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OUCHTERLONY DOUBLE DIFFUSION IMMUNIZATION METHOD TO INVESTIGATION THE INTERACTION OF RABBIT ANTISERUM TO THE PITUITARY GLAND OF CAT FISH (*SYNODONTIS SCHALL)* WITH THAT OF HIGHER VERTEBRATES: WISTER RAT (*RATUS NERVICUS)* AND WHITE LEGHORN CHICKEN (*GALLUS DOMISTICUS).*

Nadia Hessein Elzaki Elhaj & Asma Abdelrahman Ahmed Ibrahim
Department of Biology and Biological Technology,
Faculty of Science and Technology of Al Neelain University, Khartoum,
Sudan.

ABSTRACT

The purpose of this studies to identity the interrelation of pituitary gland of fin-fish (*Synodontis schall*) to that of higher avian and mammalian classes**:** White Leghorn Chicken (*Gallus domisticus*) and Wister Rat (*Ratus nervicus*) respectively. Pituitary homogenate (PG H) was chosen as a tool to this investigation. Ouchterlony immunodiffusion test (1948) and Ring test were applied.

Results indicated a strong -cross identity or near identity between the vertebrates studied. Two methods of Immuno-diffusion were used: double diffusion precipitation and ring test. Ring test precipitin reactions show +ve cross- reactions between pituitary homogenate of all the experimental animals against rabbit antiserum to PGH of *S. schall* indicated that these

vertebrates immunologically identical. Moreover, this nearness was varied among the group. A clear and strong cross-reactions were between antiserum of fish pituitary homogenate and that of rats reflecting a positive and strong resemblance to the genetically make up of the two vertebrate classes, Pisces and Mammalia that restored during their evolution. The avian inter-correlation was unexpectedly weak with fish.

Keyword: Immunediffusion method, interrelation of pituitary gland.

1. INTRODUCTION:

Ouchterlony (1948)method of immunization for research purposes depends on the production of antiserum of animals include rabbits, guinea pigs, mice, goats, sheep, monkeys horses, and hens. Selection of species depends on availability

GLOBAL JOURNAL OF ADVANCED RESEARCH
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and volume of antiserum required. The mechanism regulating antibody synthesis and the reactions produced in the cells of the immune system in the presence of viral particles.

Ouchterlony double immune-diffusion also known as agar gel immune-diffusion or passive double immunodiffusion is an immunological technique used in the detection, identification and quantification of antibodies and antigens, such as immuno-globulins and extractable nuclear antigens.

The precipitation reaction is a highly specific serological reaction involving the binding of antigen by antibody. Each antibody has two antigen binding sites, and each antigen could have multiple antigenic epitopes. When soluble antigens are bound by antibody to form a cross-linked "lattice" structure, the reaction is called precipitation. Based upon this principle, the presence of specific antigens in a mixture and the relative concentration of antigens can be detected using antibodies (Boundless, 2015).

As more antigens are added, the amount of protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the zone of equivalence or equivalence point. This process can be used to isolate and concentrate a particular antigen from a sample containing many thousands of different antigens. (Bailey, *et.al.* (1996).

The synthesis of antibodies increases rapidly to a higher concentration than initially produced by the first dose of antigen is found in the blood serum of immunized animals. This serum called antiserum.(Mayer *et. al.* ,1987)

The injection of protein hormone repeatedly into rabbit without adjuvant will produce in the serum antibodies which should be capable of counteraction in vivo the biological activity of the antigen. The antigen is usually administrate with adhering agent or adjuvant to elicit maximal antibody response.(Nilsson, (1986).

Precipitation reactions are carried out in a number of ways one of these is the liquid precipitation (Vasanthakumari, 2007).

2. MATERIAL AND METHODS

The immunoassay was carried out by two ways of immunodiffusion method: the ring test and gel agar. Both the preparations of pituitary glands homogenates of the animals under study and the antiserum to the fish (S. schall) homogenate were done as follows.

2.1 Preparation of pituitary glands homogenates:

pituitary glands of the experimental animals(rat, fish and chicken) were removed weighed, quick frozen, and saved until sufficient tissue is available for extraction (10-25 glands) after original wet weights were recorded . The removed gland was quick frozen (in dry ice) and stored in deep freeze (-18 °C) until extraction is undertaken. Glands removed from freezer and crushed to a powder while they still in the frozen state. The powder was subjected to alternate slow freezing and thawing 3 or 4 times to induce cellular disruption. 0.1 ml sodium carbonate (1:9 dilution of powdered gland) added to 9 volumes the material previously subjected to a temperature of 0.0°C for 6 hours and stirred gently to avoid the production of foam. Upon completion of the gentle stirring, the extract carefully adjusted to a pH of 10.0. The final volume of the extraction was determined. The necessary dilution was calculated and done with amount of sodium chloride that required producing an isotonic solution. Then solution was filter with filter paper and placed the extract in vials and quickly frozen

2.2 Preparation of antiserum:-

An area on the rabbit's back large enough to allow 40 or 50 inoculation was shaved. 1ml syringe was used to inoculate the antigen i.e. homogenate of *S. schall pituitary* gland.

Up to 40 intramuscular inoculates on both sides of the backbone was done. Appropriate precautions were taken to avoid infection in the inoculated area. BY the fourth week after inoculation, the first bleeding is done to test the titer of the antibody produced. The rabbit was placed in an appropriate box to keep it still during the bleeding. One of the ear was shaved, the marginal vein was located , a lamb used to warm the ear and to swell the vein and an angular incision of about 45 was made .The cut was made deep to allow clean bleeding, then the blood was collected in the clean glass tube . A maximum of 30 ml of blood was collected in each session. The bleeding was stopped by using a piece of cotton , then

GLOBAL JOURNAL OF ADVANCED RESEARCH
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incision pressed for about 10 to 20 min until the bleeding stops , second immunization once the titer has been obtained , it was convenient to give regular booster immunizations to collect the maximum amount of antiserum .

2.3 Immunodiffusion Test by the Ring method:

Precipitation reaction in this method was tested by liquid agar. Rabbit antiserum to *S. schall* PGH was placed at the bottom of a standrd test tubes and antigen solution is layered over it. In the positive case a ring of white precipitate is formed at the junction of two liquids. The pituitary gland homogenate prepared separately for each experimental animal, centrifuged at low rate for 20 minutes at 4.0°C. The pure homogenates decanted and any precipitates were dissolved in 3.5ml of 0.8% Na Cl at pH 7.6. These mixtures poured separately into 4 test tubes contained 1 ml saline solution mixed with antiserum to *S. schall*. The tubes labeled according to the species and heated at 80.0° C for one hour and centrifuged .The precipitations shown as one distinct precipitin line to the specific antiserum for the positive species .The presence of precipitation was verified again after 24 hours.

2.4 Agar-gel –double diffusion:

Agar gel prepared by adding 50 ml of distilled water to 1.0 g of normal agar .Then mixture was transferred to a set of Petri dishes and auto clave for 15 minutes to purify the agar . hen Petri dishes were placed at room temperature to solidify and central well surrounded by other 6 wells were pore in agar layer in each Petri dish. The antiserum of rabbit was placed in a central well of an agar plate, and the preparations to be tested are in the small wells surrounded the central one. The appearance of localized precipitin lines between the central well and the peripheral ones is an indication of antigen antibody reactions between the diffusing materials .The result was presented by photo using Canon digital camera and/or Samsung GALAXY *S* II mobile camera.

3. RESULTS

Results shown by the two methods of immunization: ring test and agar gel precipitation indicates different responds of *S. schall* PGH antiserum with the PGH of higher vertebrates. The ring test demonstrated precipitation rings for the PGH of all the studied animals to antiserum to the fish *S. schall* PGH (plate 1A). However, the agar gel method differentiated between the responds among the different animals under study. The highly positive respond was by the Wister rat *R. nervicus* that almost equalized the respond to *S. schall'* PGH .The White leghorn chicken *G. domistica'* PGH demonstrated the least respond (plate 2A) .The sensitivity of the method was checked for confirmation by displacing *S. schall* PGH by distilled water (plate2B).The results as general indicated that all these vertebrates immunologically identical with fish pituitary homogenate. Strong cross – reactions between antiserum of *S. schall* to Wister rats gave a clear reaction identity. So the pituitary glands homogenate of Wister rat (*R. nervicus*) more immunologically identical with fish pituitary homogenate than the pituitary gland of White leghorn chicken

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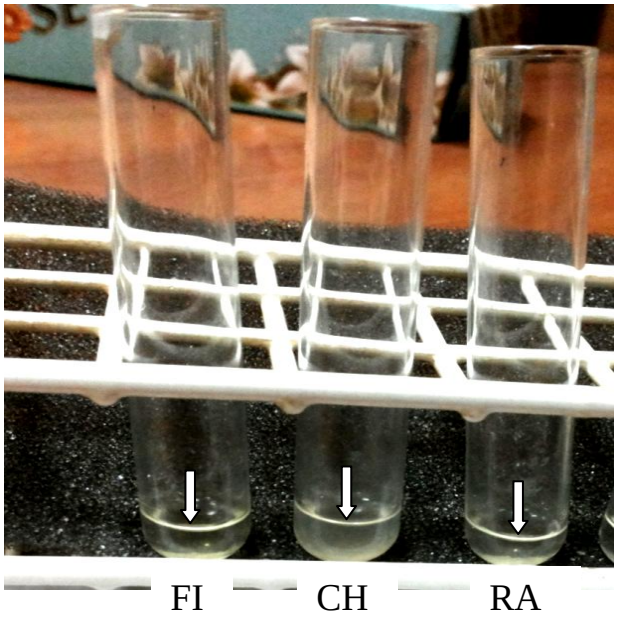
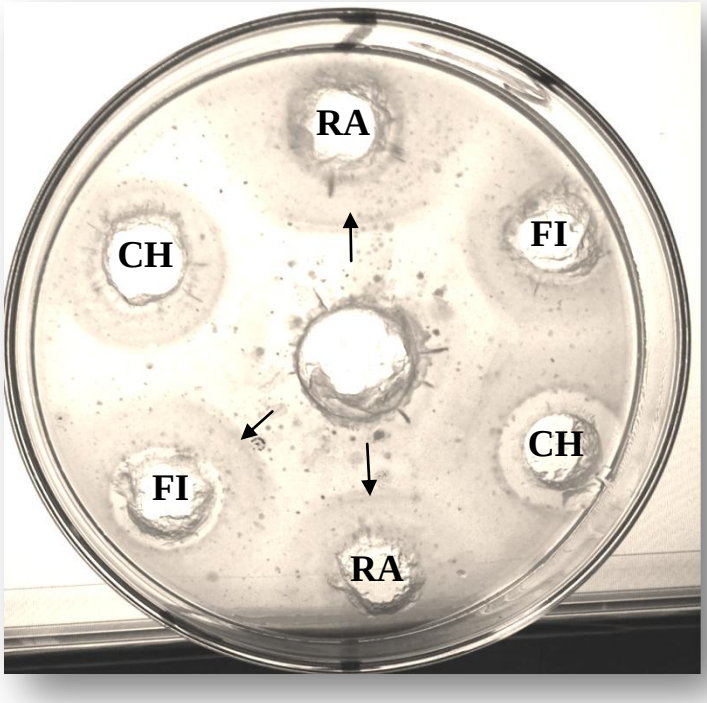
GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)

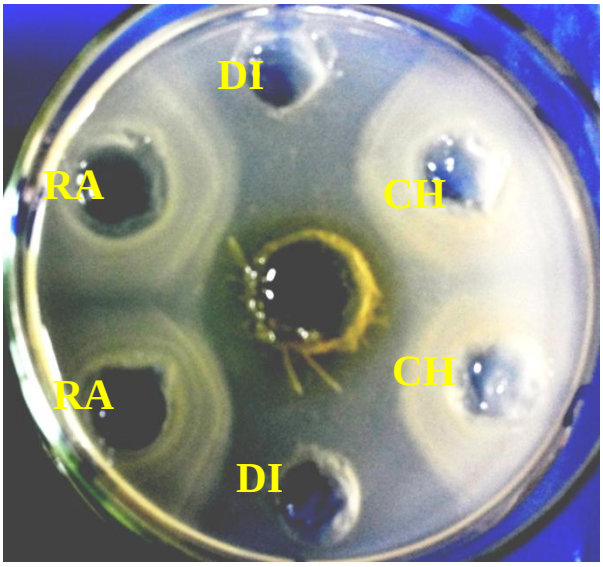


Pate (1) showing the ring precipitin (⇩) on pituitary glands homogenate of *S. schall*(FI) , *G. domisticus*(CH), and *R. nervicus* (RA) against the rabbit antiserum to *S. schall* PGH.



Plate(2A)showing Immunochemical interrelation rabbit Antiserum to pituitary homogenate of the fish *S. schall* (centre well) with pituitary glands from fish *S. schall*(FI) ,Wister rat *R. nervicus*(RA*)* and chicken *G. domistica* (CH) based on immunodiffusion tests.

GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)



Plate(2B) showing Immunochemical interrelation rabbit Antiserum to pituitary homogenate of the fish S. schall (centre well) with distilled water (DI) ,pituitary glands from Wister rat *R. nervicus*(RA*)* and chicken *G. domisticus* (CH)based on immunodiffusion tests.

4. DISCUSSION

Immunochemical relationship of PGH from the vertebrates species based on the results of Ouchterlony immunodiffusion. All vertebrates under study shown identity or near identity to fish pituitary homogenates, and shown a high degree of conservation of structure occurs during evolution. This confirmed the immunodiffusion studies by Morrow, *et. al.*(1982) with the rabbit antiserum raised against dogfish antibody for immunoglobulin demonstrated and reported that the physicochemical properties of dogfish antibody response to injected antigen bear a striking resemblance to those of immunoglobulin M(1gm) found in higher vertebrates.

Pan (1993) studies on purification of a variety of growth hormone proteins from mammalian, avian , amphibian and fish pituitary glands demonstrated reactions of identity or near identity by immunodiffusion method on a gar gel for all the studied classes of animals. However, Hayashida (1975) reported that the somatotropins of existing primitive fishes such as the shark and sturgeon, but not those of modern bony fishes (striped bass and salmon), have shown clear immunochemical relationship to those of phylogenetically higher vertebrates. Van,*et. al.* (1972) detected identity of growth hormone in Sockeye salmon(*O. nerka*) to bovine growth hormone by radioimmunoassay and immunocytochemistry, using an antiserum .

Hayashida .*et al* ,(1973) observed that the equivalent follicle stimulating effect of a rat pituitary extract with the same ovine FSH antiserum . Graham, F.W. (1980) studied on Chum and Coho salmon pituitaries also demonstrated that these protein are immunologically identical .These records confirmed the result of the present study which show close relationship of The PGH of fish to rat rather than to chicken. However , the sensitivity of immunodiffusion method was confirmed by different studies such as Moudgal, *et. al.* (1960) studies on the immunochemical relationship between bovine and ovine growth hormone been investigated by means of the quantitative precipitin technique and diffusion analysis on agar gel. It was observed according to the results of the Ouchterlony double-diffusion technique, that of the pituitary extracts from various species used as sources of growth hormone only that of the deer contains a component which cross reacts completely with bovine growth hormone.

GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)

Lunenfeld, *et al.*, (1970)studies on Antiserum to human pituitary follicle-stimulating hormone (FSH), isolated by starch-gel electrophoresis, have been produced in rabbits. The presence of antibodies to FSH was indicated by the single precipitin line obtained when the antiserum were inter-reacted with FSH isolated by starch-gel electrophoresis and a line of identity obtained between the antiserum and human FSH, HMG and equine FSH in Ouchterlony immunodiffusion in agar. This was further supported when 0.5 ml of the antiserum completely blocked the increase in the ovarian weights caused by 100 µg human FSH, 100 µg HMG and 40 IU of HCG in immature female rats.

Hayashida T.(1975) studies with Immunological rat pituitary growth hormone included four rhesus monkeys, four rabbits, and three guinea pigs immunized with rat pituitary growth hormone (RGH) in Freund's adjuvant. Antiserum from all monkeys produced good precipitin reactions with RGH in the double-diffusion procedure in agar, while antiserum from none of the rabbits showed any precipitin reaction, even after continued immunization. All guinea pigs yielded antiserum that would precipitate RGH, but the reactions were much less intense than those obtained with any of the monkey antiserum.

Ludwik *et. al.* (1960) Used ring test for detecting precipitating antibodies . He showed that an antiserum to rat pituitary is capable of inhabiting highly purified bovine STH .This inhibiting occurred even if no serological evidence of precipitating antibodies to the bovine STH was found to rat pituitary tissue.

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