COMPARISON OF POLYMERASE CHAIN REACTION AND CONVENTIONAL TECHNIQUES FOR DETECTION OF *CRYPTOSPORIDIUM* IN FECAL SAMPLES OF DIARRHEIC CALVES

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SUMMARY

Cryptosporidium parvum is a coccidian protozoon that causes diarrhea in newborn animals and human The purpose of this study was the evaluation of RT-PCR (reverse transcription-polymerase chain reaction and ELISA (Enzyme-linked immunoassay) for detection of Cryptosporidium in fecal samples derived from diarrheic calves They were compared with the routine screening procedure applied in this field study that is mZN(modified Zehle Neelsen staining) and direct method followed by microscopy. Eighty fecal samples collected from calves suffering from diarrhea were used as research materials in this study.

Overall comparison of the results showed respective sensitivities and specificities of 70% and 100% for mZN staining, 60%and 100% for the direct method, 100% and 95.71% for PCR and 100% and 87% for ELISA.

The RT-PCR technique appeared to be the most specific and sensitive diagnostic method. In spite of the lower specificity of ELISA, It is still a practical technique especially in epidemiological studies with large numbers and to exclude negative cases

INTRODUCTION

Cryptosporidium parvum is now recognized as one of the major causes of neonatal diarrhea in calves, observed primarily in young calves from birth to 30 days of age *(Luginbuhl et al., 2005; Lefay et al., 2000 and Kvac and Vitovec 2003)*

C. *parvum* oocysts, which are widely distributed in the environment of many cattle operations, are ingested by the newborn calf. Following an incubation period of 72 to 96 hours, diarrhea is observed for 2 to10 days, during which time oocysts are demonstrable in feces. Oocyst shedding continues for a variable number of days following cessation of diarrhea. Diagnosis of cryptosporidiosis is based on detection of fecal oocysts. Detection methods include concentration *(Arrowood and Sterling 1987)*, staining of fecal smears *(Arrowood, and Sterling. 1989,)*, latex agglutination

immunoassay *(Pohjola et al., 1986),* and demonstration by immunofluorescence using monoclonal or polyclonal antibodies reactive with C. *Parvum* oocysts *(Alles et al., 1995).*

These methods are not sensitive enough to detect small numbers of oocysts in which the threshold for detection may require the presence of 50,000 oocysts per gram of feces in immunofluorescence and 500,000 oocysts per gram in case of mZN staining *(Weber et al., 1991).* Also difficulties in detecting oocysts in fecal specimens are compounded by the variation in the consistency between individual fecal samples, the amount of the sample used, and oocysts losses occurred during recovery process *(Arrowood et al., 1995 and Parisi And Tierno, 1995)*

Moreover confirmation of the presence of *Cryptosporidium* in environmental and fecal samples is labor-intensive, time consuming, costly, and often difficult, being dependent upon training and expert knowledge of morphologic differentiation of this small coccidian *[Michel et al., 2000 and Fabiana et al;, 2005].*

The development of a monoclonal antibody (MAb)- based capture enzyme-linked immunosorbent assay (ELISA) for the detection of C. *parvum* oocysts in bovine feces has also been developed and used in many surveys in calves *(Krzysztof et al;, 1990 Fabiana et al;, 2005, Luginbuhl et al., 2005 and Starkey et al., 2005).*

The ability of PCR to amplify DNA thousands of times make it logical method for detecting *C. parvum* DNA even in very small amounts *(Hallier-Soulier, and Guillot. 2000).* Workers have described many PCR-based protocols for detection of *Cryptosporidium* parasites in cattle *(Kato et al., 2003; Caccio, 2004 and Coupe et al., 2005)*

The objective of this study was to determine the evaluation of PCR and ELISA for diagnosing *cryptosporidiosis* in field cases of diarrhea among calves with reference to the conventional methods mZN staining and direct methods.

MATERIALS AND METHODS

Samples:

Eighty Fecal samples were collected from calves (4 days to 6 weeks age) suffering from diarrhea. Calves were raised in small farms at Alexandria Province. Diarrheic feces were collected. Part of the sample was mixed with equal volumes of 2.5% potassium dichromate solution, and the mixtures were stored at 4 ^{0}C. The other part of the sample was kept at -20 ^{0}C without adding preservative for the application of PCR

Modified Ziehl–Neelsen (mZN)

The technique was done according to *Arrowood, and Sterling. (1989)* in which faecal smears, air dried and fixed in absolute methanol for 3 minutes.

The slides were immersed in cold strong carbol-fuchsin for 15 minutes. After that the slides were rinsed thoroughly in tap water. Then Counterstained with 0.4% malachite green for 30 seconds. The slides were air-dried and examined microscopically. A little immersion oil is spread over the smear which is then viewed with either direct or oil immersion lenses.

Direct method:

It was done according to *Gobel et al., (1982)*. Two drops of diarrheic liquid feces were spread over a slide. Before complete dryness of the sample, two drops of immersion oil was added and cover slid was applied. The slide was examined immediately by microscope

Enzyme linked immunosorbant assay (ELISA)

The ELISA were performed with unconcentrated fresh fecal specimens according to the instructions of the manufacturer (Riadascreen-*Cryptosporidium*; Biaopharm A.G., Landwehrstr, Germany). Aliquots from the original fecal specimen were diluted 1:11(100 µl of liquid feces to 1 ml of stool diluents), mixed well. the plates were developed as per the manufacturer's instructions, briefly A 100µl aliquot of the resulting samples were placed in a test well. One hundred µl of positive control and 100µl of negative control were placed in separate wells. (The plate wells are coated with antibodies against *Cryptosporidium*) 100 µl of the HRP conjugated monoclonal antibodies against *Cryptosporidium* were added and the plates were incubated at room temperature for 60 minutes. The plate was washed five times with diluted wash buffer. Then 100 µl of the substrate was added and the plate was incubated at room temperature for 15 minutes. Fifty µl of stop buffer (1M sulfuric acid) were added and the plate was read spectrophotometrically at 450 nm. Sample was considered positive if its reading was more than 10% above the cut-off (Cut-of= reading of the negative control+0.15.

Reverse transcriptase polymerase chain reaction (RT- PCR)

Preparation of feces for recovering *C. parvum* DNA. 100 µl of diarrheic fecal sample was collected in a microcentrifuge tube and centrifuged (15,000 3 *g*, 5 min); the supernatant was removed by pipetting, and the pellet was resuspended with 100 µl of *Cryptosporidium* lyses buffer (CLB) that contained 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.05 M NaOH, 1 mg of proteinase K per ml, and 1 mg of RNase per ml. The mixture was mixed, and 100 µl was pipetted to a microcentrifuge tube. If the feces were diarrheic,. Subsequently, DNA from fecal specimens was extracted by four cycles of freeze-thawing (liquid nitrogen for 5 min; 75°C for 5 min) and then by incubation (75°C for 3 h) and centrifugation (15,000 3 *g* for 5 min). Fifty microliters of the supernatant resulting from each sample was collected *(Leng et al., 1996)*.

Test procedures

The test was done according to *(Webester et al., 1996)* The PCR mixture consists of 5 µl of 10X reaction buffer (50mM KCL; 10mMTris-c\CL, pH8.4;

5.5 mM $MgCl_2$; 0.1% gelatin); 5µl of dNTP (Amersham Bioscience, UK Limited, Little Chalfont, Buckinghamshire, England) mix (2mM each base); 100 ng each primer; 2.5 units Taq polymerase (Amersham Bioscience, UK Limited, Little Chalfont, Buckinghamshire, England) 68% v/v TE buffer pH 8 10 µl of sample in a final volume of 50µl. The primers used were forward primer 5'ATC TTC ACG CAG TGA GT, reverse primer 5' CAT CAG CCG GTA GAT GTC GA. PCR reactions were initially denatured at 94 ^{0}C for 3 min., then cycled with 90 seconds at 94 ^{0}C (denature), 90 seconds at 50 ^{0}C (anneal) and 120 seconds at 72 ^{0}C (extend) This cycle was repeated 40 times followed by 10 minutes incubation at 72 ^{0}C. Amplification products were visualized by running 12 ml of the reaction mixture in 2% agarose gels in Tris-borate-EDTA buffer. Gels were run at a constant 110 V for 90 min. Gels were stained in an ethidium bromide solution (0.5 mg/ml) for 30min, destained for 30 min, and visualized under UV light

Statistical analysis:

Diagnostic test results were analyzed in two ways: In the first case, It was carried out using Epi-Info computer program designed by *Dean et al. (1994)* and produced by World Health Organization (WHO). The percentage of agreement, sensitivity and specificity were calculated according to *Knapp and Miller (1991).*

In the second case: Comparative analysis of the results were done by using the Kappa statistic that give overall measures of the agreement between two tests in the absence of the standard. It compares with the observed proportion of samples in which the test agree with the proportion that would be accepted to agree by chance. Calculation and interpretation of Kappa values were carried out according to *Landis and Koch (1977)*: kappa values that fell between 0.0 and 0.20 indicate a slight agreement, between 0.21 and 0.40 a fair agreement, between 0.41 to 0.60 moderate agreement, between 0.61 and 0.80 a substantial agreement and between 0.81 and 1.00 almost perfect agreements

RESULTS

The results of testing 80 fecal samples for the presence of *Cryptosporidium* using mZN, direct method, ELISA and PCR *(Cryptosporidium* PCR product size was 452 bp) are presented in table 1&2. According to this tabulation, all the applied tests were compatible with 3 samples and 61 negative samples. Out of the remaining 16 samples, 7 were positive in three tests, 4 were positive in two tests and 5 were positive in one test. Thus after summing up all the positive , 19 samples were qualified as positive.

According to the data of table 3, which present results of the analysis of comparing all of the applied diagnostic tests with he reference system created (mZN staining and direct method), The sensitivity and specificity was 70%

and 100%, respectively for the mZN staining method, 60 % and 100% for the direct method, 100% and 87.14% for ELISA method and 100% and 95.71% for PCR. (table 3). ELISA and PCR showed The highest sensitivity where the acid fats staining and direct method showed the highest specificity.

The value of kappa statistic for every pair of compared tests are presented in table 4. The Kappa value between mZN and direct method was 0.184 indicating slight agreement while fair agreement was recorded between mZN and PCR (0.345), between direct method and PCR (0.283). The lowest kappa value was recorded between ELISA and mZN and between ELISA and direct method.

DISCUSSION

Rapid diagnosis of *Cryptosporidium* is based on revealing the presence of cryptosporidial oocysts, their antigens or dsRNA in the clinical material samples. Numerous methods were developed such as concentration *(Arrowood and Sterling 1987),* staining of fecal smears *(Asahi et al., 1988),* latex agglutination immunoassay *(Pohjola et al., 1986),* demonstration by immunofluorescence *(Alles et al., 1995),* ELISA *(Krzysztof et al;, 1990).* And PCR *(Coupe et al., 2005)*

Each of the above mentioned methods has its advantages and disadvantages. The significant element of determining clinical usefulness of the method is the choice of a proper reference system to which the results of the evaluated system will be referred. The reference methods in our study isof the standard microscopic detection of oosysts by mZN stained slides or by the direct method. The mZN staining has been used as reference method for evaluation of techniques in *Cryptosporidium* diagnosis before *(Luginbuhl , et al., 2005 and Marques et al., 2005)*

In this study ELISA technique showed high sensitivity (100%) (Table 3) In previous studies sensitivity of ELISA in *Cryptosporidium* diagnosis was sufficient to detect oocysts in animals with clinical signs of disease (10^5 to10^7 oocysts per ml of feces as well as in calves which have recovered from diarrhea but continue to shed oocysts for several days *(Krzyszktof et al., 1990).* The test proved to be valuable diagnostic tools for *Cryptosporidium* infection with high specificity and sensitivity *(Luginbuhl , et al., 2005 and Marques et al., 2005).*

However low specificity (87%) was observed in ELISA in our study (table 3) and the lowest kappa values were observed between ELISA and other techniques (Table 4). As shown in table (2), five samples were positive in ELISA test and did not correlate positively with any other test. It may be presumed that these five positive samples by ELISA should be numbered as false positive. This is supported by. *Weitz (1995)* who recorded that ELISA

has a lower sensitivity than that of the stained methods commonly used for the diagnosis of cryptosporidiosis and *(CCDR 1999)* who interpretated false-positive test results may go unrecognized because of problems associated with the kit reagents or technician error. However, the ELISA kit is simple and rapid to use and offers a less subjective method than microscopy for detecting this protozoan in fecal samples submitted to a busy diagnostic laboratory *(Siddons, et al., 1992)*. This is a highly sensitive, and it is useful for screening large numbers of specimens in a short time period and not rely on microscopy skills *Mehta (2002)* the test also allowed cryptosporidial diagnosis, even when the parasite's integrity is compromised *(Fabiana et al., 2005)*

Table (3) showed 100% sensitivity of PCR and close to that value (95.71%) specificity. In previous studies PCR methods have been shown to be more sensitive and specific than traditional microscopic techniques for detecting *Cryptosporidium* spp. in clinical and environmental samples *(Mayer and Palmer. 1996 and Morgan et al., 1998)*. The test been used successfully in epidemiological studies in cattle *(Matsubayashi et al., 2004 Santin et al., 2004 ; Gomez-Couso et al., 2005; Nydam et al., 2005). Webester et al., (1996)* found positive PCR in feces containing 1600 oocysts per gram of bovine feces while nested PCR procedure was able to detect the equivalent 40 oocysts/ml of stools *(Coupe et al., 2005)* The RT-PCR detected 10^2 times fewer oocysts than the ELISA in serial dilutions of diarrheic bovine feces. *(Leng et al., 1996)*

As shown in table (1&2), the lowest number of positive samples were observed by mZN and direct method. This may be due to low number of oocysts in the sample as it was reported that these methods are not sensitive enough to detect small numbers of oocysts in which the threshold for detection may require the presence 500,000 oocysts per gram *(Weber et al., 1991)*. Also it is often difficult, being dependent upon training and expert knowledge of morphologic differentiation of this small coccidian *[Michel et al., 2000 and Fabiana et al;, 2005]*.

Conclusion:

The excellent sensitivity and specificity of PCR, , make this method a suitable tool for diagnosis and genotyping of Cryptosporidium in stools. for species identification . The sensitivity of PCR technology, well permit early diagnosis as well as for detection of sub clinical asymptomatic infection and analysis of environmental samples where there is small number of oocysts. In spite of lower specificity ,we believe that the ELISA test is a useful assay for ruling out cryptosporidiosis especially when there are indicative clinical signs with inconclusive microscopic diagnosis, or in large-scale epidemiological surveys.

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Table (1) Detection of *Cryptosporidium* in fecal specimens of calves by mZN, Direct method, ELISA and PCR

No of samples	mZN	Direct method	ELISA	PCR
3	+	+	+	+
3	-	+	+	+
4	+	-	+	+
1	+	-	+	-
3	-	-	+	+
5	-	-	+	-
61	-	-	-	-
Total 80	7	6	19	13

Table (2): List of positive samples by the particular methods used for *Cryptosporidium* detection in fecal specimens of calves

Samples	mZN	Direct method	ELISA	PCR	No of +ve methods
1	+	+	+	+	4
4	+	+	+	+	4
78	+	+	+	+	4
2	+	-	+	+	3
8	+	-	+	+	3
26	-	+	+	+	3
30	-	+	+	+	3
53	-	+	+	+	3
77	+	-	+	+	3
16	+	-	+	+	3
42	-	-	+	+	2
57	-	-	+	+	2
70	-	-	+	+	2
23	-	-	+	+	2
34	-	-	+	-	1
39	-	-	+	-	1
62	-	-	+	-	1
63	-	-	+	-	1
69	-	-	+	-	1
Total	7	6	19	13	

Table 3: Comparison of sensitivity and specificity of the methods used for *Cryptosporidium* detection in fecal specimens of calves

Method		True positive[a] N= 10	True negative[b] N= 70	Sensitivity[c] (%)	Specificity[d] (%)
mZN	+ve	7	0	70	100
	-ve	3	70		
Direct method	+ve	6	0	60	100
	-ve	4	70		
ELISA	+ve	19	9	100	87.14
	-ve	-	61		
PCR	+ve	13	3	100	95.71
	-ve	-	67		

[a] True positive means that sample was positive in mZN staining and direct method or at least one of these tests
[b] True negative means that samples was negative in both mZN staining and direct method.
[c] Sensitivity = number of samples in which the test was positive when mZN staining and direct method or at least one of them were positive/true positive)X100%
[d] specificity = number of samples in which the test was negative when both mZN staining and direct method were negative /true negative)X100%

Table (4):Kappa statistic value as a measure for agreement between methods used for Cryptosporidium detection in fecal specimens of calves

method	mZN	Direct method	ELISA	PCR
mZN	1	0.184	-0.0004	0.345
Direct method		1	-0.0006	0.283
ELISA			1	0.002
PCR				1

الملخص العربي

دراسة تقييم تفاعل انزيم البلمرة مع الطرق التقليدية للكشف عن الكريبتوسبوريديا في عينات براز لعجول تعاني من الإسهال

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الغرض من هذه الدراسة هو تقييم مقارنة تفاعل انزيم البلمرة و الإليزا للكشف عن الكريبتوسبوريديا في عينات براز لعجول تعاني من الإسهال وذلك بقارنتهما بالإختبارات الروتينية وهي الفحص المجهري لمسحات براز مصبوغة أو بالفحص المباشر. وقد استخدم في هذه الدراسة ثمانون عينة براز تم تجميعها من عجول رضيعة تعاني من الإسهال وقد أظهرت النتائج أن نسبة الحساسية ونسبة التحديد كانت 70% و 100% لطريقة الصبغة 60% و100% للطريقة المباشرة و100% و95.71% لتفاعل انزيم البلمرة و100% و87% للإليزا.

يدل ذلك على أن اختبار تفاعل انزيم البلمرة هو أكثر الإختبارات حساسية وتحديدا في تشخيص الكريبتوسبوريديا و بالرغم من قلة تحديد اختبار الإليزا إلا أنة يظل اختبار عملي وسهل وسريع خصوصا في الدراسات الوبائية التي تتطلب الدراسة على عدد كبير من العينات.