ISSN 1996-3351

# Asian Journal of Biological Sciences

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Asian Journal of Biological Sciences 1 (2): 77-83, 2008
ISSN 1996-3351
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# Development of an Elisa Test for Serological Diagnosis of Coccidial Infections and Studying of Resistance against Coccidiostats Based on Flock History

[1]R. Kiani and [2]H.H. Farhang
[1]Department of Clinical Sciences, Faculty of Veterinary Medicine,
Shahid Chamran University, P.O. Box 61355-145, Ahvaz, Iran
[2]Department of Pathobiology, Faculty of Veterinary Medicine,
Islamic Azad University, Tabriz, Iran

**Abstract:** With soluble antigens prepared from sporulated oocysts of *E. tenella*, a sensitive enzyme linked immunosorbent assay was developed to survey broiler chickens' sera related to 20 broiler flocks for coccidiosis. Sera were taken at random from non-vaccinated 6 and 7 week-old-broiler chickens against coccidiosis. All flocks had not natural outbreak of coccidiosis and some of them had history of prophylactic in-feed medication with currently-used coccidiostates. All sera had absorbance values very above the detection level. There was no significant difference between prophylactic in-feed medicated groups to non-medicated groups.

**Key words:** Elisa, coccidia, drug resistance, coccidiostats

## INTRODUCTION

Avian coccidiosis, an intestinal infection caused by ubiquitous protozoan species of *Eimeria*, is a major parasitic disease considered as one of the most expensive and common diseases of poultry. It costs to the world poultry industry at least US $ 2400 million annually (Shirley *et al*., 2005). This sum includes the cost of prophylactic in-feed medication of broiler and breeding stocks and if prophylaxis fails, the financial losses due to mortality and poor performance of the survival birds (Williams, 1998).

Immunity to coccidiosis is largely dependent on cellular immunity. This has been well documented in rats, mice (Smith and Hayday, 1998) and chickens (Lillehoj, 1998; Dalloul and Lillehoj, 2005; Shirley *et al*., 2005). Even though extensive works has failed to demonstrate any practical importance of circulating antibodies to *Eimeria* parasites in chickens (Talebi and Mulcahy, 1995b; Tajima *et al*., 2003), investigators suggest that antibodies may play some roles in providing protective immunity such as some reducing invasion to epithelial cells (Tierney *et al*., 2007; Wallach *et al*., 1992). Although antibodies may not play an important role in protection (Lillehoj and Trout, 1996), serum antibody titers are clear evidence of prior exposure to *Eimeria* parasites and probably develop in parallel with cellular immunity (Constantinoiu *et al*., 2008). Various methods have been used to quantitate serum antibodies to *Eimeria* parasites. These include immunofluorescence (Watanabe *et al*., 2001; Jean *et al*., 2000), flow cytometric analysis (Pakandl *et al*., 2008), agglutination (Reinap *et al*., 1999), precipitation (Rose and Long, 1962), neutralization, complement fixation (Davis, 1981) and enzyme linked immunosorbent assay (Elisa) (Nakamura *et al*., 1991; Smith *et al*., 1993, 1994; Garg *et al*., 1999; Miyamoto *et al*., 2002; Tajima *et al*., 2003; Du *et al*., 2005; Onaga *et al*., 2005; Abdul Hafeez *et al*., 2006; Constantinoiu *et al*., 2007; Pakandl *et al*., 2008). Between these procedures, Elisa test is widely used to measure antibodies against *Eimeria* parasites. Although Elisa test has not

**Corresponding Author:** Rezvan Kiani, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University, P.O. Box 61355-145, Ahvaz, Iran

yet become a routine method in most parasitological laboratories, but because this assay requires only a small amount of antigen and has short reaction time (Crowther, 2001), it is likely that Elisa is useful for large scale antibody screening for parasites.

For these reasons, the present study was conducted to develop a sensitive Elisa test for detecting antibodies against *Eimeria* parasites and surveying the seroprevalence infection due to these pathogens and resistance phenomenon against coccidiostats, based on flock history, in broiler chickens.

## MATERIALS AND METHODS

### Parasites

From April to May 2007, three field isolates of *E. tenella* were collected from the ceca of broiler chickens died due to natural outbreak of cecal coccidiosis. Samples were taken from 3 broiler farms at suburb of Amol (a city in the northern Iran). The obtained oocysts were isolated by means of flotation with saturated salt solution described by Long *et al*. (1976). The isolated samples were purified with using sodium chloride solution containing 0.2% active chloride for 45 min. Oocysts free form fecal debris and bacteria were collected in sufficient amount of 2.5% potassium dichromate solution and stored in refrigerator at 4°C until use.

### Oocysts Propagation

Eighteen Ross non-SPF chickens were obtained from a commercial hatchery, with a good reputation of producing disease free chickens, as day old. All chickens were placed in clean wire-floored cages and provided food and water containing no prophylactic medication against coccidiosis, *ad libitum*. For more confidence in absence of any *Eimeria* spp. oocysts in the food (Kiani *et al*., 2007), the prepared ration were autoclaved at 121°C for 15 min (White, 1989).

To obtain propagated oocysts, 2 week-old chickens were directly inoculated with $3\times10^3$ field sporulated oocysts (three chickens for each isolate) into the crop by oral gavage and scarified 4 days post-infection. Based on clinical signs, predominant cecal lesions, morphology and measurement of the oocysts and presence of characteristic schizonts and gametocytes only in fresh cecal mucosal smears, infection with *E. tenella* were approved (Davies *et al*., 1963). The identified propagated oocysts of all ingested chickens were obtained and pulled together and used for preparing antigens.

### Preparation of Antigens from Oocysts

Ten million sporulated oocysts were washed twice in carbonated buffer (0.05 M, pH 9.6) and resuspended in 1 mL of mentioned solution. After these procedures, oocysts were disrupted by freezing/thawing for three times and sonicated (Nissei, Model US 330, Japan) at 20 kHz for 5 min in a jacketed vessel at 4-8°C. The homogenate was centrifuged at 10.000 x g for 30 min at 4°C to remove any remaining particular matters. The supernatant was used as antigen and collected in 100 µL aliquots and stored at -80°C until use. Protein concentration of supernatant was determined by modified Lowry procedure (Wang and Smith, 1975) (BioPhotometer, Eppendorf, Hamburg, Germany) by using Bovine Serum Albumin (BSA) as standard and adjusted to 500 µg/1 mL with carbonated buffer.

### Positive and Negative Control Sera

Antibody positive and negative control sera were obtained respectively from *E. tenella* infected chickens (three chickens) placed at clean wire-floored cages, 14 days post-infection (Lillehoj and Ruff, 1987) with identified oocysts and non-infected chickens (six chickens) housed in different building (to prevent any probable contamination from infected chickens) and were raised in coccidia free conditions.

**Elisa**

A previously described procedure (Smith *et al*., 1994) was performed with some modifications as follows. Flat bottomed polystyrene microtitration plates (Immuno-plate; Nalge Nunc International, Denmark) were coated with 100 μL of soluble *E. tenella* antigen, per each well and incubated at 37°C for 2 h. After rinsing three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), each well was treated with 100 μL of 10% fetal calf serum in PBST for 1.5 h at room temperature. This procedure was done for blocking non-specific binding sites. The plates were washed three times with PBST. Test sera were diluted 1:100 in PBST and added to each well. After 1 h incubation at room temperature, plates were washed three times with PBST and 100 μL of 1:1400 dilution of rabbit anti-chicken immunoglobulin-peroxidase (Sigma-Aldrich Inc, St Louis, Missouri, USA) in PBST was added into each well. After 1 h incubation at room temperature, plates were washed three times with PBST and 100 μL of freshly prepared enzyme substrates consisting 0.5 mg 2.2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Boehringer Manheim GmbH, Mannheim, Germany) and 2 μL hydrogen peroxide 0.3%/mL with 0.1 M citrate buffer solution, pH 5.0, were added to each well and plates were allowed to stand in the dark at room temperature. After 20 min, the enzyme substrate reaction was stopped by addition of 100 μL of 2 N $H_2SO_4$ to each well. Absorbance of the reacted solution was measured at 405 nm with an Elisa reader (Molecular Devices, Sunnyvale, CA, USA).

The detection level (cut-off point) was used in the assay based on the mean absorbance (A) value of the negative sera: Detection level = Mean A value + (3×standard deviation) (Onaga *et al*., 1986).

**Serum Samples**

From July to September 2007, a total of 1000 serum samples were taken at random from 6 and 7 week-old-chicks (500 samples for each age) related to twenty broiler flocks. Samples were obtained from broiler farms located at suburb of Amol. All flocks were not vaccinated against coccidiosis and in each age, half of the samples had history of prophylactic in-feed medication with currently-used coccidiostates. Only those farms were selected that had not natural outbreak of coccidiosis.

Blood samples were taken from the wing veins and allowed to clot for 1 h at room temperature. Clotted samples were transported to the laboratory and stored overnight at 4°C and then centrifuged at 800 x g for 5 min. Sera were separated, aliquoted and heated at 56°C for 30 min for inactivation of complement and then stored at -20°C until use. All sera were analyzed with the developed Elisa Triplicate.

**Statistical Analysis**

Comparison of data was done by student's t-test and values of $p<0.05$ were considered to be statistically significant.

## RESULTS

**Detection Level**

The mean absorbance value of chickens which rose in coccidia free conditions and its standard deviation were 0.303±0.01. From these data, detection level was calculated to be 0.333.

The mean absorbance value of control positive sera and its standard deviation were 0.482±0.04.

**Field Antibody Levels to Eimeria**

All tested sera had absorbance values very above the detection level. In each age, there was no significant difference between Elisa values of prophylactic in-feed medicated group to non-medicated group ($p>0.05$) (Table 1).

Table 1: Elisa absorbance values in non-medicated and medicated groups (Mean±SD)

| Age of slaughter (weeks) | Treatments | |
|---|---|---|
| | Medicated group[1] | Non- medicated group[1] |
| 6 | 0.404±0.06[a] | 0.406±0.08[a] |
| 7 | 0.409±0.06[a] | 0.415±0.04[a] |

[a]Values in line or column with no common superscript differ significantly ($p<0.05$). [1]Two hundred and fifty chicks were examined in each group per age

In each medicated and non-medicated groups, there was no significant difference between Elisa values of 6 and 7 week groups ($p>0.05$) (Table 1).

## DISCUSSION

In Iran, the broiler industry has been developing rapidly in recent years. The broiler chickens are mostly reared in a deep-litter system and in spite of continuous use of a number of prophylactic drugs as food additives; coccidiosis has become a serious problem and remains as one of the major diseases confronting the poultry industry of country.

Among the various species of *Eimeria* which are different in their pathogenicity (McDougald, 2003; Shirley *et al*., 2005), *E. tenella* which causes cecal coccidiosis, is highly pathogenic (Yadav and Gupta, 2001) and is the commonest of the *Eimeria* species throughout the world (Trees, 1996). Chicken *Eimeria* species share many antigens and antibodies rose against one species cross-react with the antigens of other species (Xie *et al*., 1992; Uchida *et al*., 1994; Talebi and Mulcahy, 1995a; Constantinoiu *et al*., 2007). Considerable degrees of cross reaction between the oocyst antigens of *E. tenella* and antisera against other *Eimeria* species have been reported (Rose *et al*., 1984; Karkhanis *et al*., 1991; Chapman and Shirley, 2003). Therefore, when antibody levels are measured by the present developed Elisa to survey the spread of *Eimeria* infections in the field, the assay will detect antibodies against all *Eimeria* species other than *E. tenella*.

All sera prepared from prophylactic in-feed medicated and non-medicated groups (in each 6 and 7 weeks of age) had absorbance values very above the level of detection. Because the level of antibodies appears to be related to the severity of infection (Nakamura *et al*., 1991; Smith *et al*., 1993; Lee *et al*., 2007) and according to Garg *et al*. (1999) and Onaga *et al*. (1986) which proposed that the infection is substantially suppressed by medication with prophylactic drugs as evidenced by low Elisa values, results of the present study suggest that, in Amol area, the *Eimeria* infections are substantially resisted to medication with currently-used prophylactic drugs.

In broilers, peak infection of coccidiosis occurs at 4-5 weeks of age (Long and Rowell, 1975; Chapman, 2003). Because high antibody levels against *Eimeria* spp parasites are detected 2 weeks after the infection (Lillehoj and Ruff, 1987; Constantinoiu *et al*., 2007), no significant difference of Elisa values between 6 and 7 week groups is compatible to these reports.

## CONCLUSION

According to the present study it is concluded that with increasing of drug resistance of *Eimeria* spp. in the field, development of an alternative control method such as vaccines against these economically important pathogens could clearly be useful and practical.

## ACKNOWLEDGMENTS

The technical assistance of M. Rasadi and all staffs of Behdam Veterinary Hospital are gratefully acknowledged. The authors also appreciate the help of F. Niknafs (scientific manager of Zarbal Corporation) and N. Mohammadi (head manager of Par sefid Corporation). We thank R.A. Jafari for helpful comments on earlier versions of the manuscript.

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