

Production of Rabies Immunoglobulin Using Caprylic Acid Purification Method

- J. Jaya Prakash Ph.D*



Abstract

Since rabies is still a major cause of human death in many developing countries and the implementation of recommended post exposure prophylaxis by vaccination and specific immunoglobulin therapy are largely hampered by its high cost. The development of low cost rabies vaccine and immunoglobulin preparation of high priority in all the countries. In view of the disadvantages of human and equine rabies Immunoglobulin, still there is urgent need for safe and cost effective antirabies Immunoglobulin especially for person who have been severely exposed (Category III bites) to the virus. Our aim is to produce a less immunogenic and cheaper antirabies Immunoglobulin for the benefit of mankind. The animals have been immunized, and the plasma was processed with different concentration of caprylic acid to yield 90 % pure IgG. Rapid Fluorescent Focus Inhibition Test (RFFIT) is highly recommended now a day for estimation of rabies neutralizing antibodies. Our results showed that four immunized rabbit were produced 400 IU/ml of purified rabies antibody, Rabbit IgG has low immunogenic effect than human, horse and sheep when injected into the mouse. Pure concentrated rabbit antibody may serve as a possible alternative to lab scale production.

Keywords: Rabies, RFFIT, IgG, Caprylic Acid, Rabbit

Introduction

Rabies is a zoonotic, viral disease caused by *lyssa* virus of the family *Rhabdoviridae* which is responsible for acute encephalomyelitis. Once clinical symptoms appear, the disease is fatal. In developed countries rabies is spread from wild to domestic animals and consequently to humans. In countries like Africa, Asia and Latin America dogs are the main host of the disease, making them responsible for most of the rabies deaths worldwide. In 1996, the number of rabies death was estimated to be between 40,000 and 70,000. Approximately 10 million people receive post-exposure treatment each year because of contact with animals suspected with rabies. Polyclonal antibody therapeutics are widely used today in medicines for viral and toxin neutralization and for replacement therapy in patients with immunoglobulin deficiency (2). Hyper immune antibody preparation produced from animals serum have been used over the past century for the treatment of a variety of infectious agent and

medical emergencies, including dioxin of toxicity, snake envenomation and spider bites(3-4). Traditionally equine IgG has been purified by Caprylic acid based fractionation (5). This fractionation relies on selective precipitation of all non-IgG serum protein. It is also widely used for manufacturing of anti venom and similar immunological products (14). IgG purity obtained from a single stage Caprylic acid precipitation process is about 70% and recovery is about 67% (5), Caprylic acid based purification method was developed which proved to be better in terms of production time, yield, albumin contamination and turbidity (12). The aim of this research work is to purify F (ab)₂ fragment from animal serum by using Caprylic acid purification (7). The recommended treatment for category III exposures consist of both vaccine and rabies Immunoglobulin (RIG), according to WHO recognized regimens while vaccine provide long term protection but late response while Immunoglobulin provides immediate response to compensate for the time necessary for endogenous antibodies to appear (Khasoplod

et.al,1996). The life saving benefit of adding specific RIG to post-exposure treatment of subject exposed to rabies has been clearly established, particularly in case of severe wounds (8,9,10). Although more than 90% of rabies cases are encountered in developing countries (10, 11), many cannot afford human rabies Immunoglobulin (HRIG) due to its high cost therefore the use of highly purified equine rabies Immunoglobulin (ERIG) is a safe and effective alternative. Therefore, it is necessary to monitor the level of protection induced by PEP or rabies antibody so that additional booster doses may be advised if necessary. As of today only two tests are recommended by WHO (1996) for estimation of rabies antibodies i) Rapid Fluorescent Focus Inhibition Tests (RFFIT) (15) and mouse neutralization test (MNT). These tests are generally performed only in one or two centers in India. Especially RFFIT test is done in our institute.

Materials and Methods

Materials

Crude plasma, reference standard and Neuro-2A cells, Caprylic acid, Pepsin (Himedia), 96well microtitre plate, FITC conjugated immunoglobulin, UV-Vis spectrophotometer, Fluorescent microscope (Nikon model, Japan) was obtained from PIIC, Coonor.

Immunization Schedule

Four healthy, 3-months-old female and male rabbit weighing (2 to 5 kg) each were injected subcutaneously with rabies vaccine, and bleeding schedule is given in **table-1** (Abhayrab tissue cultured derived rabies vaccine, to obtained from Pasteur Institute of India, Coonor.).

Table-1 Immunization schedule

Immunization day	Dose	Route	Bleeding
0	500µl	Deep IM, FCA	5ml
7	500µl	Subcutaneous	5ml
14	500µl	Subcutaneous	5ml
21	500µl	Subcutaneous	5ml
35	500µl	Subcutaneous	5ml
70	500µl	Subcutaneous	5ml
85	500µl	Subcutaneous	10ml
100	500µl	Subcutaneous	10ml

IM-Intramuscular; FCA-Freund's Complete Adjuvant.

Preparation of F (ab)2 from hyper immune plasma

Collection of hyper immune plasma

The animal is not subjected to stress during blood collection. The use of sedative to facilitate blood sampling is usually unnecessary. However, in large scale production,

operator may find it advantageous to use sedatives to maintain low stress levels and achieve rapid blood collection. The volume of blood to be removed should not exceed 15% of the total blood volume. In practice, amount of up to 1% of the total body weight can be safely removed. Exsanguinations should be performed under general anesthesia and is best carried out by heart puncture. (13)

Hyper immunized animals were maintained as per the guidelines of CPCSEA (Committee for Purpose of Control and Supervision on Experiment on Animals) India. Animals were bled and 5ml of hyper immunized plasma was collected from each immunized animal in a sterile, non-pyrogenic glass container containing Heparin, ACD, and EDTA for antibody titre value or potency estimation in plasma by Rabies antigen neutralization test (RFFIT)

Enzymatic digestion of plasma

The hyper immunized plasma was maintained at room temperature (22° C to 25° C) and was diluted using WFI (Water For Injection). The pH was adjusted to 3.2± 0.1 with 50% Hcl. Plasma was diluted to 0.1% (w/v) and pepsin was added for digestion under constant stirring for 1 hour at 30-37°C for complete action of pepsin on IgG. The digestion was stopped by adding 10% NoaH (v/v) and pH was adjusted to 4.2± 2.

Caprylic acid purification

The pepsin-digested plasma was incubated for an hour at 55° C to 56° C. The Caprylic acid was added very carefully and slowly with constant stirring for 1 hrs at 22° C to 25° C at variable strength to reach final concentration of 3%(v/v) and the mixture was centrifuged at 4400 rpm for 30 mins. After dialysis, the supernatant was removed and incubated against PBS overnight. The proteins in supernatant were filtered using 0.22µm filter (Millipore). 0.85% sodium chloride was added to make it isotonic and 0.01µl/ml of Thiomersal was added as a preservative to make an isotonic and as a preservative. It was tested for IgG sample antibody titre using RFFIT and purity analysis with SDS-PAGE.

Protein content estimation and Electrophoresis

The protein concentration of each sample was determined spectrometrically UV-Vis spectrometrically at 650nm using standard methods followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to monitor protein purification during caprylic acid procedure. Electrophoresis was performed for 50 mins at 140V using 7.5 % polyacrylamide gel. Broad range of marker protein of known weight was run along with the protein sample and protein bands were visualized using Coomassie Brilliant Blue (CBB) R-250 Staining (16).

Challenge virus

Neuroblastoma-2Acell (PV-3462) adapted rabies virus, a fixed laboratory strain, was obtained from the Pasteur Institute of India (coonor,India). After virus propagation in Neuro-2A cells, vials containing PV-3462 suspensions were

kept at -80°C . Preparation of the challenge virus (PV-3462) seed stock was titrated according to TCID (20). The final TCID_{20} to $\text{TCID}_{100}/0.1\text{ml}$ was 10^{-4} to 10^{-6} .

Potency estimation

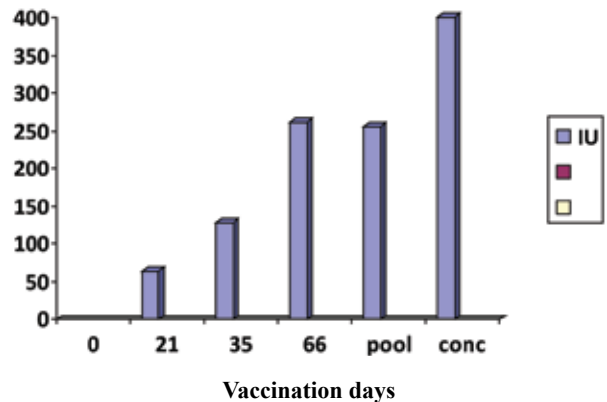
Potency of antirabies plasma was determined by rapid fluorescent focus inhibition test (RFFIT) (15). Each plasma sample was diluted two fold with maintenance medium which contained 10% of FBS (fetal bovine serum) and placed on a 96 well microtitre plate. The constant quantity of rabies virus (PV3462 Neuro-2A cell adapted) was added to each well and incubated in 4% CO_2 incubator at 37°C for 90 mins followed by addition of Neuro-2A cells to each well and incubated for 42 to 48 hrs (depends upon the monolayer formation). Finally, cells were fixed with 70% (v/v) acetone and stained with a fluorescent antibody in order to detect the presence of non-neutralized virus (fluorescent foci) using a fluorescent microscope. The RFFIT test was used to determine a value for the rabies virus neutralizing antibody concentration (IU/ml). The IU standard for international units and was calculated from titre by comparison with WHO international standard of antirabies Immunoglobulin of known concentration.

Results and Discussion

WHO has recommended post-exposure passive immunization in all category III bite patient. Currently developing countries, less than 1% of all post-exposure treatment inside vaccine and serum. This is because HRIG is not available and it's not affordable due to its high cost and there is apprehension about the side effect of animal RIG, especially anaphylaxis reaction. It has also become difficult to obtain animal RIG because the major international supplier has decided to discontinue the production of animal RIG. Therefore it's of great concern to develop a substitute for HRIG and ERIG.

Caprylic Acid Purification of IgG

The procedure previously used for the purification of IgG, based on caprylic acid precipitation of non-IgG plasma proteins (5,17,18) was successfully applied to rabbit plasma electrophoretic analysis of there preparation showed a broad predominant band 80 to 20 KDa corresponding to IgG heavy and light chain respectively.

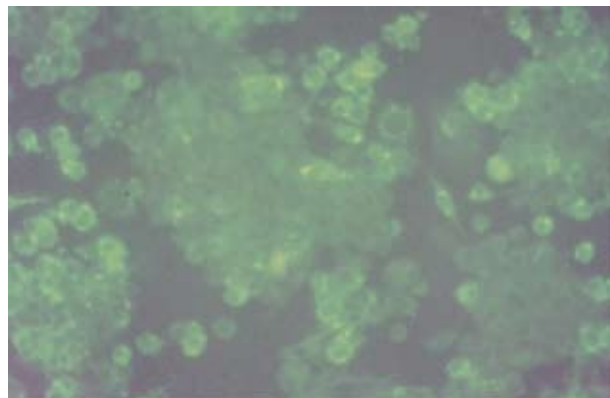


Rabbit hyper immune plasma against rabies was used as test material. A preliminary experiment was carried out to study caprylic acid fractional precipitation of rabbit serum protein using 3% of the caprylic acid. Most serum protein was precipitated at 3% Caprylic acid concentration. Protein analysis by SDS-PAGE showed that albumin was completely precipitated by 3% caprylic acid (19). The removal of small peptide fragments and / or reagents (ammonium sulfate or Caprylic acid) used in the fractional precipitation of antibody is usually carried out by dialysis in cellulose (MWCO 12–14 kDa) bags. This process is time-consuming and can take several days. It is costly in terms of pyrogen-free water, and also risks contamination with endo toxins, which could result in rejection of the valuable antivenoms (2).

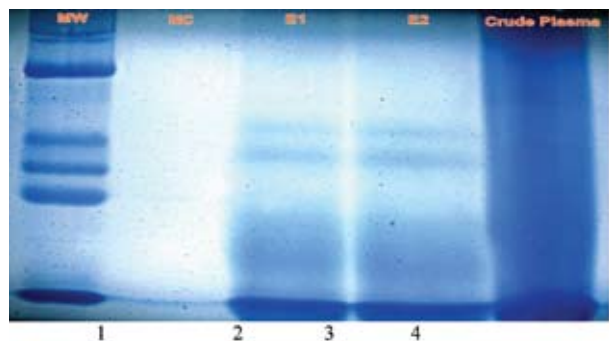
To cross these barriers Ultra filtration has been introduced to replace simple dialysis in various laboratories. In this study, the supernatant of Caprylic acid precipitation was passed through the cationic exchanger in which Caprylic acid, pepsin and numerous acidic peptides were simultaneously and effectively removed in the unbound fraction.

Hyper immunized antirabies serum digested plasma was fractionated by Caprylic acid. Most protein was precipitated at a Caprylic acid concentration more than 2%. The recovery of antibody activity in the supernatant was reduced when the Caprylic acid concentration was increased. Protein analysis by SDS-PAGE revealed that all supernatants contained mainly $\text{F}(\text{ab})_2$ resulting from the digestion of IgG by pepsin. However, the contaminated protein in the supernatant obtained by precipitation with 1% Caprylic acid seemed to be more than those with higher concentration. The precipitate was principally composed of low molecular weight digested protein other than $\text{F}(\text{ab})_2$.

Picture: RFFIT test Fluorescent Microscopic picture (Virus neutralization in Vero cell line)



The Caprylic acid concentration of 3% gave the best results. High antibody activity recovery and protein concentration estimation by Lowry's method neither the assay of antibody activity RFFIT nor evaluation of protein pattern SDS-PAGE.



SDS-PAGE of purified IgG constituted by whole IgG molecules. Non-reduced samples were loaded in a 7.5% polyacrylamide gel in the presence of SDS. Proteins were stained with silver stain. Lane 1 marker, Lane 2 and 3 corresponds to 1% of CA purified plasma, while lane 4 crude plasma. Bands corresponding to further diluted immunoglobulins are identified.

The neutralizing activity of rabbit antirabies antibody (7440 IU/mL) was analysed invitro on cultured neuro-2A cell (fig) which shows that rabbit immunized with cell cultured derived rabies vaccine contained antibody capable to neutralizing rabies virus effectively.

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg/ml)	Activity (IU/ml)	Total activity IU/ml
Hyperimmune Plasma	30	71.19	2135.7	256	7680
After pepsin digested	60	31.90	1914	120	7200
Caprylic acid purified	62	21.09	1307	120	7440
Concentrated sample	25	16.06	240.9	400	10,000

The antibody increased after booster immunization to reach its peak at 7th dose, then declined gradually up to 10th dose. This efficient in-vitro virus neutralization let us to investigate whether post-exposure administration of antibody could not prevent the disease caused by virulent virus in mice, the result indicate that the progression of the disease symptoms was significantly delayed when antibody inoculate at injection site.

In conclusion, in this study the fractionation of rabies hyper immune plasma was standardized for optimal recover of rabies immunoglobulin besides the usual fractional precipitation with ammonium sulphate. This study describes a simple and highly efficient method for the fraction of hyper immune plasma in rabies immunoglobulin production. Caprylic acid fraction gives a highly enriched immunoglobulin preparation with a high yield, adequate neutralizing potency and optimal physiochemical properties. Antibody titre of the plasma after CA purification with pepsin digestion in case of rabbit was found to be more 7440 IU/mL and concentrated sample was found to be 10000 IU/ mL. This methodology might be highly useful in antiserum production laboratories. Studies on a larger scale should be performed to evaluate its suitability for large-scale fractionation of therapeutic rabies immunoglobulin.

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