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ORIGINAL PAPER

A novel ELISA test for laboratory diagnosis of *Blastocystis* spp. in human stool specimens

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Abstract Detection of *Blastocystis* is routinely performed by microscopy, culture, and formyl-ether (ethyl acetate) concentration technique (FECT). Yet, these methods require special skilled personnel, are time consuming, and often involve processing that may cause misdiagnosis. The aim of this work is to demonstrate the usefulness of a newly introduced ELISA test for the detection of *Blastocystis* antigens in stool samples (CoproELISA^{TM} *Blastocystis*, Savyon Diagnostics) as a proper alternative to currently used methods, especially microscopy. A cohort of 179 fresh/frozen clinical stool samples was tested by the ELISA test, and results were compared to consensus methods comprised of microscopic examination of Lugol's iodine staining, culture, and immunofluorescence assay (IFA). The new ELISA test was able to detect fewer than 10^3 cells, recognized subtypes 1, 2, 3, and 5 (comprising >95 % of human *Blastocystis* infections), and exhibited similar reactivity when comparing formalin-preserved samples to fresh/frozen samples. The test demonstrated 92 % sensitivity, 87 % specificity, and 89 % accuracy when culture, and IFA or microscopy consensus results were taken as reference. When the consensus was comprised of culture and IFA, the test demonstrated sensitivity, specificity, and accuracy of 82, 86, and 84 %, respectively. In contrast, the sensitivity of Lugol staining microscopy was only 18 %. This work presents a unique ELISA test that provides an alternative to the use of microscopy, currently most widely used method. The test enables high-throughput screening and diagnosis of *Blastocystis*, adaptation to automatic procedures.

Keywords *Blastocystis* · Screening · ELISA · Diagnosis · Protozoa

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Introduction

Blastocystis is an enteric protozoan parasite highly prevalent in humans and animals (Abe 2004; Alfellani et al. 2013; Ash and Orihel 1987). Infection is associated with non-specific symptoms (i.e., diarrhea, abdominal pain, weight loss, constipation, anal itching, excess gas) and was found to be associated with irritable bowel disease (Qadri et al. 1989; Krüger et al. 1994; Pasqui et al. 2004; Biedermann et al. 2002; Dogruman-Al et al. 2009). This wide array of non-specific symptoms has confounded the understanding of the potential pathogenicity of *Blastocystis* species. As a result, many of these infections are undiagnosed. Most common approaches to the detection of *Blastocystis* include direct smear, microscopy; formyl-ether (ethyl acetate) concentration technique (FECT); and xenic in vitro culture (XIVC). Yet, these methods are time and labor intensive and require skilled personnel. *Blastocystis* has several morphological forms (vacuolar, cyst, amoeboid, granular, multivacuolar, and avacuolar) as well as numerous morphological features associated with the parasitic growth cycle. Consequently, microscopy is difficult, resulting in lower sensitivity, particularly when parasites are found in low numbers (Stenzel and Boreham 1996). FECT destroys some of the forms during stool processing, thus reduces recovery of parasites and therefore may not be efficient (O'Gorman et al. 1993). Culture requires 2–3 days for diagnosis and may allow preferential growth of specific strains

while eliminating others (Parkar et al. 2007). This is most important in view of the possibility of having mixed subtype infections (Stensvold et al. 2008), in which the pathogenic strains may be eliminated. Therefore, culture has a limited efficiency as a screening tool. Nevertheless, to date, microscopy and culture are considered as the "gold standard" methods for the detection of *Blastocystis*. PCR for diagnosis of *Blastocystis* was introduced in 2006 (Stensvold et al. 2006). Some publications refer to DNA isolation and PCR detection of *Blastocystis* directly from stool samples (Stensvold et al. 2006, 2007a; Eida and Eida 2008; Roberts et al. 2011; Forsell et al. 2012). The analysis of DNA extracted directly from stool samples is considered to be highly sensitive, providing the means for genotyping and subtyping. Nevertheless, it is aggravated by the presence of stool-associated PCR inhibitors and protozoan nucleases. In addition to apoptosis-related DNA fragmentation and the substantial genetic heterogeneity, the task of finding suitable loci that can be used for detection and differentiation is challenging (Chou and Tai 1996; Nasirudeen and Tan 2004). Also, Stensvold et al. (2007b) showed that conventional PCR was not significantly more sensitive than short-term XIVC and permanent staining. Diagnostic RT-PCR assays have been recently introduced, however, so far have not been used for screening (Stensvold 2013). While molecular DNA genotyping in stool has been described for many intestinal protozoa (Limor et al. 2002; Verweij et al. 2004; Peek et al. 2004), in the case of *Blastocystis*, PCR was used principally for subtyping of cultured isolates (Scicluna et al. 2006; Yoshikawa et al. 2003, 2004), phylogenetic studies, and ribotyping (Scicluna et al. 2006; Noël et al. 2003; Abe 2004; Rivera and Tan 2005; Rivera 2008). The genus *Blastocystis* includes highly diversified isolates, and it was recently proposed that any of the isolates from mammals and birds may be assigned to one of 17 subtypes (Alfellani et al. 2013). It was shown that variations of commonly found *Blastocystis* subtypes of human infections further confound and limit detection efficacy (Stensvold et al. 2007b; Vennila et al. 1999). Genotyping and subtyping of isolates may be used to differentiate between symptomatic and asymptomatic infections. However, isolates that have been associated with symptomatic infections in humans have also been found in asymptomatic carriers, making subtyping unsuitable for the determination of pathogenicity.

The aim of this work was to evaluate the analytical and clinical utility of a newly introduced ELISA-based test (CoproELISA™ *Blastocystis*, Savyon Diagnostics, Israel) for the detection of *Blastocystis* antigens in fresh/frozen and preserved stool samples. This ELISA is intended to be used for the detection of *Blastocystis* antigens in specimens collected from patients with gastrointestinal (GI) symptoms and is suggested to be utilized as a proper alternative for the diagnosis and screening of *Blastocystis* infections.

Materials and methods

Clinical samples Fecal specimens were obtained from routine fecal examinations in the microbiology laboratory of Numune Education and Research Hospital and Gazi University Hospital (both from Ankara, Turkey) from September 1 to October 30, 2012 and from Clalit Health Services Microbiology Laboratory, Nesher Regional Laboratory (Nesher, Israel). Samples were stored at −20 °C and were preserved with or without Formalin/SAF. The clinical samples were used under the authorization of the Gazi University Clinical Research Ethics Committee in Turkey and the Clalit Health Services Ethics Committee in Israel, according to the origin of the samples.

Culture In Turkey, pea-sized fecal samples were cultured in 8-ml culture medium (Ringer's solution containing 10 % horse serum and 0.05 % asparagine) (Dogruman-Al et al. 2009, 2010; Roberts et al. 2013) at 37 °C for 3 days followed by standard microscopic analysis. Negative samples were reanalyzed by microscopy at 4-, 5-, and 7-day culture. A sample was designated as negative if *Blastocystis* spp. growth was not obtained within 7 days. In Israel, all fresh fecal samples were inoculated into two culture systems upon receipt. A pea-sized stool sample was put into two different diphasic systems. One was an in-house growth medium consisting of a modified Boeck and Drbohlav's diphasic growth media consisted of egg base with an overlay of 3-ml Ringer-Lockes solution (Ash and Orihel 1987). The other medium was HY ENTAMOEBA KIT, purchased from Hy Laboratories Ltd. (Rehovot, Israel). Tubes were incubated at 37 °C in anaerobic conditions, and a drop of sediment was examined every 2 days by microscopy.

Microscopy All stool samples were examined by direct wet smear, prepared by mixing a small amount of stool (about 2 mg) with a drop of 0.85 % NaCl and a drop of Lugol's iodine on the same slide. These mixtures were covered by a 22×22-mm coverslip and were screened by microscopy (Forsell et al. 2012).

Molecular genotyping Extraction of genomic DNA from clinical isolates was carried out by QIAamp DNA Mini Kit (Qiagen, Germany). *(1)* Amplification: A set of primers was used for PCR amplification and sequencing. These primers consisted of the forward primer BhRDr (GAGCTTTTTAAC TGCAACAACG) and the reverse primer RD5 (ATCTGGTT GATCCTGCCAGT) (Scicluna et al. 2006), synthesized by Integrated DNA Technologies Ltd. (Israel). The primers were used in a standard PCR reaction using a FastTaq DNA polymerase (Roche Ltd, Germany) comprising denaturation at 95 °C for 3 min, 30 cycles of 1 min each at 94, 55, and 72 °C for 60 s, followed by a final extension step at 72 °C for 2 min. Amplicons of 619 bp long were observed after

electrophoresis on a 1 % agarose gels. *(2)* Sequencing: DNA sequence analysis was performed on all PCR-positive samples. The PCR products were purified using QIAquick™ PCR Purification Kit (Qiagen). The PCR products were then sequenced in both directions by Hy Laboratories Ltd. (Rehovot, Israel). The SSU rDNA sequences were then compared with those available in GenBank using the BLASTN program run on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). Following PCR and sequencing, clinical *Blastocystis* isolates were identified and classified according to Stensvold et al. (2007c).

Immunofluorescence assay (IFA) Fecal specimens were evaluated using Blasto-Fluor (Antibodies Inc., Davis, CA, USA), a commercially available FITC-tagged antibody stain specific for *Blastocystis* prepared from whole cell *Blastocystis* antigen (subtype 3). Staining was performed by combining 200 μl of fecal sample, 200 μl of phosphate buffered saline (PBS), and 4 μl of stain, followed by incubation for 60 min at 37 °C. The sample was viewed under a fluorescence microscope using 495- and 515-nm excitation and emission filters, respectively.

CoproELISA™ Blastocystis ELISA All procedures were according to the manufacturer instructions (http://savyondx.com/_Uploads/dbsAttachedFiles/CoproELISA_*Blastocystis*_V4.pdf). The CoproELISA™ *Blastocystis* ELISA test is based on a mixture of rabbit polyclonal antibodies raised against a pool of *Blastocystis* subtypes (1, 2, 3, and 5).

Analytical sensitivity assay A titration (10^2–5×10^4 cells) was performed on subtype-defined clinical isolates using the kit's stool diluent. The negative control included all the test components excluding cells. The specific limit of detection (LoD) for each isolate was quantified separately.

Preservative compatibility assay The suitability of the assay to be used with fresh/frozen or preserved specimens was determined using a cohort of 23 positive fecal samples (fresh/frozen or preserved in 10 % formalin) for 6 days.

Clinical performance A cohort of 179 stool samples from symptomatic patients were collected and tested by the ELISA. Microscopic examination of Lugol's iodine staining, culture, and IFA were used as a consensus reference. In a cohort of 89 stool samples, the performances of the ELISA and standard microscopic examination of Lugol's iodine staining were assessed separately, each against a consensus reference composed of culture and IFA.

Cross-reactivity Cross-reactivity with other GI pathogens was determined using stool specimens which have been confirmed as positive by routine ova and parasite (O&P) analysis, culture, or enzyme immunoassays (EIA). The organisms tested included *Entamoeba* strains, *Endolimax nana*, *Dientamoeba fragilis*, *Cryptosporidium* spp., *Giardia lamblia*, *Clostridium difficile*, *Helicobacter pylori*, *Salmonella* spp., *Shigella* spp., and *Campylobacter jejuni.*

Results

As previously reported, infections in humans are caused by certain *Blastocystis* subtypes which are more prevalent than others. Figure 1a demonstrates the analytical sensitivity of subtypes commonly found in human blastocystosis. The calculated LoDs were 230, 380, 250, and 1100 cells for subtypes 1, 2, 3, and 5, respectively.

The compatibility of the ELISA test with common preservation media and procedures was assessed (Fig. 1b). High correlation (R^2=0.96) was found in the readouts of fresh/frozen vs. 10 % formalin-fixed samples, thus indicating that the test is compatible with both fresh/frozen and formalin-fixed samples. In a different set of experiments (data not shown), similar results were obtained with SAF preservation. Furthermore, neither formalin nor SAF had influence on the signal of negative samples, i.e., no increase in background signals was observed.

The clinical performance of the ELISA test in a cohort of 179 stool samples from symptomatic patients is presented in Table 1. The ELISA exhibited 92 % sensitivity, 87 % specificity, and 89 % accuracy as compared to the consensus techniques. Tables 2 and 3 present the performances of the ELISA and the standard microscopic examination of Lugol's iodine staining in a cohort of 89 stool samples, respectively, both assessed against a consensus reference composed of culture and IFA. While the ELISA demonstrated sensitivity, specificity, and accuracy levels of 82, 86, and 84 %, respectively, the commonly used microscopic analysis demonstrated sensitivity, specificity, and accuracy levels of 18, 100, and 64 %, respectively.

Finally, the ELISA was examined for cross-reactivity with relevant GI parasites and bacteria as listed above and had shown no reactivity with any of the tested pathogens.

Discussion

Blastocystosis has been associated with a myriad of non-specific and confounding symptoms, also exhibited by infection with other GI pathogens. The shared symptoms together with the current limitations of existing diagnostic techniques make the management of *Blastocystis* challenging and provide ambiguous interpretation in regard to its pathogenicity.

The purpose of this work was to evaluate the suitability of a newly marketed ELISA (and currently the only commercial

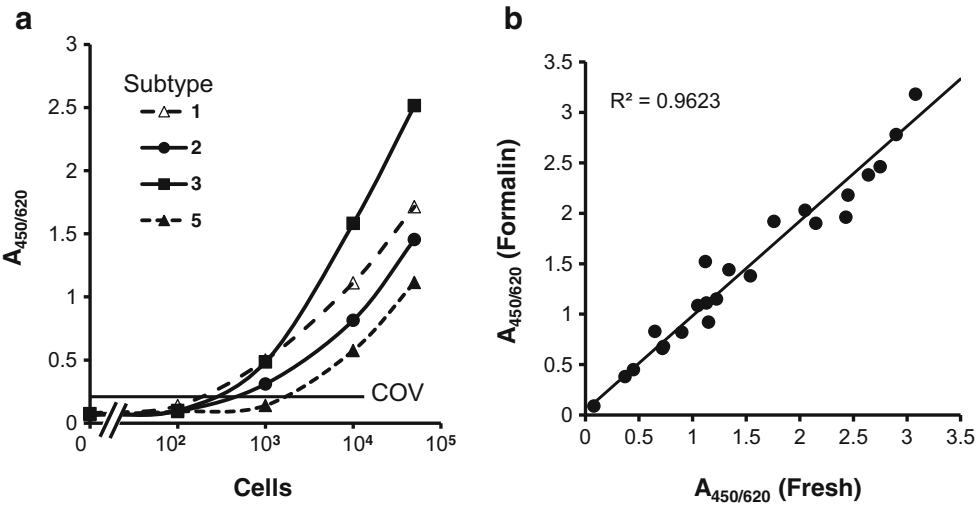

Fig. 1 Analytical performance of CoproELISA™ *Blastocystis*. **a** Analytical sensitivity of the test for *Blastocystis* subtypes 1 (*white up-pointing triangle*), 2 (*black circle*), 3 (*black square*), and 5 (*black up-pointing triangle*) representing limits of detection (LoD) of 230, 380, 250, and 1100 cells, respectively. **b** Linear correlation between absorbance values (OD, $A_{450/620}$) achieved with fresh vs. formalin-fixed samples (R^2>0.96). Similar results were obtained with SAF-fixed samples (data not shown)

test in this field) to be adopted in clinical laboratories for routine diagnosis and screening of *Blastocystis* in stool specimens. In this regard, an efficient screening assay is expected to (i) exhibit high performance parameters, (ii) be easy to perform, (iii) be compatible with fresh/frozen and fixed specimens, (iv) be relatively rapid (offering same day results), and (v) enable the use of automatic processing.

Assessment of the analytical sensitivity of the test for common human-associated *Blastocystis* subtypes revealed LoDs in the range of 200–1100 cells/well. The theoretical analytical sensitivity of microscopic examination is 1 cell/HPF (high-power field), which is equivalent to 1000 cells/well in ELISA. This lower LoD of the ELISA as compared to microscopy elucidates the favorable clinical performance of the ELISA, when the performances of both methods were examined against culture and IFA. Subtype variations of commonly found *Blastocystis* subtypes in human infections is considered to further confound and limit detection efficacy. Subtypes 1, 2, 3, and 5 were reported to account for over 95 % of human blastocystosis cases. The observation that these *Blastocystis* subtypes are well detected increases the ELISA utility for diagnosis and screening.

Storage conditions may influence the detection of *Blastocystis* spp. These conditions affect the ability of *Blastocystis* to maintain its morphology during laboratory processing, are important for the detection of co-infections, and permit the characterization of infection intensity (Stensvold et al. 2007b; Stensvold 2013). Formalin/SAF fixation is a routine preservation method which is used both for shipment of samples as well as for preservation of samples by the laboratory itself for batching or confirmation. The compatibility of the ELISA test with routine procedures was shown by its capability to provide reliable detection of *Blastocystis* spp. in fresh/frozen specimens as well as formalin/SAF-preserved stool samples. This is important since maintaining detection capability in the presence of preservatives as well as freeze/thaw cycles is not obvious. Preservative agents and freeze/thaw cycles affect parasitic recovery and abrogate antigenicity. As reflected in Fig. 1b, detection of *Blastocystis* spp. by the ELISA test is independent of storage conditions or preservation medium and emphasizes the suitability of this ELISA test in this aspect.

The clinical performance of the test was examined against a consensus of three available methods, namely microscopic

Table 1 Overall clinical performance of CoproELISA™ *Blastocystis* vs. culture + IFA or culture + microscopy as reference methods

		Culture + IFA or microscopy		
		Positive	Negative	Total
CoproELISA™ *Blastocystis*	Positive	67	14	81
	Negative	6	92	98
	Total	73	106	179

Sensitivity=92 %
Specificity=87 %
Accuracy=89 %

Table 2 Clinical performance of CoproELISA™ *Blastocystis* vs. consensus comprised of culture + IFA

		Culture + IFA		
		Positive	Negative	Total
CoproELISA™ *Blastocystis*	Positive	32	7	39
	Negative	7	43	50
	Total	39	50	89

Sensitivity=82 %
Specificity=86 %
Accuracy=84 %

Table 3 Clinical performance of Lugol staining microscopy vs. consensus comprised of culture + IFA

		Culture + IFA		
		Positive	Negative	Total
Lugol staining microscopy	Positive	7	0	7
	Negative	32	50	82
	Total	39	50	89

Sensitivity=18 %

Specificity=100 %

Accuracy=64 %

examination of Lugol's iodine staining (Stenzel and Boreham 1996), culture (Tan 2004), and IFA (Tan 2008). Overall performance of the ELISA indicated a high degree of sensitivity (92 %), specificity (87 %), and accuracy (89 %) providing the clinician with a comparative and efficient tool by which to assess the clinical probability of *Blastocystis* infection. We have previously reported on the performance and clinical usefulness of the FITC-conjugated anti-*Blastocystis* antibody for usage in IFA (Dogruman-Al et al. 2010). As reported, the IFA stain exhibited high sensitivity and specificity levels (86.7 and 97.3 %, respectively) when using culture as the gold standard. We have therefore used culture and IFA as a consensus in a second set of experiments where we assessed the clinical performance of ELISA vs. regular microscopic analysis (the most prevalent method for detection of *Blastocystis* spp. in routine laboratory setup). This assessment revealed that while ELISA exhibited 82 % sensitivity, Lugol staining microscopy was only able to detect 18 % of the positive samples. This is in agreement with previous reports exemplifying the insensitivity of microscopy.

The relatively high prevalence of *Blastocystis* that can be found in the colonization state calls for cross-reaction study with other human intestinal pathogens in order to ensure specific detection of *Blastocystis* as being the cause of the disease especially in cases of mixed infections. This is important in particular in view of the similar clinical symptoms between many of the pathogens. The lack of cross-reactivity with other GI pathogens emphasizes the ability of the ELISA to specifically detect the *Blastocystis* in these cases or alternatively to eliminate it as the cause of illness.

All in all, our results suggest that the CoproELISA™ *Blastocystis* test may provide a reliable screening tool for blastocystosis, thus overcoming few of the drawbacks of current techniques for the diagnosis of *Blastocystis*. The test exhibits superior analytical and clinical performances over routinely used microscopic techniques and allows for the detection of the most prevalent human *Blastocystis* subtypes from a myriad of sample processing types. It enables medium-high-throughput testing capabilities as well as adaptation to automation, offering sample-to-answer results within the same day and without the need for highly skillful personnel. We surmise that the ELISA assay may possess a niche in the clinical setting for rapid screening and detection of *Blastocystis* in the clinical microbiology laboratory.

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