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Crypto-*Giardia* antigen rapid test *versus* conventional modified Ziehl-Neelsen acid fast staining method for diagnosis of cryptosporidiosis

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ABSTRACT

Objective: To evaluate the validity of Crypto-*Giardia* antigen rapid test (CA-RT) in comparison with the conventional modified Ziehl-Neelsen acid fast (MZN-AF) staining method for the diagnosis of cryptosporidiosis. **Methods:** Fifteen preserved stool samples from previously confirmed infections were used as positive controls and 40 stool samples from healthy people were used as negative control. A total of 85 stool samples were collected from suspected patients with cryptosporidiosis over 6 months during the period from January till June, 2011. The study was conducted in the department of parasitology, central laboratory, Alnoor Specialist Hospital, Makkah, Saudi Arabia. All samples were subjected to CA-RT and conventional MZN-AF staining method. Validation parameters including sensitivity (SN), specificity (SP), accuracy index (AI), positive predictive value (PPV), and negative predictive value (NPV) were evaluated for both tests. **Results:** Out of 15 positive controls, CA-RT detected 13 (86.7%) while MZN-AF detected 11(73.3%) positive cases. However, CA-RT detected no positive case in 40 normal controls but MZN-AF detected 2(5%) as positive cases. Based on the results, the SN, SP, AI, PPV and NPV were high in CA-RT than MZN-AF staining method, *ie*., 86.7% *vs*. 73.3%, 100% *vs*. 95%, 96.4% *vs*. 89.1%, 100% *vs*. 84.6% and 95.2% *vs*. 90.5%, respectively. Out of a total of 85 suspected specimens, CA-RT detected 7(8.2%) but MZN-AF detected 6(7.1%) cases as positive. **Conclusions:** CA-RT immunoassay is more valid and reliable than MZN-AF staining method.

1. Introduction

Parasitic diseases especially enteric protozoan continue to cause significant morbidity and mortality throughout the world irrespective of the patient's immune status. *Cryptosporidium* species have a worldwide distribution and *Cryptosporidium parvum (C. parvum)* and *Cryptosporidium hominis (C. hominis)* are the species most commonly associated with human cryptosporidiosis that have the fecal-oral route[1]. These are recognized globally as important causes of diarrhea in children as well as in adults with several water and food borne outbreaks[2,3].

Diagnostic methods that can provide rapid and accurate differential identification and assessment of *Cryptosporidium* infection, are very important for the epidemiological studies of the disease and its control[4]. Conventional methods for detection of cryptosporidiosis include microscopic examination of fecal smears with modified Ziehl-Neelsen acid fast (MZN-AF) staining method[1]. The growing interest in rapid diagnostic testing along with the lack of well-trained microscopists forced clinical laboratories to consider the options with regard to immunoassay kits that can be adopted and included as routine diagnostic protocols[5]. The current study, therefore, aimed to evaluate the validity of a rapid immunoassay test, *ie*., Crypto-*Giardia* antigen rapid test (CA-RT) in the diagnosis of cryptosporidiosis as compared to conventional MZN-AF staining method.

2. Material and methods

A total of 85 stool samples of suspected patients with cryptosporidiosis were collected over 6 months period from January 2011 till June 2011 by the parasitology unit

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of the department of laboratory and blood bank of Alnoor Specialist Hospital, Makkah, Saudi Arabia. All diarrheic patients under 12 years with immune-suppression were taken. In addition, 15 preserved stool samples from previously confirmed infections were used as positive control and 40 stool samples from healthy people were used as negative control. All samples were divided into 2 parts, one part used immediately for conduction of CA-RT and the other part were concentrated and preserved in 10% formalin and kept at 4 ℃ for conventional MZN-AF staining method.

2.1. Crypto-Giardia antigen rapid test

The Crypto-*Giardia* device is produced by (DIA. PRO, Diagnostic Bioprobes S.r.l Via Columella n° 31, 20128 Milano, Italy. E-mail: diapro@tin.it) and is a rapid chromatographic immunoassay for the qualitative detection of *Cryptosporidium* and *Giardia* antigens in human stool specimens. The test has been used in the current study as a rapid test for diagnosis of cryptosporidiosis. The procedure of the test was conducted as instructed by the manufacturer. Briefly, a separate specimen collection vial was used for each sample with 1 mL of the buffer. The cap of the vial was unscrewed and the stick was introduced two times into the fecal specimen to pick up a small amount of sample (150 mg) and then the vial was closed with the buffer. Stool sample and the vial were shacked in order to assure good sample dispersion. For liquid stool samples, the fecal specimen was aspirated with a dropper and 150 μL was added into the specimen collection vial with buffer. The tests (stool sample and buffer) were allowed to reach to room temperature (15-30 ℃) prior to testing. For testing, a separate Crypto-Giardia Device was used for each sample. The device was removed from its sealed pouch and used as soon as possible. The specimen collection vial was shacked to assure good sample dispersion then the tip of the vial was broken off and exactly 4 drops or 100 μL were dispensed into the specimen well (S). The result was recorded 10 min after dispensing the sample.

Figure 1. CRYPTO positive: Two lines appear across the central window, in the result line region (red test line) and in the control line region (green control line).

2.2. Fecal parasite concentrator

Fecal parasite concentrator (FPC) is a simple, clean and efficient device used to concentrate helminthes eggs, larvae and protozoan cysts or oocysts in stool samples. This approach is applied to increase the sensitivity for the detection of parasites, especially in mild infections. The FPC has been used in the current study to concentrate *Cryptosporidium* oocysts in stool samples. Briefly, 9 mL of 10% formalin were added to the flat-bottomed tube, to which, one spoonful of fresh stool was added and mixed thoroughly. Three drops of Triton ×-100 and 3 mL of ethyl acetate were added to the mixed specimens. The 15 mL centrifuge tube was securely attached to the FPC strainer. The FPC strainer was attached tightly to the flat-bottomed tube containing the fecal specimen and was shacked vigorously for 30 s. The conical end was pointed downword and the specimen was poured through the strainer into the 15 mL centrifuge tube. The FPC strainer was unscrewed from the flat-bottomed tube that attached. The transport tube and strainer were discarded in an appropriate manner. The 15 mL tube was caped and centrifuged at 500×*g* for 10 min. After centrifugation, the specimen was appears clearly separated into 4 layers. The debris layer was rimed by using an applicator stick. The debris and supernatant fluid were poured off. With the inverted tube, a cotton-tipped applicator stick was used to clean and remove the remaining debris. The tube was returned to an upright position and 2 to 3 drops of 10% formalin were added, and the sediment was thoroughly mixed and kept for further testing[6].

2.3. Modified Ziehl-Neelsen acid fast staining method

MZN-AF stain kit (Crescent Diagnostics (Ireland) Ltd. Invent, Dublin City University, Dublin 9, Ireland. VAT number: IE 9568341B, Company number: 405107. E-mail: info@crescentdx.com) is useful for the identification of oocysts of the coccidian species (*Cryptosporidium*, *Isospora*, and *Cyclospora*), which may be difficult to detect with routine microscopic examination as previously described[7]. MZN-AF stain has been used in the current study for identification of *Cryptosporidium* oocysts. Briefly, a small drop of the concentrated preparation of the stool sample was used to prepare thin smears on a clear microscopic glass slides. The slides were allowed to air dry and then fixed for 3-5 min in absolute methanol and the slides were allowed to air dry. Fixed slides were placed on staining racks and flooded with ZN carbol fuchsin for 20-25 min (slide desiccation was avoided by adding more stain when needed) and then the slides were rinsed under slow ran tape water. Five percent acid alcohol was added on slides approximately 20-30 s for de-colorization and then the slides were rinsed under slow ran tape water. The slides were then flooded with methylene blue for 2-3 min for counter staining and then the slides were rinsed under slow ran tape water and allowed to air dry. After drying, slides were examined microscopically with a drop of oil under high power (100 × oil immersion) lens. Positive *Cryptosporidium* oocysts slides stain bright red

with a blue background[5].

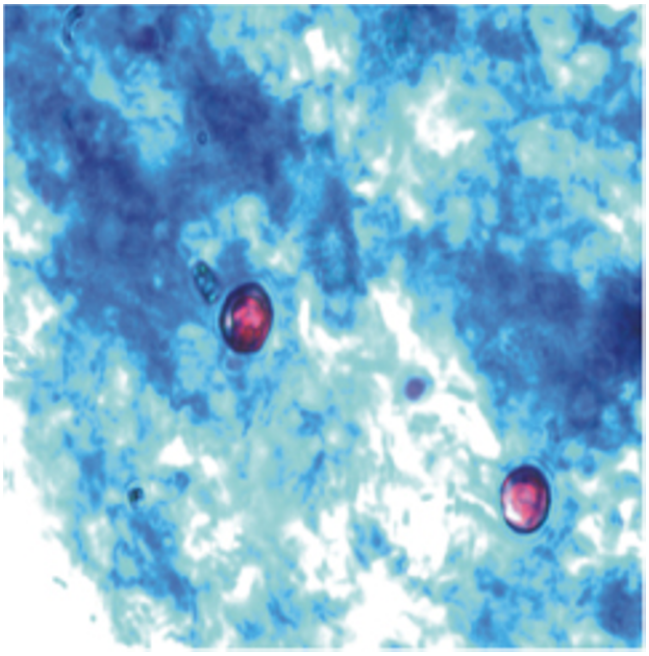

Figure 2. Modified ZN stained slide showing *Cryptosporidium* oocyst with methylene blue counter stain.

2.4. Evaluation of diagnostic tests

Based on the results of both CA-RT and MZN-AF staining method with both the positive and negative control samples, true and false positive (TP & FP) and true and false negative (TN & FN) values of both test were obtained. These values were used to evaluate the validity of both tests for the diagnosis of *Cryptosporidium* infection. The evaluation parameters included sensitivity (SN), specificity (SP) and accuracy index (AI). In addition, positive predictive value (PPV) and the negative predictive value (NPV) were also evaluated[8].

Sensitivity = (TP) / (TP + FN) ×100
Specificity = (TN) / (TN + FP) × 100
Accuracy index = (TP + TN) / (Total count of samples) × 100
Positive predictive value = (TP) / (TP + FP) × 100
Negative predictive value = (TN) / (TN + FN) × 100

2.5. Ethical issues

Hospital research ethical committee endorsement was taken for this research. We declare that we have no financial or personal relationship(s) which may have inappropriately influenced us in writing this paper.

3. Results

Out of 15 positive control specimen, 13(86.7%) were diagnosed positive by CA-RT while 11(73.3%) by MZN-AF staining method and remaining specimen showed negetivity. However, CA-RT diagnosed all negative control samples as negative cases but MZN-AF staining method diagnosed 2(5%) cases as positive. Based on the results, the SN, SP, AI, PPV and NPV were high in CA-RT than MZN-AF staining method, *ie*., 86.7% *vs*. 73.3%, 100% *vs*. 95%, 96.4% *vs*. 89.1%, 100% *vs*. 84.6% and 95.2% *vs*. 90.5%, respectively.

Out of a total of 85 suspected specimens, CA-RT detected 7(8.2%) cases as positive while MZN-AF detected 6(7.1%) positive cases. On the other hand, both tests were able to detected 4(4.7%) common positive cases.

Table 1
Evaluation parameters of both CA-RT and MZN-AF staining method.

	TP(*n*)	TN(*n*)	FP(*n*)	FN(*n*)	SN(%)	SP(%)	AI(%)	PPV(%)	NPV(%)
CA-RT	13	40	0	2	86.7	100	96.4	100.0	95.2
MZN-AF	11	38	2	4	73.3	95	89.1	84.6	90.5

TP=True positive; TN=True negative; FP=False positive; FN=False negative; SN=Sensitivity; SP=Specificity; AI=Accuracy index; PPV=Positive predictive value; PV=Negative predictive value; CA-RT=Crypto-*Giardia* antigen rapid test; MZN-AF= Modified Ziehl-Neelsen acid fast staining.

Table 2
Results of both CA-RT and MZN-AF staining method with suspected clinical samples of cryptosporidiosis (*n*=85).

	CA-RT		MZN-AF		CA-RT / MZN-AF	
Suspected Specimen	+ve	-ve	+ve	-ve	+ve	-ve
	7(8.2)	78(91.8)	6(7.1)	79(92.9)	4(4.7)	76(89.4)

Data has been presented in number and percentage. CA-RT = Crypto-*Giardia* antigen rapid test, MZN-AF = Modified Ziehl-Neelsen acid fast staining.

4. Discussion

The aim of the current study was to evaluate the validity of CA-RT as an example of rapid immunoassays for the diagnosis of cryptosporidiosis in comparison with conventional microscopic examination using MZN-AF staining assay. The currently obtained validation parameters, based on testing control positive and control negative samples, revealed higher sensitivity (86.7%), specificity (100%) and AI (96.4%) of CA-RT than those of MZN-AF staining method. The lower sensitivity, specificity and AI of the conventional microscopy was expected and attributed to the fact that detection of the parasite by this method depends largely on the experience and skills of the microscopist. In addition, the detection limits of conventional microscopical

techniques have been estimated as 50 000 to 500 000 oocysts per gram of feces[9]. The currently reported absolute specificity (100%) of CA-RT is obviously attributed to the use of monoclonal antibodies in this immunoassay. Assays uses monoclonal antibodies directed against a specific *Cryptosporidium* antigen greatly reduce the possibility of cross-reaction. This fact was confirmed by Silva *et al* 2003, where no cross-reaction was detected with other intestinal parasites[4]. In addition, previous study reported the absence of any cross-reaction activities with other intestinal protozoa or helminthes using four different commercial antigen-capture kits[10]. On the other hand, regarding sensitivity, the currently reported sensitivity of the evaluated CA-RT was relatively low as compared to previously reported sensitivity of Nested polymerase chain reaction (PCR) [11]. This could be attributed to the use of monoclonal antibody that although increased the specificity of the test, it reduced its sensitivity. In addition, antigenic variability within clinical isolates of *Cryptosporidium* could have result in some infections to escape detection[12,13].

The predictive values of CA-RT for diagnosis of cryptosporidiosis as compared to conventional methods using MZN-AF staining revealed the better accountability of the antigen detection immunoassay over conventional methods. The predictive values usually give an indication about the detection ability of a given assay of disease's probability in suspected population during further investigation. Regarding the PPV, CA-RT revealed 100% PPV that is considerably higher than that (84.6%) of MZN-AF method. These results indicate higher ability of CA-RT to detect positive cryptosporidiosis cases as being positive in suspected population as compared to conventional methods during further investigation of the disease. In addition, microscopy has suffered from problems of sensitivity and specificity as variable results between laboratories is common, these problems due to inability in many cases to distinguish *Cryptosporodium* from the other fecal components of similar size and shape such as yeasts[14]. On the other hand, the NPV of CA-RT test as revealed in the current study was low (95.2%), however it was relatively higher than that (90.5%) of conventional method.

The overall results of the current study indicated the presence of considerable difference in the effectiveness and validity of the two tests. One strong advantage of these immunoassays over conventional methods was the simplicity and objectivity in reading the results. This indicated the suitability of those rapid antigen detection-based immunoassays for use in developing countries where more sophisticated equipments as spectrophotometers are usually unavailable.

Rapid stool antigen detection-based immunoassays offers simple and objective alternative to conventional microscopy for routine diagnosis of cryptosporidiosis in suspected patients. It is simple to perform, requires minimal training and can be used for single-specimen or batch-testing approaches. Therefore, antigen detection-based immunoassays provide an alternative diagnostic assay, especially for those who present cryptosporidiosis-like symptoms with repeatedly negative results based on conventional methods. In addition, rapid antigen detection-based immunoassays are especially useful when screening children in day cares, during suspected outbreak or post treatment testing of previous patients.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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