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EASYSCREEN™ ENTERIC PROTOZOA ASSAY FOR THE DETECTION OF INTESTINAL PARASITES: A RETROSPECTIVE BI-CENTER STUDY

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KEY WORDS

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Dientamoeba fragilis
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Giardia intestinalis

ABSTRACT

Gastroenteritis caused by single or multiple pathogens remains a major diagnostic challenge for the laboratory, as diagnosis is achieved using different techniques with variable sensitivity and specificity. The aim of this study was to evaluate the EasyScreen™ Enteric Protozoa Detection Kit, a multiplex PCR assay for the detection and identification of the 5 most common protozoan parasites in fecal samples. A total of 632 fecal samples, submitted for routine screening to 2 centers in north-eastern Italy, was included in the study. The results of the molecular assay were compared to those of the standard diagnostic procedures, represented by microscopy and immunoassays. Out of 32 samples testing positive by conventional tools, 31 were detected as concordantly positive using the EasyScreen Kit. Additionally, 91 out of 632 samples only tested positive by the molecular test, therefore increasing the positive detection rate by 275%. Finally, the EasyScreen assay detected 14 co-infections compared to 3 co-infections identified by conventional methods. The EasyScreen Kit provided a rapid and sensitive simultaneous identification of the most important diarrhea-causing protozoa that infect humans. Additionally, this molecular assay presents several advantages compared to conventional tools, such as the standardization and near-total automation of the process. Although critical issues related to the employment of molecular assays are still evident, the system is suitable for clinical parasitological diagnosis as long as it is used in association with conventional tools.

Protozoan diseases significantly contribute to the burden of gastrointestinal illness worldwide (Petri et al., 2000), with most infections and deaths affecting people in developing countries. The World Health Organization (WHO) estimates that about 50 million people worldwide suffer from amoebic infection each year, resulting in 40,000–100,000 deceased (WHO, 2008).

Despite the fact that the mortality due to gastrointestinal infections is much lower in industrialized countries, disease caused by enteric protozoa may potentially result in a greater economic burden in this setting due to higher medical and treatment costs (Fletcher et al., 2012). On the other hand, estimation of the disease burden is often complicated by a lack of reliable data as a result of under-diagnosis and the lack of monitoring programs (Newell et al., 2010).

Several species of enteric protozoa are associated with diarrheal illness in humans, with some causing severe debilitating illness, especially in the immunocompromised host (Stark et al., 2009). For example, *Cryptosporidium* is mainly associated with watery diarrhea, which may become chronic and potentially life-threatening in immunocompromised individuals, especially acquired immunodeficiency syndrome (AIDS) patients (Davies and Chalmers, 2009).

Depending on age and geographical exposure, specific patient populations are at risk to acquire infections with certain protozoan species. These factors also influence the severity and duration of the clinical presentation (van Lieshout and Verweij, 2010). In industrialized countries as well as in the developing world, infections with *Giardia intestinalis* and *Cryptosporidium* spp. are more common in children. Infections caused by *Dientamoeba fragilis* are also often observed in children, usually below 5 yr of age (Tam et al., 2012); in industrialized countries, these infections are associated with relatively mild, but often persistent, gastrointestinal manifestation although their pathogenicity is still debated (Roxström-Lindquist et al., 2006; Davies and Chalmers, 2009).

According to the U.S. Center for Disease Control (CDC) guidelines (CDC, 2009), some types of *Blastocystis* species are more likely than others to be associated with symptoms. According to data present in literature, the pathogenicity of this protozoan is controversial: patient recovery has been reported

without any treatment, excluding patients such as children and immunocompromised adults (Sekar and Shanthi, 2013). However, identification of *Blastocystis* spp. in a stool sample should be investigated in any case, if associated with symptoms of disease (CDC, 2009). Another at-risk population is represented by travelers visiting countries with low levels of sanitation and hygiene, particularly tropical regions, where *G. intestinalis*, *Cryptosporidium* spp., or *Entamoeba histolytica* are common infections (Fotedar et al., 2007; Shah et al., 2009; Ximénez et al., 2009).

The diagnosis of these pathogens has traditionally been challenging; conventional detection of enteric protozoa usually relies on either microscopic examination of fecal material or on immunoenzymatic or immunochromatographic assays, neither of which exhibit an optimal sensitivity (Ryan et al., 2017). In addition to this, accurate determination of the incidence of these infections is hampered by inappropriate or insufficient test prescription (Polage et al., 2011; Fletcher et al., 2012). The microscopic examination of cysts and trophozoites remains the cornerstone of diagnostic testing for the intestinal protozoa, although this method is labor-intensive and requires a high level of skill for optimal interpretation. A pressing concern for most laboratories is the shortage of skilled technologists capable of reliably performing the parasitological examination (McHardy et al., 2014).

Microscopic diagnosis is particularly troublesome in cases of *D. fragilis* infection; because a known cyst-stage of the parasite is lacking, stool samples should be promptly examined or fixed to preserve the fragile trophozoites. Furthermore, permanent staining procedures are needed for accurate identification, and the interpretation of slides requires experienced personnel as *D. fragilis* may be difficult to distinguish from other non-pathogenic protozoa (Stark et al., 2010). Unfortunately, no enzymatic or chromatographic immunoassays are available to detect *D. fragilis* antigens.

To overcome the lack of sensitive diagnostic techniques in detecting enteric parasites in clinical specimens, several polymerase chain reaction (PCR) assays have recently been developed and clinically assessed (Liu et al., 2013; Siah et al., 2014; Vocale et al., 2015). Molecular tools may help to clarify the etiology of prolonged diarrhea, gastrointestinal disturbance, and fatigue, and enteric panel assays are now commercially available, presenting the advantage of greater sensitivity and better standardization compared to microscopy and immunoassays (Stark et al., 2010; Ryan et al., 2017).

Among others, the EasyScreen Enteric Protozoa Detection Kit (Genetic Signatures, Sydney, Australia) is a PCR assay with the ability to detect different kinds of parasites in multiplexed reactions. This assay is based on a patented technology, which modifies the 4 customary DNA nitrogenous bases into only 3 bases, A, T, and G. The change in target sequence is able to limit post-amplification contaminations and allows an improved efficiency of multiplex real-time PCR (RT-PCR) detection, as there is less competition between different primers. In addition to that, the 3 base™ conversion makes the genomes of different subtypes more similar to each other, and therefore primers and probes that target 3 base sequences contain fewer mismatches, are more homologous, and are less cross-reactive in assays designed to detect multiple subspecies of pathogens (Stark et al., 2014).

The aim of this study was to retrospectively evaluate the EasyScreen Enteric Protozoa Detection Kit on 632 fecal samples in which diagnosis of enteric protozoa was achieved by conventional methods. While a gold standard is still lacking, microscopy is still largely used and, in many laboratories, the use of PCR tests in parasitology has been rare or absent. The importance of this work is in the standardization of the diagnostic process in parasitology allowed via molecular methods compared to traditional microscopy or immunoenzymatic assays. Molecular technologies, while well known in research, are still novel in clinical laboratories and need to be further evaluated on a consistent amount of data and settings to be able to establish them as a consolidated diagnostic approach.

MATERIALS AND METHODS

Fecal samples were submitted for routine screening of parasitic infection at 2 hub laboratories located in north-eastern Italy, i.e., the Unit of Microbiology of the Hub Laboratory of the Great Romagna Area (Pievesestina di Cesena, FC, Italy) and the Unit of Microbiology, St. Orsola-Malpighi University Hospital (Bologna, Italy). From January to October 2015, fecal samples were collected, stored at –80 C, and retrospectively tested with the EasyScreen Enteric Protozoa detection assay. Prior to testing, all samples underwent the anonymization procedure used at the Unit of Microbiology of the Hub Laboratory of the Great Romagna Area in order to adhere to the regulations issued by the local Ethical Board (AVR-PPC P09, rev.2; based on Burnett et al., 2007). The results of the molecular assay were compared to those of the standard diagnostic procedures represented by microscopy and immunoassays.

Routine screening for detection of enteric protozoa

Detection of intestinal protozoa was performed at the Unit of Microbiology of the Hub Laboratory of the Great Romagna Area and at the Unit of Microbiology of Bologna following international guidelines (CLSI, 2005; McHardy et al., 2014). Briefly, stools collected in a plastic disposable tube were macroscopically investigated regarding form and consistency of feces and the presence or absence of blood or mucus. Specimens were concentrated using Mini System Paragreen (Biolife Italiana, Milan, Italy) or Para-Pak® PLUS EcoFix (Meridian, Bioscience Europe, Milan, Italy). Fresh and concentrated stools were microscopically investigated with Lugol's iodine solution staining (Farmitalia Carlo Erba, Milan, Italy). At least 300 microscopic fields were examined with ×25 and ×40 magnifications per slide. If the presence of *D. fragilis* or *Blastocystis* spp. was suspected by microscopy examination of Lugol's stained slides, fecal smears were prepared from fresh stools, fixed, and stained with Giemsa solution (Merck KGaA, Darmstadt, Germany) (oil examination ×100). Antigens of *Cryptosporidium* spp. and *G. intestinalis* were detected using an immunoenzymatic test (Giardia/Cryptosporidium Chek, Techlab Inc., Blacksburg, Virginia) and/or an immunochromatographic test (Immunocard STAT C/G, Meridian Bioscience Europe).

For *Cryptosporidium* spp., positive results were confirmed through Kinyoun staining. While both Kinyoun staining and antigen tests can be conducted on both fresh and preserved

Table I. Comparative identification of intestinal protozoan parasites using conventional methods and EasyScreen™ Protozoa Detection Kit.

	Conventional methods					
Easy Screen Protozoa Detection Kit	Negative	*Blastocystis hominis*	*Dientamoeba fragilis*	*Giardia intestinalis*	*Cryptosporidium* spp.	*Entamoeba histolytica*
Negative	523	0	0	0	0	0
Blastocystis hominis	42	17	1	0	0	0
Dientamoeba fragilis	39	0	3	0	0	0
Giardia intestinalis	8	0	0	11	0	0
Cryptosporidium spp.	2	0	0	0	1	0
Entamoeba histolytica	0	0	0	0	0	0

material, the analysis was conducted on fresh samples due to the internal guidelines standing in our laboratory.

Entamoeba histolytica antigens were detected with an immunoenzymatic assay (*E. histolytica* II Techlab Inc.) or an immunochromatographic test (RIDA®QUICK Crypto/Giardia Entamoeba – Combi, R-Biopharm Italia Srl, Melegnano, Italy).

EasyScreen Enteric Protozoa detection assay

Sample preparation: The specimens were prepared by dipping a sterile flocked FecalSwab™ (Copan Diagnostics, Murrieta, California) into the fecal matter and mixing it inside a tube containing liquid transport medium.

The nucleic acid was extracted using the EasyScreen sample processing kit. One hundred and fifty microliters of liquid specimen were mixed into a lysis buffer containing bisulfite reagent and then vortexed and incubated at 95 C for 15 min; this step ensured the lysis of the microorganism and the universal modification of its nucleic acid into its 3 base form, which is achieved by converting all cytosine bases to thymine through a uracil intermediary. The nucleic acid was then purified through magnetic beading on the GS1 Automation System, based on the Nimbus platform (Hamilton Robotics, Reno, Nevada), on which the PCR setup was performed as well.

RT-PCR: The real-time amplification reaction was performed on a CFX96 thermal cycler (Bio-Rad Laboratories, Milan, Italy). Along with the reagents needed for the amplification and detection of the target sequences, the kits are supplied with an internal positive control to check for any PCR inhibition and primers and probes which target a universal bacterial sequence that should be endogenously present in all stool specimens (extraction control). Each target amplified during the PCR reaction is labelled with a specific fluorescent probe and identified through its unique fluorophore in a specific emission channel by the RT-PCR detector, which measures the fluorescence signals and distinguishes them from each other. The RT-PCR software then interprets all data collection and provides a result (Stark et al., 2014).

Statistical analysis: McNemar statistical hypothesis testing with Yates correction was used to evaluate the significance of the results obtained. The chi-square test was applied both separately to each target pathogen when applicable and globally to the performance of the 2 methods.

RESULTS

The results obtained using the EasyScreen Enteric Protozoa Detection Kit were compared with the routine diagnostic algorithm on 632 fecal specimens. Thirty-two samples tested positive by both techniques with 31 concordant findings; a single sample was recognized as positive with both techniques, but the pathogen was identified as *D. fragilis* by microscopy and as *Blastocystis hominis* using the EasyScreen assay. Ninety-one samples out of 632 (14.4%) were identified as positive only by the molecular test (Table I). In 14 specimens, a dual detection was reported by the molecular assay; 11 out of 14 were characterized by the presence of *D. fragilis* and *B. hominis* while the remaining 3 samples were double-positive for *G. intestinalis* and *B. hominis*. On the other hand, only 3 of the 14 co-infections were detected by microscopy; all showed the coexistence of *D. fragilis* and *B. hominis*.

As shown in Table I, 523 samples tested negative with both techniques. *Blastocystis hominis* was detected in 17 specimens by microscopy while the EasyScreen assay identified this protozoan parasite in 60 samples; *D. fragilis* was detected in 4 samples using conventional methods while 42 samples tested positive for this protozoan species with the molecular assay. *Giardia intestinalis* and *Cryptosporidium* spp. were identified by microscopy in 11 and 1 cases, respectively, while the EasyScreen assay detected 19 and 3 cases, respectively. There were therefore 8 additional *G. intestinalis*, 39 additional *D. fragilis*, 2 additional *Cryptosporidium* spp., and 42 additional *B. hominis* positive samples detected by the molecular test.

No sample tested positive for *E. histolytica* with either conventional or molecular methods.

The chi-square statistics allowed us to reject H_0 for *B. hominis*, *D. fragilis*, and *G. intestinalis*, with a *P*-value <0.01; this demonstrates a significant difference in the number of detections by employing conventional or molecular tests. On the other hand, the statistical test showed a non-significant difference regarding *Cryptosporidium* spp., which was likely due to an insufficient positive pool size. The EasyScreen assay demonstrated higher performances when compared to conventional tools as a global method as well (*P*-value <0.01), showing a significant increase in sensitivity in the detection of most target pathogens.

DISCUSSION

Prevalence and clinical impacts of diseases caused by enteric protozoa are underestimated due to lack of suitable detection methods (Ryan et al., 2017). Adequate diagnosis of intestinal protozoa is limited by many factors because conventional microscopy is time-consuming, requires a highly trained microscopist, entails the collection of multiple stool specimens, and

exhibits insufficient sensitivity (Yansouni et al., 2014). Therefore, there is an increasing demand for low-complexity, high-throughput, and cost-effective complements to (or replacements for) the microscopy-based approaches to protozoan diagnosis.

The EasyScreen Enteric Protozoan Detection Kit evaluated here is a multiplex RT-PCR targeting common enteric parasites. This method uses a novel chemistry that universally modifies the nucleic acid genomes of pathogens by converting all cytosine bases to thymine. This technology has been previously applied to the detection of high-risk human papilloma virus and demonstrated greater specificity compared to traditional methods (Baleriola et al., 2008).

The EasyScreen molecular assay is able to detect 5 different protozoan pathogens responsible for gastroenteritis; the clinical performance of this test was assessed on 632 specimens which were previously characterized through microscopy and/or immunoassays according to the guidelines for enteric parasite detection in stool samples locally in use. Although all 32 samples previously identified as positive according to conventional methods were detected as positive using the Easy-Screen Kit as well, one of those samples was identified as a *D. fragilis* by microscopy and as *B. hominis* using the EasyScreen assay. Unfortunately, the latter stool specimen was not available for a second microscopy evaluation.

Furthermore, on the 600 stool samples that tested negative by microscopy, 91 tested positive by the molecular test; 8 were identified as *G. intestinalis*, 38 as *D. fragilis*, 43 as *B. hominis*, and 2 as *Cryptosporidium* spp. This resulted in an increase of the detection rate of 252% for *B. hominis*, 950% for *D. fragilis*, and 73% for *G. intestinalis*, respectively. Additionally, dual detection was reported by the EasyScreen molecular assay in 14 specimens while only 3 of these co-infections were detected using conventional tools.

Even though this study was not designed to evaluate the prevalence of protozoan infections detected at the Microbiology laboratories that serve as a diagnostic hub in a large area (about 3.0 million inhabitants) in north-eastern Italy, the observed distribution of enteric protozoa was comparable to other European countries (Röser et al., 2013; Alexander et al., 2017). In fact, *B. hominis*, *D. fragilis*, and *G. intestinalis* were the most-frequently detected protozoa in this study whereas the detection of *E. histolytica* and *Cryptosporidium* spp. was scarce. These results were expected: *E. histolytica* is predominantly found in patients from tropical regions (Keddy et al., 2005) while the lower prevalence of *Cryptosporidium* spp. compared to other industrialized countries, such as the United States, Canada, or the U.K. (Checkley et al., 2015) is related to deep aquifers. Our findings are in line with previous data from northern Italy; by examining fecal samples from 2,962 patients in the Milan area during 2007–2009, only 1 sample tested positive for *Cryptosporidium* spp., suggesting a low circulation of this protozoan parasite in the northern part of the country (Grande et al., 2011).

As with any other molecular technique, the EasyScreen Enteric Protozoan Detection Kit is subjected to some limitations, such as the failure to detect pathogens that were not included in the panel due to the primers' specificity. Difficulties in determining the clinical value of a pathogen by mere genome detection, as opposed to identification of the microorganisms by microscopy, are also a critical point, as results of molecular tests may lead to overtreatment. Furthermore, although the costs required to perform molecular methods are steadily decreasing, they remain elevated compared to conventional techniques; this could represent a major impairment, especially for laboratories that do not perform a sufficient amount of tests to cushion the expenses.

A major limitation of this study was the lack of a third technique to settle discrepancies between the rate of positive samples detected by conventional and molecular methods, although previous studies show that this discrepancy is due to the increased sensitivity of molecular assays rather than aspecific reactions (Stark et al., 2014). Nonetheless, our results showed an overall better performance of the EasyScreen molecular method, which presents additional advantages compared to microscopy such as the rapidity to accomplish the results and the standardization and near-total automation of the process. It could be useful to evaluate if PCR commercial kits can be conducted on preserved material.

The best use of the reagent kits is to run tests in multiples of 20; in our case, the samples were run in batches of 80, providing the results within a working day, with a hands-on time shorter than 3 hr. The same amount of samples would be processed through conventional methods in no less than 3 days, and by the full-time employment of 1 skilled microscopist. The pre-analytical steps allow for an appropriate collection and storage of the specimen, which is more likely to be representative of the whole sample. In addition to that, automation removes most variables linked to parasite identification through microscopy, which is dependent on the expertise and knowledge of the laboratory personnel. Furthermore, an automated analytical platform such as the one of an EasyScreen assay allows for interconnection with the lab information system and therefore the automatic and traceable transfer of results.

An accurate parasitological diagnosis from fecal samples cannot typically be made based on the results of a single laboratory test. Several proposals have been made regarding this, such as the possibility of analyzing every sample with both PCR and conventional microscopy. Alternatively, analysis with a single method has been proposed to be sufficient for complete parasitological diagnosis when an algorithm based on clinical information is applied (Bruijnesteijn van Coppenraet et al., 2009). In our opinion, collecting 1 sample in 2 tubes would probably be more suitable when comparing different techniques while clinical data is crucial to reach an accurate diagnosis. This was supported by unpublished data collected in our laboratory upon testing 100 healthy subjects, which demonstrated the presence of specific pathogenic protozoan genes from *G. intestinalis*, *B. hominis*, and *D. fragilis*.

In conclusion, the EasyScreen assay appears suitable for clinical parasitological diagnosis as long as it is used in association with conventional tools which overcome the limitations intrinsic in molecular methods. Considering the high impact of the automation process and the elevated cost of the molecular methods, these assays would provide the deeper benefit to the workflow of the hub laboratories where large numbers of samples are processed daily.

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