of Abidjan, the economic capital of Côte d'Ivoire. The study was part of a site assessment to identify a suitable setting in Côte d'Ivoire for a subsequent multi-country investigation on the aetiology of persistent diarrhoea and persistent abdominal pain.[37,38] This research is coordinated by the European research network with the acronym NIDIAG, which aims to develop simple and cost-effective diagnosis-treatment algorithms for three clinical syndromes (i.e., digestive syndromes, persistent fever and neurological disorders) in tropical settings.[33,38–41] In the site assessment reported here, a case-control approach was adopted. Hence, individuals aged ≥1 year presenting with persistent diarrhoea (≥2 weeks) and individuals without any gastrointestinal symptoms (control group) were invited to participate. Definitions put forth by WHO were used to define diarrhoea, i.e., the passing of three or more loose stools within 24 hours.[38] The prevalence of various diarrhoeagenic bacteria, helminths, intestinal protozoa and viruses in cases and matched controls has been presented elsewhere.[37]

Field and laboratory procedures

Fresh stool samples were obtained in the early morning and transferred to the local hospital laboratory in Dabou. Upon arrival, an RDT indicating the presence of GDH (Clostridium *K*-SeT, Coris BioConcept, Gembloux, Belgium)[42] was performed to screen for *C. difficile*. Additionally, several microscopic techniques were employed for the diagnosis of intestinal protozoa and helminth infections (i.e., Baermann funnel, formalin-ether concentration technique, Kato-Katz thick smear and Koga agar plate).[37]

For later confirmatory testing of *C. difficile* in a specialised laboratory, approximately 0.3 g of each stool sample was transferred into a small vial and aerobically stored under 'real life conditions' in a fridge without proper maintenance of the cold chain due to power cuts. After a storage period between 8 and 19 days (mean 11 days), the samples were transferred at ambient temperature to the German National Advisory Laboratory for *C. difficile* in Homburg, Germany. All GDH-positive stool samples were analysed for the presence of *C. difficile* by anaerobic culture on a selective solid medium (CLO agar, BioMérieux, Marcy l'Étoile, France), and by multiplex PCR of stool samples (GenoType CDiff, Hain Lifescience, Nehren, Germany). The multiplex PCR detects the following *C. difficile*-associated genes: *gluD* (encoding for GDH), *tpi* (encoding for triose phosphate isomerase), *tcdA* (encoding for toxin A), *tcdB* (encoding for toxin B) and *cdtA/B* (encoding for the binary toxin). Additionally, characteristic deletions in the regulatory gene *tcdC* and resistance to moxifloxacin (mutations in the *gyrA* gene) are detected. The PCR assay was regarded as the internal diagnostic reference standard in our study due to stability of culture-independent assays, and results obtained by anaerobic stool culture after the prolonged sample storage were compared to this method. Suspected culture-grown colonies were identified based on typical morphology and distinct odour. Diagnosis of *C. difficile* was subsequently confirmed by matrix-assisted laser desorption ionisation time-of-flight mass

spectrometry (MALDI-TOF MS with BioTyper software, Bruker Daltonics, Bremen, Germany). Phenotypic characterisation was performed by antimicrobial susceptibility testing using Etests (metronidazole, moxifloxacin and vancomycin) and disk diffusion assay (clarithromycin and rifampicin), according to previously described standard protocols.[32,43] Species-specific EUCAST breakpoints (http://www.eucast.org) were used, if available.[44] PCR ribotyping using capillary gel electrophoresis was performed in Homburg, Germany to achieve genotypic differentiation and the results were independently confirmed in Leiden, The Netherlands. Files of untypeable *C. difficile* strains with previously undescribed ribotype patterns were also sent to Leeds University, UK for analysis using an in-house database.

To further analyse the influence of storage conditions on the performance of diagnostic tests, ten stool samples stemming from symptomatic, hospitalised patients with laboratory-proven toxigenic *C. difficile* infections were prospectively collected at the Saarland University Medical Center in Homburg, Germany. The faecal samples were split into two aliquots and stored in parallel at 4°C and at 20°C (ambient temperature) for at least 28 days. Diagnostic testing for *C. difficile* using toxigenic culture and an RDT for detection of GDH and toxins A/B (C. Diff Quik Check Complete, Alere, Köln, Germany) was repeated after 7, 14 and 28 days. The resulting RDT line intensities were documented using a semi-quantitative grading scheme, i.e., 3+, strong;

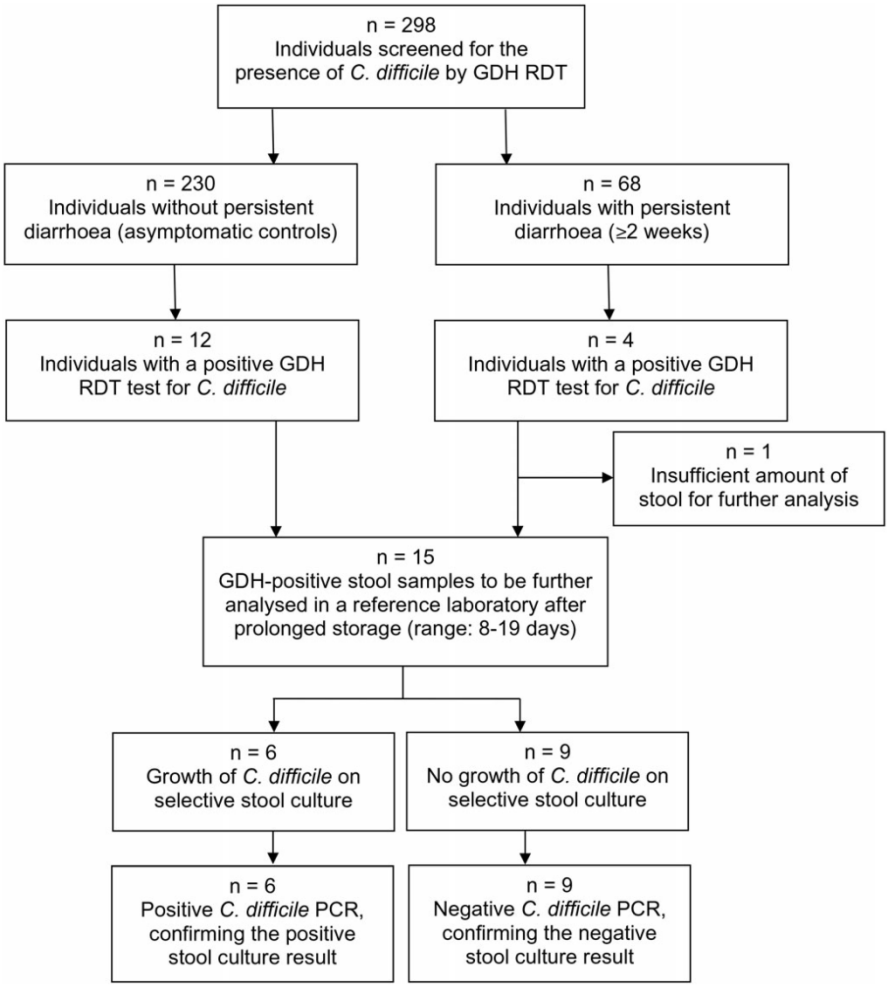

Figure 1. Study flowchart on the occurrence of *Clostridium difficile* in 298 individuals in Dabou, south Côte d'Ivoire, in October 2012. GDH: glutamate dehydrogenase; RDT: rapid diagnostic test.

2+, moderate; 1+, faint; and –, negative. The culture isolates of *C. difficile* were further characterised by PCR-based detection of toxin genes and ribotyping.

Statistical analysis

Data of the RDT screening results in Côte d'Ivoire and of subsequent laboratory procedures were entered twice and cross-checked in Excel version 14.0 (edition 2010, Microsoft Corp., Redmond, WA, USA). Analysis of *C. difficile*-specific RT patterns was performed using the software BioNumerics version 7.1 (edition 2013, Applied Maths, Sint-Martens-Latem, Belgium). In brief, a Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) algorithm were employed to compare closely related *C. difficile* isolates and to infer their genetic relatedness.

Ethics statement

The study protocol was approved by the institutional research commissions of the Swiss Tropical and Public Health Institute (Swiss TPH, Basel, Switzerland) and the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (CSRS; Abidjan, Côte d'Ivoire). Study approval was given by the Directorate of the Hôpital Méthodiste in Dabou. The study is registered on Current Controlled Trials (http://www.controlled-trials.com; identifier ISRCTN86951400). Individuals aged above 12 months with residency in Dabou or surrounding villages with written informed consent (parents/guardians signing for individuals aged below 18 years) were eligible to participate.

Results

The study flowchart is shown in Figure 1. In brief, GDH RDTs were performed on 298 stool samples for screening of *C. difficile* directly on site. Sixty-eight samples were provided by individuals with persistent diarrhoea (≥2 weeks) and 230 specimens originated from individuals without diarrhoea (asymptomatic controls). Positive test results were found on 16 samples. Four faecal specimens stemmed from symptomatic patients with persistent diarrhoea (5.8%). Of these, two patients stated to have received antibiotic treatment with cotrimoxazole and metronidazole, respectively, in the two preceding months. The remaining 12 RDT-positive samples were found in healthy controls (5.2%). Of note, samples with a faintly discernible test band were also considered as GDH-positive to increase the sensitivity of the screening test (Figure 2).

Fifteen GDH-positive samples were available for subsequent culture and molecular diagnosis in a European reference laboratory. Anaerobic culture yielded six *C. difficile* isolates which were independently confirmed by direct stool-based PCR. No specific DNA of *C. difficile* could be amplified in the nine culture-negative samples, thus leading to a 100% concordance between stool culture and direct molecular testing. Among the six isolates, none was a toxigenic strain, as determined by toxigenic culture and PCR for *tcdA* (toxin A), *tcdB* (toxin B) and *cdtA/B* (binary toxin). Four *C. difficile* isolates originated from individuals without gastrointestinal disorders (1.7%), while two isolates stemmed from symptomatic patients with persistent diarrhoea (2.9%). The difference between cases and matched controls was not statistically

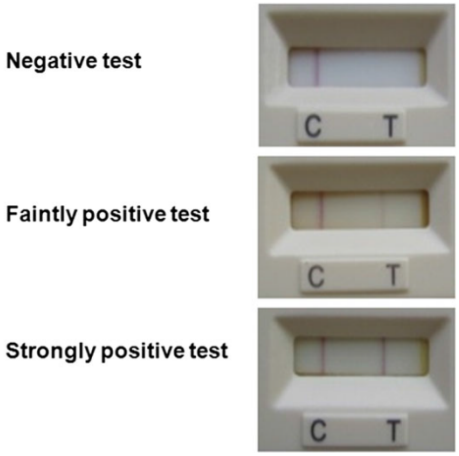

Figure 2. Results given by the rapid diagnostic glutamate dehydrogenase (GDH) screening test (Clostridium *K*-SeT) employed for diagnosis of *Clostridium difficile* in Dabou, south Côte d'Ivoire in October 2012: negative, faintly positive and strongly positive.

significant (Fisher's exact test, p=0.622) and the lack of toxin production excludes *C. difficile* as causative agent in the two symptomatic patients.

We assessed all GDH-positive isolates for parasitic co-infections. An infection with intestinal protozoa or helminths was found in five out of nine RDT-positive and culture-negative samples (three *Endolimax nana*, one *Giardia intestinalis*, one triple infection with *E. nana*, *Entamoeba coli* and hookworm). Among the six culture-positive *C. difficile* samples, only one parasitic co-infection was detected (triple infection with *Blastocystis* spp., *E. coli* and *Entamoeba hartmanni*).

The genotypic differentiation of the six *C. difficile* isolates via ribotyping determined one isolate as RT199 and one isolate as RT390, while the patterns of the remaining four *C. difficile* isolates differed from those of the >500 previously described RTs. Hence, these isolates could not be typed in Homburg, Leiden and Leeds and represent new RTs. A dendrogram (Figure 3) depicts the specific ribotyping patterns of these *C. difficile* isolates from Côte d'Ivoire in comparison to some closely related RTs. Details on the antimicrobial susceptibility of all isolates are presented in Table 1.

Additionally, ten toxigenic *C. difficile* isolates originating from clinically relevant infections of hospitalised patients in Homburg were analysed to assess the influence of storage conditions on antigen detection and culture of preserved stools. All samples, whether stored at 4°C or 20°C, tested positive for both GDH and toxins after 7, 14 and 28 days, and *C. difficile* could always be recovered in toxigenic culture during the study period. In some samples, there was a trend towards reduced signal intensities of RDT after prolonged storage, and this observation was slightly more pronounced if stool samples were stored at ambient temperature. However, *C. difficile* RDTs are designed as qualitative assays, and both GDH and toxins remained detectable in all stool samples. Stability of testing after prolonged storage of

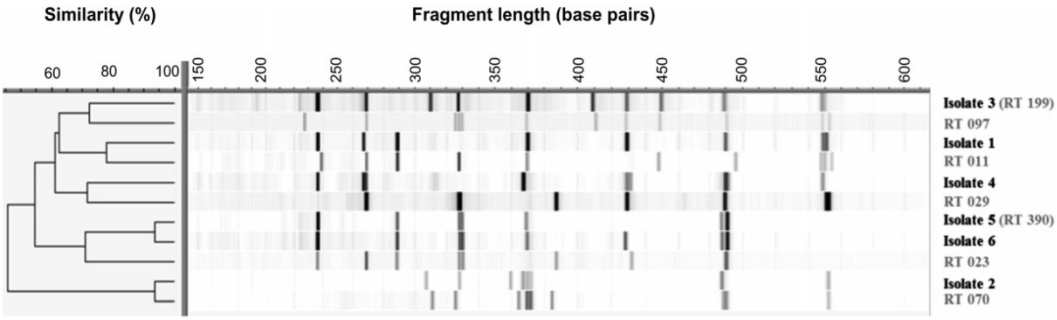

Figure 3. Dendrogram showing the specific ribotype (RT) profiles of six *Clostridium difficile* isolates from Dabou, south Côte d'Ivoire, obtained in October 2012, in comparison to related RTs, as determined by capillary ribotyping. Ribotyping displays a high diversity of the isolates. The dendrogram was generated by the software BioNumerics version 7.1 (edition 2013, www.applied-maths.com).

Table 1. Genotypic and phenotypic characterisation of *Clostridium difficile* isolates from Dabou, south Côte d'Ivoire, obtained in October 2012. PCR ribotyping, toxigenic culture and antimicrobial susceptibility testing (S, susceptible; R, resistant) were performed. The results of toxigenic culture were independently confirmed by multiplex PCR for toxin genes (*tcdA*, *tcdB* and *cdtA/B*). The minimal inhibitory concentration (MIC, expressed as μg/ml) is given, if susceptibility testing was performed by Etest

			Antimicrobial susceptibility testing (MIC, expressed as μg/ml)				
C. difficile	Ribotype	Toxigenic culture	Metronidazole	Vancomycin	Moxifloxacin	Clarithromycin	Rifampicin
Isolate 1	New (unknown)	Non-toxigenic	S (0.5)	S (1.0)	S (1.5)	R	S
Isolate 2	New (unknown)	Non-toxigenic	S (0.75)	S (0.5)	S (1.5)	S	S
Isolate 3	RT199	Non-toxigenic	S (0.5)	S (0.5)	S (1.0)	S	S
Isolate 4	New (unknown)	Non-toxigenic	S (0.75)	S (0.75)	S (1.5)	S	S
Isolate 5	RT390	Non-toxigenic	S (0.5)	S (0.5)	S (1.5)	R	S
Isolate 6	New (unknown)	Non-toxigenic	S (0.75)	S (0.75)	S (1.5)	R	S

stool samples was confirmed for a variety of clinically important ribotypes (Table 2).

Discussion

Our results indicate that the two-step diagnostic algorithm with a point-of-care GDH screening on the spot, followed by prolonged storage under disrupted cold chain conditions and subsequent sample transfer to a reference laboratory for selective anaerobic stool culture and PCR ribotyping is feasible to investigate *C. difficile* in faecal samples obtained from resource-constrained settings. Importantly, stool culture results were not affected by prolonged storage (up to 19 days) under aerobic atmosphere and varying, non-standardised temperature conditions. Cultural growth of *C. difficile* was observed in all PCR-confirmed specimens, thus showing an excellent agreement between both methods and confirming the stability of infectious spores of *C. difficile* over prolonged time periods. Additional experiments performed on stool samples stemming from hospitalised patients with toxigenic *C. difficile* infections in Germany further underscored our findings, as both GDH and toxins remained detectable in the stool over a period of at least 28 days, regardless of storage conditions.

The use of GDH RDT is an easily applicable, rapid and sensitive tool to screen for *C. difficile* infection and is suitable for use in remote and resource-constrained settings. It is thus of particular importance in areas where prompt diagnosis is crucial and more sophisticated laboratory work-up is not feasible.[45] Notably, of 15 GDH-positive stool samples in our study, only six revealed *C. difficile* by culture. However, even samples with a faintly positive test band in the RDT for GDH were included for further diagnostic work-up to maximise the diagnostic sensitivity of the initial screening, acknowledging that this strategy might decrease the specificity of the RDT. On the other hand, we cannot exclude that low amounts of *C. difficile* may indeed have been present in these culture-negative samples, and unfavourable storage conditions (intermittent cold chain, no preservation medium) might have negatively influenced the recovery of bacteria.[46] However, our finding that all culture-negative samples were also negative for *C. difficile* when employing a stool-based PCR strongly suggests false-positive RDT results as the more likely explanation for this discrepancy.

Table 2. Results of a stool-based rapid diagnostic test (RDT) detecting *Clostridium difficile* glutamate dehydrogenase (GDH) and toxin A/B, employed on stool samples from symptomatic patients and performed after prolonged aerobic storage at 4°C and 20°C over a storage period of 28 days. Resulting RDT line intensities are presented using a semi-quantitative grading scheme: 3+, strong; 2+, moderate; 1+, faint; –, negative

Sample	Ribotype	Toxin genes		Day 0	Day 7	Day 14	Day 28
1	001	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	3+	3+	2+	2+
			Toxin RDT (20°C)	3+	3+	2+	1+
2	005	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	2+
			Toxin RDT (4°C)	2+	2+	1+	1+
			Toxin RDT (20°C)	2+	2+	1+	1+
3	005	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	2+	2+
			GDH RDT (20°C)	3+	3+	2+	2+
			Toxin RDT (4°C)	3+	3+	2+	2+
			Toxin RDT (20°C)	3+	3+	2+	2+
4	011	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	2+	2+	1+	1+
			Toxin RDT (20°C)	2+	1+	1+	1+
5	013	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	1+	1+	1+	1+
			Toxin RDT (20°C)	1+	1+	1+	1+
6	014	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	2+	2+	2+
			Toxin RDT (4°C)	3+	3+	1+	1+
			Toxin RDT (20°C)	3+	1+	1+	1+
7	017	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	2+
			Toxin RDT (4°C)	2+	2+	2+	2+
			Toxin RDT (20°C)	2+	2+	2+	2+
8	027	*tcdA*, *tcdB*, binary toxin	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	3+	3+	3+	3+
			Toxin RDT (20°C)	3+	3+	3+	3+
9	029	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	3+	3+	3+	3+
			Toxin RDT (20°C)	3+	3+	3+	3+
10	029	*tcdA*, *tcdB*	GDH RDT (4°C)	3+	3+	3+	3+
			GDH RDT (20°C)	3+	3+	3+	3+
			Toxin RDT (4°C)	3+	3+	3+	2+
			Toxin RDT (20°C)	3+	3+	3+	2+

When using an RDT, the resulting line intensity can vary and pale, weak lines may lead to false-positive results. Guideline systems on how to read test results in a reliable, standardised way have been developed e.g., for malaria RDTs, and may also be useful to objectify the interpretation of *C. difficile*-specific RDTs.[47] With regard to its use as a first-line screening test, the Clostridium *K*-SeT RDT showed moderate specificity in this study, which underscores the need for a confirmatory test to avoid false-positive results.[48] Moreover, GDH assays cannot discriminate between toxigenic and non-toxigenic infections. However, new research has elucidated that asymptomatic *C. difficile* carriage per se profoundly alters the intestinal microbial diversity,[49] and further studies assessing this infection in both asymptomatic carriers and symptomatic patients from tropical settings will provide additional insights into the pathogenesis of CDAD.

An optimisation of the culture-based recovery of *C. difficile* from human stool has been identified as an important research need for epidemiological studies.[50] However, only few investigations have

addressed this issue with conflicting results, and most studies did not employ independent molecular testing to elucidate ambiguous results obtained by culture and RDT. In contrast to our findings, a previous study reported a rapid decrease in the recovery rate of *C. difficile* from 26 equine samples kept under aerobic conditions with no *C. difficile* isolate being culturally detectable after 12 days of aerobic storage at 4°C, whereas all isolates could still be recovered when stored anaerobically.[51] Another study found that the recovery of *C. difficile* on selective culture is only minimally affected by storage conditions, whereas toxins in the stool samples rapidly become undetectable after repeated freezing at −20°C.[52] A Canadian study reported no influence of temperature and storage conditions on the recovery of *C. difficile* kept over a period of 8 weeks.[53] This finding is in agreement with our results and may be explained by the bacterium's characteristic formation of durable endospores that persist in the environment before germinating again under optimised growth conditions. Future investigations should thus include environmental examinations and assess the effects of appropriate selective media that may facilitate the conversion from durable *C. difficile* endospores into the vegetative, cultivable form after prolonged storage.

Our study has several limitations. First, the low number of positive samples limits the generalisability of our findings. Second, none of the *C. difficile* strains in Côte d'Ivoire was toxigenic. However, we tried to address these constraints by the additional analysis of toxigenic isolates from clinically relevant infections from Homburg. In all samples, the prolonged storage did not negatively impact on the diagnostic yield of *C. difficile* stool culture, which underscores the reproducibility of our approach. Third, also healthy controls were tested in our study for the presence of *C. difficile*, despite many clinical guidelines stating that only symptomatic patients should be tested.[3,34] While this is true for clinical settings, investigations on the bacterium's occurrence in the environment, animals and healthy humans will contribute to an improved understanding of the largely unknown epidemiology of *C. difficile* in Africa. Indeed, a recent study from Côte d'Ivoire showed the presence of *C. difficile* in 12.4% of cooked beef meat sold by street vendors in Abidjan.[21]

Conclusions

A two-step diagnostic approach consisting of GDH screening on site, followed by in-depth genotypic and phenotypic characterisation in specialised laboratories is a promising strategy to investigate *C. difficile* in tropical settings. The recovery of *C. difficile* in human faecal samples from Côte d'Ivoire remained unaffected by prolonged storage and transport conditions that lacked standardisation and cold chain. This observation is of considerable importance for resource-constrained settings where accurate diagnosis cannot be ascertained and stool samples might be transported over several days to specialised laboratories. The present pilot study identified only non-toxigenic apathogenic isolates, both in symptomatic cases and matched asymptomatic controls. Further studies are warranted to deepen our knowledge of the transmission patterns, strain diversity and clinical relevance of *C. difficile* in the humid tropics. Our results should also encourage surveillance studies to CDAD on the African continent to obtain more insights into the global epidemiology of *C. difficile* infections.

Authors' contributions: SLB, JU and LvM conceived the study; SLB, JTC, BB, EKN, JU and LvM designed the study protocol; SLB and JKC carried out the clinical assessment; SLB, MH, EJK and LvM carried out the laboratory diagnostic tests, and analysis and interpretation of these data. SLB, JU and LvM drafted the manuscript; JTC, PM, BB, MH and EJK critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. SLB and LvM are guarantors of the paper.

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Competing interests: P. Mertens is Director for Research and Development (R&D) at Coris BioConcept (Gembloux, Belgium). The RDT employed in the current study (Clostridium K-SeT) is produced by Coris BioConcept. The company had no role in study design, data collection, data analysis, decision to publish or preparation of the manuscript.

Ethical approval: The study protocol was approved by the institutional research commissions of the Swiss Tropical and Public Health Institute (Basel, Switzerland) and the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (Abidjan, Côte d'Ivoire). Study approval was given by the Directorate of the Hôpital Méthodiste in Dabou. The study is registered on Current Controlled Trials (http://www.controlled-trials.com; identifier ISRCTN86951400).

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9 Application in Europe of a urine-based rapid diagnostic test for confirmation of *Schistosoma mansoni* infection in migrants from endemic areas

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RAPID COMMUNICATIONS

Application in Europe of a urine-based rapid diagnostic test for confirmation of Schistosoma mansoni infection in migrants from endemic areas

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In February 2015, a male patient from Eritrea with persistent abdominal pain and rectal bleeding was diagnosed with *Schistosoma mansoni* infection upon examination of a rectal biopsy. In May 2015, repeated stool microscopy identified *S. mansoni* infection in another Eritrean patient with abdominal pain and considerable eosinophilia (34%). Use of point-of-care circulating cathodic antigen (POC-CCA) tests on urine confirmed *S. mansoni* infection in both patients. Wider application of non-invasive POC-CCA urine tests will improve schistosomiasis diagnosis and clinical management in migrants.

We report the application of a urine-based antigen detection test for rapid, non-invasive diagnosis of intestinal schistosomiasis in two migrants from Eritrea. The potential implications of this rapid and highly sensitive diagnostic test with a short turn-around time for improved migrant health in European hospital settings and travel clinics are discussed.

Descriptions

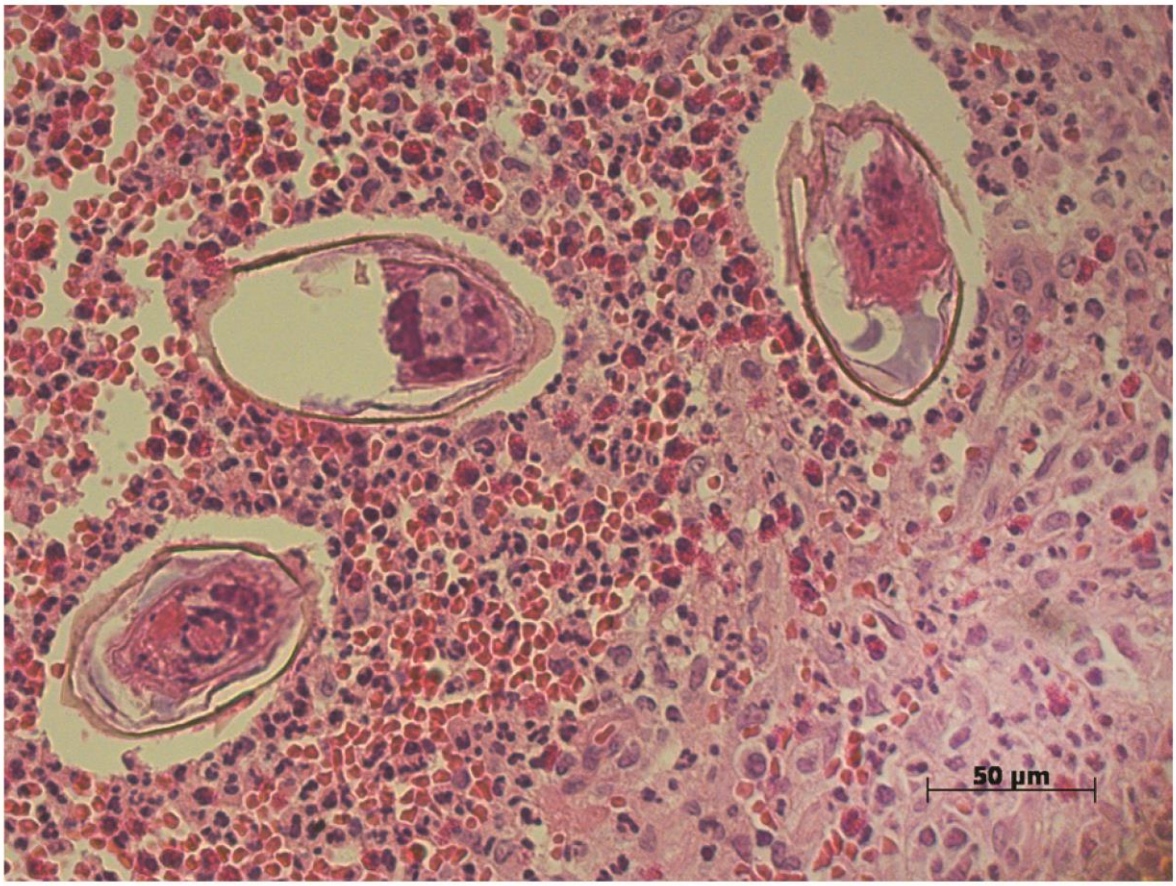
Patient 1

At the beginning of February 2015, a previously healthy male patient from Eritrea in his late teens presented to a German hospital with a history of rectal bleeding during the preceding days and persistent abdominal pain that had lasted for several weeks. No diarrhoea and no further digestive symptoms were reported at the time of presentation. The patient did not take any medication and past medical history was unremarkable. He was of Eritrean origin and had migrated to Germany approximately one year before presenting at the hospital. On physical examination, the patient was afebrile, vital signs were normal and no abdominal abnormalities were noted. However, rectal examination revealed a small, palpable mass, located approximately 7 cm from the anal verge. Blood tests showed a normal white blood cell count (5,800 cells/mL) with 6% eosinophils. Liver function tests and C-reactive protein were normal. Flexible colonoscopy identified two polyp-like lesions in the rectum, measuring up to 20 mm and 3 mm, respectively, while the rectal mucosa appeared macroscopically normal. Polypectomy was performed and biopsies were taken for histological workup, which showed erosive lesions and chronic intestinal inflammation. Upon microscopic examination of both polyps, typical pathognomonic features of eggs of the blood fluke *Schistosoma mansoni* were observed (Figure 1). Distinct histopathological features were the unique shape and size of parasite eggs (measuring 130–140 x 50–60 µm and lateral spine) and the eosinophil infiltration around the granulomas.

A stool sample could not be obtained from the patient. However, a urine sample was subjected to a rapid point-of-care (POC) antigen test. This test detects a schistosome-excreted circulating cathodic antigen (CCA). This POC-CCA test is commercially available (Rapid Medical Diagnostics; Pretoria, South Africa) and has been validated in various schistosomiasis-endemic settings of sub-Saharan Africa, where it proved more sensitive for *S. mansoni* than stool microscopy using the Kato-Katz technique [1]. Indeed, the reported sensitivity and specificity of the POC-CCA urine test for *S. mansoni* diagnosis during a multi-centre evaluation in five African countries were in the range of 78–92% and 56–94%, respectively, whereas the estimated sensitivity of the Kato-Katz technique was 44–77%. The POC-CCA in our patient with histologically proven schistosomiasis gave a faintly positive test line (termed ‘trace’ and

Figure 1

Biopsy of a rectal polyp, with typical granulomatous lesions with eosinophil infiltration, in a patient with intestinal schistosomiasis, Germany, February 2015



Haematoxylin and eosin, original magnification x40.

considered to be positive by the manufacturer) (Figure 2), thereby confirming the suitability of this non-invasive test to be employed in the diagnostic workup of patients with suspected schistosomiasis.

Abdominal ultrasound examination was performed to exclude hepatic fibrosis and other indicators of *Schistosoma*-induced chronic morbidity. The patient was treated with praziquantel, 40 mg/kg for three consecutive days [2], which led to complete resolution of clinical symptoms.

Patient 2

While preparing this report, we observed in May 2015 another young male patient from Eritrea with long-lasting abdominal pain and considerable peripheral blood eosinophilia (34%; norm: ≤5%).

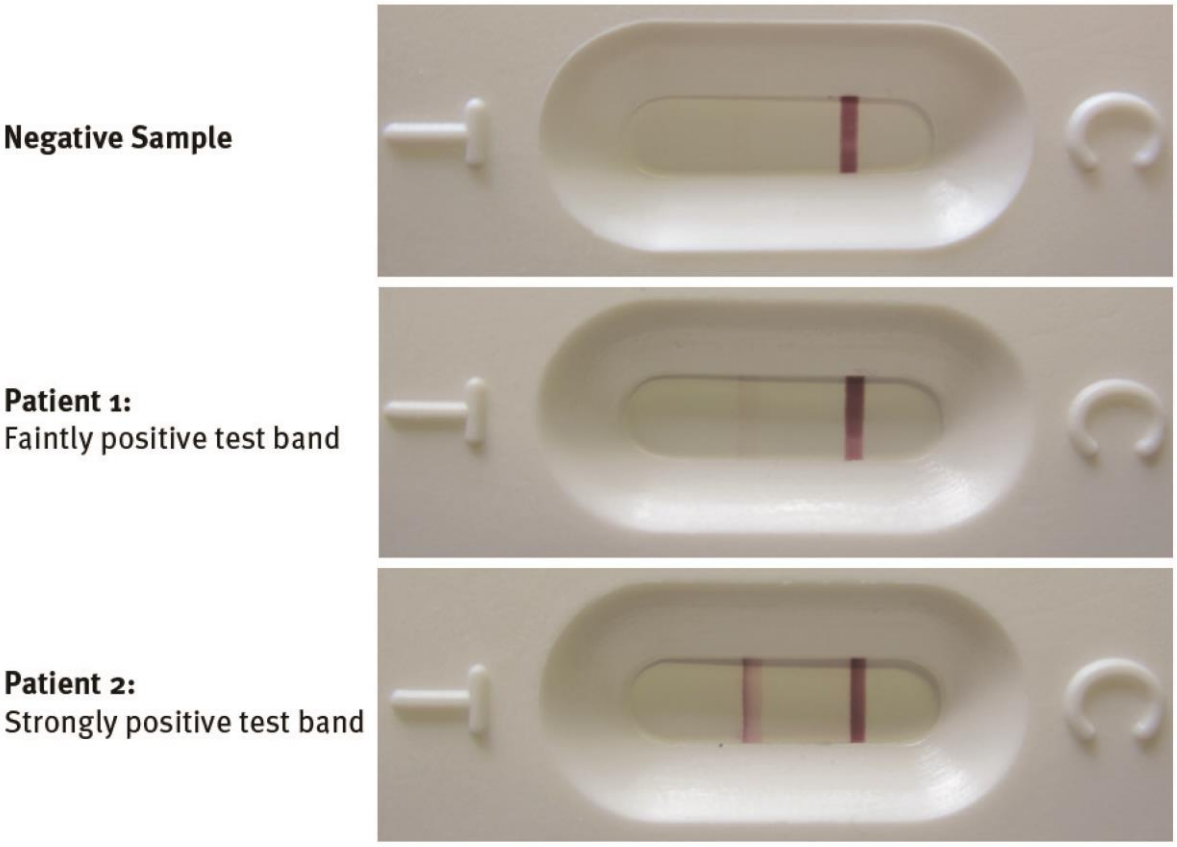
The patient, who was in his early twenties, had migrated to Germany 10 months before presentation and did not report any diarrhoea or rectal bleeding. Upon clinical examination and abdominal ultrasound, no abnormalities were noted. A POC-CCA test was applied on urine on the day of presentation and gave a strongly positive test result (Figure 2), while the initial stool microscopy was negative and only few eggs of *S. mansoni* were detected upon repeated stool examination. No additional parasitic or bacterial intestinal infections were found during the further diagnostic workup. Based on the positive test result in the POC-CCA urine test, intestinal schistosomiasis was diagnosed and treatment with praziquantel promptly initiated. A follow-up visit for this patient is scheduled in June 2015.

Discussion

Schistosomiasis is a parasitic disease that is endemic in large parts of sub-Saharan Africa, including Eritrea. The two main schistosome species in Africa are *S. mansoni* (causing intestinal schistosomiasis) and *S. haematobium* (causing urogenital schistosomiasis)

FIGURE 2

Results obtained by application of the point-of-care circulating cathodic antigen (POC-CCA) test for rapid diagnosis of *Schistosoma mansoni* infection on two patients' urine samples, Germany, February–May 2015



The control band (C) is present in all three specimens, while a test band (T) is only present in positive samples and its strength may depend on the infection intensity.

[3-5]. Infection is acquired through contact with cercariae-infested freshwater bodies. More than 250 million people are infected with schistosomes [6,7]. While long-term morbidity from chronic *S. mansoni* infection can result in hepatic fibrosis, portal hypertension and hypersplenism [8], many infections may be missed due to the unspecific clinical presentation and the low sensitivity of the most widely used diagnostic assays (i.e. stool microscopy) [3].

Persistent abdominal pain and occasional blood in stool were the only symptoms reported by our patient 1, despite the significant inflammation observed in the intestinal mucosa. Inflammatory rectal polyps have been described as a feature of chronic *S. mansoni* infection [9-11], but schistosomiasis is rarely considered in the differential diagnosis of intestinal polyposis outside endemic areas [12]. In patient 2, the incidental finding of considerable peripheral blood eosinophilia was the main reason to perform diagnostic tests pertaining to parasitic infections.

POC rapid diagnostic tests (RDTs) have the potential to improve the diagnosis and management of schistosomiasis patients. Thus far, repeated stool microscopy on several faecal specimens is the recommended diagnostic 'gold' standard, but light-intensity infections (i.e. ≤100 eggs per gram of stool) are often missed [3] and technical expertise in microscopic recognition of intestinal parasites is waning in many laboratories worldwide [13]. The urine-based POC-CCA is increasingly used in risk mapping and epidemiological surveys in schistosomiasis-endemic countries [1,14-16]. Previous studies have shown the positive and negative predictive values of a single POC-CCA to be 77% and 72–89%, respectively, if compared to multiple Kato-Katz thick smears as diagnostic reference standard [17,18]. The test has, however, not yet entered clinical practice in European hospitals and laboratories [7].

In our patient 1, the POC-CCA reliably detected the *S. mansoni* infection and confirmed the previously established histopathological diagnosis. Although polypectomy needed to be performed, an earlier use of a non-invasive urinary RDT may have provided evidence of active schistosomiasis more promptly. Indeed, in patient 2, the POC-CCA gave a positive test result on the first day of presentation, while helminth eggs of *S. mansoni* could only be detected upon repeated stool microscopy some days later. The clinical presentation of persistent abdominal pain and rectal bleeding is indicative of schistosomiasis in patients from endemic areas. A rapid diagnosis of this infection may allow faster adequate treatment, which in turn may lead to a resolution of the symptoms and avoid the need for further invasive diagnostic workup. While the POC-CCA is a highly sensitive and rapid tool to complement parasitological diagnostics, it cannot however replace a thorough microscopic examination of stool and urine, as the sensitivity for detection of *S. haematobium* is not sufficiently high [1], and no other parasites (e.g. soil-transmitted helminths) can be detected by POC-CCA.

Over the past decade, there has been a significant increase in migration from Africa and the Arabian Peninsula into Europe, with new arising challenges for the healthcare systems in various European countries [19]. Previous research has shown that inadequate communication and cultural barriers negatively affect the health-seeking behaviour of newly arrived Eritrean asylum seekers in Europe [20]. These immigrants may frequently present with imported infectious diseases that are not typically considered by European physicians in the differential diagnosis. A report from Sweden in 2014, for instance, has highlighted a dramatic increase of imported *Plasmodium vivax* malaria that was closely linked to newly arrived refugees from Eritrea [21]. While unexplained fever in African migrants usually prompts diagnostic testing for malaria, it is conceivable that rather unspecific gastrointestinal complaints, which are a key feature of many infections with helminths, including schistosomiasis, will not always lead to repeated stool sampling and in-depth diagnostic workup. Hence, we speculate that wider use of POC-CCA urine tests could improve the *S. mansoni* detection rate and, in turn, may reduce the number and costs of invasive diagnostic procedures.

Conclusions

Imported infections with *Schistosoma* spp. might increase in Europe due to a rise in migration from endemic settings, particularly from Africa. Due to the frequently unspecific clinical presentation of intestinal schistosomiasis and the insufficient sensitivity of stool microscopy, infections may easily be missed. We describe the successful use of POC-CCA urine tests for diagnosis of schistosomiasis and encourage physicians caring for migrants from endemic areas to consider implementing this rapid, relatively inexpensive (single test costs approximately EUR 1.50), and highly sensitive test as part of the diagnostic workup for gastrointestinal disorders.

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Conflict of interest

None declared.

Authors' contributions

Patient examination, history taking and endoscopy: SZ, DV. Histopathological analysis: PAS, RMB. Microbiological diagnostics: SLB, HM, MH. Wrote the manuscript: SLB, MH, JU. All authors have read and approved the final version of the manuscript.

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10 Discussion

10.1 Summary of research objectives and key findings

The current Ph.D. thesis was embedded in the multi-country, EC-funded NIDIAG study (NIDIAG is the acronym for 'Better **DIAG**nosis of **N**eglected **I**nfectious diseases; see http://www.nidiag.org). The overarching goal of the thesis was to elucidate the aetiology of persistent digestive disorders in resource-constrained tropical settings, to evaluate new diagnostic approaches and to contribute to the development of novel, evidence-based clinical algorithms that may be employed for the management of persistent digestive disorders at the peripheral healthcare level. While diarrhoeal diseases are among the most important infectious causes of disease worldwide (second only to lower respiratory infections), prior research mainly focussed on acute diarrhoea or single pathogens rather than considering persistent digestive disorders as a distinct clinical syndrome. Hence, the main specific objectives of the current thesis were (i) to review the set of pathogens that may cause persistent diarrhoea (≥14 days; in all age groups) and persistent abdominal pain (≥14 days; in individuals aged 1-18 years) in tropical and subtropical countries; (ii) to develop a study protocol for a prospective, multi-country investigation pertaining to this clinical syndrome; and (iii) to assess the aetiology of persistent digestive disorders by the application and diagnostic comparison of conventional stool microscopy, RDTs and multiplex PCR.

The research mission of the Swiss Tropical and Public Health Institute (Swiss TPH) is based on three major pillars, all of which are equally important to advance science: innovation, validation and application. The various studies carried out during the current Ph.D. thesis have contributed in several ways to this value chain. Table 10.1 summarises the main findings and contributions of this Ph.D. thesis within the nexus of innovation, validation and application. In brief, the systematic review elucidated that more than 40 bacterial, parasitic (helminths and intestinal protozoa) and viral pathogens may cause persistent abdominal pain and/or persistent diarrhoea. The subsequently performed case-control study in Dabou, south Côte d'Ivoire identified a considerable setting-specificity and a high number of asymptomatic intestinal infections in healthy controls, which were taken into account for the development of the NIDIAG study protocol for a multicentric investigation pertaining to persistent digestive disorders. While the application of some innovative molecular diagnostics (e.g. multiplex PCR) in Côte d'Ivoire elucidated the insufficient diagnostic accuracy of

conventional diagnostic methods, the unexpected detection of many pathogens even in asymptomatic controls calls for an urgent revision of previously well-established concepts regarding the aetiology and pathogenesis of infectious diseases. Importantly, the diagnostic accuracy of available RDTs for the detection of *C. difficile*, *Cryptosporidium* spp., *G. intestinalis* and *S. mansoni* was assessed to define their potential suitability to be used for clinical decision-making at the peripheral healthcare level where sophisticated laboratory facilities are often not available.

While the specific technical limitations of the research studies carried out during this Ph.D. thesis have been presented in detail in the respective chapters, four implications of the current work shall here be discussed in a broader context and in further detail: (i) the burden and local epidemiology of persistent digestive disorders; (ii) implications of rapid diagnostic tests for epidemiological studies and individual patient management; (iii) concepts in transition: the complex pathogenesis of intestinal infections; and (iv) novel approaches for a syndromic management of digestive disorders.

Table 10.1 Overview of the major findings and contributions of the respective chapters in the current Ph.D. thesis, stratified by the three main research pillars at the Swiss TPH, namely innovation, validation and application.

Chapter	Title	Innovation	Validation	Application
3	Persistent digestive disorders in the tropics: causative infectious pathogens and reference diagnostic tests	✓		
		Explanation: First systematic review specifically targeting pathogens that may cause persistent diarrhoea, persistent abdominal pain and blood in the stool, with a particular focus on reference tests and diagnostic tools that could be utilised in resource-constrained tropical settings.		
4	Diagnosis of neglected tropical diseases among patients with persistent digestive disorders (diarrhoea and/or abdominal pain ≥14 days): a multi-country, prospective, non-experimental case-control study	✓	✓	
		Explanation: Detailed protocol of a multicentre study to elucidate the aetiology, clinical features, diagnosis and existing management algorithms for persistent digestive disorders in Africa (Côte d'Ivoire and Mali) and Asia (Indonesia and Nepal).		
5	Experiences and lessons from a multi-country NIDIAG study on persistent digestive disorders in the tropics			✓
		Explanation: Complete set of 33 SOPs for the NIDIAG digestive study that give detailed information on the application of clinical, laboratory, quality control and data management activities.		
6	Combined stool-based multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire	✓	✓	
		Explanation: The first case-control study in south Côte d'Ivoire that employed a combination of multiplex PCR, rapid diagnostic tests, conventional microscopic methods and clinical features to assess bacterial, parasitic and viral pathogens and their association with persistent diarrhoea.		

Chapter	Title	Innovation	Validation	Application
7	Real-time PCR for detection of *S. stercoralis* in human stool samples from Côte d'Ivoire: diagnostic accuracy, inter-laboratory comparison and patterns of hookworm co-infection	✓	✓	
		Explanation: Rigorous diagnostic comparison of two microscopic techniques and real-time PCR methods (independently employed in two laboratories) for detection of *S. stercoralis* in human stool samples, including a word of caution pertaining to misidentification of hookworm larvae as *S. stercoralis* in diagnostic laboratories.		
8	Molecular and culture-based diagnosis of *C. difficile* isolates from Côte d'Ivoire after prolonged storage at disrupted cold chain conditions	✓	✓	
		Explanation: Presentation of the feasibility and diagnostic accuracy of a two-step algorithm to investigate *C. difficile* in West Africa, consisting of an RDT screening on site and subsequent transfer of RDT-positive samples to a specialised laboratory for in-depth investigations (stool culture and PCR).		
9	Application in Europe of a urine-based rapid diagnostic test for confirmation of *S. mansoni* infection in migrants from endemic areas	✓		✓
		Explanation: First-ever application of a diagnostic tool (POC-CCA urine cassette test), which has previously been validated in epidemiological studies in endemic settings, for individual patient diagnosis of individuals with suspected schistosomiasis in Europe ('reverse innovation').		

10.2 The burden and local epidemiology of persistent digestive disorders

Persistent diarrhoea and persistent abdominal pain are important causes of morbidity and mortality worldwide, particularly in areas of poor sanitation and weak health systems [1,2]. While acute diarrhoea is a commonly recognised cause of child mortality, it has recently been shown that persistent diarrhoea accounted for 56.3%, 44.9% and 12.2% of diarrhoea-associated deaths in children aged between 1 and 4 years in Ethiopia, Uganda and the United Republic of Tanzania, respectively [3]. Persistent diarrhoea may cause severe sequelae such as malabsorption, pancreatic and gastric dysfunction and specific nutritional deficiencies [4-6]. Indeed, an Ethiopian study reported that persistent diarrhoea constituted only 5% of all diarrhoea cases in children that were seen at a referral hospital in Addis Ababa, but 86% of these patients had signs of accompanying malnutrition [7]. According to recent data stemming from the Taabo health and demographic surveillance system (HDSS) in south-central Côte d'Ivoire, diarrhoeal diseases account for 5.5% of all deaths in children aged below 5 years [8,9]. The importance of persistent digestive disorders in resource-constrained settings has been confirmed during the ongoing NIDIAG study. During the first study performed in Dabou (chapter 6 of this Ph.D. thesis), 68 patients with persistent diarrhoea could be enrolled within 3 weeks [10]. At the time of writing, the patient recruitment for the multi-country NIDIAG digestive study has just been completed, and the obtained results further underscore the frequency of persistent digestive disorders. Indeed, 553 patients with persistent digestive disorders and 553 asymptomatic controls have been recruited at the NIDIAG study site in Niono, Mali during an enrolment period of less than 10 months.

As shown in this Ph.D. thesis (chapter 3), persistent digestive disorders may be caused by more than 40 infectious agents, but there is also considerable setting-specificity with regard to the predominant pathogens. During our NIDIAG site assessment study in Dabou, Côte d'Ivoire, only four pathogenic agents were identified in ≥10% of all symptomatic patients, namely enterotoxigenic *E. coli* (ETEC; 34% prevalence), *G. intestinalis* (27%), *Shigella* spp. (27%) and *S. stercoralis* (12%). These results confirm previous studies in Côte d'Ivoire that reported *S. stercoralis* to be endemic in rural areas, with prevalences as high as 48% in some villages [11]. Additionally, a study employing morbidity questionnaires and a standardised medical examination showed that both *S. stercoralis* and hookworm are significantly associated with gastrointestinal symptoms (e.g. abdominal pain and diarrhoea) in rural Côte d'Ivoire [12]. Unfortunately, the correct diagnosis of *S. stercoralis* is cumbersome and a

combination of laborious microscopic methods and PCR should be employed to achieve the highest diagnostic accuracy. Due to the unavailability of these techniques in many resource-constrained settings, it is conceivable that a considerable number of *S. stercoralis* infections are missed. Hence, the development of a sensitive RDT for this pathogen is warranted.

G. intestinalis, the second commonly detected intestinal parasite in patients with persistent diarrhoea in Dabou, was known to be endemic in Côte d'Ivoire, as it had been studied mainly in the context of polyparasitism with multiple helminthic and other intestinal protozoon infections [13-16]. Indeed, self-reported morbidity could be linked to such intestinal polyparasitism [17] and intestinal parasitic infections reduced the self-rated quality of life of infected individuals significantly [18]. Furthermore, there is a clear link between poor sanitation, lack of hygiene, defecation behaviour, unsafe sources of drinking water and higher prevalences of soil-transmitted helminths and intestinal protozoa, thus underscoring the close connection between these NTDs and poverty [19,20]. Asymptomatic carriage of *G. intestinalis* is a common feature in low-income countries, which renders the attribution of clinical symptoms to the finding of this protozoon in the stool difficult. However, a systematic review and meta-analysis have elucidated a significant association between *G. intestinalis* and persistent diarrhoea (adjusted odds ratio 3.18, $p<0.001$), while this was not observed for acute diarrhoeal episodes and *G. intestinalis* infection [21].

In contrast to intestinal parasites, only little emphasis has been placed on bacterial and viral pathogens in the context of gastrointestinal disorders in resource-limited countries, which may partially be explained by the lack of well-equipped diagnostic laboratories in these settings [22]. However, considerable efforts have been made in recent years to capture the whole aetiological spectrum of acute diarrhoea, including bacterial and viral pathogens, in low-income countries. Most noteworthy is the Global Enteric Multicenter Study (GEMS), an international research consortium that performed a 3-year prospective case-control study in seven sites in sub-Saharan Africa and southern Asia to gain insights on the prevailing pathogens in children aged below 5 years with acute moderate-to-severe diarrhoea [23]. Similar to our findings from Côte d'Ivoire, at least one pathogen was identified in 83% of cases and 72% of controls. Despite the large number of pathogens being investigated, only four infectious agents were significantly associated with moderate-to-severe diarrhoea in all sites, namely rotavirus, *Shigella* spp., ETEC and *Cryptosporidium* spp. [24]. Likewise,

Shigella spp. and ETEC constituted also the most prevalent pathogens in our study in Dabou and it will be interesting to see whether similar results will be obtained when analysing the data from the currently ongoing, multi-country NIDIAG study that is carried out in Côte d'Ivoire, Indonesia, Mali and Nepal. Indeed, the importance of *Shigella* spp. as diarrhoea-causing agent has also been acknowledged by a case-control study from Ecuador [25]. A recent study in the People's Republic of China assessed differences between the urban area of Beijing and a rural area in the Henan province, an area of poor sanitation and hygiene. Strikingly, *Shigella* spp. was by far the predominant bacterial pathogen in the rural area and its prevalence was more than 30-fold higher than in Beijing [26]. Hence, the currently ongoing development of a vaccine against shigellosis is of crucial importance, as it might have a significant beneficial impact on child health in low-income countries [27], similar to what has been observed after the introduction of the rotavirus vaccine [28,29]. Yet, the considerable setting-specificity of digestive disorders and the varying range of causative pathogens in many settings may limit the suitability of approaches targeting single pathogens to decrease the disease burden in communities [30], and interventions promoting improved access to safe water, sanitation and hygiene (WASH) should concurrently be encouraged [31-33].

Several barriers exist that hinder research on persistent digestive disorders in resource-constrained settings. First, the multi-faceted aetiology of these infections requires a battery of microbiological laboratory investigations to be carried out, which is logistically challenging and often impossible in tropical countries. Second, there is no objective standard definition for the entity 'persistent abdominal pain', which may influence on the comparability of different studies [34]. Third, the health-seeking behaviour of patients suffering from persistent digestive disorders also needs to be considered. Indeed, researchers from Yemen performed a qualitative study to assess local concepts of illness and found that diarrhoeal diseases were usually not judged by the local population as illnesses that would require medical treatment [35]. Hence, traditional medicine was much more frequently used to treat these conditions, and physicians were rarely consulted. A similar study conducted in Bamako, Mali assessed the health-seeking behaviour for paediatric diarrhoea and found that the more severe diarrhoea cases were more likely to present to traditional healers than to trained health professionals at governmental healthcare centres [36]. It follows that the burden of diarrhoeal and other digestive diseases may be underestimated if only patients

presenting to major health centres or hospitals are considered. Similar experiences have been made during the NIDIAG digestive study in Côte d'Ivoire and Mali and it will thus be interesting to analyse the results of the NIDIAG study also in light of the patients' health-seeking behaviour, so that clinical management algorithms can subsequently be implemented in those settings where most patient with persistent diarrhoea and abdominal pain present.

10.3 Implications of RDTs for epidemiological studies and individual patient management

In the different chapters of the current Ph.D. thesis, various diagnostic tools have been utilised that can be grouped into three major categories: (i) conventional microscopic tests that allow for direct visualisation of a pathogen; (ii) pathogen-specific RDTs, which use dye-labelled antibodies to detect antigen of a target pathogen; and (iii) nucleic acid amplification tests that directly detect genetic material (DNA or RNA) of a specific pathogen via PCR. RDTs and PCR assays targeting NTDs and other major pathogens in tropical settings have been developed relatively recently. While PCR and microscopy require a rather well-equipped diagnostic laboratory, RDTs can be performed even under harsh field conditions, such as in peripheral, underequipped healthcare centres in rural sub-Saharan Africa [37]. The most prominent example for a widespread implementation of RDTs in resource-limited areas is malaria, an infection caused by blood parasites of the genus *Plasmodium* [38]. The implementation of these tests allowed for malaria testing in many rural health centres without laboratory facilities, where case management previously relied exclusively on clinical judgment [39]. In 2010, WHO recommended that all patients with suspected malaria should undergo testing for malaria (instead of starting presumptive treatment), using RDTs in most areas where light microscopy was not available. However, strategies that rely exclusively on treating RDT-positive patients for malaria have been repeatedly criticised for a number of reasons [40], e.g. the possibility of other causative diseases despite a positive malaria RDT in highly endemic settings [41], false-negative results due to the prozone effect [42] and cost-effectiveness concerns [43].

RDTs have also been developed for intestinal pathogens and have found their way into daily clinical practice in many laboratories in Europe and North America. The most commonly used tests are those targeting the three intestinal protozoa species *Cryptosporidium* spp.,

E. histolytica and *G. intestinalis* [1]. However, it is important to note that there is no commercially available RDT that can reliably distinguish *E. histolytica* from the apathogenic, yet morphologically identical *E. dispar* [44]. For *G. intestinalis*, the sensitivity of RDTs has been reported to be superior to that of microscopy in samples stemming from patients with acute diarrhoea. In the current Ph.D. thesis, an RDT for *G. intestinalis* detection (Crypto/Giardia DuoStrip; Coris BioConcept, Gembloux, Belgium) has been applied in Côte d'Ivoire for persistent diarrhoea, but the results were disappointing. Indeed, the sensitivity of the RDT in comparison to PCR and microscopy was only 40% [10]. This might partially be explained by a relatively low quantity of *Giardia* cysts being shed in the faeces of patients with persistent diarrhoea, because the sensitivity of stool microscopy was also unexpectedly low (51%). However, a recent diagnostic comparison study from Belgium confirmed the insufficient diagnostic accuracy of the RDT employed here (Crypto/Giardia DuoStrip), with a reported sensitivity for *G. intestinalis* detection of only 58-66% [45].

RDTs for *Cryptosporidium* detection are particularly useful, because the small oocysts of these coccidian parasites are easily missed upon stool microscopy (e.g. when a formalin-ether concentration technique is employed) and are only visible when staining techniques (e.g. acid-fast staining) are utilised. Hence, it is conceivable that infections due to *Cryptosporidium* spp. are underreported worldwide, despite the growing evidence concerning the substantial contribution of cryptosporidiosis to the burden of diarrhoeal diseases, which has recently been confirmed in the GEMS study [24]. In contrast to previous concepts, it is now widely acknowledged that cryptosporidiosis is not limited to HIV-infected individuals [46-48]. Hence, a wider application of diagnostic tests targeting *Cryptosporidium* spp. in resource-limited settings is recommended. Besides RDTs for intestinal protozoa, it would also be useful to enhance the availability of bacterial RDTs for detection of the *C. difficile*-specific GDH in tropical areas. This RDT is a highly sensitive, validated tool that can be used as a reliable screening test for *C. difficile*-associated infections, e.g. in patients who develop diarrhoea while receiving antibiotic treatment. While *C. difficile* is estimated to account for more than 95% of all diarrhoea-associated deaths in elderly people in the Western world [49], its epidemiology and medical importance in Africa are poorly understood. Preliminary findings from this Ph.D. thesis point towards significant differences regarding the predominating *C. difficile* strains in sub-Saharan Africa. Hence, hospital-based and epidemiological studies investigating the causes of diarrhoeal diseases in the tropics should

consider screening for *C. difficile* using an RDT; positive samples could then be sent to a reference laboratory for further diagnostic work-up.

In contrast to the aforementioned protozoal and bacterial RDTs, the POC-CCA urine cassette test for diagnosis of *S. mansoni* is the only validated and commercially available RDT for helminths. Its diagnostic accuracy and superior sensitivity compared to stool microscopy have been shown in multi-country studies [50-52], even though recent research has questioned whether there may be a certain level of false-positive test results in pregnant women [53]. In chapter 9 of this Ph.D. thesis, it has been shown that the POC-CCA test can also be utilised for the confirmation of suspected intestinal schistosomiasis in migrants from endemic areas or returning travellers [54]. Surprisingly, such a broader implementation of the POC-CCA test has not yet occurred in travel clinics and hospitals. However, by the time of writing the current work, we have identified a total of six RDT-positive cases in migrants from Eritrea in the Institute of Medical Microbiology and Hygiene (IMMH) in Homburg/Saar, Germany, out of which some patients would not have been detected by stool microscopy. In addition to the high sensitivity, this non-invasive RDT may also considerably reduce the costs for unnecessary diagnostic investigations in patients with schistosomiasis-associated morbidity (e.g. colonoscopy in patients with persistent abdominal pain). Hence, a wider application of the POC-CCA test for the clinical management of patients from endemic areas should be encouraged in Europe and elsewhere, because an early detection and subsequent treatment of intestinal schistosomiasis might avert considerable morbidity in infected individuals.

10.4 Concepts in transition: the complex pathogenesis of intestinal infections

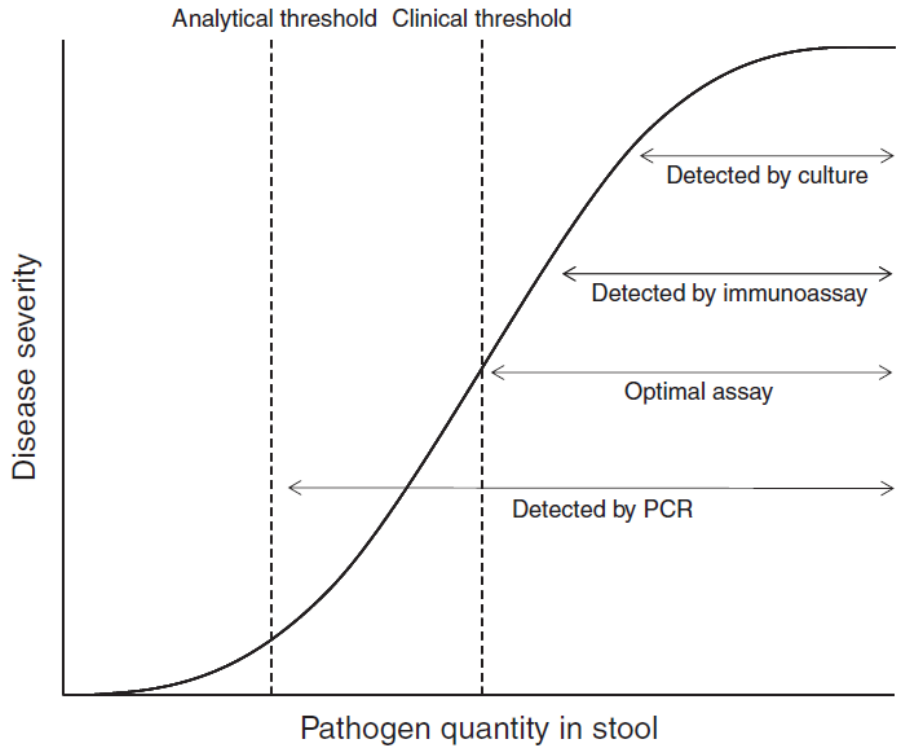
PCR-based techniques are characterised by high diagnostic sensitivity, which has been confirmed in the current work [10,55], even though the diagnostic accuracy of PCR assays for some parasitic pathogens (e.g. *S. stercoralis*) is at present not high enough to replace conventional microscopic methods [56]. However, in the current work, the application of PCR techniques on stool samples also detected high rates of intestinal pathogens in asymptomatic individuals. Surprisingly, for most bacterial, parasitic and viral pathogens, no significant prevalence differences among patients and controls could be identified. In agreement with these findings, recent studies employing multiplex PCR on stool samples of

asymptomatic individuals confirmed this observation [57]. Apparently, this phenomenon is not limited to the gastrointestinal tract, because similar results have also been reported from studies that employed PCR-based techniques to investigate patient blood samples for pathogens giving rise to systemic infections. Indeed, a study from Gabon identified common bacterial pathogens such as *S. aureus* and fastidious bacteria such as *Borrelia* spp. and *Rickettsia felis* in a certain amount of afebrile children [58]. It has thus recently been suggested to include, whenever possible, asymptomatic controls in epidemiological studies to improve our understanding regarding the aetiology of infections and the role of detected pathogens [59,60].

In the case of diarrhoeal diseases and digestive disorders, several considerations might help to explain the finding of considerable pathogen prevalences in healthy controls. First, asymptomatic colonisation with infectious agents may occur and could be explained by (i) the recent ingestion of very small amounts of the pathogen, which are not sufficient to cause human disease; (ii) the ingestion of animal pathogens that cannot cause human disease, but cannot reliably be differentiated by PCR (e.g. porcine ETEC pathotypes); and (iii) the fact that PCR detects nucleic acids stemming from a pathogen, but cannot distinguish between live and dead organisms, meaning that PCR-positive signals due to prolonged nucleic acid shedding may persist for weeks or months after a resolved infection [61]. Additionally, bacteria such as ETEC just cause disease when they are located in the small intestine, while apathogenic colonisation in the large intestine may occur [62]. For some pathogens (e.g. *Blastocystis* spp., *Campylobacter* spp., *G. intestinalis*), there is growing evidence that only some strains, which can only be differentiated by detailed molecular analysis, may cause disease [61]. ‘Environmental contamination’ with small amounts of potentially disease-causing pathogens as well as dietary habits and the composition of the intestinal flora may also play a decisive role whether nucleic acids of a pathogen can be detected by PCR. In summary, while the sensitivity of conventional diagnostic techniques is frequently too low to detect all clinically meaningful infections, PCR techniques are characterised by a very high analytical sensitivity, which may in turn ‘overdiagnose’ pathogens due to detection of irrelevant quantities of nucleic acids. Hence, there is a need to define the characteristics of an optimal diagnostic assay and to establish ‘clinical thresholds’ for PCR-based testing methodologies (Figure 10.1).

In a recent proof-of-concept study, we have shown that a metagenomics approach, i.e. the application of molecular sequencing techniques to characterise whole microbial communities without isolating or culturing the individual pathogens, may provide additional information on the intestinal microbiome [63]. Four stool samples stemming from our study on persistent diarrhoea in Dabou were purposefully selected and subjected to a metagenomics approach. Strikingly, up to 11 pathogen classes could be identified in a single stool specimen, which sheds further doubt on the established concept that the presence or absence of one single causative agent may determine the onset of infectious pathologies of the intestinal tract. Hence, it is conceivable that the whole composition of the gut microbiome, i.e. the entire intestinal flora, as well as its interactions with the human host influence whether or not an individual develops overt disease [64,65]. Future research in high-income and low-income countries is needed to further our understanding of these complex interactions and to develop innovative concepts pertaining to the pathogenesis of intestinal infections.

Figure 10.1 Detection limit of different diagnostic assays in relation to a 'clinical threshold' (published in [66]).



10.5 Novel approaches for a syndromic management of digestive disorders

One of the main objectives of the ongoing NIDIAG study is the development of novel, evidence-based clinical algorithms that enable an improved diagnosis and management of individuals presenting with persistent digestive disorders (≥14 days) at the peripheral healthcare level. Based on the findings reported in this Ph.D. thesis, several lessons can be learned, which will be further influenced by the final study results of the NIDIAG study. Indeed, at the time of writing, the enrolment of patients and controls has successfully been completed in all four study countries (Côte d'Ivoire, Indonesia, Mali and Nepal) and an 'algorithm development workshop' has been held in Bamako in mid-November 2015. Study findings from Nepal are currently being entered into an electronic database and have not yet been analysed, while the number of patients with persistent digestive disorders encountered in Indonesia was very low, so that the original study design had to be adapted. Hence, the following considerations will mainly address NIDIAG data from Côte d'Ivoire and Mali as well as the results from the studies that were performed as part of this Ph.D. thesis.

There is a clear need for the development of setting-specific algorithms, as the major aetiological agents causing persistent digestive disorders as well as the associated signs and symptoms may vary considerably in different regions. With regard to the clinical symptomatology, significant differences were observed during the NIDIAG study. Indeed, more than 90% of all enrolled patients in Mali complained about persistent abdominal pain, whereas persistent diarrhoea was more frequently reported in Côte d'Ivoire. While *S. stercoralis* is one of the most important helminths contributing to this syndrome in Côte d'Ivoire [10,56], there have been hardly any cases of strongyloidiasis during the NIDIAG study in neighbouring Mali, where schistosomiasis (both due to *S. mansoni* and *S. haematobium*) seems to play a major role in the pathogenesis of persistent abdominal pain in children and adolescents. Giardiasis is highly prevalent across both countries and the systematic follow-up of patients during the NIDIAG study revealed a substantial clinical improvement or complete resolution of symptoms after treatment with metronidazole in patients infected with *G. intestinalis*. The role of bacteria as contributors to persistent digestive disorders remains unclear. The multiple positive PCR signals obtained in both patients and controls render an attribution of morbidity to the different pathogens very difficult. Additionally, most patients with confirmed parasitic diseases and positive PCR

signals for bacterial pathogens improved on antiparasitic treatment alone (i.e. albendazole, ivermectin, praziquantel or metronidazole). Hence, it seems to be justified to focus mainly on helminthic and protozoal agents in the context of persistent digestive symptomatologies that occur in tropical settings.

The novel diagnosis-treatment algorithms shall be utilised in primary healthcare centres, where few adjunctive diagnostic tools besides clinical judgment are available. Hence, RDTs would constitute the only available diagnostic tests. Indeed, two RDTs seem to be particularly useful for the management of persistent digestive disorders and the decision whether to initiate targeted treatment, i.e. the POC-CCA urine cassette test for *S. mansoni* (an important pathogen in the NIDIAG setting in Mali) and an RDT for *G. intestinalis* (high prevalences in both Côte d'Ivoire and Mali). However, the overall prevalence of a given infection in symptomatic patients is also important to decide whether diagnostic testing should at all be implemented; if the prevalence of a specific pathogen in a given area is very high, the clinical signs and symptoms alone may be sufficient to initiate presumptive treatment. The 'decision threshold' when to start such an empirical medical treatment greatly depends on (i) the potential toxicity of the drug; and (ii) the potential long-term morbidity caused by an untreated infection. With regard to schistosomiasis, for example, treatment with praziquantel has few adverse events, is efficacious and potentially prevents long-term morbidity such as hepatic fibrosis and subsequent portal hypertension [67,68]. Hence, the 'decision threshold' is conceivably relatively low in the context of persistent digestive disorders. The application of RDTs could also be important to decide whom not to treat with anti-infective drugs. Due to the high number of observed co-infections with *S. mansoni* and *G. intestinalis* in the digestive syndrome study site in Mali, the application of an RDT for e.g. *G. intestinalis* may help to decide whether an anti-protozoal and an anthelminthic drug should be administered concurrently or whether there is no need for anti-protozoal treatment.

The clinical features of digestive infections are usually unspecific, and hence, do not allow to distinguish between specific pathogens. However, diarrhoea and nausea have been repeatedly linked to strongyloidiasis and hookworm disease in Côte d'Ivoire, and intestinal helminth infections in general may also cause extraintestinal disease manifestations such as wheezing on pulmonary auscultation and cutaneous manifestations (e.g. itching and rash) [69]. After having analysed the entire NIDIAG dataset from the study countries, it will be interesting to

see whether a clear link between intestinal helminthiasis and such suggestive clinical findings can be elucidated, which would be very valuable for subsequent clinical algorithms. The final NIDIAG algorithm for the management of persistent digestive disorders will most likely be relatively simple and will consist of a clinical assessment to establish whether the definition of persistent digestive disorders is met, followed by either a presumptive treatment with one or two drugs that would cover the most important pathogens (particularly in areas where a limited number of pathogens are highly prevalent), or by application of one or two RDTs to determine the potential cause of the disorders (e.g. to rule out intestinal schistosomiasis in settings of low endemicity). A clinical improvement should normally be seen within one week after starting medical treatment; hence, persisting symptomatologies would require referral to the next healthcare level; thus a district or reference hospital. However, based on a preliminary analysis of findings from the NIDIAG study, it seems that such a referral will be required for less than 5% of all patients.

Insights gained from previous studies pertaining to the implementation of clinical algorithms in resource-limited settings revealed a clear need to critically validate and evaluate these algorithms in collaboration with the local caregivers, e.g. for the diagnosis and management of dengue fever [70], human African trypanosomiasis [71], malaria [72] and pulmonary tuberculosis [73]. Indeed, the NIDIAG algorithms will only be utilised if they prove to be valid and helpful under ‘real life conditions’. An integrated management of common clinical syndromes has the potential to improve the clinical care of symptomatic patients and to concurrently decrease the irrational use of anti-infective drugs [74]. Hence, the first NIDIAG algorithm for an evidence-based management of persistent digestive disorders will be developed after complete data analysis and will then be validated in a subsequent study in Niono, Mali in early 2016. Eventually, the algorithm might be implemented at multiple primary healthcare centres in this area to provide evidence-based guidance for an improved clinical management of persistent diarrhoeal and digestive diseases.

10.6 Recommendations and research needs

The current work assessed persistent diarrhoea and persistent abdominal pain to investigate the significance of these symptomatologies in resource-limited settings of the tropics, to elucidate the potentially implicated pathogens and to compare several clinical and laboratory

diagnostic features. Several suggestions and research needs arise from the results obtained in the present work:

1. Persistent digestive disorders are of considerable importance in resource-limited settings, but a particular health-seeking behaviour needs to be considered. If compared to acute and more rapidly progressing diseases, minor attention is directed towards persistent digestive disorders and hence, patients rarely present to hospitals or large health centres. Therefore, future studies investigating this clinical syndrome should closely collaborate with peripheral healthcare centres in rural areas. There is considerable setting-specificity and persistent digestive disorders should thus also be investigated in other areas of sub-Saharan Africa, Asia and Latin America.

2. Multiple pathogens can be detected in stool samples from patients and controls, which renders estimates of the contribution of a single pathogen to the development of intestinal disorders difficult. Future studies trying to explore the aetiology of infectious diseases should therefore employ a case-control approach.

3. Future research should address new concepts pertaining to the complex aetiology of intestinal infectious diseases. Such an approach should include the use of highly sensitive molecular diagnostic methods (e.g. PCR). Additionally, multiple consecutive stool specimens should be obtained and analysed to document the excretion patterns in relation to clinically apparent infections, nutritional and environmental factors. The application of metagenomics and an accurate description of the intestinal microbiome will be important adjunctive steps.

4. RDTs are important tools that help to manage intestinal infectious disorders. RDTs for detection of *Cryptosporidium* spp. and *G. intestinalis* have varying diagnostic accuracy. The urine-based POC-CCA cassette test is a useful and sensitive tool to detect *S. mansoni* infections and its suitability for case detection and clinical management of individual patients in endemic and non-endemic settings should be further assessed.

5. The epidemiology of *C. difficile* in sub-Saharan Africa is poorly understood. RDTs should be more widely employed to investigate the presence of this bacterial pathogen in

samples stemming from patients with diarrhoea, asymptomatic controls, animal stool specimens and environmental samples. RDT-positive samples can be stored for weeks at ambient temperature before being sent to a laboratory for further diagnostic characterisation. Hospital- and community-based studies will help to estimate the significance of *C. difficile* as the causative agent of intestinal infections in sub-Saharan Africa.

6. Strongyloidiasis is an important infection and has the potential to cause life-long persistence if left untreated. *S. stercoralis* is endemic in many parts of sub-Saharan Africa, but the diagnostic accuracy of currently available laboratory tests is not sufficient. Hence, concerted efforts should be made to develop a stool-based RDT for detection of *S. stercoralis*, which could be used for both mapping studies in tropical settings and individual patient management.

10.7 Conclusions

Persistent diarrhoea and persistent abdominal pain constitute important public health problems in many resource-limited tropical settings and may be caused by multiple intestinal pathogens. In the frame of the international NIDIAG research consortium, the current Ph.D. thesis investigated persistent digestive disorders in Côte d'Ivoire. A systematic review was performed to assess the aetiological spectrum of pathogens causing persistent digestive disorders. Subsequently, a detailed study protocol and more than 30 specific SOPs were developed to investigate this clinical syndrome prospectively in a multi-country study. A case-control study was carried out in south Côte d'Ivoire to evaluate clinical features and infectious agents associated with persistent diarrhoea. Further emphasis was placed on the epidemiology, clinical importance and diagnostic techniques for three pathogens deemed of particular relevance, i.e. *C. difficile*, *S. mansoni* and *S. stercoralis*. The following main conclusions can be drawn from the current work.

1. Persistent diarrhoea and persistent abdominal pain are of considerable public health relevance in Côte d'Ivoire and elsewhere in the tropics. Most affected individuals live in rural, remote areas with limited access to healthcare facilities. The aetiological spectrum is broad and includes more than 40 bacterial, parasitic (helminths and intestinal protozoa) and viral pathogens. The predominating pathogens differ from one setting to another. Co-

infections are frequently detected in both symptomatic patients and healthy controls, especially if highly sensitive PCR techniques are employed. The clinical relevance of these findings remains to be elucidated and the inclusion of asymptomatic controls should be encouraged in future epidemiological studies.

2. *S. stercoralis* is endemic in Côte d'Ivoire and its reported prevalence in two different settings varied between 10% and 20%, depending considerably on the employed diagnostic techniques. Standard copromicroscopic tools (e.g. Kato-Katz technique) fail to detect *S. stercoralis*, and a combination of the Baermann funnel concentration technique and the Koga agar plate technique yields more accurate results. A novel, stool-based real-time PCR for *S. stercoralis* has been validated in the current work and is the single most sensitive diagnostic technique. However, PCR alone should not replace the microscopic techniques. There is a need for a *Strongyloides*-specific rapid antigen detection test that can be applied on stool samples.

3. *C. difficile* can be found in human stool samples in Côte d'Ivoire. Non-toxigenic strains predominate in patients and asymptomatic controls and the molecular ribotype patterns differ considerably from those seen in other geographical areas. An RDT-based diagnostic two-step algorithm has been successfully validated in Côte d'Ivoire to facilitate the screening of *C. difficile* in resource-limited settings without immediate access to a diagnostic laboratory. Indeed, prolonged storage of RDT-positive samples without properly maintained cold chain only minimally affects the subsequent recovery of *C. difficile* and its toxins in stool culture.

4. Intestinal schistosomiasis is an important cause of persistent abdominal pain in individuals from endemic settings, even in the absence of diarrhoea or blood in the stool. The urine-based POC-CCA cassette test is more sensitive than standard microscopic techniques (e.g. Kato-Katz and formalin-ether concentration technique) for the diagnosis of *S. mansoni*. In the current work, it has been shown that this RDT can also be a useful tool for individual patient management outside endemic areas, e.g. in European hospitals caring for immigrants from Africa or returning travellers. Its use in patient groups with a clinical or epidemiological history suggestive of schistosomiasis is strongly encouraged.

10.8 References

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