# Inhibition of *Eimeria acervulina* Sporozoite Invasion by Rabbit and Chicken Antisera Using ISI Assay

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***Summary***

Hyperimmune antisera from rabbits, which immunized with sporozoite antigens of *E.acervulina* and chickens that infected with homologous sporulated oocysts were used for invasion experiments in MDBK cell. Both hyperimmune antisera were able to decrease significantly (P=0.002 for rabbit sera and P=0.004 for chicken sera) MDBK cell invasion by sporozoites of *E.acervulina*. Comparison of the effects in various conditions (with and without pre-treatment) demonstrated that the percentage inhibition for the rabbit antisera with pre-treatment was higher than that without pre-treatment (P=0.05). Overall, the degree of inhibition of sporozoite invasion varied between the antisera and the rabbit anti-sporozoite sera in both conditions had a greater degree of inhibition than the chicken homologous antisera (P=0.003). These observations are another proof of the effectiveness of serum antibodies on sporozoites of *Eimeria* species and may suggest that rabbits recognize more key epitopes of sporozoites than the natural host.

***Key words*****:** *Eimeria acervulina*, rabbit, chicken, antisera, MDBK cells, inhibition, sporozoite invasion.

## *Introduction*

To inhibit sporozoite invasion (ISI) into host cells rather than impair the replication of the developmental stages is an ideal immunological approach for control of

coccidiosis when the direct and indirect losses following entrance of sporozpoites into the cell are considered. The invasion process consists of at least three phases including recognition of suitable cells, attachment to them and entrance into the cells (induction of parasitophorous vacuole and translocation) (Russell 1983, Dubremetz 1993). The organelles of sporozoites or merozoites are involved in the invasion process, for example, the conoid serves in penetration (Ryley 1973, Augustine 1989), the micronemes act in recognition/binding of the target cells, and the rhoptries participate in parasitophorous vacuole formation (Dubremetz 1993). In natural coccidial infections, site-specificity for each species is determined before the invasion takes place (Shiotani *et al* 1992) and certain molecules present on caecal epithelium of chickens are involved in attracting of the sporozoites towards the site of entry (Vervelde *et al* 1993). The ability of sporozoites to invade various cell cultures (Doran & Augustine 1973, Millard & Long 1974, Augustine 1985) provides an ideal model for study of biological aspects of *Eimeria* species. However, the aim of this study was to compare the effectiveness of antibodies from hyperimmune sera raised in rabbits (immunized with sporozoite antigens of *E.acervulina*) and in chickens (following repeated infections with homologous sporulated oocysts) on inhibition of sporozoite invasion of MDBK cells using ISI assays, with and without pre-treatment of the sporozoites with the antisera.

## *Materials and Methods*

**Preparation of purified sporozoites of *E. acervulina*.** Two groups of one-day-old chicks (Cobb 500) were fed on non-medicated broiler diet *ad libitum*, raised in wire-floored cages under coccidia-free conditions and their faeces examined daily for presence of oocysts in order to monitor environmental contamination. At 5 weeks of age, the chickens were leg-banded and each group was kept in a separate room. At 6 weeks of age, the chickens one group (6 chickens) were inoculated (2 doses at 3 weeks intervals) with ($1\times10^5$) sporulated oocysts (Johnson & Long 1989) of *E.acervulina* strain W119 (Central Veterinary Laboratory, Weybridge, Surrey, UK) into the crop as previously described (Mockett & Rose 1986), while chickens of other group was

kept as a control. Following the primary inoculation, faeces of infected chickens were collected for four days starting from the 5th day post-inoculation (pi). Unsporulated oocysts were harvested, sporulated, purified and stored at 4°C (Ryley & Wilson 1976, Hofman & Rather 1990). For preparation of purified sporozoites, $1\times10^8$ sporulated oocysts were excysted (Wang 1978, Sutton *et al* 1989, Hofmann & Rather 1990), the sporocysts purified (Dulski & Turner 1988), sporozoites liberated (Smith & Strout 1979, Sutton *et al* 1989), purified (Dulski & Turner 1989), resuspended in medium and counted.

**Preparation of chicken hyperimmune antisera.** Blood samples were taken from the vena cutanea ulnaris (wing vein) of the chickens on day 0 (before inoculation) and weekly post-inoculation in order to determine the antibody level of the sera using ELISA. When titers of specific antibodies of infected chickens were high, chickens of booth groups were bled and sera were collected and stored at –20°C until used. Sera of control chickens were used for comparative studies with those of infected groups.

**Preparation of rabbit anti-sporozoite sera.** A group of rabbits (New Zealand white strain) were kept in a cage system in a coccidia-free environment and fed on non-medicated diet. Two rabbits were injected with 4ml of emulsified suspension of $1\times10^7$/ml homogenized sporozoites (*E.acervulina*) in Freund's complete and incomplete adjuvant (FCA and FIA) at 2 weeks intervals. Antibody responses of the rabbits were evaluated by measurement of antibody titers in sample sera taken on different occasions. Two weeks after the second injection, at the peak of antibody production, the rabbits were bled, the sera collected and stored at –20°C until used. A group of rabbits was also kept as control for monitoring environmental contamination and their sera were compared with those of the immune rabbits.

**Culture of MDBK cells on cover slip.** A total number of $35\times10^4$ MDBK cells in 1ml Dulbecco's minimum essential amino acids medium (DMEM, Gibco) [supplemented with 1% L-glutamine (Sigma), 1% gentamicin (Gibco), 7.5% fetal bovine serum (Sigma), 1% non-essential amino acids (Gibco), 100 IU/ml penicillin and 100μg/ml streptomycin (Gibco)] were cultured on 13mm round Thermonox plastic cover-slips

(Nunc Inc.) using 24-flat bottom well sterile tissue culture plates (Nunc Inc.). The cultured plates were incubated for 24 h at 37°C.

**ISI assay using without pre-treatment.** When the cover slip had a confluent monolayer cell sheets, which was ≥85%, the media of each well (culture unit) was discarded using a sterile pipette. After dispensing 0.5 ml of each ditution (1/10, 1/50, 1/100, 1/1000, 1/2000, and 1/5000) of hyperimmune chicken or rabbit antisera in DMEM into each well (triplicate cultures per each serum dilution), the cultures were inoculated immediately with $1\times10^6$ purified sporozoites of *E.acervulina* in 0.5ml DMEM. For each assay, three cell cultures without serum and sporozoites, three cultures with cells and sporozoites, and three cells cultures containing only the highest concentration of serum were used as controls. The plates were incubated for 24 h at 40°C in 5% $CO_2$ and a humidified atmosphere. In order to count the number of sporozoites, which had invaded the cultured cells, haemotoxylin & eosin staining was used. Briefly, the cover-slips are removed from the wells of the tissue culture, placed in a 13mm wide agar aids staining rack and immersed in a bath of phosphate buffered saline (PBS) for 2-5min. Acetone solution was used for fixation of the cells and after 5min, the cells were then given a quick rinse in distilled water before being placed into haemotoxylin for 2min. The cover-slips are washed in tap water before being dipped twice (10-15s) in 1% acid alcohol solution and then washed again under running tap water for 5 to 10min so as to allow bluing to occur. The cover slips were placed in eosin for 1-2min and then the excess stain was washed with tap water for 2-3min. The cover slips were placed sequentially in 90% alcohol, absolute alcohol and xylene each lasting approximately 2min. The surface of the cover slip bearing the cells was placed facing upward on top of a drop of mounting fluid (D.P.X) using a glass slide. A second drop of D.P.X. is then placed on the cover slip before another glass cover slip was placed on top (the refractive index of the plastic make microscopic examination difficult if the cover slip with surface bearing the cells face downward). The inhibition of sporozoite invasion was quantified by counting the number of intracellular sporozoites in 10 microscopic fields per culture (three cultures for each sample) at 600X magnification (approximately an area of

10041μm$^2$/field) and percentage of inhibition for each culture was determined using the following equation:

$$\% \text{ Inhibition} = 100 \times \frac{\text{Sporozoites of control culture} - \text{sporozoites of culture with serum}}{\text{Sporozoites of control culture}}$$

**ISI assay with pre-treatment.** In general, apart from treating of sporozoites with the antisera or normal sera, the procedures for this assay were same as described for previous ISI assay. Briefly, $6\times10^6$ (twice of required number, $1\times10^6$/culture and triple cultures/each serum dilution) purified sporozoites of *E.acervulina* were incubated in 6ml of each serum dilution (1/10, 1/50, 1/100, 1/1000, 1/2000, and 1/5000) of the antisera or normal sera in DMEM for 1 h at room temperature. The suspensions were centrifuged at 800*g* for 15min in a Beckman model TJ-6 centrifuge using a TH-4 rotor. The supernatant was discarded and the sediment for each sample was re-suspended in fresh growth DMEM medium in a concentration of $1\times10^6$ sporozoites. A volume of 1ml DMEM containing $10^6$ pre-treated sporozoites was used for inoculation of the cultured as described for previous ISI assay.

## *Results*

*ISI assay using without pre-treatment conditions.* During these assays, the rabbit antisera raised against sporozoite antigens of *E. acervulina* had deleterious effects on sporozoites but degree of prevention of sporozoite invasion into MDBK cells differed among various dilutions of the antisera and as shown in Figure 1, the 1/10 dilution had the highest percentage inhibition effects (97%). Sera from the control rabbits did not significantly affect the invasion process, the differences in the mean percentage ISI by the rabbit antisera and those from the control rabbits was significant (P=0.002). In the case of the chicken homologous antisera or sera from the control chickens, the kinetic of inhibition effects was similar to that observed for the rabbit sera and differences in the mean percentage inhibition between the sera from the hyperimmune and control chickens was significant (P=0.004). Comparison of the effects of the antisera from rabbits and chickens revealed that the rabbit antisera were slightly more effective than those of the chickens in inhibition of sporozoite invasion but the differences between them was not significant (P>0.5).

Figure 1. *Effects of antibodies from hyperimmune rabbit (immunized with sporozoites of E. acerevulina antigens) and chickens (infected with homologous sporulated oocysts) sera on inhibition of sporozoites invasion into MDBK cells using ISI assays without pre-treatment conditions*

*ISI assay using with pre-treatment conditions.* The kinetics of inhibition effects of the rabbit and chicken antisera on sporozoites of *E.acervulina* in these assay were similar to those observed in ISI assay using without pre-treatment conditions. As shown in figure 2, the 1/10 dilution of both the antisera from the rabbits or chickens had also the highest inhibitory effects (92% and 74%, respectively). While sera from the control rabbits or chickens did not significantly affect the invasion process and the differences in the mean percentage ISI by the rabbit or chicken antisera with comparison to those of corresponding control sera were also significant (P=0.002 and P=0.004, respectively) in pre-treatment conditions. The mean percentage inhibition of sporozoites by the rabbit antisera significantly (P=0.003) differed from that for the chicken hyperimmune sera.

Comparison of the inhibitory effects of the antisera or the control sera within these two conditions (with and without pre-treatment) indicated that these conditions do not

affect significantly (P>0.05) the inhibitory effects of the normal sera. In the case of antisera, pre-treatment of sporozoites by the rabbit antisera was more effective at higher dilution (1/1000-1/5000) in comparison to that observed with ISI assay using without pre-treatment, and the difference between them was significant (P=0.05).



Figure 2. *Effects of antibodies from hyperimmune rabbit (immunized with sporozoites of E. acerevulina antigens) and chickens (infected with homologous sporulated oocysts) sera on inhibition of sporozoites invasion into MDBK cells using ISI assays with pre-treatment conditions*

## *Discussion*

The results of this study in regard to the effectiveness of antibodies on sporozoites are in agreement with previous studies which have reported passive immunization against some species of *Eimeria* (Wallach *et al* 1990, Smith *et al* 1994) or have demonstrated that antibodies have deleterious effects on the developmental stages of these parasites (Crane *et al* 1986, Rose, 1987), despite lack of correlation between protection and serum antibody level (Gilbert *et al* 1988, Lillehoj *et al* 1989, Talebi & Mulcahy 1955) following natural coccidial infections. Pre-treatment of sporozoites with antibodies affects the ability of sporozoites to invade cells *in vitro* (Augustine 1985) and prolong incubation period of sporozoites with monoclonal antibodies

increases the inhibitory effect with respect to both the penetration and development of the parasites in cell cultures (Danforth 1983). Differences in the degree of inhibition of sporozoites by chickens and rabbits anti-*E.acervulina* sera observed during this study may be related to different mechanisms involved in the induction of immune responses by the natural host and a laboratory animal. It has been suggested that antigen recognition may differ between hosts (Rose & Mokett 1983) and so far in the case of *Eimeria* species, rabbits and chickens have been shown to recognize different antigens of *E.tenella* (Vervelde *et al* 1992, Talebi & Mulcahy 1944). In addition to the fact that rabbits were injected with homogenized sporozoites and the chickens infected in a natural conditions, is likely to have a bearing on the results because development of parasites inside of host cells or substances produced during the life-cycle may affect the extent of T-cell involvement and other factors in the immune response.

***References***

Augustine, P.C. (1985). A study of the invasion of cells by *Eimeria* sporozoites using monoclonal antibodies generated against sporozoites and cultured host cells. In: L.R. McDougald, L.P. Joyner & P.L. Long (Eds), *Research in Avian Coccidiosis, Proceedings of the Georgia Coccidiosis Conference*. Pp:602-608. Department of Poultry Science, University of Georgia, Georgia, USA.

Augustine, P.C. (1989). The *Eimeria*: Cellular invasion and host cell parasite interaction. In: P. Yvore (Ed.), *Coccidia and Intestinal Coccidiomorphs, Proceedings of the Vth International Coccidiosis Conference*, Tours (France). Pp:205-212. Service des Publications, INRA, Paris.

Crane, M.S.L., Norman, D.J., Gnozzio, M.J., Tate, A.C., Gammon, M. and Murray, P.K. (1986). *Eimeria tenella*: quantitative *in vitro* and *in vivo* studies on the effects of mouse polyclonal and monoclonal antibodies on sporozoites. *Parasite Immunology* 8:467-480.

Danforth, H.D. (1983). Use of monoclonal antibodies directed against *Eimeria tenella* sporozoites to determine specificity and *in vitro* effect on parasite penetration and development. *American Journal of Veterinary Research* 44:1722-1727.

Doran, D.J., Augustine, P.C. (1973). Comparative development of *Eimeria tenella* from sporozoites to oocysts in primary kidney cell cultures from gallinaceous birds. *Journal of Protozoology* 20:658-661.

Dubremetz, J.E. (1993). Apical organelles (rhoptries, micronemes, and dense granules) and host cell invasion by coccidia: what do we know now? In: J.R. Barta and M.A. Fernando (Eds), *Proceedings of the VIth International Coccidiosis Conference*, Guelph (Canada). Pp:3-9. Department of Pathology, Ontario Veterinary College, University of Guelph, Canada.

Dulski, P., Turner, M. (1988). The purification of sporozoites from *E. tenella* oocysts using percoll density gradients. *Avian Diseases* 32:235-239.

Gilbert, J.M., Bhanushall, J.K. and McDougald, L.R. (1988). An enzyme-linked immunosorbent assay for coccidiosis in chickens: correlation of antibody level with prior exposure to coccidia in the laboratory and in the field. *Avian Diseases* 32:688-694.

Johnson, J.K., Long, P.L. (1989). The fecundity and drug sensitivity of precocious lines of avian *Eimeria*. In: P. Yvore (Ed.), *Coccidia and Intestinal Coccidiomorphs, Proceedings of the Vth International Coccidiosis Conference*, Tours (France). Pp:671-676. Service des Publications, INRA, Paris.

Hofmann, J., Raether, W. (1990). Improved techniques for the *in vitro* cultivation of *E. tenella* in Primary chick kidney cells. *Parasitology Research* 76:479-486.

Lillehoj, H.S., Ruff, M.D., Bacon, L.D., Lamont, S.J and Jeffers, T.K. (1989). Genetic control of immunity to *Eimeria tenella*. Interaction of MHC genes and non-MHC linked genes influence levels of disease susceptibility. *Veterinary Immunology and Immunopathology* 20:135-148.

Millard, J., Long, P.L. (1974). The viability and survival of sporozoites of *Eimeria in vitro*. *International Journal of Parasitology* 4:423-432.

Mockett, A.P.A., Rose, M.E. (1986). Immune responses to *Eimeria*: Quantification of antibody isotypes of *E. tenella* in chicken serum and bile by mean of the ELISA. *Parasite Immunology* 8:481-489.

Rose, M.E. (1987). Immunity to *Eimeria* infections. *Veterinary Immunology and Immunopathology* 17:333-343.

Rose, M.E., Mockett, A.P.A. (1983). Antibodies to coccidia: detection by the enzyme-linked immunosorbent assay (ELISA). *Parasite Immunology* 5:479-489.

Russell, D.G. (1983). Host cell invasion by *Apicomplexa*: an expression of the parasite's contractile system. *Parasitology* 87:199-209.

Ryley, J.E. (1973). Cytochemistry, physiology, and biochemistry. In: D.M. Hammond and P.L. Long (Eds), *The Coccidia*. Pp:145-181. University Park Press, Baltimore, USA.

Ryley, J.E., Wilson, R.G. (1976). Drug screening in cell culture for the detection of anticoccidial activity. *Parasitology* 73:137-148.

Shiotani, N., Baba, E., Fukata, T., Arakawa, A. and Nakanishi, T. (1992). Distribution of oocysts, sporocysts and sporozoites of *Eimeria tenella* and *Eimeria maxima* in the digestive tract of chickens. *Veterinary Parasitology* 41:17-22.

Smith, C.K., Strout, R.G. (1979). *E. tenella*: accumulation and retention of anticoccidial ionophores by extracellular sporozoites. *Experimental Parasitology* 48:325-330.

Smith, N.C., Wallach, M. Miller, C.D., Morgaenstern, R., Braun, R. and Eckert, J. (1994). Maternal transmission of immunity of *Eimeria maxima*: Enzyme-linked immunosorbent assay analysis of protective antibodies induced by infection. *Infection and Immunity* 62:1348-1357.

Sutton, C.A., Shirley, M.W. and Wisher, M.H. (1989). Characterization of coccidial proteins by two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Parasitology* 99:175-187.

Talebi, A., Mulcahy, G. (1994). High-resolution mapping of B-cell epitopes within an antigenic sequences from *Eimeria tenella*. *Infection and Immunity* 62:4202-4207.

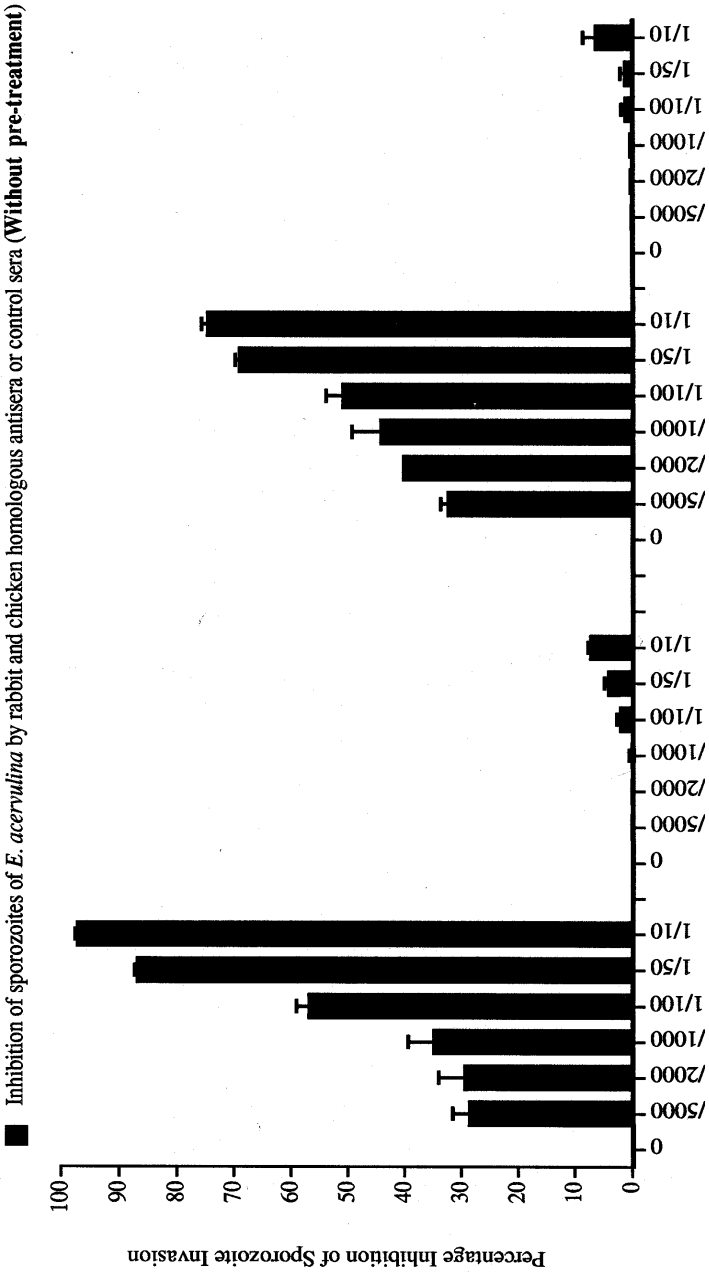
Talebi, A., Mulcahy, G. (1995). Correlation between immune responses and oocyst production of chickens monospecifically infected with *E. maxima*. *Avian Pathology* 24:485-495.

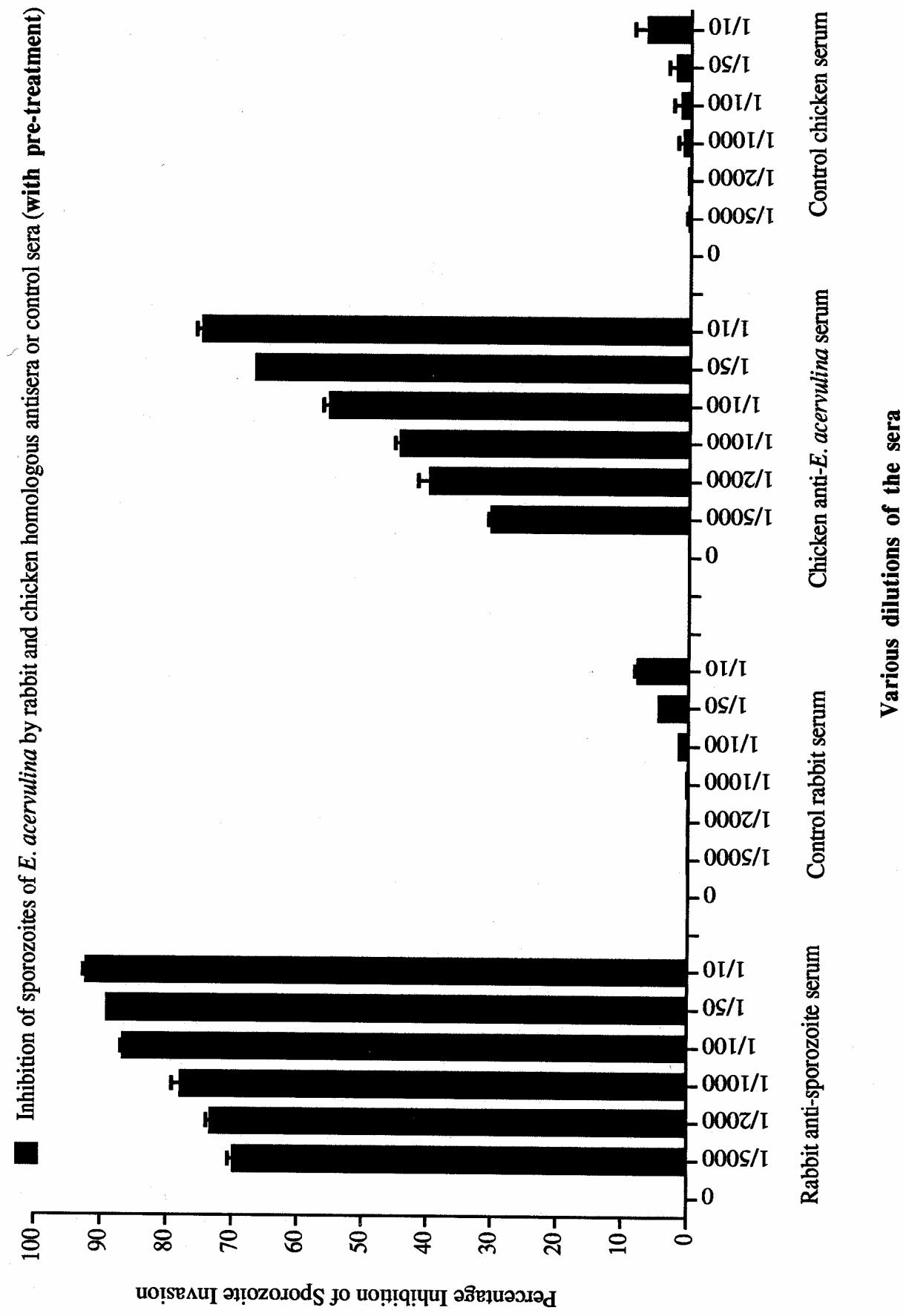
Vervelde, L., Vermeulen, A.N. and Jeurissen, S.H.M. (1992). *In situ* immunocytochemical detection of cells containing antibodies specific for *Eimeria tenella* antigens. *Journal of Immunological Methods* 151:191-199.

Vervelde, L. Vermeulen, A.N. and Jeurissen, S.H.M. (1993). Common epitopes on *Eimeria tenella* sporozoites and caecal epithelium of chickens. *Infection and Immunity* 61:4504-4506.

Wallach, M.G., Pillemer, G., Yarus, S., Halabi, A., Pugatsch, T. and Mencher, D. (1990). Passive immunization of chickens against *E. maxima* infection with a monoclonal antibody developed against a gametocyte antigen. *Infection and Immunity* 58:557-562.

Wang, C.C. (1978). Biochemical and nutritional aspects of coccidia. In: P.L. Long, K.N. Boorman and B.M. Freeman (Eds), *Avian Coccidiosis, Proceedings of the thirteenth Poultry Science Symposium*. Pp:135-184. British Poultry Science Ltd., Edinbrugh, UK.




Inhibition of sporozoites of E. acervulina by rabbit and chicken homologous antisera or control sera (with pre-treatment)
Percentage Inhibition of Sporozoite Invasion
100
90
80
70
60
50
40
30
20
10
0
Rabbit anti-sporozoite serum
Control rabbit serum
Chicken anti-E. acervulina serum
Control chicken serum
1/10
1/50
1/100
1/1000
1/2000
1/5000
0
Various dilutions of the sera