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A Field Investigation on the Correlation Between Reproductive Disorders and Eimeriosis in Female Buffaloes with Emphasis on Use of Partially Purified Oocyst Antigen for Diagnosis

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Abstract: A total number of 1379 female buffaloes reared at Lower Egypt was gynecologically examined by ultrasonography and blood and fecal samples were collected during a period of three years (2004 - 2006). Out of these animals 896 (64.97%) were suffering from reproductive disorders. The recorded reproductive disorders were ovarian inactivity, endometritis, delayed puberty, mastitis, repeat breeding, retained placenta and abortions, vaginal/uterine prolapses and cystic ovaries. Eimeriosis was detected by coprological examination in 62.87% of the total examined samples. The incidence was high in animals having genital disorders (81.70%) as compared with healthy animals (27.95%). High infection rate was recorded in animals suffering from inactive ovaries (87.90%), abortion (78.57%), repeat breeding (72.41%) and endometritis (66.91%). Low incidence was reported in animals suffering from mastitis (48.72%), retained placenta (51.85%) and delayed puberty (57.89%). *Eimeria* sp oocyst antigen was partially purified by ionexchange chromatography in which DEADE cellulose was utilized. Two isolated fractions and crude antigen were characterized by SDS polyacrylamide gel electrophoresis which showed 14 bands in crude extract. While the isolated fractions revealed simple electrophoretic profile, fraction I resolved into 8 bands of molecular weight ranged from 15.1 to 180 KD and fraction II resolved into 6 bands of molecular weight ranged from 21.2 to 87.7 KD. Employing naturally infected animal sera in immunoblot assay, six immunogenic bands were detected in crude extract, four of them were relative in FI of molecular weight ranged from 25.1 to 124.6 KD, while the immunogenicity of F II reside in only two bands of 45.9 and 28 KD. Fraction I was more sensitive than fraction II by ELISA. Hence, fraction I was utilized for diagnosis of Eimeriosis with 100% sensitivity. It could be concluded that a tight association is found between the occurrence of reproductive disorders and instantaneous infections with Eimeriosis. Fraction I of *Eimeria* sp.oocyst partially purified antigen could be of helpful diagnostic value.

Key words: Buffaloes · Reproductive disorders · *Eimeria* · Ionexchange Chromatography · ELISA · Immunoblot

INTRODUCTION

In Egypt, reproductive disorders cause great economic losses, especially in animals kept in small holder farms and exposed to stressful conditions such as mal-nutrition, bad hygiene, parasitism and pollution [1].

Bovine Eimeriosis is an important disease caused by protozoan parasites of the family Eimeridae. This protozoan can cause infection at any age of animals [2], leading to considerable intestinal damage, weaken the immune system, slow weight gain and subsequent economic losses [3,4].

Diagnosis of Eimeriosis depends on the presence of oocysts in fecal samples. Microscopical examination is laborious and inaccurate. Alternative immunodiagnostic approach would represent considerable advantage for clinical and epidemiological studies; ELISA assay was useful in the detection of antibody response against *E. bovis* and *E. zurnii* in calves [5,6]. In addition IgM, IgG and IgG2 antibodies to *E. bovis* first generation merozoite antigen were determined by ELISA and Western blotting in naturally infected cows [7]. Moreover, coproantigen was considered as a reliable diagnostic potential antigen for serodiagnosis of bovine Eimeriosis [8,9].

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Previous studies [5,8,9] proved the potency of *Eimeria* oocyst antigen and coproantigen in the diagnosis of Eimeriosis in calves and cattle. No available data were be traced in the available literature regarding serodiagnosis of Eimeriosis in buffaloes.

The current investigation intended to correlate between the occurrence of reproductive disorders and Eimeriosis in female buffaloes. Also, evaluation of the use of partially purified antigen from *Eimeria* sp. oocyst for diagnosis of Eimeriosis in buffaloes was another task.

MATERIALS AND METHODS

Animals: A total number of 1379 female buffaloes (2-9 years), reared at Lower Egypt was used to execute this investigation throughout a period of 3 consecutive years (2004-2006). These animals were kept in smallholder farms and fed mainly on green fodder (*Trifolium alexandrinum*) with an inadequate amount of concentrate mixture and no regular system of vaccination or veterinary intervention.

A complete data on case history, owners complain, clinical examination and reproductive status were recorded for each animal. All female buffaloes were subjected to rectal examinations using Ultra sound apparatus (Pia Medical Falcs e`Saote, the Netherlands) with an endorectal linear array 6-8 MHz transducer and reproductive status and/or disorders were recorded.

Sampling: Blood samples without any anticoagulant were collected through the jugular vein puncture from each of examined buffaloes kept to clot, centrifuged at x1500g for 20 minutes, sera were separated and kept at -20°C until analyzed. Fecal samples were collected during rectal examination in polyethylene bag. Sera were screened for brucellosis using Rose Bengal test [10].

Coprological examination: The incidence of Eimeriosis in female buffaloes either in apparent healthy animals or in those having reproductive disorders was recorded after coprological examination according to [11].

Serodiagnosis

***Eimeria* sp. oocyst collection:** Oocysts were collected from positive fecal samples as the method described by [12]. The collected oocysts were placed in 2.5% solution of potassium dichromate and incubated at 28°C for sporulation.

Sera collection: Serum samples from 69 infested and 15 from coprologically negative animals were analyzed for antibodies detection.

Antigen preparation: Sporulated oocyst antigen was prepared according to [13]. The oocysts were homogenized in 0.15M phosphate buffer saline pH 7.2 using a tefflon glass homogenizer followed by sonication for 5 minutes to disrupt remaining intact oocysts. The homogenates were centrifuged at 15000 rpm for 45 mins at 4°C. The protein content of the supernatant was determined according to [14]. The supernatant was aliquoted and stored at -20°C until use.

Ionexchange chromatography: Purification of *Eimeria* sp. oocyst antigen was performed by ionexchange chromatography in which DEAE cellulose was adopted. The elution pattern was recorded by UV transmission at 280 nm [15]. Eluted fractions were lyophilized and stored until used.

Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE of crude extract and two isolated fractions was performed according to [16] under the reducing conditions. High and low molecular weight markers were used. After separation, gels were fixed in 50% methanol and stained with silver stain according to [17].

Enzyme Linked Immunosorbent Assay (ELISA): The assay was adopted to evaluate *Eimeria* sp. oocyst fractions activities utilizing naturally infected sera of buffaloes. The assay was performed as described by [18] and the cut off point of optical density values was determined [19]. It was calculated as mean + 3SD of the OD values for sera from coprologically negative buffaloes. The assay was also used to evaluate the potency of the selected fraction in diagnosis of Eimeriosis.

Immunoblotting: Protein bands of crude as well as pure fractions of *Eimeria* sp. oocyst antigens were electrophoretically transferred from SDS-polyacrylamide gel to nitrocellulose sheets according to [20] in a blotting system. Nitrocellulose paper was incubated with buffaloes naturally infected sera. After washing, the paper was incubated with alkaline phosphatase conjugated anti-bovine IgG, then exposed to substrate solution for 30 mins. Nitrocellulose paperwas washed with distilled water to stop the reaction.

Statistical analysis: Data were computed and statistically analyzed [21].

RESULT

Incidence of reproductive disorders: Out of 1379 female buffaloes, examined in Lower Egypt during a period of 3years (2004- 2006), 896 (64.97%) suffered from reproductive disorders (Table 1). The main recorded reproductive disorders were ovarian inactivity, endometritis and delayed puberty. However, Rose Bengal test indicated that all of these animals were negative reactors for brucellosis.

Prevalence of Eimeriosis infection: Eimeriosis was detected by coprological examination in 62.87% of the total examined samples (Table 2), the incidence was high (P <0.001) in animals suffering from genital disorders (81.70%) as compared with healthy animals (27.95%).

Prevalence of Eimeriosis infection in relation to reproductive disorders: The percentage of buffaloes with reproductive disorders and simultaneously positive for Eimeriosis was 81.70%. Meanwhile, the percentage in buffaloes showing no reproductive disorders was 27.95% (Table 2). The incidence (%) was high in animals suffering from ovarian inactivity (87.90), abortion (78.57), typical repeat breeders (72.41) and endometritis (66.91) and was low in animals having mastitis (48.72), retained placenta (51.85) and delayed puberty (57.89) and cystic ovaries (60.00).

Table 1: Incidence of reproductive disorders in examined female buffaloes (%)

Animals	No	%
Healthy	483	35.03
Reproductive disorders	896	64.97
• Ovarian inactivity	504	56.25
• Endometritis	136	15.18
• Delayed puberty	95	10.60
• Mastitis	78	8.71
• Typical Repeat breeders	58	3.24
• Retained placenta	29	3.01
• Abortions	14	1.56
• Vaginal/uterine prolapses	13	0.89
• Cystic ovaries	11	0.56
Total number	1379	100

Table 2: Correlation between Eimeriosis infection and reproductive disorders in female buffaloes

		Eimeria positive	
Reproductive disorder	Number	Number	%
Healthy	483	135	27.95
Reproductive disorders	896	732	81.70
• Ovarian inactivity	504	443	87.90
• Endometritis	136	91	66.91
• Delayed puberty	95	55	57.89
• Mastitis	78	38	48.72
• Typical repeat breeders	29	21	72.41
• Retained placenta	27	14	51.85
• Abortions	14	11	78.57
• Vaginal/uterine prolapse	8	5	62.50
• Cystic ovaries	5	3	60.00
Total number	1379	867	62.87

Purification of *Eimeria* sp. antigen: Purification of *Eimeria* sp oocyst crude extract by ionexchange chromatography using DEAE cellulose revealed two fractions (Fig. 1).

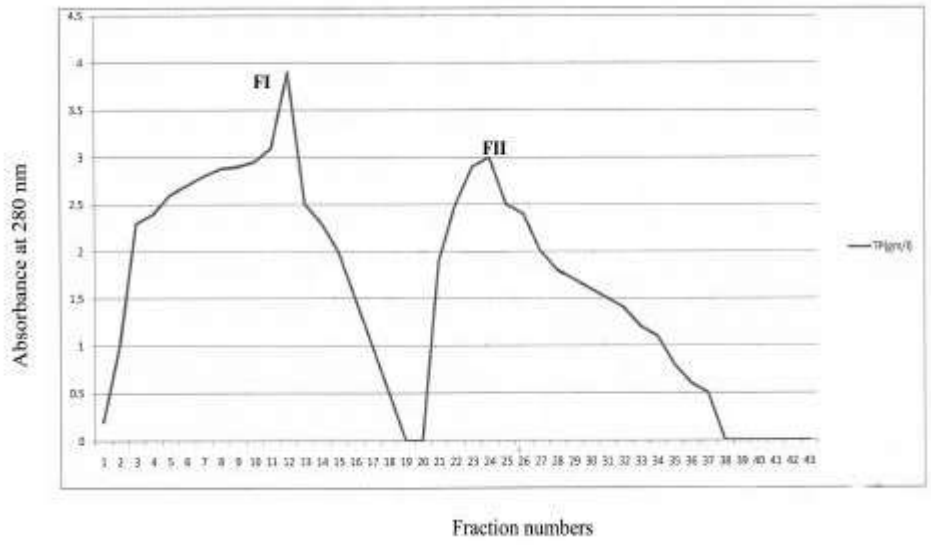

Fig. 1: Purification of *Eimeria* sp. oocyst crude antigen by DEAE-cellulose column ionexchange chromatography

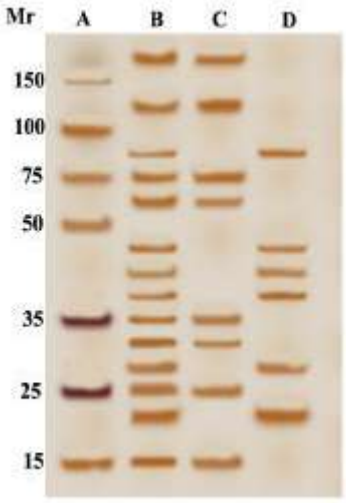

Fig. 2: Electrophoretic pattern of *Eimeria* sp. oocyst antigens: Lane A: Molecular weight standards, Lane B: Crude antigen, Lane C: F I antigen, Lane D: F II antigen

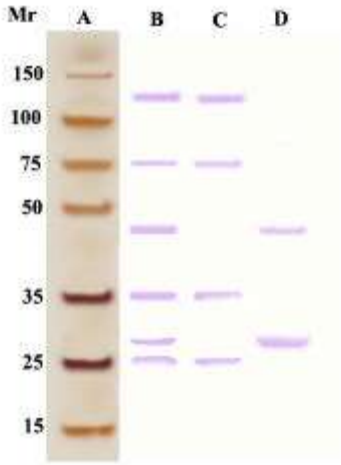

Fig. 3: Immunogenic polypeptides of *Eimeria* sp antigens identified in immunoblot assay using serum from naturally infected water buffaloes. Lane A: molecular weight standards, Lane B: crude antigen, Lane C: FI antigen, Lane D: FII antigen

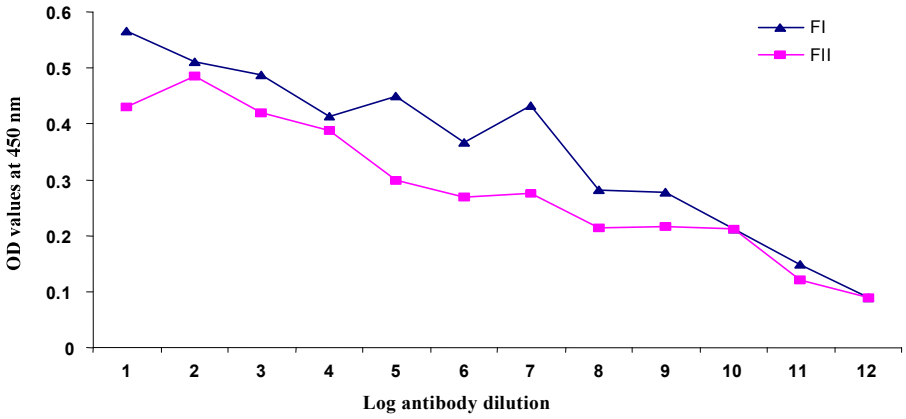

Fig. 4: Evaluation of antigenic activities of *Eimeria* sp. oocyst fractions resulted from DEAE cellulose column against naturally infected sera of female buffaloes

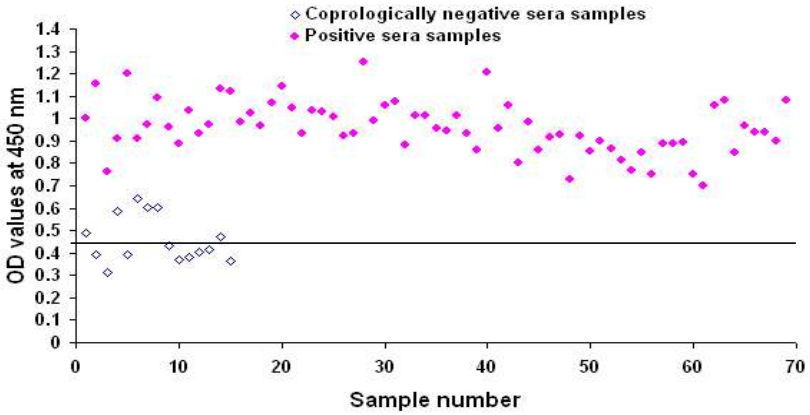

Fig. 5: ELISA evaluation of the potency of *Eimeria* sp. isolated fraction in the diagnosis of Eimeriosis in female buffaloes. - Horizontal line shows cut off value

Electrophoretic profile of *Eimeria* sp oocyst crude and pure antigens: Crude extract and two isolated fractions were separately electrophoresed on SDS-PAGE. The crude extract showed 14 bands of molecular weights ranged from 15.2 to 181.7 KD (Fig. 2-laneB). While fraction I revealed 8 bands of molecular weights ranged from 15.1 to 180 KD (Fig 2- lane C). The electrophoretic profile of fraction II showed 6 components ranged from 21.2 to 87.7 KD (Fig. 2-laneD).

Identification of antigenic components: Protein bands responsible for the immunogenicity in crude extract and pure fractions were identified by immunoblot assay using naturally infected sera (Fig. 3). Six immunogenic bands were observed in crude extract of molecular weight ranged from 25.3 to 126.6, four of them were reactive in FI with molecular weights of 124.6, 75.6, 35.3 and 25.1 KD, while the immunogenicity of F II reside in only two bands of 45.9 and 28 KD.

Antigenic potency of the isolated fractions: The antigenic activity of each fraction was evaluated by ELISA in which naturally infected sera of buffaloes were utilized. As shown in Fig. 4 fraction I showed the most potent activities at low sera dilution compared with the fraction number II. According to this result fraction number I was selected for diagnosis of Eimeriosis among naturally infected animals.

Diagnosis of Eimeriosis using fraction I of *Eimeria* sp oocyst: Sera collected from naturally infected animals, as proved by coprological examination were evaluated against fraction number I in ELISA. The assay confirmed the infection and recorded 100% sensitivity (Fig. 5). The fraction detected IgG regardless the intensity of infection where the absorbance value ranges were 0.314 -1.25. Also 6 serum samples from 15 coprologically negative animals showed positive results by ELISA (Fig. 4). The cut off value was 0.45.

DISCUSSION

Out of the world 160 million heads of buffaloes, 3.9 million heads are found in Egypt and produced 65% of meat and milk used by local population. Despite these animals are bred under harsh socioeconomic conditions and suffer from reproductive disorders and parasitic infection [1].

In the current study, case history, owner complain, clinical examination and ultrasonographic scanning of the genital organs of 1379 female buffaloes, revealed that 64.97% suffering from reproductive disorders. In this respect, it was reported that buffaloes suffering from a lot of problems, mainly reproductive disorders which cause great economic losses, especially in animals kept in small holder farms and exposed to stressful conditions such as mal-nutrition, bad hygiene, parasitism and pollution [1]. However, it was not aimed here to discuss these problems, but intention was directed to investigate the possible correlation between the occurrence of these disorders and Eimeriosis in buffaloes.

In this study, a clear association was observed between the increased incidences of Eimeriosis and the occurrence of reproductive disorders in the examined buffaloes. In this respect, it was reported that Eimeriosis infected buffaloes suffering from loss of condition, retarded growth, intermittent diarrhea and anemia [22,23]. 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[24]. Lack of trace elements correlated well with the occurrence of genital disorders in buffaloes, mainly inactive ovaries [25].

The multiplicity of parasitic antigens might be considered as the major difficulties in immunological studies of parasites [26]. Thus, to increase the diagnostic potency of antigens, isolation of their immunogenic fraction will be useful [27]. In the current research, it is good target to isolate partially purified *Eimeria* sp oocyst antigen from naturally infected buffaloes and used it in the diagnosis of this parasitic infection. Ionexchange chromatography technique was used to purify *Eimeria* sp oocyst crude extract. This method resulted in two fractions F I and F II. Moreover, the evaluation of the isolated fraction was done by ELISA. It revealed that FI was more potent than F II using buffaloes naturally infected serum. In previous studies, many authors purified sporozoites and sporulated oocycts of some intestinal Eimeriosis in chicken and rabbits using gel column chromatography [28-30].

The two isolated fractions in the present study were characterized electrophoretically to allow a comparison with the crude extract. The electrophoretic profile of crude extract that presented in the current research showed 14 bands of molecular weights ranged from 15.2 -181.7 KD. These data were contrary with those of [31,32], they reported that the *E. stiedae* sporulated oocyst antigen revealed 9 bands. While [33] found that polypeptide bands of *E. stiedae* sporulated oocyst were 2 bands. This

difference might be due to the difference of *Eimeria* sp. and procedure of antigens preparation. The two isolated fractions showed the most simple electrophoretic profile, where fraction I consisted of 8 bands of molecular weight ranged from 31.5 to 180-KD, while fraction II had only 6 bands of molecular weights ranged from 21.2 to 87.7 KD. No available data were traced concerning this contribution.

The immunoreactive bands of both crude and two isolated fractions were identified by immunoblot assay in which buffaloes naturally infected sera were utilized. Six immunogenic bands were identified in crude extract. In contrast, these data were contrary with [34] who reported that one polypeptide band of molecular weight 20 KD was an immunodominante antigen on the surface of *E. bovis* sporozoites in which calves hyperimmune sera were utilized. Such variation might be due to they used *E. bovis* sporozoites and hyperimmune sera, while in the present study *Eimeria* sp oocyst and naturally infected sera were utilized. In the present study, the most immunogenic fraction (FI) have 4 immunogenic bands of molecular weights 124.6, 75.6, 35.3 and 25.1 KD and probably responsible for the highest diagnostic potency. One of these bands of molecular weight 25 KD were detected in purified antigen of *E. tenella* and it can protect chickens against sever Eimeriosis [30].

In the current research ELISA test in which partially purified fraction I, the most potent fraction, has been used to diagnose infection among buffaloes. Fraction I which proved 100% sensitivity could detect anti-*Eimeria* oocyst IgG in all infected sera regardless its infection intensity or days post infection. Moreover, 6 sample sera from 15 coprologically negative samples showed positive result in ELISA. This result proved the advantage of antibody detection assay over coprological test.

It could be concluded that a tight association is found between the occurrence of reproductive disorders and instantaneous infections with Eimeriosis in buffaloes. The use of partially purified fraction in ELISA might be of great value in the control and epidemiological studies of this important and economic parasite. Further purification might be necessary for obtaining high purified antigens with satisfactory results.

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