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COMPARE MICROSCOPY STAINING AND POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF CRYPTOSPORIDIUM INFECTION AMONG FRISIAN CALVES IN MINUFIYA GOVERNORATE

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ABSTRACT

Cryptosporidiosis infection could be considered as a causative agent of diarrhea in 60.69 of pre-weaned calves. Diarrhea in beef cattle has an important impact in economic productivity. Infectious diarrhea is mainly caused by four agents: Enterotoxigenic Escherichia Coli (ETEC), Rotavirus, Corona virus and Cryptosporidium parvum. The aim of the study was to compare microscopy staining method and polymerase chain reaction (PCR) as methods for diagnosis of Cryptosporidium species. Fecal samples were collected from three hundred and seventy one naturally infected Friesian calves. and modified trichrome blue. The obtained result showed incidences of cryptosporidium in naturally infected Friesian calves, 117 (31.67%), 127 (34.33%) and 116 (31.33%) out of 371 by Modified Ziehl-Neelsen technique, Safranin-methylene blue and modified trichrome stains respectively. Safraninh-methylene blue staining is better technique if compared with Modified Ziel-Neelsen technique and modified trichrome technique from 47 postive calves. Safranin-methylene blue staining, Cryptosporidium spp.was found in 20 fecal samples from 47 Friesian calves aged (1-3 months). By using PCR, cryptosporidium specific bands were found in 22out of 47 calves of these cases. It can be concluded that microscopic staining method, which can be used as a diagnostic method has some limitations, while, PCR is more sensitive and specific, which allows the identification of parasite oocyst.

Keywords: Cryptospordium, Modified Ziehl-Neelsen, Safranin, Trichrome methylene blue, PCR.

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1. INTRODUCTION

Cryptosporidiosis is caused by a protozoan parasite and it is one of several pathogens causing neonatal diarrhea in calves (Tzipori, 1983).

Cryptosporidial infections were reported among cow calves in Egypt for the first time (Iskander, 1985).

Cryptosporidium parvum mostly infects the intestine of the neonatal calves and cause short term diarrhea and shedding oocyst only 1-2 weeks (Naciri et al., 1999). It was concluded that, the C. parvum constitutes the major etiological agent of neonatal diarrhea (Naciri et al.,1999). Because of high number of oocysts are shedding during parturition, neonatal calves acquired the infection mainly at birth (Faubert and Litvinsky, 2000). The appearance of enteropathogenic E.coli associated with cryptosporidium may

increase the mortality rate in calves affected with diarrhea (Nagy et al., 1980). The first time in Egypt, cryptosporidium oocysts were detected by using Modified Trichrome stain (Hammouda et al., 1996). The oocysts in the faeces of calves were identified by using modified Ziehl-Nelsen stain and appeared as spherical in shape within a clear halo and their size ranged from 5-7 µ diameters (Otify et al., 1993 and Abdel-Salam et al., 1993). Oocysts were collected by concentration floatation technique and appeared as tiny subspherical, colourless bodies with thick wall. Safranin, Giemsa, methylene blue-eosin and modified Ziehl-Neelsen to stain the faecal smears. The dimensions of the detected oocysts were 2.8-5 x 3.2-5.8 µ (El-Akabaway, 1993). It was recorded that Safranin-methylene blue stain with 0.5% methylene blue as a counter stain was not only more simple and rapid but also more sensitive than the modified Ziehl-Neelsen technique. Safranin-methylene blue stain was recommended for diagnosis of cryptosporidiosis (Bogaerts et al., 1984). The detection of cryptosporidium with Safranin methylene blue stain was nearly similar to modified Ziehl-Neelsen stain while both staining were accurate than the Giemsa stain (Khalil, 1993). Polymerase Chain Reaction (PCR) was used for detection and identification of Cryptospordium species from faecal samples due to its sensitivity and specificity (Amar et al., 2004). Molecular methods such as polymerase chain reaction followed by restriction fragment length polymorphism (RFLP) or gene sequencing are useful for identification of Cryptospordium species and genotypes. A multiplex PCR assay was developed for detection of four species of Cryptosporidium that commonly infect cattle (Kirkpatatrick and Farrell 1984).

2. MATERIALS AND METHODS

2.1. Animals:

Between December 2011 and November 2012, this study was conducted on 371 Friesian calves from different localities at Menufyia Governorate (Table I). The animals were exposed to full parasitological and clinical examination. The examined calves were classified into 2 groups.

Group 1: 261 Friesian calves of less than one month old (122 calves up to 10 days, 74 calves of 11-19 days and 65 calves from 20-29 days)

Group 2: One hundred and ten Friesian calves aged one month to less than 3 months.

The two animal groups were sub-grouped according to the consistency of the feces as follows: Non diarrheic calves: 134 Friesian calves and Diarrheic calves: 237 Friesian calves.

2.2. Faecal samples:

Fecal samples were collected from Friesian calves. Macroscopic and Microscopic examinations were applied on each fecal sample. The feces were filtered through two layers of gauze to remove the coarse particles and stored in an equal amount of 2.5% potassium dichromate solution at 4°C till the time of examination (Santin and Zarlenga, 2009).

2.3. *Clinical examination of investigated calves:*

All animals under investigation were exposed to full clinical examination according to (Radostits, et al., 2007).

2.3.1. Macroscopic examination:

it was carried out to detect the abnormalities in consistency, color of the feces and presence of other abnormalities.

2.3.2. Microscopic examination:

The fecal samples were examined by ordinary direct smear method and saline smear method; these methods were carried out according to (Belding, 1952).

2.3.3. Staining procedures:

The fecal samples were subjected to Modified Ziehl-Neelsen staining technique. This technique was performed according to (Casemore et al., 1985a). Briefly, the procedure as follows. The dried fecalfilms were fixed by methyl alcohol for 3 minutes. The fixed slides were immersed in concentrated cold carbol fuchsin for 15 minutes. The slides were then rinsed with tap water for 2 minutes, decolourized with 3% acid alcohol 10-15 seconds, then rinsed in tap water for 2 minutes. Counter-staining with 0.4% malachite green was done for 3 seconds, finally rinsed with tap water and air dried. As well as this slid another smeared fecal slids were subjected to Safranin- methylene blue Technique. This technique was performed according toBaxby et al., (1984). The modified trichrome staining technique was applied on the same fecal samples according to Weber et al., (1992).

2.3.4. Examination and measurement of the oocysts:

The stained slides were examined by the light microscope using high power (40x) and oil immersion lens (l00x). The Measurements were made by ocular micrometer calibrated against a stage micrometer slide (OIE, 2008).

2.3.5. DNA Extraction

Forty seven fecal samples were obtained from infected Feresian calves (1-3 months age) containing oocysts. Oocysts were concentrated from feces by Potassium dichromate washed off fecal specimens with distilled water by centrifugation at 1500 xg for 10 minutes at room temperature. Genomic DNA was extracted from 200 mg of each specimen using QIAamp DNA Mini

Stool Kit (Qiagen, Clinilab, Egypt) (Usluca and Akosy 2011). DNA was extracted in accordance with the procedures suggested from the manufacture Usluca and Akosy (2011)

2.3.6. Polymerase chain reaction (PCR):

The primers were used for detection of Cryptospordium spp. oocyst of the gene Cryptospordium Oocyst Wall Protein (COWP) which are Cry9 (5'-GACTGAATACAGGCATTATCTTG-3') and Cry 15 (5'-GTAGATAATGGAAGAGATTGTG-3') (Amar et al., 2004). The PCR reaction was 50 ul which contained the mixtures of 10 ul of DNA sample, GoTaq Green Master mix of 2x, 25 ul, upstream primer ,10uM 1 ul, down stream primer 10 uM, 1 ul, then adjust the volume to 50 ul by adding 13 ul of Nuclease free water (Promega). PCR was performed under the following conditions: 35 cycles at 94 oC for 1 min, 55 oC for 30 sec, and 72 oC for 1 min, followed by 72 oC for 10 min. Positive and negative controls were included in each batch of tests. A 10 ul aliquot of PCR product was analyzed for COWP gene (550 bp) frgments by electrophoresis in 1% agarose/ethedium bromide gels.

3. RESULTS

3.1. Clinical Examination

Diseased Frezian Calves showed watery diarrhea of whitish or yellowish coloration. Fever, anorexia, depression, tenesmus, colic and emaciation , while some of them revealed normal feces.

3.2. Parasitological examination of the fecal samples:

3.2.1. Macroscopic examination:

Most of the fecal samples which were collected from calves 56.32% showed watery diarrhea, sometimes mixed with blood, mucous and whitish to yellowish in coloration.

3.2.2. Microscopic examination.

The oocysts isolated from the feces of naturally infected calves were fully stained

by modified Ziehl-Neelsen technique. The oocysts were spherical to ovoid in shape and the wall was smooth. It contains 4 sporozoites and a residuum. The measurement of oocysts was varied from 3.7-5.4 x 4.5-5.6 Urn (mean 4.6 x 5, 4 Urn).

The oocysts appeared as acid fast (red-pink) on a blue background, By using Safranin-methylene blue staining technique, the oocysts appeared orange in color on a blue background, while the oocysts appeared pink with dark granules when stained by Modified trichrome (Figure 1).

3.2.3. Incidence of cryptosporidium in naturally infected Frisian calves

The results revealed that 117 (31.67%), 127 (34.33%) and 116 (31.33%) out of 371 Friesian calves examined by Modified Ziehl-Neelsen technique, Safranin-methylene blue and Modified trichrome stains were infected with C. oocysts respectively. (Table I).

3.2.4. The relation between the incidence of cryptosporidium infection and the age of Frisian calves.

The present study showed that out of 190 and 47 diarrheic calves of less than one month and 1-3 months only 102 (54%) and 10 (21.3%) were infected with cryptosporidium oocysts respectively. While examination of 71 non diarrheic faecal material from calves of less than one month revealed that 5 (7.5%) had cryptosporidium infection. In addition C. oocysts were detected in 3 (4.7%) out of 63 non diarrheic faecal materials from calves aged 1-3 months; (Table1). The incidence of cryptosporidium infection among Friesian calves using three different stains was shown in (Table 2 and Figure 1).

PCR products which were produced by using primers designed according to COWP gene of Cryptospordium parvum and the size of the fragment was 550 bp. Diagnosis of cryptosporidium parvum by PCR was more accurate and sensitive than microscopic diagnosis which were (46.8%), 42.55%) respectively in young age calves as shown in Table 3 and Figure 2.

4. DISCUSSION

Cryptosporidium spp. mostly infect the intestine of the neonatal calves and cause short term diarrhea. It was concluded that, the *C. parvum* constitutes the major etiological agent of neonatal diarrhea (Naciri et al., 1999). Modified Ziehl-Neelsen , Safarnin methylene blue, and Trichrome methylene blue were used to stain Oocysts which were collected by concentration floatation technique (El-Akabaway1993). Safranin methylene blue is more simple and rapid as well as, more sensitive 34.33% if compared with Modified Ziehl-Neelsen and Trichrome methylene blue 31.67% , 31.33% respectively.Bogaerts et al,(1984) reported that Safranin was used and sensitive for diagnosis of cryptosporidiosis. Molecular methods can be used to diagnose Cryptospordiosis in fecal samples in calves and stool samples in Humans. The sensitivity of PCR method is about 20 oocysts in 1 ml of stool sample (Lind). For Genotyping of *Cryptospordium* spp, COWP and 18S rDNA genes are frequently used. In our study work the primers of *Cryptospordium* wall protein coding gene (COWP) was used. This gene has high sensitivity and selectivity Morgan et al, (1998) they were mentioned that PCR was considered as an alternative method to microscopic examination. A comparison with PCR the percentage of positive cases was 22 while in microscopic staining method was 10 out of 47 calves fecal samples respectively. In the light of these results, selectivity Weber et al, (1992),

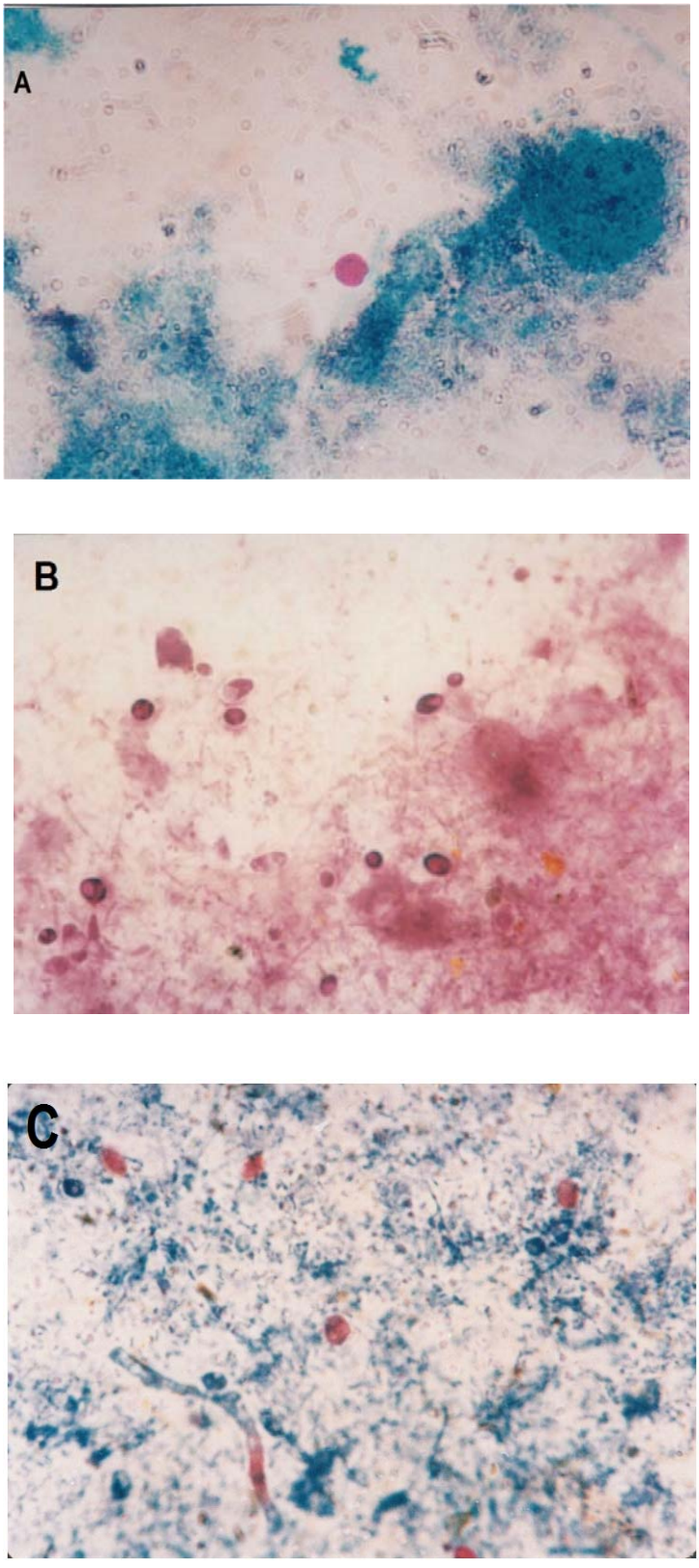

Fig. 1. (A): Modified Ziehl-Neelsen, (B): Safranin- methylene blue, (C): modified trichrome staining

Table (1): The incidence of cryptosporidium infection among Frisian calves in fecal samples by using microscopic staining technique.

Calves and their ages	Faecal consistency					
	Diarrhoeic			Non Diarrhoeic		
	No. of examined calves	Positive	Percentage	No. of examined calves	Positive	Percentage
less than one month	190	107	56.32	71	7	9.89
1-2 month	30	8	26.67	39	2	5.13
2-3 month	17	3	17.65	24	1	4.17
Total	237	118	49.79	134	10.8	7.46

Table (2): The incidence of cryptosporidium infection among Friesian calves by using three different stains.

Animal	Total number of fecal samples	Modified zeal nelson		Safranin methylene blue		Modified trichrome	
		Positive numbers	Percentage %	Positive numbers	Percentage %	Positive numbers	%
Friesian calves	371	117	31.53	127	34.23	116	31.36

Table (3): The incidence of cryptosporidium infection among frisian calves by using PCR and Microscopic staining techniques.

Technique	Number of examined samples	No. of positive samples	%
PCR	47	22	46.8
Microscopic	47	20	42.55

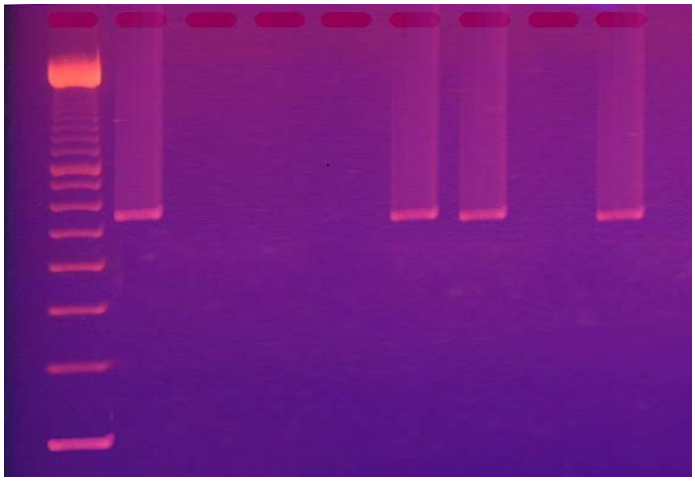

Fig .2. 1% Agarose Gel electrophoresis of PCR products produced by using primers designed according to COWP gene of of Cryptospordium spp. Lane 1, 100 bp DNA Ladder (marker) ranged from 100 to 2000 bp, Lane 2, Positive control, Lane 3, Negative control, Lanes 4, 5 and 8 are negative while, Lanes 6, 7 and 9 are positive and the size of fragment 550 bp.

Morgan et al, (1998)and Usluca, (2011) agree with our result It is concluded that PCR is a reliable method for identification of spp and could be used in place of the Microscopy method.

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مقارنة آستخدام الصبغات الميكروسكوبي وتفاعل البلمرة المتسلسل في تشخيص عدوى الكربتوسبرديوم بين العجول الفرزيان في محافظة المنوفية

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الملخص العربي

الإصابة بالكريبتوسبوريديوم تعتبر من أحد العوامل المسببة للإسهال في العجول الفريزيان قبل الفطام .حيث نسبة الإصابة بسبب الكريبتوسبوريديوم تصل الي .60% .69والأسهل في عجول التسمين له تأثير اقتصادي علي الإنتاج .الإسهال المعدي يسببه أربع ميكروبات أساسية وهي أي كولاى، فيروس الروتا، فيروس الكورونا، والكريبتوسبوريديوم .والهدف من هذ البحث دراسة المقارنة بين الصبغات المختلفة باستخدام الميكروسكوب وتفاعل البلمرة المتسلسل في تشخيص الكريبتوسبوريديوم .عينات البراز تم تجميعها من 371عجل فرزيان مصابة طبيعيا بالكريبتوسبوريديوم .تم صبغ شرائح من مسحات برازيه باستخدام زيل نيلسون المعدلة وظهرت البويضات المتحوصلة حمراء-دم غزال والخلفية زرقاء وكذلك عند استخدام صبغة السفرانين ظهرت البويضات المتحوصلة برتقالية اللون والخلفية زرقاء داكنة ولكن عند ما تم صبغ البويضات المتحوصلة بصبغة ثلاث كروم ظهرت بلون دم الغزال .وأظهرت هذه الدراسة أن نسبة الإصابة بالكريبتوسبوريديوم في العدوى الطبيعية في عجول الفرزيان باستخدام الصبغات الثلاث 117 بنسبة (31.67٪ (و 127بنسبة 34.33)٪ (و 31.33) 116٪ (من أ جمالي 371عجل فريزيان عندما صبغت بزيل نيلسون المعدلة والسفرانين الازرق والثلاث كروم على التوالي .وتعتبر صبغة السفرانين أفضل من زيل نيلسون المعدلة وثلاث كروم في تشخيص الكريبتوسبوريديوم .باستخدام صبغة السفرانين الازرق تم تشخيص 10حالات إصابة بالكريبتوسبوريديوم من 47عجل فرزيان عمره 3-1)شهور (، بينما باستخدام تفاعل البلمرة المتسلسل تم تشخيص 22حالة من إجمالي 47عجل فرزيان .ويمكن تلخيص ذلك بأن استخدام طريقة الصبغة الميكروسكوبي لها حدود في التشخيص بينما طريقة تفاعل البلمرة المتسلسل حساس وخاص ويشجع معرفة وتحديد البويضة المتحوصلة الكريبتوسبوريديوم.

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