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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;

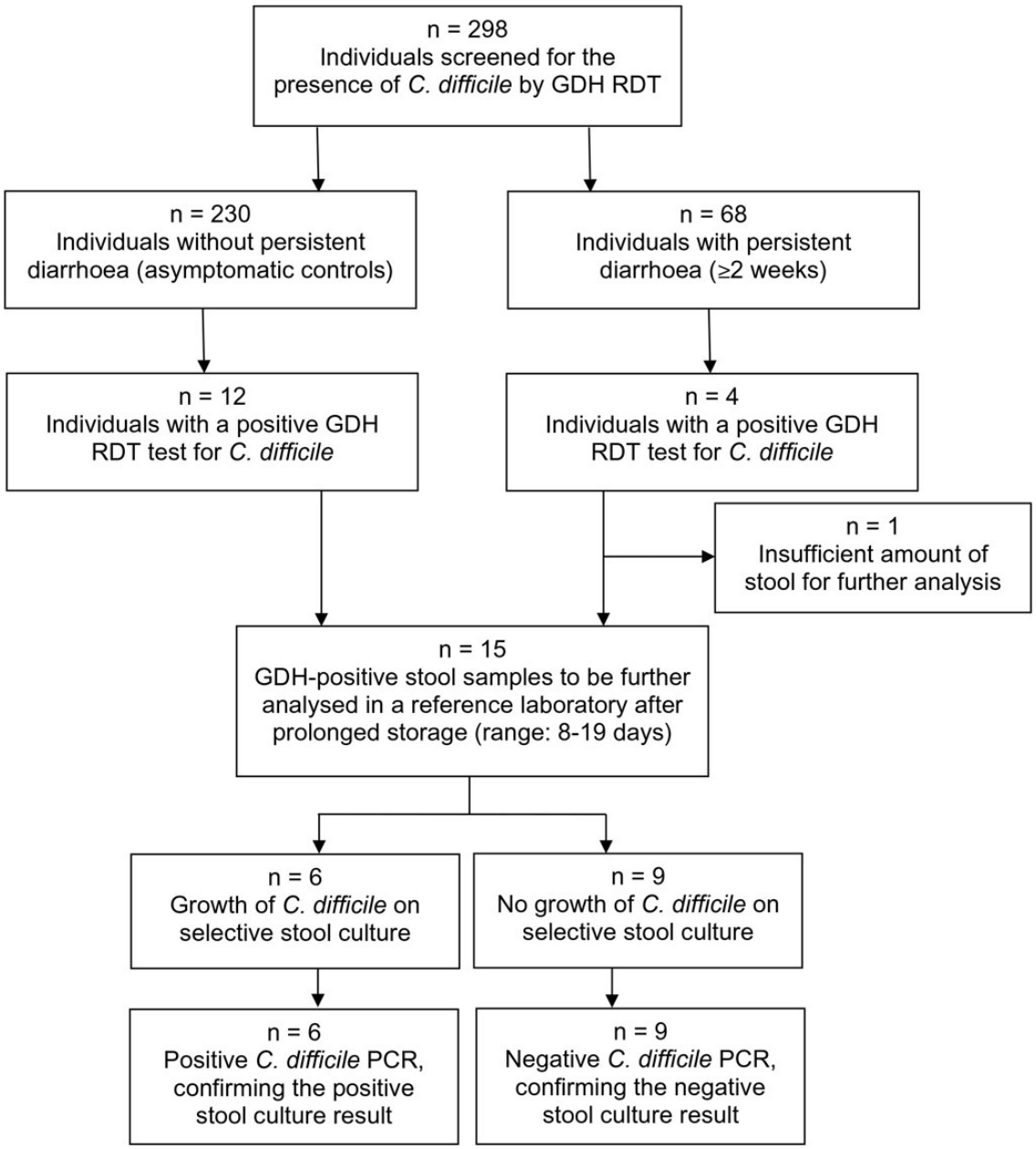

Figure 1. Study flowchart on the occurrence of *Clostridium difficile* in 298 individuals in Dabou, south Côte d'Ivoire, in October 2012. GDH: glutamate dehydrogenase; RDT: rapid diagnostic test.

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

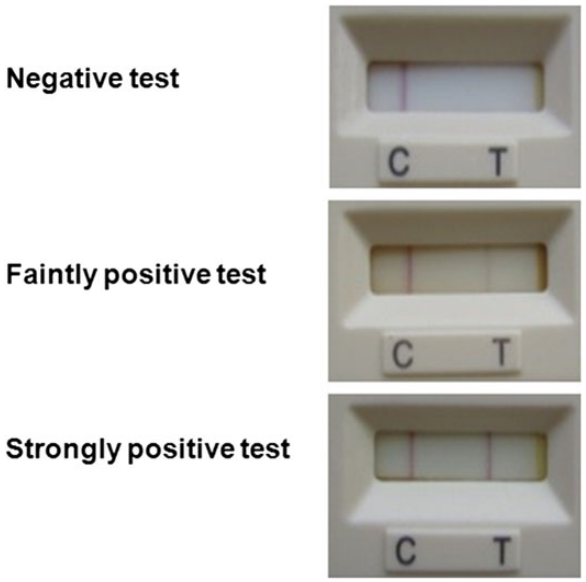

Figure 2. Results given by the rapid diagnostic glutamate dehydrogenase (GDH) screening test (Clostridium *K*-SeT) employed for diagnosis of *Clostridium difficile* in Dabou, south Côte d'Ivoire in October 2012: negative, faintly positive and strongly positive.

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

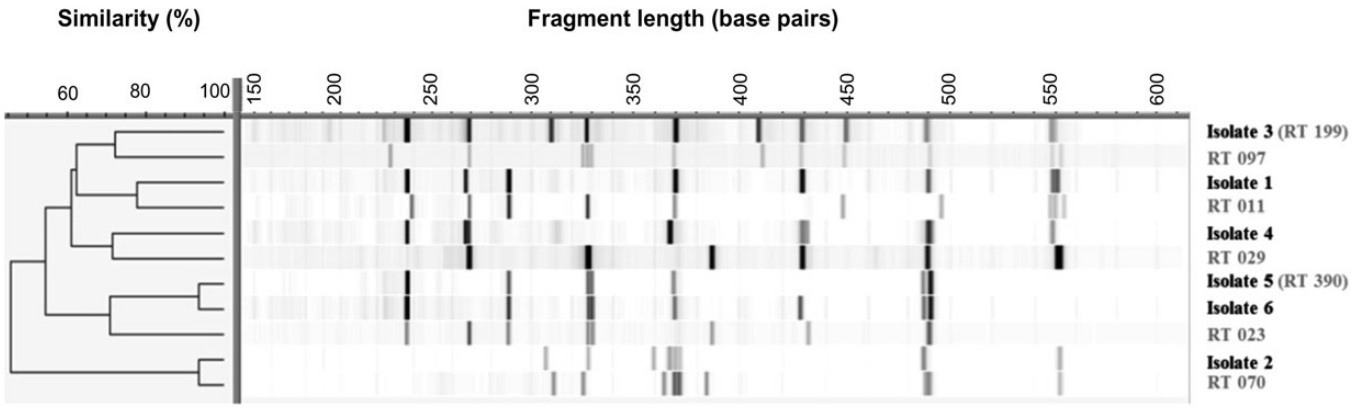

Figure 3. Dendrogram showing the specific ribotype (RT) profiles of six *Clostridium difficile* isolates from Dabou, south Côte d'Ivoire, obtained in October 2012, in comparison to related RTs, as determined by capillary ribotyping. Ribotyping displays a high diversity of the isolates. The dendrogram 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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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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