



# ***Plasmodium gallinaceum*: Specifically Recognized Antigens by Infected Sera**

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## **Abstract**

**T**his study aimed to separate crude proteins of *Plasmodium gallinaceum* antigen (PgAg) by SDS-PAGE and detect reactive antigens with infected chicken sera by Western blot. Antigen was prepared from 80% parasitized chicken blood, differentially centrifuged to separate parasite cells from frozen lysed red blood cells. The parasite cells were sonicated and centrifuged. The soluble crude PgAg samples were separated and analyzed into protein bands with SDS-PAGE. Several proteins of blood-stage extract were in the molecular-weight (MW) range 22-205 kDa, with some protein bands at higher and lower MWs than the standard proteins. By Western-blot analysis, PgAg-blotted membranes reacted with sera from inoculated chickens with blood stage *Pg*; chickens in an endemic area diagnosed with malaria by symptoms and positive ELISA; chickens with malaria symptoms in a fresh-poultry market, and other diseases; a blood protozoan (*Leucocytozoon sabrazezi*) co-infected with other parasites; a coccidian (*Eimeria tenella*); and Newcastle virus, including negative serum. The results showed 2 malarial protein bands, *ie*, 32.5 kDa reacted with all *Pg*-inoculated sera, and some positive sera by ELISA and endemic area. Another antigen was MW 72 kDa, with all *Pg*-inoculated sera and endemic sera, but not with ELISA-positive sera. This study showed that malaria-infected chickens produced specific antibodies against two interesting avian malaria antigens, of MW 32.5 and 72 kDa, which can be used in Western blot detection.

**Keywords:** *Plasmodium gallinaceum* antigen, chicken sera, SDS-PAGE, immunoblot

## **Introduction**

The spread of avian malaria (*Plasmodium gallinaceum*) in different parts of Thailand is affecting both poultry- and egg-production industries [1]. Chemotherapy may not be the most effective solution for this parasite

infection, because the currently used drugs cannot completely kill the parasite [2,3]. Drug residues, which can remain in poultry products, meat and eggs, also raise concerns about consumer safety. Vaccination has long been known as the best method of disease control in farm animals, and has been proven safe for both animals and consumers [4,5].

Immunity plays an important role in protection against parasitic and other

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microorganism infections. Antibodies against those harmful organisms are soluble proteins generated by host immunity as a result of antigen induction. However, some of these antibodies may be crucial to the host for protection or for fighting invading organisms. For this reason, the study of antigens, recognized by infected or immune animals, may help to identify important antigens as potential vaccine candidates [6]. The objective of this project was to study the parasite-antigen profile of the avian malaria parasite, *P. gallinaceum*, and its specific antigen to the infected chickens.

## Materials and methods

### Cultivation of laboratory animals and malarial parasites

The parasite used in this study was *P. gallinaceum* (*Pg*) isolate MNTH43 (originating from Minburi District, Bangkok, Thailand, in AD 2000). Subcutaneous serial passages were maintained in the blood of male 3-week-old chickens (Babcock B380) under a light:dark cycle of 12:12 hours, at 27 °C; the animals were fed on commercial chicken feed (Betagro101; Betagro Group, Thailand) and water *ad libitum*. The degree of parasitemia was monitored daily; each chicken was gently restrained in dorsal decumbency, with wings extended. Parasitemia was evaluated by thin blood film stained with 10% Giemsa. A chicken with 50% parasitemia was used as the blood donor for the experimental groups of chickens. The experimental groups were used to achieve high *P. gallinaceum* parasitemia levels, to prepare antigen, and for collecting *Pg*-immune serum samples. Another group was maintained as healthy animal subjects, clear of malaria by blood-smear examination, for preparation as non-*Pg* immune sera samples. All experimental chickens were reared at the Animal Care Unit, Faculty of Veterinary Science, Chulalongkorn University.

### Antigen and serum samples

A total of 50 chickens were included in this stage of the experiments; 10 were used for raising blood-stage *P. gallinaceum*, to prepare antigen, and 30 were sources of *Pg*-immune serum samples via

subcutaneous-infection passages with blood-stage *P. gallinaceum*. Two weeks' post-infection, when blood-smear examinations indicated parasitemia clearance, immune-serum samples were collected from the surviving chickens. The 10 remaining chickens were kept healthy for the preparation of the non-*Pg* immune sera samples.

All serum samples were taken from 5 ml of blood from the experimental chickens, drawn from the jugular vein by 22 g x 1" needle. The blood samples were centrifuged at 3,000xg for 5 min; serum samples were collected and aliquoted to 0.5 ml each and frozen at -80 °C until needed. Other sera with *P. gallinaceum* infection derived from symptomatic chickens in an endemic area (Chachoengsao Province, Thailand), and proven positive by ELISA in parallel. In addition, serum samples were collected from malaria-symptomatic chickens in a fresh-poultry market (Khlong Toei District, Bangkok, Thailand). Heterologous sera were proven by 1) detection of the blood protozoan (*Leucocytozoon sabraezesi*), where chickens co-infected with intestinal parasites were detected by the finding of eggs of *Capillaria*, *Ascaridia/Heterakis*, *Spirurida* and *Cestoda*; 2) chickens experimentally infected with the coccidian (*Eimeria tenella*) and Newcastle virus.

### Parasite isolation and antigen preparation

Ten infected chickens were used as the source of blood-stage antigen. Recipients with 80% parasitemia were selected for blood collection; 5 ml of blood was drawn from the jugular vein of each chicken, using syringes coated with heparin anticoagulant (2,500 IU). The collected blood was washed in PBS solution (0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 2 M NaCl, pH 7.4), thrice, while centrifuged at 1,157xg for 5 min at 4 °C (Varifuge 20 RS; Heraeus Sepatech). Washed blood samples were kept frozen at -20 °C overnight, and parasite cells isolated by differential centrifugation, as described by Saiwichai *et al* [7]. Parasite cells from the final step were broken down by sonication (Vibracell) at 40% amplitude, with on/off pulses every 2 seconds at 10 °C for 5 min. Crude soluble antigens of *P. gallinaceum*

were obtained by centrifugation at 41,657xg, at 20 °C, for 30 min. The supernatant was collected and protein quantitated by spectrophotometer at 750 nm, as described by Lowry *et al* [8]. One ml of crude antigen was aliquoted and frozen at -20 °C until needed. A volume of uninfected chicken red blood cells was also extracted to determine protein content, as a control.

### **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Antigen and blood extract were separated by SDS-PAGE, with 12% separating and 5% stacking mini-gels, as described by Laemmli [9], with the application of 120 volts for 65 min. The gel was stained with Coomassie brilliant blue (R-250) for 1 hour and then destained in destaining solution. Photographs were taken as needed. The molecular weights of the protein bands were determined by comparison with standard protein markers (BioRad®), according to Weber and Osborn [10].

### **Immunoblotting**

To identify the antigens specific for the different antibody clones, the separated proteins in the gel were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell) using a BioRad Trans Blot Cell, at a constant 350 volts for 3 hours. After transfer, the nitrocellulose membrane was incubated in PBS containing 0.05% Tween 20 (PBS-T) as the blocking solution for 1 hour, then dried and kept at 4 °C until further use.

The stripped membrane was immersed in PBS-T for 5 min and then incubated in primary antibody (chicken sera from different infections) diluted in PBS-T (1:100) at room temperature for 2 hours. The membrane was washed in PBS-T (3 times, 5 min each) and then immersed in diluted secondary antibody (rabbit anti-chicken IgG linked with horseradish peroxidase) in PBS-T at 1:5,000 for 1 hour. The strip was washed, as above. To indicate bound antibodies, the membrane was incubated for 3-5 min in a solution of 2, 6-dichloroindophenol. The reaction was stopped using distilled water. *P. gallinaceum* antigen MW were determined as above [10]. Since the *Eimeria*

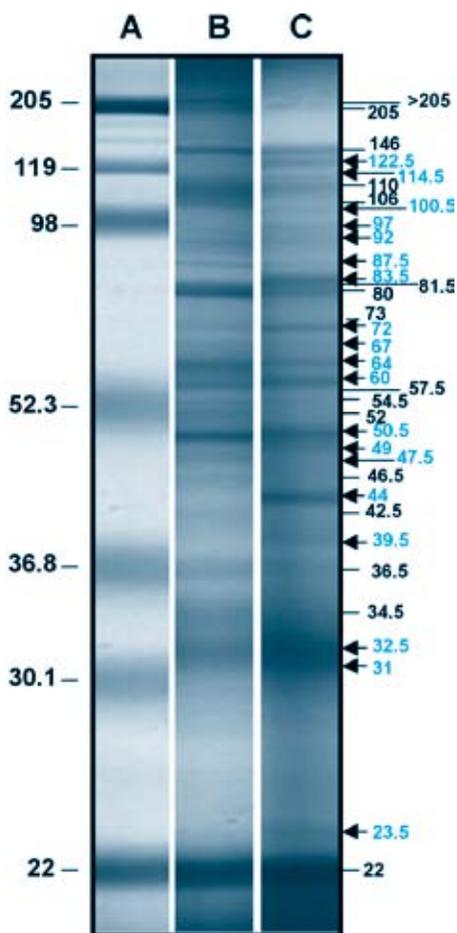
*tenella* and Newcastle-virus infection serum samples yielded below-cut-off OD-ELISA values (unpublished data), their serum samples and that of the blood-protozoan infection were individually pooled to encounter strip antigens.

### **Results**

*P. gallinaceum*, after subcutaneous serial passages through the blood of male three-week-old Babcock chickens, showed 100% morbidity and 67% (20/30) mortality rates. Parasitemia < 1% was initially found at Day 4 post-infection. The common clinical signs in the present study were depression, fever, anorexia, reduced weight gain, poor feed conversion, anemia, green feces, and finally, death. Ten of 30 surviving chickens were selected and provided enough blood samples to prepare sera.

Coomassie blue staining and SDS-PAGE separated several protein bands of crude *Pg* and blood extracts; most of the protein bands had the same molecular weights. Several protein bands from blood-stage *Pg* extract had MWs in the range of protein standard markers, as follows: 22, 23.5, 31, 32.5, 34.5, 36.5, 39.5, 42.5, 44, 46.5, 47.5, 49, 50.5, 52, 54.5, 57.5, 60, 64, 67, 72, 73, 80, 81.5, 83.5, 87.5, 92, 97, 100.5, 106, 110, 114.5, 122.5, 146, and 205 kDa. Nineteen bands (arrowed in Fig 1) probably represented malarial proteins with strong and weak contents, *ie*, 23.5, 31, 32.5, 39.5, 44, 47.5, 49, 50.5, 60, 64, 67, 72, 83.5, 87.5, 92, 97, 100.5, 114.5 and 122.5 kDa, because they were not found in red blood cell extract (Fig 1).

Under Western blot analysis, the separated *Pg* antigens were probed with serum antibodies from *Pg*-inoculated chickens; *Pg*-infected chickens from an endemic area and positive *Pg*-sera by ELISA; chickens with *Pg*-symptomatics from a fresh-poultry market; uninfected chickens; and chickens infected with *E. tenella*, Newcastle virus, and *L. sabrazesi*, respectively. Several antigens were detected by serum antibodies from *Pg* infections, especially reacted bands with strong backgrounds from the sera of chickens with *Pg* symptomatics. It appeared that pooled sera from *E. tenella* infections did not react with this antigen. When considering



**Fig 1 Protein banding patterns in gel stained with Coomassie brilliant blue. A) protein standard markers, B) uninfected red blood cells, C) crude *P. gallinaceum* proteins.**

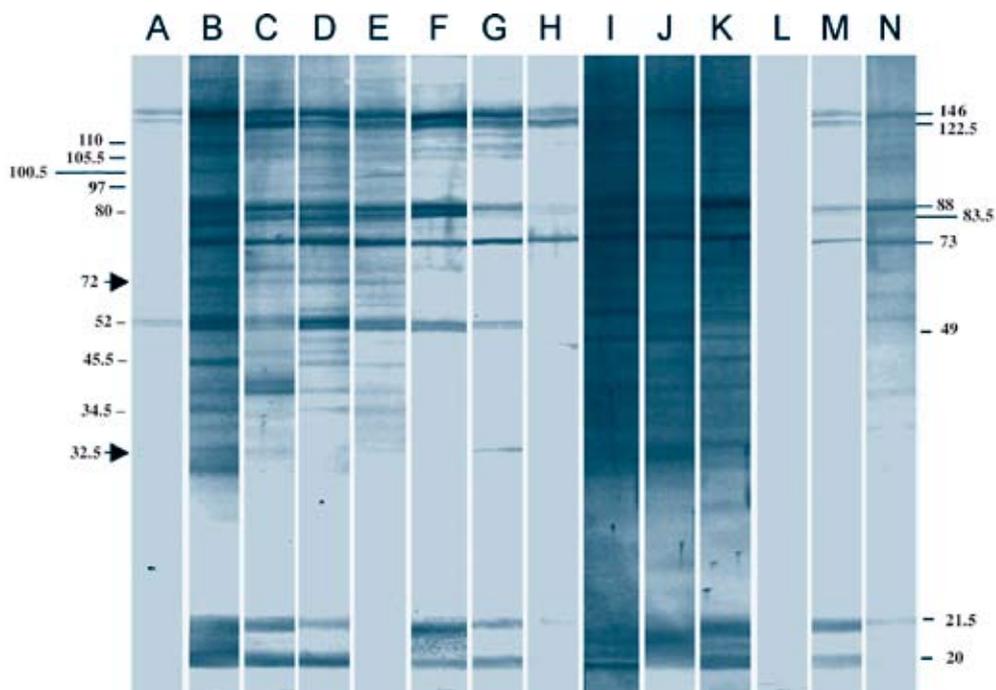
specific detective antigens, there were 2 bands of interest: 72 and 32.5 kDa, although 32.5 kDa was found in reactions of all *Pg*-inoculated sera, 1 of 3 serum samples by positive ELISA, and 1-2 serum samples from *Pg*-symptomatics. The 72 kDa antigen was also found in reactions with all *Pg*-inoculated sera and *Pg*-symptomatics, but not with sera from positive ELISAs (Fig 2).

## Discussion

The experiments with Babcock chickens infected with *P. gallinaceum* by serial blood passages showed 100% (30/30) morbidity and

67% (20/30) mortality rates. This indicated the high susceptibility of this chicken strain (Babcock B380), which showed early parasitemia at Day 4 post-infection by subcutaneous inoculation. In contrast, White Leghorn chickens received intraperitoneal inoculation with blood-stage *P. gallinaceum*, resulting in first parasitemia at Day 6 [11]. Peak parasitemia (~80%) among the infected chickens occurred on Day 6 [11]. Differences between inoculation dose and severity of disease were related to clinical signs, mortality, pre-patent period, exo-erythrocytic parasites, and severity of pathological changes in the organs [12]. In sero-tests, although the more serum was used in the Western blot than the ELISA, pooled antibodies against *E. tenella* did not react with any *P. gallinaceum* antigenic bands. This was related to individual serum samples giving low OD-ELISA values (unpublished data). In contrast, pooled antibodies of Newcastle virus infection showed a few reactive antigen bands at 146, 122.5, 88, 73, 21.5, and 20 kDa. Antibodies against the blood protozoan, *L. sabrazesi*, reacted with many antigenic bands, and those sera showed higher OD-ELISA values than the cut-off point (unpublished data). However, some cross-reacted bands may occur due to antibodies against those parasites—*Capillaria*, *Ascaridia/Heterakis*, *Spirurida*, and *Cestoda*. Observation of the antibodies from inoculation and natural infections with *P. gallinaceum* indicated that antibodies from *Pg*-immune sera (blood stage passages) and sera from the fresh-poultry market had a similar antigen-antibody banding pattern. This was somewhat different from the pattern from chicken sera, which were detected by symptoms from endemic areas and positive ELISA. These sera showed a few reactive bands, from 52 to 205 kDa, and low MW at 21.5 and 20 kDa. This might be attributable to individual immune responses and different ages of the chickens, which were obtained from the laboratory and from the local fresh-poultry market in an endemic area.

This study of blood-stage *P. gallinaceum* examined the protein profile and differentiated *Pg*-antigen using antibodies against *P. gallinaceum*



**Fig 2 Western blot analysis; reactions between *P. gallinaceum* antigen and serum samples from different sources: A) uninfected chicken serum, B-E) *Pg* immune serum by inoculation, F-H) *Pg*-positive serum in an endemic area detected by ELISA, I-K) serum from symptomatic chickens in a fresh-poultry market, L) pooled sera from *Eimeria tenella* infection, M) pooled sera from Newcastle virus infection, N) pooled sera from *Leucocytozoon sabraezesi* infection.**

from experimental and naturally infected chickens. Unfortunately, published data on this malarial parasite have been reported for different stages and for different purposes from our study. However, this study indicated 2 bands of interest for the specific detection of *P. gallinaceum* infection in chickens. The antigenic bands 72 and 32.5 kDa need further investigation in an experimental model for their antigenic properties and roles against *P. gallinaceum*.

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