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CRYPTOSPORIDIUM PARVUM IN CLINICALLY EXAMINED DAIRY CALVES; MOLECULAR AND BIOCHEMICAL STUDIES

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Abstract

Cryptosridiosis is a life threatening illness of neonatal calves, leading to mal-absorption and diarrhea. The present study was carried out on 25 Holstein dairy calves, 3 to 15 days old in El-Eman farm, Ismailia Governorate, Egypt. Animals were assigned into two groups; G1: included 20 calves suffered from watery to mucoid diarrhea and G2: included 5 calves were proved to be clinically healthy and used as a control one. The diseased calves were treated by oral administration of Halofuginone at a dose rate of 100 µg per kg once daily for 7 successive days. A total number of 90 blood and fecal samples were obtained from all animals during the study; 40 blood samples from diseased calf (at the onset of the appearance of the symptoms and on the 8th day of the treatment), 40 fecal samples at the same pattern from the same animals and 10 samples (blood and feces) were collected from the control one. Forty-five blood samples were analyzed for serum enzyme activities of ALT, GGT and LDH and for serum levels of urea, creatinine, total protein, albumin, globulin, IgA, TC, TGs, HDL-C and LDL-C. The later, forty-five fecal samples were examined to detect *Cryptosporidium* infection utilizing ICT, microscopical and molecular identification based on *18S rRNA* Gene. Clinically infected dairy calves showed clinical signs of profuse watery diarrhea with yellowish colored feces (40%) and other calves showed mucoid diarrhea (60%) which persist for 7 days resulting in dehydration, weakness and loss of body weight. The study revealed a significant increase in levels of ALT, GGT, LDH and urea with a highly significant reduction in concentration of IgA in the diseased calves. Meanwhile, a non-significant change in levels of total protein, albumin and globulin were reported among all groups. Lipid profile of the diseased calves revealed a significant increase in both levels of T.C and LDL-C with a highly significant reduction in the level of T.Gs in the diseased calves. Meanwhile, HDL-C level showed a non-significant change in all groups.

Keywords: Cryptosporidium, Calves, diarrhea, hepatic, renal, IgA, Halofuginone, PCR

Introduction

Cryptosporidiosis is becoming one of the important zoonotic diseases causing a respiratory and gastrointestinal illness which caused by an apicomplexan protozoan parasite called *Cryptosporidium* (Yagita *et al,* 2001). It is considered a life threatening illness of neonatal calves leading to malabsorption and diarrhea, which in turn result in economic losses in animal husbandry (Azami, 2007). *C. parvum* an obligate intracellular parasite commonly affect newborn calves 5 to 15 days old showed varying degrees of apathy, reduced feed intake associated with a signs of diarrhea, which persists for several days, feces are yellow or pale, watery and can contain mucus. Persistent diarrhea can result in dehydration, a marked loss of body weight and emaciation (Radostits *et al,* 2007). Diarrhea can cause dehydration, metabolic acidosis and impaired function of many organs, manifested by several metabolic disturbances expressed by biochemical and enzymatic alterations (Ghanem *et al,* 2012). Abdel Megeed, *et al.* (2015) mentioned that cryptosporidium infected calves showed yellowish, greenish or clay colored profuse watery diarrhea, anorexia, colic pain, normal body temperature, reduced milk suckling and with progression of the disease, animals dehydrated with evidence of lying down posture.

Diagnosis of cryptosporidiosis usually depends on the morphological identification which needs time, effort and experience, as well as, is insufficient to confirm infection.

Consequently, the use of molecular techniques for *Cryptosporidium* identification was more common to solve a lot of problems accompanied with morphological identification (Ghoneim *et al*, 2017). Although PCR is as a very sensitive method to detect *Cryptosporidium*, yet it is too expensive for routine diagnosis (Bialek *et al*, 2002).

Various treatment trials were tried to test the efficiency of some agents as bovine hyper immune colostrum, halofuginone and paromycin against cryptosporidiosis (Harp *et al*, 1996; Lefay *et al*, 2001).

The study aimed to monitor the changes in clinical signs and biochemical parameters in dairy calves cryptosporidiosis, to compare between the immune chromatographic test (ICT), microscopic and molecular examination to detect cryptosporidiosis and to evaluate effect of Halocur (Halofuginone lactate) in treatment.

Material and Methods

The present study was carried out on 25 Holstein dairy calves (5 male & 20 female), their ages were ranged from 3 to 15 days old in El-Eman farm, El-Manayf, Ismailia Governorate, Egypt. These animals were assigned into two groups; the first group (G1), include20calves suffered from watery to mucoid diarrhea and the second group (G2) include 5calves were proved to be clinically healthy and used as a control one. Calves during the period of study were kept together under open half shelter system and fed on a milk diet twice daily and tap water was provided *ad libitum*. Physical examination of diseased and healthy calves was conducted according to the clinical examination procedures (Radostits *et al*, 2000).

A total number of 90 blood and fecal samples were collected from all animals during the study; 40 blood samples from diseased calf (at the onset of the appearance of the symptoms and on the 8th day of the treatment) and 40 fecal samples at the same pattern from the same animals and 10 samples (blood and feces) were collected from the control one.

Blood was collected from the jugular vein from all calves under the study. The blood was placed in plain tubes, left at room temperature for clot formation, and centrifuged at 3000 rpm for 15 min. The obtained clear sera were collected and stored at -20°C until used for the biochemical analysis (Coles, 1986).

The fecal samples were collected directly from the rectum using examination gloves and stored closed in fecal cups at 4°C until being examined.

All analytical biochemical kits were obtained from Biodiagnostic Company (Cairo, Egypt). Meanwhile, Bovine IgA ELISA kit was obtained from MyBioSource, USA. Halocur® drug (each ml contain 0.05 mg Halofuginone lactate) was obtained from MSD, Animal health, New Zeland. For rapid detection of *cryptosporidia*; Rapid BoviD-5 Ag test Kit supplied by BioNote Company, Korea was used.

Biochemical analysis: Hepatic and renal biomarkers: Serum alanine aminotransferase (ALT) enzyme activity was investigated (Reitman and Frankel, 1957), gamma glutamyl transferase (GGT) (Szasz, 1969) and lactate dehydrogenase (LDH) (Zimmerman and Henery, 1979). Serum level of urea was determined as (Coulombe and Favreau, 1963) and creatinine(Henry, 1974).

Serum proteinogram & IgA: Total serum protein level was determined (Gornall *et al*, 1949), albumin level was assessed (Bartholomew and Delaney, 1964). Serum level of globulin was calculated by subtracting the serum albumin from total serum protein (Kaneko *et al*, 1997). IgA level was determined according to the enclosed pamphlet.

Lipid profile: Total cholesterol (TC) was determined by enzymatic colorimetric method (Allain *et al*, 1974), triglycerides (TGs) was performed (Fossati and Prencipe, 1982). Serum concentrations of High density lipoprotein cholesterol (HDL-C) (Tiez, 1990) and low density lipoprotein cholesterol (LDL-C) was assessed (Bachorik, 2000).

Fecal examination: A total of 45 fecal samples were utilized, the first 20 samples before treatment were positive by immune chromatographic test (ICT), and then examined microscopically and kept at -20ºC until processed for DNA extraction.

Morphological detection and identification of *Cryptosporidium* oocysts: 45 fecal samples were examined by concentration floatation technique using sugar (Georgi and Georgi, 1990).Emulsified fecal specimens were dried, fixed in methanol and stained by modified Ziehl-Neelsen technique (Henriksen and Pohlenz, 1981) and examined under oil immersion.

Molecular identification: The first group fecal samples before treatment were utilized, and kept at -20ºC until processed for DNA extraction.

DNA Extraction: DNA was extracted from fecal samples using Genomic DNA purification kit (Applied biotechnology) after manufacturer's recommendations. DNA concentration and purification were measured using a Nano Drop, and stored at -20 ºC until use.

C. parvum molecular identification based on *18S rRNA* Gene: *C. parvum18S rRNA* was amplified from each sample using nested conventional PCR methodologies, the reaction mixtures and primers (Bialek *et al*, 2002), the outer primer set CPr I (5´- AAA CCC CTT TACAAG TAT CAA TTG GA-3´) & CPr II (5´- TTC CTATGT CTG GAC CTG GTG AGT T-3´) is complementary to a region of the small subunit ribosomal RNA gene of *C. parvum*, amplifying a 676 by sequence. In addition to the inner primer set CPr III (5´- TGC TTA AAGCAG GCA TAT GCC TTG AA-3´) &CPr IV (5´- AACCTC CAA TCT CTA GTT GGC ATA GT-3´). PCR was ran following the cycling condition: initial denaturation at 94ºC for 5 minutes followed by 30 cycles of 94ºC for 30s, 72ºC for 1 min.& 72ºC for 5 min. High melting temperatures of inner primer set allowed a two-step nested PCR with high stringency. Amplification products were separated on 1.6% agarose gel containing 0.4µg/ml of ethidium bromide (Bio-Rad Laboratories Inc., Hercules, CA) and the gel was run at 90 volts for 40-60 min. and then imaged.

Sequence analysis: PCR products were purified and sent for sequencing, the Sequences were analyzed through BLAST using MEGA7 software.

Phylogenetic analysis: Phylogenetic analysis of samples and the other *Cryptosporidium* samples of the same genus from the GenBank were performed using UPGMA approaches and the tree was constructed using MEGA7 methods (Fig. 3).

Treatment: Calves were treated by oral administration of Halofuginone at a dose rate of 100µg/ kg once daily for 7 successive days (Radostits *et al*, 2007).

Statistical analysis: Data were analyzed using independent and paired T-test through SPSS, version, 20 (Levesque, 2007).

Results

Clinical examination of diseased calves showed profuse watery diarrhea with yellowish colored feces (8/20) 40% and mucoid diarrhea (12/20) 60% which persist for 7 days resulting in dehydration, weakness and loss of body weight. Symptoms were highly detected in young calves aging less than one month and decreased with increasing age. Biochemical assessment of serum manifested a significant increment in levels of ALT, GGT, LDH and urea in diseased calves as compared with the control healthy one with a significant decrement in the treated calves when compared with the diseased one. Meanwhile, a non-significant change in creatinine level was reported in all groups (Tab. 1). Also, a non-significant change in levels of total protein, albumin and globulin were reported among all groups. Meanwhile, concentration of IgA revealed a highly significant reduction in the diseased calves as compared with healthy one, with a highly significant rise in the treated calves when compared with the diseased one (Tab. 2). Lipid profile of diseased calves revealed a significant increase in both levels of cholesterol and LDL-C in the diseased calves as

compared with healthy one without significant difference in treated calves as compared to diseased one. T.Gs level showed a highly significant decrease in the diseased calves as compared with healthy one, without significant difference in treated calves as compared to diseased one, HDL-C level without significant change in all groups (Tab. 3). They showed a complete cure 8 days after starting treatment that continued for seven successive days. Fecal 20/45 samples were ICT positive in diseased calves before treatment, 20 samples were negative in treated and healthy calves. Detection of oocysts showed that freshly prepared unstained *Cryptosporidium* was tiny, bright, refractile spherical to subspherical, colorless with distinct wall. Post-staining was densely stained red bodies onblue-green fecal debris background and measured 2.8-5x3.2-6µm with an average of 3.99x4.6µm(Fig. 1).17th positive samples analyzed based on 18S rRNA PCR products (Fig. 2),proved by sequencing to be 100% similar to sequences of *C. parvum* (isolate BP185), the sequence were submitted in the Genbank under access No. MG869612, in addition to the phylogenetic tree (Fig. 3), sequenced sample clustered with *C. parvum.*

Table 1: M±S.E for serum hepatic & renal biomarkers in cryptosporidiosis calves before & after treatment.

Analytes	Groups	Mean values ± S.E		P-value
ALT (U/L)	Control vs D.Pre	13.67± 0.88	26.7± 1.54	0.001**
	D.Pre vs D.Post	26.7± 1.54	17.62± 1.78	0.014*
GGT (U/L)	Control vs D.Pre	10.37± 0.53	14.45±0.58	0.003 **
	D.Pre vs D.Post	14.45±0.58	11.8± 0.41	0.014*
LDH (U/L)	Control vs D.Pre	1228± 71.5	1345± 77.5	0.014*
	D.Pre vs D.Post	1345± 77.5	1112± 93.2	0.015*
Creatinine (mg/dL)	Control vs D.Pre	1.0 ± 0.06	0.84 ± 0.04	0.160^{NS}
	D.Pre vs D.Post	0.84 ± 0.04	0.85± 0.05	0.844^{NS}
Urea (mg/dL)	Control vs D.Pre	20.33± 1.20	25.38± 1.24	0.047*
	D.Pre vs D.Post	25.38± 1.24	21.5± 1.46	0.032*

D.Pre: diseased calves pre-treatment, D.post: diseased calves post-treatment, vs: versus. ^{ns}: non-significant ($p > 0.05$), *: significant ($p \leq 0.05$). **: highly significant ($p < 0.01$).

Table 2: M ± S.E for serum proteinogram and IgA concentration in cryptosporidiosis calves before and after treatment.

Analytes	Groups	Mean values ± S.E		P-value
Total proteins (gm/dL)	Control vs D.Pre	5.9± 0.17	5.68 ±0.17	0.508^{NS}
	D.Pre vs D.Post	5.68 ±0.17	6.0 ± 0.15	0.266^{NS}
Albumin (gm/dL)	Control vs D.Pre	2.93 ± 0.07	2.53 ± 0.2	0.260^{NS}
	D.Pre vs D.Post	2.53 ± 0.2	2.78± 0.17	0.262^{NS}
Globulin (gm/dL)	Control vs D.Pre	2.96 ± 0.12	3.16 ± 0.09	0.308^{NS}
	D.Pre vs D.Post	3.16 ± 0.09	3.21± 0.11	0.714^{NS}
IgA(mg/dL)	Control vs D.Pre	294.6 ±1.9	277.4 ±1.4	0.009**
	D.Pre vs D.Post	277.4 ±1.4	350.9 ±5.1	0.006**

Table 3: M ± S.E for serum lipid profile in cryptosporidiosis calves before and after treatment.

Analytes	Groups	Mean values ± S.E		P-value
TC (mg/dL)	Control vs D.Pre	91.5± 2.02	131.6± 7.6	0.012*
	D.Pre vs D.Post	131.6± 7.6	128.3± 12.6	0.847^{NS}
T.Gs (mg/dL)	Control vs D.Pre	104± 1.5	78.38± 1.3	0.001**
	D.Pre vs D.Post	78.38± 1.3	74.88± 3.6	0.356^{NS}
LDL-C (mg/dL)	Control vs D.Pre	27.37± 1.79	50.58± 3.1	0.002*
	D.Pre vs D.Post	50.58± 3.1	65.68± 11.3	0.251^{NS}
HDL-C (mg/dL)	Control vs D.Pre	43.33± 0.33	47.75± 1.5	0.123^{NS}
	D.Pre vs D.Post	47.75± 1.5	43.63± 1.7	0.145^{NS}

Discussion

In this study, symptoms were highly detected in young calves of age less than one month and decreased with age progress. Similar observations were reported by Göz *et al.* (2006) and Osman and Sadiek (2008). Moreover, Radostits *et al.* (2007) reported that cryptosporidium infection in calves associated with varying degrees of villus atrophy suggest that digestion and absorption may be impaired, conducting diarrhea.

In the present study, *Cryptosporidium* was detected firstly with ICT which is a sensitive and rapid method for cryptosporidiosis detection (Vanathy *et al,* 2017), then with microscopical and molecular examination. The morphological character of detected *Cryptosporidium* species was similar to *C. parvum*, the diameter of *Cryptosporidium* oocyst was 3.99x4.6um, similar to those obtained by Fayer *et al.* (2000), morphological diagnosis of *Cryptosporidium* is difficult and needs more experience so molecular techniques are considered successful and more popular for *Cryptosporidium* diagnosis and also help in the species identification, in the herein study, PCR was nested proved to be more sensible and more specific to detect *Cryptosporidium* due to presence of a secondary round of amplifications Minarovičová *et al.* (2007), present sequence gave 100% identity with *C. parvum* and also clustered with *C. parvum* in the phylogenetic tree, so our morphological results were assured by the molecular analysis results which detected strongly positive samples with *Cryptosporidium parvum*, accompanied with the sequencing and the phylogenetic analysis.

Serum biochemical analysis manifested that liver enzyme activities including ALT and GGT were significantly elevated in diseased group in comparison with the control healthy group indicating pathological affection in liver or inflammation of gastrointestinal tract (Ghanem and Abd El-Raof, 2006), which agreed with Ghanem *et al.* (2012) and Youssef *et al.* (2017). GGT, a cell membrane attached enzyme in liver, kidney and small intestine, elevated with hepatocellular damage (Boonprong *et al,* 2007).

Lactate dehydrogenase (LDH) enzyme is a non-specific cytoplasmic enzyme found in different tissues of the body. LDH act as a catalyst converting lactate into pyruvate. Elevated serum level of LDH enzyme may refer to acute cell damage. Increased ALT, GGT & LDH activities in the blood of cattle might be due to liver injury induced by acidosis (Mori *et al,* 2007).

The present study showed non-significant change in the levels of total proteins, albumin and globulin with significant decrement of IgA level in diseased group as compared with healthy one. Non-significant changes in the serum total protein level in all groups were in line with those results obtained by Youssef *et al.* (2017) and Ghanem *et al.* (2012). Normal values of total protein and albumin were even in liver injury unless hepatitis embraced a massive portion and intense enough to cause organ failure (Pincus and Schaffner, 1999). Fisher *et al.* (1975) and (Balikci and Al, 2014)found decreased serum IgA in diarrheic calves. Ghanem *et al.* (2012) deduced a non-significant change in α1 and α2 globulin but recorded significantly reduced serum levels of ß-globulin and ɣ-globulin in the diarrheic buffalo calves compared to the control calves. ɣ -globulins have a remarkable role in humeral immunity that guard against pathogens. So, decrease in their level gives a sign of the suppressive effect of pathogens on the immune system.

The increased serum urea level and a non-significant change in creatinine level in diseased calves agreed with Osman and Sadiek (2008). Blood urea concentration depends on various factors as physiological state, nutrition, diarrhea and renal disease. Creatinine is a sharp index of renal insufficiency; therefore, there is no evidence of renal injury. The increased urea level might be due to decreased renal tubular flow rate, as a body trial to save fluids lost with diarrhea (Fisher, 1965).

Liver is responsible for cholesterol synthesis and excretion. Hypercholesterolemia may result from decreased hepatic uptake or excretion into the bile. It is cleared from blood by forming steroids or removed by hepatocytes (Thrall *et al*, 2004). The present lipid profile results disagreed with those recorded by Bozukluhan *et al.* (2017). Fortuoso *et al.* (2018) reported lowered serum T.Gs levels and unchanged cholesterol levels in diarrheic calves. As formerly reported oocysts caused gut epithelial disruption; resulting in diarrhea (Chen *et al*, 2002). Mal nutrition may lead to decreased triglyceride level, as they are manufactured in the liver from dietary food or by being absorbed from the intestine (Ankur *et al*, 2012). Triglycerides are fats stored in the body due to ingested carbohydrates that were not instantly used (Smelt, 2010), so anorexia developed in diarrheic calves and mobilization of fat stores occurred with degradation of T.Gs into fatty acids & glycerol. Akgün *et al.* (1998) found that many acute disease cases displayed decreased levels of plasma triglyceride.

In the present study, Halofuginone treated calves gave significant improvements in all analysis in comparison to diarrheic calves. So, Halofuginone lactate treated *C. parvum* infected calves. This agreed with Giadinis *et al.* (2008) who reported that Halofuginone lactate at a dosage of 100µg/kg for 7 consecutive days was a potential treatment of cryptosporidiosis in goat kids and reduced diarrhea and fatality rate and minimized the environmental contamination.

Conclusion

Cryptosporidium parvum induced diarrhea in calves and a major problem of neonate calves. It caused significant change in hepatic biomarkers, lipid profile and IgA level of diseased calves. Halocur proved a promising treatment in dairy calves. ICT is a sensitive and rapid method for cryptosporidiosis detection with difficult morphological diagnosis. So, molecular techniques are the must.

Conflict of interest: Authors declared that they neither have competing interests, nor received any financial support.

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Explanation of figures

Fig. 1: *Cryptosporidium* oocysts stained with Modified Ziehl-Neelsen stain.

Fig. 2: Electrophoretic separation of *18S rRNA* gene. L1: ladder (100bp); lane L2, L3, L6, L7: positive samples; L4: negative control, L5: positive control.

Fig. 3: Phylogenetic analysis constructed using UPGMA methods, to construct phylogenetic tree of *Cryptosporidium* species sequences from Genbank and sequence sample (AHNE1).