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PCR-based verification of positive rapid diagnostic tests for intestinal protozoa infections with variable test band intensity

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ABSTRACT

Stool-based rapid diagnostic tests (RDTs) for pathogenic intestinal protozoa (e.g. *Cryptosporidium* spp. and *Giardia intestinalis*) allow for prompt diagnosis and treatment in resource-constrained settings. Such RDTs can improve individual patient management and facilitate population-based screening programmes in areas without microbiological laboratories for confirmatory testing. However, RDTs are difficult to interpret in case of 'trace' results with faint test band intensities and little is known about whether such ambiguous results might indicate 'true' infections. In a longitudinal study conducted in poor neighbourhoods of Port Elizabeth, South Africa, a total of 1428 stool samples from two cohorts of schoolchildren were examined on the spot for *Cryptosporidium* spp. and *G. intestinalis* using an RDT (Crypto/Giardia DuoStrip; Coris BioConcept). Overall, 121 samples were positive for *G. intestinalis* and the RDT suggested presence of cryptosporidiosis in 22 samples. After a storage period of 9–10 months in cohort 1 and 2–3 months in cohort 2, samples were subjected to multiplex PCR (BD Max™ Enteric Parasite Panel, Becton Dickinson). Ninety-three percent (112/121) of RDT-positive samples for *G. intestinalis* were confirmed by PCR, with a correlation between RDT test band intensity and quantitative pathogen load present in the sample. For *Cryptosporidium* spp., all positive RDTs had faintly visible lines and these were negative on PCR. The performance of the BD Max™ PCR was nearly identical in both cohorts, despite the prolonged storage at disrupted cold chain conditions in cohort 1. The Crypto/Giardia DuoStrip warrants further validation in communities with a high incidence of diarrhoea.

1. Introduction

According to the 2010 Global Burden of Disease (GBD) study, intestinal protozoa infections were responsible for more than 10 million disability-adjusted life years (DALYs) (Hotez et al., 2014; Murray et al., 2012). Intestinal protozoa are mainly acquired through ingestion of contaminated water or food and they affect the gastrointestinal tract (Shirley et al., 2012; Speich et al., 2016). *Cryptosporidium* spp. and *Giardia intestinalis* (syn.: *G. lamblia* and *G. duodenalis*), for instance, cause a wide variety of clinical manifestations, ranging from asymptomatic carriage to severe diarrhoea with subsequent exsiccosis (DuPont, 2016; Sow et al., 2016). Immunocompromised individuals (e.g. with HIV infection or alpha1-antitrypsin deficiency) are particularly vulnerable to intestinal protozoa infections (Becker et al., 2014; Stark et al., 2009). The long-term effects of chronic or multiple infections, particularly among children in areas of poor sanitation, remain controversial. While some studies from high-endemicity settings suggest a causal role of both cryptosporidiosis and giardiasis to the development of malnutrition and persistent diarrhoea (Muhsen and Levine, 2012), other studies found no clear associations (Donowitz et al., 2016). Hence, a recent review highlighted the need for additional, high-quality epidemiological and clinical data on giardiasis and

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cryptosporidiosis in Africa (Squire and Ryan, 2017). Within the frame of a cohort study conducted in deprived urban settings of Port Elizabeth, South Africa, which assessed the influence of such parasitic infections on the physical fitness, cognitive performance and psychosocial health of schoolchildren (Gall et al., 2017; Müller et al., 2016; Yap et al., 2015), there was an opportunity to compare different diagnostic tests.

Asymptomatic shedding of *G. intestinalis* among household members might give rise to frequent (re-)infections in a community (Oliveira-Arbex et al., 2016). Hence, it has been speculated that mass screening programmes for human intestinal protozoa might help to elucidate the actual infection rates, disease burden and related transmission patterns (Huang and White, 2006; Santos et al., 2012; Turkeltaub et al., 2015). However, the observed prevalence in any endemic setting highly depends on the employed diagnostic approach (Ignatius et al., 2012). Different laboratory techniques are available for the diagnosis of *Cryptosporidium* spp. and *G. intestinalis*, including stool microscopy (direct faecal smear and sedimentation-based concentration procedures), immunochromatographic rapid diagnostic tests (RDTs) that detect pathogen-specific antigens, and nucleic acid-based molecular diagnostic techniques, mainly polymerase chain reaction (PCR) assays (Becker et al., 2013; McHardy et al., 2014; Soares and Tasca, 2016). RDTs and PCR usually show a higher sensitivity than stool microscopy (Laude et al., 2016; van Lieshout and Roestenberg, 2015). An important additional feature of RDTs is that they allow diagnostic testing on the spot without specialised laboratory equipment. Hence, RDTs seem particularly useful in resource-constrained healthcare centres in low- and middle-income countries to improve patient management and community-based screening programmes. Nevertheless, the interpretation of results obtained by RDTs is subjective because pathogens are not directly visualised. Instead, a positive RDT leads to a test line of specific intensity. In the absence of any test line or the presence of a strong test line, interpretation does not pose problems and the RDT is regarded as negative or positive, respectively. However, a faintly discernible test line may be interpreted as either positive or negative (Coulibaly et al., 2013; van der Palen et al., 2009). Standardised RDT grading schemes and automated readers have been developed to harmonise the interpretation criteria (e.g. for malaria RDTs), but controversies remain as to whether weakly positive test lines represent 'true' positives or not. Additionally, most diagnostic accuracy studies comparing microscopy, RDTs and PCR were performed on symptomatic individuals with diarrhoea, whereas there is a need to evaluate these techniques for *Cryptosporidium* spp. and *G. intestinalis* also in non-diarrhoeic individuals.

In the current study, stool samples were obtained from schoolchildren in disadvantaged neighbourhoods of Port Elizabeth and were analysed on the same day using an RDT for concurrent detection of *Cryptosporidium* spp. and *G. intestinalis* (Crypto/Giardia DuoStrip; Coris BioConcept) (Müller et al., 2016; Yap et al., 2015). RDT-positive samples were transferred to Europe and examined by a multiplex PCR for intestinal protozoa. The study also enabled the determination of (i) the effect of prolonged storage of stool samples on the results obtained by multiplex PCR; and (ii) the correlation between RDT test band intensity and DNA loads, as assessed by cycle threshold (C_t) values.

2. Materials and methods

2.1. Ethics statement

The study was approved by the institutional research commission of the Swiss Tropical and Public Health Institute (Swiss TPH; reference no. FK #121). Ethical approval was granted by the ethics committees of Northwest and Central Switzerland (EKNZ; reference no. 2014-179, approval date: 17 June 2014), the Nelson Mandela Metropolitan University (NMMU; study number H14-HEA-HMS-002, approval date: 4 July 2014), the Eastern Cape Department of Education (approval date: 3 August 2014) and the Eastern Cape Department of Health (approval date: 7 November 2014). The study is registered at ISRCTN registry on Current Controlled Trials (unique identifier: ISRCTN68411960, registration date: 1 October 2014).

Oral assent was sought from all participating children and individual written informed consent obtained from parents/guardians. For illiterate parents/guardians, the information sheet available in English, Xhosa and Afrikaans was read aloud in the appropriate language. Participation was voluntary. To ensure confidentiality, study participants were given unique identification numbers.

2.2. Study location and population

The study was carried out in 2015, targeting 8–12-year-old schoolchildren (some older children were also included) at eight quintile 3 primary schools situated in socioeconomically disadvantaged communities of Port Elizabeth. The quintile system categorises the degree of poverty in the neighbourhood around school locations (quintile 1 denotes the poorest and quintile 5 the least poor schools). Port Elizabeth is located in the Western region of the Eastern Cape province of South Africa (geographical coordinates: 33°57′29″ to 34°07′54″S latitude and 25°36′00″ to 25°55′49″E longitude).

2.3. Study design

The work presented here forms part of a larger longitudinal cohort study entitled 'Disease, activity and schoolchildren's health' (DASH), during which repeated surveys are being conducted (Yap et al., 2015). The sample size calculation for the DASH study aimed at achieving a precise prevalence estimate for intestinal parasites and recommended to recruit at least 1200 children (Müller et al., 2016; Yap et al., 2015). For the current retrospective diagnostic study, stool samples were obtained during the first two regular DASH surveys in February/March and September/October 2015. Many children were asymptomatic, but a brief survey was carried out to assess self-reported morbidity and collect clinical data. In clinically stable patients, there were no distinct exclusion criteria. Yet, children found to be infected with either *Cryptosporidium* spp. or *G. intestinalis* who reported abdominal pain, blood in the stool or diarrhoea, and/or who had abnormal findings on clinical examination (i.e. pulmonary wheezing or crackles, cutaneous ringworm infection, tachycardia) were referred to the local clinic for medical work-up and treatment.

2.4. Field and laboratory procedures

Children provided one fresh morning stool sample (approximately 20 g) in collection containers that had been distributed by health care professionals the day before. The stool containers did not contain any fixative. Stool samples were transferred to the laboratory of the Department of Medical Laboratory Sciences at NMMU in Port Elizabeth. On the day of collection, an immunochromatographic RDT was carried out for the concurrent detection of *Cryptosporidium* spp. and *G. intestinalis* (Crypto/Giardia DuoStrip, Coris BioConcept; Gembloux, Belgium) (Nguyen et al., 2012). In brief, a small amount of the stool sample (approximately 10 µl) is taken with a loop and mixed with a buffer. Subsequently, the RDT dipstick is put into this solution, and results are read after 15 min. Of note, no microscopic examination for intestinal protozoa was performed. The results of RDT test line intensities were documented as follows: −, negative; 1+, faintly positive/borderline positive; 2+, moderately positive; and 3+, strongly positive.

2.5. Multiplex PCR

Approximately 3–5 g of those stool samples that had a positive RDT result for *Cryptosporidium* spp. and/or *G. intestinalis* were stored in a fridge at a temperature of 4–6 °C. However, interruptions of the

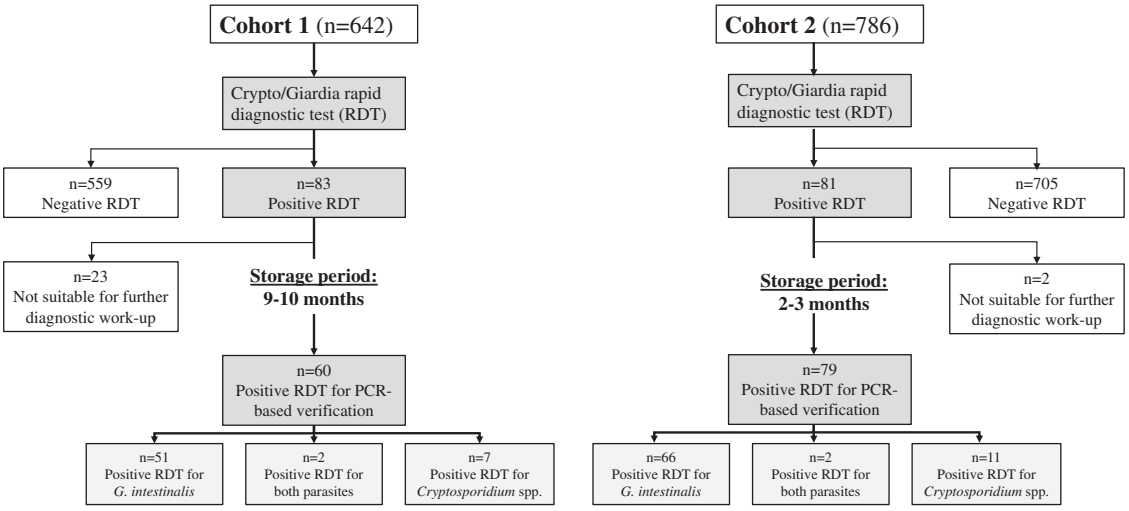

Fig. 1. Diagnostic sample flow and results obtained by an immunochromatographic rapid diagnostic test (Coris BioConcept; Gembloux, Belgium) employed on stool samples of school-aged children from Port Elizabeth, South Africa in 2015.

electrical power supply occurred occasionally and were not systematically documented. All positive RDTs were kept in a fridge at NMMU until late November 2015 when they were transferred to the Institute of Medical Microbiology and Hygiene at Saarland University (Homburg/Saar, Germany) for *post-hoc* molecular diagnostic PCR testing. Hence, stool samples were stored either for about 2–3 months or 9–10 months. Of note, only RDT-positive samples were kept and forwarded to Europe because the main objective of the study was to verify whether RDT-positive samples are confirmed by PCR.

The samples were processed using the BD Max™ system with the BD Max™ Enteric Parasite Panel for the concurrent detection of *Cryptosporidium* spp. (*C. hominis* and *C. parvum* only), *Entamoeba histolytica* and *G. intestinalis* (Becton Dickinson Diagnostics; Heidelberg, Germany). Details of the BD Max™ system and stool sample processing have been described elsewhere (Mölling et al., 2016). In brief, the nucleic acid extraction and subsequent real-time PCR (including all controls) were carried out in one machine, the BD Max™ instrument, thereby minimising the actual hands-on workload for laboratory staff, while providing results within 3 h. A sample processing control is included in each run. Possible results for each target are positive (POS), negative (NEG) or unresolved (UNR). All PCR examinations were carried out by the same experienced laboratory technician who was not informed about the results obtained by the previously employed RDT.

2.6. Treatment of infected children

Children infected with soil-transmitted helminths were treated with a single oral dose of albendazole (400 mg), free of charge, according to World Health Organization (WHO) and national treatment guidelines. Subjects with a positive *Cryptosporidium* spp. or positive *G. intestinalis* RDT result, in combination with related clinical symptoms (e.g. diarrhoea, abdominal pain or blood in the stool), were referred to the nearest local clinic for treatment. Of note, PCR assays were conducted several months after collection and transfer of stool samples to a specialised laboratory abroad; hence, these results did not guide the clinical management.

2.7. Statistical analysis

Data were double-entered and cross-checked using Microsoft Excel version 14.0 (edition 2010, Microsoft Corporation). Statistical analysis was performed using STATA version 12.0 (StataCorp.; College Station, USA). Due to the different duration of sample storage at suboptimal conditions, samples obtained in February/March and September/October 2015 were analysed independently. Mean C_t values were calculated and these were related to the band intensity of the RDT results to assess potential correlations pertaining to infection intensity. A *P*-value of < 0.05 was considered as statistically significant.

3. Results

3.1. Study cohort

During the longitudinal DASH study, stool samples from two cohorts, consisting of 642 and 786 schoolchildren, respectively, were collected and screened using the Crypto/Giardia DuoStrip RDT in February/March 2015 (cohort 1) and September/October 2015 (cohort 2). Positive results for either *Cryptosporidium* spp. or *G. intestinalis* were found in 83 (12.9%) and 81 (10.3%) individuals, respectively. These samples were subjected to *post-hoc* molecular diagnostics in December 2015, corresponding to storage periods of either 9–10 or 2–3 months. Out of 83 RDT-positive samples obtained during the first sampling period, 23 were judged unsuitable for further diagnostic work-up due to fungal overgrowth or other signs of poor sample conservation. In the 2–3-month storage group, only two out of 81 samples were deemed unsuitable, as assessed by visual inspection. Hence, the final study cohorts consisted of 60 samples (specimens stored for 9–10 months; cohort 1) and 79 samples (specimens stored for 2–3 months; cohort 2). The diagnostic sample flow and the results obtained by RDT are presented in Fig. 1.

Cohort 1 consisted of slightly more females than males (31 *vs.* 29) with age ranging from 9 to 13 years (mean age: 10.1 years). In cohort 2, there were more males than females (48 *vs.* 31) with age ranging from 9 to 14 years (mean age: 10.6 years).

3.2. Overall PCR performance

Overall, PCR detected the presence of *G. intestinalis* in 82.7% of all samples used for the final analysis (115/139), whereas neither *Cryptosporidium* spp. nor *E. histolytica* were found. In 12 out of the 139 individuals used in the final analysis (n = 8, 8.6% in cohort 1; n = 4, 5.1% in cohort 2), PCR yielded an invalid result (UNR) during the first run, but worked well upon first PCR repetition with a slightly different sample quantity.

Table 1
Two-way contingency tables comparing the results obtained by BD Max™ Enteric Parasite Panel PCR and the immunochromatographic Coris Crypto/Giardia Duo-Strip rapid diagnostic test (RDT) for the diagnosis of *Giardia intestinalis* in stool samples from schoolchildren in Port Elizabeth, South Africa, in 2015.

(a) Cohort 1 (samples collected in February/March 2015, PCR performed in December 2015)

RDT	BD Max™ Enteric Parasite Panel PCR		
	Positive	Negative	Total
Positive	49	4	53
Negative	1	6	7
Total	50	10	60

(b) Cohort 2 (samples collected in September/October 2015, PCR performed in December 2015)

RDT	BD Max™ Enteric Parasite Panel PCR		
	Positive	Negative	Total
Positive	63	5	68
Negative	2	9	11
Total	65	14	79

3.3. Comparison of RDT and PCR in cohort 1 (prolonged storage period of 9–10 months)

In cohort 1, there were 53 samples with a positive RDT result for *G. intestinalis* and nine samples with an RDT result indicating a *Cryptosporidium* spp. infection. Co-infections with both pathogens were suspected in two cases. Multiplex PCR performed on the 60 samples from cohort 1 identified *G. intestinalis* infection in 50 specimens, while no *Cryptosporidium* spp. was detected. When comparing PCR and RDT results for *G. intestinalis*, PCR confirmed 49 out of 53 RDT-positive samples, owing to a confirmation rate of 92.5%. Of note, PCR identified one additional *G. intestinalis* infection in the seven samples that had tested negative on the RDT for *G. intestinalis*, but positive for *Cryptosporidium* spp. A 2-way contingency table for the diagnostic agreement between RDT and PCR is displayed in Table 1a .

3.4. Comparison of RDT and PCR in cohort 2 (short storage period of 2–3 months)

Out of the 79 samples analysed in cohort 2, 68 had a positive RDT result for *G. intestinalis* and 13 samples were positive for *Cryptosporidium* spp. Cohort 2 comprised two co-infections according to RDT results. In the PCR analysis, 63 RDT-positive samples were confirmed to contain *G. intestinalis*, owing to a concordance of 92.7% (63/68) between both techniques. Furthermore, PCR identified two additional *G. intestinalis* infections in the 11 RDT-negative samples (Table 1b). PCR for *Cryptosporidium* spp. was negative in all samples, thus none of the 13 positive RDT results was confirmed. Of note, all samples testing positive on RDT for *Cryptosporidium* spp. had faintly visible test band intensity.

3.5. Comparative assessment of infection intensity

According to a semi-quantitative RDT band intensity scheme for the diagnosis of *G. intestinalis* (Fig. 2), 46 samples (38%) had a faintly visible test line (1+), whereas 53 (44%) and 22 (18%) specimens were recorded as 2+ and 3+, respectively. The PCR confirmation rate for RDTs with a band intensity of 1+ was 89.1% (41/46), whereas it was 94.7% (71/75) for RDT results with stronger band intensities ($P = 0.245$). The mean C_t value for samples found positive for *G. intestinalis* on multiplex PCR was 27.1 (standard deviation (SD): 4.8) in cohort 1 and 26.1 (SD: 5.0) in cohort 2 ($P = 0.330$). In both cohorts, the mean C_t values were < 26.0 if the RDT band intensity was 2+ or 3+, whereas they were > 28.0 in case of RDT band intensities of 1+. In samples that were exclusively positive by PCR, but negative on RDT, the mean C_t values were > 30.0 in both cohorts, thus indicating a lower pathogen load in these samples. The correlation between C_t values and RDT band intensity is displayed in Fig. 3.

4. Discussion

Intestinal protozoa infections are important causes of acute and persistent diarrhoea and abdominal pain (Turkeltaub et al., 2015). Transmission occurs through the faecal-oral route and high carriage rates in asymptomatic individuals facilitate a continued spread of protozoal infections such as giardiasis and cryptosporidiosis. In many endemic settings, however, there is a lack of even basic laboratory infrastructure, such as centrifuges, and microscopes that are required for standard diagnostic tests (Becker et al., 2016; Boelaert and The NIDIAG Consortium, 2016). Hence, RDTs offer a unique opportunity for point-of-care diagnosis in the absence of basic laboratory infrastructure. Accuracy of an RDT is a central feature. Here, we show that 93% of RDT-positive samples for *G. intestinalis* tested on the day of stool production were confirmed by *post-hoc* PCR several months later in a reference laboratory in Europe. Importantly, we found a positive correlation between RDT test band intensity and the pathogen quantity in the stool.

Most RDTs for intestinal protozoa detection are considered more sensitive than microscopy. However, the reported diagnostic accuracy of a certain RDT may differ considerably from one study to another, and may also yield different results if applied to diarrhoeic stool samples or to specimens obtained from asymptomatic individuals (Bouyou-Akotet et al., 2016). In a recent review, the authors concluded that RDTs should complement microscopy whenever possible to improve the diagnostic accuracy of stool-based diagnosis of human intestinal protozoa infections (Soares and Tasca, 2016). When assessing the test characteristics of RDTs, it is important to note that most studies were performed before the advent of molecular PCR techniques, i.e. comparing RDT results to those of stool microscopy, but in the absence of a highly sensitive diagnostic reference standard. It follows that the observed diagnostic accuracy may vary significantly, which is illustrated by five published studies of the RDT used here. Indeed, these studies reported a sensitivity and specificity for *G. intestinalis* detection in the range of 44.4–96.2% and 97.7–100%, respectively (Becker et al., 2015a; Nguyen et al., 2012; Oster et al., 2006; van den Bossche et al., 2015; Weitzel et al., 2006).

In most published reports, PCR is the single most sensitive diagnostic technique for detection of *Cryptosporidium* spp. and *G. intestinalis*. Many experts thus conjecture PCR to complement and, potentially, substitute stool microscopy for intestinal protozoa diagnosis. However, commercially available multiplex PCR kits for intestinal protozoa detection such as the BD Max™ Enteric Parasite Panel used here have only recently become available (Verweij, 2014). To our knowledge, only three previous studies from Sweden (Mölling et al., 2016), the United Kingdom (Batra et al., 2016) and the United States of America (Madison-Antenucci et al., 2016) have assessed the technique's potential for diagnosis of intestinal protozoa. In the largest of these studies, a total of 2495 specimens from eight centres were examined and results obtained by the BD Max™ Enteric Parasite Panel were compared to standard diagnostic techniques. The reported sensitivity and specificity of the molecular assay for *G. intestinalis* was 98.2% and 99.5%, respectively, and 95.5% and 99.6% for *Cryptosporidium* spp. Nevertheless, it is important to note that PCR may also yield false-negative results in some instances, e.g. if inhibitors are present in the faecal sample. In the study presented here, we report a high confirmation rate between RDT and PCR for *G. intestinalis* (93%), and such high agreement did not differ whether samples were stored for 2–3 or 9–10 months, i.e. indicating that the BD Max™ Enteric Parasite Panel provides accurate

Fig. 2. Results obtained by an immunochromatographic rapid diagnostic test (Coris BioConcept; Gembloux, Belgium) for the diagnosis of *Giardia intestinalis*, stratified by test band intensity: (a) negative; (b) 1+; (c) 2+; and (d) 3+. Of note, the test used in this study is double-sided and the results for *Cryptosporidium* spp. can be read on the back side of the test strip.

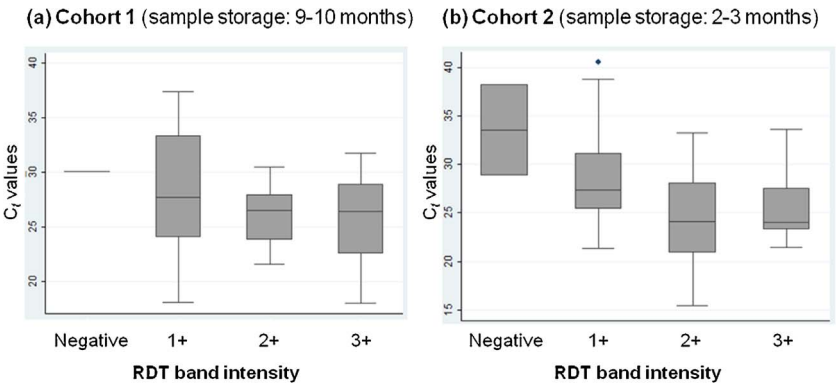

Fig. 3. Correlation between C_t values obtained by PCR and band intensity on an immunochromatographic rapid diagnostic test (RDT) for the diagnosis of *Giardia intestinalis* in two cohorts of schoolchildren in Port Elizabeth, South Africa, in 2015. Note: ◊ represents outlying values.

results even in the case of suboptimal storage conditions, which might have important considerations for future studies in resource-constrained settings.

In our study, the correlation between strong RDT band intensities and higher pathogen load, as expressed by lower C_t values, is particularly noteworthy. Pertaining to the application of RDTs for an improved diagnosis of, for example, malaria (van der Palen et al., 2009) or schistosomiasis (Becker et al., 2015b), there has been considerable debate whether faintly visible band intensities should be interpreted as 'positive' or 'negative'. Of note, no correlation between pathogen load and RDT band intensity was observed in a study comparing three different RDTs from other manufacturers than the test used here (Johnston et al., 2003). Based on our findings, we recommend that any visible test band for *G. intestinalis* on the Crypto/Giardia DuoStrip RDT should be interpreted as a positive signal, whereas the test for *Cryptosporidium* spp. needs further optimisation and faint test bands should be regarded as negative.

Our study has several limitations. First, only samples with a positive RDT result for either *Cryptosporidium* spp. or *G. intestinalis* were further analysed with multiplex PCR, whereas RDT-negative specimens were not re-examined by PCR. Hence, our study does not allow to independently assess sensitivity and specificity of the employed diagnostic techniques. However, the main purpose of the current study was to verify the proportion of positive RDTs from an endemic area being confirmed by a highly sensitive PCR technique, because previous studies had reported variable accuracy of stool-based RDTs when used in endemic settings. Second, no additional diagnostic techniques have been performed, most importantly microscopy for *G. intestinalis* and *Cryptosporidium* spp., which could have been helpful (i) to reveal the 'true' infection status in samples with conflicting results on PCR and RDT; and (ii) to provide information on the pathogen quantity being present in a sample (in addition to C_t values obtained by real-time PCR). However, sufficiently sensitive microscopic techniques (e.g. formalin-ether concentration technique and Kinyoun staining) were not available locally when the study was carried out. Third, we cannot exclude that the prolonged storage at partially disrupted cold chain conditions could have negatively influenced the PCR results. However, we did not observe a significant difference between the two cohorts in our study, so that the BD Max™ system's performance seems to be only marginally affected by prolonged and suboptimal storage conditions. Yet, we cannot exclude the possibility that the nucleic acids of *Cryptosporidium* spp. might have been unstable, and thus not detectable even in cohort 2. However, this speculation seems unlikely because previous studies with a shorter storage period revealed similar discrepancies between results obtained by the employed RDT and PCR testing for *Cryptosporidium* spp. (Becker et al., 2015a).

5. Conclusions

The stool-based Crypto/Giardia DuoStrip RDT has been designed to concurrently detect the intestinal protozoa *Cryptosporidium* spp. and *G. intestinalis*. Ninety-three percent of positive results for *G. intestinalis* were confirmed by *post-hoc* PCR and the intensity of the RDT test band correlated with the infection intensity. In contrast, trace RDT results for *Cryptosporidium* spp. should be regarded as negative. The BD Max™ Enteric Parasite Panel is a suitable stool-based PCR technique that can be reliably used when employed on native stool samples that have been stored at disrupted cold chain conditions for up to 10 months.

Conflict of interest

P. Mertens is Director for Research and Development (R & D) at the company Coris BioConcept (Gembloux, Belgium). All other authors declare no competing interests.

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