Detection of Serum Antibodies in *Eimeria tenella*-Infected Chickens by Enzyme Linked Immunosorbent Assay (ELISA) with Merozoite and Oocyst Antigens

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ABSTRACT. Soluble antigens prepared from sporulated oocysts and second generation merozoites of *E. tenella* were used for enzyme linked immunosorbent assay (ELISA) to investigate antibody in sera of two breeds of chickens, i.e. commercial broilers and SPF single comb white leghorn layers, which were experimentally infected with *E. tenella*. In broilers inoculated with oocysts at 15 days of age, ELISA values increased rapidly after day 19 post inoculation (PI) and reached the maximum lebel on days 29 and 32 PI against both merozoite and oocyst antigens. The values against merozoite antigen were significantly higher than those against oocyst antigen. In SPF layers infected at 15 days of age, the values increased gradually after 7 days PI. There were no significant differences between values against two antigens. Generally, the values in broilers tended to be higher than those in SPF layers, especially against merozoite antigen. In broilers inoculated with oocysts at 1 and 15 days of age, ELISA values increased rapidly and reached the maximum level on days 11 and 20 post second inoculation (PSI) against merozoite and oocyst antigens respectively and then the values against merozoite antigen decreased. The values against merozoite antigen were markedly higher than those against oocyst antigen. In SPF layers inoculated twice, the values reached the highest on day 11 PSI as in the case of broiler; however, after that day, the values against both antigens decreased. The sera reacted similarly against both antigens. The values against merozoite antigen were significantly higher in broilers than in SPF layers. The values of maternal antibodies were comparatively high in broiler chicks and decreased gradually with the age of bird to the negligible level at 10 days of age.—KEY WORDS: coccidia, ELISA, *Eimeria tenella*, oocyst, second generation merozoite.

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Immunity against avian coccidiosis has been studied by many authors [8–9, 15, 17]. It has been reported that chickens acquired protective immunity after coccidian infection and the immunity is specific to coccidian species [9–10]. The mechanism of the immunity, however, is still obscure, and especially the role of serum antibodies in protective immunity is unclear [11–13].

Enzyme Linked Immunosorbent Assay (ELISA) has been used in the diagnosis and antibody determination in various animal and human diseases [22]. ELISA is one of the techniques widely used to determine antibody against a variety of protozoal diseases [22, 24].

In the present study, we investigated serum antibodies of infected chickens by a modified ELISA technique by Gilbert *et al.* [7], and Rose and Mockett [21]. Antibodies were determined by using soluble antigens from second generation merozoites and sporulated oocysts of *E. tenella*, and the optical density (OD) of serum antibodies was investigated in the course of infection.

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MATERIALS AND METHODS

Birds: Newly hatched commercial broiler (Arbor-Acres) and single comb white leghorn chickens were used. The latter breed of bird derived from the parent flock which had been reared under specific pathogen free (SPF) condition in Aburahi Laboratories, Shionogi and Co., Ltd. Chickens were kept in steam sterilized, wire floored cages and provided with feed and water *ad libitum*.

Parasite: Fresh sporulated oocysts of *Eimeria tenella (E. tenella)* of NIAH strain originated from the National Institute of Animal Health, Japan were used [2, 23].

Experimental designs: Twelve broiler and 20 layer chickens were used. The two breeds of chickens were divided into two groups according to the method of inoculation. In the first group, 6 broiler and 10 layer chickens were inoculated with a single dose of 5×10^4 sporulated oocysts each at 15 days of age. The chickens were bled 7 days, and after then at 3 or 4 day intervals until 35 days after inoculation. In the second group, 6 broiler and 10 layer chickens were inoculated with a dose of 5×10^4 sporulated oocysts each at the age of 1 and 15 days after birth.

The chicken were bled seven days and after then at 3 day intervals until 15 days after the first inoculation. After the second inoculation at day 15 the chicken were bled again at intervals of 3 or 4 days until 35 days.

To detect maternal antibody by ELISA, birds were kept free of coccidia and 3 birds each were sacrificed at 0, 3, 5, 7, 10, 14 and 21 days of age. Sera were heated at 56°C for 30 min for inactivation of complement and stored at −20°C until use.

Isolation and purification of second generation merozoite of E. tenella: Twenty five of 15-day-old commercial broiler chickens were inoculated orally with 1×10^5 sporulated oocysts of *E. tenella*. Chickens were sacrificed 94–96 hr after inoculation, and second generation merozoites were purified by the methods of Fernando *et al.* [3] and McDonald *et al.* [16] with slight modification. The cecum was removed from the chickens immediately after slaughter and put into petridishes containing phosphate-buffered saline (PBS, pH 7.2). The ceca were cut open longitudinally and large blood clots were removed. The ceca were rinsed with PBS and put into another petridish containing PBS, and the mucosa was scraped with a spatula. The suspension of the scraped tissue was pooled and centrifuged at 1,100×g for 10 min. The sediment was suspended in an appropriate amount of PBS and the suspension was homogenized in a glass teflon homogenizer (Labo stirrer, Yamato Scientific., Co., Ltd., Tokyo) until most of second generation merozoites were freed from schizonts. The homogenate was centrifuged at 70×g for 10 min and the supernatant was stored. The procedure was repeated three times. All the supernatant fluid was stored and centrifuged at 1,100×g for 10 min. The sediment was resuspended with 3 m*l* of 70% Percoll solution (Pharmacia, Upsala, Sweden). The suspension was carefully overlaid on 30 m*l* of 70% Percoll solution in a centrifuge tube and the tube was centrifuged at 24,000×g for 20 min. Second generation merozoites, which concentrated above the erythrocytes layer, were pipetted and mixed with five parts of PBS. The mixture was centrifuged at 1,100×g for 10 min and the sediment was suspended in 0.5 m*l* of PBS to prepare as merozoite suspension.

Preparation of merozoite antigens: The merozoite suspension was centrifuged at 1,100×g for 5 min and the sediment was resuspended with 0.1 M Tris-HCl (pH 6.8) containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF) and aprotinin (protease inhibitor, 200 kallikrein units/m*l*). This procedure was repeated three times. Finally, the sediment was suspended gently with 0.5 m*l* of 0.1 M Tris-HCl (pH 6.8). After glass beads of 0.5 mm in diameter were added about one-third (V/V) of its volume, the suspension was sonicated (Sonicator, Ohtake Works, Tokyo) 6–7 times at 25 watt for 30 sec in an ice bath. The sonicated merozoite suspension was transferred to a small Eppendorf tube (Eppendorf, Netheler, Hamburg) and was mixed with an equal volume of 0.1 M Tris-HCl containing 3% of Nonidet-P40 and 10 mM ethylene diamine tetraacetic acid (EDTA). The mixture was allowed to stand overnight at 4°C. The supernatant was collected after centrifuging at 6,500×g for 10 min. Protein included in the supernatant fluid was determined with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA.) and the fluid was stored at −20°C until use.

Preparation of oocyst antigens: Ten-day-old broiler chickens were infected with *E. tenella* oocysts. Sporulated oocysts were prepared from the feces of chicken collected 7 days after the infection by the method of Davis [2]. From the purified oocysts, the antigen was prepared by the same procedure as used for merozoite antigen mentioned above.

ELISA: ELISA was performed in 96-well flat-bottomed high binding type ELISA plates (Costar, Cambridge, MA). Appropriate dilutions and concentrations of reagent were determined by checkerboard titration reported by Gilbert *et al.* [7] with slight modification. Each well was coated with 5 μg merozoite or oocyst antigen in 100 μl of 0.015 M carbonate-buffered solution (pH 9.6) and incubated at 4°C over night. After discarding the antigen, each well was coated with 200 μl of 3% bovine serum albumin (RIA grade, Sigma, St. Louis, U.S.A.) in PBS and incubated for 2 hr at room temperature to block non-specific adsorption. Wells were emptied and then into them was poured 100 μl sera 100 fold diluted in PBS containing 0.05% Tween 20 (PBST) each. After 2 hr incubation at room temperature, wells were rinsed five times with PBST, and then was added 100 μl of peroxidase-conjugated rabbit anti-chicken IgG (H+L chains specific, Cappel, West Chester, PA) 1,000 fold diluted in PBST. After 2 hr incubation at room temperature, wells were washed five times with PBST and then was added 100 μl freshly prepared substrate solution of 2,2-azino-di(3-ethyl-benzithiazoline) sulfonate (AB-TS, Kirkegaard & Perry Laboratories Inc., Mary-

land). After 30 min of incubation at room temperature in darkness, absorbances were measured at 405 nm with an automatic Micro-ELISA reader (Bio-Rad, Richmond, CA.). To obtain positive control sera 5 broilers were inoculated twice with 5×10^4 sporulated *E. tenella* oocysts each at 10 and 17 days of age. Eleven days after the second inoculation the broilers were sacrificed and bled. After inactivated by heating at 56°C for 30 min, sera were stored at −20°C until use. Negative control sera were obtained from three-week-old SPF chicken.

Statistical analysis: Mean±SD values of OD were analyzed by Student's T test.

RESULTS

Antibody levels of chicken sera after a single inoculation with *E. tenella* were assayed by ELISA (Figs. 1 and 2). In broiler chickens (Fig. 1), ELISA values against both merozoite and oocyst antigens reached the maximum levels on days 29 and 32 post inoculation (PI) respectively following lower values on days 11, 15 and 19 PI. The values against merozoite antigen were markedly higher than those against oocyst antigen ($p<0.05$, asterisks) on days 15, 19 and 26 PI. In SPF layer chickens (Fig. 2), ELISA values increased gradually after day 7 PI and the highest values were observed on days 32 and 35

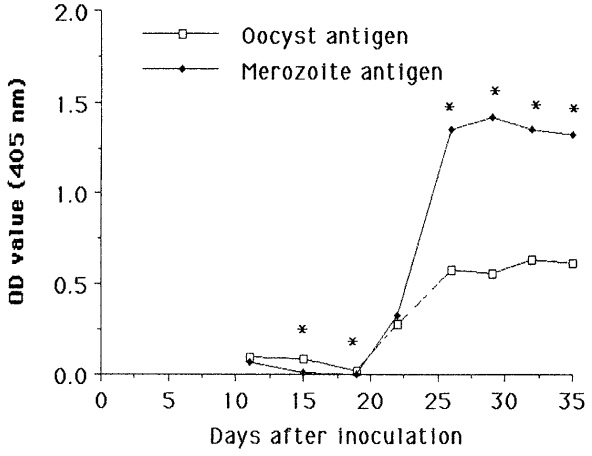

Fig. 1. Mean OD values in the serum of six broiler chickens (Arbor-Acres) after a single inoculation with 5×10^4 sporulated oocyst of *E. tenella* at 15 days of age. The OD value was determined by ELISA using two different antigens. Asterisk (*) show significant differences ($P<0.05$) between ELISA values by merozoite antigen and those by oocyst antigen. OD values of the sera of the treated chickens were obtained by subtracting the mean OD values with the control value of 0.124.

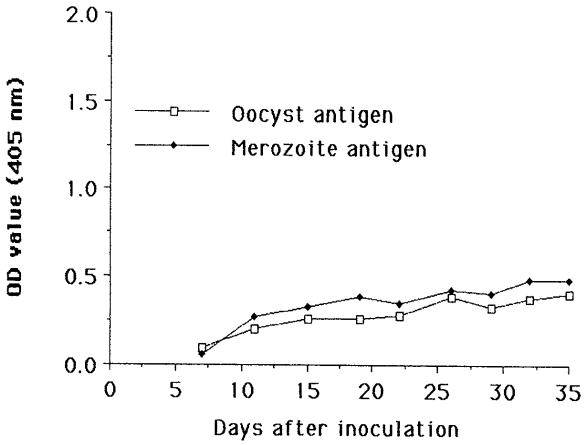

Fig. 2. Mean OD values in the serum of 10 single comb white leghorns (layer, SPF) after a single inoculation with 5×10^4 sporulated oocysts of *E. tenella* at 15 days of age. The OD value was determined by ELISA using two different antigens. No significant difference was observed between the values of OD against the two antigens ($P>0.05$). OD values of the sera of the treated chickens were obtained by subtracting the mean OD values with the control value of 0.124.

PI against merozoite and oocyst antigens respectively. There are no significant differences between the values against merozoite and oocyst antigens. When the results in broilers and SPF layers were compared to each other, ELISA values of SPF layers against merozoite and oocyst antigens were significantly higher than those of broilers on day 19 and 15 PI respectively; however, the values of broilers against merozoite and oocyst antigens were significantly higher than those of layers on day 26 PI.

Antibody levels were observed in the course of twice inoculation by ELISA (Figs. 3 and 4). ELISA values against both merozoite and oocyst antigens were low in broilers after the primary inoculation at one day of age (Fig. 3). After the secondary inoculation at 15 days of age, ELISA values increased rapidly and reached the maximum levels against merozoite and oocyst antigens on days 11 and 20 post second inoculation (PSI) respectively. The values against merozoite antigen decreased after day 11. The ELISA values against merozoite antigen were markedly higher than those against oocyst antigen after day 7 PSI. In SPF layers (Fig. 4), ELISA values increased after the secondary inoculation and reached the highest on day 11 PSI similar to the case of broiler; however, after day 11, the values decreased against both antigens. No significant differences were observed between the values against merozoite and oocyst antigens. EL-

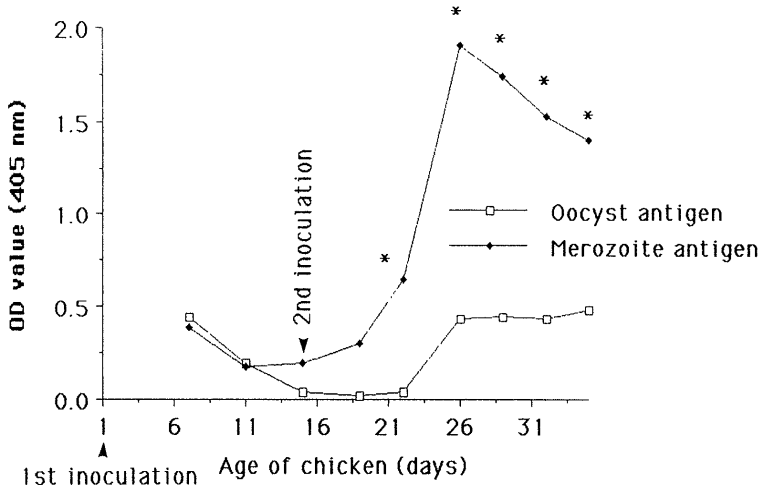

Fig. 3. Mean OD values in the serum of six broiler chickens (Arbor-Acres) after double inoculation with 5×10^4 sporulated oocyst of *E. tenella* at one day of age. The OD value was determined by ELISA using two different antigens. Asterisks (*) show significant differences ($P<0.05$) between ELISA values by merozoite antigen and those by oocyst antigen. OD values of the sera of the treated chickens were obtained by subtracting the mean OD values with the control value of 0.124.

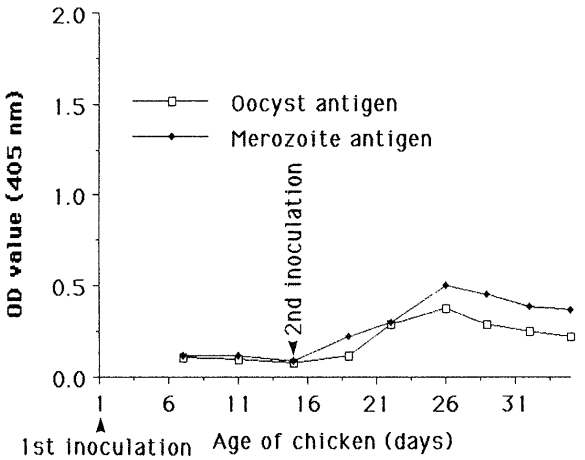

Fig. 4. Mean OD values in the serum of 10 single comb white leghorns (layer, SPF) after double inoculation with 5×10^4 sporulated oocysts of *E. tenella* at one day of age. The OD value was determined by ELISA using two different antigens. No significant difference was observed between the values of OD against the two antigens ($P<0.05$). OD value of the sera of the treated chickens were obtained by subtracting the mean OD value with the control value of 0.124.

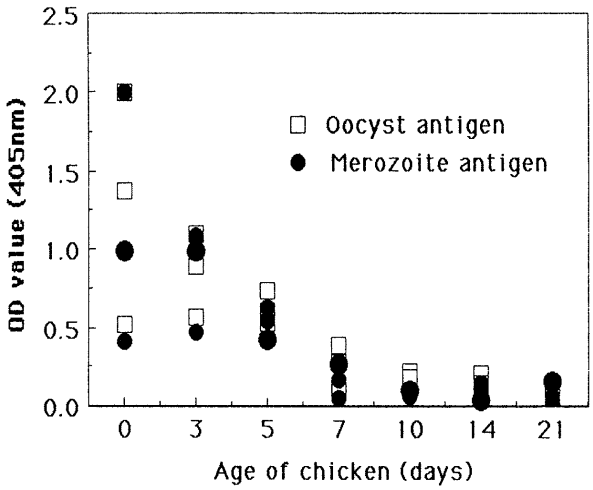

Fig. 5. Maternal antibodies against second-generation merozoite and sporulated oocyst antigens of *E. tenella* detected by ELISA in broiler chicks of different ages.

ISA values of broilers against merozoite and oocyst antigens were higher than those of SPF layers on days 26 and 35 PI respectively (Figs. 3, 4), however, the values of SPF layers against merozoite antigen were lower than those of broilers through the course of experiment, and values against oocyst antigen was higher than those of broilers on day 22 PI.

Maternal antibodies in broilers were investigated by ELISA using merozoite and oocyst antigens (Fig. 5). The chicken sera showed high ELISA values at hatching, which thereafter decreased gradually with the age of bird and were negligible in level after 21 days of age.

DISCUSSION

The present results showed that antibodies were produced after single or multiple inoculations of *E. tenella*. Antibody response was first detected 7 days after inoculation and gradually increased in parallel with the duration of infection. However, weak antibody response was detected for 15 days after the first inoculation. These results were similar to those described by other investigators [14, 19–20], who reported that antibody is consistently developed in chicks after single or multiple inoculations. These results suggest that the underdeveloped immune response in broiler chickens for 15 days after birth may be caused by maternal antibody which was transferred to young chicks. Gilbert *et al.* [7] reported that maternal antibody detected in serum of broiler chicks frequently interferes with the development of immunity in very young. SPF chickens which were negative for antibody against *E. tenella*. This result was similar to those reported by previous investigators [1, 4–7].

In the present results the antibody level in sera of broiler chickens was higher against second generation merozoite antigen than against oocyst antigen after single or multiple inoculation. Rose *et al.* [19–21] reported that in the life cycle of *E. tenella* second generation merozoites are highly immunogenic compared with the other forms of parasite, and that different stages in their life cycle probably have different antigenic structures and compositions, and also differ in immunogenicity and ability to induce protective immune response. On the contrary, as reported by Jenkins and Dame [9], infective sporozoites may not induce strong immune response owing to their relatively smaller numbers and the absence of sporozoite antigen in the later stage of infection. Our results suggest that the second generation merozoite and sporulated oocyst antigens were different to each other in reactivity, and agreed with those of Jenkins and Dame [9] and Rose *et al.* [19–21]. Furthermore, in our experiments, the second generation merozoite and sporulated oocyst antigens of *E. tenella* were compared to each other and analysed by the western blotting technique, and the result revealed that second generation merozoite and sporulated oocyst antigens had similarities and dissimilarities in polypeptide bands that could react specifically to sera from chicken infected with *E. tenella*. As mentioned above, there were some differences in reactivity between second generation merozoite and sporulated oocyst antigens of *E. tenella* either by ELISA or immunoblotting. Our results suggest that the second generation merozoite antigen is more reactive than sporulated oocyst antigen. As reported by Nolan *et al.* [18], merozoite antigen is noticeably more reactive than oocyst antigen. Finally, we suggest that the effect of maternal antibody on the development of active immunity in young chicks, and, in addition, the correlation between high levels of antibody and polypeptide bands detected by ELISA and immunoblotting are needed to be clarified.

REFERENCES

1. Danforth, H. D. and Augustine, P. C. 1983. Specificity and cross-reactivity of immune serum and hybridoma antibodies to various species of avian coccidia. *Poult. Sci.* 62: 2145–2151.
2. Davis, L. R. 1973. Techniques. pp. 413–458. *In*: The Coccidia: *Eimeria, Isospora, Toxoplasma* and Related Genera (Hammond, D. M. and Long, P. L. eds.), Univ. Park Press, Baltimore.
3. Fernando, M. A., Al-Attar, M. A., and Bowles, G. H. 1984. Preparation of second generation merozoites of *E. necatrix* free of host tissue. *J. Parasitol.* 70: 154–155.
4. Giambrone, J. J., Klesius, P. H., Eckamm, M. K., and Edgar, S. A. 1981. Influence of hormonal and chemical bursectomy on the development of acquired immunity to coccidia in broiler chickens. *Poult. Sci.* 60: 2612–2618.
5. Giambrone, J. J., Klesius, P. H., and Edgar, S. A. 1980. Avian coccidiosis: Evidence for a cell-mediated immune response. *Poult. Sci.* 59: 38–43.
6. Giambrone, J. J. and Johnson, L. W. 1984. Development of cell-mediated immunity and resistance to clinical coccidiosis infection in chickens selected for resistance and susceptibility to *E. tenella*. *Poult. Sci.* 63: 2162–2166.
7. Gilbert, J. M., Bhanushali, J. K., and McDougald, L. R. 1988. An enzyme-linked immunosorbent assay for coccidiosis in chickens: Correlation of antibody levels with prior exposure to coccidia in the laboratory and in the field. *Avian Dis.* 32: 688–694.
8. Jeffers, T. K. and Shirley, M. W. 1982. Genetic, specific and infraspecific variation. pp. 63–100. *In*: The Biology of the Coccidia (Long, P. L. ed.), Univ. Park Press, Baltimore.
9. Jenkins, M. C. and Dame, J. B. 1987. Identification of immunodominant surface antigens of *E. tenella, acervulina* sporozoites and merozoites. *Mol. Biochem. Parasitol.* 25: 155–164.
10. Lillehoj, H. S. 1987. Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates hosts protective immunity. *Infect. Immun.* 55: 784–791.
11. Lillehoj, H. S. 1986. Immune response during coccidiosis in SC and FP chickens. I. In vitro assessment of T cell proliferation response to stage specific parasite antigens. *Vet. Immunol. Immunopathol.* 13: 321–330.

12. Lillehoj, H. S. 1988. Influence of inoculation dose, inoculation schedule, chicken age, and host genetics on disease susceptibility and developement of resistance to *E. tenella* infection. *Avian Dis.* 32: 437–444.
13. Lillehoj, H. S. and Ruff, M. D. 1987. Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with *E. tenella, acervulina* or *maxima. Avian Dis.* 31: 112–119.
14. Long, P. L., Rose, M. E., and Pierce, A. E. 1963. Effects of fowl sera on some stages in the life cycle of *E. tenella. Exp. Parasitol.* 14: 210–217.
15. Krassner, S. M. 1963. Factors in host susceptibility and oocysts infectivity in *E. acervulina* infections. *J. Protozool.* 10: 327–332.
16. McDonald, M., Rose, M. E., and Jeffers, T. K. 1986. *E. tenella* immunogenicity of the first generation of schizogony. *Parasitology* 93: 1–7.
17. Mockett, A. P. A. and Rose, M. E. 1986. Immune response to *Eimeria*: Quantification of antibody isotypes to *E. tenella* in chicken serum and bile by means of the ELISA. *Parasit. Immunol.* 8: 481–489.
18. Nolan, A. and Goldring, O. L. 1986. Demonstration of antibodies to *Eimeria* species in lambs by an enzyme-linked immunosorbent assay. *Res. Vet. Sci.* 42: 119–123.
19. Rose, M. E. 1971. Immunity to infection: Coccidiosis is as an example. pp. 93–108. *In*: Poultry Disease and World Economy (Gordon, R. F. and Freeman, B. M. eds.), British Poultry Science, Edinburgh.
20. Rose, M. E. and Long, P. L. 1962. Immunity to four species of Eimeria in fowls. *Immunology* 5: 79–92.
21. Rose, M. E. and Mockett, A. P. A. 1983. Antibodies to coccidia: detection by enzyme-linked immunosorbent assay (ELISA). *Parasit. Immunol.* 5: 479–489.
22. Ruitenberg, E. J., Sterrenberg, P. A., Brosi, B. J. M., and Buys, J. 1976. Reliability of the ELISA for the diagnosis of *Trichinella spiralis* infections in conventionally raised pigs. *J. Immunol. Methods* 10: 67–83.
23. Stotish, R. L. and Wang, C. C. 1975. Preparation and purification of merozoites of *E. tenella. J. Parasitol.* 61: 700–703.
24. Voller, A., Bidwell, D., Huldt, G., and Engvall, E. 1974. A microplate method of ELISA and its application to malaria. *Bull. WHO* 51: 209–211.