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CHARACTERIZATION OF ANTIBODIES FROM EGG YOLK OF SOME BIRDS

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ABSTRACT

The objective of the study on IgG antibody in Egg yolk of some birds, is to characterize by comparing and contrasting, the IgG antibody precipitated from the egg yolk of immunized bird (chicken) and local birds (chicken and Guinea fowl).IgG antibody from the egg yolk of the birds, were purified by ethanol fractionation. The purity of the antibody was monitored by SDS-PAGE and Immunoelectrophoresis, and the immunological activity was tested by Ouchterlony technique. Protein concentrations of the egg yolk from immunized chicken, local chicken and Guinea fowl were 0.87 mg/ml, 2.37 mg/ml and 0.29 mg/ml respectively, while the molecular weights were 70,598, 70,598 and 74,405 KD. From the figures obtained, a conclusion was drawn that egg yolks of immunized bird and local birds are good sources of immunological active IgG antibody.

Keywords: IgG antibody, SDS-PAGE, Immunoelectrophoresis, Ouchterlony technique

INTRODUCTION

Antibodies are gamma globulins which are characteristically formed when antigens are introduced into body. They are specialized proteins made by lymphocytes which can recognize and precipitate or neutralize invading bacteria, viruses, or foreign proteins from specie. They appear in the serum of a given vertebrate mostly in the gamma globulin fraction, in response to injection of an antigen [1]. Antibodies are detected by their ability to combine with antigen. The distribution of the immunoglobulin IgG, IgM and IgA in serum, bile, egg white, egg yolk, intestinal secretion in chicken, pigeon, turkey, Japanese quail, bovine have been studied [2-3] . Chicken immunoglobulin is the most studied of all avian immunoglobulin. The ability of chickens to produce high levels of precipitating antibodies following injections of heterologous serum proteins makes this species useful for immunologic investigation. Chickens, pheasants and Japanese quail possesses at least two distinct classes of immunoglobulin each based upon the antigenic differences in the H chains. The two distinct classes of immunoglobulin were designated as IgM and IgG in an attempt to relate them to mammalian immunoglobulin.

However, serum antibodies of hens are efficiently transferred and accumulated in egg yolk [4-7] providing a valuable source of antibodies. Using chicken as the immunization host for producing egg yolk antibodies (IgY) instead of IgG from mammalian species brings a number of advantages: [8-9] the animal suffering is reduced (no bleeding), as antibodies are obtained directly from the egg and only egg collection is required upon immunization, [10] antibody isolation is fast and simple; [11] very low quantities of antigen are required to obtain high and long-lasting immunoglobulin titers in the egg yolk from immunized hens, and [12] a single egg contains as much antibodies as an average bleed from a rabbit [13-15].The

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globulin-like protein in the egg yolk of the domestic hen has been termed gamma livetin which was believed to be IgY. [16-19]. Specific antibodies including immunoglobulins from egg yolk have increasing applications in virus diagnosis [20-22] in detection and estimation of specific molecules [22-23] in food analysis and as an alternative approach in determination of structure and function of complex molecules. Chicken antibodies may be used in varieties of procedures i.e radioimmunoassay, as first or second antibodies and in immunoelectrophoretic techniques. Egg yolk antibody (IgY) has been successfully used for protection of experimental animals against infectious diseases and toxemias [23].

Successful development of efficient methods to isolate immunoglobulins from egg yolk was the basic requirement of route application of yolk antibodies in microbiology [23]. Egg yolk antibodies have been used in agriculture and veterinary medicine to control germs of infectious intestinal diseases of piglets[24-25] Other applications of yolk antibodies include protection of low birth weight or immunodeficient infants or the treatment of diarrhea of AIDS patient or women during pregnancy . Fowl has been used in immunologic studies for more than 50 years.but little is known about the structure of their immunoglobulins. The aim of this study is to characterize by comparing and contrasting, the IgG antibody precipitated from the egg yolk of immunized bird (Chicken) and local birds (Domestic hen and Guinea Fowl).

MATERIALS AND METHODS

Collection of Samples

Eggs from eight months old chicken, (Broilers) immunized against Newcastle disease at a day old, Gumboro disease at 10days old, Lacota disease at 3 weeks old, Fowl pox disease at 4 weeks old and Newcastle disease 6 weeks old, were collected from University of Ibadan, Nigeria Poultry Farm. Eggs from local chicken and Guinea fowl was purchased at Mokola and Oje market Ibadan, Nigeria respectively. All the eggs collected were stored at 4^0C. Three 4 months old male rabbits, purchased from the Animal House, Sango, Ibadan were fed with pellets under a controlled environment.

Isolation and Purification of IgG From Egg Yolk

Removal of lipoprotein from Egg Yolk

50ml of pooled egg yolks from immunized chicken, local hen and Guinea Fowl after separation from the albumin, was diluted with 400ml of distilled water (10^oC). The mixture was thoroughly mixed for 10min at 10^0C by magnetic stirrer and centrifuged at 3200rpm for 10min by Sorvall RC-5C authomatic superspeed Refrigerated Centrifuge. The supernatant (390ml) was separated from the light yellow precipitate and 650ml of Precooled 96% ethanol (-20^0C) was added to the supernatant to a final concentration of 60% (v/v). The mixture was stirred for 30min at 4^0C by a magnetic stirrer, and the mixture was centrifuged at 12000rpm for 20min. The resultant precipitate was collected after the centrifugation and dissolved in 390ml of 30mM NaCl solution and then mixed for 30mins by stirring at 4^0C. The homogeneous suspension was passed through filter (No I, whatmann) at 4^0C to remove precipitates (Lipoproteins) then the filtrate was obtained.

Purification of IgG Antibody

Precooled 50% ethanol (555ml) was added to the filtrate (370ml) to final concentration of 30 %(v/v) and stirred for 30min at 4oC by a magnetic stirrer. The mixture was centrifuged at 12000rpm for 20min. The resultant precipitate was collected and suspended uniformly in 50ml of 30mM Nacl (pH 6.6) stored at 4^0C.

Protein Determination

Bradford's Method [26] of quantitative determination of protein was used to determine the protein concentration of the suspended precipitates. Bradford method of quantitative determination is a rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. This method involves the binding of Coomassie brilliant blue G-250 to protein.

Preparation of Samples

Serial dilutions were made from final precipitates obtained from the yolk fractions of the bird's egg. Four mls of Bradford reagent was added to each tube and vortexed. After 30min the absorbance was determined at a wavelength of 595nm from the Beckman: Du (R) 65uv spectrophotometer along side with the standard solution.

Electrophoretic Separation of the Protein Samples Collected From the Bird's Egg Yolk by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done under a reducing condition. α-Lactoalbumin, Trypsin inhibitor, Carbonic anhydrase, Ovalbumin, Albumin, and Phosphorylase whose molecular weights are 14,000, 20,000, 30.100, 43,000, 67,000 and 94,000 respectively, were used as standard proteins. The standard proteins and the three samples from egg yolks of the three birds were treated for 30min at 100^0C in 50mM Tris-HCl buffer (pH 6.8), containing 2% (w/v) SDS, 100mM dinitrothreitol, 10% glycerol, and 0.1% bromophenol blue. Reduced protein samples were analyzed by electrophoresis in a 12% (w/v) acrylamide gel, containing water, 30% acrylamide, 1.5M Tris (pH 8.8), 10% ammonium per sulphate and TEMED (NNNN-Tetramethl ethylene diamine). Stacking gel (4ml) containing 2.7ml of water, 0.67ml of 30% acrylamide mixture, 0.5ml of 1.0M Tris (pH 6.8), 0.04ml of 10% and 0.004 ammonium per sulphate of TEMED (NNNN-Tetramethyl ethylene diamine) was prepared and used. The gel was mounted in the electrophoresis apparatus (i.e Mini-Protean 11 cell) Tris-glycine electrophoresis buffer containing 25mM Tris, 250mM glycine (pH 8.3) and 0.1% SDS was added to the top and bottom reservoirs.

Loading Of the Wells

Ten wells were loaded in this order, 3µl of the marker was loaded into the bottom of the second well, 5µl of the first sample (From immunized chicken) into the third well, 5µl of second sample (Local hen) into the fourth well, 10µl of the third sample (Guinea fowl) into the fifth well. The sixth well was left and the seventh well was loaded with 2µl of the first sample, 3µl of the second sample into the eighth well and 5µl of the third sample into the ninth well. The unused wells i.e first, sixth and the tenth well were loaded with SDS loading buffer. The electrophoresis apparatus was attached to an electric power supply for 54mins at maximum voltage of 150 until bromophenol blue reaches the bottom of the resolving gel. Proteins separate into different bands according to their molecular weights. Finally, the gel was stained with Coomassie Brillant Blue R-250(Sigma chemical Co).The Molecular weights of the samples from the three birds were determined. This was done by calculating the refractive value.

$$\text{Refractive value} = \frac{\text{Distance Migrated}}{\text{Dye Front}}$$

Distance migrated is calculated by measuring each band of the samples. Dye front is calculated from the marker proteins i.e the total length of the marker protein bands. The refractive values were plotted against the log molecular weight.

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Immunodiffusion Assay of the Samples Extracted From the Yolk of the Birds

The recovery of immunoglobulin (IgG) from the egg yolk of the three birds were monitored and tested by immunodiffusion using rabbit anti chicken IgG .

Raising Of Igg Antibody in Rabbit Materials

Precipitated IgG antibody (2.37mg/ml) from the egg yolk of immunized chicken (0.87mg/ml),local chicken (2.37mg/ml) and Guinea fowl (0.29mg/ml) were suspended in corresponding volume of Phosphate buffer saline (pH 7.2) to a concentration of 300µg/ml.This solution was emulsified in equal volume i.e 2ml of complete friend's adjuvant (Difco Lab. Detroit Michigan, U.S.A). Three, four months old rabbits labeled A, B, C were fed with pellets and injected with 1ml each of precipitated IgG antibody (300µg/ml) from egg yolk of local hen(A), immunized chicken(B), and Guinea fowl (C) r espectively .The precipitated antibody was emulsified in complete Freund,s adjuvant. Booster immunization was done two weeks after the primary immunization by giving 1ml each of the same of precipitated antibody (300µg/ml) from egg yolk of the birds. After one week, each rabbit labeled A,B,C received 1ml each of the precipitated IgG antibody (500µg/ml) from egg yolk of the three birds. The precipitated antibody (500µg/ml) was also emulsified in complete Freud's adjuvant. The weights of the three rabbits were monitored and the serum collected from the rabbit for further tests.

Collection of Serum

Serum was collected two weeks after each injection. This was done by collecting two milliliters of blood from each rabbit. The blood was allowed to clot at room temperature for 1hr. The expressed serum was collected the following morning and centrifuged at 3000rpm for 10min to sediment the erythrocytes. The serum collected was stored at 4^0C.

Ouchterlony Technique

Quchterlony technique is a simple and extremely useful technique. It is based on the principle that when antigen and antibody diffuse through a semi solid medium (e.g agar) they form a line of precipitation where the two reactants meet. As the precipitate is soluble in excess antigen, a sharp line is produced at equivalence, its relative position being determined by the concentration of the antigen and antibody in the agar. 1.5% agar was prepared in Phosphate buffer saline (pH 6.4) which was melted on a Magnetic Stirrer hot plate (Gallenhamp). The molten agar was poured on two slides placed on a leveler (LKB 6821B leveler) and allowed to solidify. Small wells were sucked out of agar, a few millimeters apart and the wells were filled with 5µl of antibody or antigen as shown. The slides were placed in a humid chamber and left overnight at room temperature. The following day, the slides were dried by covering with moist filter paper and left for 24hrs and later stained with Coomassie Brilliant Blue R-250 (Sigma chemical Co, London.

Immunoelectrophoresis

1.5% Agar was prepared in Barbitone buffer (pH 8.6). The agar was melted on a magnetic Stirrer Hot Plate (Gallenhamp). The slide was marked positive and negative at both ends. Molten agar was poured on the slide placed on a leveler to solidify. Four small wells were punched. Each well was filled with 5µl of each precipitated IgG antibody from local, immunized chicken, and Guinea fowl egg yolk, 5µl of the mixture precipitated IgG antibody from the egg yolk of the three birds. Small amount of bromophenol was added to one of the wells labeled A. Four troughs were created on the slide Each trough was filled with 50µl of antisera from three rabbits and 50µl of the mixture of the antisera from the three rabbits (αA,

αB, αC). The slide was placed in an electrophoretic tank filled with barbitone buffer (pH 8.6). Each end of the slide was connected to the buffer chambers by filter paper and the electrophoresis tank closed. The electrophoresis tank was connected to a power supply at 80V for three hours. The slide was removed and left overnight in phosphate buffer saline (pH 7.2). The following day the slide was dried for 24hr by covering with moist filter paper and later stained with Coomassie brilliant blue R-250 (Sigma chemical Co).

RESULTS

The final precipitate obtained from the egg yolk of each bird was reactive with corresponding IgG antibody raised in rabbit against the precipitate , i.e forming a single precipitate band in agar immunodiffusion is as shown in Fig 1. There was a cross reaction between the final precipitate obtained from the egg yolks of local chicken and immunized chicken i.e the final precipitate from the egg yolk of local chicken (precipitated IgG antibody) was reactive with antisera labeled A (IgG antibody raised against precipitated IgG antibody from egg yolk of local chicken) and Antisera B (IgG antibody raised against precipitated IgG antibody from egg yolk of immunized chicken).

The immunoelectrophoresis analysis of the final precipitate obtained from the egg yolk of each bird yielded distinct precipitation arc in the gamma (γ) region. The analyses of the extracts from egg yolk of local chicken and immunized chicken yielded, a single precipitation arc, each in the gamma (γ) region, while extract from the egg yolk of guinea fowl yielded two separate precipitation arc; in the gamma (γ) region. The mixture of the final precipitate or extract obtained from the egg yolks of the three birds gave three precipitation arcs, all in the gamma region (Fig 2).

The protein concentration of the final precipitate obtained from egg yolk fractionation in immunized chicken, local chicken and Guinea fowl were 2.37mg/ml, 0.87mg/ml and 0.29mg/ml.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis, yields a single band, but close double bands in local chicken and Guinea fowl respectively.the molecular weights of the precipitated IgG from egg yolks of local chicken, immunized chicken and Guinea Fowl, determined by SDS-PAGE analysis were 70,958, 70,958, 74.405. (Fig 3)

DISCUSSION

Egg yolk is composed of granules (Lipoproteins and Phosvitin), dispersed in a soluble fraction(livetins and low density lipoprotein) [27] and low density lipoproteins; [28].The major protein present in the soluble fraction are α-,β-γ-livetin and low density lipoprotein. Γ-livetin is the protein of interest in this study, and it was shown from studies to be IGY (12, 17) or igG [27-28] or yIgG [28].

Out of the egg yolks from three birds used in this study, local chicken has the highest protein concentration (2.37mg/ml), followed by the immunized chicken (0.87mg/ml), the extract from the egg yolk of guinea fowl has the lowest protein concentration (0.29mg/ml). There are some factors which might be responsible for the difference in protein concentration in the egg yolk of the birds. The difference is likely to be due to γ-livetin fraction of the egg yolk.

Many factors might have contributed to the high protein concentration of local chicken (2.37mg/ml). For example, the exposure of the local chicken due to its free movement, to various kinds of diseases, and their encounter with the real virus without being vaccinated. Also local chicken has more tendency to raise more antibody naturally against disease, since the life span (2years) is more than that of immunized chicken(One year). The protein

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concentration in the egg yolk from immunized chicken (0.87mg/ml) was lower than that of local chicken (2.37mg/ml) because immunized chicken is usually restricted to a particular environment where vaccination is made available against some specific diseases.

SDS-PAGE analysis under reducing condition yielded two bands as previously described by [36, 43]. The first band represents the heavy chain protein band while the second band represents the light chain. The light chain protein band was not clearly shown when compared with SDS-PAGE analysis of [43]. An extra band close to the heavy chain was observed in the SDS-PAGE analysis of each extract from egg yolk of the two local birds (Local chicken and Guinea fowl).As previously described by [42, 46] the extra band indicates the presence of low density lipoprotein, which shares the observed in the SDS-PAGE analysis of the extract from the egg yolk of immunized bird.

The immunoelectrophoretic analysis of extract from egg yolk of Guineafowl yielded two separate precipitation arcs; both arcs were within the γ-region. A single precipitation arc was observed, when the extracts from the yolk of immunized and local chicken were used. The two bands observed in the immunoelectrophoretic analysis of the egg yolk from Guinea fowl might have shown some degree of impurity, since SDS-PAGE revealed only one band.

Comparing results from SDS-PAGE analysis and immunoelectrophoresis, egg yolk from Guinea fowl contained more low density lipoprotein than that of local chicken, while small amount was found in the egg yolk from immunized chicken. Antigenic analysis of the egg yolk from local chicken, immunized chicken and Guinea fowl can be used to characterize IgG antibody obtained from their egg yolks. This analysis brought out the similarities and differences between the precipitated IgG antibodies from egg yolks of the three birds. The final precipitates obtained from the egg yolk of each bird were reactive with Rabbit anti-chicken raised against the precipitates. Presence of precipitation line between the precipitated antibody from the egg yolk of each bird and the corresponding Rabbit anti-chicken IgG raised against them, confirms the formation of an Antibody-Antigen complex (Fig 2).

Results from Ouchterlony technique confirms the stability of the immunological activity of the purified IgG extracted from the egg yolk of each bird. It has been shown from studies, that antiserum raised against a given antigen can react with particular related antigen which bears one or more identical determinant [1]. Also, it is possible for antibodies to possess binding sites that are complementary to specific structural features of the antigen molecule [47]. Ouchterlony technique used in this study, revealed a cross reaction between the IgG antibody from the egg yolk of local chicken and that of immunized chicken (Fig 1).The cross reaction between the IgG antibody from the egg yolk of local chicken and immunized chicken shows similarity in the structure of IgG antibody extracted from two birds. Also the cross reaction might be due to the possibility of the antibody raised against precipitated IgG from the egg yolk of immunized chicken possessing binding site that are complementary to the structural features of precipitated IgG from the egg yolk of local chicken . Such observations were reported from studies of [1] there was no cross reaction between the IgG from the egg yolk of Guinea Fowl and that of immunized chicken (Fig 1). Lack of such cross reaction could be due to differences in the structure of IgG antibody extracted from the egg yolk of Guinea Fowl and that of immunized chicken. There may be possibility of the antibody raised against IgG antibody from the egg yolk of immunized chicken, possessing binding sites that are not complementary to the structural features of IgG antibody from the egg yolk of guinea fowl.

The structures of IgG antibody extracted from egg yolk of each bird was not denatured because it was active against the rabbit anti-chicken IgG raised against each IgG antibody from the egg yolks of the Birds.

CONCLUSION

Serological tests are commonly used in screening of Poultry Health [37] These tests are expensive because trained staff or skilled personnels are required to collect blood samples. Studies have shown that immunoglobulin IgG content of the egg yolk is the same or even higher than IgG in serum of the chicken.[5, 48]. IgG isolated from egg yolk can be employed for further studies.

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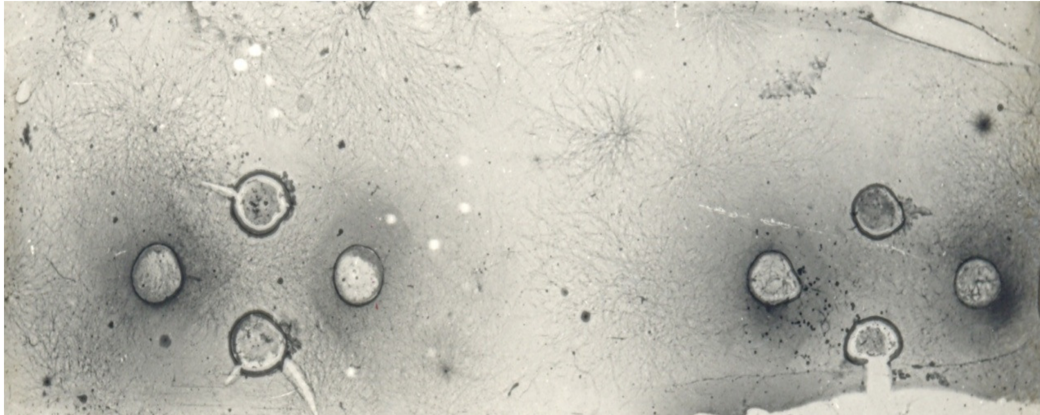

Fig.1a Result obtained from Oucterlony Test.

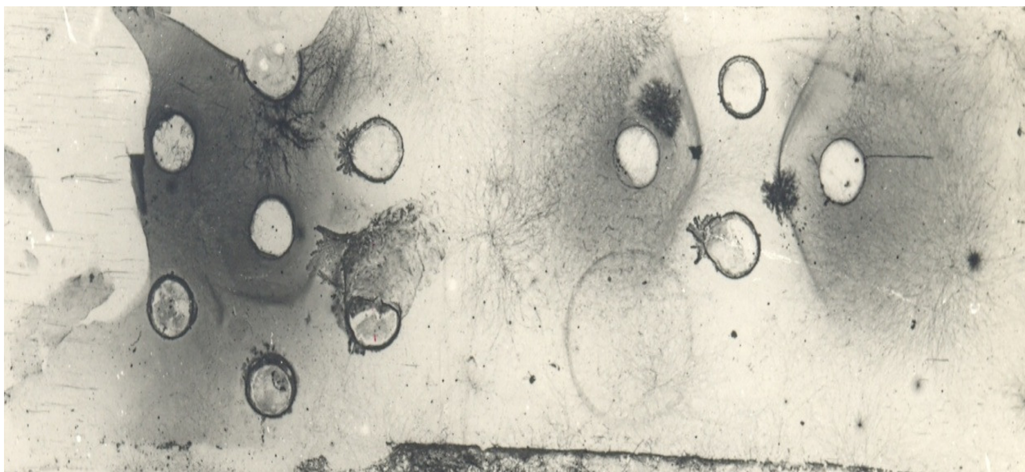

Fig.1b Result obtained from Ouchterlony Test

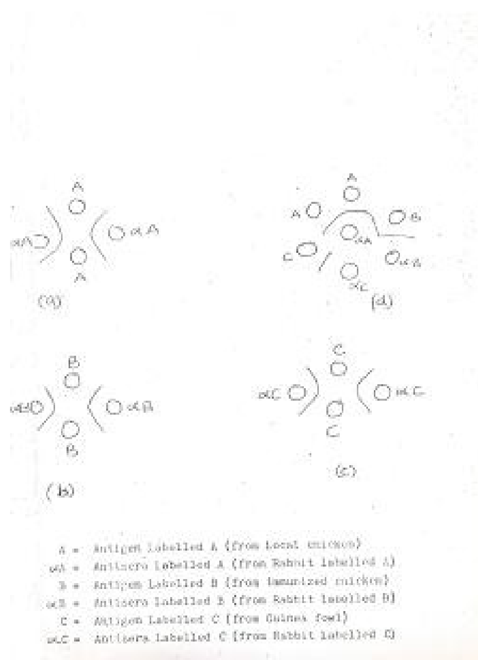

Fig.1c Schematic diagram of result obtained from Oucterlony Test.

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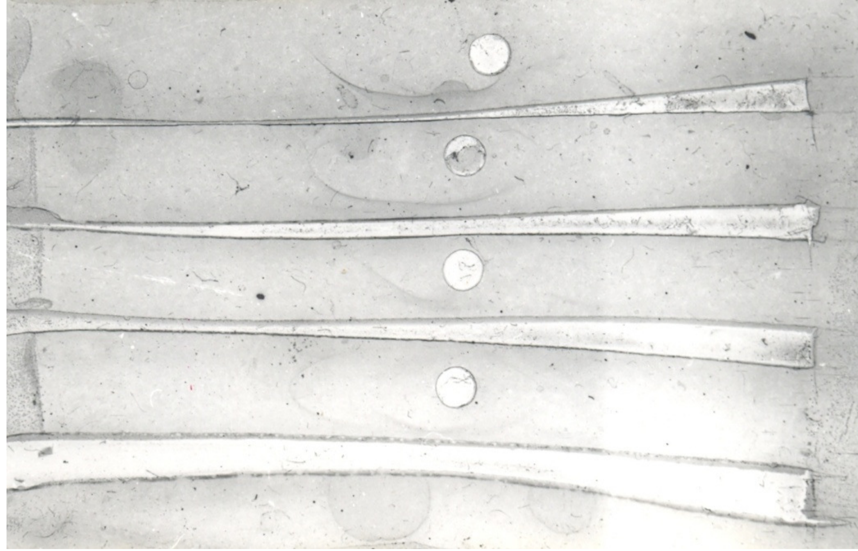

Fig.2a Result obtained from immunoelectrophoresis test.

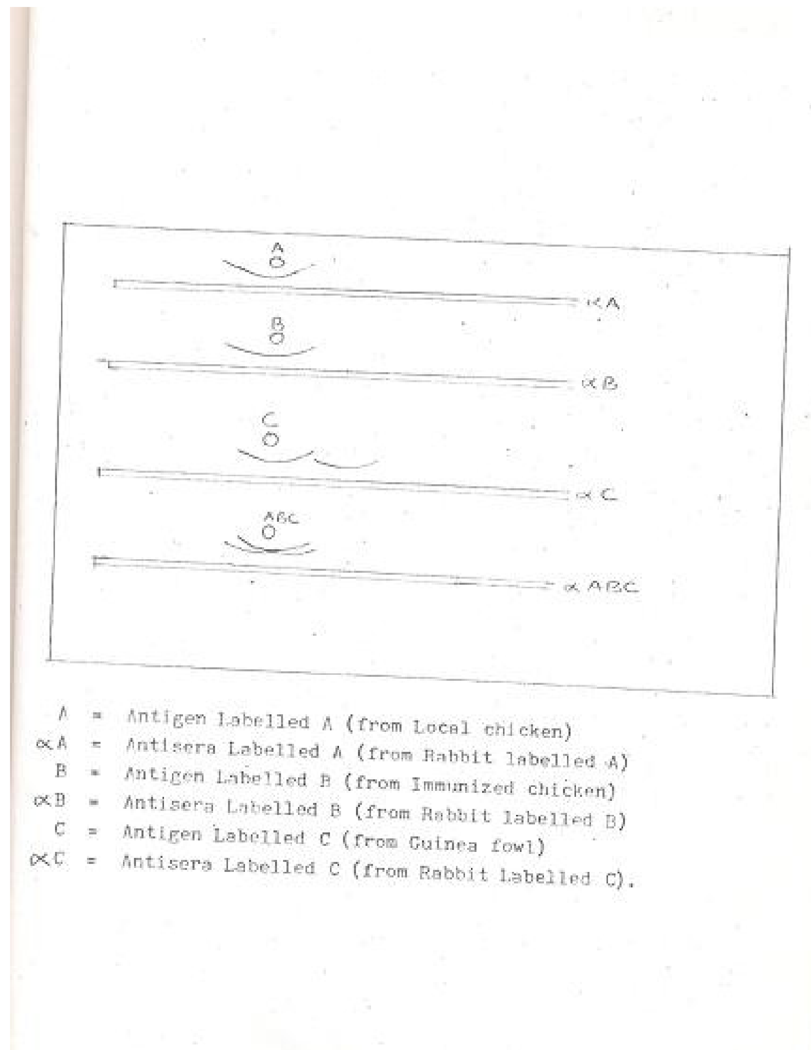

Fig.2b Schematic diagram of result obtained from immunoelectrophoresis Test.

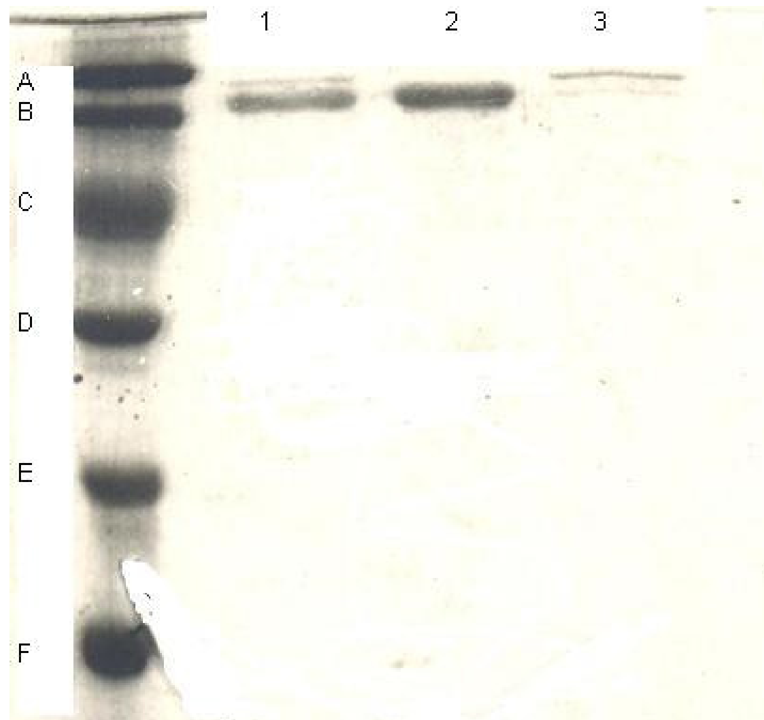

Fig.3 Sodium dodecylsulphate Polyacrylamide Gel (SDS-PAGE) Electrophoresis of samples from Egg Yolk. Markers A: lactolbumin (14,000KD), B: Trypsin inhibitor (20,000KD), C: Carbonic anhydrase (30,100KD), D: Ovalbumin (43,000KD), E: Albumin (67,000KD), F: Phosphorylase (94,000KD). 1: Immunized chicken, 2: Local chicken, 3: Guinea fowl.

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