Bull. Soc. Pathol. Exot. (2015) 108:171-174
DOI 10.1007/s13149-015-0434-5

CLINICAL BIOLOGY / *BIOLOGIE CLINIQUE*

Evaluation of the new ImmunoCard STAT!® CGE test for the diagnosis of Amebiasis

Évaluation d'un nouveau test ImmunoCard STAT !® CGE pour le diagnostic de l'amibiase

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Received: 28 January 2015; Accepted: 16 April 2015
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Abstract For many years, microscopic examination of stool samples has been considered to be the "gold standard" for diagnosis of intestinal parasites although the Polymerase Chain Reaction (PCR) analysis is increasingly utilized due to its high accuracy. Recently, PCR has been approved by the World Health Organization as the current method of choice for the diagnosis of *Entamoeba histolytica* infection. In this study we evaluated a novel immunochromatographic antigen detection rapid test, ImmunoCardSTAT CGE (Meridian Bioscence, Milan, Italy), which has been proposed for the diagnosis of infections caused by *Cryptosporidium parvum-Giardia intestinalis-Entamoeba histolytica*. There is another rapid test with a similar name, the ImmunoCard STAT! *Crypto/Giardia*, but it is just for *Cryptosporidium* and *Giardia*. We aimed to compare *E. histolytica* results obtained from the rapid test with those of a rt-PCR for the detection of *E. histolytica / E. dispar* DNA. The new ImmunoCard rapid antigen detection test exhibited 88% sensitivity and 92% specificity (if assessed on rt-PCR negative samples) but showed a high proportion of cross-reaction between the pathogenic *E. histolytica* and the non pathogenic *E. dispar*.

Keywords *Entamoeba histolytica* · Real Time (rt) PCR · Rapid antigen detection test · Amebiasis · Diagnosis

Résumé Depuis de nombreuses années, l'examen microscopique des selles a été considéré comme le « gold standard » pour le diagnostic des parasites intestinaux, bien que l'analyse de réaction en chaîne par polymérase (PCR) soit de plus en plus utilisée en raison de sa grande précision. Récemment, la PCR a été approuvée par l'Organisation mondiale de la santé comme méthode de choix pour le diagnostic de l'infection par *Entamoeba histolytica*. Dans cette étude, nous avons évalué un test rapide de détection d'antigène par immunochromatographie, ImmunoCardSTAT CGE (Meridian Bioscence, Milan, Italie), qui a été proposé pour le diagnostic des infections causées par *Cryptosporidium parvum-Giardia intestinalis-Entamoeba histolytica*. Nous avons cherché à comparer, pour *E. histolytica*, les résultats obtenus à partir du test rapide avec ceux d'une rt-PCR pour la détection de ADN de *E. histolytica / E. dispar*. Le nouveau test de détection rapide ImmunoCard présentait 88 % de sensibilité et 92 % de spécificité (si appréciée sur les échantillons négatifs en RT-PCR), mais a montré une forte proportion de réactions croisées entre la pathogène *E. histolytica* et la non pathogène *E. dispar*.

Mots clés *Entamoeba histolytica* · PCR temps réel · Test de détection rapide des antigènes · Amibiase · Diagnostic

Introduction

Amebiasis is a parasitic infection caused by *Entamoeba histolytica* and one of the most common parasitic infections world-wide, infecting about 50 million people and resulting in 10,000 to 40,000 deaths per annum [13].

The diagnosis of *E. histolytica* infection has traditionally relied upon microscopic examination of fresh or fixed stool specimens [4]. However, microscopy has several limitations [5-7]; the most important is the inability to distinguish the pathogenic species *E. histolytica* from the morphologically identical non-pathogenic species *E. dispar* and *E. moshkovskii* [1,3,5,9,11] (Fig. 1). Detection of antibodies is insensitive in early disease and unable to distinguish active infection from previous exposure in individuals who migrate from, or currently reside in, an area of endemicity [2]. Several newer diagnostic tests are now available, including stool antigen tests and molecular assays. Stool antigen tests have been reported to outperform microscopy and to be as sensitive

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and specific as culture with isoenzyme analysis [8]. Recently, molecular analysis like rt-PCR assay has been reported to show excellent sensitivity and specificity [4-6,9] and it has been approved by the World Health Organization as the current method of choice for the diagnosis of *Entamoeba histolytica* infection [13]. Unfortunately rt-PCR methods are not routinely available in most laboratories, especially in low-middle income countries. A rapid fecal antigen detection test could be a valid, alternative method for the diagnosis of *E. histolytica* in resource-limited settings.

Using our in home rt-PCR as gold standard, we collected fresh stool samples of patients presenting gastrointestinal symptoms and fluid from liver abscess, with the objective to evaluate a new antigen *E. histolytica* detection test, based on immunochromatographic technology.

Material and methods

A total of 114 specimens were collected on the basis of: unclear/altered cysts at microscopy, compatible with *E. histolytica/ E. dispar/ E. moshkovskii* (Fig. 1) and/or positive serology and/or high clinical suspect. All the patients were from the Centre for Tropical Diseases at Sacro Cuore Hospital (Negrar, Italy). Informed consent was obtained from all the patients. Of the 114 specimens, 106 were stool samples and 8 were fluid samples drained from liver abscess.

Subjects were requested to provide fresh stool samples for antigen and rt-PCR.

Antigen test with ImmunoCard STAT® CGE test

ImmunoCard STAT® CGE is a new test which uses specific monoclonal antibodies against *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica* that detects all forms of the parasites during their life cycle. The test is based on the use of three types of microspheres: blue microspheres covalently linked to a monoclonal anti-*Cryptosporidium parvum* antibody, red microsphere covalently linked to a monoclonal anti-*Giardia* antibody, green microspheres covalently linked to a monoclonal anti-*Entamoeba histolytica* antibody as well as purple microspheres used as test control.

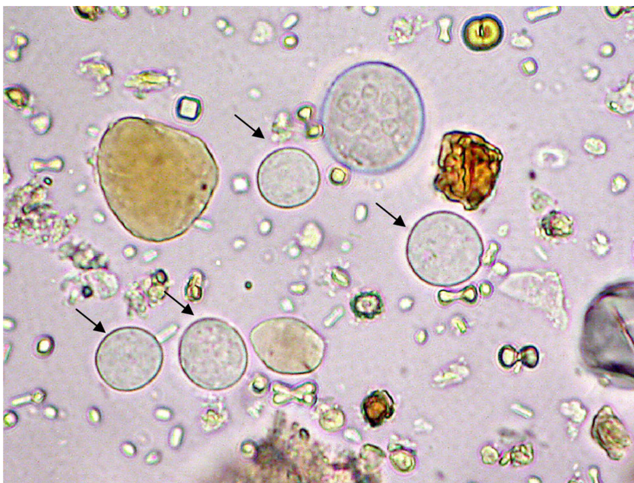

Fig. 1 Cysts compatible with *E. histolytica* / *E. dispar* / *E. moshkovskii*. (400 x) / *Kystes s'apparentant à E. histolytica / E. dispar / E. moshkovskii (400x)*

The parasites present in stool samples react with the latex particles which are coated with specific monoclonal antibodies against the antigen. This latex particles/antibodies/parasites complex migrates through a chromatographic process towards the reaction area. In this area the anti-antibodies react with the latex particles particles/antibodies/parasites complex are present.

This reaction leads to the appearance of a blue and/or red and/or green lines. These lines are used to interpret the result, following a ten minute room temperature incubation

Test was performed according to the manufacturer's instruction, using about 50 mg of fresh or frozen stools. The sensitivity declared by Meridian Bioscence for the ImmunoCard test is in the range between 75% - 93.8% and the specificity between 94.6% - 99.5%.

DNA isolation

Stool specimens were collected according to the routine procedure of our laboratory. In detail, about 200 mg of stools were stored at -20 °C overnight in a solution of PBS with 2% PvPP (polyvinylpolypyrrolidone, Sigma-Aldrich, Milan, Italy) before starting the DNA isolation.

Two-hundred ul of liver abscess fluid were stored at -20°C until the molecular analysis. In each sample, Phocine Herpes Virus type-1 (PhHV-1, kindle provided by Dr. Pas S., ErasmusMC, Department of Virology, Rotterdam) was added within the S.T.A.R. buffer (Roche), serving as an internal control for the isolation and amplification steps. Prior to start the DNA extraction, all the samples were boiled for 10 min. at 100° C, to allow the disruption of all the eventual cysts.

The DNA was extracted using MagnaPure LC.2 instrument (Roche Diagnostics, Monza, Italy), following the protocol "DNA I Blood_cells High Performance II", using the kit "DNA isolation kit I" (Roche).

The DNA was eluted in a final volume of 100 µl.

In house Real-time PCR assay

The rt-PCR assay was performed as described by Verweij et al [12]. The small-subunit (SSU) rRNA gene sequences for *E. histolytica* and *E. dispar* (Genebank accession n°. X64142 and Z49256 respectively) were chosen as an amplification target. The specific-primers E. histolitica/E. dispar are able to

amplify a fragment of 172 bp; the probes *E. hisolitica*-specific and *E.dispar*-specific were designed within the same region. Primers were synthesized by MWG Biotech S.r.l. (Ebersberg, Germany) and probes were synthesized by Lifetechnologies (AppliedByosistems, Monza, Italy). Amplification reactions were performed in a volume of 25 µl with 2x MM (SsoFast master mix, Bio-Rad Laboratories, Milan, Italy), 2.5 µg of BSA (Sigma-Aldrich), 60 nM of each *E. histolytica*/*E. dispar* specific primer, 80 nM of each PhHV-1-specific primer, 100 nM of *E.histolytica*-specific MGB probe, 100 nM of *E.dispar*-specific MGB probe and 200 nM of PhHV-1- specific probe, and 5 µl of the DNA samples. The rt-PCR cycle protocol consists of 3 min at 95°C followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C, and 30 sec at 72°C. The reactions, detection and data analysis was performed with the CFX96 detection system (Bio-Rad Laboratories) using the white plates. Appropriate positive and negative controls were included in all the experiments. As control for PCR inhibitors and amplification, the PhHV-1 DNA was amplified with the appropriate primers/probe mix in the same reaction of *E. histolytica/E. dispar* in a multiplex PCR setting.

Results

One-hundred fourteen samples were tested with the new rapid diagnostic test.

Of the 106 stool specimens tested, 18 were positive for E. *histolytica* by rt-PCR, of which 16 were positive by Immunocard STAT, indicating a sensitivity of 88%. Of the 49 negative stool samples at the rt-PCR, 46 were confirmed by the Immunocard STAT assay (specificity 93.8% if assessed on this denominator). Of 39 samples positive for E. *dispar* at rt-PCR, 31 (79%) resulted positive at the Immunocard STAT assay (Table 1).

If we compare all the positive results at the analysis in rt-PCR (n=57) to those obtained with the rapid test (n=47), regardless the species, the concordance was good (k = 0.756, with 95% CI 0.632 - 0.879) (Table 3).

Liver abscess samples: of 8 specimens obtained from drainage of liver abscess, 7 were positive for *E. histolytica* by rt-PCR, of which 2 (28%) were positive by Immunocard STAT (Table 2).

Discussion

Microscopy is often the only available method for diagnosis of amebiasis in resource-limited setting and it is highly dependent on the skill of the microscopist [10]. Therefore, antigen detection tests are being more widely used, and are generally considered to have a comparable sensitivity to that of stool culture, while being much easier to perform. However, it is often difficult to find an antigen detection test with a good combination of sensitivity and specificity. Currently no commercial antigen test can be considered the mainstay in the diagnosis of *E. histolytica*. A new attractive methodology for the laboratory diagnosis of infections is rt-PCR, a tool with high sensitivity and specificity, leading to shorter turnaround times and able to differentiate the species, but it is still a comparatively expensive procedure. Thus, poor regions of the world, where *E. histolytica* is most prevalent, will be unfortunately less likely to benefit from rt-PCR and could take advantage by the use of rapid antigen detection tests.

The latter are usually easy to perform, do not need any expensive instruments and the result can be available in 10-30 min. Besides ImmunoCardSTAT CGE, which we have analyzed in this work, we are aware of only another rapid antigen detection test claimed to be able to distinguish the infection from *E.histolytica* and *E.dispar*: RIDA® QUICK

Table 2 Comparison of results of rt-PCR and ImmunoCard-STAT assay performed on liver abscess / *Comparaison des résultats entre la PCR temps réel et le test ImmunoCard-STAT sur des abcès du foie.*

rt-PCR	**ImmunoCartSTAT**	
	Positive	Negative
E. histolytica (7)	2	5
Negative (1)	0	1

Table 1 Comparison of results of rt-PCR and ImmunoCardSTAT assay performed on stool specimens / *Comparaison des résultats entre la PCR temps réel et le test ImmunoCardSTAT sur des échantillons de selles.*

rt-PCR		**ImmunoCardSTAT**		
		Positive	Negative	
E. histolytica (18)		16	2	
E. dispar (39)		31	8	
Negative (49)		3	46	
Total	106	50	56	106

Table 3 Evaluation of concordance (κ Cohen) between rt-PCR and ImmunoCardSTAT on stool samples. (considering *Entamoeba* complex as a whole) / *Évaluation de la concordance (κ de Cohen) entre la PCR temps réel et le test ImmunoCardSTAT sur des échantillons de selles (en considérant le complexe Entamoeba dans son ensemble).*

	ImmunoCardSTAT			
rt-PCR		+	-	Total
	+	47	10	57
	-	3	46	49
	Total	50	56	106

K = 0.756; 95% confidence interval: From 0.632 to 0.879

Cryptosporidium/Giardia/Entamoeba Combi (R-biopharm-Germany).

The results of our study showed a reasonable sensitivity (88%). Specificity was also good (93.8%) if assessed over the denominator of negative samples at rt-PCR, but the test was not able to distinguish the species within the *Entamoeba* complex in stool samples, probably detecting a common antigen of *E. histolytica*/*E. dispar* rather than a specific Ag for the former. For RIDA® QUICK test, R-biopharm declares a sensitivity and specificity for *E. histolytica* of 84,8% and 87,4% respectively.

Concerning liver abscess, the performance of ImmunoCardSTAT was not good; only 2 out of 7 (28%) positive rt-PCR samples were detected by the antigen test. A possible reason could be that the test recognizes the antigen expressed on cysts surface, while the invasive trophozoites are the form that is present in liver abscesses.

We duly acknowledge some limitations of this study, especially in the patient selection that was performed with non uniform criteria. The reason was, primarily, that we meant to collect potentially positive samples in a short time in order to be able to assess the new test on a sufficient number of samples. This should be intended as a pilot study, preliminary to a prospective diagnostic study on amoebiasis. However, we believe that the main results are so clear that they are unlikely to be caused by a selection bias.

In conclusion, our results suggest that ImmunoCardSTAT may be considered a good test for *Entamoeba* complex as a whole, but not for species identification, that should best rely on rt-PCR in order to correctly diagnose patients, reducing the morbidity and mortality of amoebiasis and minimizing unnecessary treatments of infections with non-pathogenic species.

Conflict of interest The authors declare no conflict of interest

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