Detection of *Cryptosporidium parvum* antigen by co-agglutination test and ELISA

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التعرُّف على مستضدّ خفيات الأبواغ الصغيرة باختبار التراصّ المشترك ومقايسة الإليزا

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خلاصة: إن تأكيد وجود خفيات الأبواغ في العينات البيئية عمل معقّد ومكلّف وكثيراً ما يصعب أداؤه. ونعرض في هذه المقالة اختباراً للتراصّ على الشريحة يتّسم بالبساطة والاقتصاد (اختبار التراصّ المشترك) من أجل اكتشاف مستضدّ خفيات الأبواغ في البراز والمصل والماء. وتُظهر النتائج أن التراصّ المشترك كوسيلة للتحرّي أفضل بوضوح من مقايسة الإليزا (المازّة المناعية المرتبطة بالإنزيم) ومن التلوين بملوّن تسيل نيلسن المعدل، وإن كانت مقايسة الإليزا أكثر دقة. ونحن نوصي باختبار التراصّ المشترك كوسيلة جديدة لاكتشاف مستضدّ خفيات الأبواغ في المسوحات الوبائية الواسعة النطاق.

ABSTRACT Confirmation of the presence of *Cryptosporidium* in environmental samples is laborious, costly and often difficult. We report here a simple and economic slide agglutination test (co-agglutination test) for detecting cryptosporidial antigen in stool, serum and water. The results show that as a screening method co-agglutination is clearly superior to enzyme-linked immunosorbent assay (ELISA) and modified Ziehl–Neelsen staining, although ELISA is more accurate. The co-agglutination test is recommended for application as a new tool for detecting cryptosporidial antigen in large-scale epidemiological surveys.

Détection de l'antigène de *Cryptosporidium parvum* par l'épreuve de coagglutination et le dosage ELISA

RESUME La confirmation de la présence de *Cryptosporidium* dans les échantillons environnementaux est laborieuse, coûteuse et souvent difficile. Nous présentons ici une épreuve d'agglutination sur lame simple et économique (test de coagglutination) pour détecter l'antigène cryptosporidien dans les selles, le sérum et l'eau. Les résultats montrent que l'agglutination en tant que méthode de dépistage est clairement supérieure au titrage immuno-enzymatique (ELISA) et à la coloration de Ziehl-Neelsen modifiée bien que la méthode ELISA soit plus exacte. Le test de coagglutination est recommandé pour l'application d'un nouvel instrument afin de détecter l'antigène cryptosporidien dans des enquêtes épidémiologiques de grande envergure.

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Introduction

The enteric protozoan parasite *Cryptosporidium parvum* is a well-documented cause of water-borne disease in humans. *Cryptosporidium* is associated with persistent diarrhoea, especially in malnourished paediatric patients, and with prolonged severe or fatal diarrhoea in patients with acquired immunodeficiency syndrome (AIDS) in whom the parasite may also invade tissues other than gut [*1*]. Even low oocyst concentrations may cause serious illness if ingested by immunosuppressed individuals [*2*].

In Egypt, cryptosporidiosis is increasing. In 1986 and 1987, its prevalence was 16.6% [*3*] and 11.6% [*4*] in Cairo and Alexandria respectively. Another study in 1995 gave a prevalence of 27.9% in diarrhoeic children in Alexandria and Behera [*5*].

Cryptosporidiosis is diagnosed by the identification of oocysts in stool specimens. The detection is most commonly performed by the acid-fast staining method or by an immunofluorescence assay that requires the presence of large numbers of oocysts [*6*]. There is evidence that these current diagnostic methods have low sensitivity, and sparse, intermittent or atypical oocyst excretion may lead to an inaccurate result. In addition, detection of cryptosporidial antibodies does not differentiate between past and more recent infection [*7*].

The detection of *Cryptosporidium* oocysts in water samples relies upon the use of fluorescent-labelled antibodies, preferably using flow cytometry and epifluorescence microscopy [*8*]. These techniques are time-consuming and are limited by the specificity and reliability of antibodies binding to epitopes on the oocyst wall [*9–11*]. Moreover, naturally occurring oxidizing conditions or disinfection treatments can produce viable oocysts that are undetectable using standard protocols [*11*]. Thus other tests that are rapid, simple to process, inexpensive and at the same time highly sensitive and highly specific are needed [*12*].

The co-agglutination (Co-A) test is a novel immunological method, which has already found some applications in parasitology [*13*]. The test is based on the immunological reaction between specific parasite antibodies bound to protein A-bearing *Staphylococcus aureus* Cowan I strain (SAPA) cells and parasite antigen [*14*].

The present study attempts to compare the Co-A test as a new tool for the detection of cryptosporidial antigen in different specimens (stool, serum and water) with two other commonly used techniques: enzyme-linked immunosorbent assay (ELISA) and a modified Ziehl–Neelsen (mZN) staining method.

Materials and methods

Stool and sera of humans

Human stool and sera were collected from 40 acute and 10 chronic diarrhoeic children (diarrhoea for more than 2 weeks, suffering from leukaemia or nephrotic syndrome, or malnourished) aged 2–12 years in El-Chatby Hospital, Alexandria. Samples were also collected from children (< 12 years) with other diagnosed parasitic diseases (*Giardia lamblia*, *Entamoeba histolytica*, *Isospora belli*, or *Microspora*) and from 10 healthy asymptomatic children (< 12 years) proven negative for parasites by complete stool examination (sedimentation and staining by mZN staining).

Stool and sera of animals

Swiss albino mice were immunosuppressed using Endoxan, 70 mg/kg/mouse/day for 3 weeks [*15*]. The mice were in-

fected 1 week later with 10^6 oocysts of *C. parvum*/mouse [*16*]. Stool and sera were collected on days 1, 3, 5 and 7 post-infection, and then weekly for 2 weeks.

Stool samples were preserved in phosphate buffered saline (PBS) according to Chapman et al. [*17*], and all samples were stored at –35 °C.

Water

Water samples were randomly collected from 20 different sources in Alexandria including tap water, water tanks, swimming pools from different districts, and from the El-Mahmoudia and El-Noubareya canals.

Antigen preparation

Cryptosporidium oocysts originally isolated from human diarrhoeic stool and diagnosed by a modified acid-fast staining and mZN stain [*18*] were purified according to the method of Kilani and Sekla [*19*] to be used as antigen for preparation of hyperimmune sera.

Hyperimmune sera

Hyperimmune sera were produced in both rabbits and guinea pigs using cryptosporidial antigen, following a pilot study that indicated a dose of 5×10^3 oocysts/rabbit every 2 weeks for 6 weeks, or 2×10^3 oocysts/guinea pig according to the same schedule.

One week following the final dose, sera were collected and stored at –35 °C to be used in ELISA. Only rabbit hyperimmune sera were used in the Co-A technique.

Examinations

Microscopic examination

Stool and water samples were screened by modified acid-fast (mZN) staining, after Sheather's sucrose concentration flotation method, and examined by microscopy to detect cryptosporidial oocysts in stool and water [*8,15*].

Co-agglutination test

Co-A was used to detect cryptosporidial antigens in stool, serum and water samples [*14*]. The antigen concentration was calculated according to Boulos et al. [*20*]. Cryptosporidial antigen was used as a positive control. Normal stool, sera and distilled water were used as negative controls. The specific parasite antigens present in specimens combine with SAPA cells (Sigma, No. 9151), resulting in visible clumping of cells if the reaction is positive [*14*].

Enzyme-linked immunosorbent assay

Double sandwich ELISA was performed to detect cryptosporidial antigen in stool, serum and water samples. Reaction optical density was read by spectrophotometry at 492 nm [*21*]. Cryptosporidial antigen was used as a positive control and normal stool, sera and distilled water as negative controls. The cut-off value for a positive reaction was calculated to be double the optical density value of the negative control.

Statistical methods

Means and standard deviation were calculated according to Knapp and Miller [*22*].

Results

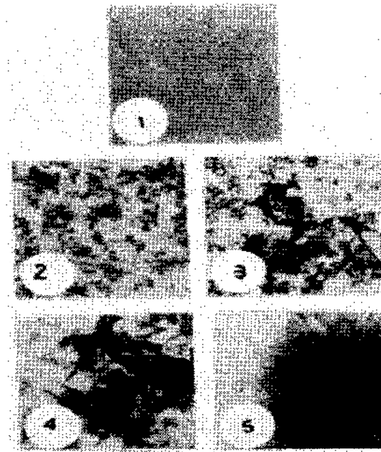
The results of stool samples from human cases are summarized in Table 1. Of 40 acute diarrhoeic paediatric patients, 7 were positive by mZN staining, while 13 were positive by Co-A and 14 by ELISA. The results from chronic diarrhoeic patients were similar, with mZN and Co-A giving 2 patients positive and ELISA giving 3 positive. In the 10 healthy individuals, only 1 sample gave a positive reaction by ELISA. The results of the Co-A and ELISA tests on sera from acute diarrhoeic patients were similar. ELISA was able to detect cryptosporidial antigens in 2 serum samples, whereas Co-A detected none.

Table 2 shows the mean number of *Cryptosporidium* oocysts in stool and their antigen concentrations in stool and sera by Co-A and ELISA in positive cases.

The results of the experimental infection of immunosuppressed mice are shown in Table 3. mZN staining gave a negative result early on the first day post-infection, when the stool sample was positive by both Co-A and ELISA. The sera were negative by the latter two tests. ELISA showed that the antigen concentration in sera increased with the duration of infection.

As regards the 20 water samples, 14 were negative and 6 positive by mZN staining, 10 were negative by Co-A, and 8 negative and 12 positive by ELISA. Table 5 shows the intensity of cryptosporidial water pollution by different techniques.

Figures 1 to 5 illustrate variation in the reaction of the Co-A test.



Figures 1–5 **Varying reactions with Co-A test are shown: 1) illustrating a negative reaction; 2) illustrating a very weak positive reaction (+); 3) illustrating a weak positive reaction (++); 4) illustrating a moderately positive reaction (+++); 5) illustrating a highly positive reaction (++++).**

Table 1 **Detection of *Cryptosporidium* oocysts and antigens in stool and sera of paediatric patients**

Patient group	No. of patients	mZN staining		Co-A				ELISA			
				Stool		Sera		Stool		Sera	
		–ve	+ve	–ve	+ve	–ve	+ve	–ve	+ve	–ve	+ve
Acute diarrhoeic patients	40	33	7	27	13	30	10	26	14	28	12
Chronic diarrhoeic patients[a]	10	8	2	8	2	8	2	7	3	7	3
Patients with other parasitic diseases[b]	10	10	–	10	–	10	–	10	–	10	–
Healthy individuals	10	10	–	10	–	10	–	9	1	10	–

[a] *Patients had leukaemia, nephrotic syndrome or were malnourished.*
[b]*Patients had giardiasis or amoebiasis, isosporiasis or microsporidiosis.*
Sensitivity of Co-A in stool = 92.8%. *Sensitivity of ELISA in stool = 100%.*
Specificity of Co-A in stool = 81.8%. *Specificity of ELISA in stool = 78.7%.*
mZN = modified Ziehl–Neelsen.

Table 2 **Mean number of *Cryptosporidium* oocysts in stool and antigenic concentration in stool and serum by Co-A and ELISA in positive cases**

Patient group	No. of positive cases	Cryptosporidial oocyst no. mZN staining count/HPF	Cryptosporidial antigen concentration			
			Co-A		ELISA (optical density at 492 nm)	
		Stool Mean ± *s*	Stool	Sera	Stool Mean ± *s*	Sera Mean ± *s*
Acute diarrhoeic cases	1	1.01 ± 0.05	+	–	0.351 ± 0.025	0.280 ± 0.022
	2	2.15 ± 0.19	++	+	0.405 ± 0.078	0.312 ± 0.121
	3	5.65 ± 1.14	+++	++	0.733 ± 1.124	0.548 ± 0.177
	4	–	+	–	0.271 ± 0.037	0.162 ± 0.025
	5	4.97 ± 0.98	+++	++	0.399 ± 0.088	0.370 ± 0.038
	6	–	+	–	0.297 ± 0.091	0.158 ± 0.012
	7	–	–	–	0.245 ± 0.021	0.223 ± 0.078
	8	1.85 ± 0.23	+	+	0.252 ± 0.015	0.387 ± 0.123
	9	–	+	+	0.265 ± 0.018	0.271 ± 0.025
	10	2.58 ± 1.22	+++	+++	0.433 ± 0.145	0.410 ± 0.144
	11	–	++	++	0.331 ± 0.13	0.352 ± 0.05
	12	3.13 ± 0.71	+++	+	0.655 ± 0.125	0.541 ± 0.187
	13	–	+	+	0.423 ± 0.099	0.372 ± 0.122
	14	–	++	+	0.782 ± 0.051	0.533 ± 0.431
Chronic diarrhoeic cases	1	5.78 ± 1.36	+++	+++	0.746 ± 0.152	0.744 ± 0.015
	2	4.33 ± 1.09	+++	+++	0.701 ± 0.143	1.132 ± 0.157
	3	–	–	–	0.251 ± 0.098	0.175 ± 0.014
Healthy individuals	1	–	–	–	0.222 ± 0.058	0.160 ± 0.011
Positive control				++++	1.544	0.123
Negative control				–	0.097 ± 0.013	0.082 ± 0.05

Cut-off value of stool sample for ELISA was 0.194.
+ = very weak positive. *++ = weak positive.*
HPF = high power field.
Cut-off value of serum sample for ELISA was 0.164.
+++ = moderately positive. *++++ = highly positive.*
mZN = modified Ziehl–Neelsen. *s = standard deviation.*

Discussion

The laboratory diagnosis of cryptosporidiosis relies on the recognition by conventional light microscopy of morphological features specific to the parasite oocyst. However, this technique is laborious, and can be insensitive and prone to error when performed by inexperienced staff [7]. We report here a simple and economic slide agglutination test for detecting cryptosporidial antigen. The test was performed on stool and sera from human cases and from experimentally infected immunosuppressed mice as well as on water samples taken from different locations. The mZN staining procedure was used for initial screening of stool and water samples, followed by Co-A and ELISA for antigen detection in stool, sera and water samples.

In the acute human cases, Co-A and ELISA were able to detect the antigen (sensitivity 92.8% and 100% respectively) in stool samples that were negative by mZN staining. This could be explained by intermittent shedding of the oocysts in the stool [23]. Chapman et al. [17] suggested that the presence of soluble antigen(s) or of other stages in the life cycle of *Cryptosporidium* is responsible for false negative results by mZN staining.

The antigen was not detected in all the sera from patients that were positive by Co-A and ELISA tests on stool samples. This could be attributed to the absence of antigen in the circulation early in infection. Our results from the experimentally infected mice support this finding, since sera were negative on the first day of infection. Light infection could also explain this re-

Table 3 Detection of *Cryptosporidium* oocysts and antigens in stool and sera of infected immunosuppressed mice at different times post-infection

Time post-infection (PI)	Cryptosporidial oocyst no. mZN staining count/HPF	Cryptosporidial antigen concentration			
		Co-A		ELISA (optical density at 492 nm)	
	Stool Mean ± s	Stool	Sera	Stool Mean ± s	Sera Mean ± s
1 day PI	–	+++	–	0.583 ± 0.05	0.202 ± 0.017
3 days PI	1 ± 0.05	++	+++	1.023 ± 0.18	0.315 ± 0.13
5 days PI	2.75 ± 0.11	++++	++	0.615 ± 0.24	0.595 ± 0.33
7 days PI	5.49 ± 0.16	++++	+++	0.644 ± 0.35	0.687 ± 0.47
2 weeks PI	5.57 ± 0.09	++	+++	0.432 ± 0.11	0.755 ± 0.45
3 weeks PI	4.35 ± 0.23	++	+++	0.348 ± 0.09	1.195 ± 0.21
Positive control		++++		1.544 ± 0.123	
Negative control	–	–		0.097 ± 0.013	0.082 ± 0.05

Cut-off value of stool sample for ELISA was 0.194. Cut-off value of serum sample for ELISA was 0.164.
+ = very weak positive. *++ = weak positive.*
+++ = moderately positive. *++++ = highly positive.*
mZN = modified Ziehl–Neelsen. *s = standard deviation.*
HPF = high power field.

sult, as indicated by negative mZN-stained stool samples.

In 2 cases, chronic diarrhoeic patients showed similar results by all 3 techniques. This could be explained by the infection being heavy enough that patients shed a lot of organisms in the stool [*24,25*]. The high level of serum antigen observed in our study was therefore expected. The case of a chronic immunosuppressed patient who was positive only by ELISA is in accordance with the work done by Ungar et al. [*24*], who reported positive ELISA serologies before the actual detection of *Cryptosporidium* organisms in AIDS patients. They added that this made such assays particularly useful in the AIDS population.

There was no evidence in our work of cross-reactivity with other intestinal protozoan parasites by either Co-A or ELISA.

In the healthy human controls, ELISA was found to be a very sensitive technique, detecting a low antigen level in the stool of one healthy control. Cryptosporidial infection in this case could be asymptomatic, according to Casemore [*26*].

The study of experimentally immunosuppressed, infected mice enabled us to determine the exact day of infection and to follow the course of the disease. mZN

Table 4 Intensity of cryptosporidial water pollution by different techniques

Positive water samples	Cryptosporidial oocyst no. mZN staining count/HPF Mean ± *s*	Cryptosporidial antigen concentration Co-A	ELISA (optical density at 492 nm) Mean ± *s*
Tap water	–	+	0.355 ± 0.126
Swimming pool	–	–	0.166 ± 0.085
Water tank sample 1	1.02 ± 0.58	++	0.253 ± 0.052
Water tank sample 2	1.54 ± 0.22	+++	0.744 ± 0.135
Water tank sample 3	–	++	0.486 ± 0.014
Water tank sample 4	1.08 ± 0.27	++	0.427 ± 0.77
Water tank sample 6	2.87 ± 0.05	++++	0.632 ± 0.178
El-Mahmoudia sample 1	0.95 ± 0.04	+	0.177 ± 0.088
El-Mahmoudia sample 2	2.42 ± 0.35	+++	0.512 ± 0.105
El-Noubareya sample 1	–	–	0.161 ± 0.015
El-Noubareya sample 2	–	+	0.198 ± 0.004
El-Noubareya sample 3	–	+	0.210 ± 0.121
Positive control		++++	1.254 ± 0.178
Negative control	–	–	0.075 ± 0.055

Cut-off value was 0.150.
+ = very weak positive.
++ = weak positive.
+++ = moderately positive.
++++ = highly positive.
mZN = modified Ziehl–Neelsen.
s = standard deviation.
HPF = high power field.

staining failed to demonstrate the parasite at early stages of infection, while the other two tests were able to detect the antigen in the stool as early as the first day post-infection. Sera were negative by both Co-A and ELISA at this point. This indicates that the parasite was still in the intestine and no parasitic penetration had yet occurred. Penetration of *Cryptosporidium* through the wall of the intestine after the 7th day of infection [*16*] could explain the high levels of the antigen detected from the 7th day post-infection onwards. Similarly, Gomez Morales et al. [*27*] reported that antigen was detectable by ELISA from 2 to 22 days post-infection in AIDS patients. Our ELISA results are in line with the work of Anusz et al. [*28*], who developed an ELISA using monoclonal antibodies and tested it on bovine faecal specimens; they found that ELISA was more sensitive than acid-fast staining.

Waterborne spread of *Cryptosporidium* is possible [*29*]. Ordinary detection methods are poor at detecting this parasite in drinking water [*30*]. In the present work, most of the positive samples were from water tanks or the El-Mahmoudia and El-Noubareya canals, and only 1 from tap water. Co-A and ELISA were able to detect the antigen in negative mZN-stained water samples. Very low oocyst concentrations or the presence of degenerated oocysts as a soluble antigen(s) might be the cause of the negative mZN results. Youssef et al. [*31*] used the direct fluorescent monoclonal antibody and mZN to detect *Cryptosporidium* in water samples. They reported that the former technique was more sensitive and specific. Furthermore, Siddons et al. [*32*] described a commercial ELISA with a sensitivity similar to that of the immunofluorescent antibody test for detecting *Cryptosporidium* oocysts in water.

The high sensitivity of Co-A and ELISA (92.8% and 100% respectively) in this study could be attributed to the absence of preservatives in processing the specimens. This is in line with Dagan et al. [*12*], who stated that ELISA was specific and sensitive (98%) when preservatives were not used. Weitz [*33*] used paraffin-fixed samples and reported a lower sensitivity of ELISA than mZN.

The results of our study are in accordance with many previous reports of the superiority of ELISA. It is characterized by high sensitivity even in the presence of very low quantities of antigen [*28,32*]. The results obtained with the Co-A test approached the ELISA test results. Similarly good results were obtained by Karki and Parija [*14*], who used Co-A to detect circulating antigen in amoebic liver abscess. They recommended its use as a routine parasitological test. Furthermore, when Co-A was used to detect antigen in either sera or urine from experimental and human toxoplasmosis, satisfactory results were obtained [*34*].

In summary, we consider Co-A and ELISA to be important tools in the detection of *Cryptosporidium* in specimens from different sources. These tests are superior to mZN staining, with ELISA having greater accuracy than Co-A as it can be read by spectrophotometry. However, Co-A is simpler to perform, the results can be obtained within 30–45 minutes of receiving the specimens, and it does not require much technical skill. Therefore, the Co-A test is suitable for detecting cryptosporidial antigen in different samples in epidemiological surveys and in poorly equipped routine clinical laboratories.

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