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Applied Studies on Coccidiosis in Growing Buffalo-Calves with Special Reference to Oxidant/Antioxidant Status

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Abstract: Due to the special clinical and economic importance of *Eimeria sp* infection in livestock and the non availability of enough data in buffaloes, the present study was carried out. Blood and fecal samples were collected from 191 growing buffalo-calves to detect the prevalence of *Eimeria sp* infection and to investigate the association between infection and changes in general health condition and oxidant/antioxidant status. *Eimeria* sp. oocysts were detected using both light microscopy and coproantigen ELISA test. The isolated coproantigen was structurally characterized by SDS polyacrylamide gel electrophoresis, isoelectric focusing and immunobloatting. Clinical symptoms, complete blood picture, hemoglobin biophysical properties, oxidant/antioxidant markers and some trace element concentrations were recorded. Results indicated that coproantigen ELISA test detects more positive cases (92.10%) than light microscopy (64.90%) in examined calves. High prevalence of infection was recorded in 6 - 9 month old calves, male calves and during the green season of the year. Structural characterization of isolated coproantigen showed that it consists of 9 polypeptides of 24 - 201 KDs with isoelectric points of 5 - 8.6 and had immunoreactive bands of 176, 35 and 24 KDs. Infected calves suffered from rough hair coat, retarded growth, diarrhea, inferior appetite, anemia and skin lesions. These animals showed increased malondialdehyde (MDA, $p<0.001$) and nitric oxide (NO, $p<0.01$) with decreased catalase (CAT,$p<0.001$), superoxide dismutase (SOD $p<0.001$), ascorbic acid (ASCA<0.01), total antioxidant capacity (TAC, $p<0.001$), glutathione-reduced (GSH, $p<0.001$), zinc (Zn, $p<0.05$), copper (Cu, $p< 0.001$), iron (Fe, $p<0.001$) and selenium (Se, $p<0.001$)values in their blood as compared to the negative control group. In conclusion, *Eimeria* coproantigen has 3 immunoreactive bands which can be used for accurate diagnosis of coccidiosis in buffalo-calves. The infection has negative effects on the health condition and the growth performance of growing buffalo calves and it is associated with disturbed oxidant/antioxidant status.

Key words: Buffalo-calves · coccidia · *Eimeria* · Oxidant /antioxidant status · Diagnosis · Characterization · Blood constituents

INTRODUCTION

Buffaloes are the prime source of good quality meat and milk in Egypt and some other developing countries. These animals are mainly reared in small holder farms and suffer from a lot of stressful conditions such as mal-nutrition and parasitism and characterized by inferior productive and reproductive potentials [1].

Coccidia are intracellular parasites of the intestinal epithelium in domestic animals. Genus *Eimeria* is of special clinical and economic importance in livestock. Nearly, all cattle are infected with coccidia, but only a limited number suffers from coccidiosis. This disease occurs mainly in young animals whereas; the immune status plays a role in the protection of older animals. Infection occasionally occurs in calves over 6 months of age or even in adult cattle [2]. Many cattle are subclinically infected, resulting in considerable economic losses. Coccidiosis costs cattle ranches more than $ 400 million annually in lost profit due to reduction of feed efficiency, slower weight gain and increases susceptibility to other diseases [3]. For efficient control, exact diagnosis of the *Eimeria species*, the evaluation of animal management and husbandry practices are of most importance [4].

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In growing calves, fecal examination indicated *Eimeria* sp. infection rates varied from 70-100% [5,6] and 30.90-59% [7, 8]. In buffalo calves, an incidence of 100% was reported in Egypt [9], while a low incidence of 7.70% was recorded in India [10]).

A coproantigen ELISA test has been used successfully for immunodiagnosis of a number of protzoal infections including *Eimeria* sp. [11].

Oxidative stress results in the faster production of reactive forms of oxygen than it's safely neutralization by antioxidant mechanisms. It has a negative effect on animal health and production [12] and is implicated as a major initiator of tissue damages [13]. Antioxidants scavenge free radicals to obtain an optimal redox balance and include vitamins, trace elements and enzymes [1, 14]. Zinc, copper and selenium have significant roles in maintaining good health condition in farm animals [15]. Low blood copper and zinc concentrations in growing animals result in general weakness, stunted growth, anemia, delayed puberty and infertility [16].

Unfortunately, no enough data regarding coccidiosis in buffaloes could be traced in the available literature. Therefore, it is planned to investigate this topic with special references to the health, productive and reproductive drawbacks in infected animals. The present study was designed to throw light on coccidiosis in growing buffalo-calves with special reference to evaluation of the two commonly used diagnostic techniques; light microscopy and coproantigen ELISA test. Also, a trial was carried out to detect the immunogenic band/s which might be responsible for detection of *Eimeria* antibodies. Moreover, clarification of the associations between infection and changes of health condition and oxidant/antioxidant status in infected buffalo-calves was a further target.

MATERIALS AND METHODS

The present study was carried out during the period from September 2004 to June 2006 as a part of the National Research Centre Project No. 7120106.

Animals: One hundred and ninety one heads of buffalo-calves aging 1week to 12 months were used in this study. These animals were kept in small holder farms at villages of Lower Egypt. They were fed on Barseem (December-May), concentrates, crops residue and rice straw. Owner complains were recorded and animals were clinically examined.

Samples collection: Samples of blood (with and without EDTA) and feces were collected from calves. Uncoagulated blood samples were used for performing complete blood picture as well as determination of some biophysical properties of hemoglobin (electric conductivity and derivatives), R-GSH and Se values. Serum was separated from coagulated blood samples by centrifugation (x 3000 g, 15 minutes at 4°C) and kept at –20° C for assaying some oxidant/antioxidant markers as well as for serodiagnosis of *Eimeria* sp. Fecal samples were subjected to parasitological examination.

Analyses

Fecal examination: Fecal samples were subjected to fecal examination using concentration flotation technique [17] to detect the oocysts of different *Eimeria* sp.

Serodiagnosis

preparation of Coproantigen: Coproantigen was prepared from fecal samples of infected calves with *Eimeria* sp. [18]. Briefly, fecal samples were vigorously shaked with an equal volume of 0.15M phosphate buffer saline (PBS) containing 0.3% tween 20 until slurry was formed followed by centrifugation at 10000g for 30 minutes. The supernatant was examined for protein content [19] and stored at -20 oC until use.

Enzyme linked Immunosorbent Assay (ELISA): The assay was adopted to evaluate the diagnostic value of coproantigen in the detection of *Eimeria* sp. antibodies. The optimum antigen, serum and conjugate concentration were determined by checkerboard titration and the test procedures were carried out as described by [20] and the cut off values of optical density (OD) were calculated according to [21].

Antigen characterization

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS -PAGE): The crude extract was mixed with reducing buffered samples and electrophoresed on SDS-PAGE slab gels as outlined by [22]. After separation, the gel was fixed in 50% methanol and stained with silver stain [23]. Molecular weight standards were electrophoresed on the same gel to calculate the relative molecular weight of the examined antigens.

Isoelectric focusing: Isoelectric focusing (IEF) was performed in slab gel supplemented with urea. The ampholine gels were stained with Commasie blue and

wetly photographed [24]. Isoelectric point (P_I) of a particular protein is determined by running a mixture of proteins of known PIs on the same gel (IEF mix-sigma, USA 3.6-9.3).

Immunoblot: The assay was carried out to identify the immunoreactive components recognized in the antigen using naturally infected calf sera which was previously investigated by ELISA. The assay was carried out as the method described by [25].

Blood picture: Complete blood picture including erythrogram and leukogram was carried out and anemia indices were calculated [26].

Biophysical properties of hemoglobin: The electrical conductivity of hemoglobin was measured using conductivity digimeter [27]. Also, 4 hemoglobin derivatives were determined using simultaneous multicomponent spectrophotometry [28].

Oxidant/antioxidant markers: Oxidant/antioxidant markers including MDA[29], nitric oxide, NO [30], CAT ([31], SOD[32], ascorbic acid, ASCA[33], R-GSH [34] and TAC[35] were colorimetrically assayed using chemical kits (Biodiagnostic Co., Egypt).

Trace elements concentration: Concentration of Zn and Cu in diluted serum samples and Se in whole blood samples were determined using atomic absorption spectrophotometry (Perkin Elmer, 2380) as outlined by [36].

Statistical analysis: Data were computed and statistically analyzed [37].

RESULTS

Prevalence of *Eimeria* sp. in growing buffalo calves: Table (1) shows the comparative efficiency of the used techniques for diagnosis of *Eimeria* sp. in growing buffalo-calves. The prevalence was also depicted (Fig. 1). Coproantigen ELISA test gave a higher prevalence (92.10%) as compared with light microscopy (64.90%).

Table (2) reveals the age distribution for the prevalence of infection. Calves aged 6-9 months exhibited the highest infection rate. However, those aged less than 3 months revealed the lowest rate. Moreover, the results demonstrated that the incidence of *Eimeria sp* was higher in male calves than female calves (Table 3).

Table 1: Comparative efficiency of techniques used for detection of *Eimeria* sp. in growing buffalo- calves

	Prevalence of infection	
Technique	No.	%
Light microscopy	124	64.90
Coproantigen ELISA test	176	92.10

Table 2: Effect of age on the prevalence of *Eimeria sp* in growing buffalo-calves (%)

		Prevalence of infection	
Age groups	Examined Calves No	No.	%
1 week – 3 month	18	4	22.20
3 – 6 month	47	24	51.10
6 – 9 months	70	45	64.30
9 - months	56	32	57.10

Table 3: Effect of sex of calf on the prevalence of *Eimeria* sp. in growing buffalo-calves

		Prevalence of infection	
Sex	Examined animals No	No.	%
Males	61	43	70.50
Females	130	80	61.50

Table 4: Effect of feeding season on the prevalence of *Eimeria sp* in growing buffalo- calves (%)

		Infection rate (%)	
Feeding seasons	Examined animals No	No.	%
Green	125	118	94.40
Dray	66	58	87.90

Total examined buffalo-calves number =191

In the sametime, it was found that the prevalence of *Eimeria sp* infection was affected by feeding season. It was higher during the green than the dry seasons (Table 4).

Characterization of coproantigen: The electrophoretic profile of coproantigen was recorded (Fig. 2). The crude extract was resolved into 9 bands of molecular weights; 201, 176, 116, 68, 51, 35, 30, 29 and 24 kDs. For further characterization, PIs of coproantigen were identified by IEF technique (Fig. 3).

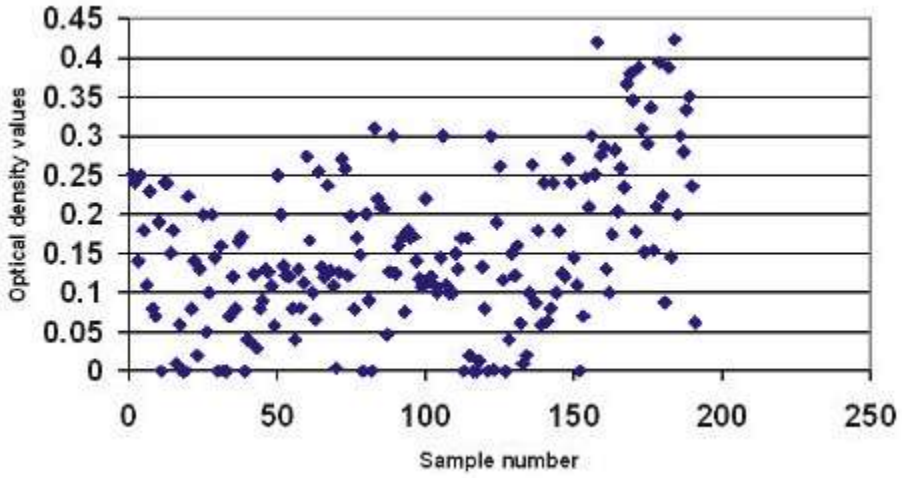

Fig. 1: Scater graph representing the potency of *Eimeria* sp. Coproantigen in the diagnosis of coccidiosis

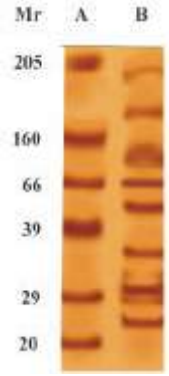

Fig. 2: Electrophoretic pattern of *Eimeria* sp. Coproantigen
Lane A, molecular weight standards
Lane B, Coproantigen fractions

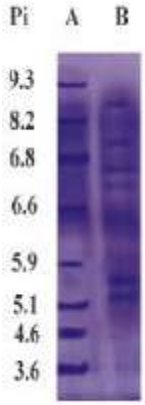

Fig. 3: Isoelectric focusing of *Eimeria* sp. Coproantigen
Lane A, isoelectric focusing standards
Lane B, Coproantigen fractions

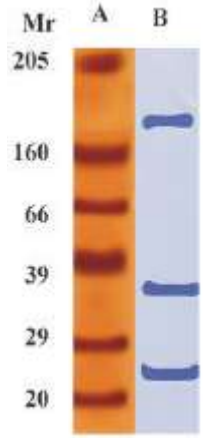

Fig. 4: Immuno-blotting analysis of *Eimeria* sp. Coproantigen
Lane A, molecular weight standards
Lane B, Immunogenic bands

The antigen revealed 8 bands having Pis of 8.6, 7.3, 6.8, 6.7, 6.5, 5.6, 5.3 and 5.0 kDs. Immunoreactive bands of *Eimeria* coproantigen were identified using naturally infected calf serum and were depicted (Fig. 4). The recognized immunogenic bands were 176, 35 and 24 kDs.

Clinical symptoms associated with *Eimeria sp* infection: Table (5) shows the main recorded symptoms in *Eimeria sp* infected buffalo-calves. In general, the infected calves had more than one symptom. The main observed clinical symptoms are rough hair coat, retarded growth and diarrhea.

Table 5: Main clinical symptoms associated with *Eimeria* sp. infection in buffalo-calves

Symptoms *	No.	%
Rough hair coat	52	29.50
Retarded growth	45	25.60
Diarrhea	38	21.60
Inferior appetite	25	14.20
Skin lesions	19	10.80

Total infected buffalo-calves number =176

*Some animals revealed more than one symptom

Table 6: Blood picture of buffalo- calves naturally infected with *Eimeria* sp. (Mean ± SE)

Item	Parameters	Negative control calves^N	*Eimeria* sp. infected calves^N
Erythrogram	RBCs (10^6/ml^3)	6.09±0.13	4.17±0.05***
	Hb (g/dl)	14.41±0.77	12.96±0.23
	PCV (%)	38.61±0.47	36.31±0.26**
	MCV (fl)	63.40±2.17	87.07±1.98***
	MCHC (%)	37.32±1.17	35.69±0.84
Leukogram	Total WBCs (10^3/ml^3)	7.57±0.23	9.72±0.98
	Lymphocytes (%)	62.52±0.73	62.28±1.17
	Neutrophil (%)	35.04±2.11	32.04±3.17
	Monocytes (%)	1.25±0.08	2.09±0.04***
	Esinophil (%)	0.99±0.08	2.63±0.04***
	Basophil (%)	0.20±0.04	0.96±0.06***

* Significant at p< 0.05 ** Significant at p< 0.01 *** Significant at p< 0.001 N = Number of samples= 5 (aged 9-12 months)

Table 7: Some biophysical properties of hemoglobin in buffalo- calves naturally infected with *Eimeria sp* (Mean ± SE)

Hemoglobin properties		Negative control calves^N	*Eimeria* sp. infected calves^N
Electric conductivity (Siemens/Cm)		83.14±1.29	79.17±1.93
Derivatives	Oxy -hemoglobin (%)	76.13±4.89	73.66±3.19
	Met- hemoglobin (%)	7.18±0.87	8.11±0.10
	Carboxy- hemoglobin (%)	4.11±0.17	5.16±0.18
	Sulf- hemoglobin (%)	2.96±0.08	3.57±0.08

N = Number of samples= 5 (aged 9-12 months)

Table 8: Concentration of oxidant /antioxidant markers in buffalo- calves naturally infected with *Eimeria sp* (Mean ± SE)

Item	Parameters	Negative control calves^N	*Eimeria sp* infected calves^N
oxidant	Malondialdhyde (MDA, mmol/ml)	1.77±0.05	3.63±0.13***
	Nitric oxide (NO, imol/L)	20.18±2.82	32.19±1.80**
antioxidant	Catalase CAT, U/ml)	2.77±0.03	0.78±0.04***
	Superoxide dismutase (SOD,U/ml)	407.20±8.24	356.35±2.13***
	Ascorbic acid (ASCA, μgm/L)	147.14±5.26	117.53±6.14**
	Total antioxidant capacity (TAC, mmol/L)	1.78±0.06	0.77±0.04***
	Glutathione reduced (R-GSH,mmol/L)	8.07±0.11	4.33±0.22***

** Significant at p< 0.01 *** Significant at p< 0.001 N = Number of samples= 5 (aged 9-12 months)

Table 9: Concentration of some trace elements in buffalo-calves naturally infected with *Eimeria sp* (Mean ± SE)

Trace elements	Negative control calves^N	*Eimeria* sp. infected calves^N
Zinc (μg/dl)	145.13±8.13	118.71±2.63*
Copper (μg/dl)	127.17±2.76	99.12±4.14***
Iron (μg/dl)	227.61±5.10	193.16±3.13***
Selenium (μg/l)	155.65±2.16	117.19±0.99***

* Significant at p< 0.05 *** Significant at p< 0.001

N = Number of samples= 5 (aged 9-12 months)

Blood picture: Table (6) reveals that *Eimeria sp* infected calves had significantly low red cell count (p<0.001), packed cell volume (p<0.01) and mean corpuscular volume (p<0.001) indicating the presence of hypochromic macrocytic anemia. The total white cell increased with obvious monocytosis, esinophilia and basophilia (p<0.001) as compared with the control group.

Physical properties of hemoglobin: Analysis of some physical properties of hemoglobin indicated that the electric conductivity and oxyhemoglobin derivative were slightly decreased in *Eimeria* sp. infected calves as compared to healthy calves (Table 7).

Oxidant/antioxidant markers: Concentrations of some oxidant/antioxidant markers were recorded (Table 8). Significant increase of MDA (p<0.001) and NO (p<0.01) and decreases of CAT (p<0.001), SOD (p<0.001), ASCA (p<0.01), TAC (p<0.001) and R-GSH (p<0.001) values were observed in *Eimeria* sp. infected buffalo-calves as compare to negative control calves.

Trace elements: Table (9) shows the concentration of studied trace elements in. *Eimeria sp* infected buffalo-calves. Infected calves had significant low blood Zn (p<0.05),Cu (p< 0.001), Fe (p<0.001) and Se (p<0.001) as compared to the control group.

DISCUSSION

Coccidiosis causes great economic losses for livestock as a result of reduction in feed efficiency, slow weight gain and increased susceptibility to other diseases [3]. Due to there is no enough data regarding coccidiosis in buffaloes were traced in the available literature, this study was designed to throw light on coccidiosis in growing buffalo-calves.

In the present study, light microscopy and coproantigen ELISA test indicated that 64.90 and 92.10% of the examined Egyptian buffalo- calves are positive for *Eimeria sp*, respectively. However, variable frequency of *Eimeria* sp. infections was recorded.

Regarding light microscopy, [9] recorded that 100% of Egyptian buffalo-calves are infected with *Eimeria* sp. However, a lower incidence of 7.7% was recorded in India [10]. Moreover, [38] concluded that *Eimeria sp* was considered the most common parasites that infecting buffalo calves in Brazil. In cattle, an incidence of 70 – 100% was recorded by [5,6,39]. Meanwhile, a low prevalence (30 – 59%) was recorded by [7, 8, 40]. Differences in the recorded prevalence may be attributed to environmental conditions, management way and feeding strategies.

Concerning seroprevalence, coproantigen ELISA test recorded a higher prevalence of *Eimeria sp* than that reported by light microscopy. Similar trend was obtained by [11] who found respective prevalence of 36.80 and 10.00% in calves. Furthermore, the use of PCR technique allowed the detection of more coccidia oocysts than light microscopy [41]. High results obtained by coproantigen ELISA test could be attributed to its high specificity and sensitivity for the diagnosis of the gastro intestinal tract infection [42].

In this study, high prevalence of *Eimeria* sp. was obtained in 6-9 months old calves, males and during the green season. On the contrary, [39] reported that calves until 7 weeks showed increased prevalence of *Eimeria* sp. that followed by slight decrease. Such contradictions could be attributed to the species, housing condition and system of management. In this respect, [7] concluded that sex of calves has no significant effect on the prevalence or intensity of infection. High incidence of infection during the green season coincides with the result of [7,39]. This finding may be related to dominance of the suitable environmental condition for sporulation of *Eimeria* oocysts during that season.

The important facet of the present study is the structural characterization of *Eimeria sp* coproantigen which was found to be consisted of 9 bands ranging from 201-24 kDs. This result was comparable to that of [43] who revealed that coproantigens of *Eimeria stiedae* resolved into 9 bands.

Given the fact that the structural characterization of component according to more than one dimension adds to its structural profile, *Eimeria* coproantigen in the present attempt was also characterized according to its isoelectric points by isoelectric focusing technique. The assay resolved into 8 bands of PIs (8.6 -5.05). In the previous studies [44], isolated aminopeptidase from *Eimeria tenella* with Mr106 kD and an P_I of 5.1. This P_I is similar to one of those bands isolated in the current study. This similarity is probably referred to the common component between those *Eimeria* sp.

In the current research, immunoblot assay was used to identify the immunoreactive bands in the coproantigen that might be responsible for the detection of *Eimeria* sp. antibodies. This assay revealed only 3 reactive components of molecular weight (176, 35 and 24 kDs). In this respect, [43] identified 6 reactive components in *Eimeria stiedae* coproantigen by immunoblot in which rabbit sera after 3 weeks post vaccination was used. However, these polypeptides had different molecular weights than those observed in the present study using naturally infected calf sera.

In the current investigation, *Eimeria* infected buffalo-calves were suffering from loss of condition as indicated by rough hair coat, retarded growth, diarrhea, skin lesions and anemia. Similar clinical findings were reported in growing calves by [45]. Moreover, [16] found that animals showed general weakness and stunted growth are mostly suffered from anemia. The damage of the intestinal epithelium caused by the multiplication of *Eimeria* stages resulting in loss of blood and marked anemia as well as impaired absorption, utilization and assimilation of some elements such as iron and copper[46].

The recorded changes in the leukogram of *Eimeria* sp. infected buffalo-calves in this study coincide with the finding of [46] in coccidia infected ewes and indicated tissue damage and allergic condition due to parasitism [26, 46] Moreover, the alteration in the studied hemoglobin biophysical properties, electric conductivity and derivatives could be attributed to changes in the activity of methemoglobin reductase system due to increased stress [47]. Also, this condition could be explained in light of the behavior of hemoglobin molecule which acts as semiconductor material under certain condition [48]. It was reported that the electric conductivity was reduced in diseased animals mainly due to mechanism of folding and unfolding of the hemoglobin molecules [49].

In the present study, *Eimeria* infected buffalo-calves had high concentration of free radicals (MDA and NO) and depleted antioxidant system (CAT, SOD, ASCA, TAC and R-GSH). This implies that the affected calves are under stress condition. Oxidative stress has been implicated as a major initiator of tissue damages and can affect enzymatic activity, signal transcription and gene expression, especially apoptotic gene [13]. In animals suffering from stressful conditions, due to parasitism, [50] reported high level of MDA in rats following *Fasiola* infestation. In the same time, it was reported that growing animals show increased oxidative stress as indicated by the significant higher concentration of lipid peroxides in serum than in adult animals [51].

In this study, serum Zn, Cu, Fe and Se concentrations decreased in *Eimeria* infected calves as compared to normal healthy calves. In this respect, it was concluded that animals have Cu and Zn deficiency are usually suffering from general weakness and stunted growth[16,52], while in Cu deficient animals, increased susceptibility to infection and growth retardation were the main causes [53].

It could be concluded that high incidence of growing buffalo-calves harbor *Eimeria* oocysts. *Eimeria* coproantigen had 3 immunogenic bands which can be utilize in the diagnosis of coccidiosis. Buffalo-calves infected with *Eimeria sp* showed disturbed health condition and oxidant/ antioxidant status which of course affect their productivity.

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