Field and laboratory diagnosis of *Cryptosporidium parvum* and *Escherichia coli* in newly born buffalo calves suffering from diarrhea

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Abstract
The present study was carried out on 97 faecal samples from newly born buffalo calves (1 week- 3 month) in two private farms at Giza governorate in Egypt. All samples were examined for evidence of *Cryptosporidium* spp. and *E. coli* as czauses of diarrhea. Immunochromatographic (IC) method by RIDA Quick *Cryptosporidium* coproantigen detection kit was used. *Cryptosporidium* infection was detected in 21(21.6 %) by using IC. Infectivity rate of *Cryptosporidium* was affected by the age of calves. Calves (1-3 weeks) showed the higher rate of incidence than the older ones (1-3 month). Also the infectivity was higher in diarrheic calves more than apparently healthy calves. Also all samples were cultured bacteriologically on ordinary and specific medium. All the isolates were identified morphologically and biochemically. The present study revealed the presence of *E. coli* with a percentage of 23.7 %. All the isolated strains were identified serologically. Only 15 out of 23 strains were typed serologically as O25:K11, O55: K59, O124: K72. In addition, 12 (12.4 %) samples were positive for both *Cryptosporidium* and *E. coli*. The combination of *Cryptosporidium* and *E.coli* were present especially in young newly born calves. *Cryptosporidium* and *E.coli* infection were two of the important causes of diarrhea in newly born calves and RIDA Quick antigen detection test proved to be a useful mean for diagnosis of cryptosporidiosis in fresh faecal samples because it was rapid, sensitive and simple to perform.

Key words: *Cryptosporidium , E .coli - Diarrhea in calves, Coproantigen detection*

Introduction

Diarrhea is a complex disease in human and causes a significant economic impact in animals due to the heavy losses. The infection can be in combination with other micro-organisms **(Acres *et al*., 1999).**

Diarrhea in newly borne calves causes many economic losses directly through the high mortality and the need for expensive treatment and indirectly as poor growth after repeated clinical disease conditions and treatment **(Gomez – Cousa *et al*., 2005)**.

Cryptosporidium spp. affects the digestive tract of many vertebrates, including humans, farm animals (cattle, sheep, goat, and horses), pets (dogs and cats), laboratory animals (rats and mice) and birds. *Cryptosporidium* spp. grow and multiply in the microvillus borders of the enteric epithelium cells. The parasite may additionally infect other tissues such as the respiratory and renal epithelia, especially in immune compromised humans **(Mercado *et al.,* 2007).** When oocysts are ingested by a suitable host, the endogenous phase of the parasite life cycle begins by invasion of the target cells. The sporulated oocyst is the only exogenous stage in the life cycle of the parasite and being excreted with the faeces of an infected host which is highly relevant for diagnosis of *Cryptosporidium* infection **(Fayer and Xiao, 2007).**

Transmission of *Cryptosoporidium*, both within and between host species including humans, is by faecal oral spread of the environmentally resistant oocysts, which are fully sporulated and infective when excreted in faeces **(Abebe *et al*., 2008).**

Laboratory diagnosis of Cryptosporidiosis is usually achieved by microscopic detection of *Cryptosporidium* oocysts in stool specimens; staining techniques include acid fast stains and immunofluoresence **(Schnyder *et al*., 2009)**. Microscopy is time consuming and required well trained observer to identify the organisms. In an attempt to establish sensitive and cost-effective methods to diagnose intestinal infections with *Cryptosporidium*, a number of coproantigen tests have been developed based on the detection of parasite antigens **(Regnath *el al*., 2006).** These tests are rapid to perform manually during a time of 1 to 2 minutes and results are obtained within 9 to 10 minutes. Moreover, it does not require experienced staff or special equipments **(El- Helaly 2012)**.

Escherichia coli among other enteric bacteria was the main encountered microorganism in calves diarrhea **(Aziz and Daph, 2005)**. *Escherichia coli* diarrhea is considered as one of the major problems fac-

ing live stock production in Egypt. **(Refai, 1980)**. Diarrhea due to *Escherichia coli* is one of the most common diseases of young calves, despite vaccinations programs and management measures, necessitating treatment with antibiotics and fluid therapy **(Gyles, 1993)**. *Escherichia coli* diarrhea in newborn calves is usually characterized by watery white or yellowish diarrhea, rapid onset and short time course with high mortality **(Abd-Elrahman, 2011)**.

The aim of this study was to diagnose *Cryptosporidium* spp. by commercially qualitative rapid coproantigen test by RIDA® Quick *Cryptosporidium* kit, as well as laboratory diagnosis of corresponding *E.coli* infection and detection of virulence activities of *E.coli* isolated strains.

Material and Methods

Collection of samples:

Ninety- seven faecal buffalo calves samples were collected from two private farms (F1 and F2) at Giza Governorate in Egypt. These farms have problems of diarrhea. The samples were collected under aseptic condition in sterile disposable plastic bags that were closed tightly and labelled by their age, place, date of collection and the clinical status of the animal, diarrheic or nondiarrheic (apparently healthy) and sent to the laboratory as quick as possible for the parasitological and bacteriological investigations.

These samples (97 samples) consisted of 68 diarrheic and 29 nondiarrheic (apparently healthy) samples, classified according to age (Table 1).

Table (1): Number of faecal samples collected from buffalo calves conducted for parasitological and bacteriological examination in two farms.

Age	Farm 1		Farm 2	
	Diarrheic	Non Diarrheic (apparently healthy)	Diarrheic	Non Diarrheic (apparently healthy)
1 Week	9	4	8	3
2 Weeks	8	3	6	3
3 Weeks	7	4	7	2
1 month	4	3	4	1
2 months	2	2	5	1
3 months	6	1	2	2
Total	36	17	32	12

Parasitological examination:

The 97 collected faecal samples (68 diarrheic and 29 nondiarrheic) were tested to detect the antigen of *Cryptosporidium* spp. by using a commercially qualitative immunochromatographic (IC) assay by RIDA® Quick *Cryptosporidium* kit (R-Biopharm Ag, Germany). The test was performed according to **Ghattas (2012).**

Bacteriological examination:-

All faecal samples were directly streaked onto the surface of blood agar, nutrient agar, Macconkeys agar and eosin methylene blue (EMB) agar plates. The inoculated plates were incubated at 37oC for 24 – 48 hours and examined for bacterial growth. Suspected colonies on different media were subcultured, purified and subcultured in semisolid agar and kept as stock culture. Pure culture colonies were studied for their morphological, colonial appearance according to **Koneman *et al*., (1997)**.

Biotyping of the isolates:

Biotyping of isolates was determined according to **Burrows (1985)** by using of carbohydrated fermentation, decarboxylation of lysine and ornithine and hydrolysis of esculin tests (Zaki and El-Mahrouk, 2005)

Serological identification of enterotoxigenic *E coli :*

Serological identification for *Escherichia coli* isolates was carried out using slide agglutination test for both O and K antigen. This identification was officially and kindly done by Serological Department, Bacteriological Laboratory, Ministry of Health, Cairo, Egypt. .

Virulence factors of *E.coli* isolates:-

Haemolytic activity: It was observed by blating isolated *E.coli* on sheet blood and recorded as α, β haemolysis and non haemolytic.

Congo red (CR) Binding test: All covered *E.coli*

isolates were tested for its growth status on congo red medium according to **Berkhoff and Vinal (1986).** Plates were incubated at 37°C for 24 hours, after wards were left at room temperature for additional 2 days. Congo red positive (CR+) *E.coli* strain was indicated by growth of red colonies .

Detection of enterotoxigenic *E.coli* isolates: AII isolates of *E.coli* were tested for their ability to produce heat stable enterotoxin (ST) by mouse suckling technique.

Samples preparation for recovery of enterotoxin :The isolated *E .coli* strains were grown over night in brain- heart infusion broth and then inoculated in culture medium (10 ml of casamino acids yeast extract medium) in a 250 ml Erlenmeyer flask for production of toxins according to **Evans *et al* . (1973)**. After inoculation, the flasks were incubated with shaking on rotatory shaker at 37°C for 24 hours. The culture fluid was centrifuged at 250 xg for 30 min. at 4 ◌̊C. The supernatant was taken and subjected to heat treatment (80° C for 30 min .) before storing at – 70 ◌̊C for detection of heat stable enterotoxin (ST) according to Giannella (1976) .A 0.1 ml. of culture filtrate was injected through the abdominal wall into the milk filled stomach of 3 infant mice (2-4 days old). Another 3 infant mice were injected with 0.1 ml. saline as a negative control. All mice were kept for 3 hours then were scarified. The ratio of the intestines weight to the remaining body weight was determined. A ratio greater than 0.083 was recorded as positive **(Dean, 1972).**

Results

Parasitological results :-

Examination of the 97 faecal samples by the ″ RIDA® Quick *Cryptosporidium* kit″ revealed that the incidence of *Cryptosporidium* spp. was 21 samples (21.6%) The distribution of positive cases in accordance to the farms and calves age are given in table (2) .

In farm 1, the present study showed that the infection rate in buffalo calves was 25 % in 9/36 diarrheic and 11.8% in 2/17 non diarrheic apparently healthy calves. While in farm 2, the present study recorded that the infection rate in buffalo calves was 25% in 8/32 diarrheic and 16.7% in 2/12 of non diarrheic apparently healthy calves.

The positive and negative results of the RIDA® Quick *Cryptosporidium* kit are shown in Fig (1). Positive test showed the control blue band and red (positive) band, while negative sample showed the control blue band only.

Table (2): Incidence of *Cryptosporidium* spp. infection using RIDA® Quick *Cryptosporidium* kit.

Age	Farm 1		Farm 2	
	Diarrheic	Non diarrheic	Diarrheic	Non diarrheic
1 Week	3/9	1/4	2/8	1/3
2 Weeks	2/8	0/3	2/6	0/3
3 Weeks	2/7	1/4	2/7	1/2
1 month	1/4	0/3	1/4	0/1
2 months	0/2	0/2	1/5	0/1
3 months	1/6	0/1	0/2	0/2
Total	9/36 (25 %)	2/17 (11.8%)	8/32 (25 %)	2/12 (16.7%)

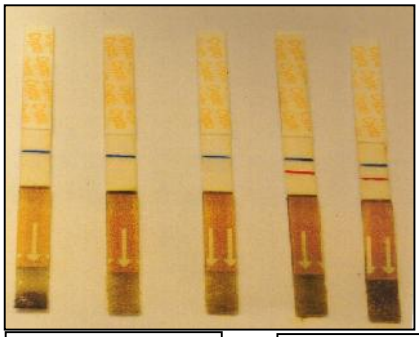

Fig. (1). **(A) Negative test:** showing control blue band only.
(B) Positive test: showing control band (blue band) and test band (red band).

Bacteriological results:

Out of 97 faecal samples 23 (23.7%) strains of *E.coli* were isolated from farm (1) and farm (2) (table 2). Out of *E .coli* strains 15 (65.2 %) were serotyped in three groups. O25 :K11 , O55:K59 ,O124:k72 with a number of strains 4,7,4 respectively. .The rest 8 (34.8%) strains were untyped (table 4).

Results of haemolysin activity for serotyped *E. coli* strains are shown in table (5).

A total of 10 strains out of 23 tested isolates (43.5 %) showed positive results. Results of Congo red activity of serotyped *E.coli* strains are shown in table (5) A total of 7 strains out of 23 tested isolates (30.4%).

Testing the enterotoxin production (ST) of the same isolates showed that 13 isolate were positive out of 23 strains (56.5%) tested as shown in table (5).

In addition 12 (12.4%) samples were positive for both *Cryptosporidium* and *E.coli*. The combination of *Cryptosporidium* and *E.coli* were present especially in young newly born calves. Ten samples at 1 – 3 weeks and 2 samples at 1 – 3 month were positive for both *Cryptosporidium* and *E.coli* in a percent of 10.3 % and 2.1 % respectively.

Table (3). Incidence of *E.coli* infection in the two farms.

Age	Farm 1	Farm 2
1 week	4/13	3/11
2 weeks	2/11	2/9
3 weeks	2/11	6/9
1 month	2/7	1/5
2 months	0/4	1/6
3 months	0/7	0/4
Total	10/53 (18.9%)	13/44 (29.6%)

Table (4). Distribution of *E.coli* serotypes in farms in relation to the age of calves.

Age	O25		O55		O124		Untyped	
	F1	F2	F1	F2	F1	F2	F1	F2
1 Week	1	--	1	1	1	--	1	2
2 Weeks	--	--	--	--	1	1	1	1
3 Weeks	--	1	1	3	--	--	1	2
1 month	1	--	1	--	--	1	--	--
2 months	--	1	--	--	--	--	--	--
3 months	--	--	--	--	--	--	--	--
Total	2	2	3	4	2	2	3	5

Table (5). Virulence factors of *E.coli* isolates .

Serotypes	No.of isolates	Haemolysin (+V)	Congo red (+V)	Enterotoxin (+V)
O25: k11	4	2	2	2
O55: k59	7	3	4	5
O124: k72	4	2	1	4
untyped	8	3	0	2
Total	23	10 (43.5%)	7 (30.4%)	13 (56.5%)

Discussion

A complex variety of factors considered as life threatening causes for animals. Environment immunity and microbial contamination play an important role in disease condition such as diarrhea. The diagnosis of the etiological agent of diarrhea can only be performed by laboratory examination because of the variety of causative agents **(Cic *et al.*, 2008).**

In the present study, the incidence of *Cryptosporidium* spp. infection among buffalo calves was 21 (21.6%) by the immunochromatographic (IC) method using RIDA® Quick *Cryptosporidium* kit. The infectivity rate of *Cryptosporidium* was affected by the age of calves and the clinical status of the faecal samples (diarrheic and non diarrheic). Calves (1 – 3 weeks) shed the higher rate of incidence (31.2%)

in diarrheic and 30% in non diarrheic while, the older ones 1-3 months showed the lowest rate of infection (17.3% in diarrheic and 10% in non diarrheic).

The present results came in harmony with **Liorente *et al.,* (2002).** They stated that IC assay is more sensitive for the detection of *Cryptosporidium* infection. They found the sensitivity and specificity were 98% and 100% respectively. **Luginbiihl *et al*., (2005)** applied the IC rapid test in the field for the diagnosis of *Cryptosporidium* infection in calves and compared their results to that obtained with Modified Ziehl Neelson stain (MZN) and Auramine (AU) method. They found the IC method had a very high diagnostic specificity 100% and diagnostic rate was 75%. Also, **Lanz Uhda *et al.,* (2008)** used the fastest strips for the diagnosis of *Cryptosporidium* in faecal samples of 147 calves. They found the incidence of infection was 55%. They stated that the commercial test for field use is rapid and satisfactory for the detection of *Cryptosporidium* in faecal samples.

Diaz – Lee *et al.,* (2011) stated that the combination of stain and IC methods might give an excellent sensitivity level and confidence for *Cryptosporidium* detection in calves. The diagnostic efficiency of the staining (traditional) methods is extremely operator dependent. The IC test is an alternative for rapid and operator independent diagnosis of cryptosporidiosis, although more expensive.

The present results showed that *E.coli* were recovered from samples with incidence of 23.7%. These results agree with that reported by **Aziz** and **Daph (2005)** which is 23.2% and with that reported by **Tanios *et al*., (2000)** and **El-shabury *et al*., (1999)** who found that E.coli is 24.4% and 24.7% respectively. The present results disagreed with results reported by **Abd El Rahman (2011)** who found that the incidence of *E.coli* diarrhea is 39.23%. *Escherichia c0li* is considered as one of the major problems all over the world **(Pohl *et al*., 1992)** and also in Egypt **(Refai, 1980).**

Serologically, out of 23 *E.coli* strains from samples, 4 strains were serotyped as O25: K11, 7 strains were serotyped as O55: K59, 4 strains were serotyped as O124: K72. The rest 8 strains were untyped with the available diagnostic sera. These results agreed with **Aziz** and **Daph (2005)** who isolated O55:K59 from diarrheic cases. Also, the present results agreed with **Rajkhowa *et al.,* (2009)** and **Sharma *et al*., (2004)** as they isolated O25: K11 and O55: K59 .

It is interesting to mention that the incidence of haemolysin activities in *E.coli* showed 43.5% which agreed with available data from **Aziz** and **Daph (2005)** and **El Shaboury *et al.,* (1999).**

Congo red dye could be used as a detective of virulence for *E .coli* strains and distinguish between virulent and avirulent *E.coli* strains . The present results showed that 30.4% of the isolated *E.coli* could bind actively Congo red and this observation coincides with that mentioned by **El -Mahrouki *et al.,* (2006).**

Out of 23 recovered *E.coli* strains tested by infant mouse test 13 were toxigenic with an incidence of 56.5 %.This result relatively agreed with that mentioned by **Aziz** and **Daph (2005)** who stated incidence of 71.8% and with the previous result reported by **El-Shabaury *et al*., (1999).**

The combination of *Cryptosporidium* and *E.coli* were present especially in young newly born calves. *Cryptosporidium* and *E.coli* infection were two of the most important causes of diarrhea in newly born calves. The rapid antigen detection test RIDA proved to be a useful mean for diagnosis of cryptosporidiosis in fresh faecal samples because its quickness, sensitivity and simplicity to perform.

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التشخيص الحقلى والمعملى لطفيل الكريبتوسبوردیا بأرفام والميكروب القولونى فى العجول الجاموسى حديثة الولادة التى تعانى من الاسهال

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الملخص العربى

في هذة الدراسة تم تجميع 97 عينة براز من العجول الجاموسى حديثة الولادة من مزرعتين بمحافظة الجيزة تمفحص جميع العينات لكل من طفيل الكريبتوسبوردیا بارفام والمكروب القولونى و هما من أهم الامراض المسببة للاسهال فى العجول حديثة الولادة .

تم فحص جميع عينات البراز لطفيل الكريبتوسبوردیا بارفام بطريقة الاستشراب اللونى المناعى السريع (الكروماتوجرافي المناعي) للكشف عن الانتجين فى عينات البراز . أظهرت النتائج ان نسبة الاصابة لطفيل الكريبتوسبوردیا بارفام (21.6%) وتعتبر طريقة الاستشراب اللونى المناعى السريع طريقة حديثة وسريعة وموفرة للوقت واكثر حساسية لانها تستخدم طريقة للكشف عن الانتجين الموجود فى عينات البراز.

يمكن استخدام هذه الطريقة فى الحقل وذلك لسرعة التشخيص وبالتالى سرعة العلاج لتقليل النفوق الذى يحدث فــى العجول حديثة الولادة.

تــم في هذة الدراسة عـــزل الميكروب القولونى من عينات البراز فى عدد 23 عينة بنسبة(23.7%)منهم 15عينة تم تصنيفهم الى ثلاثة انواع سيرولوجية هى O124:k72, O55:K59, K11: O25وقد تم اجراء الاختبارات الممرضة للمعزولات المشخصة سيرولوجيا و ذلك للتمييز بين العترات الممرضة و الغير ممرضة واظهرت النتائج ان عدد 10 عينات بنسبة 43.5% لها القدرة على ذوبان الدم .وعدد 7 إيجابى بنسبة 30.4% لإختبار صبغة الكونغو الحمراء. كما جرى إختبار المعزولات للقدرة على إفراز السموم المقاومة للحرارة باستخدام اختبار الفئران الرضيعة و اظهرت النتائج أن 13عتره إيجابية بنسبة 65.5%.ومن خلال هذه الدراسة يتضح اهمية الكشف السريع عن مسببات الاسهال فى العجول حديثة الولادة التى منها طفيل الكريبتوسبورديام و الميكروب القولونى .