MATERIAL AND METHODS

Experimental Birds

Plasmodium (*Garnhamella*) *coturnixae*, obtained from a game bird *Coturnix coromendelica*, was the malaria parasite, which was used in the present investigation. The host is a bird of the quail family, commonly known as black breasted quail or rain quail. These birds were purchased from the local market and were kept in laboratory cages with leg bands. Other birds of the quail family, Gray quail (*Coturnix coturnix*), the Jungle bush quail (*Perdicula asiatica*), the Rock bush quail (*Perdicula argoondah*), the painted bush quail (*Cryptoplectron erythrorhyncum*) and the partridge (*Francolinus sp.*), the duck (*Anser sp.*), the pigeon (*Columba livia domestica*), were also purchased from the same market for cross infection experiments. The chickens were purchased from a Government Poultry firm as seven days old bird, for the same purpose and also maintained in our laboratory. These seven days old chickens were maintained in cages provided with overhead lamps for another 15 days.

Diet of the Birds

Kakni dana, a locally available bird's food grain was the food for all the birds except chicken. One handfull of this grain was given to each cage once daily. The chicken were nurished with chicken mesh available in the poultry shop.

Maintainance of the Parasite in Laboratory

The blood of all the birds were tested just after they were brought to the laboratory and then once a week by blood film preparation, stained with Leishman stain. When it was made sure that the birds did not harbour any naturally occurring malaria parasite, they were inoculated with blood containing infective forms of the Plasmodium (Garnhamella) coturnixae. The method of inoculation was intravenous for heparinized blood and intramuscular for citrated blood.

Four to six days after intravenous inoculation and 13-16 days after intramuscular inoculation the parasites were found to appear in the circulating blood of the inoculated bird. The parasite is maintained in our laboratory by needle passage for the last seven years.

For cross-infection experiments, birds other than Coturnix coromendelica, were maintained in the laboratory by the same procedure. They were inoculated either intravenously or intramuscularly in the same way as the Coturnix coromendelica.

Blood Film Preparation and Staining

A drop of blood from the wing vein of the bird was put on a clean slide and a blood film was drawn and dried. The slide was covered with Leishman stain for one minute and then covered with distilled water (pH 7.8) or, water containing Geimsa three drops

per ml. After 3 minutes the slides were washed and dried. These slides were observed under oil immersion microscope.

Preparation of Leishman stain

One gm. of Leishman stain was dissolved in 100 ml. methanol, in a pestle and mortar. The solution was used after two days.

Experiment to prove the non-susceptibility of other birds

1. Repeated inoculation of the parasite to chick

Four chickens were inoculated for seven days daily with 0.5 ml heavily infected blood subcutaneously.

Two chickens were inoculated for 20 days with 0.5 ml of infected blood daily subcutaneously.

2. Use of an immunosuppressive drug

Before inoculation of the parasite eight chickens were treated with subcutaneous injection of different doses of effcorlin, a cortico-steroid drug. They were then inoculated with 2 ml of heavily parasitized coturnix blood.

3. Parasitized cells were washed with sterile saline and then incubated either with normal Coturnix serum or, with normal chick serum for five minutes. These bloods were inoculated into normal coturnix birds.

Serum protein was estimated by the method of Lowry et al. (1951)

Reagents used :

1. 2% Na_2CO_3 in 0.L. N NaOH.

2. 0.5% $CuSO_4$, $5H_2O$ in 1% Na or K tartarate.

3. Alkali-Copper solution, - 50 ml. reagent (1) was mixed with 1 ml. reagent (2).

4. Phenol reagent was titrated with NaOH to thepphenopthalin end point (Folin, 1927). The Folin reagent was diluted to 1%.

5. Bovine serum albumin - Standard solutions of different strengths were prepared in normal saline.

0.1 ml. serum was mixed with 0.9 ml. distilled water. 0.1 ml. of this diluted serum was taken for estimation. Working standard was prepared by dissolving 100 mg. albumin in 10 ml distilled water. 10 ml. was usually used. Distilled water was used as blank. All the samples were first made upto the same volume. To each test tube 1 ml. reagent (3) was added, mixed well and was allowed to stand at room temperature for 10 min. 0.1 ml. reagent 4 was added very rapidly and mixed within a second. After 30 minutes the sample was read in the Beckman spectrophotometer at 750 mμ.

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Serum sodium and potassium was determined in a flame photometer following the method of MacIntyre (1961)

The emission spectra of sodium and potassium produced by heat were studied. Serum was diluted to different dilutions. The hemolyzates of different birds' RBC showing the same optical density at 540 mμ were also taken. Sodium and potassium were read at a particular dilution only and was calculated against the standard. The flame photometer was checked from time to time to establish the effect of sodium and potassium on each other's emission.

Standards were prepared in the following dilution

1. Sodium standard : 0, 120, 130, 140, 150 mEq. of sodium chloride per litre of water.

2. Potassium standards : 0, 2, 4, 6 mEq. of Potassium chloride per litre of water.

Estimation of serum gamma globulin concentration by turbidity was carried out according to the method of De la Huerga and Popper (1950)

Reagents

189 gm of $(NH_4)_2SO_4$ and 29.3 gm of NaCl were dissolved in distilled water and the volume of the solution was made to a litre.

With a micropipette, 0.1 ml of serum was transferred to a test tube or colorimeter cuvette and 5 ml of the reagent were added. After allowing the mixture to stand for 30 min., the test tube or cuvette was inverted twice without shaking and the turbidity was

read in a spectrophotometer using a wavelength of 650 mμ. The reagent was used as a blank. The percent of transmission was interpolated on the standard curve used for the thymol turbidity test. The results were expressed in grams gamma globulin per 100 ml. serum.

Qualitative analysis of serum proteins by disc electrophoresis was carried out according to the method of Ornstein and Davis (1964)

Reagents used :

Small pore gel

1)	Acrylamide	30.0 gms	
	N"-N'-methylene bisacrylamide	0.8 gms	
	Distilled water to make	100 ml	
2)	1N HCl	24 ml	
	Tris base	18.15 gm	
	Tetraethymethylene diamine (TEMED)	0.23 ml	
	Distilled water to make	100 ml	
3)	Ammonium persulfate	0.14 gms	Freshly prepared
	Distilled water to make	100 ml	

The solutions were added in following proportion

1:1:2 (1, 2, 3 solutions)

Large pore gel

1) Acrylamide	5 gms
N"-N'-methylene bisacrylamide	1.25 gms
Distilled water to make	100 ml
2) 1M H_3PO_4	12.8 ml
Tris base	2.85 gms
TEMED	0.1 ml
Distilled water to make	100 ml
3) Riboflavin	2.0 mg
Sucrose	40.0 gm
Distilled water to make 100 ml	

The solutions were added in the following proportions :

2:1:1 (1, 2, 3 solutions)

Materials

Glass tubes (8 mm OD 60 mm long) were marked at 40 mm and 50 mm from one end. These tubes were acid cleaned and stored in a closed vessel to keep free of dust.

Tris Glycine buffer for reservoir

Tris base	6.0 gm
Glycine	28.8 gm

Distilled water to make 1 litre

Just before use, the solution were diluted 1/10 and chilled. The pH of the solution was 7.4.

Fixative

Naphthol blue black (Amido-swarz)	1 gm
7% Acetic acid upto	100 ml

Tubes were inserted into rubber holders on plexi glass plate. The tubes were filled to the 40 mm mark with freshly mixed small pore gel solution with a pasteur pipette. Without delay distilled water was layered over the 'SP' gel solution to a depth of 10 mm. In order to make a sharp interface a pasteur pipette was pulled to produce a very fine orifice. The flow of the distilled water from this pipette were checked in a vertical position the water was released at a rate of about 1 drop every second. In order to layer the water over the gel solution the outside of the tip pipette were wiped while holding the pipette in horizontal position. This holding of the pipette in horizontal position was continued while approaching the tube and contact was established between the tip of the pipette and the inside wall of the tube near the top. The water was allowed to trickle down the side of the tube without forming large drop. The tube was filled with water upto 10 mm.

The tubes were allowed to stand for thirty minutes. During this period the interface disappeared and the acrylamide polymerized to formaa gel with a visible surface at a height of

about thirty seven millimeter from the bottom of the tube. When the gels were well formed the tubes were inverted, the water was drained, on a tissue paper.

A few drops of large pore gel solution were introduced to rinse the space above the gel and the tubes were inverted again, drained off and wiped. The large pore gel solutions were introduced with pasteur pipette upto the next mark; water was carefully layered as previously over the 'LP' gel solution.

The tubes were then placed before a fluorrescent lamp at a distance of about two to three inches from the surface of the tube. Photopolymerization was completed in about five minutes. Its completion was evident by disappearance of the yellow colour of the riboflavin in the gel solution and the appearance of a faint opalescence in its place.

Application of the sample

Sample of serum containing 100 ug of protein was alayered over the 'LP' gel, covered with one drop of 50% sucrose and the remaining space of the tubes were filled with buffer. The tubes were inserted through the grommets in the upper chamber with the top of the tube protruding about 5 mm above the grommet. A drop of tris glycine buffer was dropped to the tube to prevent lodgement of air bubbles on insertion into the lower buffer compartment. The upper and lower chamber were filled with tris-glucine buffer (pH 7.4).

The electrophoretic separation was started with a current not exceeding one milliampere per tube. Just before starting the separation a drop of bromophenol blue was added to the buffer in the upper chamber. When all of the tracking dye had entered the 'LP' gel the current was increased to an upper limit of three milliampere per tube and were kept at about this level throughout the rest of the electrophoretic separation. The experiments were terminated at the moment when the blue disc of the tracking dye reached the bottom of the tube.

After completion of the run the gels were taken out by rolling a needle in the gel tube in distilled water. The gel was placed in a wide tube and filled with fixative stain. After fifteen minutes the stain was poured off and the gel was washed with 7% acetic acid, for three days.

The protein bands were counted from large pore junction.

<u>The serum protein was analysed by paper electrophoresis following the method described by Tiselius modified by Block et al. (1958)</u>

Charged, or ionized molecules migrated through a buffered supporting material in a potential gradient. The mobility depends upon molecular characteristics, such as net charge, size and conformation; ionic strength of the buffer etc.

Reagents required

1. Veronal buffer (diethyl barbituric acid) .. 3.12 gm
   Sodium diethylbarbiturate .. 17.1 gm
   } 0.05 M pH 8.6 1000 ml distilled water freshly prepared.

2. Stain : 100 mg of bromophenol blue in 100 ml. of methyl alcohol.

3. Whatman paper : No.I

Tests

Buffer was poured into the compartments. The strips (1" broad) of Whatman paper No.I was soaked in the buffer and placed in horizontal position for 15 minutes to reach equilibrium. 0.05 ml to 0.1 ml serum was applied equally along a pencil line near the negative pole. The current was on for 16 to 18 hrs. The strips were then dried and stained with bromophenol blue and washed with 5% acetic acid.

Estimation of hemoglobin

1. Hemolyzate preparation

Blood was drawn from the wing vein and centrifuged and the packed red cells were washed with cold 0.85% saline thrice. The packed erythrocytes were lysed with equal amount of distilled

water and a few drops of toluene; the mixture was shaked vigorously for 5 min and then centrifuged for 15 minutes. The clear red supernatant fluid was taken and stored in the deep freeze at-20°C.

2. Quantitative estimation

0.04 ml. of the hemolyzate was mixed with 5 ml. of distilled water and the optical density was read at 540 mμ.

3. Qualitative analysis by disc electrophoresis

Acrylamide gel tubes were prepared following the previously described method. Hemoglobin preparation was done in such a way that each hemoglobin solution had equal optical densities at 540 mμ. One hundred microgram of hemoglobin was added to each tube. The electrophoretic run was carried out following the previously described methods. Just after the run was completed, a photograph was taken of the different bands of hemoglobin.

Paper electrophoresis was also done with the same hemoglobin samples.

4. Quantitative estimation of each fraction of hemoglobin by the process of elution

From the junction of large pore and small pore, 2 mm pieces of the gel were cut and each piece was eluted in 2 ml. tris glycine buffer for 48 hours. The eluted fluid were read by spectrophotometry at 540 mμ.

5. Urea treatment for the breakdown of hemoglobin chain according to Ingram (1957)

Urea dissociates hemoglobin into its component chains. Hemoglobin solutions of different birds were treated with Urea solutions and then separated by disc electrophoresis.

Reagents required

1) Urea 2M buffer solution

120 gm. of urea was added to one litre cold tris-glycine-buffer

2) 8M Urea solution

36 mg. urea was added to 0.1 ml. hemoglobin solution.

Before application on the acrylamide gel the urea treated hemoglobin solutions were incubated for 30 min. at 37°C with frequent shakings.

Tests

Equal quantity of urea treated hemoglobin samples were added to acrylamide gel tubes and the electrophoretic run was carried out in 2M urea tris glycine buffer to isolate dissociated chains of hemoglobin.

Techniques of free parasite preparation

The malaria parasites were freed following the method of Trager (1954).

Material required for the experiments were

1) Anti Coturnix erythrocyte rabbit serum

A fresh rabbit was bought from the market. The antisera were prepared following the method of Speck et al. (1946) and Trager (1950). The normal Coturnix erythrocytes were washed with saline and were inoculated in the ear vein of the rabbit. The inoculation was done twice weekly, at an interval of two or three days. The doses of injection were gradually increased from 0.1 ml to 1 ml over a period of three weeks. Generally, after two weeks antibody against Coturnix erythrocytes was formed. If not, the inoculation was repeated for another week. Blood was collected from the ear vein, and serum was separated and stored at -20°C.

2) Guinea pig serum

This serum was freshly collected as a source of complement.

3) Trypsin solution

200 mg of twice recrystallised trypsin was dissolved in 20 ml of 0.003 N HCl.

4) DNAse solution

10 mg lyophilized deoxyribonuclease was dissolved in 2 ml 0.8% sterile saline.

All these reagents were stored in the deep freeze.

5) Saline albumin glucose solution (Freshly prepared)

NaCl	:	0.33 gm
KCl	:	0.44 gm
NaH_2PO_4	:	0.014 gm
K_2HPO_4	:	0.156 gm
$NaHCO_3$	:	0.09 gm
Glucose	:	0.25 gm
Bovine albumin	:	0.6 gm
Water upto	:	100 ml

Procedure

One ml. of heparinized blood was collected from a highly infected bird and centrifuged. The packed red cells were washed thrice with 0.85% cold saline. To the packed cells was added four times their volume of saline albumin glucose solution and mixed with 30 µl Guinea pig serum and 90 µl anti coturnix serum and incubated with shaking for half an hour at 37°C. Forty µl trypsin solution was then added. After an hours incubation at 38°C. 30 µl DNAse solution was added and the tube was again incubated for half an hour at 37°C. The mixture was centrifuged for 3 minutes at 500 rpm. The supernatant suspension was collected and centrifuged at 2000 rpm for 20 minutes. The dark brown colour precipitate was washed in saline glucose albumin solution and resuspended in saline glucose albumin and the preparation was observed under a microscope.

Precipitin reaction in gel

The precipitin reaction can be carried out in a gel system when antibodies and antigens were introduced in different region of the agar gel and allowed to diffuse freely toward each other; precipitin bands form where they meet at equivalent proportion. We followed the double diffusion technique in two dimensions, developed mainly by Ouchterlony in Sweden (1958).

Reagents used

1) I.D. agar (Code BR27G, made in England by Oxoid Ltd. London).

2) Normal saline

3) Sodium azide

4) Ouchterlony glass plate - Small glass petri-dishes (2" diameter) were cleaned by acid and distilled water and then sterilized.

Preparation of agar gel

One gram of agar was dissolved in 100 ml. of normal saline. In order to avoid growth of contaminating micro-organisms in the gel, 0.02 gm % sodium azide was added to the solution. To dissolve the agar the solution was autoclaved and stored at room temperature. Just before use, it was melted in a boiling water bath.

Preparation of Ouchterlony gel plate

At first the plate was coated with a thin layer of 0.1 % agar solution and then four ml. of hot agar solution was poured on this thin layer. It was essential to have the plates on a horizontal surface in order to produce a uniform agar layer. It was then kept at room temperature for the settlement of the gel. The plates were then covered and after two hours they were kept in the refrigerator in an inverted position overnight. The holes of suitable size and shape were made by punching the agar gel, with a standard gel cutter.

Tests

Antibody was added to the central hole with a pasteur pipette to fill it up. Antigens were added to the peripheral holes. The plates were placed horizontally in a humid atmosphere for 24 to 72 hours, at room temperature.

The reactants diffused from the wells and white precipitin bands formed where they met at equivalent proportions.

Hemagglutination test was carried out according to the method of Bray et al. (1973) and Stavitsky (1954) with a little modification

Materials used

1) Sheep blood in Alsever's solution.

2) Physiological saline - 0.85% saline was used.

3) Phosphate buffered saline :

(PBS) pH 6.4,	0.154M - KH_2PO_4	:	6.460 ml
	0.154 M - Na_2HPO_4	:	13.540 ml
	Normal saline	:	80 ml
(PBS) pH 7.2,	0.154 M - KH_2PO_4	:	7.17 ml
	0.154 M - Na_2HPO_4	:	22.8 ml
	Normal saline	:	120 ml

4) Tannic acid solution

Tannic acid (Fisher scientific company) 1:40,000, i.e. 25 mg in one litre of PBS (pH 7.2). Freshly prepared.

5) Antigen solution.

Malaria parasites, separated from the red cells were homogenized in a glass homogenizer in 0.5 ml. of 0.85 % saline. The suspension was centrifuged in a high speed centrifuge. After centrifugation, the supernatant was stored as 0.85% saline extract of parasite, (Extract no. I, containing the soluble proteins), and the pallet was resuspended in 0.5 ml of 1 M saline, homogenized and centrifuged. The supernatant was stored as 1 M saline extract (Extract II containing membrane protein). These two extracts were used in the tests as antigen solution.

6) Antibody solution

Sera of susceptible and non-susceptible birds were used

in the test as antibodies. These sera were inactivated at 56°C for 1 hour and absorbed with sheep erythrocytes.

7) Bovine serum albumin solution

BSA 1% - 1 gm of bovine albumin was dissolved in PBS (pH 7.2)

BSA 0.1% - 0.1 gm bovine serum albumin was dissolved in 100 ml (pH 6.4).

These BSA solutions were always freshly prepared and were used instead of normal rabbit serum. These solutions were inactivated at 56°C for half an hour.

Sheep cell suspension

Ten ml. of sheep blood was collected in Alsever's solution and centrifuged. The packed cells were washed three or four times in saline (by centrifugation in 1000 rpm for 15 min.), until the supernatant became clear. Then 2.5% cell suspension was prepared by taking 2.5 of packed cells (by volume) in 10 ml (pH 7.2).

Preparation of tanned RBC suspension

Equal amounts of 2.5% cell suspension and tannic acid solution (1:40,000) were mixed and incubated at 37°C for 15 minutes and then centrifuged and washed with 0.1% BSA to avoid Panagglutination (Eidinger, 1964). The cells were then washed with 0.85% saline and suspended in 0.85% saline to make 2.5% cell suspension.

Antigen

Protein of extract I and extract II were estimated by the method of Lowry, F. L. The antigen solution was diluted by PBS (pH 6.4) to give a protein concentration 0.1 mg in 20 ml (1:20,000).

Preparation of antigen coated tanned red cell suspension

Equal volumes of 1:20,000 diluted antigen, and 2.5% tanned RBC suspension were mixed together, and incubated at room temperature for 30 minutes. The mixture was centrifuged at 1000 rpm for 10 to 15 min. The supernatant was removed and the cells were washed with double amount of 1% BSA. The solution was centrifuged at the same speed and for the same time. The cells were resuspended in the original volume in 1% BSA to make 2.5% cell suspension.

Absorption of antisheep antibody in the antiserum

All the inactivated sera were mixed with sheep cell suspension to absorb the antisheep antibody present in the experimental antisera. The mixture was done in the following proportions :

Sera 1 ml. were mixed with 4 ml. (2.5%) cell suspension to make 1/5 dilution. Sera 1 ml. were mixed with 1 ml. 2.5% cell suspension to make 1/2 dilution. After one hour these sera were centrifuged. The supernatants were taken for the test and different dilutions were made with BSA (7%.).

Test

In each test tube 0.5 ml of 1% BSA was added. One fifth diluted antigen 0.5 ml. was taken in the first empty test tube and added to the next tube containing 0.5 ml. BSA and mixed well. From this test tube 0.5 ml. solution was added to the corresponding

tubes serially, so that two fold dilutions beginning either from 1:5 to 1:80 or 1:2 to 1:16 were obtained.

Two drops (0.05 ml) antigen coated cell suspension was added to each tube and mixed well. Then the tubes were placed in room temperature for two hours and then in the refrigerator overnight.

The results were read on next day. Positive reaction was indicated by 'matt' surface of the red cells at the buttom of the tubes and negative by 'button' formation.

Incubation of free parasites with normal erythrocytes

Materials required

1. Washed free parasites were prepared following the method previously described.

2. Glucose-albumin-buffer

3. Parasite suspension

The free parasites were washed with glucose-albumin-buffer and then suspended in 0.5 ml of this buffer to make approximately 20% - 30% cell suspension.

4. Normal erythrocyte preparation

Blood of normal Coturnix and chick were drawn. The erythrocyte of each sample was washed twice, first with 0.85% saline and then with buffer. Only the packed cells were then taken.

Fifty micro liter parasite suspension were incubated with five micro liter erythrocyte of each sample separately for thirty minutes at thirty seven degree with frequent shaking. Then centrifuged at 1000 rpm for 10 min. and the supernatant was discarded. Each sample was washed with normal saline and slides were prepared. The slides were fixed with methanol and dried. The dry slides were stained with Geimsa. The number of erythrocytes to which the parasites attached themselves were counted and expressed as a percentage of the total number of erythrocytes.

Parasite inoculation in chick embryo

Materials required

Eggs

Fertilized eggs were bought from Government poultry firm and placed in a 37°C incubator on cotton pad. To keep the chamber moist a petridish containing water and cotton, was placed in the chamber. The eggs were rolled twice daily.

Instruments and other materials

Syringe Ampoule cutter saw Small forceps Small scissors	Sterile in an autoclave

Pipette

Celotape

Liquid paraffin : Sterile by boiling

Parasites

The blood of one infected Coturnix, was drawn from the wing vein directly with sterile 1 cc syringe just before inoculation.

Tests

Fertilized hen's egg were incubated at 37°C in an egg incubator. On the 11th and 13th days of incubation the embryoes were viewed through a hole in the egg candle box in a dark room. Eight embryoes were selected in each of these two days. The blood vessels, rendered visible through the view box were marked with a pencil. The egg shells were cleaned with alcohol and placed on a pad of sterile cotton wool. A window 1 cm. square which included the marked blood vessel was outlined by means of a thin saw; the egg shell was then cut through with the help of the saw and lifted by means of a sterile forceps. A tiny care was taken to leave the shell membrane intact. A tiny drop of sterile liquid paraffin was then put in through the window and the blood vessels were thus rendered prominent. A small quantity of coturnix blood, 0.01 ml containing the parasitized erythrocytes was inoculated into the embryonic blood vessel intraveneously without rupturing the shell membrane. The window, then was covered with a small piece of cellopape and the egg was replaced in the incubator.

Four eggs were opened in the same way as above but no incubations were given. They were covered with cellotape and replaced in the incubator. They as well as four other unoperated eggs were treated as controls for the experiment. Two such experiments were carried out on each of these two days of incubation.