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Whatman Protein Saver Cards for Storage and Detection of Parasitic Enteropathogens

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Abstract. Current methods to identify the etiology of diarrhea require laboratory facilities for storage of pathogens, which is often challenging in low-resource settings. This study evaluated the efficacy of a low-cost method for preserving stool specimens for the detection of parasitic enteropathogens using Whatman 903 protein saver cards (Sigma-Aldrich, St. Louis, MO). Stool samples known to be positive by multiplex real-time polymerase chain reaction for *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica* parasites were preserved on 232 Whatman cards. DNA was then extracted from cards using Chelex and Qiagen extraction protocols, and tested for these parasites using multiplex real-time PCR. We included stool samples known to have a higher parasite load (cycle threshold [ct]-value < 30) and those with a lower parasite load (ct values 30–35). Sensitivities and specificities were determined using DNA extracted directly from whole stool samples using Qiagen kits (QIAGEN, Hilden, Germany). For whole stool samples with ct values < 30, preserved directly on Whatman 903 protein saver cards for *Giardia* analysis, the sensitivity was 100% for both Qiagen and Chelex DNA extraction. For *E. histolytica*, this was 100% for sensitivity for Qiagen and 80% for Chelex DNA extractions, and for *Cryptosporidium*, this was 80% for Qiagen and 50% for Chelex DNA extraction. The specificity was 100% for all parasites for all extraction procedures. Given the high sensitivity for stool samples with higher parasite loads, we recommend the use of the Whatman 903 protein saver card for preserving fecal specimens for the analysis of *Giardia* and *E. histolytica* using Qiagen DNA extractions in low-resource settings.

INTRODUCTION

Diarrheal diseases are a leading cause of mortality for children under 5 years of age and was attributed to approximately 446,000 deaths in this age group in 2016.[1] The most common enteropathogens in a recent global multicenter study of pediatric populations were *Shigella* spp., heat-stable enterotoxin-producing *Escherichia coli* (ST-ETEC), *Campylobacter* spp., *Cryptosporidum* spp., rotavirus, and adenovirus 40/41. Seventy-eight percent of diarrheal cases among children enrolled in the study were attributable to these six pathogens.[2] Diarrheal infections due to the parasite *Cryptosporidium* are the highest among children less than 1 year of age.[3] In addition, the parasite *Giardia lamblia* has been shown to be a common enteropathogen among children, associated with diarrheal disease and childhood stunting.[4–6] The parasite *Entamoeba histolytica* is also a common etiological cause of diarrhea among older children in several countries in Asia and Africa.[7]

The implementation of pathogen-specific control measures is contingent on the availability of robust epidemiological data that characterizes the predominant enteropathogens in a given population. With advancements in the area of diagnostics, a range of laboratory methods are currently available for determining microbial etiology in the field of diarrheal diseases. The major methods are broadly classified as culture-based, microscopic, antigen-based, and molecular methods, with each having variable efficacy and costs. Culture-based methods are widely considered the standard for diagnosing diarrhea infections caused by bacteria.[8] However, factors such as the laboratory expertise required and cost per test can limit the use of this method in low-resource settings.[9–12] Furthermore, this method cannot be used to distinguish between toxin-producing and nonproducing bacterial strains, required for the implementation of pathogen-specific surveillance and targeted interventions.[13]

Similar issues arise for the detection of parasitic enteropathogens using microscopic methods. The sensitivity of this method, which involves the identification of the parasite and ova in stool samples, often depends on the burden of disease in the patient and microscopic skills of the laboratory technician performing the analysis.[13] Furthermore, it is not possible to discern species-level differences using this technique, which makes it difficult to distinguish between pathogenic and nonpathogenic parasite strains.[13] In comparison, emerging technologies such as antigen-based assays can be used to specifically identify toxin-producing and pathogenic strains of enteric microbes.[11] These assays also have the potential of being more field-adaptable, especially in low-resource settings.[14,15] For instance, the rapid dipstick test that is used for cholera detection requires minimal laboratory facilities and less technical skill for implementation compared with the culture method.[16] However these assays show varying levels of effectiveness in the field, often displaying lower sensitivity and specificity compared with the culture method.[14,15,17] Hence, more research is needed to improve the performance of these technologies in field settings.

Molecular methods such as PCR have consistently shown higher sensitivity as well as higher potential for standardization across study sites compared with culture-based, microscopic, and antigen-based assay methods.[18–20] However, this technique requires a PCR machine, which is often not available in low-resource settings.[11] Furthermore, there are several sample storage and processing steps that need to be performed before conducting PCR to analyze diarrheal pathogens. For instance, samples that are collected typically need to be stored under freezing conditions and transported using a cold chain. However, it may not be feasible to set up cold storage facilities in low-resource settings. Methods that allow sample storage and transport under room temperature conditions would be ideal for these settings.

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Previous work in Cameroon has shown that fecal specimens can be preserved on Whatman 903 protein saver cards for subsequent DNA extraction and PCR analysis for *Vibrio cholerae*.[21] Furthermore, another study has demonstrated that Whatman FTA Elute cards (Sigma Aldrich, St. Louis, MO) can be used to preserve fecal samples containing bacterial and parasitic enteropathogens for PCR detection.[22] Whatman 903 protein saver cards have the advantage of being a third of the cost of Whatman FTA Elute cards (1.5 USD versus 4.60 USD per card), which will allow for a more economically feasible option in low-resource settings. This is compared with the additional cost of 5–8 USD per sample for shipping the stool samples on dry ice from, for example, Bangladesh to the United States. The differences between the cards are that Whatman 903 protein saver cards provide an untreated matrix for storage of biological samples, whereas Whatman FTA Elute cards contain a matrix treated with chaotropic salts to facilitate cell lysis and preservation of DNA. Preserving fecal specimens on these Whatman protein saver cards removes the need for cold storage in resource-limited settings and allows for shipment to facilities with a PCR machine without a cold chain. To date the method of storing stool samples on Whatman 903 protein saver cards has only been validated for *Vibrio cholerae*.[21] Research is needed on the efficacy of this method for other enteric pathogens.

The goal of our study is to evaluate the efficacy of Whatman 903 protein saver cards in preserving parasite DNA compared with the standard method of storage of raw stool samples in a –80 freezer. The use of Whatman protein saver cards has the potential to reduce costs associated with sample transport and storage, and allow for the analysis of parasitic enteropathogens in resource-limited settings.

METHODS

Stool samples positive for at least one of the following three parasites were included in our study: *G. lamblia*, *Cryptosporidium* spp., or *E. histolytica*. Sixty stool samples were collected from individuals not reporting diarrhea at the time of stool collection (non-diarrhea stool collection) at International Centre for Diarrhoeal Disease Research, Bangladesh surveillance sites in Dhaka, Bangladesh. All these individuals were confirmed to have these parasites by real-time PCR. Non-diarrhea stool samples were analyzed to determine if the Whatman cards could be used to identify asymptomatic parasite infections, which could be contributing to intestinal inflammation among susceptible pediatric populations.[17] Ten negative control samples were also included from this surveillance site and confirmed by real-time PCR to not have these parasites. We stratified stool samples based on cycle threshold (ct) values from PCR. For parasite-positive stool samples, we stratified samples based on a ct value < 30 and ct values between 30 and 35. For parasite-negative stool samples, we selected samples with ct values > 35. The ct value cutoff of 30 has been used in a previous study evaluating the impact of sample storage on ct values.[20] Furthermore, the ct value of 35 has been widely used as the threshold for determining negative samples.[23] Two hundred milligrams of each stool sample was transferred either directly to Whatman 903 protein saver cards or after suspension in 300 µL of sterile distilled water (stool suspension), 232 Whatman cards in total were included in this analysis. Stool suspension was included to study whether there are differences in the efficacy of DNA extraction from whole stool versus stool suspension stored on Whatman cards. After transferring the whole stool or stool suspension to the Whatman cards, the cards were dried overnight and were stored for 48 hours before conducting DNA extractions and real-time PCR analysis.

The Qiagen and Chelex DNA extractions methods were used in this study according to previously published methods.[23,24] The Qiagen method was modified from the Qiagen QIAamp Fast DNA Stool Mini Kit protocol (QIAGEN, Hilden, Germany). Dried stool samples from the Whatman protein saver cards were cut and placed in 2-mL screw cap tubes (USA Scientific, Orlando, FL) prefilled with 340 mg of Sigma-Aldrich acid-washed glass beads. The samples were incubated with 1 mL InhibitEX buffer for 15 minutes at room temperature. The samples were then run in a bead beater for 3.5 minutes and incubated at 95°C for 5 minutes. After centrifugation, 600 µL of sample lysate was transferred to 2-mL microfuge tubes (USA Scientific) containing 25 µL proteinase K. This was followed by the addition of 600 µL of lysis buffer, a brief vortex for 15 seconds, and incubation of samples at 70°C for 10 minutes. After incubation, 600 µL of 100% ethanol was added to the samples and the total lysate was transferred to spin columns for subsequent washes (500 µL of wash buffers 1 and 2) and elution with 200 µL of elution buffer. The eluted DNA was then stored at –20°C for further experiments. In the Chelex method, dried stool samples from the Whatman protein saver cards were cut and placed in 1.5 mL microfuge tubes (USA Scientific). One milliliter of sterile 1× phosphate buffered saline (PBS) was added and samples were incubated at room temperature for 10 minutes. After incubation, the supernatant was discarded and the samples were washed with 1× PBS once more. The sample was then incubated in 2% Chelex solution (200 µL) at 100°C for 8 minutes. After incubation, samples were spun down and supernatants were collected for subsequent DNA quantification and PCR reactions. DNA concentration was measured using the Nanodrop instrument (Thermo Fisher Scientific, Waltham, MA). The presence or absence and quantification of parasite DNA was determined using real-time multiplex PCR according to previously published methods.[18] Briefly, the multiplex real-time PCR assay was performed to identify *Giardia*, *Cryptosporidium*, and *E. histolytica.* Positive and negative controls were used in each run of qPCR. Amplification consisted of 15 minutes at 95°C followed by 40 cycles of 20 seconds at 95°C and 60 seconds at 60°C. Amplification, detection, and data analysis were performed with the CFX96 Real-time detection system (Bio-Rad Laboratories, Hercules, CA). Fluorescence was measured during the annealing step of each cycle. The sequences of primers and probes used in these experiments are indicated in Supplemental Table 1.

The data from the Real-time PCR experiments were analyzed using CFX manager, Version 3.1 software (Bio-Rad Laboratories). Samples with ct values less than 35 were considered positive for the respective parasite. The sensitivity and specificity were estimated for preserving stool directly on the Whatman card and for stool suspension using whole stool (not on a protein saver card) as the gold standard. The 95% confidence intervals (CIs) for sensitivity and specificity were calculated using the exact method. We estimated *P*-values using the Wilcoxon signed-rank test for comparisons made of DNA concentrations.

TABLE 1

Comparison of average ct values obtained by conducting multiplex real-time PCR on parasite-positive stool samples stored on Whatman protein saver cards for 48 hours using Qiagen or Chelex DNA extraction protocols

Sample type	DNA extraction method	Average ct values ± standard deviation (min-max)		
		Giardia lamblia (*N* = 20)	*Cryptosporidium* (*N* = 20)	*Entamoeba histolytica* (*N* = 20)
Whole stool	Qiagen	30 ± 4 (22–35)	29 ± 5 (19–35)	30 ± 3 (22–34)
Whole stool directly on Whatman cards	Qiagen	31 ± 3 (23–36)	32 ± 4 (26–38)	33 ± 4 (24–38)
	Chelex	33 ± 3 (27–39)	36 ± 2 (31–39)	33 ± 3 (25–36)
Stool suspension on Whatman cards	Qiagen	31 ± 4 (23–39)	33 ± 4 (28–40)	33 ± 3 (25–36)
	Chelex	32 ± 3 (25–37)	33 ± 4 (27–39)	33 ± 3 (26–36)

ct = cycle threshold.

RESULTS

The Chelex extraction method led to significantly higher DNA concentrations compared with the Qiagen method for both direct whole stool and stool suspensions preserved on Whatman cards ($P < 0.0001$) (Supplemental Table 2). For the Chelex DNA extraction method, the concentration of DNA from whole stool samples transferred directly to the Whatman cards was significantly greater than from stool suspensions for all three parasites tested ($P < 0.0001$), whereas for the Qiagen method a significant difference was only found for *Cryptosporidium* spp. ($P < 0.0004$). Table 1 presents the average ct values obtained after whole stool or stool suspension was preserved on Whatman cards. For stool directly preserved on the Whatman card, the overall sensitivity for *G. lamblia* using the Qiagen method was 95% (95% CI: 75%, 100%) and 80% (95% CI: 56%, 94%) using the Chelex method (Table 2). For stool suspension, the overall sensitivity for *G. lamblia* was 85% (95% CI: 62%, 97%) for using the Qiagen method and 80% (95% CI: 56%, 94%) using the Chelex method. When only samples that had a higher parasite load were considered (ct value < 30), all methods for direct stool and stool suspension on Whatman cards had a sensitivity of 100%. For lower parasite loads (ct value ≥ 30) for direct stool on Whatman cards, this ranged from a sensitivity of 90% using the Qiagen method to 60% for the Chelex method. For stool suspensions, the sensitivity was 70% using the Qiagen method and 60% using the Chelex method for lower parasite loads. All methods had a specificity of 100% for *G. lamblia*.

The sensitivity and specificity for *E. histolytica*–positive samples is presented in Table 3. For whole stool directly preserved on the Whatman card, the overall sensitivity for *E. histolyica* using the Qiagen method was 60% (95% CI: 36%, 81%) and 70% (95% CI: 46%, 88%) using the Chelex method. For stool suspension, the overall sensitivity for *E. histolytica* for the Qiagen method was 85% (95% CI: 62%, 97%) and 75% (95% CI: 51%, 91%) for the Chelex method. When only samples that had a higher parasite load were considered, whole stool and stool suspension on Whatman cards for the Qiagen method had a sensitivity of 100%. For the Chelex method both whole stool and stool suspension on Whatman cards for high–parasite load samples had a sensitivity of 90%. Lower parasite load samples for whole stool on Whatman cards had a sensitivity of 20% for Qiagen and 50% for the Chelex method. For stool suspensions, the sensitivity was 70% for Qiagen and 60% for the Chelex method for lower parasite loads. All methods had a specificity of 100% for *E. histolytica*.

For stool directly preserved on the Whatman card, the overall sensitivity for *Cryptosporidium* using the Qiagen method was 40% (95% CI: 19%, 64%) and 25% (95% CI: 9%, 49%) using the Chelex method (Table 4). For stool suspension, the overall sensitivity for *Cryptosporidium* for both the Qiagen and Chelex method was 35%. When only samples that had a higher parasite load were considered, direct stool on Whatman cards had a sensitivity for the Qiagen method of 80% (95% CI: 44%, 97%) and only 50% (95% CI: 19%, 81%) for the Chelex method, whereas the sensitivity was 70% for stool suspension for both DNA extraction methods. For lower parasite loads, this was 0% for stool suspension for both DNA extraction methods for both direct stool and stool suspension on Whatman cards. All methods had a specificity of 100% for *Cryptosporidium* spp.

DISCUSSION

Our study represents the first evaluation of Whatman 903 protein saver cards for the preservation and detection of parasitic enteropathogens. This method displayed a high specificity (100%) for all three parasitic pathogens that were tested. The sensitivity of this method varied depending on the parasite. For high parasite loads, both *G. lamblia* and *E. histolytica* were detected with a sensitivity of 100% when stool was added directly on Whatman cards using the Qiagen method for DNA extraction, whereas for *Cryptosporidium* this ranged from 70% to 80%.

TABLE 2

Comparison of *Giardia lamblia* sensitivity between whole stool samples and stool samples stored on Whatman protein saver cards

Application method	DNA extraction method	High parasite load* (*N* = 10)	Low parasite load† (*N* = 10)	All samples (*N* = 20)
		Sensitivity (95% CI)	Sensitivity (95% CI)	Sensitivity (95% CI)
Whole stool directly on Whatman cards	Qiagen	100% (95% CI: 69%, 100%)	90% (95% CI: 56%, 100%)	95% (95% CI: 75%, 100%)
	Chelex	100% (95% CI: 69%, 100%)	60% (95% CI: 26%, 88%)	80% (95% CI: 56%, 94%)
Stool suspension on Whatman cards	Qiagen	100% (95% CI: 69%, 100%)	70% (95% CI: 35%, 93%)	85% (95% CI: 62%, 97%)
	Chelex	100% (95% CI: 69%, 100%)	60% (95% CI: 26%, 87%)	80% (95% CI: 56%, 94%)

CI = confidence interval.
* Whole stool ct value < 30.
† Whole stool ct value ≥ 30.

TABLE 3
Comparison of *Entamoeba histolytica* sensitivity between whole stool samples and stool samples stored on Whatman protein saver cards

Application method	DNA extraction method	High parasite load* (*N* = 10) Sensitivity (95% CI)	Low parasite load† (*N* = 10) Sensitivity (95% CI)	All samples (*N* = 20) Sensitivity (95% CI)
Whole stool directly on Whatman cards	Qiagen	100% (95% CI: 69%, 100%)	20% (95% CI: 3%, 56%)	60% (95% CI: 36%, 81%)
	Chelex	90% (95% CI: 56%, 100%)	50% (95% CI: 19%, 81%)	70% (95% CI: 46%, 88%)
Stool suspension on Whatman cards	Qiagen	100% (95% CI: 69%, 100%)	70% (95% CI: 35%, 93%)	85% (95% CI: 62%, 97%)
	Chelex	90% (95% CI: 56%, 100%)	60% (95% CI: 26%, 88%)	75% (95% CI: 51%, 91%)

CI = confidence interval.
* Whole stool ct value < 30.
† Whole stool ct value ≥ 30.

The concentration of DNA extracted using the Chelex method was significantly higher than the Qiagen method. The Chelex method and similar boiling methods have been previously validated for the extraction of DNA from samples containing bacterial pathogens preserved on Whatman cards or filter paper.[21,25] In these previous studies, diarrheal stool or water samples were analyzed. However, to our knowledge, our study is the first to investigate this method for parasites on non-diarrheal stool. The reason for the higher concentration of DNA extracted using the Chelex method compared with Qiagen method is possibly because the former method does not use a separate purification step using silica gel based–columns. This method of purification has been associated with low recovery and loss of DNA during the extraction process.[26]

The concentration of DNA from stool samples transferred directly to the Whatman cards was significantly greater than from stool suspensions for all parasites tested for the Qiagen DNA extraction method. This is likely because stool suspension leads to dilution of the stool sample. However, the Chelex method using stool suspensions yielded a higher sensitivity than for stool directly on the Whatman cards for *Cryptosporidium* spp. and *E. histolytica.* Previous studies have demonstrated that stool contains PCR inhibitors and that the dilution of stool samples may improve PCR sensitivity.[27–30] Hence, it is possible that the preparation of stool suspensions in our study reduced PCR inhibitors in the sample. This could explain the improved sensitivity of the Chelex method with stool suspension compared with stool directly on the Whatman cards. Because the Chelex method is more cost-effective compared with the Qiagen method, this method is a promising option in low-resource settings.

In this study, we evaluated the sensitivity of the Whatman card method for sample preservation using samples stratified by higher and lower parasite loads based on ct value. We observed lower sensitivity of this method for ct values > 30 for all parasites tested. However, for *G. lamblia* preserving stool directly on Whatman cards with Qiagen DNA extraction still had a sensitivity of 90% at ct values > 30. This finding suggests that Whatman cards can be used to detect Giardia at low quantities. However, for *Cryptosporidium* and *E. histolytica*, the Whatman card does not appear to have adequate sensitivity to be used as a surveillance tool for low parasite loads. To our knowledge, our study is the first to compare higher and lower parasite load detection using Whatman protein saver cards. This analytical approach has been used to understand the sensitivity of other storage methods such as the preservation of diarrheal pathogens on cotton swabs.[20] Similar to our observations, this study demonstrated that sensitivity of the swab storage method was lower for samples with a low enteropathogen load compared with those with a higher load. This was similar for both bacteria-positive stool samples such as *Campylobacter*, *Shigella*, *ETEC*, and parasite-positive stool samples such as *Giardia* and *Cryptosporidium*.

The sensitivity of the Whatman card method for *Cryptosporidium* was low. The highest sensitivity was observed for stool directly on the Whatman card using the Qiagen method for ct values less than 30 at 80%. One potential explanation is that our DNA extraction protocol was not effective in disrupting the *Cryptosporidium* oocyst wall, which is needed for the release of DNA. DNA extraction protocols for isolation of *Cryptosporidium* DNA often require steps such as freeze–thaw in liquid nitrogen, sonication, or high-speed bead-based homogenization to lyse the oocyst wall.[18,31,32] We only performed high-speed bead-based homogenization in our DNA extraction protocol, it is possible freeze–thaw in liquid nitrogen was also needed. Another explanation is that *Cryptosporidium* oocysts respond in unique ways to environmental stresses such as cold and dehydration.[33] Therefore, it is possible that the drying of the stool sample on Whatman cards affected the ability of the oocysts to be lysed by the bead-beating step. Future studies should explore whether modifying our DNA extraction protocol would improve the detection of *Cryptosporidium* DNA from Whatman cards.

A study by Lalani et al.[22] evaluated the limits of detection for various enteropathogens, including *Giardia* and

TABLE 4
Comparison of *Cryptosporidium* spp. sensitivity between whole stool samples and stool samples stored on Whatman protein saver cards

Application method	DNA extraction method	High parasite load* (N=10) Sensitivity (95% CI)	Low Parasite Load† (N=10) Sensitivity (95% CI)	All samples (*N* = 20) Sensitivity (95% CI)
Whole stool directly on Whatman cards	Qiagen	80% (95% CI: 44%, 97%)	0% (95% CI: 0%, 31%)	40% (95% CI: 19%, 64%)
	Chelex	50% (95% CI: 19%, 81%)	0% (95% CI: 0%, 31%)	25% (95% CI: 9%, 49%)
Stool suspension on Whatman cards	Qiagen	70% (95% CI: 35%, 93%)	0% (95% CI: 0%, 31%)	35% (95% CI: 15%, 59%)
	Chelex	70% (95% CI: 25%, 93%)	0% (95% CI: 0%, 31%)	35% (95% CI: 15%, 59%)

CI = confidence interval.
* Whole stool ct value < 30.
† Whole stool ct value ≥ 30.

Cryptosporidium following the storage of suspensions of stool samples on Whatman FTA Elute cards. Consistent with our results, Whatman FTA elute cards were more effective for the preservation and detection of *Giardia* DNA compared with *Cryptosporidium* DNA. Previous studies have also evaluated the sensitivity of filter paper in detecting diarrheal parasites such as *Giardia* and *Cryptosporidium*.[34,35] This method was comparable with the Whatman cards in detecting both *Giardia* and *Cryptosporidium*.[34]

Our study has a few limitations. First, in our study we stored stool samples for 2 days before analysis and did not test the effects of long-term storage of stool samples on sensitivity. It is possible that longer storage periods may result in DNA degradation, which could reduce the sensitivity of this method. This is relevant particularly in low-resource settings, where there may be a delay between sample collection and analysis by PCR. The study by Lalani et al.[22] demonstrated that storage of parasite-positive stool samples for 1–3 months on Whatman FTA Elute cards did not affect detection of *Giardia* but adversely impacted the detection of *Cryptosporidium*. Hence, it is critical to understand the effects of long-term storage on the sensitivity and specificity for detecting various parasitic diarrheal pathogens. Second, our study was conducted with a small sample size, which could affect the precision of the sensitivity and specificity calculations in the study. Third, our study does not provide information regarding the efficacy of Whatman 903 protein saver cards in comparison with Whatman FTA Elute cards and filter paper methods that have been previously evaluated. Future studies should compare these methods for sample preservation at the same time.

In conclusion, given the high sensitivity and specificity for stool samples with higher parasite loads as well its low cost, we recommend the use of the Whatman 903 Protein Saver Card for preserving fecal specimens for the analysis of *Giardia* and *E. histolytica* in settings with limited access to laboratory and cold storage facilities. Future studies should focus on evaluating the efficacy of this method with long-term storage of samples and on improving the sensitivity of this method for storage and detection of *Cryptosporidium* parasites.

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