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ENZYME-LINKED IMMUNOSORBENT ASSAY AND INDIRECT HAEMAGGLUTINATION TECHNIQUES FOR MEASUREMENT OF ANTIBODY RESPONSES TO *EIMERIA TENELLA* IN EXPERIMENTALLY INFECTED CHICKENS

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ABSTRACT: An indirect haemagglutination test (IHA) and ELISA technique were developed to detect antibodies against *Eimeria tenella*. The ELISA technique was relatively easy to perform, more sensitive than the IHA test, and needed only a fraction of the antigen required for IHA. The highest titers using ELISA were 1:16,384 compared to the IHA titers of 1:64 for the same sera. The ELISA titers depended upon the age of the birds when they were infected, the number of oocysts inoculated and the number of inoculations. Immunodeficient birds (cyclophosphamide-treated), when inoculated with several doses of oocysts of *E. tenella* (350, 3,000, 30,000) showed no IHA or ELISA antibody titers. The immuno-competent chickens of the same age, which received identical doses of oocysts responded with readily detectable antibody levels. Chickens inoculated with *E. maxima* or *E. necatrix* had sera titers of 1:50 or 1:400, respectively, when reacted with *E. tenella* antigen. The *E. tenella* inoculated birds had titers as high as 1:3,200 with the same antigen.

Circulating antibodies to *Eimeria tenella* infection have been demonstrated by different methods. McDermott and Stauber (1954) reported the agglutination of second-generation merozoites by sera of chickens recovered from cecal coccidiosis. Rose and Long (1962) demonstrated precipitins in immune sera, but showed that their presence was not required for resistance to infection. Long et al. (1963) and Burns and Challey (1965) reported the *in vitro* lysis of *E. tenella* sporozoites and merozoites by sera of resistant chickens. Morita et al. (1972) detected antibodies against *E. tenella* by a dye test, while Abu Ali et al. (1976) demonstrated circulating antibody response to *E. tenella* by fluorescent antibody. Later, Kuil et al. (1977) measured antibodies against *E. maxima* and *E. acervulina* by indirect fluorescent antibody test.

The present investigation was conducted to compare 2 methods for detection of the humoral immune response to *Eimeria tenella* in experimentally infected chickens. A semipurified soluble antigen derived from sporulated oocysts was used in the enzyme-linked immunosorbent assay (ELISA) and indirect haemagglutination test (IHA). Since its introduction by Engvall and Perlmann (1972) for use in the detection of serum antibodies, the ELISA has found wide ap-

plication in parasitology (Voller et al., 1976) and may have a potential use for the diagnosis of infections with coccidia.

MATERIALS AND METHODS

Animals

One-day-old Rhode Island Red or White Leghorn chicks were obtained from the Poultry Science Department, University of Minnesota (breed based on availability) and placed in a coccidia free room. They were raised in clean wire-floored cages and provided food with no coccidiostat and water *ad lib*. Uninfected chickens were kept in the same room throughout the study.

Inoculation of experimental birds

The oocysts of *E. tenella*, *E. necatrix* and *E. maxima* were originally provided by Eli Lilly and Co. Fresh cultures of oocysts of *Eimeria* spp. were prepared routinely from the ceca or intestines of donor birds. Eight days after oral inoculation of infective oocysts, the infected birds were killed, the ceca (*E. tenella* and *E. necatrix*) or small intestine (*E. maxima*) were removed, and the contents were homogenized. The oocysts were sporulated at room temperature in 2.5% aqueous potassium dichromate and stored at 4 C until used. The sporulated oocysts of *Eimeria* spp., in each stock suspension, were counted in a haemocytometer and the required dose calculated. Experimental chickens, from 2 wk to 16 wk old, were orally inoculated with oocysts of *E. tenella* (300 to 450,000, with an interval of 2 wk between inoculations). Each experiment utilized only 1 breed of chicken. We found no difference between Rhode Island Red and White Leghorn chickens in their response to coccidia. Infected chickens were kept in a separate room in isolation cages (Bemrick and Hammer, 1979) and provided with food and water *ad lib*. Each bird was identified with a leg band.

Eight days after the last inoculation, the birds were

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TABLE I. *Minimum and maximum IHA and ELISA titers obtained from sera of chickens infected with E. tenella.*

Exp.	Group	No. birds used for		Age first inoculated (in weeks)	Doses of oocysts used for sensitization and challenge ($\times 10^3$)	Range of reciprocal titers	
		ELISA	IHA			ELISA	IHA
1	A	51	36	2	0.3	4-64	0-2
	Co*	8	8			0	0
2	B	22	19	3	0.3	16-256	0-2
	Co	10	4			0	0
3	C	42	28	3	0.3, 3	64-4,096	2-4
	Co	8	4			0	0
4	D	4	4	8	225	1,024-4,096	2-64
	Co	2	2			0	0
5	E	4	4	8	450	256-4,096	4-8
	Co	2	2			0	0
6	F	30	26	14	0.5, 5, 69	64-16,384	4-64
	Co	5	5			0	0

* Co = Control uninoculated chickens.

bled via cardiac puncture and killed by cervical dislocation. Serum from each bird was collected separately and stored at -20°C . Each sample was inactivated at 56°C for 30 min, prior to use.

Ceca or small intestines were removed immediately. The cecal lesions induced by *E. tenella* were scored using a modification of the technique of Johnson and Reid (1970). The difference between our method and that cited was the use of 0 to +5. The 0 was for birds with no lesions or detectable oocysts. Our score of +1 was for birds with no visible lesions, but oocysts were present in the fresh smears. This addition made the technique somewhat more accurate for the detection of an infection. It raised the number of the categories from the original 5 of Johnson and Reid to 6 in the current investigation.

Cross-reaction experiments

These experiments were conducted to determine the immunologic cross-reactivity between *E. tenella* and 2 other avian coccidians, *E. maxima* and *E. necatrix*.

Commercial White Leghorn chickens were inoculated twice with about 1,200 and 12,000 sporulated oocysts of *E. maxima* or *E. necatrix*. Their sera were evaluated for antibodies using the ELISA technique utilizing the antigen prepared from *E. tenella* oocysts. The infection with each species was conducted separately to avoid possible contamination.

Immunosuppressed birds

One-day-old chicks received 4 mg cyclophosphamide (Sigma Chemical Co., St. Louis, Missouri) intramuscularly as a single dose for 4 days, in order to suppress their immune responses (Nathanson et al., 1981). Chickens in this experiment were divided into 4 groups: A) untreated inoculated, B) untreated uninoculated, C) treated inoculated birds, and D) treated uninoculated. Inoculated birds received 2 immunizing doses of 350 and 3,000 sporulated oocysts of *E. tenella* with a 2-wk interval between the doses. All birds were challenged with 30,000 oocysts.

Preparation of antigens

Sporulated oocysts of *E. tenella* were centrifuged in a saturated salt solution and then separated from debris

by passing through a glass bead column (modified from Wagenbach, 1969). The column was made of an empty 50-ml syringe filled to 20-ml level with 200- μm glass beads. Nytex mesh (60 μm) was used for the bottom layer to prevent a loss of the beads. The oocysts were simply washed through the glass beads with distilled water. For further purification oocysts were cleaned in a solution of 2.5% sodium hypochlorite followed by several washes in water.

Oocysts were frozen and thawed 3 times and then disrupted with a Sonifier (Branson Sonic Power Co., Danbury, Connecticut), at a setting of 7 for 9 min in an ice bath. The disruption was confirmed by microscopic examination. Soluble antigen was separated from the debris by centrifuging at $12,000 \times g$ for 30 min. The protein concentration was 1.15 mg per ml (Bio Rad) using bovine serum albumin as the standard. The soluble antigen eliminated false positives obtained with IHA or ELISA when compared to the complete antigen or the sedimented pellet obtained after centrifuging.

Indirect haemagglutination

The indirect haemagglutination (IHA) technique utilized chromium chloride sensitized sheep cells (Jandl and Simmons, 1957). Titration was performed in round-bottomed microtiter plates and Titertek Microtitration equipment (Flow Laboratories, McLean, Virginia). Sensitized sheep red blood cells were coated with soluble antigen (protein concentration of 616 μg per ml). The titer was determined to be the serum dilution in the last well giving complete haemagglutination, using 2-fold dilutions.

Enzyme-linked immunosorbent assay (ELISA)

The sandwich ELISA technique was performed according to Voller et al. (1976) with some modifications. The amounts of antigen, conjugate and substrate used in this assay were optimized before they were used in the present study. Soluble antigen was diluted in carbonate buffer, to contain 11 μg antigen protein per ml. It was dispensed (100 μl) into each well of flat-bottomed Immulon micro ELISA microtitration plates (Dynatech Laboratories, Inc., Alexandria, Virginia). The plates were incubated overnight at 4°C . The plates were then washed 3 times with 0.85% phosphate buffered

TABLE II. Reciprocal ELISA titers and lesion scores for chickens treated with cyclophosphamide and inoculated with oocysts of *E. tenella*.

Groups	Cyclo* treated	Oocyst inoculated	Weeks of age	Total chicks in a group	Mean lesion score after challenge‡	ELISA titers after challenge							
						0	50	100	200	400	800	1,600	3,200
A	—	+	8	20	0	0	2	5	6	5	1	0	1
B	—	—	8	12	0	12	0	0	0	0	0	0	0
C	+	+	8	20	3.87	20	0	0	0	0	0	0	0
D	+	—	8	12	0	12	0	0	0	0	0	0	0

* Cyclophosphamide, 4 mg once a day for 4 days.

† Inoculation with 350, 3,000, 30,000 oocysts for *E. tenella*, starting at 2 wk old.

‡ Challenged with 30,000 oocysts at 6 wk old.

saline (PBS) containing 0.05% Tween 20. Serum was diluted 4-fold with a starting dilution of 1:4. After 1.5 hr, the serum was removed and the plates were washed 3 times with PBS. Peroxidase conjugated rabbit anti-chicken IgG (Cappel Laboratories, West Chester, Pennsylvania) was added at a dilution of 1:1,000 in PBS. The substrate O-phenylenediamine (Eastman Kodak Co., Rochester, New York) in phosphate-citrate buffer was then added. To stop the reaction, 50 μ l of 2 N H₂SO₄ were added to each well. The optical densities were read at 490 nm on an ELISA reader (Dynatech, Lab Inc., Alexandria, Virginia). Optical densities of less than 200 were considered negative, because the known negative sera were below 200 and the known positives gave higher optical densities.

RESULTS

The results of experiments 1–6 are shown in Table I. It has included the IHA and ELISA titers for the birds of 3 wk to 19 wk old, that had been inoculated with 1 to 3 doses of oocysts of *E. tenella*. The doses varied from as low as 300 up to 450,000. Sera from 153 chickens inoculated with *E. tenella* and 35 uninfected control sera were analyzed for antibody using the ELISA technique. Sera from all of the inoculated birds were positive, and 3 control sera showed optical densities of ≥ 200 at 1:4 dilution. The false pos-

itive sera were all derived from 19-wk-old chickens. Sera from 117 *E. tenella* inoculated chickens and 25 uninfected control birds were tested by the IHA technique. All of the control sera and 29 (25.2%) from inoculated birds had no hemagglutination titers. The highest titer obtained with IHA was 1:64 (2 birds), compared to titers of 1:16,384 (5 birds) detected with the ELISA technique.

In order to decrease the chance of false positives, all subsequent sera dilutions started at 1:50 and continued with 2-fold dilutions. This procedure eliminated false positive reactions in our control sera.

In the immunosuppression studies, none of the suppressed birds which had been inoculated, regardless of the number of oocysts in the inoculum, had any ELISA antibody titers. The uninoculated birds, both treated and untreated, were all negative. The untreated-inoculated chickens had titers (1:50–1:3,200) after 3 inoculations. All of the untreated-inoculated birds were protected against the challenge dose and had no visible cecal lesions, or detectable oocysts in their cecal smears. The challenged immunosuppressed birds were unprotected, and had mean lesion scores of

TABLE III. Reciprocal ELISA titers obtained with *E. tenella* antigens and sera from birds inoculated with either 1 or 2 doses of *E. maxima*, *E. necatrix*, or *E. tenella*.

Experiment	First inoculation						Second inoculation									
	No. birds	No. oocysts per birds ($\times 10^3$)	ELISA titers				No. birds	No. oocysts per birds ($\times 10^3$)	ELISA titers							
			0	50	100	200			0	50	100	200	400	800	1,600	3,200
<i>E. maxima</i>	18	1.2	14	4	0	0	15	12	10	5	0	0	0	0	0	0
	12	0	12	0	0	0	12	0	12	0	0	0	0	0	0	0
<i>E. necatrix</i>	10	1.2	7	2	1	0	10	12	4	3	0	2	1	0	0	0
	12	0	12	0	0	0	12	0	12	0	0	0	0	0	0	0
<i>E. tenella</i>	22	0.3	18	3	0	1	42	3	0	21	0	6	0	9	0	6
	10	0	10	0	0	0	8	0	8	0	0	0	0	0	0	0

3.87. One of them died after the third inoculation as a result of the coccidia infection (Table II)

In the cross-reaction studies, data from 18 chickens inoculated with 1,200 oocysts of *E. maxima* (first inoculation) indicated that 14 sera (72.2%) had no antibody titers and 4 sera (22.2%) had titers of 1:50 against the *E. tenella* antigen. Of the 15 surviving chickens 10 (66.6%) birds had no antibody titers after a second inoculation of 12,000 oocysts. Sera from 5 chickens had titers of 1:50.

Seventy percent of the 10 chickens inoculated with 1,200 oocysts of *E. necatrix* had no titers and the highest titer obtained was 1:100. Of the birds which received a second inoculating dose of 12,000 oocysts, 4 sera (40%) had no titers and 1 bird had a titer of 1:400. The highest titer for the chickens inoculated with 300 and 3,000 oocysts of *E. tenella* were 1:200 and 1:3,200 respectively (Table III).

DISCUSSION

The ELISA technique and the IHA technique were used to measure the serum antibody response of chickens orally inoculated with oocysts of *E. tenella* when they were between 2 wk to 16 wk old. The IHA was less sensitive than the ELISA technique and required 56 times more antigen than the ELISA. All *E. tenella* inoculated chickens used in the experiments, plus sera from 3 normal birds, were determined to be positive using the ELISA technique. These false positives have not been attributed to accidental exposure to oocysts, because the birds were protected against such exposure through the utilization of our modification (Bemrick and Hammer, 1979) of the special cages designed by Patton and Sapsanski (1974), and special handling facilities. Random fecal and necropsy samples collected periodically from all uninoculated birds in our facilities were examined for *Eimeria* spp., and were consistently negative. There has always been a danger of accidental infection in any experiment, but because of the extreme precautions taken and the isolated titer found in the older uninfected birds we are unable to explain these positive responses. In an attempt to eliminate these false positive reactions, sera were diluted to 1:50 and subsequent 2-fold dilutions prior to testing.

Recently 2 other similar papers have been published by Rose and Mockett (1983) and Danforth and Augustine (1983). These papers support the

presence of some cross-reactivity between *E. tenella*, *E. maxima*, and *E. necatrix*.

Danforth and Augustine (1983) could eliminate the cross-reactivity by using hybridoma technology and detected induction of antibody by the immunofluorescent antibody (IFA) technique. This cross-reactivity was assumed to be due to the antigen(s) shared by these species of coccidians.

Antibody titers could not be detected in any of the chickens injected with cyclophosphamide and inoculated 1, 2, or 3 times with sporulated oocysts of *E. tenella*. This may have demonstrated the effect of cyclophosphamide in the removal of the B-cells from these chickens. Nathanson et al. (1981) reported that cyclophosphamide treated chickens suppressed antibody production to *Pasturella multocida* for at least 10 wk, and the cell-mediated immune response for less than 1 month. Untreated birds of the same age, that had been inoculated with the same numbers of oocysts of *E. tenella*, all had detectable titers ranging from 1:50 to 1:3,200.

To determine the cross-reactivity between antigenic determinants of *E. tenella* and other chicken coccidians, sera from chickens inoculated with *E. maxima*, *E. necatrix* or *E. tenella* were collected and evaluated for antibodies that reacted with antigens prepared from *E. tenella* oocysts.

The cross-reactivity of sera collected from *E. maxima* inoculated birds, thought to be the most immunogenic of the chicken coccidia, was low. The highest titer obtained with either 1 or 2 inoculations, was 1:50. Sera from chickens inoculated with *E. necatrix* had positive titers up to 1:400 after the second sensitizing inoculation. Birds in this study were inoculated with larger numbers of oocysts in an attempt to increase the antibody response and therefore to increase the chances of obtaining a cross-reaction with the *E. tenella* antigen. Chickens receiving oocysts of *E. tenella* had titers as high as 1:200 or 1:3,200 after first and second inoculations, respectively.

The ELISA technique, with appropriate modifications, may provide a useful method for the serological diagnosis of other important coccidians of large animal species, such as cattle or swine.

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ANNOUNCEMENT . . .

Immunoparasitology Symposium

The Third International Immunoparasitology Symposium will be held 19–21 June 1985 in Lincoln, Nebraska. The symposium will be technique/approach oriented. Topics covered will stress immunodiagnosis, parasite modulation of host immune responses, separation and characterization of substances of parasite origin, immune modulations, and genetic engineering/biotechnology for vaccination against parasites. Protozoans, arthropods, cestodes, trematodes, and nematodes will be addressed. Several new systems will be demonstrated. Poster presentations by participants are encouraged.

Proceedings of the meeting will be available to participants at registration. Attendance will be limited to 300 persons. A fee of \$75.00 (\$50.00 for graduate students) will cover registration costs, proceedings, and lunches for each day. For more complete information, including speakers and topics, and registration forms, contact Dr. Gary L. Zimmerman, Symposium Chairman, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331 (telephone [503] 754-2927 or 2141).