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ARTICLE

Evaluation of an immunochromatographic dip strip test for simultaneous detection of *Cryptosporidium* spp, *Giardia duodenalis*, and *Entamoeba histolytica* antigens in human faecal samples

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Abstract Immunochromatographic (IC) tests may play an important role in the future diagnosis of parasitic diseases because of their speed and simplicity of use. A recently developed test to detect *Cryptosporidium* spp, *Giardia duodenalis* and *Entamoeba histolytica* was evaluated. Microscopy and PCR were the "gold standard" reference techniques and the results of this IC test were compared with those obtained with ELISA and IC single test for the three parasites. One hundred sixty stool samples were assayed. Using microscopy, 22 samples were diagnosed as positive for *Cryptosporidium* spp., 31 for *Giardia duodenalis*, 41 for *Entamoeba histolytica/dispar*, and 68 had a negative diagnosis for the three parasites. Results of IC tests show sensitivities of 70–72% for *Cryptosporidium*, 90–97% for *Giardia* and 62.5% for *Entamoeba histolytica*. Specificities were of 93.6–94.9%, >99% and 96.1%, respectively. In all diagnoses, agreement with microscopy and PCR was over 90%, except in the triple test and microscopy in *E. histolytica* detection that was 76.3%, due to the inability of microscopy to differentiate *E. histolytica* from nonpathogenic species such as *E. dispar* or *E. moshkovskii*. The triple stool immunoassays provide adequate sensitivities and specificities for use in outbreak situations, for screening proposals and for massive assays in endemic areas where a large number of samples must be analysed or as complementary test for individual diagnosis.

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Introduction

Cryptosporidium spp., *Giardia duodenalis* and *Entamoeba histolytica* have been recognised as causative agents of diarrhoeal disease in humans worldwide [1, 2]. All these protozoa could be transmitted through consumption of contaminated water or foods, person to person, and also zoonotic transmission is relevant [3]. Therefore, outbreaks sometimes occur and, as indicated by some studies and our experience, it is not unusual to find several of these protozoa simultaneously, mainly in patients from developing countries where infections are endemic [4].

Traditional diagnostic methods for these infections use faecal samples and must include concentration procedures along with specific staining techniques for proper microscopic detection and identification of the parasite. These methods are laborious, take a long time and require specialized and trained personnel. In addition, microscopic

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examination of three samples obtained on different days is required to achieve sensitivity up to 85% [5, 6].

Other techniques such as immunofluorescence microscopy (IFM) improve sensitivity (about 97.4% for *Cryptosporidium*), but they are expensive and laborious techniques, and are not routinely available in all laboratories [7–9].

Also, in the case of *Entamoeba*, differentiation between the pathogenic *E. histolytica* and non-pathogenic *E. dispar* is essential [10, 11], and direct microscopic examination does not allow this because both species are morphologically indistinguishable. Differentiation may be achieved using molecular techniques, isoenzyme analysis or ELISA assays, although the low prevalence of *E. histolytica* in faecal samples from patients which have been found to contain *Entamoeba histolytica/dispar* by microscopic examination, makes the use of molecular techniques in the routine investigation of *Entamoeba* positive patients recommendable [11].

Molecular techniques to detect *Cryptosporidium*, *Giardia* or *E. histolytica* include PCR or real-time PCR and provide great sensitivity and specificity, but they are time-consuming and require expensive qualified equipment for routine PCR diagnosis [11–15].

In recent years, antigen detection assays, such as enzyme immunoassays (EIAs) and immunochromatography (IC), to detect *Cryptosporidium*, *Giardia* and *E. histolytica/dispar* have been developed [16–21]. Some of them are able to differentiate between *E. histolytica* and *E. dispar*. A number of products with a good range of sensitivity and specificity are commercially available.

The aim of this study was to evaluate a new IC test that incorporates antibodies for simultaneous detection of *Cryptoporidium* spp, *G. duodenalis*, and *E. histolytica* (triple IC). Direct microscopic examination was considered as "gold standard" for parasite diagnosis, and PCR techniques were also considered as reference because of their sensitivity and their ability to differentiate *Entamoeba* species. The usefulness of the triple IC test was compared with ELISA assay and with single IC test for the three parasites.

Materials and methods

Stool samples

One hundred sixty stool samples were collected from patients with intestinal discomfort or diarrhoea, in which parasitic infections should be rule out, in the Microbiology Service of Lozano Blesa Hospital and in the Laboratory of Parasitology, Faculty of Medicine, University of Zaragoza (Spain). Samples were taken and analyzed by microscopic examination and by all different IC tests, from July 2008 to December 2008. Fresh specimens were processed within 24 hours of collection. At least two aliquots of each sample were immediately frozen and stored at −20°C for PCR and ELISA assays.

Microscopic analysis

A formalin-ethyl acetate concentration procedure was performed on all samples. Direct microscopic examination of a few microliters of each concentrated sample was performed, along with microscopic visualization of trichrome and modified Zielh-Neelsen staining of each sample, in order to detect cysts or throphozoites of *Giardia* and *Entamoeba*, and *Cryptosporidium* oocysts, respectively**.** A Nikon Eclipse 80i microscope was used. A previous observation was performed with the 10X objective examining all the possible fields in a smear of 22x22, later moving to 40X and 100X. All observations were performed by a skilled and experienced microscopist.

IC analysis

The following immunoassay diagnostic kits were used according to the manufacturer's instructions: Rida®Quick *Cryptosporidium* (N1202), *Giardia* (N1102) and *Entamoeba* (N1702) single tests (SC, SG and SE) for the detection of individual parasites and Rida®Quick *Cryptosporidium/Giardia* (N1122) for simultaneous detection of *Cryptosporidium* and *Giardia*, and *Cryptosporidium/Giardia/Entamoeba* (N1722) combi tests (CG and triple CGE) for simultaneous detection of all three parasites. All the tests used were provided by R-Biopharm (Darmstadt, Germany).

A total of 30–50 mg of solid samples or 100 microliters of liquid ones, were placed into 1.0 ml of extraction buffer provided by the manufacturer, and then shaken vigorously. The suspension was left at room temperature for 5 minutes until solid particles had settled. Then 150 μl of supernatant were transferred to another tube, and a reaction strip was placed into this tube. The test was incubated at room temperature and the results were read after 5 minutes for single test (SC, SG and SE) and 10 minutes for multiple test (CG and CGE). A control line was visible on the strip each time the test was completed successfully. A positive reaction appeared as a different colour band. For SC, SG and SE a positive test appeared as a red band; for the CG test a blue band indicated a positive test for *Cryptosporidium* spp. and a pink-red band indicated a positive test for *Giardia duodenalis*; for CGE positivity the colour of bands were blue for *Cryptosporidium* spp, red-pink for *Giardia duodenalis* and green for *Entamoeba histolytica*. Any reaction in the test, regardless of colour intensity, was interpreted as a positive result. No reaction in the test and a visible control line was interpreted as a negative result.

ELISA analysis

ELISA references were: ELISA R-Biopharm RidaScreen® Cryptosporidium (C1201), ELISA R-Biopharm RidaScreen® Entamoeba (C1701) and ELISA R-Biopharm RidaScreen® Giardia (C1101). A calibration curve with a pure concentrated standard of each antigen was included in all assays. A Labsystems iEMS microplate absorbance reader and Ascent Software version 2.6 were used for absorbance measurement and analysis. To establish the cut-off, 0.15 extinction units were added to the measured extinction for the negative control, and the mean of ELISA's cut-off was 0.2 absorbance units. As indicated by the manufacturer instruction's, samples were considered positive when their extinction was more than 10% above the calculated cut-off, equivocal when their extinctions were within ± 10% of the cut-off and negative when their extinctions were 10% below the calculated cut-off.

PCR analysis

DNA was extracted from freeze aliquots of stool samples, using Ibian DNA Stool kit. DNA of *G. duodenalis* and *Cryptosporidium* spp were detected by a heminested polymerase chain reaction (PCR) of the triose phosphate isomerase (*tpi*) gene performed using previously described protocols [12] (primers TPIAF: 5'- CGAGACAAGTGTTGAGATGC-3', TPIAR: 5'-GTCAAGAGCTTACAACACG-3', TPIAIF: CCAAGAAGGCTAAGCGTGC, TPIBF: GTTGCTC CCTCCTTTGTGC, TPIBR: CTCTGCTCATTGGTCTCGC, TPIBIF: GCACAGAACGTGTATCTGG), and by a nested PCR of a small-subunit (SSU) rDNA gene fragment as described by Xiao et al. [13] (primers SSU1: 5'-TTCTAGAGCT AATACATGCG, SSU2: 5'-CCTAATCCTTCGAAA CAGGA-3', SSU3: 5'-GGAAGGGTTGTATTTATTAGA TAAAG, SSU4: AGGAGTAAGGAACAACCTCCA), respectively. *Entamoeba histolytica* and *E. dispar* were detected and differentiated by means of nested PCR, using primers and conditions described by Gutierrez-Cisneros et al. [22] and Evangelopoulos et al. [14] (primers E1: 5'TGCTGTGATTAAAACGCT-3', E2: 5'-TTAACTATTT CAATCTCGG-3', Eh-1: 5'-ACATTTTGAAGACTTTATGT AAGTA-3', Eh-2: CAGATCTAGAAACAATGCTTCTCT-3', Ed-1: 5'-GTTAGTTATCTAATTTCGATTAGAA-3', Ed-2: 5'-ACACCACTTACTATCCCTACC-3').

Statistical methods

Sensitivity, specificity, PVP (positive predictive value), and NPV (negative predictive value) values were calculated with an Openoffice (Sun Microsystem) spreadsheet with the following formula to analyse data: sensitivity: $100\cdot[a/(a + c)]$; specificity: $100\cdot[d/(b + d)]$; PVP: $100\cdot[a/(a + b)]$; and NPV: $100\cdot[d/(c + d)]$, where 'a' represents real positive samples, 'b' are false positive samples, 'c' are false negative samples and 'd' are real negative samples

Concordance was calculated, with 95% confidence intervals, and results for IC and ELISA test were compared by Yate's corrected χ^2 test, considering p values$\leq$0.05 to be statistically significant.

Results

A total of 160 stool samples were microscopically examined for the presence of *Cryptosporidium*, *Giardia* and/or *Entamoeba histolytica/dispar*. Of these specimens, 22 were positive for *Cryptosporidium*, 31 for *Giardia* and 41 were positive for *Entamoeba histolytica/dispar* using direct or staining microscopic analysis. Two samples were positive for *Giardia* and *E. histolytica/dispar* simultaneously. The remaining 68 samples were negative for all three parasites studied. Also, one sample contained *Hymenolepis nana* eggs, another one *Endolimax nana* cysts and three were positive for *Blastocystis hominis*.

Table 1 Sensitivity, specificity and NPV data in IC diagnosis of *Cryptosporidium* spp, with microscopy and polymerase chain reaction (PCR) as reference methods

Test	Reference method							
	Microscopy				PCR			
	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
SC	72.7	95.7	95.7	72.7	70	94.3	95.7	63.6
CG	63.6	97.1	94.4	77.7	65	96.4	95.1	72.2
CGE	72.7	94.9	95.6	69.5	70	93.6	95.6	60.8
ELISA	72.7	94.9	95.6	69.5	65	92.9	94.9	56.5

SC Single Crypto IC test, *CG* Crypto-Giardia Combo IC test, *CGE* Crypto-Giardia-Entamoeba Triple IC Test, *PPV* positive predictive value, *NPV* negative predictive value

Table 2 Sensitivity, specificity and NPV data in IC diagnosis of *Giardia duodenalis*, with microscopy and polymerase chain reaction (PCR) as reference methods

Test	Reference Method							
	Microscopy				PCR			
	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
SG	96.8	>99.5	99.2	100	90.6	99.2	97.7	96.7
CG	96.8	>99.5	99.2	100	90.6	99.2	97.7	96.7
CGE	96.8	>99.5	99.2	100	90.6	99.2	97.7	96.7
ELISA	93.5	97.7	98.4	90.6	87.5	96.9	96.9	87.5

SC Single Crypto IC test, *CG* Crypto-Giardia Combo IC test, *CGE* Crypto-Giardia-Entamoeba Triple IC Test, *PPV* positive predictive value, *NPV* negative predictive value

Tables 1, 2, and 3 show sensitivity and specificity rates obtained for commercial rapid and ELISA tests, with reference to the microscopy and PCR techniques. In addition, the positive predictive value (PPV) and negative predictive value (NPV) are shown in these tables.

In *Cryptosporidium* diagnosis, five samples were positive by microscopy but negative by PCR, and only one of these samples was negative for all IC tests. Three of these samples contained a very low number of oocysts. In contrast, three samples were negative by microscopy but positive by PCR, and again, only one was negative by all IC tests. However, the results obtained by both techniques showed statistically significant concordance (95%; 95% CI range 90.5–97.4%; $p<0.05$). Between the triple IC test and microscopy and PCR, the concordance was 91.9% (95% CI 86.6–95.2%) and 90.6% (95% CI 85.1–94.2%), respectively. Comparing with the results obtained with ELISA, SC and CG test, all show statistically significant association ($p<0.05$), but the agreement was better between triple and SC IC assays (99.4%).

When the presence of *Giardia duodenalis* was analysed, concordance between microscopy and PCR reached 98.1% (95% CI 94.7–99.3%; $p<0.05$). ELISA showed 96.9% (95% CI 92.9–98.6%) agreement with microscopy and 95% (95% CI 90.5–97.4%) with PCR. The triple IC test reached 99.4% of concordance with microscopy, SG and CG test, and slightly lower for PCR and ELISA (97.5%; 95% CI 93.8–99%).

Concordance between microscopic detection of *Entamoeba* and PCR was 92.5%, but decreased to 78.8% when the specific PCR to detect *E. histolytica* was considered. Similar values were obtained comparing the triple IC test with microscopy (76.3%), and increased when comparing with ELISA (95.6%), specific PCR to *E. histolytica* (93.8%) or SE single IC test (97.5%). Agreement between ELISA and PCR for *E. histolytica* was 89.4% (95% CI 83.7–93.2%).

Discussion

The rapid and visual IC diagnostic methods are easy to implement and do not require sophisticated equipment or experienced staff to conduct them. The choice of a diagnostic test should be based on two fundamental premises: sensitivity and specificity. Therefore, both parameters must be analysed when a new diagnostic test is developed. A triple IC test which includes antibodies for detection of *Cryptosporidium*, *Giardia* and *Entamoeba histolytica* was evaluated using microscopy and PCR as reference techniques, and compared with ELISA test and single similar IC test for each parasite.

Table 3 Sensitivity, specificity and NPV data in IC diagnosis of *Entamoeba histolytica/dispar*, with microscopy and polymerase chain reaction (PCR) as reference methods

Test	Reference method											
	Microscopy				*E. histolytica* PCR				*E. dispar* PCR			
	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
SE	19.5	95.8	77.6	61.5	62.5	94.7	98	38.4	28.6	97.6	83	76.9
CGE	17.1	96.6	77.2	63.6	62.5	96.1	98	45.4	25.7	98.4	82.6	81.8
ELISA	24.4	97.5	78.9	76.9	75	92.8	98.6	35.3	40	97.6	85.3	82.3

SC Single Crypto IC test, *CG* Crypto-Giardia Combo IC test, *CGE* Crypto-Giardia-Entamoeba Triple IC Test, *PPV* positive predictive value, *NPV* negative predictive value

Microscopy was undoubtedly the "gold standard" for parasitological diagnosis, while PCR techniques were used as reference in this work for their recognized sensitivity and their ability to differentiate *Entamoeba* species, although both have limitations [23–25].

For diagnosis of *Cryptosporidium*, discrepancies found between microscopy and PCR can be interpreted as follows: in samples which were positive by microscopy and negative by PCR, this technique could have failed because the low number of oocysts present in the samples and/or because inhibitions produced in PCR for starting material. On the other hand, in samples which were negative by microscopy, with low number of oocysts, and without presence of PCR-inhibitory substances, the greater sensitivity of this technique permits the detection of *Cryptosporidium*. All IC tests studied were similar in sensitivity, specificity, NPV and PPV, with both microscopy and PCR methods as reference. Various rapid tests are commercially available, with variable sensitivities and specificities [19, 20, 26–28]. Sensitivity found for IC tests and ELISA is lower than that found by Chalmers et al. [8] and by Llorente et al. [29] for enzyme immunoanalysis (EIA) test or by Regnath et al. [26], who obtained 100% sensitivity and specificity in *Cryptosporidium* diagnosis with Rida®Quick Crypto/Giardia combi. Other authors, with the same test, obtain 92% and 97% sensitivity in *Cryptosporidium* and *Giardia* diagnosis [28]. Our results agree with those obtained by Weitzel et al. [27], also for Rida®Quick IC tests, with sensitivities about 75–80%, but PPV of 69–77%, indicating that the tests could be used in epidemiological studies but in individual diagnosis, positive samples should be confirmed by microscopy to avoid false positive results. The high agreement between triple and SC single tests indicates that their effectiveness was not affected by the presence of anti-*Giardia* and anti-*E. histolytica* antibodies.

In the case of *Giardia* detection, sensitivity (93.5–97%) and specificity (97–100%) were very high for all IC tests, and slightly better than those obtained with the ELISA test, and agree with those described by Regnath (100% sensitivity and 99% specificity) [26]. There were no significant differences between the IC tests. The high NPV and PPV values reflect a low number of false positives and false negatives and concordance with microscopy and PCR was good, showing the usefulness of triple IC, CG, and SC IC tests to detect *Giardia*, and suggesting that the IC test could replace the ELISA copro-antigen test for diagnosis purposes. Several other immunochromatographic tests already exist for *G. duodenalis*, with sensitivities between 58 and 100% and specificities between 99 and 100% [17, 19, 20, 25, 30, 31], but only detect *Giardia* or at most *Giardia* and *Cryptosporidium*.

When microscopy was the reference technique, sensitivity, NPV and PPV of both IC and ELISA tests for *E. histolytica* detection, was low, because microscopy only diagnosed *Entamoeba* and is not able to differentiate *E. histolytica* and *E. dispar*. PCR was found to be 100 times more sensitive than ELISA for detection of the two species [32]. In this case, sensitivity of the triple IC test was lower than ELISA, however, specificity was higher with PCR as "gold standard", and also the technique is easier and faster to perform.

The combination of several tests in one strip may provide a more convenient mode of testing for many laboratories. Another enzymatic rapid immunoassay (EIA) combines detection of *Cryptosporidium*, *Giardia* and *Entamoeba histolytica /dispar*, but requires more manipulations and is thus more laborious than the IC test. It is not able to differentiate between *E. histolytica, E. dispar*, and *E. moshkovskii* and its sensitivity is not high [33, 34]. Sensitive and specific detection of *Entamoeba histolytica* infection is required in order to ensure that patients receive the proper treatment. Solaymani-Mohammadi reported a 100% agreement between the TechLab antigen detection kit and traditional PCR for diagnosis of *E. histolytica* [35]. In this study, agreement between the triple IC test and PCR was lower, and sensitivity, specificity, NPV and PPV obtained for the IC tests to detect *E. histolytica* indicate that they cannot replace analysis with PCR or ELISA. DNA extraction from faecal specimens is complex and PCR remains expensive and requires skilled technicians; therefore, if the use of PCR is not possible, a combination of several methods such as ELISA and immunochromatography might be useful to diagnose *E. histolytica*.

The stool immunoassays provide adequate sensitivities and specificities with clinically relevant cost-effectiveness. They yield rapid results, for outbreak situations, for screening proposals and for massive assays in endemic areas where a large number of samples must be analysed, since the entire test could be performed in 15–20 minutes. Further studies must be carried out to demonstrate the effectiveness and efficiency of IC tests in the analysis of samples containing multiple parasites.

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