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Detection of *Cryptosporidium parvum* in calf feces using microscopical, serological, and molecular methods

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Abstract

Cryptosporidiosis is a critical gastrointestinal disease in calves. This study examined 150 fecal samples of diarrheic calves collected from the eastern region of Saudi Arabia for detection of *Cryptosporidium parvum* using the Modified Ziehl-Neelsen (MZN) method, Enzyme-Linked Immunosorbent Assay (ELISA), and conventional Polymerase Chain Reaction (PCR). The performance of these methods was assessed using diagnostic accuracy tests. The present study identified *C. parvum* oocysts in fecal samples by modified Ziehl-Neelsen 40/150; 26.66%, ELIZA 60/150; 40%, and PCR 78/150; 52% methods, respectively. The microscopic method revealed higher specificity 65.27% than the ELIZA 51.38%, while the ELIZA showed higher sensitivity 32.05% than MZN method 19.23%. However, MZN and ELIZA methods were unsatisfactory diagnostic tools compared with the PCR as the area under the curve values in Receiver Operator Characteristic (ROC) analysis were less than 0.6. Furthermore, using the kappa analysis test revealed no agreement between MZN and ELISA methods compared with PCR at P<0.05.

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Introduction

The *Cryptosporidium* parasite is a vital protozoan parasite, and over 150 species of mammals have been identified as hosts of nearly 20 genotypes of *C. parvum* and also considered a worldwide problem in humans and animals (1,2). The parasite is considered a significant cause of neonatal profuse watery diarrhea due to its life cycle of *C. parvum* is done in the epithelial cells in gut canal (3). Infection by *C. parvum* results in intercellular colonization of the extra-cytoplasmic microvillus of the small intestine (4) and leads to production losses and a high mortality rate in pre-weaning calves during the first 28 days of life (5). *Cryptosporidium parvum* is easily transmitted to humans, especially immune-compromised individuals, and is therefore considered an anthroponotic zoonotic disease (6). Different methods were used in the diagnosis of apicomplexan protozoal infection included cryptosporidiosis. A direct microscopic method is obtained using morphological identification with a 100× oil immersion lens. It uses different staining techniques such as hot or cold modified Ziehl-Neelsen (MZN) and auramine phenol to detect the oocyst containing four mature sporozoites (7). The microscopical method is an excellent tool and highly economical but requires expertise and experienced diagnostic personnel to reduce false-positive results and thus can be time-consuming and also parasites species and multiple infections the microscopic examination cannot detected these issues (8,9). Indirect serological methods, like latex agglutination test (LAT), modified

agglutination test (MAT), ELISA enzyme-linked immunosorbent assay which, depend on coproantigen detection. Coproantigen detection assays are rapid and useful for screening large numbers of specimens simultaneously but do not provide details concerning the species of apicomplexan protozoal included *Cryptosporidium* detected (10,11). Molecular methods, e. g. conventional polymerase chain reaction (PCR), are used to detect parasite DNA but needed amplification before PCR test to overcome eliminate or inactivation inhibitors (12,13). PCR is a sensitive method compared to microscopical and serological diagnosis in humans and animals for detecting *Cryptosporidium* and is used to differentiate the species of helminthes but incurs a high cost in developing counties (14). The main advantage of the PCR method is detecting various *Cryptosporidium* species at the species, genotype, and subtype levels. Therefore, choosing the most effective diagnostic technique relies on multiple factors, including accessible resources, trained personnel, available time, and the number of specimens tested. Each technique also has different levels of specificity and sensitivity (15).

In Saudi Arabia, no studies guide the diagnosis of *Cryptosporidium* species in calves. Therefore, this study aimed to detect *C. parvum* using microscopic (hot MZN staining), ELISA, and PCR methods and subsequently estimate the diagnostic accuracy of each method.

Materials and methods

Ethical approve

The study is not an animal experiment, but a diagnostic study, using common sampling methods for diagnostic purposes. The methods were performed in accordance with the guidelines of ethical committee of ministry of environment, water, and agriculture, KSA, the authors confirm the study was carried out in compliance with the ARRIVE guidelines. Also All respective animal protocols were reviewed by state ethics commission and have been approved by competent authority (Ethical committee of Alexandria university, Egypt, serial number (0305796) at 20-10-2022, FWA No: 00018699 and IRB No: 00012098). Write the name of scientific or institutional board that give the ethical approve to conduct this scientific work and give the approval issue number and date.

Sample collection

One hundred fifty fecal samples from different private farms were collected from calves (> 3 months old). All 150 calves were clinically examined for body temperature, mucus membranes, and degree of dehydration. The calves were suffering from profuse watery diarrhea. Fecal samples were collected from profuse watery diarrheic calves. Each sample was divided into two parts: one part was preserved in 10% formalin prepared via the formol ether concentration technique for later staining with the hot MZN method (16). The second part was preserved in laboratory tubes at −20°C for further investigation.

Hot MZN staining

The reagents needed to perform hot MZN staining were Carbol-Fuchsin (10 gm basic fuchsin, 100ml absolute ethanol, 50 gm carbol, and one-liter distilled water), sulfuric acid-ethanol solution (3ml concentrated sulfuric acid and 100 ml methanol 95%) was put in a glass stopper. (sulfuric1%) Moreover, methylene blue (100ml glycerol, 1ml 3%aqueous Mb, 100ml distilled water). Furthermore, the procedure of hot MZN staining was done through three steps: carbol fuchsin, decolorization, and counter-stain. Respectively in between each step rinsed, the slides with tap water were drained and air drying, so the practical procedures were done as follows; the first step was a thin fecal smear was made from the sediment of the centrifuged formalized specimen and was allowed to air dry, then the slide was placed on a staining rack and flooded with carbol fuchsin for five minutes. The slide was heated gently with a Bunsen burner. Then the slide was rinsed with tap water. The second step was decolorization with 1% sulfuric acid ethanol solution for about 2 minutes, then rinsing with tap water, draining, and air drying. The final third step was the slide was flooded with methylene blue (counter stain) for one minute, and then rinsed with tap water; draining and air drying was done. The smear was examined microscopically using a high-power magnification to detect oocysts and oil immersion objective to identify them as *Cryptosporidium* oocyst retained a red/pink color due to *Cryptosporidium* was acid-fast versus blue or clear background (17).

ELISA method

The method was performed using a multiscreen Antigen ELISA kit for antigenic detection of *Cryptosporidium* in feces (Bio-X Diagnostics S.A., Belgium) according to the manufacturer's instructions. The microtitration plate was coated with specific antibodies. Fecal samples were diluted and incubated in the coated wells. After 1 h incubation at 21°C +/- 3°C, tetramethylbenzidine was added, and the absorbance was read at 450 nm using a microplate ELISA reader (Thermo Scientific, USA) (18).

Conventional PCR method

Specific primers for C. parvum were as follows: forward primer 5`-GCCCACCTGGATATACACTTTC-3`; reverse primer 5`-TCCCCCTCTCTAGTACCAACAGGA-3`. Amplified DNA was separated using agarose gel electrophoresis and was visualized using a U.V. transilluminator (320 nm) (19).

Isolation of *C. parvum* DNA from fecal samples

Fecal specimens were collected from the rectum of all selected animals and were prepared according to Johnson *et al*. Fecal samples stored in 2.5% potassium dichromate were

washed several times (4 times) with PCR buffer (10 mM Tris, 50 mM K Cl, 3.5 mM Mg Cl2) by centrifugation. A 20% Chelex 100 (Bio-Rad Lab., Calif.) was mixed with fecal samples (20 µl of chelex stock to 100µl of samples). Then, samples were subjected to six cycles of freezing and thawing to release the DNA from oocysts using a dry ice ethanol bath for freezing and a water bath (98 oC) for thawing with incubation time from 1 to 2 minutes in each bath. In the next step, samples were centrifuged, and 50 µl of supernatant was used as the template in the PCR assay (20).

DNA amplification

The primer set described by Laberge *et al*. was used in PCR which is specific for *Cryptosporidium parvum*. Sequences of primers were as follows: forward - 5'GCC CAC CTG GAT ATA CAC TTT C3`; reverse - 5'TCC CCC TCT CTA GTA CCA ACA GGA 3`. The size of the amplified product was 358 bp. (Figure 1) The PCR mixture contained PCR reaction buffer (10 mM Tris-HCl, 1.5 mM MgCl, 50 mM KCl, pH 8.3) and contained 1.0 mM each of forward and reverse primers, 0.2 mM each dATP, dGTP, dCTP and dTTP, 100 mg/ml BSA and 2.5 U Taq DNA polymerase (Boehringer Mannheim Canada, Laval, Que´bec, Canada). 10 µl of template DNA was added in the case of fecal samples and 30 µl in the case of filtered environmental water pellet suspensions. Reaction mixtures were initially denaturated at 94ºC for 1 min and then subjected to 40 cycles of denaturation at 94 ºC for 15 s, annealing at 50oC for 1 min, and extension at 72ºC for 1 min, with an additional 7-min extension at 72 ºC (21).

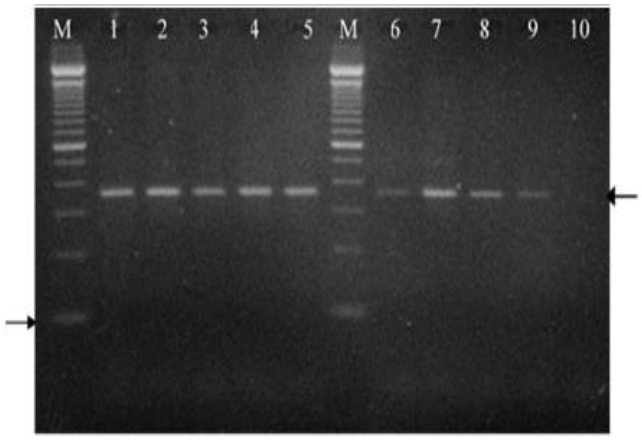

Figure 1: Sensitivity of the PCR assay for detecting *Cryptosporidium parvum* as determined by 2 % agarose gel electrophoresis. M: 100 bp ladder, 358 PB positive samples lines 1-9, line 10 negative.

Statistical analysis

Data in a Microsoft Excel® (Microsoft® office 2013) spreadsheet were recorded and analyzed using SPSS (version 22, IBM Corp., Armonk, NY). The Kappa test (cross-tabulation) measured the agreement values between microscopic and ELISA methods and PCR. Diagnostic accuracy of any diagnostic procedure or a test gives discriminates between certain two conditions of interest (healthy or negative and disease or positive cases). This discriminative ability can be quantified by the measures of sensitivity, specificity, positive and negative predictive values (PPV, NPV), likelihood ratio, diagnostic efficiency%, and discrimination ability. Receiver operating characteristic (ROC) curve values were calculated using the area under the curve (AUC) as a diagnostic accuracy test to validate the prediction of cryptosporidiosis; a level of 95% was considered statistically significant.

Results

The current study reported infection rate percentages based on the hot MZN staining method, ELISA, and conventional PCR at 26.66%, 40%, and 52%, respectively. In this study, the ELISA technique diagnosed more positive samples (25 true positive samples) than those found via microscopic examination (15 true positive samples) (Tables 1 and 2); however, microscopic examination diagnosed more negative samples (47 true negative samples) more than the ELISA technique did (37 true negative samples). Analysis of the two screening techniques was performed via kappa testing. This revealed no agreement between screening tests and PCR, with a kappa value of -0.152 for the hot MZN staining method and -0.164 for ELISA, although there was a significant difference between the screening test results and that for PCR (Table 3).

Table 1: Correlation of Ziehl-Nielsen staining results and conventional PCR results for detection of *C. parvum* from fecal samples investigated in the present study

Detection method		Conventional PCR method		
		Nr. of positive (%)	Nr. of negative (%)	Total
MZN	Nr. of positive	15 (37.5)	25 (62.5)	40
	Nr. of negative	63 (57.3)	47 (42.7)	110
	Total	78 (52.0)	72 (48.0)	150

Table 2: Correlation of ELISA results and conventional PCR results for detecting *C. parvum* from calf fecal samples

Detection method		Conventional PCR method		
		Nr. of positive (%)	Nr. of negative (%)	Total
ELISA	Nr. of positive	25 (41.7)	35 (58.3)	40
	Nr. of negative	53 (58.9)	37 (41.1)	90
	Total	78 (52.0)	72 (48.0)	150

Table 3: Measure of the agreement by kappa test between both screening techniques compared with conventional PCR as the gold standard for *C. parvum* diagnosis in calf fecal matter

Detection methods	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
MZN	-0.152	0.071	-2.144	0.032
ELISA	-0.164	0.079	-2.068	0.039

AUC values of 0.577 and 0.5833 (Table 4) were obtained for the microscopy and ELISA, respectively (Figure 2). These values of less than 0.6 indicated that both screening techniques were unsatisfactory diagnostic tests compared with the gold standard PCR technique.

Table 4: Diagnostic performance of the microscopic examination of hot Ziehl-Nelsen stain and ELISA as diagnostic screening techniques for *C. parvum* diagnosis compared to the conventional PCR as the gold standard in the fecal matter among calf

Diagnostic accuracy test	Screening tests	
	Hot MZN	ELISA
Diagnostic efficiency %	41.33	41.33
Sensitivity % (95%Cl)	19.23 (11.50-30.04)	32.05 (22.18-43.70)
Specificity % (95%Cl)	65.27 (53.05-75.85)	51.38 (39.40-63.22)
PPV % (95%Cl)	37.50 (23.17-54.19)	41.66 (29.31-55.08)
NPV % (95%Cl)	42.72 (33.45-52.51)	41.11 (31.00-51.98)
PLR (95%Cl)	0.55 (0.31-0.96)	0.65 (0.44-0.98)
NLR (95%Cl)	1.23 (1.09-1.39)	1.32 (1.10-1.58)
DA %	19.78	17.23
AUC	0.577 (0.486-0.669)	0.583 (0.491-0.674)

PPV, positive predictive value; NPV, negative predictive value; PLR, likelihood ratio for positive results; NPL, likelihood ratio for negative results; DA, discrimination ability (PPV+ NPV-100)100%; AUC area under the curve.

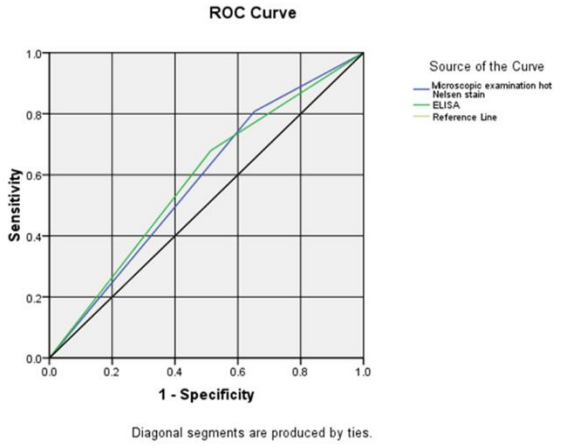

Figure 2: ROC curve for predicting the effectiveness of ELISA or microscopic staining as diagnostic tools by using PCR as a gold standard.

Discussion

The incidence rate of *C. parvum* infection is 100% in neonatal calves with diarrhea has been previously reported by Avendaño (22). Similar results have also been reported among diarrheal dairy calves in France by Mammeri *et al*. (23). Elsafi and Rashmi (24,25), revealed that; false negatives reported in the ELISA test (as compared with gold standard PCR results) have been attributed to different reasons: first, the sensitivity of the test may be low because of the antigenic variability of the *Cryptosporidium* isolates; and second, the variable density of the parasite, where low parasite densities could be due to late infections In this study, the percentage of positive samples detected by ELISA was higher than those detected via the MZN staining method, and these results agree with several other studies that have reported that ELISA-based methods are more sensitive than microscopy methods by Chalmers (26). Furthermore, Ezzaty *et al*. (27) found that; the infection rate of *Cryptosporidium* oocysts in cattle fecal samples via PCR was 35% higher than that determined using ELISA 18.7%. Conversely, several studies by Khurana *et al.* (28) have indicated that ELISA was less sensitive than the microscopic examination method. Here, the sensitivity of the hot MZN staining method 19.23% was significantly lower than that of ELISA 32.5%. This result agrees with Elgun *et al.* (29), who reported a lower sensitivity for the MZN staining method than other techniques, including immunofluorescence assays.

Furthermore, Sumeeta *et al.* reported a higher sensitivity for ELISA (95.35%) than found for the MZN stain method 79.06% and also reported 100% specificity for both the MZN method and ELISA.ELISA is a simple and easy method and can be rapidly performed for many samples; furthermore, the ELISA method does not require as much diagnostic and technical skill as the microscopy method (30). The specificity of the hot MZN staining method 65.27% was

higher than that for ELISA (51.38%), which is in agreement with other studies that reported that MZN staining was more specific 98.9-100% with lower sensitivity by Tuli (31). However, the current study regarding the agreement between screening tests and PCR is inconsistent with that determined by Ghaffari and Kalantari, who reported a moderate agreement between PCR and ELISA (kappa = 0.55) and poor agreement between microscopic examination and PCR and the used of hot MZN staining to identify *Cryptosporidium* oocysts in 10.80% of samples versus a much higher PCR-determined infection rate of 66.4% in the same calf fecal samples (32). Furthermore, Goñi *et al.* demonstrated a good agreement between microscopy and PCR. Regarding the diagnostic accuracy test, the current finding agrees with a study by Kar *et al*, However, developing countries may be less able to perform PCR assays because of the limited resources (33). Therefore, the ELISA method is recommended instead, as this demonstrates high sensitivity and specificity (34).

Bhat *et al*. reported that PCR detected significantly more *Cryptosporidium* infection than that found via microscopic examination. The c*ryptosporidium* infection rate in calves using PCR was twice as much as those inferred by the direct fecal smear used in the hot MZN staining method and which requires highly experienced diagnostic expert personnel to reduce the misdiagnosis due to artifacts or other intestinal apicomplexan parasites (35). Similarly, Clarke and McIntyre reported that false-positive samples were detected by microscopy because of the presence of artifacts, such as yeasts and debris in the stool; however, false-negative samples were also found to be due to poor uptake of stain by the oocytes (36). Furthermore, by Alseady revealed that; the overall prevalence of infection with *Cryptosporidium* is 21% (21/100) by conventional microscopic (modified Ziehl-Neelsen staining) method on the other hand, PCR diagnostic technique the *Cryptosporidium* infection is detected in 38 samples 38% with sensitivity 100% which the differences of infection attributed to multiple factors included management systems and rearing methods, non modified risk factors likes' age, environmental conditions and breed of cattle and modified risk factors as, the sampling techniques and diagnostic methods (37). The most common species of Cryptosporidiosis are *C. parvum* in rural area then *C. hominis* in urban area and the lowest is *C. ryana* and *C. bovis* and also there are fact that *C. parvum* is not specific to a host (38). There are subtype family is widespread of Cryptosporidiosis that cause infection among both human and animal, and *C. hominis* in animal, conceivably a source of human infection with same species (39). Furthermore, there are three subtypes of *C. parvum* IbA21G2, IbA19G2 and IbA13G3 but the second subtypes were recorded in Iraq previously (39). So, the calves its age less than six weeks during pre-weaning the *C. parvum* is the key enteropathogens of neonatal calf led to diarrhea (40). In Kuwait the molecular identification of *C. parvum* is 62.8% in pre-weaned calves (41).

Conclusion

We found that microscopy and ELISA were unsatisfactory diagnostic tests compared with PCR. Microscopy is an affordable technique but has lower sensitivity in diagnosing the positive samples alone and, therefore, should be accompanied by ELISA or PCR to obtain an accurate diagnosis of *Cryptosporidium* infection based on fecal samples. We found that PCR was the most accurate and sensitive diagnostic tool for *Cryptosporidium* infection, especially in samples of low fecal matter density.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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مقارنة الطرق المختلفة لتشخيص طفيل الأبواغ الخبيئة في براز العجول باستخدام التقنيات المجهرية والمصلية والجزيئية

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الخلاصة

يعد طفيل الأبواغ الخبيئة هو مرض رئيسي في الجهاز الهضمي في العجول. فحصت هذه الدراسة ١٥٠ عجلاً مصاباً بالإسهال من المنطقة الشرقية من المملكة العربية السعودية باستخدام طرق تشخيصية مختلفة، بما في ذلك الفحص المجهري باستخدام صبغة الزيل نيلسون الساخنة، تحليل الاليزا، وتفاعل السلسلة المتبلمرة. تم تقييم أداء هذه الطرق في الكشف عن طفيل الأبواغ الخبيئة في عينات براز العجول من خلال اختبارات دقة التشخيص. حددت هذه الاختبارات أكياس بيض طفيل الأبواغ الخبيئة في ٤٠ عينة من العجول (٦٦,٢٦٪) بواسطة صبغة الزيل نيلسون الساخنة، ٦٠ (٤٠٪) بواسطة تحليل الاليزا و ٧٨ (٥٢٪) بواسطة وتفاعل السلسلة المتبلمرة. كان للفحص المجهري خصوصية أعلى ٦٥,٢٧٪عن تحليل الاليزا ٥١,٣٨٪ بينما تحليل الاليزا كان له حساسية أعلى (٣٢,٠٥٪) من صبغة الزيل نيلسون الساخن (١٩,٢٣ ٪) ؛ ومع ذلك، كانت كل من طرق صبغة الزيل نيلسون الساخن وطريقة الاليزا اختبارات تشخيصية غير مرضية مقارنة بالتقنية القياسية الذهبية الى تفاعل السلسلة المتبلمرة حيث كانت المنطقة الواقعة تحت قيم المنحنى في تحليل خصائص مشغل جهاز الاستقبال أقل من ٦,٠ علاوة على ذلك، لم يكن هناك اتفاق (اختبار كابا) بين صبغة الزيل نيلسون الساخن المجهري وتقنيات الاليزا مقارنة مع تفاعل السلسلة المتبلمرة عند مستوى ثقة إحصائي ٠,٠٥.