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A quadriplex PCR assay for rapid detection of diarrhoea-causing parasitic protozoa from spiked stool samples

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Abstract. Diarrhoea is a leading killer of children, accounting for 9% of all deaths among children under age 5 worldwide and 3% in Malaysia in 2015. A large proportion of diarrhoea illnesses among children in developing countries are ascribed to an unknown etiology because microscopic examination was the only available technique which has low detection limits. The proposed study aimed to evaluate a new quadriplex PCR assay to detect parasitic pathogens namely *E. histolytica*, *G. lamblia* and *C. parvum* which considered responsible for the majority of human infections. Three set of specific primer pairs were designed for detection of parasitic pathogens. Quadriplex PCR assay was optimized and an internal amplification control was incorporated to check for PCR inhibitors in samples. The PCR assay was evaluated using spiked stool samples. Specific primer pairs were successfully designed and simultaneously amplified the targeted genes. The analytical sensitivity of the quadriplex PCR at the DNA level was found to be 50 ng DNA. The analytical specificity was evaluated with 11 reference protozoal and bacterial strains and was found to be 100%. We concluded that the developed quadriplex PCR assay was rapid and gave results within 5 hours which is essential for the identification of parasitic pathogen and might be useful as an additional diagnostic tool whenever time is important in the diagnosis of parasite that cause diarrhoea.

INTRODUCTION

In 2015, it was reported that diarrhoea had killed 9% of children under the age of 5 worldwide and 3% in Malaysia, making it the leading cause of death among children. (UNICEF Data, 2018). Since microscopic examination has a low detection limit and it was the only available technique in developing countries, a large proportion of diarrhoea recorded cases among children were ascribed to an unknown etiology (Ajjampur *et al.*, 2008). Direct observation of protozoa by microscopy is frequently employed as a rapid and simple diagnostic method. However, it has frequently been shown to offer variations in results due to low sensitivity and depend to a great extent upon the skill of the person carrying out the analysis (Ajjampur, Rajendran, 2008, Al-Kubaisy *et al.*, 2014). Although, many commercial kits are available and used as alternative cost-effective diagnostic methods (Goni *et al.*, 2012; Minak *et al.*,

2012). However, the specificity and sensitivity of these kits have been reported to be lower than those obtained using the PCR based detection assays. Indeed, molecular detection methods by PCR is simple and can offer a better turnaround time (Al-Talib *et al.*, 2014a; Haque *et al.*, 2007). Although many PCR-based diagnostic assays have been reported, to the best of our knowledge none of these assays have been introduced into the clinical laboratory as routine diagnostic tests. This may be due to several reasons. First, most of these assays rely on multi-step procedures for the protozoal DNA extraction and subsequent PCR amplification (Hawash *et al.*, 2016). Second, most of these assays lack standardization and proper clinical evaluation (McHardy *et al.*, 2014). Finally, for poor countries where parasitic infections predominate, PCR is still considered an expensive technique in comparison to the conventional diagnostic methods (Hawash, Ghonaim, 2016).

This study aimed to evaluate a new quadriplex PCR assay to detect parasitic protozoa namely *Entamoeba histolytica-which* (*E. histolyticawhich*), *Giardia lamblia* (*G. lamblia*) and *Cryptosporidium parvum* (*C. parvum*) which were responsible for the majority of human infections. The study protozoa are globally distributed due to the high infectivity rates and the availability of various modes of transmission (Al-Kubaisy, Al-Talib, 2014). Since early identification of diarrhoea causing pathogens is vital for immediate and prompt treatment, the proposed quadriplex PCR assay includes three primer pairs which make it able to detect any pathogens in a short duration of time and at a lower cost. Various molecular methods have been used to identify faecal parasites however; these methods do not detect all target pathogens simultaneously. Hence, the newly developed PCR assay was a modified and inclusive method which can identify any of these parasites simultaneously. The proposed assay was evaluated by comparing the results with the conventional methods, and an internal amplification control was included to detect the presence of any PCR inhibitors in samples as done in previous study (Al-Talib *et al.*, 2014b). This study helps to incorporate the DNA amplification technology into the diagnostic parasitology laboratory to detect the causative pathogens.

MATERIALS AND METHODS

Study design and protozoal stock

This was a cross-sectional study conducted in Institute of Medical Molecular Biotechnology - Faculty of Medicine – UiTM from October 2016 to June 2017. The Research and Human Ethics Committee, Universiti Teknologi MARA approved the study protocol (REC/62/16). Protozoal strains used in this study for positive and negative control were obtained from American Type Culture Collections. *E. histolytica*, strain HM 1: IMSS (ATCC 30459), *G. lamblia*, strain WB, clone 6 (ATCC 30957), and *C. parvum*, strain Iowa (P102).

The stock culture of *E. histolytica* HM1: IMSS strain was obtained from Pusat Pengajian Sains Kesihatan, Universiti Sains Malaysia. The trophozoite of *E. histolytica* HM1: IMSS strain were then axenically cultivated in TYI-S-33 medium in culture flasks and incubated anaerobically by using Anaerocult A (Merck) for 48 hours. The culture flasks were placed in ice-cold bath for 5 minutes and trophozoites were centrifuged and re-suspended in phosphate buffered saline pH 7.2 and the parasite number was determined. The stock of *G. lamblia*, strain WB, clone 6 (ATCC 30957 was axenically grown anaerobically in ATCC® Medium 2155: LYI Giardia Medium in 13 ml glass tubes at 35°C, in a horizontal position at a slight incline and were subcultured at interval of 72 hours. The cells were adhered on the surface of the glass tubes and were dislodged by repeated inversion of the culture tubes after chilling for 10 min in an ice water bath. Cell concentrations were estimated with a Coulter counter. A purified preparation of 1×10^6 *C. parvum* oocysts lowa (P102) in 5% formalin in 4 ml was purchased from

Dr. Henry Stibbs (Waterborne Inc., New Orleans, LA, USA). Each 1 µL contain about 250 oocysts.

Spiked stool samples

Stool samples obtained from healthy donors were collected and confirmed by microscopic examination as negative for any protozoa. About 0.2 g of stool materials was aliquot in microcentrifuge tubes and preserved in 10% formalin and 75% ethanol for further PCR use.

Primer design for pentaplex PCR assay

The 16S rRNA of *E. histolytica*, 16S rRNA of *G. lamblia* and 18S rRNA of *C. parvum* genes sequence were obtained from GenBank. Primers were designed using the NCBI Primer Blast with the parameters set to create a product of 100-500bp and melting temperature 70°C. The specificity of the designed primers was checked using BLAST, which is available at the GenBank website. The primer sequences for the four genes and expected PCR product sizes are shown in Table 1.

DNA extraction

All strains were subjected to DNA extraction using QIAmp® Stool Mini Kit (Qiagen, UK) following the manufacturer's protocol with minor modification. This is needed because the genetic material of protozoa to be isolated is enclosed mainly in oocysts/cysts which possess very robust cell membranes. This includes boiling at 100°C temperature for 10 minutes and proteinase K treatment for 2 hours at 55°C.

Quadriplex Polymerase Chain Reaction

The uniplex and multiplex PCR amplifications were carried out with Eppendorf Mastercycler Gradient Thermal Cycler ((Eppendorf AG, Hamburg, Germany). GoTaq® Hot Start Polymerase (Promega) and other PCR reagents were used in amplification reactions with the final concentrations given at Table 2. For the quadriplex PCR, optimized primers concentration for each protozoa are 0.15 µM for *E. histolytica*, 0.25 µM for *G. lamblia* and 0.25 µM for *C. parvum* genes. The amplification was carried out using an initial denaturing cycle at 95°C for 5 min and the subsequent cycles as follows: denaturation, 30 s at 95°C; annealing, 20 s at 68°C; and extension, 30 s at 72°C. PCR products (6 µl per lane) were electrophoresed using 1% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen, Cergy Pontoise, France).

Evaluation of quadriplex PCR

The analytical sensitivity was evaluated using various concentrations of genomic DNA starting from 50 ng to 600 ng obtained from a reference strain. Analytical specificity was evaluated using DNA lysates prepared from pure strains of *E. histolytica*, *G. lamblia* and *C. parvum* and other 3 related intestinal protozoa and 5 non-protozoal Gram negative-bacteria (Table 3). *E. histolytica*, *G. lamblia*, and *C. parvum* oocysts were decimally diluted in PBS and seeded into 200 mg of uninfected human stool samples. Genomic DNA were extracted using QIAmp® Stool Mini Kit (Qiagen, UK) following the manufacturer's

Table 1. Oligonucleotides (primers) used in the study

Primer	Region	Sequence	Product size
C. parvum	18s rRNA	5'-GGTATTGGCCTACCGTGGCAATG-3' 5'-TAATTTGCGCGCCTGCTGCC-3'	112
E. hystolitica	16s like	5'-AGGATGAAACTGCGGACGGCTC-3' 5'-GTAGCCATCTGTAAAGCTCCCTC-3'	327
G. lamblia	16s like	5'-TACCGGCCGGGGACGGGTGA-3' 5'-GGCCCAGGGCGTCTGAGGGC-3'	524
Internal control		5'-GCATTGCTCTTCACAGGGCCGT-3' 5'-GGTAATGCCCCCAAACCGGGC-3'	677

Table 2. The final concentration of PCR reaction mix

Reagent	Concentration	Final concentration
ddH20		
Buffer	5×	1×
MgCl2	25 mM	1.5 mM
dNTP	10 mM	0.2 mM
DMSO	100%	5%
Primer-F	10 µM	0.25 µM
Primer-R	10 µM	0.25 µM
Taq polymerase	5 U/ul	0.05 U/ul
DNA template	100 ng	

Table 3. Enteric pathogens used in this study and results of multiplex PCR

Reference strains	18s rRNA	16S rRNA	16S rRNA	Internal control
1. *Salmonella enterica* (ATCC 14028)	–	–	–	+
2. *Shigella flexneri* (ATCC 12022)	–	–	–	+
3. *Escherichia coli* (EHEC) (ATCC 43889)	–	–	–	+
4. *Campylobacter jejuni* (ATCC 33559)	–	–	–	+
5. *Klebsiella pneumoniae* (ATCC 13883)	–	–	–	+
6. *Cyclospora cayetanensis* (ATCC PRA-3000S)	–	–	–	+
7. *Dientamoeba fragilis* (ATCC 30948)	–	–	–	+
8. *Blastocystis hominis* (ATCC 50177).	–	–	–	+
9. *Entamoeba histolytica* (ATCC 30459)	–	+	–	+
10. *Giardia intestinalis* (ATCC 30957)	–	–	+	+
11. *Cryptosporidium parvum* (ATCC 87667)	+	–	–	+

protocol with minor modification as mention previously. All spiked samples were thoroughly mixed and 1 µl of each aliquot was subjected to amplification by the quadriplex PCR.

RESULTS

In the present study, the quadriplex PCR was optimized successfully to identify the 16S rRNA of *E. histolytica*, 16S rRNA of *G. lamblia* and 18S rRNA of *C. parvum* genes simultaneously. Throughout the optimization process, no PCR amplification products were obtained using standard protocol. However, when 0.5% of DMSO added to the master mix, we managed to get PCR amplification bands for all protozoa (Figure 1). The quadriplex PCR gave the best results when 1.0 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U *Taq* polymerase and 0.5% DMSO with 68°C annealing temperature (Figure 1). The first sets of *E. histolytica* primer which is Forward 5'ATGGCCAATTCA TTCAATGAATTGAG 3' and Reverse 5' GCCCTCCAATTGATTTCGTAGGAG 3' failed to give a specific band when quadriplex PCR were performed even though its showed specificity during BLAST (Figure 2). Other optimizations performed include reduced primer concentration and increased annealing temperature but still failed to get specific band. We speculate that, there is a presence of another binding site of this primer in another species that is not updated in GenBank. The whole operating period from stool extraction till obtaining PCR results is 5 hours which can be considered as fast compared to conventional methods.

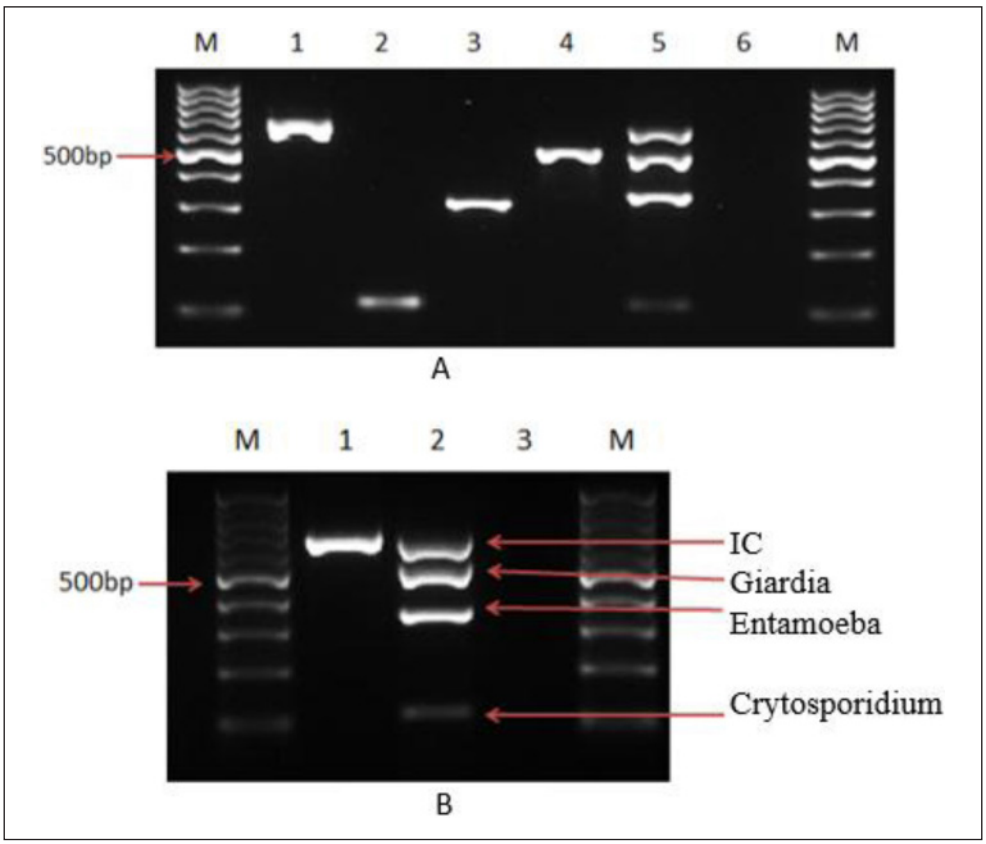

Figure 1. Representative SYBR Safe stained 3 % agarose gel picture. (A) PCR amplification products for singleplex PCR. M, GeneRuler™ 100 bp DNA marker; Lane-1, Internal Control; Lane-2, Cryptosporidium; Lane-3, Entamoeba; Lane-4, Giardia; Lane-5, positive control; Lane-6, no-template master mix sample (PCR negative control). (B) PCR product for multiplex PCR. M, GeneRuler™ 100 bp DNA marker; Lane-1, Internal Control; Lane-2, multiplex PCR product, Lane-3, no-template master mix sample (PCR negative control).

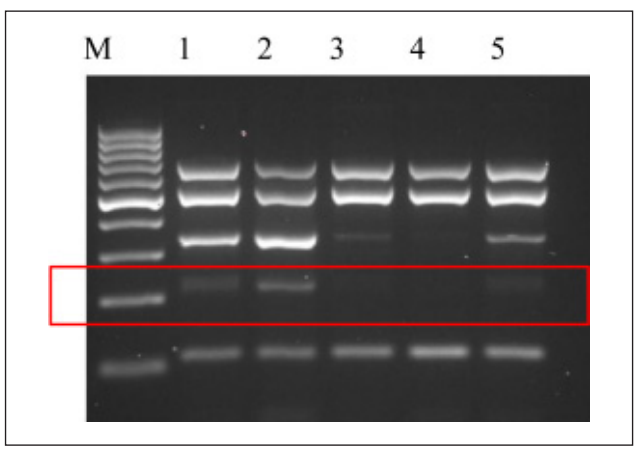

Figure 2. SYBR Safe stained 3 % agarose gel picture showed PCR amplification products for the first sets of *E. histolytica* primer. M, GeneRuler™ 100 bp DNA marker; Lane 1-5, Optimization using various primer concentrations.

The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets.

The analytical sensitivity of the quadriplex PCR at the DNA level was found to be 50 ng (Figure 3). The analytical specificity of the quadriplex PCR assay found to be positive for the *E. histolytica* (16S rRNA), *G. lamblia* (16S rRNA) and *C. parvum* (18s rRNA). A representative gel picture of quadriplex PCR assay with reference protozoal strains is shown in Figure 1A. However the other 3 related intestinal protozoa and 5 Gram negative-bacteria were negative (Table 1). Overall, the analytical specificity of quadriplex PCR was 100% for the detection of study protozoa.

Further evaluation of the quadriplex PCR assay was made using ten normal stools to determine specificity of the assay. No amplification bands were detected indicate 100% assay specificity (Figure 4).

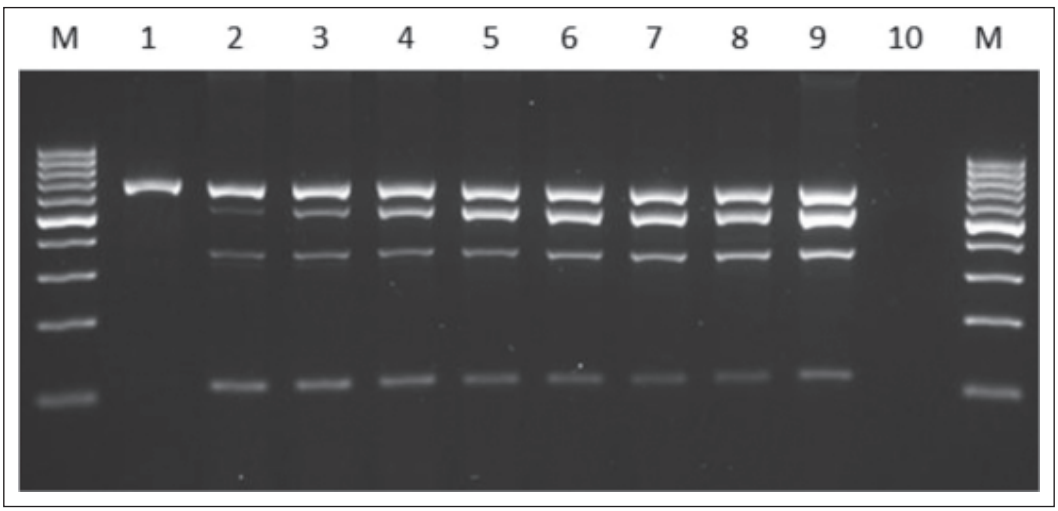

Figure 3. Representative SYBR Safe stained 3 % agarose gel picture showing PCR amplification products for Limit of detection test from negative stool sample spiked with the three protozoal genomic DNA. M, GeneRuler™ 100 bp DNA marker; Lane-1, Internal Control; Lane-2, 50 ng genomic DNA; Lane-3, 100 ng genomic DNA; Lane-4, 200 ng genomic DNA; Lane-5, 300 ng genomic DNA; Lane-6, 400ng genomic DNA; Lane-7, 500ng genomic DNA; Lane-8, 600 ng genomic DNA, Lane-9, positive control; Lane-10, no-template master mix sample (PCR negative control).

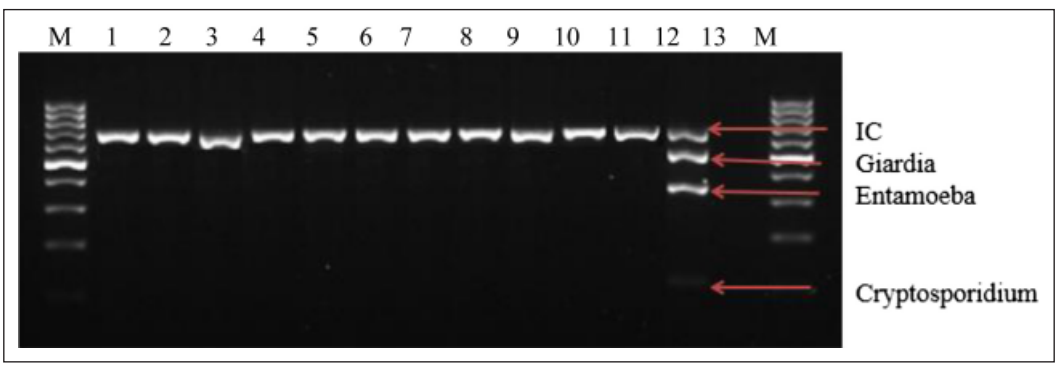

Figure 4. Representative SYBR Safe stained 3 % agarose gel picture showing PCR amplification products for specificity test from negative stool samples. M, GeneRuler™ 100 bp DNA marker; Lane 1, Internal Control; Lane 2 to 11, 100 ng negative stool genomic DNA from 10 independent samples, Lane 12, positive control; Lane 13, no-template master mix sample (PCR negative control).

DISCUSSION

In this study, a multiplex PCR assay was developed for synchronous detection of three important enteric protozoa which cause human diarrhoea including *E. histolytica*, *G. lamblia* and *C. parvum*. In this study, we design three specific primers sets with additional primer set as internal control. We design our primers to be highly specific to avoid cross-reaction with other organism's DNA. We considered specific features in designing our primers to achieve successful multiplexing of all primers together. In particular, the narrow primers melting temperatures (Tm) and short lengths targeted DNA sequences which can be differentiated easily by the agarose gel electrophoresis.

The used of Go Taq® Hot start polymerase (Promega) to drive the diagnostic multiplex PCR assay has shown to be advantageous in this PCR assay. This includes (1) it is a comparatively inexpensive polymerase compared to commercially available hot start polymerase; (2) it does not require early preliminary heating steps for its activation as required by other hot start polymerases; (3) the green buffer has a tracking dye which helps unload the PCR products directly into the agarose gel which save significant of time. In establishing the multiplex PCR, the ultimate effort is to minimize the preferential amplification of one DNA target over another as reported previously (Shum and Paul, 2009). The correct annealing temperature, final concentration of $MgCl_2$, and careful balancing of the primer concentrations minimized preferential amplification. In addition, inclusion of 0.5% DMSO (Sigma) in the reaction proved to be a very effective step in improving the sensitivity and the specificity of the assay. All primers sets work well at 68°C.

The low DNA yield was one of the challenges that encountered during isolation of DNA from stool samples. To improve the DNA yield, few modifications have been used such as increasing the incubation period of proteinase K from 10 minutes to 2 hours and additional incubation for 10 minutes at 100°C.

In this study, we managed to obtain higher DNA yield than non-modified protocols. However, it takes too long for the extraction to be completed and this could be one of the limitations for diagnostic purposes. For the purposes of detection, DNA is performed at the same efficiency from all oocysts / cyst, the lower detection limit of the PCR diagnostic assay is 50ng genomic DNA. Despite this promising result, the methods used for estimating the lower detection limit of the diagnostic assay experienced by one major weakness. The faecal-derived oocysts /cysts suspensions used for the seeding experiments were not entirely purified. As a result, the actual calculation of concentration of DNA is very challenging.

The comparison of analytical sensitivities with previous studies will not be precise due to using different methods to evaluate the lower detection limits in addition to using various species-specific primers.

Analytical specificity was evaluated using DNA lysates prepared from 10 enteric pathogens including bacteria and protozoa (Table 3). Nevertheless, in this study, PCR amplification was always target-specific when the assays were applied to faecal-derived stools that contained DNA from various defined sources.

PCR detection of enteric protozoa is largely dependent upon the method used for DNA extraction from the stool specimens. Most of the previously developed PCR assays have reported high levels of sensitivity and specificity using pure genomic DNA samples (Won *et al.*, 2016). This analytical sensitivity is actually estimating the performance of PCR amplification step and not the overall diagnostic process. The current study has limitation due to lack of comparison with other methods such as microscopy and coproantigen assays. This study also needs further evaluation using larger clinical samples size.

In conclusion the developed single-round quadriplex PCR assay for simultaneous detection of the three important enteric protozoa affecting humans was effective and performed well using extracted DNA from faecal samples. The quadriplex PCR assay was highly sensitive and the results could be obtained in same day. The developed quadriplex PCR assay has real potential to replace the conventional routine tests in diagnostic microbiology laboratories, particularly among countries with high prevalence of enteritis. More studies are required to assess the cost-effectiveness of the present test and to contain more pathogens including enteric bacteria in the same test that make this assay more inclusive and valuable.

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