Study on Modulation of Vaccine Immune Response with Probiotic Supplementation in Animal Models

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A thesis submitted to the Department of Microbiology, Quaid-i-Azam University Islamabad, in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in

Microbiology By: Amina Najam

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Pakistan

2023

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Study on Modulation of Vaccine Related Immune Response by using Probiotics in Animal Models"** was conducted by **Ms. Amina Najam** under the supervision of **Prof. Dr. Safia Ahmed.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

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IN THE NAME OF ALLAH,THE MOST MERCIFUL ,THE MOST BENEFICENT

DEDICATIONS

This thesis is dedicated to my teachers and especially to our beloved teacher and mentor, **Prof. Dr. Abdul Hameed (late**), who have given me wings to rise higher in professional life.

To my **Parents, Teachers, Family and Friends** who were always been there for me and supported me throughout my life.

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"There is always something to hope for." —The Catcher in the Rye by J.D. Salinger.

All glory to Allah, the kindest and beneficent. He, whose glory encompasses the entire cosmos and is omnipresent. Then I humble myself before the Holy Prophet (PBUH), the undeniable symbol of compassion and kindness for humanity.

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Amina Najam

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

Abbreviations	Description
PEP	Post Exposure Prophylaxis
PrEP	Pre-Exposure Prophylaxis
CCRV	Cell culture Rabies Vaccine
PVR	Purified Vero-cell Rabies vaccine
NZW	New Zealand White
PB	Probiotics
PBA	Probiotic A
PBB	Probiotic
EQ	Equate(Proprietary blend)
TF	Top Formula (Proprietary blend)
LB	Lab Strain
VAS	Vitamin A supplementation
O.C	Organic carrots
PPZ	Prepro-Z (Proprietary blend)
RVNA	Rabies virus neutralizing antibodies
RABV	Rabies Virus
RIGs	Rabies immunoglobulins
ARS	Anti Rabies Serum
PAb	Polyclonal Antibodies
C.A	Caprylic Acid
NTD	Neglected Tropical Disease
IgG	Immunoglobulin G
pH	Power of hydrogen ion

WHO	World Health Organization
NIH	National Institute of Health
TFF	Tangential Flow Filtration
UF	Ultra Filtration
TRC-ID	Thai Red Cross Intradermal
ID	Intradermal
IC	Intracerebral route
IPC	Institut Pasteur du Cambodge (Vaccination regimen)
IM	Intramuscular
ELISA	Enzyme Linked Immuno Sorbent Assay
OD	Optical Density
HSC	High Sero-Conversion
SSC	Sufficient Sero-conversion,
SOP	Standard Operating Procedures
SVN	Serum Virus Neutralization
DFAT	Direct Fluorescent Antibody Test Method
MNT	Mouse Neutralization Test
MICLD50	Mouse Intracerebral 50 Per Cent Lethal Dose
CVS	Challenge Virus Standard
HPLC	High Performance Liquid Chromatography
RP- HPLC	Reverse Phase High Performance Liquid Chromatography
UV	Ultraviolet
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

2-S	2 sites
Da	Dalton (unit of Molecular Weight)
ANOVA	Analysis of Variance
IU	International Units
EU	Equivalent Units
RMP	Revolutions Per Minute
WBC	White blood cells
RBC	Red Blood Cells
HGB	Hemoglobin
PLT	Platelets
HCT	Hematocrit
MVC	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
NEUT	Neutrophils
LYMPH	Lymphocytes
MONO	Monocytes
EO	Eosinophils
BASO	Basophils
ALT	Alanine Transaminase
ALP	Alkaline Phosphatase
TVC	Total Viable Count
BRM	Biological Response Modifiers

EDTA	Ethyl Diamine Tetra Acetate
WFI	Water For Injection
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
ILs	Interleukins
IFNs	Interferons
TGF	Transforming Growth Factors
DCs	Dendritic Cells
QSMI	Queen Saovabha Memorial Institute

ABSTRACT

Background: Probiotics besides several health benefits on the host, are also considered as growth promoters on a large scale to improve the productivity of many animal species for human consumption. New Zealand white rabbits are the ideal laboratory animals for their use as experimental models in research and bio-medicine due to economic feasibility. This study aimed to evaluate the potential adjuvant-like properties of three strains of commercial probiotics on the growth performance (biochemical and hematological parameters and body weight of rabbits) and humoral response against rabies vaccination.

Secondly by further secondary hyper-immunization strategy using ID route of immunization, higher antibody titers could be obtained which can further be purified with the use of caprylic acid-based fractionation techniques to produce high-quality anti-sera from animal plasma. The modern purification techniques are simple, practical, and cost-effective with the potential to alleviate Pakistan's chronic shortage of rabies vaccine and antiserum in the local market.

Methodology: New Zealand white rabbits were chosen as animal models to study the effects of antibiotics, the mineral zinc, and organically carrot dietary supplements on general growth and immunological response to the rabies vaccine. The rabbits were immunised using concentrated rabies vaccine "CCRV" using Thai red crosses intradermal "TRC-ID" immunization. The feed supplementation started one week prior to vaccination till the completion of the experiment at day 60th. The primary outcome was to achieve improved sero-conversion with simple dietary interventions when contrasted with a control group in testing groupings.

Results: Our results mainly showed a comparison of the humoral immunological reaction as specific titre of antibodies against vaccination among different groups with dietary interventions. The experimental probiotic groups (G1-G6) displayed high mean titre values of 3.506-3.896, 3.804-3.962 and 4.144-4.336 EU/L at day 14, 35 and 60 respectively. While control group C had lowest titre value with 3.089, 3.505 and 3.7462 EU/L at 14th, 35th and 60th day respectively.

The optimal results were achieved by introducing caprylic acid solution into plasma,at pH 5.8, and 5% final caprylic acid concentration . Subsequently, the mixture was stirred for an additional 60 minutes and passed through different filters. This downstream production method involves a single precipitation stage and dialysis. The comparison of caprylic acid-based fractionation and classical ammonium sulphate purification techniques revealed that the former produces a final product with superior yield and quality, meeting the recommended criteria for rabies antiserum.

Conclusion: Our research has determined that implementing low-cost dietary interventions, such as probiotics and vitamins, in conjunction with improved immunization strategies can effectively decrease the overall expense of rabies vaccinations. The use of this technology presents a promising solution for locally producing anti-sera products, offering both efficiency and simplicity. Therefore, the incorporation of probiotics and vitamin-rich diets for rabbits significantly influences their overall growth and immunity levels – an approach that can be applied successfully to produce animal-derived rabies antiserum products.

The study aimed to discover further economical strategies to produce animal-derived Rabies immunoglobulins/antiserum (RIGs). Adopting intradermal vaccine administration schedules can reduce vaccine shortages, increase treatment affordability, and provide equitable access to rabies PEP. The hyper-immunization approach was also utilized to enhance PAb production in animals. To purify the final product, the caprylic acid fractionation technique was implemented to improve downstream processing, thus yielding a purified immunoglobulin fraction that surpasses classical salt precipitation techniques. These cost-effective strategies could prove advantageous to commercial manufacturers producing large-scale rabies immunoglobulins from animal plasma in developing nations.

These approaches are particularly relevant for countries with lower to moderate incomes where more efficient measures are necessary to alleviate human suffering caused by rabies. With efforts already underway, adopting various upstream and downstream strategies can assist developing countries like Pakistan in achieving goal of eliminating dog-mediated rabies deaths and reducing the global burden of rabies by 2030.

RATIONALE/ AUTHOR'S SUMMARY

Rabies is the oldest recorded and most fatal of all infectious diseases, yet this zoonotic disease remains a neglected considering an elevated prevalence of bites from dogs, it is a disregarded indigenous illness in Pakistan in both rural and urban areas. Unfortunately, there are insufficient epidemiological statistics on the disease, and no proper control program exists in Pakistan at national level. The National Health Management Information System (NHMIS) recorded roughly more than ninety-seven thousand bites from dogs incidences from three main cities in Pakistan in 2016 with estimated (59.7%) cases from Karachi, 13% from Peshawar and 11% from Hyderabad cities (Ahmad et al., 2016). However timely intervention including proper prevention, control of canine rabies with immediate treatment can save human lives (Kumar & Bakhru, 2022).

In locations where dog attacks are a particularly frequent and biggest cause of populace misery, rabies vaccination constitutes one of the most critical prophylactic interventions. However, the shortage of vaccine and rabies immunoglobulins (RIG) causes approximately 2000-5000 deaths in Pakistan (HRI, 2023). This high incidence of human rabies is mainly coupled with a lack of awareness about the disease complications, shortage of locally produced vaccines and anti-sera and the overall costs of imported cell culture vaccines and imported human rabies immunoglobulins (HRIG) (Table-7). For the strengthening of indigenous antiserum production, there is dire need to develop cost-effective and novel immune-enhancing strategies for optimization of production methodologies at different manufacturing stages, to get better product yield at the end. In this study simple dietary interventions were applied to enhance the NZW rabbits' humorous immunological reactions to rabies immunization. According to the findings of this investigation, treatment of probiotics and vitamin A rich diet along with rabies vaccination of animals is a simple, cost-effective and unique approach for production of animal derived poly-clonal antibodies (anti-sera). Moreover, the new caprylic acid fractionation method described for protein purification from plasma provided better recovery yield of purified immunoglobulin fraction in a short period of time.

GRAPHICAL ABSTRACT(UPSTREAM PROCESS FLOW)

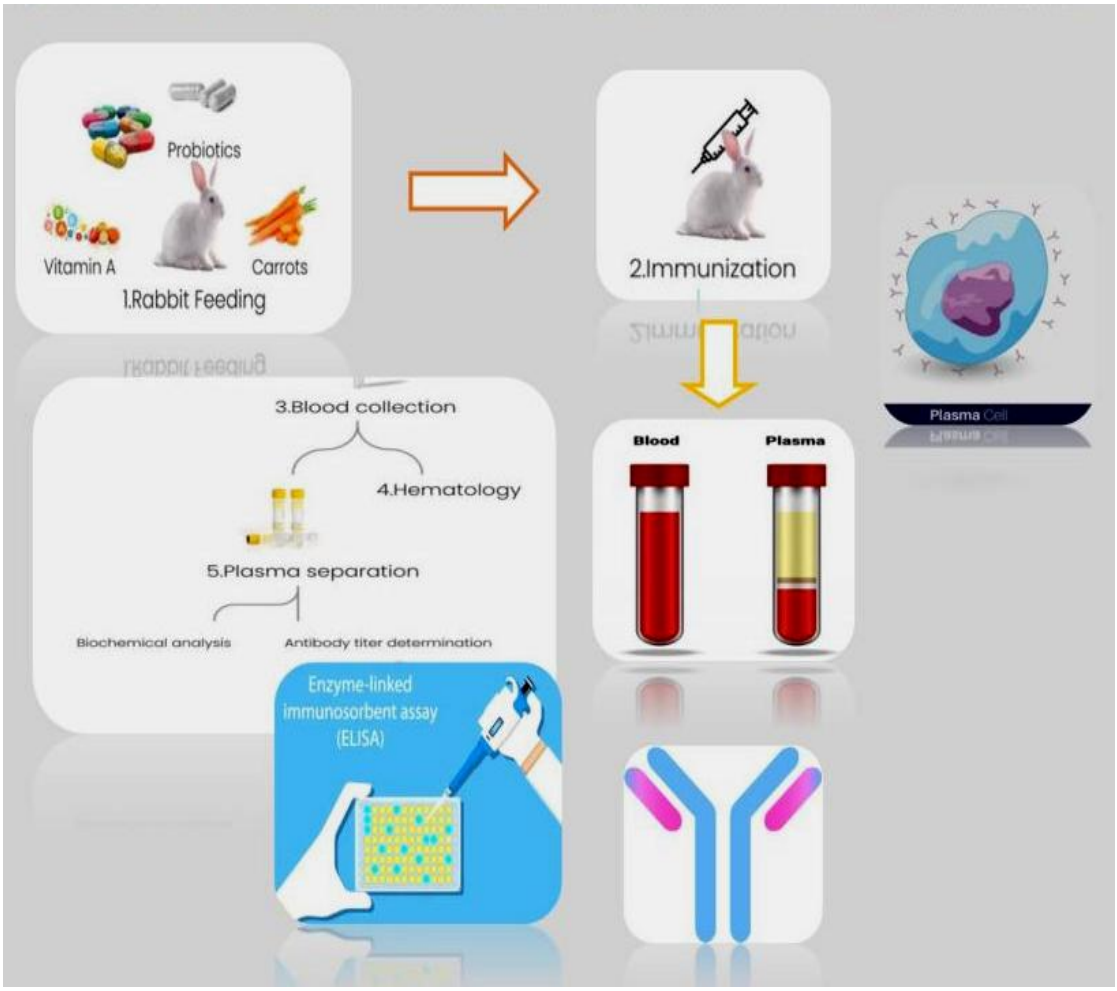


Figure 1.1: Experimental design and upstream process flow chart for production of poly-clonal antibodies against RV in NZW rabbits.

1-Animal feeding with probiotics and vitamin A, two weeks prior to start immunization, 2- primary immunization of rabbits, 3- blood sample collection and 4-antibody titer determination by ELISA kit. This study included 2-week probiotics and vitamin A feeding of animals prior to four doses primary rabies immunization of 54 New Zealand white rabbits, (n=6) in each group. Blood sampling was carried out at regular intervals (days 0,14,35) to check blood parameters and in process antibody titer determination with Platellia II^{TM}ELISA kit for rabies.

CHAPTER 01

INTRODUCTION & LITERATURE REVIEW

1.1 Rabies, A neglected tropical disease (NTD) : the global scenario

Rabies is a serious global health issue, mostly in tropical areas and subtropical areas of African and Asian countries, killing around 59,000 poor victims around the globe. The sickness is brought about by an infectious agent of the family Lyssavirus having 100 % mortality due to fatal encephalitis in host including humans. Once the deadly symptoms of rabies appear, the victim dies within one week. Therefore, several rabies prevention and control programs are actively working around the globe to control this deadly disease at the earliest (Abedi et al. 2022). Rabies is one of the most significant neglected tropical diseases (NTDs) in terms of prevalence and severity, and its clinical features have readily been used as a foundation for its advocacy. Of course, it also happens outside the tropics and in developed countries, although more than 70 % occur only in Asian and African developing countries.

Anderson and Shwiff (2015) estimated the economic impacts of canine rabies in Latin America, Africa and Asia. The article presents updated estimates of human mortality due to rabies in Africa and Asia, which are 31,329 and 38,090 respectively. The number of rabies diagnostic tests performed each year in Africa and Asia are 5,300 and 16,500 respectively. The expected total annual cost of canine rabies is approximately $124.2 billion with human mortality risk accounting for most of the total cost ($123.4 billion or 99.3% of all costs). Human mortality in Asia accounts for about 55% of the total human mortality costs, while Africa accounts for nearly all the remainder. Excluding the cost of human mortality risk, monetary expenses associated with canine rabies total $832 million, with about 82% occurring in Asia.

Figure 1.2 shows that both rural and urban populations are equally vulnerable due to dog bites, and the cycle of poverty is compounded by rabies mortality. The mortality associated with rabies are quite high in Low- and middle-income countries (LMICs) of Asia i.e. approximately 30,000 annually with India at the highest fatality.

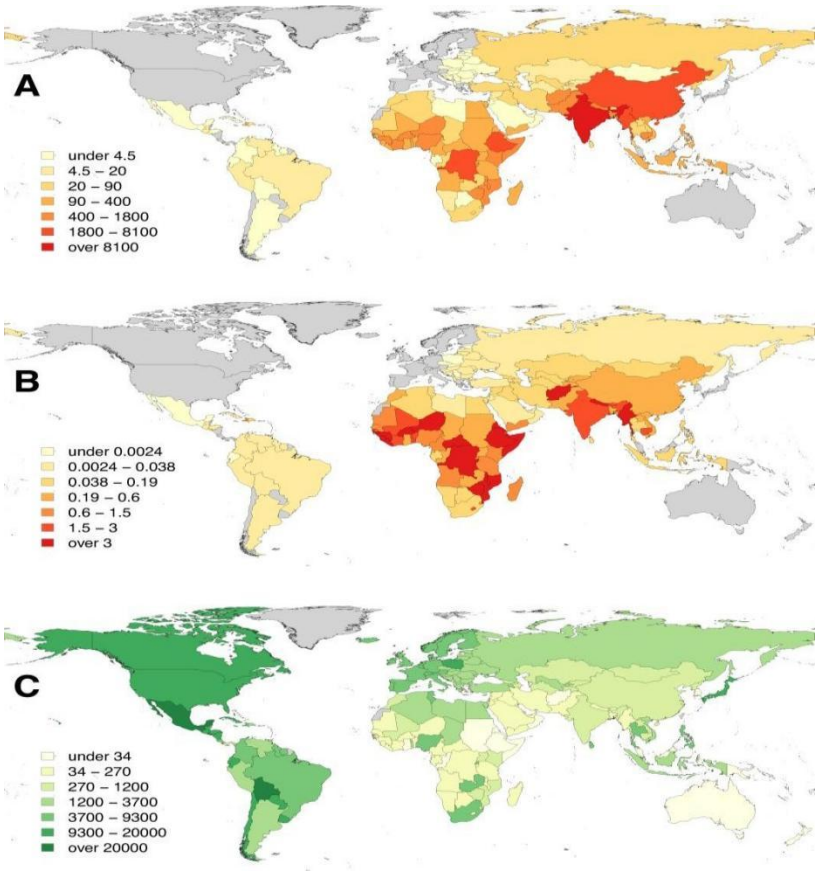


Figure 1.2 The global rabies burden distribution A) rabies-related human fatalities, B) average per capita mortality rates (per 100,000 individuals), and C) spending on dog vaccine (per 100,000 people). Areas colored in dark are unaffected by canines' rabies (Hampson et al, 2015).

In 2023, a comprehensive study was conducted to analyze the global burden of rabies across 204 countries and territories between 1990 and 2019 (Gan et al., 2023). The research team meticulously gathered epidemiological data and disease burden statistics for three decades, conducting an extensive examination. According to the study's findings, by 2019, there were approximately 14,075.51 (95% uncertainty interval:6124.33-21,618.11) incident cases of rabies worldwide with fatalities at13,743.44(95% uncertainty interval:6019.13-17,938.53). Both estimates showed a decrease from those recorded in1990 indicating progress over time in reducing global disease burdens caused by Rabies virus infection. Furthermore, the decline trend in disease burdens associated with Rabies virus infection globally was closely linked to the level of socio-demographic index (Figure 1.2).

Unfortunately, many victims of rabies don't get access to treatment due to vaccine shortages or high-cost treatment in LMICs. Many countries throughout Asia and Africa have successfully adopted intradermal (ID) schedule of vaccine administration that requires less vaccine doses than intramuscular injections. Countries including India, Pakistan, Nepal, Bangladesh, Philippines, Sri Lanka, Madagascar and the United Republic of Tanzania have therefore successfully shifted to adopt ID schedule of rabies immunization to manage shortage of vaccine in their countries (Ghai & Hemachudha, 2022; WHO, 2017).

India has the highest number of dog-mediated human rabies deaths in the world, yet rabies is not considered a public health priority and is not reported in India. The historical treatment of rabies in British India has received relatively less attention in Indian medical history literature (Radhakrishnan et al., 2020).

A study was conducted by Rimal et al., 2020 in Nepal to detect virus-neutralizing antibodies against rabies in vaccinated pet dogs. Blood samples were collected from 110 vaccinated pet dogs and tested using an indirect immune-enzymatic assay to detect rabies virus anti-glycoprotein antibodies. Of the total samples, 89.09% exceeded the required sero-conversion level, while 9.09% did not reach the sero-conversion level. The study unequivocally determined that vaccination stands as the most effective and efficient means of preventing and controlling the spread of rabies. This finding is significant not only for public health officials but also for individuals who seek to protect themselves and their loved ones from this deadly disease. By prioritizing vaccination efforts, communities can take a critical step toward minimizing the impact of rabies on both human and animal populations.

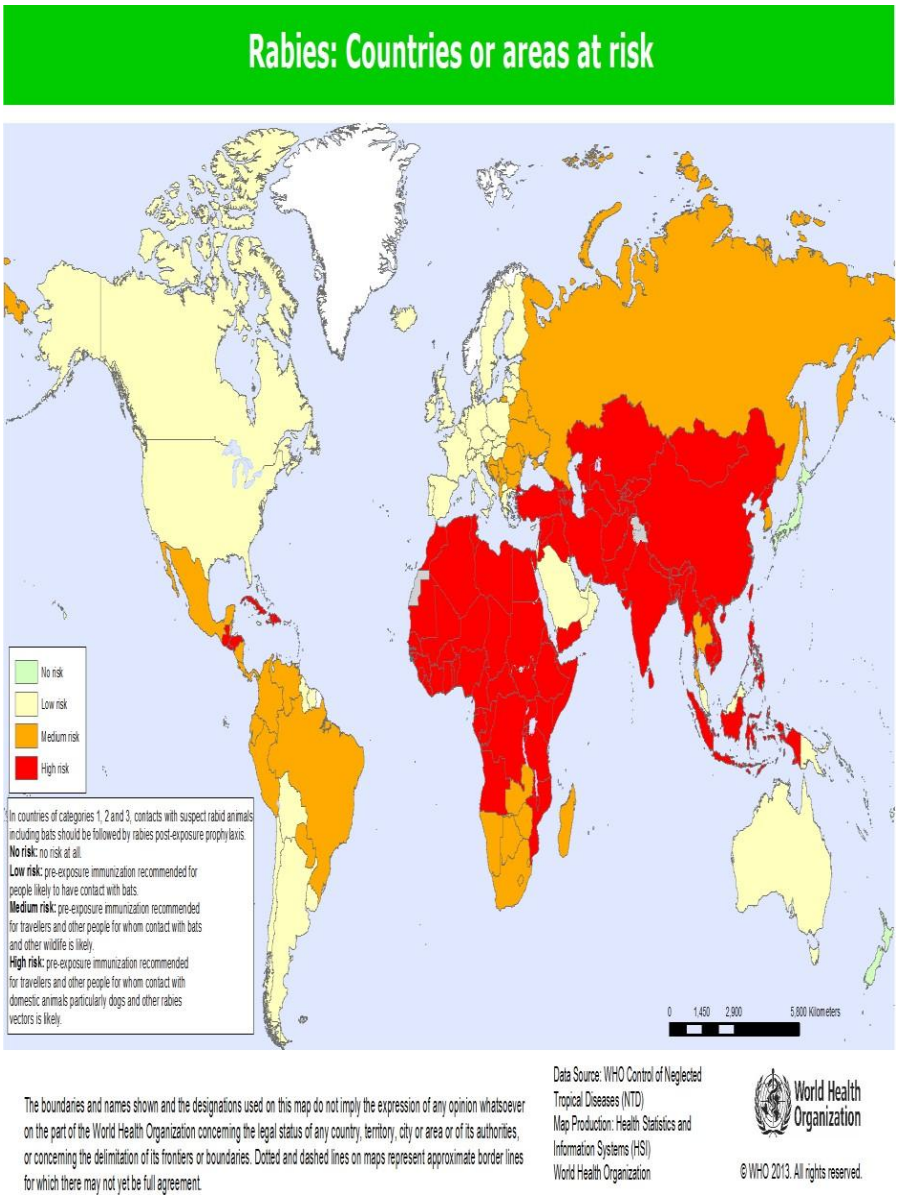


Figure1.3 Geographical distribution and global burden of dog-transmitted human rabies; high risk countries shaded red. Rabies is a fatal zoonotic disease and a neglected tropical pandemic globally. Various regions are highly affected by rabies. 70% of rabies cases are still reported from Asian and African continents (Adapted from WHO TRS 3rd report, 2017).

1.2 Rabies epidemiology in the context of Pakistan

Rabies continues to be a neglected illness in Pakistan, with fewer cases reported groups from private sector have taken the lead to start awareness about the disease and to

control the spread of rabies by taking various measures. However, these efforts are still insufficient to cater a large population of Pakistan (220 million) alone without active participation of health ministry of the country. Rabies puts a huge pressure on the country's health-care sector. Rabid dog bites are more common throughout the summer months of May to August. Men and children were more vulnerable to dog bites in general. While other countries of southeast Asia have successfully reduced the canine rabies by using various preventive tools such as mass awareness, vaccination programs etc. Rabies remains a serious public health issue in Pakistan due to various factors.

i) Free roaming of stray dogs in rural and urban areas.

ii) Dense population.

iii) Vaccine availability issues / Shortage of vaccine.

iv) High treatment cost

v) Lack of awareness about disease complication plus completion of vaccination treatment.

Pakistan is among the top five nations globally in terms of dog-mediated rabies prevalence, with an estimated annual fatality rate ranging from two thousand to five thousand individuals. Nevertheless, these estimates fail to provide a precise depiction of the actual burden due to inadequate and unreliable rabies data, inadequate preventive and therapeutic knowledge, non-existent monitoring programs targeted at this malady, insufficient testing centers for rabid cases and lack of control or mass vaccination strategies for dogs as implemented by countries with robust preventive measures. Most instances remain under-reported; victims either do not seek proper treatment timely or are unaware of the associated risks linked with this disease. The overall medical expenses incurred because of dog bites are exorbitant and beyond the means of ordinary people, thereby further exacerbating financial strains on already disadvantaged high-risk communities. (Daumerie et al., 2010; Gongal and Wright 2011; Savioli and Daumerie 2013; Siddiqui, et al., 2021).

Estimating the morbidity, disability, and mortality rates of neglected tropical diseases (NTDs), particularly rabies, poses a significant challenge. The highest incidence of dog bites occurs in low- to middle-income communities with limited access to healthcare facilities. Despite its status as the oldest recorded and deadliest infectious disease, this zoonotic ailment remains overlooked and endemic in Pakistan's rural and urban areas with high prevalence for canine attacks. Unfortunately, there is an

insufficient amount of epidemiological data on this disease, coupled with a lack of proper control programs at the national level in Pakistan. In 2016, statistics from the National Health Management Information System (NHMIS) reported around 97,000 cases of dog bites across three major cities in Pakistan - Karachi accounting for approximately 59.7%, Peshawar contributing 13% while Hyderabad registering 11% (Ahmad et al., 2016). Nevertheless, timely intervention through adequate prevention measures such as controlling canine rabies coupled with prompt treatment can save human lives (Kumar & Bakhru,2022).

1.3 Rabies Pre (PrEP) and Post exposure prophylaxis (PEP)

The rabies is 100 % fatal but fortunately a vaccine preventable disease. Vaccination for Rabies is advised in two distinct circumstances: as a preventative measure before exposure and after exposure. generally recommended for veterinarians, forest rangers, travelers who are prone to be infected with rabid and have a high risk of getting it or following contact with wildlife known or suspected to be rabid (PEP) (Masters, 2016). After the exposure to rabies virus all wounds from bites and scrapes ought to be thoroughly washed with water and soap so as quickly. The effective PEP, including administering multiple doses of vaccine and of RIG, is the only treatment for category II and III dog bite cases as described by WHO. Different regimens are being used for rabies immunization in humans or animals, including intramuscular (IM) regimen with 5 vaccine doses or intradermal (ID) regimen with 3 to 4 vaccine shots at more than one site. Thai-red cross (TRC) is an ID regimen that has gained more attention due to several advantages over the IM route (Figure 1.4; Rahman & Isloor, 2018; WHO 2018).

World health organization recommends a one week, 2 site intradermal PEP schedule with 0.1mL of vaccine injected on days 0, 3 and 7. However if the patient has already been immunized against rabies, then this can be reduced to a 1-site intradermal injection of 0.1mL on days 0 and 3 The reduction of the PEP intradermal schedule to one week, reduces the cases of required clinic visits by patients and enhances compliance of patient and further improves cost effectiveness. However, any of WHO-recommended PEP Individuals with biting infections in categories two and three can utilize this treatment plan. In third-degree exposures, rabid antibody should be delivered in addition to the vaccine (Figure 1.5; WHO, 2018a, 2018b).

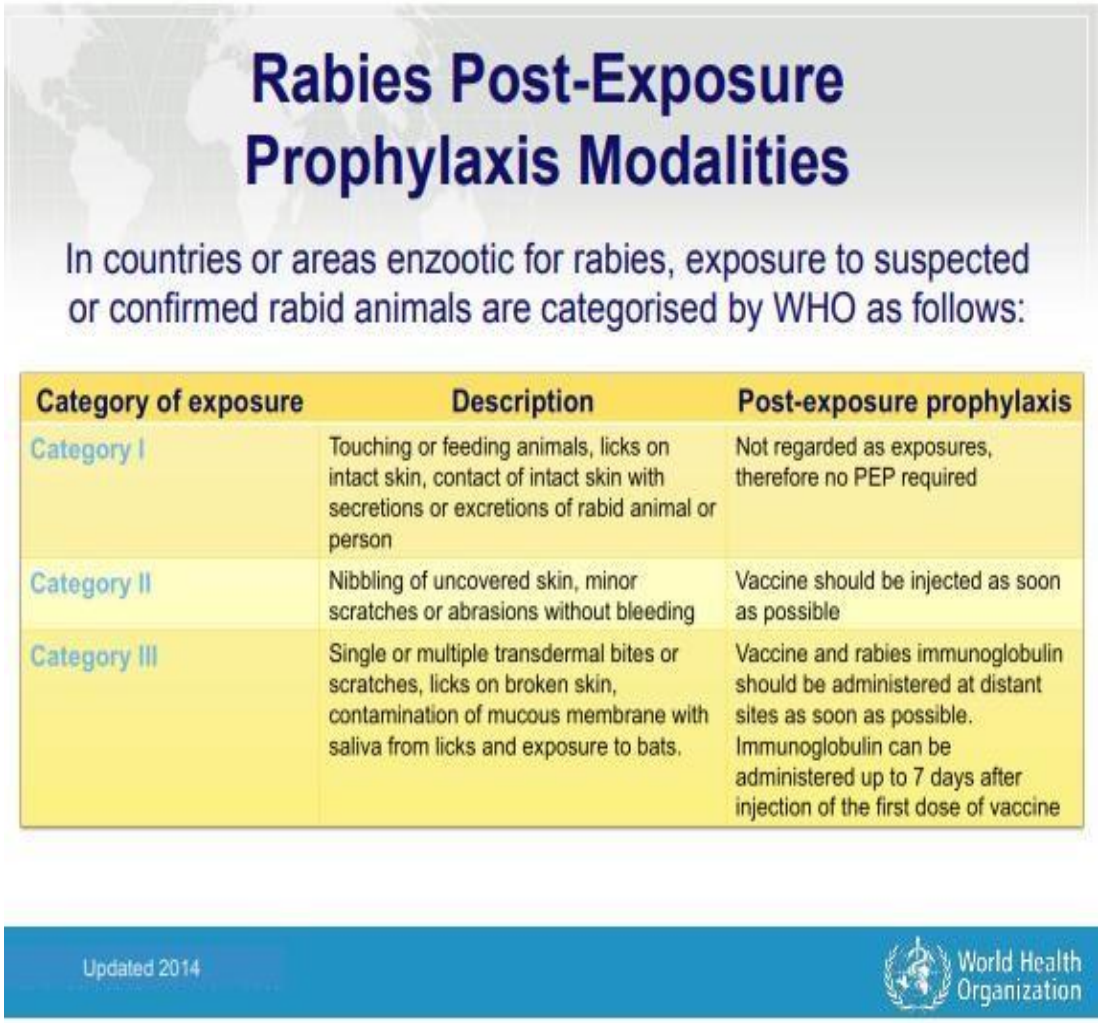


Figure1.4 WHO recommended schedule for category I, II and III exposures.

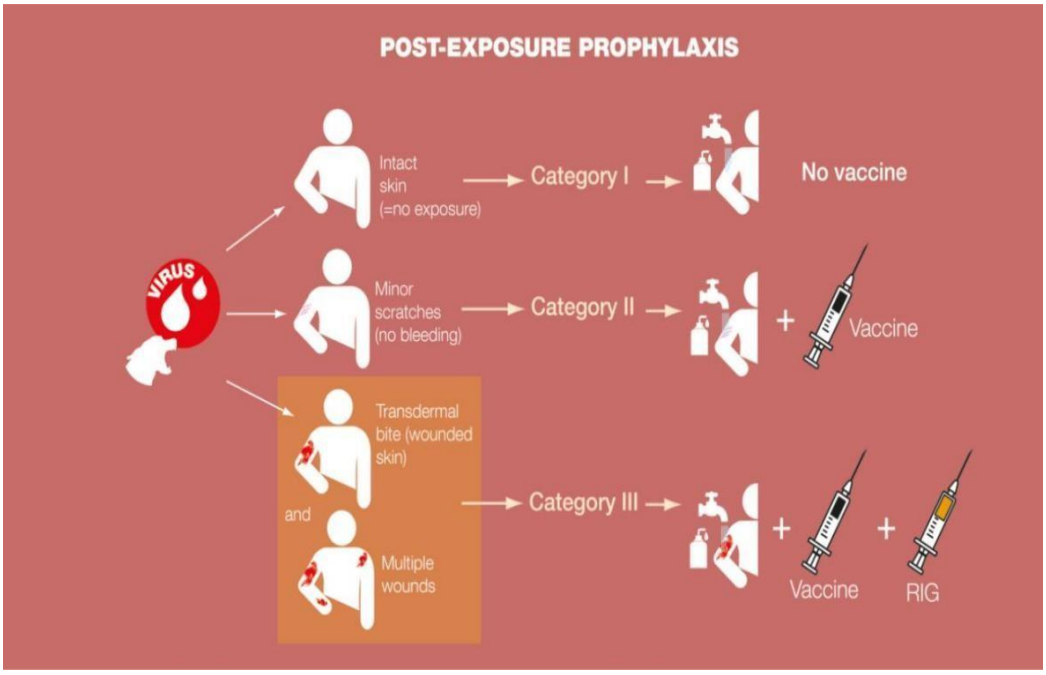


Figure 1.5 Post exposure prophylaxis for rabies recommended by WHO.

CDC suggests PEP consists of a dose of genuine human rabies immunoglobulin (HRIG) and rabies vaccine administered on the same day as rabies virus exposure, as well as vaccination with a culture of cells rabid vaccine administered on days zero three, seven, and fourteen. For those who weren't previously immunized against a disease called PEP with an injection of rabies vaccination and RIGs is indicated for both bites and non-bite circumstances, regardless of the period between exposure and medical therapy beginning. However, sufferers who have already been infected or are getting prior to being exposed rabies immunization ought to receive just the vaccine (CDC, 2022).

Adverse responses to the rabies vaccine and immunoglobulins are uncommon. Current cell-based vaccinations have fewer side effects as prior neurological cell vaccinations. However, modest local responses to the rabies vaccine are being observed, involving soreness, swelling, inflammation, or stinging at the location of the injection. It is possible to have minor discomfort and a mild fever after receiving rabid immunological immunoglobulin. Headache, feeling sick, stomach pain, cramps in the muscles, and disorientation are uncommon concerns.

1.3.1 WHO guidelines for rabies immunization using the intradermal (ID) pathway.

The WHO promotes the use of intradermal (ID) administration of modern cell culture rabies vaccines (with a potency of >2.5IU for every intramuscular treatment) for effective immune response. Numerous investigations showed that ID protocols offer an equally secure and efficient option to intravenous vaccine delivery, and it substantially decreases vaccine dose utilized by sixty to eighty percent, is less costly and has potential to rationalize vaccine shortage in developing countries. Moreover, cost effectiveness modelling also shows that intradermal schedules are cost effective when compared with intramuscular vaccination schedules. Therefore, ID regimen can be better option for immunization of hosts (humans or animals). ID schedule requires1-2 vaccine vials for finishing a full program. In rabies-endemic countries, in these neighborhoods, when the median daily earnings per individual is in dollars US1-2, this can place a huge financial burden and would cost up to US$100 on poor patients for treatment (Léchenne, M.et al. 2021, Thumbi et al., 2022).

1.3.2 Vaccines and their role in disease prevention

The history of vaccinations is said to begin in the latter part of the nineteenth century with Edward Jenner's invention of a smallpox vaccine, which is the first viral disease eliminated by this remarkable discovery in the medical sciences. More vaccines were produced against deadly disease since then and several improvements are still going on to face emerging challenges like recent pandemic of “severe acute respiratory syndrome” (COVID-19). Hence the administrations of vaccinations encompass one of the most illuminating examples of medical advancement of the well-being of humans and their life spam.

Therefore, vaccination constitutes one of the most essential preventive measures, providing safeguards against lethal infectious illnesses through immunization of children under the threshold of five. In accordance with global estimates, the use of six common vaccines for kids through various immunization programs protects over five million lives each year from horrible illnesses such as malaria, hepatitis B virus,

haemophilus strains of influenza b, polio, tetanus, and rota-virus. Vaccines are defined as "biological preparations that provide active immunity against a specific disease." On a yearly basis, many individuals die from vaccine-preventable illnesses such as bronchitis and diarrhoea (Walker et al., 2013).

Two types of vaccines were previously used for treatment of rabies, nerve tissue and cell culture-based vaccines. WHO advises that spinal nerve tissue vaccinations be replaced promptly with safer and more efficient vaccinations made using cell cultivation methods. Cell-cultured vaccines for rabies (CCRVs) of several sorts are accessible on the international market, comprising human cells that are diploid, Vero cells, and chick embryonic cellular-based vaccinations. Cell culture rabies vaccine (CCRV) has been successfully used since 19th century for protection against deadly zoonotic disease Rabies. However, Rabies prevention continues a problem in effective control of diseases in countries that are developing owing to vaccine shortages as well as financial issues related to high comprehensive therapy costs. The cultured cell rabies vaccine (CCRV) has been one of the most efficient human-to-human rabies vaccines ever discovered, and it has altered the way rabies preventive programs are implemented. Rabies vaccine (an disabled viral vaccine) is often produced using either individuals diploid cell cultivation (HDCV) or pure chick embryonic cell technology (PCEC).The most widely available (CCRVs) include human diploid cells, Vero cells; and chick embryo cells-based vaccines (Desai, 2016; Rupprecht et al., 2018). Cell culture rabies vaccines (CCRVs) initially licensed in the 1980s as an alternate to sheep brain Semple type rabies vaccine (SRV), which had many neurological and safety issues and was less immunogenic, cause of post-vaccination encephalomyelitis (PVE) and also required 14 subcutaneous injections to get complete protection (Briggs, et al., 2013; Hajela et al., 2020; Hicks et al., 2012; Masters, 2016).

1.3.3. Immune response & Humoral response to vaccines

Vaccines comprise of the agents that look like pathogenic microorganism these are made by either weakening or killing the pathogenic microorganisms or from their chemicals or one of its surface antigens can cause this. A vaccination is a pharmacological treatment that boosts resistance to a specific illness. Vaccination are

common biological substances that are delivered into the body to stimulate immune system reactions. The body's immune system is activated in such a way that stored antibody are created without really causing sickness. A vaccination often comprises an agent that is similar to a bacterium that causes illness and is frequently manufactured from weakened or destroyed forms of the microbes, its toxins, or one of the outermost proteins. The agent stimulates the immune system, causing it to recognize the chemical as foreign, eliminate it, and "keep in mind" it for future confrontations.

Vaccines provide protection by generating effector systems (cells or chemicals) that are capable of rapidly regulating disease replication or eliminating harmful pathogen elements. Vaccine-induced immunological receptors resemble antigens that are generated by B cells and have the ability of precisely attaching to a poison or virus (Siegrist, 2008). Virus neutralizing antibodies (VNA) provide specific defense against invading viral agents. Neutralizing antibodies bind to attacking virus and prevent it to cause infection. These neutralizing antibodies either bind to a viral capsid thus by inhibiting un-coating of the viral genome or they block interactions with the receptor. Only a small group of many antibodies that bind to a virus are capable enough of neutralization. After an infection or by vaccination, the host body takes some time to produce highly effective VAN and also persevere memory to protect against future encounters with the same infectious agent (Pollard & Bijker, 2021).

1.3.4 Adjuvants for enhancement of humoral response of vaccines

Thus far, the focus has concentrated on discovering new vaccine antigens or additives that elicit better protection responses from the immune system. Vaccine sensitivity can be impacted by a variety of circumstances, from antigen and adjuvant selection in vaccine composition to host factors age, microbiota, and genetics. **“Adjuvants”** are the compounds can boost the "dedicated immunological reaction" to vaccination proteins. There may be a problem with vaccine efficacy in current vaccines created with purely transgenic or manufactured targets because they are not as immunological than traditional either live or dead whole animal vaccinations. Supplements are typically included in vaccine formulations to improve antibodies, lower the amount of protein or the variety of vaccination injections necessary for preventive immunization,

and improve vaccine efficacy in immune compromised individuals, young children, and elderly population, or as antigen delivery systems (García & De Sanctis, 2014). Since 1920s Alum remains the most utilized a human-approved additive used globally, despite many side effects such as local swellings, pain etc. While alum can boost excellent antibody manufacturing, (Th2) response, its ability induce cellular (Th1) immune responses is not very efficient which is important for defense against many pathogens. This has resulted in an urgent demand to enhance as well as more potent adjuvants for the application with mucosal-delivered vaccines, DNA vaccination, cancer, and autoimmunity vaccines as a tool for immune enhancement.

Effective adjuvants may aid in the development of successful contemporary vaccinations versus emerging infectious diseases. Unfortunately, many product development attempts with adjuvants have not been successful due to stability issues, lack of effectiveness, safety concerns and manufacturing problems.

1.3.5. Probiotics, their health benefits and role as potential vaccine adjuvants

Probiotics are generally recognized as safe bacteria and are defined as "living microbes that provide beneficial health effects whenever delivered in sufficient quantities." They are defined as "biological reaction enhancers which serve as supplements for increasing vaccine-specific protection and have a chance to encourage and regulate central and gastrointestinal defensive responses." Probiotics research has gained considerable attention since last two decade due to their well described health benefits on the hosts including humans. Probiotics are usually termed as nutritional supplements or functional foods that play a major role to change, adjust, and reinstall the already present intestinal bacteria of recipient. lactic acid bacteria, *Bifidobacterium*, *S. boulardii*, and *B. coagulans* are the most regularly utilised antibiotics (Pandey et al., 2015).To date, the focus has been on discovering new vaccine antigens or supplements that elicit better preventive responses from the immune system. Researchers are very interested in the use of microorganisms as stimulating components in immunization. Due to their enhanced epidemiological comprehension and the readily accessible of numerous approaches to augment pre-existing vaccine specific-immune answers, the concept of using beneficial bacteria as a fresh gastric augmentation has sparked a lot of

attention in recent years. Specific varieties of microorganisms have been proven in experimental animals to have modulatory implications for gastrointestinal and systemically immune reactions, and this is the foundation behind human vaccination trials (Peroni and Morelli, 2021; Myneni et al., 2020).

The increasing demand for functional foods has spurred researchers to explore the development of nutritious dairy products using well-characterized strains of bacteria. Locally isolated strains of *Lactobacillus fermentum* derived from milk sourced from Bubalus bubalis (Nilli Ravi buffalo) and evaluated their potential as probiotics in fermented milk-based food products. The study aimed to find the most promising probiotic strains for milk fermentation that can improve the production of functional foods. Fifteen Lactobacillus strains were initially isolated, a, two Lactobacillus strains (NMCC-14 and NMCC-17) isolated from buffalo milk showed the best probiotic potential and can be used for probiotic product preparation. The study provided a cost- effective means for exploring LAB for the development of functional dairy products (Abid et al., 2022b).

Recent studies with COVID-19 have shown that probiotic supplements with antibacterial and immuno-modulatory abilities are being promoted as pharmacological supplements for illness treatment and virus prevention. During current COVID-19 vaccine development, several vaccine platforms were used therefore it is encouraging to use probiotic supplementation as adjuvants in boosting immunity and in enhancing vaccine-specific responses (Cuzzubbo et al., 2021). The implementation of probiotics has been ascertained to be highly advantageous in enhancing immunity. Lactobacillus-derived probiotics are advised for the prevention of influenza-like viral infections.

Probiotics play a pivotal role in the immune response by regulating immune cells within the mucosa and epithelial cells of the intestines. Although the precise mechanism of action of probiotics is not fully understood yet, but several animal and human studies have proven immuno-modulatory effects involving both the humoral and cellular components of the host's immune system (Berggren et al., 2011). A healthy gut means a healthy body. Antibiotics help boost your immune system through the combination of harmless and nutritious bacteria. They aid in fighting against harmful bacteria and

aid cells as well as tissues in their vital process of recovery. Best Supplements can also help with the generation of good bacteria and the eradication of pathogenic microbes that contaminated our circulatory systems.

Thus, microorganisms offer an extremely low-cost way of management to increase vaccine efficacy and duration of protection. Combining immunization with mucous immunological boosters and microorganisms may increase both innate and adaptive epithelial immune system responses were observed. Certain varieties of microorganisms are being proven in experiments with animals to possess modulatory consequences for gastrointestinal and peripheral immunity, and this is the foundation of prospective human vaccination trials. Due to our enhanced epidemiological awareness and the affordability of numerous approaches to augment previous vaccine specific-immune answers, the concept of using probiotic microbes as an innovative mucosal augment has sparked a lot of attention in the last two decades. Despite the exact mechanism of action of probiotics is unknown, various human and animal experiments have demonstrated immuno-modulatory responses implicating both humorous topics and cell-based parts of the host's defense mechanism.

Saccharomyces cerevisiae var. boulardii is a probiotic yeast that is effective in treating various gastrointestinal diseases. It can protect the normal microbiota of the human gut and prevent different diarrheal infections (Abid et al., 2022a). Friendly yeast and bacteria have and create many effector compounds that interact with their host cells to generate the outcomes that are observed. The effects of effector chemicals can influence the way the host's intestinal membrane barrier operates, the immunity of the host, the host's microbiota in the intestines, the host's physiological reactions, and the host's neurological systems (Figure. 1.5).

These simple vaccine adjuvant approaches using probiotics and dietary interventions has many advantages and may have great benefits of safe and effective immunization practices in underdeveloped locations and could eliminate the need for several immunization doses, particularly in kids as well as elderly people.

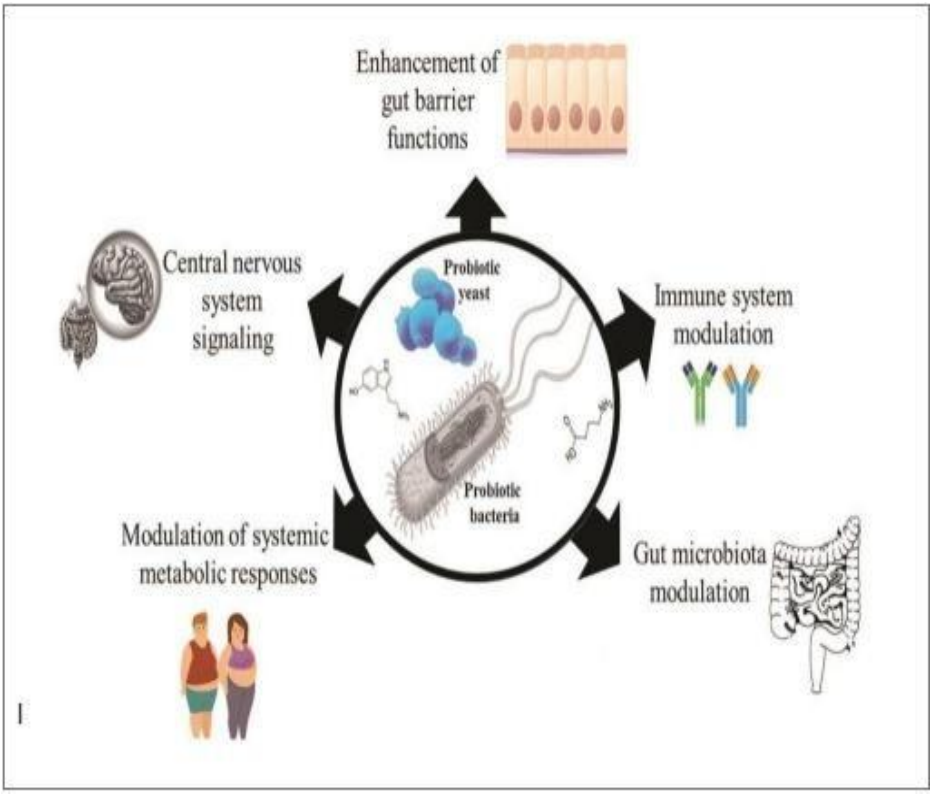


Figure1.6 Effect of different compounds produced by yeast and bacteria on different systems of host (extracted from Daliri et al., 2021).

1.3.6. Mechanism of interaction of probiotics with host /gut Microbiota

A person's gastrointestinal is host of trillions of microorganisms known as the microbiota of the gut, which play an important part in their well-being including their preservation of a healthy digestive immune system.

The human gut is a dynamic environment for several immunological interactions. The gut immune system responds to the pathogens entering the body through digestive system and hence gut microbiota has a significant role in protection against these intruders. Host mucosal immune system involve complex interactions between immune cells, epithelium, and gut microbiota of host. Intestinal epithelial cells (IEC's) play the very important role in host-microbial interactions and tissue homeostasis of mammalian gastrointestinal tract. Hence, they are the mediators in establishment of a balanced environment (Okumura & Takeda, 2017).

There are several possible mechanisms by which microbiome could regulate the performance and susceptibility of vaccination as shown by Figure 1.7 (Lynn et al., 2022) and include:

a. Immuno-modulatory substances generated by intestinal microbes, including flagellum and a protein called have been demonstrated in animal studies to influence vaccine replies by acting as organic supplements observed by receptors that recognize patterns (PRRs) with the value as toll-like receptors, also known as TLRs, and NOD2, which are conveyed by cells that present antigens. Other immune-regulating chemicals, such as lipo-polysaccharide, may likewise affect reactivity in the same way. PRRs produced by T and B cells may also physically perceive these compounds.
b. Dendritic cells, also known as DCs, play an important role in vaccine-induced immune reactions by exposing vaccine antigens to T lymphocytes and emitting immune-modulating cytokines. The microbial community influences the generation of interferon of the type I by plasmacytoid DCs (pDCs), which in turn educate ordinary DCs (cDCs) into a certain biochemical and epigenomic condition that promotes T cell priming.
c. Immunomodulatory substances generated by the microbes, like shorter-chain fatty acids (SCFAs), can improve B cell energy expenditure to meet the energy requirements of creating antibodies and may boost the activity of mutations that govern the plasma, cell division and class shifting, which could influence vaccination replies.
d. Growing evidence suggests that microorganisms may contain targets that are cross-reactivity with pathogen- or vaccine-encoded epitopes. The existence of interfering B or T cells could possibly modify vaccination outcomes.

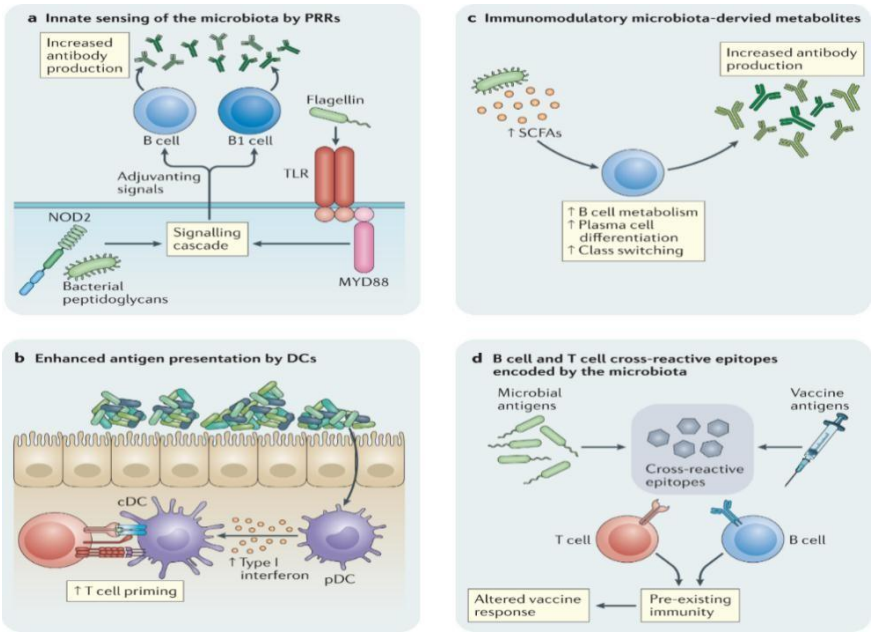


Figure 1.7 Potential mechanisms by which the microbiome could regulate vaccination susceptibility and performance (Lynn et al., 2022).

Many activator chemicals are produced by probiotic organisms, which communicate with the human host to drive their observable implications, such as hosting gut mucosal barrier operations, regulating host immune system functions and intestinal microbiota, the host's metabolism reactions, and affecting the central nervous system. Probiotics use biochemical signaling to connect with their host cells. As a result, the microbiota of the intestines is often regarded as an appealing target for improving vaccine effectiveness in populations that are at risk in nations with low or middle incomes (LMIC). Supplements to the diet, such as pre-biotics, or and the probiotics, offer a variety of cost-efficient and scale-able strategies for modulating the microbiota in the gastrointestinal tract and achieving sought successful immunization effects (Lynn et al., 2022).

The gut microbiome makeup varies greatly between people and across populations of different geographical regions. Moreover, this variability can be observed from early childhood to old age, hence correlating with reduced vaccine immunogenicity with passage of time. Therefore, as an affordable and scale able treatment to regulate the

microbiota in one's the microbiota in the gastrointestinal tract is becoming a more appealing method to improve vaccine effectiveness in populations at greatest risk. Changing the microbiota using antibiotics that benefit bacteria opens the possibility of developing dietary or medicinal treatments to sustain health in the years to come. As a result, additional research is required in order to comprehend the fundamental functions of beneficial bacteria and intestinal microorganisms (Aziz et al., 2013).

1.3.7 Influence of supplements with vitamin A on health, immunity, and vaccine responses

Vitamin A have shown promising benefits when administered with many vaccines and have shown improved responses to the Measles vaccine and Tetanus Toxoid in children. However further detailed to comprehend the fundamental processes, research conducted by immune modulation by VAS (Penkert et al., 2019; WHO 2021). A study was conducted on forty healthy human volunteers receiving VAS with five intramuscular rabies vaccine doses over period of 30 days (Siddiqui et al., 2001) Similar studies from England and South Africa also reported reduction in measles related child morbidity due to VAS. Therefore, an immediate rise in the production of antibodies could be due to enhanced proliferation of lymphocytes (Penkert et al., 2019). The effect of folic acid supplements (VAS) on immunological responses is well established. Some of vitamins A's immune-modulating processes have been investigated identified in research studies and can be linked to the clinical effects of administration. Vitamin A fortification in young babies and kids has been consistently incorporated with routine immunization campaigns such as the Measles vaccine in multiple nations to boost immunological state and minimize the incidence of illness within toddlers (Aguayo & Baker 2005, WHO guidelines 2021). Similarly, the organic carrots are natural source of several vitamins and are rich in carotenoids which helps to boost our immunity and prevents from acquiring repeated infections. Although studies on health effects of organic products such as naturally grown carrots are still limited, yet it has been reported that organic carrots are superior to conventional carrots with several improved quality characteristics. Therefore, consumption of organic foods like organic carrots in diet reduces the risk of catching diseases thus by boosting host's immunity and preventing several infections (Roselli et al.2012; Rosales-Mendoza 2015 Eggersdorfe & Wyss 2018; Figure 1.8).

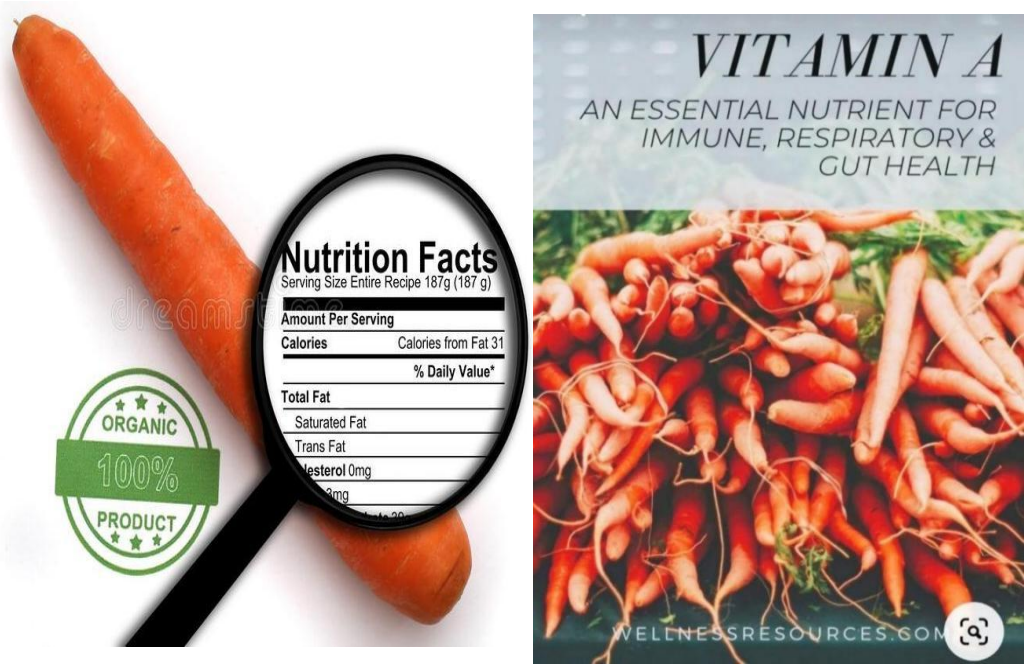


Figure 1.8 Vitamin A supplementation and its role in health and immunity.

Vitamin A supplements are available in the market and normally recommended for Vitamin A deficiency and with childhood vaccination programs. B) Carrots are the most important natural food source of Vitamin A, and rich in beta carotenoids, they are considered as cheap source of Vitamin A.

Carrots are highly nutritious due to their phytochemical content, including phenolic, vitamin C, carotenoids, and polyacetylenes. These compounds have immune-modulating and anti-inflammatory properties. Carrots are a good source of vitamins and minerals, including vitamin A, vitamin K, potassium, fiber, vitamin C, iron, and calcium. Carrot juice can increase vaccine effectiveness by promoting the activity of immune cells. Organic carrots have superior quality characteristics compared to conventional carrots. Adding carrots to the diet can boost immunity by interfering with immune system function. Vitamin C in organic carrots helps prevent infections (Johnson 2002; Roselli et al., 2012, Rosales- Mendoza & Tello-Olea 2015).

Several advantages of carotene have already been documented (Eggersdorfer and Wyss, 2018). Thus, as earlier indicated in several clinical investigations, probiotic, vitamin A, and carrots rich meals seemed to be linked with the regulation of host mammal microbiota in the gastrointestinal tract. Carrots are regarded as one of the most nutritious vegetables due to their substantial botanical compound's material, which includes phenolic, antioxidant ascorbic acid, carotenoids, and polyacetylenes, all of which have anti-inflammatory effects. The inclusion of vegetables in our diet improves our immunity by influencing several components of the immune system (Szydłowska & Sionek, 2022).

1.4 Vaccine efficacy related global challenges.

Several variables determine vaccine efficacy, notably host characteristics that differ among individuals. In overall, gender, age, inadequate food intake, intestinal microbiota variety and structure, and the relationship among personal microbiome makeup and vaccination reaction. Moreover, high occurrence of other co-morbidities is also connected with poor vaccine responses especially in elderly population. Vaccines

are reported to be less successful in countries with low or middle incomes than in nations with high incomes (Ozawa et al., 2012). Even after so many advancements, issues and worries about security exist in the discipline of vaccinology, potency and last but not the least effectiveness of vaccines. As a result, creating affordable vaccine techniques that can give a better immune system reaction with fewer injections and the greatest reach is crucial.

1.4.1 Rabies Immunoglobulins (RIGs) or rabies antiserum

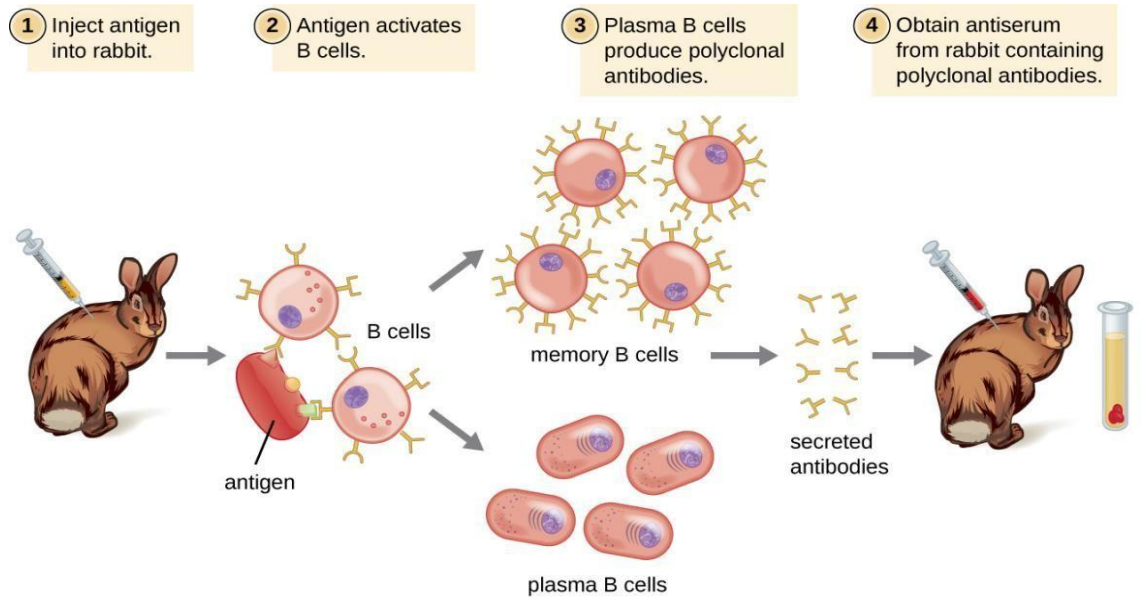
The lifesaving treatment for protection against rabies include immediate anti-rabies vaccination along with antiserum therapy. Mammalian and livestock (mostly equine) immune globulin are accessible for latent rabies immunization. In nations that are developing, the consumption of extremely refined equine immunoglobulin is which is healthier than the preceding generation's heterogeneous goods, provides an interim solution to cost difficulties as well as accessibility to more costly medicines i.e., the human derived immunoglobulin (HRIGs).

1.4.2 Production of poly-clonal antibody in animals; upstream strategy

Antiserum is made from hyper-immune animal serum or plasma containing poly-clonal immunoglobulins. The antiserum is effective in neutralizing rabies virus that is causative agent for life-threatening encephalitis. Rabbits, sheep, or horses are generally used for production of animal derived PAbs (Figure 1.9).

Different forms of commercially available anti-rabies immunoglobulin like horse-derived RIGs (ERIG) are being economically preferred in low income countries. In Pakistan four to five types of rabies anti-sera are available in the market that include (a) Human derived purified immunoglobulins (HRIGs) b) Equine derive immunoglobulins imported from other countries (ERIGs) and c) Locally produced animal derives purified immunoglobulins (ERIGs) which is produced in very less amount at NIH. However, the cost of imported products is much higher as compared to local product (Table 1.1,Figure 1.12).

The Queen Saovabha Memorial Institute (QMSI), Bangkok, Thailand, has developed effective strategies for manufacturing of therapeutic anti-rabies immunoglobulin for



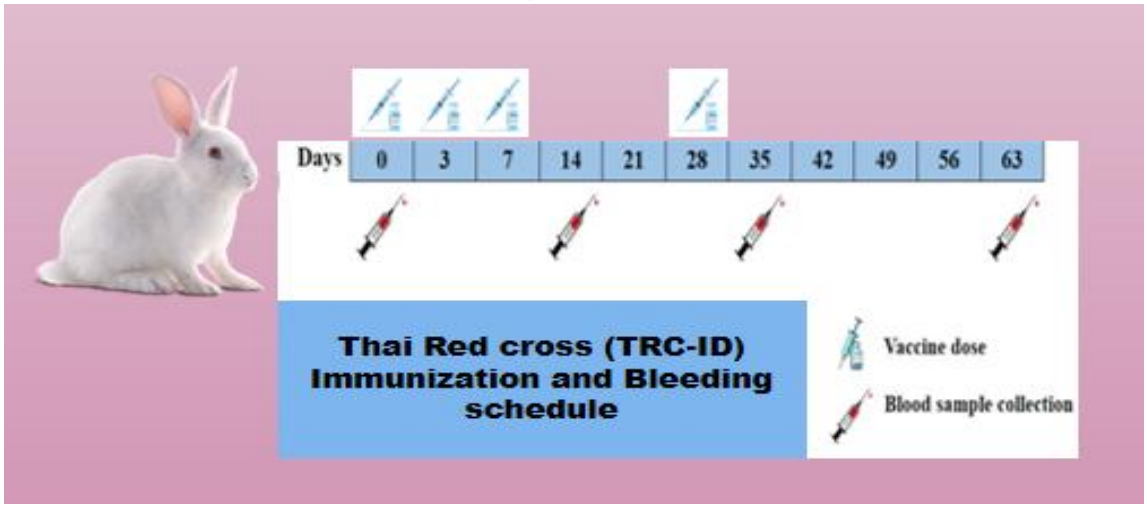


Figure 1.9 The method of extracting poly-clonal antibodies that are generated upon exposure to an epitope is depicted (a). Schematic representation of the 63 **a** y hyper-immunization protocol for poly-clonal antibody production in rabbits. TRC-ID regimen was selected for primary immunization of animals at day 0,3,7 & 28 (2-site injections) and 3 blood samples were taken for serology testing at days 0,14 and 35. Hyper-immunization was carried out after day 28 with weekly vaccine boosters till day 63. Finally, blood was collected at day 63 for further processing, purification and test analysis (b).

b

human use. Equines are usually immunized using a pure Vero cellular rabies (PVR) vaccine. The donor animals undergo a succession progressively higher doses of the vaccination. The medications are each administered through the skin into the left side of the head. The immunization phase is 105 consecutive days long, and the initial bleeding occurs fourteen weeks afterwards (Rupprecht et al., 2018).

1.4.3 Downstream Processes for transgenic rabies antiserum production.

By dint of the costly nature for human rabies immunoglobulins (HRIG), WHO still recommends heterogeneous immunoglobulins for the prevention of rabies in category III bite victims who were extensively contaminated by the influenza virus. Thus, urgent need to create more advanced methods of producing antiserum has become imperative, as the current conventional methods lead to the production of impure anti-sera that can trigger adverse reactions in patients. Therefore, it is crucial to replace conventional approaches with more innovative and internationally recognized methods of antiserum production to meet the country demand in Pakistan (Pagliusi et al., 2019). Animal derived anti-sera products often pose risk of causing sensitization and are removed more rapidly than HRIG. To combat this issue advance purification techniques may minimize the danger of blood exposure to maximize the immune system reaction while minimizing allergic components in the final product antiserum. While using these methods, it is good to follow the guidance of the World Health Organization's Expert Committee on Biomedical Standardization. Traditionally, immune globulin was purified from human blood by specific precipitation of amino acids with chilled ethanol, which has been modified for cleaning heterologous immune globulin and outlined in the third version of the laboratory processes for rabies (Rupprecht et al., 2018). Conventional procedures for the identification of animal-derived RIGs that can be used to clear horse blood or blood plasma technique using salt precipitation method of multi-step ammonium sulphate technique and more recently introduced caprylic acid technique for protein purification.

Sulphate of ammonium has been historically used to extract non-immunoglobulin protein (McKinney & Parkinson, 1987; Perosa et al., 1990; Steinbuch & Audran, 1969). However, in 1994 (Rojas et al., 1994) proposed a new and more effective technique for

anti-venom serum processing utilizing caprylic acid. This simple approach using single step processing resulted in a high recovery yield, enriched IgG preparation, minimal aggregation, and better neutralizing activity (Rojas et al., 1994). However, caprylic acid interacts insolubly with plasma proteins, leaving the majority of IgG in a soluble form. As a result, fractions of this caprylic acid are present in the final formulation of immunoglobulins, which needs to be removed. The researchers have used various purification techniques for the removal of caprylic acid. This approach may be helpful in regions with limited access to anti-sera products such as sub-Saharan Africa. The use of caprylic acid fractionation to produce high-quality whole IgG antivenoms from horse hyper-immune plasma is a simple, practical, and cost-effective process with the potential to alleviate Pakistan's chronic shortage of antiserum.

The classical method for producing anti-sera is still used in many developing countries, including Pakistan. This method based on salting out procedure uses Ammonium sulfate salt to purify immunoglobulins from hyper-immune plasma. Specific Immunoglobulins (IgGs) are separated from other non-specific proteins like Albumin, that may cause potential adverse effects when introduced into the human body. Different concentrations of the salt are added step wise with changing specific gravity and this method involves time consuming multi step processing followed by different stages of dialysis to get final purified IgGs (Figure 1.9).

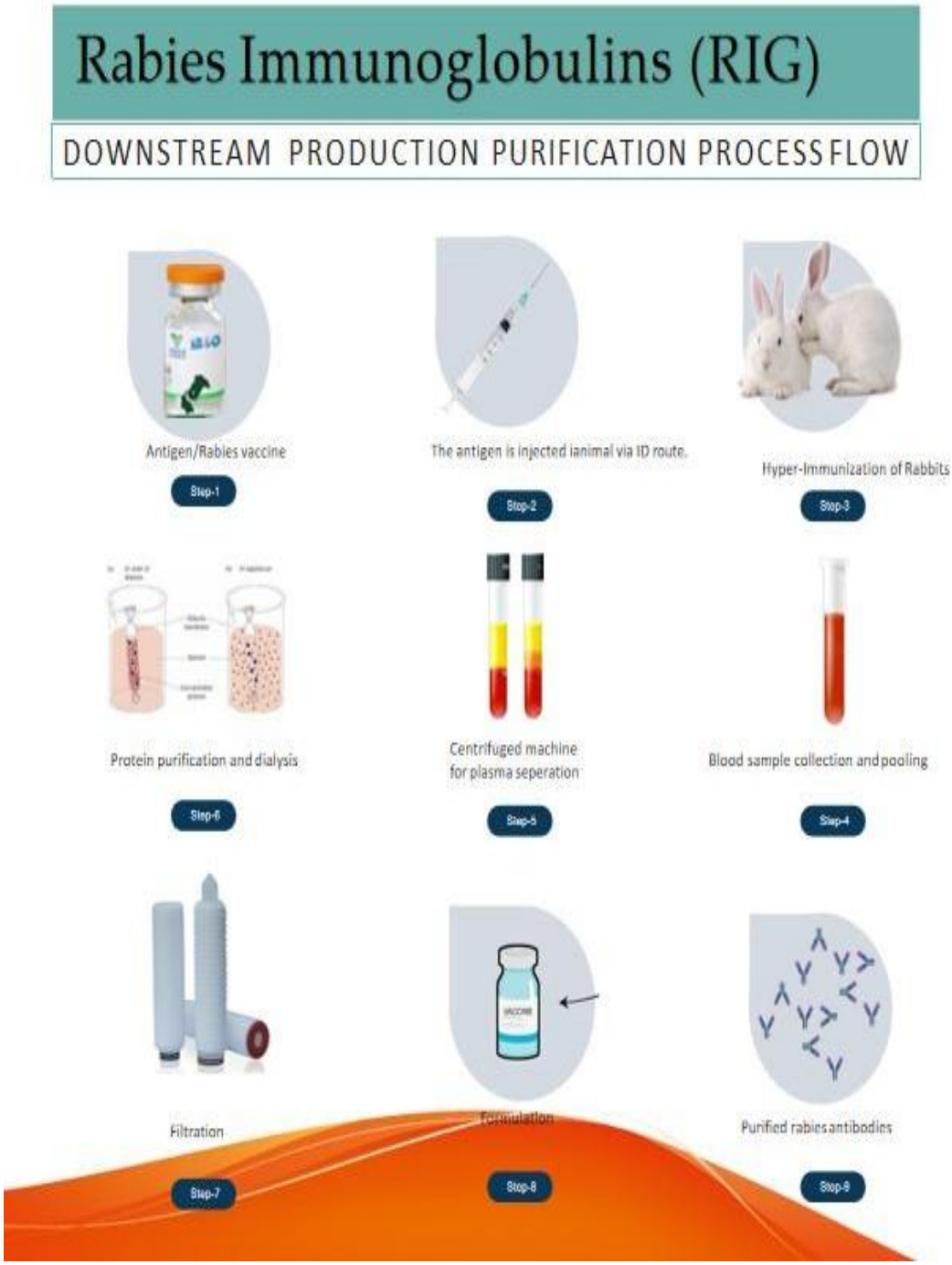


Figure 1.10 Traditional method for anti-serum production. Process flow chart for downstream processing for poly-clonal antibodies production against rabies vaccine in NZW rabbits for purified anti serum recovery.

1.4.4 Caprylic acid purification technique for antiserum production

A modified method for producing antiserum using caprylic acid has been developed by antivenom manufacturers in Costa Rica (Rojas et al., 1994) to solve the issue of non-specific plasma proteins in final product encountered with traditional methods. This approach may be helpful in regions with limited access to anti-sera products such as sub- Saharan Africa. The use of caprylic acid fractionation to produce high-quality whole IgG antivenoms from horse hyper-immune plasma is a simple, practical, and cost- effective process with the potential to alleviate Pakistan's chronic shortage of antiserum.

The manufacturing process non-IgG proteins from the plasma are precipitated in a solution of caprylic acid, resulting the purified IgG fraction in single step. This antiserum downstream production process involves a single precipitation stage and dialysis, utilizing HPLC analysis to monitor residual caprylic acid concentrations in the fractionated product. The comparison between caprylic acid-based method and previous method of ammonium sulphate fractionation showed that the caprylic acid-fractionated antivenom has a superior quality in all respects. This technology presents a promising solution to produce antiserum, offering both efficiency and simplicity. Additionally, its cost-effectiveness and practicality make it a highly attractive option for addressing the ongoing shortage of anti-sera in Pakistan and potentially in other regions facing similar challenges. By using this optimized protocol for antivenom production, healthcare professionals can have more access to lifesaving antiserum and provide better protection to those in need (WHO, 2007).

The antiserum manufactured by this optimized protocol is highly purified and potent, making it suitable for laboratory and commercial production. However, the method is manual and labor-intensive, and the removal of ammonium sulphate by dialysis takes one to two weeks, making it time-consuming and economically less feasible. Additionally, the process often precipitates other compounds, requiring Ion chromatography or sizing-exclusion chromatography are further cleansing procedures (Gutiérrez et al., 2010).

1.4.5 Dialysis against water for injection (W.F.I) for removal of chemicals

Dialysis against distilled water is the most ancient technique used to remove salts or chemicals from purified immunoglobulins, first reported by (Rojas et al., 1994). Dialysis against W.F.I or distilled water is performed by pouring the filtered final formulation in molecular porous membrane tubing (width:120 mm, diameter:76 mm used mostly for large IgGs molecules) for 48 hours is a fractionation method that keeps larger molecules in the retentate while washing smaller molecules across a membrane without affecting concentration. It can be applied to exchange buffers or eliminate salts. Organic acids or other tiny solvents or additives can be eliminated by this method. It is the most common and economical procedure for removing caprylic acid from the final product.

1.4.6 Tangential flow filtration

Tangential flow filtration (TFF) has been developed as an industrial-scale technique for purifying physiologically active proteins. It is a fast and efficient technique for separating and purifying bio molecules yielding approx. 99% pure product from hyper- immune serum. Its use can greatly benefit various areas of biology, including immunology, protein chemistry, molecular biology, biochemistry, and microbiology. A reliable and affordable technology that can process maximum bulk product from 10 to 400 Liters approximately. The operational cost of TFF can be lower than that of depth filtration by using membranes that can be reused more than 20 times (Reis et al., 1991). TFF used on industrial scale for bulk purification is shown in Figure 1.11.

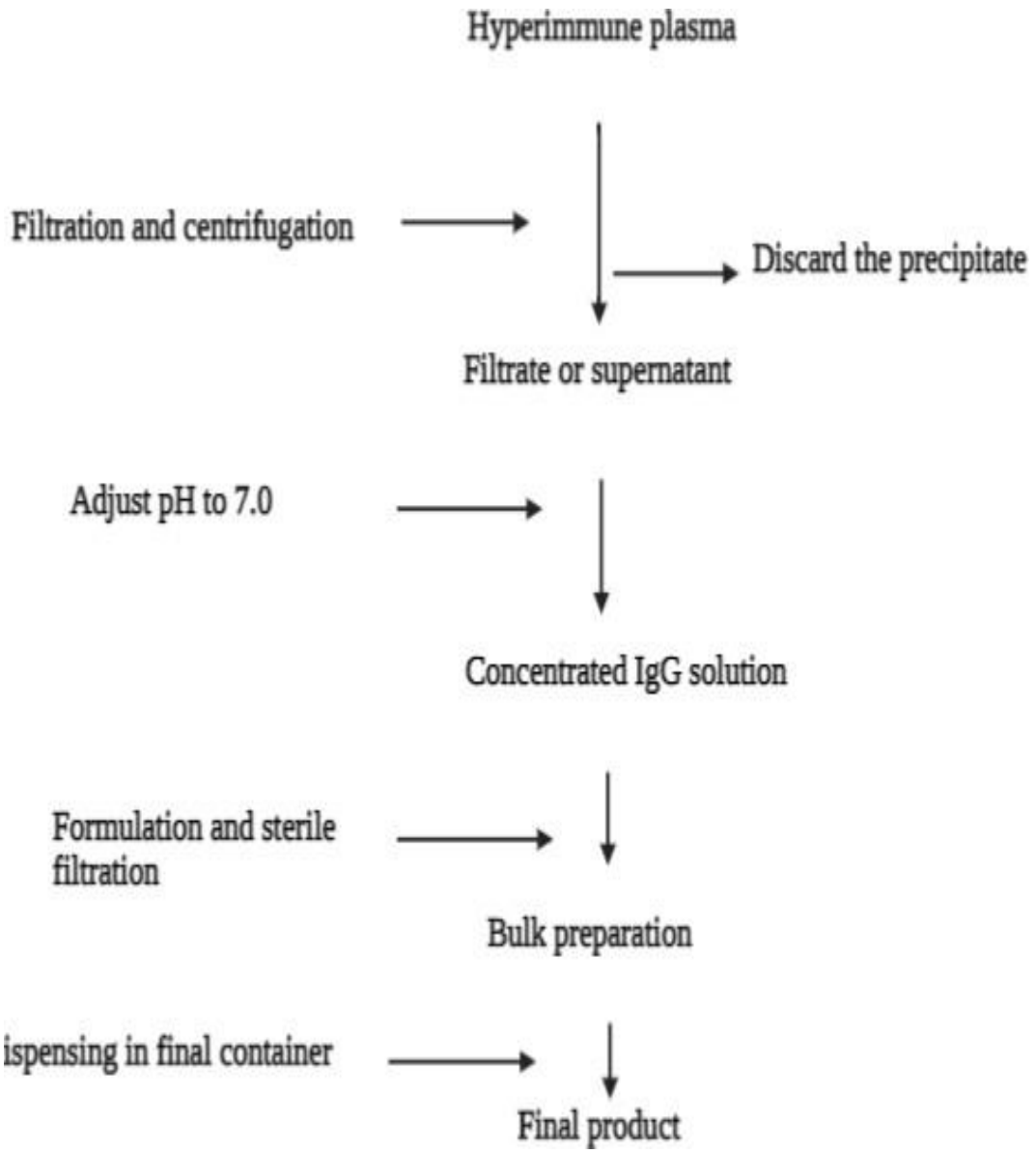


Figure 1.11 Caprylic acid-based technique for purification of plasma derived immunoglobulins. Flow chart explaining the step-wise procedure for purifying iimmunoglobulins starting from hyper-immune plasma processing to the final product formulation.

1.5 Global strategy for rabies control: Context and Rationale

The World Health Organization (WHO), the International Organization for the Health of Animals (OIE), the Food and Agriculture Organization (FAO), and the Global Alliance for Rabies Control (GARC) endorsed an international structure in 2015 for reducing rabies-related fatalities in humans with worldwide collaboration by the year 2030.The framework, is a global strategic plan involving 100 countries that require progressive implementation of programmatic changes to achieving the goal of nil rabies-related mortality by the year 2030. The main planned activities include dog vaccination reduce the risk and better control and also to increase access of high-risk population to safe and effective human vaccines and rabies antiserum (WHO, 2016).

1.5.1 Rabies vaccine and antiserum shortage in developing countries.

Vaccination, one of the greatest essential measures for prevention in locations where dog attacks are among the greatest and biggest cause of human misery is rabies vaccination. Nevertheless, many health issues face emerging countries, particularly Pakistan, whereby growing numbers of people, epidemics of diseases, and difficulty in vaccine coverage and distribution generate significant deaths and morbidity. The high expense associated with these vaccinations is also leading to insufficient and ineffective immunization programs in nations with low or middle incomes. However, there is a scarcity of vaccine and rabies antibody molecules Immunoglobulin (RIG) causes approximately 2000-5000 deaths in Pakistan (Iqbal et al., 2019). This high incidence of human rabies is mainly coupled with a lack of awareness about the disease complications, shortage of locally produced vaccines and anti-sera and the overall costs of imported cell culture vaccines and imported human rabies immunoglobulins (HRIG). Today, many potential vaccinations are currently being produced and have begun used with good efficacy results in developed countries, but they fail to provide protection in poor populations. Therefore, there is need to evaluate different vaccines and aggressive preventative measures evaluates with financial damage management (Sunohara et al., 2021).

1.5.2 Global requirement and market of rabies biologicals (vaccine and anti-serum).

According to world health organization / global market study 2 global market study human rabies working document December 2020, the rabies vaccine industry has 24 manufacturers and 27 products, with 85% supply in China and India. Only 14% of supply is pre-qualified, but there is enough to meet demand. Shortages in 2018-19 were due to one-time events. Intradermal administration can reduce annual vaccine needs from 60M vials to about 20M vials, leading to cheaper costs per person in urban areas. This may prompt manufacturers to reconsider their market presence. Global health authorities use available data estimates and country-provided information to predict the demand for rabies vaccine (WHO 2020). ID is preferred for Gavi support as it needs fewer doses than IM. Most countries still use IM for rabies vaccines, but six use both methods. Some have fully transitioned to ID, but some still follow WHO's IPC ID regimen (Figure1.13).

1.5.3 Market analysis of available rabies biologicals in Pakistan

Because there are several loopholes in the marketplace's accessibility of rabid biological substances in various parts of the country, severe strategic preparation is essential for eradicating human rabies by 2030. As lifesaver treatments for people following rabies contact, those who are impoverished should have easy availability of vaccines against rabies and immunoglobulin, and so these drugs should be accessible all year. To examine the accessibility of several types of people's rabies medicines in the nation market surveys, mapping and landscape analysis of is essentially required by the country. There are up to three kinds of cultivation of cells for rabies vaccinations accessible in the country which are imported and distributed by different pharmaceutical companies; however, some amount of indigenously produced vaccines and anti-sera are available in public organization, but they are not produced in sufficient quantity as per the market demands of the rabies biologicals. There are two distinct kinds of immune globulin readily accessible: horse rabies immunoglobulins (RIGs), that are made locally, and humans RIGs, which originate from abroad and tend to be more extensive. Nevertheless, vaccines against rabies and anti-sera are frequently in low supply in emerging nations such as the nation of Pakistan, where rabies vaccinations and anti-sera are largely acquired from other nations. The high costs of

these imported products cause an economic burden on the pocket of the poor victims as shown in Table 1.1; Figure 1.12.

Table 1.1 Cost comparison of imported human derived rabies immunoglobulins (HRIG) and animal derived rabies immunoglobulins, usually equine based (ERIG) available in Pakistan for rabies post exposure prophylaxis (PEP) recommended for category II & III bites.

Rabies immunoglobulins	Brand	Volume per vial	Average cost of unit vial
HRIG 300 IU/ml	Imported	2ml	PKR 5000 to 9000 (USD 22-39)
ERIG 150 IU/ml	Imported	1ml	PKR 5000-6000 (USD 22-26)
ERIG 200 IU/ml	Locally manufactured	10ml	PKR 2000-2500 (USD

Key: HRIG Human rabies immunoglobulin; ERIG Equine rabies immunoglobulin; PKR Pakistan Rupee; USD US dollars; ID, Intra-dermal; IM, Intramuscula; TRC ,Thai Red Cross regimen.

Figure 1.12 currently available brands of rabies immunoglobulins in Pakistan

1.6 Strategies to enhance humoral immune response in donor animals for poly-clonal antibody production.

Manufacturing cost effective anti-sera to treat fatal diseases like rabies is quite challenging due to several technical issues starting from poor immune response in the donor animals, lack of production facilities and resources to manage it, financial constraints of LMICs to purchase high-cost vaccine to immunize animals etc. An effective animal vaccination is dependent on several parameters, namely the antigen chosen, the method of management, the immuno-stimulatory character of the payload and/or adjuvant, plus the state of the immune system in the host animal. Vaccine efficacy is an indicator of the extent to which vaccine operate to generate the desired protection antibody in the host. This is determined by a variety of host characteristics such as age, gender, and medical status. Several reactions from the host have been documented following vaccination that impede the formation of immunity that defends against illness or vaccine.

Modulating host immune systems concomitantly with immunization is a good approach to increase vaccination effectiveness to boost immune outcomes and protection against specific diseases. The desired antibody level for PAb production is much higher than simple recommended protective titer values. Therefore, it is desirable to use various strategies including dietary interventions to boost the immunity of animal, vaccination regimen selection, hyper-immunization and optimization of purification techniques to get better yield of final product.

The World Health Organization (WHO), the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO) and the Global Alliance for Rabies Control (GARC) have endorsed global plan called "Zero by 30". Plan is focused to end human deaths from dog-mediated rabies by 2030. The plan involves using more dog vaccines and providing better access to human vaccines for those at risk. People at high risk of exposure should get pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) after potential exposure. WHO recommends the use of intradermal (ID) route of administration, which requires less vaccine, and shorter PEP regimens to improve program access to human rabies vaccine.(WHO 2020)

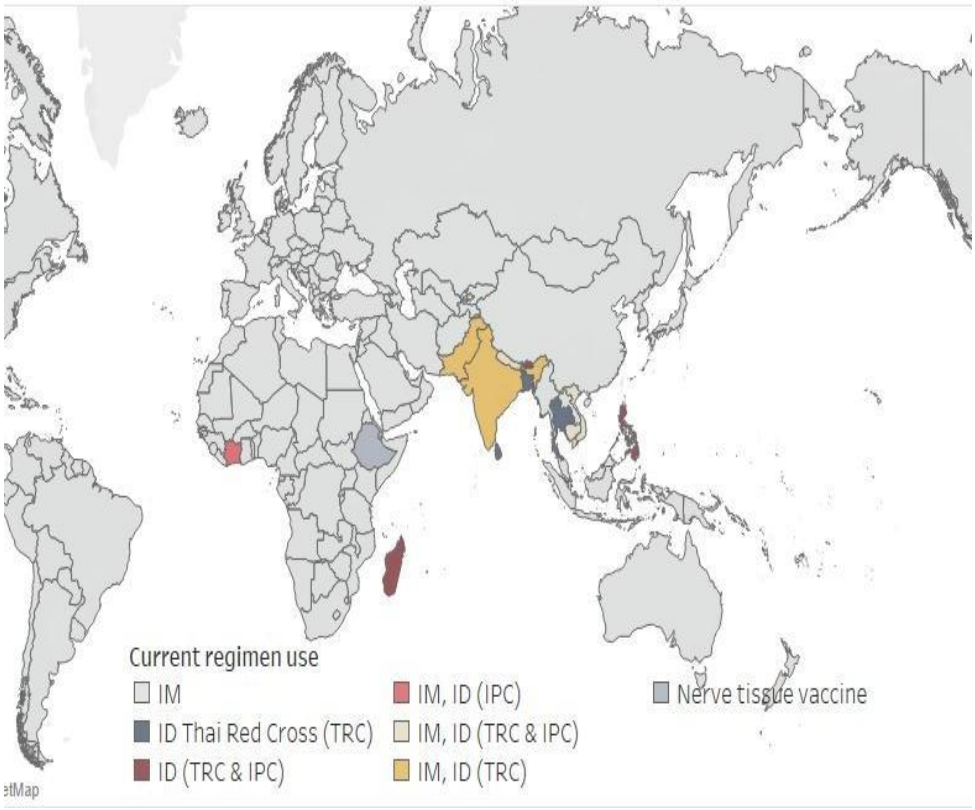


Figure 1.13 Current uses of rabies vaccine regimens in the world . The intradermal (ID) regimen is recommended for Gavi support as it requires fewer vaccine doses than the intramuscular (IM) route. However, most countries still use IM administration for rabies vaccine, with only six using both IM and ID regimens. Some countries have shifted to using ID (Thai red cross TRC-ID) regimens exclusively, but a few still use the WHO-recommended 6-dose Institut Pasteur du Cambodge (IPC) ID regimen as the most vaccine- and cost-reducing regimen Extracted from global market study human rabies vaccines ,working document 2020.(WHO 2020)

(Source :https://cdn.who.int/media/docs/default-source/immunization/mi4a/humanrabies-vaccine- market-study_public_summary.pdf?sfvrsn=2dee3e4_1&download=true)

1.7 Summary of literature review

Steps of the Process	The Methodology Used by Other Research Groups	Current Study
Step 1: pH adjustment of horse plasma	pH adjusted to 4.8 with 1N acetic acid (Habeeb & Francis, 1984) pH adjusted to either 4.5,4.9 or 5.5with 1.76N acetic acid. (Rojas et al., 1994), (Nudel et al., 2012), (Gutiérrez et al., 2005) Plasma was digested by pepsin at pH 3.2 and then pH adjusted to 6 with 1.76N acetic acid (Segura et al., 2009)	pH adjusted to 5.8 with 1.76N acetic acid
Step 2: Caprylic acid addition to plasma with continuous stirring	7.5% caprylic acid with continuous stirring for 50 mins (Habeeb & Francis, 1984) or 3%,5%, and 7%, for 1 hr (Nudel et al., 2012), (Rojas et al., 1994), (Gutiérrez et al., 2005). 2% Caprylic acid added to pepsin-digested plasma (Segura et al., 2009) Caprylic Acid at 5.5 with vigorous stirring for one h (Eursakun et al., 2012)	5% caprylic acid addition to the plasma with continuous stirring for 1 hr.
Step 3: Fractionation on and purification n of IgGs	IgG was fractionated on DEAE cellulose, concentrated by ultra-filtration or diafiltration in a Millipore ultra-filtration device to yield pure IgG. (Habeeb & Francis, 1984), (Gutiérrez et al., 2005), (Nudel et al., 2012),Dialysis against distilled water for 48 hours. (Rojas et al., 1994)	Dialysis against W.F.I for 48 hours, and then IgG was concentrated by TFF (tangential flow diafiltration).
Final pH adjustment	NaCl addition to final concentration Of 0.15M and Final pH of 7.2 by NaOH.(Rojas et al., 1994).The product was formulated with 8.5 g/l NaCl, 2.5 g/l phenol, and 0.05 g/l thimerosal, with a final pH of 7.0 by NaOH. (Gutiérrez et al., 2005)	NaCl addition to final conc. Of 0.15M and Final pH adjustment to 7.2 by 1N NaOH.
Purity check of the final product	Chemical analyses of antivenom: Determination of total protein, albumin concentration, turbidity analysis, antivenom antibody titre, and tests for pH and sterility were performed. (Nudel et al., 2012), (Eursakun et al., 2012), (Rojas et al., 1994) IgG was confirmed by either disk electrophoresis, immuno-electrophoresis, gel filtration, or electrophoresis (SDS—PAGE) under non-reducing conditions. (Habeeb & Francis, 1984), (Nudel et al., 2012), (Rojas et al., 1994) Efficacy of anti-venom and neutralization of AVS determined by animal model studies.(Gutiérrez et al., 2005), (Rojas et al., 1994)	QC/Physiochemic al analysis of Antivenoms. 1. Total protein concentration n: (Biuret Tests) 2. Electrophor etic analysis 3. Quantification on of turbidity 4. Phenol concentration 5. Caprylic acid detection

AIM AND OBJECTIVES

The study aims to explore the simple dietary strategies to enhance the rabies vaccine specific antibody titers to produce animal derived cost effective "Rabies Immunoglobulins" (RIGs) and strengthen local production of Poly-clonal antibodies (PAbs) in Pakistan.

Objectives

1 Overall growth & immunity improvement and specific humoral response enhancement against rabies vaccine (RV) with cost effective dietary interventions (Probiotics, Vitamin A supplements, Organic carrots).

2 Rabies specific Ab titer determination using Platellia II^{TM} ELISA kit (Bio- rad) & classical *in vivo* Mouse Neutralization Test (MNT) for comparison.

3 Selection of suitable vaccination strategy for RV to get improved titer in less time. Enhancement of PAb production in animals with weekly hyper-immunization

4 Optimization of downstream processing for production of purified rabies immunoglobulins (RIGs) with caprylic acid fractionation technique and Quality analysis of final product

CHAPTER 02

MATERIALS AND METHODS

2.1 Study design and procedures

The study aims to explore the simple dietary strategies to enhance the rabies vaccine specific antibody titers to produce animal derived cost effective (RIGs) and strengthen local production of poly-clonal antibodies in Pakistan. The study design is focused on the primary and secondary objectives as follows:

Primary objective was overall growth, health & immunityimprovements and specific humoral response enhancement against rabid vaccine with cost effective dietary interventions like probiotics, vitamin A and organic carrots.

Secondary objective of the study was to adopt cost effective strategies to enhance humoral response in donor animals for production of poly-clonal antibodies (PAb). These strategies include selection of suitable vaccination regimen to get improvedtiter in less time, antibody titer determination using *in-vitro* test such as Platellia II ELISA kit (Bio-Rad) instead of classical mouse neutralization (MNT) that is time consuming and requires large number of animals. Similarly, enhancement of PAb production in animals with weekly hyper-immunization strategy and further purification with new caprylic acid fractionation technique to get highly purified rabies antiserum as the final product.

A six-week research endeavor was orchestrated involving 54 New Zealand White rabbits, with weights spanning from 1.5 to 2.0 kg, and an age range of approximately 5 to 6 weeks. Each experimental group consisted of six animals (n=6), resulting in a total of six experimental and three control groups. The research protocol secured ethical clearance from the National Institute of Health (NIH), Islamabad, under Ethical Approval No. F.1-5/ERC/2020.

The rabbits were administered a cell culture rabies vaccine (CCRV) procured from the NIH. Rabbits displaying any indications of lethargy or reduced activity were excluded

from the study. The allocation of all 54 animals into various groups was randomized, leading to the following categorizations:

a) Group1 PBA (b) Group 2 PBB (c) Group 3 PBA/Vitamin A (d) Group 4 PBB/Vitamin A (e) Group 5, PBA/carrot (f) Group 6, PBB/carrot ((g) Control Group 1 (C1) (h) Control Group 2 (C2) (i) Control Group 3 (C3)

Briefly, the animals were fed with three types of probiotics including two commercially available mixed culture (mostly containing *Lactobacilli*, *Bifidobacterium* and *Saccharomyces* strains) were purchased from the market. Probiotics were diluted in 1 ml of sterile water and added into feed and fed daily from day 0 to day 35 in the early morning as per the manufacturer's instructions for a total of eight weeks.

A weekly administration of 10,000 IU of Vitamin A was provided to each animal within the designated groups over the span of 6 weeks, in conjunction with probiotics. Exclusive inclusion of organic carrots in the diet was limited to specific groups, aimed at investigating the potential effects of a naturally sourced Vitamin A on overall health and immunity. Additionally, parameters such as physical fitness, mortality ratio, and clinical observations were meticulously recorded to establish the potential impact of the interventions on the general well-being of the rabbits.

For a comprehensive analysis of blood-related factors, marginal ear vein blood samples were collected from the rabbits. These samples were then subjected to a detailed examination of various blood parameters. To evaluate the immune response of the rabbits to the rabies vaccination, rabies antibodies were assessed on the 14th and 35th days using a specialized ELISA kit known as Platellia II, specifically designed for the detection of rabies antibodies (Figure 2.1 and 2.2)

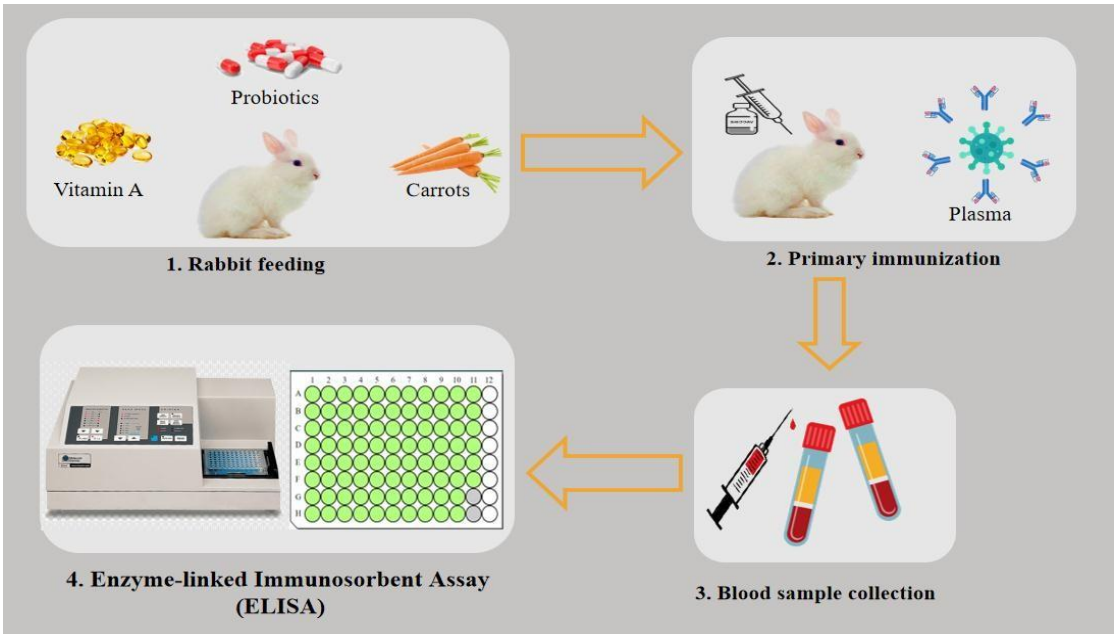


Figure 2.1 Process flow chart of primary immunization and in process titer testing for production of poly-clonal antibodies against RV with concomitant dietary supplementation with rabies vaccination in NZW rabbits. Experimental design and step wise process flow: This study included 2 week probiotics and vitamin A feeding of animals prior to four doses primary rabies immunization of 54 New Zealand white rabbits, (n=6) in each group. Blood sampling was carried out at regular intervals (days 0,14,35) to check blood parameters and specific antibody titre determination with Platellia II ™ELISA kit for rabies.

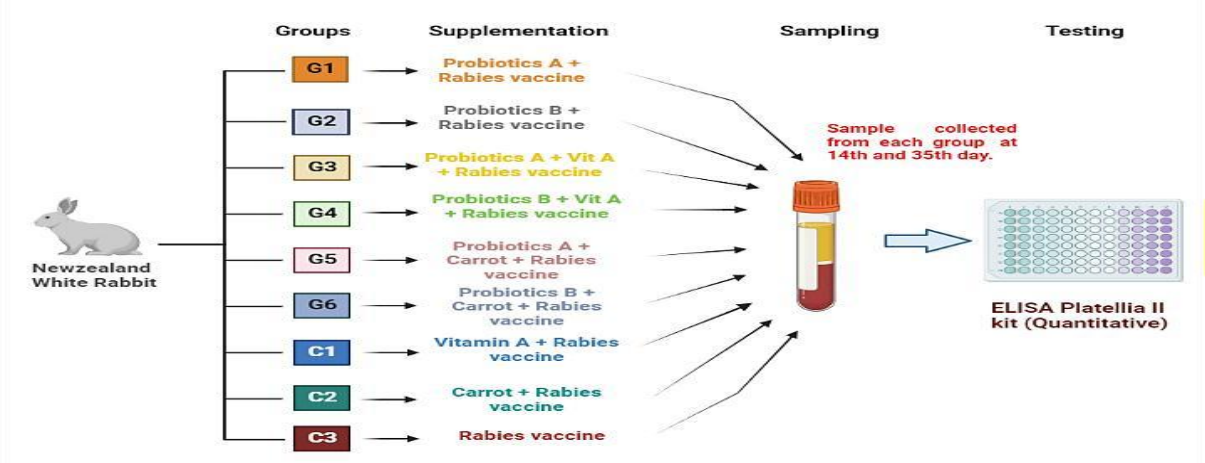


Figure 2.2 The arrangement of the animal groups was structured based on distinct dietary interventions, the process of blood collection, and the ELISA testing executed for the determination of antibody titers.

The grouping was as follows**:** Groups G1 and G2 were provided with separate probiotics, denoted as PBA and PBB respectively. For groups G3 and G4, the supplementation included vitamin A in combination with both PBA and PBB probiotics. Likewise, groups G5 and G6 had their diet enriched with carrots alongwith PBA and PBB probiotics correspondingly. In contrast, control groups C1 and C2 solely received vitamin A supplements and carrots, without the inclusion of probiotics. Acting as the primary control, group C3 underwent the study without any supplementary interventions. Uniformity was maintained across all groups in terms of the rabies vaccine administration schedule. This entailed the provision of two-site vaccine doses on days 0, 3rd, 7th, and 28th, adhering to the TRC-ID regimen for rabies vaccine administration.

2.1.1 Ethical Approval

The procedures implemented in this study were granted approval by the Institutional Ethical Committee for animal utilization at the National Institute of Health (NIH), Islamabad. Furthermore, these procedures align with global standards for biomedical research involving animals.

2.1.2 Experimental Animals, Grouping and Housing conditions.

Male New Zealand White rabbits, aged between 5 and 6 months, and with weights ranging from 1.5 to 2 kg, were carefully chosen for this study. This selection was based on the established history of enhanced antibody responses. All rabbits were nurtured within the controlled animal care facility at NIH, adhering to established standard operating procedures (SOPs). Animal care and management adhered to the institutional guidelines set for the ethical treatment and use of laboratory animals. To ensure appropriate nourishment, each rabbit was individually housed and accommodated in dedicated compartments, complete with regulated heating and cooling provisions.

Grouping of animals

Total 54 New Zealand white rabbits (Figure 2.3) were allocated to 06 experimental groups and 3 control groups marked as (1) PBA group, (2) PBB group, (3) PBA/Vit A group, (4) PBB/Vit A group, (5) PBA/carrot group, (6) PBB/carrot (7) C1 (8) C2, (9) C3 groups (Table 2.1). Each group comprised n=6 male rabbits. Blank sera were obtained before the immunization procedure at day zero. Animals included in this study were divided in to 9 groups (n=6) animals per group were assigned: two groups was given the probiotics only along with rabies vaccine (PB+RV), the other two groups were administered probiotics and Vitamin A capsules along with rabies vaccine (PB+ Vitamin A + RV) and the third set of groups was given probiotics and organic carrots as natural Vitamin A source along with rabies vaccine (PB+ Carrots+ RV). Control groups in this experiment includes main control group (C3), which was only administered with Rabies vaccine without any additional supplementation. While other two were the sub control groups (C1 & C2). The descriptive analysis of the experiment carried out shows that the experimental groups exhibited a comparatively elevated mean rabies antibody titer. (M=3.740±0.142, 3.690±0.133, respectively) than the control group (M=3.268±0.279).

Housing and diet of animals:

The care and nurturing of the animals adhered to the guidelines established by the institution for the ethical and compassionate treatment of laboratory animals. To ensure precise probiotics dosage, each rabbit was housed separately. Adequate water was supplied, and ambient conditions, including temperature regulation through heating and cooling systems as required, were maintained (Figure 2.4). The rabbits were fed a standardized NIH diet formulated in accordance with contemporary industry nutrient specifications. Every day, each animal received a 5 ml suspension containing 5 mg of Vitamin A, as well as a blend of commercial strains of Lactobacillus probiotics, as per the manufacturer's instructions (*Lactobacillus rhamnosus* GG gelatin capsule with 1010 organisms; i-Health Inc, Cromwell, CT). Supplementation with probiotics, Vitamin A capsules and organic carrots (18 gm/animal per day) were started along with standard diet one week prior to immunization as per designated groups for each intervention.

Table 2.1 Arrangement of subject animals according to Rabies Vaccination and diverse dietary enhancements in different sub- groups.

Group ID	Intervention details	No of animals	Remarks	Controlled parameters of experimental animals
G1	RV + PBA	n=6	**Group A** (Dietary Intervention groups)	Breed = New Zealand white rabbits
G2	RV + PBB	n=6		Age = 5-6 months
G3	RV + PBA + V. A	n=6		Sex = Male
G4	RV + PBB + V. A	n=6		Average weight = 1.5 to 2.0 kg
G5	RV + PBA + O.C	n=6		Housing temp ^{0} C = Ambient
G6	RV + PBB + O.Ct	n=6		Housing humidity % Rh = 60-70%
C1	RV + V. A only	n=6	**Group C** Control & Sub-control groups	Test vaccine = CCRV same batch
C2	RV+ O.C only	n=6		Duration of intervention = one week prior to vaccination till day 35.
C3	RV only	n=6		Selected vaccination regimen= Modified Thi-red cross (TRC), 2-site 4 shots (0,3,7,--,28). Intradermal (ID)
Total		n=54		

Key : RV (Rabies Vaccine), PB (Probiotic),V. A (vitamin A), O.C (organic carrots)

Figure 2.3 Experimental animal New Zealand white rabbits

Figure 2.4 Housing of rabbits in animal house facility and grouping of animals (n=6) in each intervention and control group.

2.2 Feeding of Animals with Probiotics and Vitamin A supplementation and organic carrots in various dietary groups composing cost and efficacy

The animals were nourished with a conventional diet sourced from the animal facility, designed according to prevailing industry nutrient standards. In specific groups involving carrots (G5, G6, and C2), a daily provision of 18 grams of organic carrots was administered to everyone. Rabbits were administered with two different mixed culture commercial Probiotics having mostly strains of the genera *Lactobacillus* or *Bifidobacterium* and were used as for overall improvement in health and immunity. As per the manufacturer's instructions (Serving size was one capsule per day/animal), each capsule serving contains more than 2 billion CFU. Vitamin A, one capsule per day (1000 IU), and organic carrots were provided to different animal groups along with a standard diet two weeks before immunization. Fresh organic carrots grown at the farmland of NIH were used to feed the rabbits (18 gm/animal per day) as natural vitamin A rich source for better health and immunity response. The control groups exclusively obtained a standardized diet with specified supplementary additions., such as rabies vaccine (RV) + Vit A (C1), RV + carrots (C2) and only rabies vaccine in group C3.

2.2.1. Basal Diet Composition for rabbits:

A recommended basal diet for New Zealand White rabbits in experimental studies was typically consist of the following components (Table 2.2):

• **Timothy hay:** Addition of good quality timothy hay provided fibre and essential nutrients for gut health; therefore, it was the main ingredient of the feed.

• **Pellets:** As recommended small portion of nutritionally complete rabbit pellets were incorporated into the diet. Pellets were fed in small quantities to prevent obesity of animals.

• **Vegetables**: A variety of fresh seasonal vegetables such as green fodder, cucumber lettuce, and parsley are usually added gradually in the basal diet but in moderate quantity to prevent digestive upsets.

• **Fresh water**: Clean, fresh water was always to be available for the experimental rabbits and routinely changed on daily basis.

Farm or lab animal's blood profiles were usually affected by their diet composition and efficiency of metabolic processes may hamper animal's nutritional state. Blood analysis clinically examined an animal's physiological, nutritional, and pathological condition possible.

Haematological examinations helped ascertaining the degree of any change on physiological functions. Additionally, alterations in haematological markers were frequently utilized to identify dietary stressors. According to reports from several researchers, the various diets administered to rabbits had varied impacts on their haematological markers, some negative while others showed improvements.

2.2.1.1 Effects of basal diet composition on growth and general health of NZW rabbits

It is well established fact that farm or lab animal's physiology is affected by several variables, including diet. It is important to note that diet of experimental animals should be kept consistent and not altered without proper consultation with a veterinarian or animal nutritionist.

Moreover, changes in the diet should be made gradually to allow the rabbit to differ insignificantly between all experimental groups. Further supplementation with the foundational rabbit diets yielded favorable outcomes in terms of weight gain, feed conversion ratio (FCR), and the activities of antioxidant enzymes. Additionally, it led to enhancements in the lipid profile, glucose levels, and the functioning of the liver and kidneys.

Table 2.2 Basal Diet Composition of Control groups and experimental of New Zealand White rabbits without supplementation

INGREDIENTS	CONTENT (%)	CONTENT (gm)
WHOLE CRUSH	13.70	100
WHEAT BRAN	4.11	30
CARROTS	54.80	400
GREENFODDER	27.40	200
SOYBEAN	5.23	15
CORN	10.00	18
TOTAL	100.01	

2.2.2. Probiotic supplementation

2.2.2.1. Composition of commercial Probiotics

Probiotic diets contain both regional and industrial strains. Local strains of Lactobacillus (Spark) were administered to rabbits, while the generic strains Enfold, preproz, and equalize were induced.

Serving sizes included 1 sachet each of Preproz and Enflor probiotics, which 1 equate capsule, and 10 ml of the local strain of *Lactobacillus* (Spark). From day 0 through day fourteen and up to day twenty-eight the aforementioned probiotic diet was consistently displayed.

2.2.2.2. Composition of Probiotic A/ EQ (Proprietary blend Equate™)

Each individual animal received a daily administration of a single capsule, which was delivered in liquid suspension and contained approximately 30 billion CFU (colony forming units). Within this count, there were 18 billion *Bifidobacteria* CFU and 12 billion CFU of *Lactobacilli* cultures (Figure 2.5).

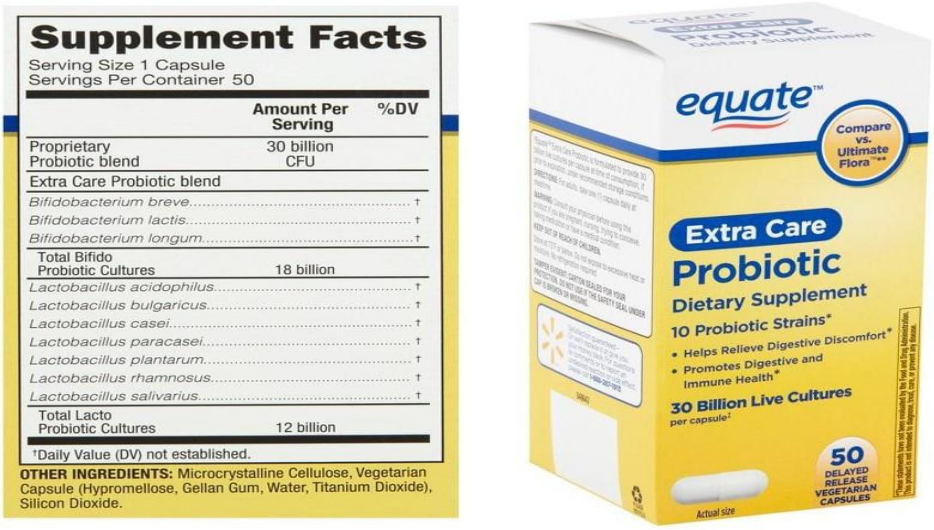


Figure 2.5 Probiotic A supplements (Proprietary blend, Equate ^{TM}) used in experiment along with composition details.

2.2.2.3. Composition of Probiotic B/ TF (Proprietary blend Top Formula^{TM})

Animals ingested a daily portion of two capsules in suspension, following the product's recommended dosage provided by the manufacturer. Every capsule encapsulated seven distinct bacterial strains, culminating in a total daily dose of 10 billion CFU. The control groups, categorized as C1, C2, and C3, received an exclusive standard diet in tandem with pre-defined dietary compositions. Specifically, C1 received Vitamin A supplementation, C2 group was supplied with organic carrots, and C3 was devoid of any dietary supplementation. Despite these distinctions, all nine groups underwent identical vaccination schedules and adhered to the same immunization protocol.

2.2.2.4. Composition of Probiotic C/PPZ (Proprietary blend PreproZTM)

Each sachet contains *Lactobacillus rhamnosus* Rosell-II (one billion CFU), Lyophilized *Saccaromyces boulardi* and added elemental Zinc.

2.2.2.5. Composition of local probiotic brand (Ecotec^{TM}) Lactobacilli strains for comparing cost and efficacy

Ecotec Probiotic Sachets are recommended as effective probiotic to improve the gut health and support a healthy immune system. Each sachet contains selected blend of live, active bacterial cultures to replenish the gut with good bacteria and promote overall well-being including improved digestion, reduced inflammation, and a stronger immunity. They are free from additives, fillers, and preservatives (Figure 2.8).

Content	2 capsules	DRI*
Lactic acid bacteria	10 billion	-- %
- of which Lactobacillus acidophilus	1.7 billion	-- %
- of which Lactobacillus plantarum	1.7 billion	-- %
- of which Bifidobacterium bifidum	1.7 billion	-- %
- of which Lactobacillus casei	1.2 billion	-- %
- of which Lactobacillus rhamnosus	1.2 billion	-- %
- of which Bifidobacterium longum	1.2 billion	-- %
- of which Bifidobacterium lactis	1.2 billion	-- %
Vitamin C	48 mg	60%
Selenium;	33 µg	60%

Figure 2.6 Probiotic B composition:(Proprietary blend, Topformula Probiotic+ Sweden): Lactic acid bacteria contain 7 strains of bacteria, with a total of 5 billion good bacteria per capsule. Daily recommended intake for a typical adult (8400 kJ/2000).

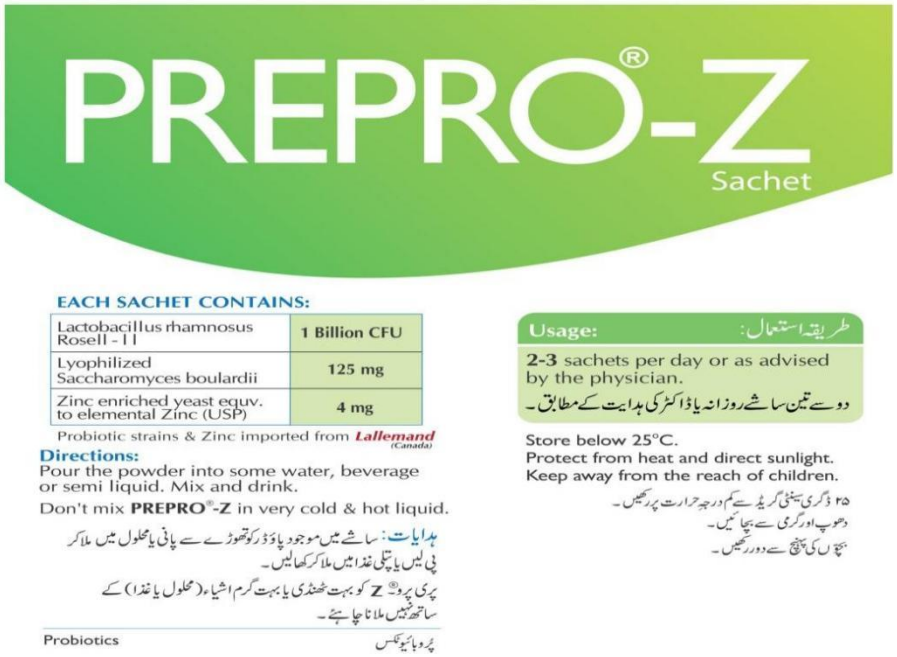


Figure 2.7 Probiotic C/PPZ (Proprietary blend Prepro-Z TM)

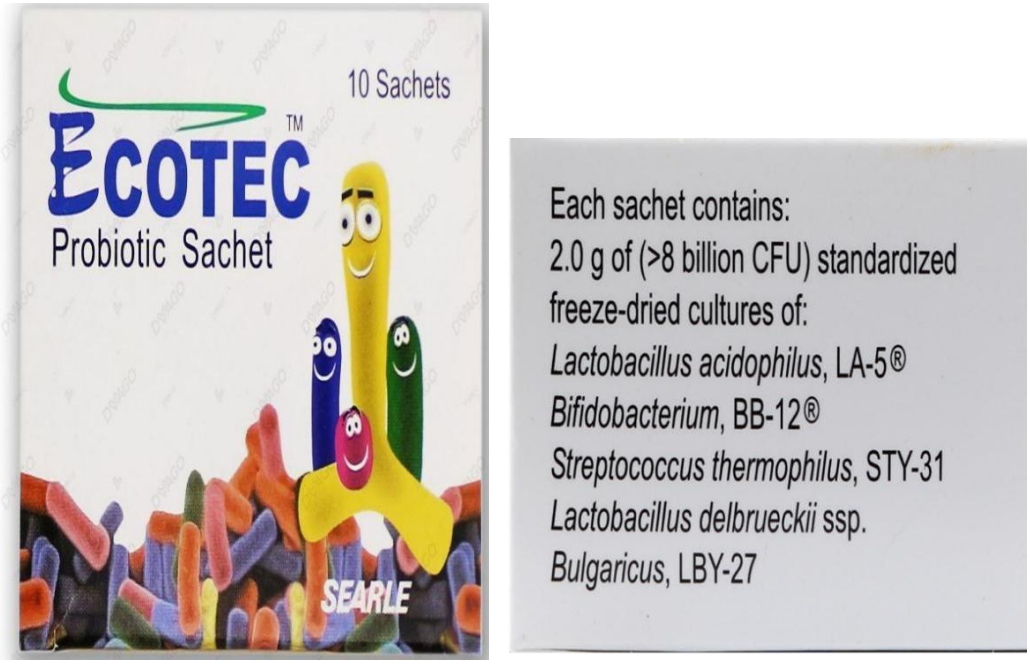


Figure 2.8 Composition of Ecotec Probiotics (Locally available brand)

2.2.2.6. Initial screening and selection of commercial probiotics for comparing cost and efficacy.

During initial experimental phase for the selection of probiotics, three freeze-dried mixed culture commercial international probiotics brands (mostly containing Lactobacilli, Bifidobacterium and Saccharomyces strains were purchased from the market and were used to feed the animals following the prescribed daily dosage, the animals were provided with their daily feed for a span of two weeks prior to day zero and continued until the 28th day. Subsequently, they were switched to a maintenance diet until the culmination of the experiment. A local probiotic brand, which offered a cost-effective solution, was chosen and procured from the nearby market. Within the experimental groups, each individual animal was administered a daily quantity of 1ml suspension. This suspension contained a mixture of commercial probiotics, adhering to the manufacturer's stipulated instructions.

Briefly, the animals were fed with three types of probiotics (2 commercially available international brands containing mixed culture were purchased from the market and one local brand was used. The probiotics contained mixed cultures of *Lactobacilli*, Bifidobacterium strains and *Saccharomyces boulardii* with added pre-biotics such as Inulin. One of the commercial probiotics contained zinc as added supplement (Figure 2.1, Table 2.1). Probiotics were diluted in 1 ml of sterile water and added into feed and fed on a daily basis from day 0 to day 35 in the early morning for a total of eight weeks.

Table 2.3: Initial Selection of probiotics cultures (Commercial vs Lab isolated). Grouping and distribution of subject animals according to different dietary intervention. Three different commercial probiotic strains were selected (EQ, PPZ and TF) and compared with locally available probiotics (LB). Probiotic supplementation was started 2 weeks prior to vaccination till day 35. All the parameters related to animal housing were kept controlled during eight-week experiment.

Groups	**Intervention details**	**No. of animals**	**Controlled parameters of experimental animals**
Group 1	RV + PB(EQ)	n=5	**Breed** = New Zealand white
Group 2	RV + PB (PPZ)	n=5	**Age** = 5-6 months
Group 3	RV + PB (TF)	n=5	**Sex** = Male
Group 4	RV + PB (LB-1)	n=5	**Average weight** =1.5-2.0 kg
Group 5	RV + PB (EQ) + O.Carrot	n=5	**Housing temp ^{0} C** = 22°C
Group 6	RV + PB(LB-1) + O.carrot	n=5	**Housing humidity % Rh** = 60- 70
Contol 1	RV only	n=5	**Test vaccine** = CCRV same batch
	Total animals	n=35	

Key : RV (Rabies vaccine), EQ (Equate^{TM}),PPZ (PreproZ^{TM}),TF (Top formula^{TM}),LB(Local straina),O.C (Organic carrots)

2.2.3 Vitamin A supplementation with selected Probiotics

Vitamin A capsules were obtained from department of Expanded program of Immunization (EPI) which are being used routinely for childhood vaccination campaign as immune enhancers, manufactured by Health Inc, Cromwell, CT Canada, (Figure 2.9).

Figure 2.9 Vitamin A supplementation in specific dietary groups.

Three commercial and one lab mixed strain lypholized cultures were used as diet supplements for enhancement of health and humoral response in NZW rabbit groups. The single human dose was provided as per manufacturer's instructions. For lab strain in Group-5 & Group-6, organic carrots were added into feed as rich natural source of vitamin A & beta carotenoid. All experimental animals from and Control group were immunized with rabies vaccine (CCRV) using intradermal TRC regimen (Table 2.4).

2.2.4 Organic carrot addition in feed of selected groups

Fresh organic carrots have been routinely purchased from the market as through procurement department as rich source of beta carotenes and vitamin A natural source in animal diet. However, we used the carrots only in the diet of designated rabbit groups for comparative analysis of dietary supplementation. Each rabbit in specific groups designated for carrot supplementation were provided fresh organic carrots (18 gm/animal per day, Figure 2.10).

Figure 2.10 Dietary supplementation with organic carrots.

Table 2.4 Grouping of animals as per probiotic brands used initial screening experiments.

Groups ID	**Probiotics brand/ nomenclature**	**Details specifications**
Group-1(EQ)	**Eqaute™ (Mixed culture)**	Serving size = 1 capsule per day/animal. Each capsule serving contains 30 billion CFU with total Bifidobacterial cultures 18 billion and Lactobacilli probiotic cultures 12 billion CFU/dose.
Group-2 (PPZ)	**PreproZ™ (Mixed culture)**	*Lactobacillus rhamnosus* Rosell-11=1 Billion CFU *Saccharomyces boulardii* =125mg
Group-3 (TF)	**Top formula™**	Each active capsule contained seven of bacterial strains with total 10 billion CFU/ 2 capsules
Group-4 (LB-1)	**Ecotec local brand***	1×10^{10} CFU/ml *Lactobacilllus* strains mixed culture with Zinc enriched yeast equivalent to elemental Zinc = 4mg
Group-5 (O.C)	**Eqaute™ + Organic carrot**	Animals fed with addition of fresh organic carrots with commercial probiotic culture.
Group-6 (O.C)	**Local brand Ecotec (LB- 1* + Organic carrot)**	Animals fed with addition of fresh organic carrots with lab derived probiotic culture.
Control group (C3)	**Nil**	Only fed with standard diet without probiotics and immunized only with rabies.

2.3 Estimation of animal growth and Clinical parameters.

Mortality and physical fitness observation, weight calculation for animal health & growth performance was monitored at regular weekly intervals from day 0, 7, 14, 21, 28, 35, 42, 49, 56 till day 63 at completion. For microbial analysis, fecal sample collection was carried out at regular intervals to observe any prominent change. Basic laboratory parameters such as ALT, ALP, Urea and creatinine were also observed during experiment on days 0,14 and 28 keeping in view the safety aspects on health of animals. The experiments were run on Cobas C311 machine using Roch standardized kits as per manufacturer's instructions.

2.3.1 Determination of physical parameters

Animals were kept under observation and strict supervision of veterinary physician of animal house of NIH for mortality on daily basis or physical fitness during experiment. Detailed checkups were conducted on weekly basis on days 0,7,14 and 28 as per SOP till completion of experiments.

2.3.2 Growth performance measurements & Food Conversion Ratio

Rabbits underwent individual weight assessments on a weekly frequency. The daily food consumption for each cage was meticulously monitored and documented.

Additionally, parameters including body weight (BW), body weight gain (BWG), and feed conversion ratio (FCR) were evaluated on a weekly basis according to Alagawany et al., 2021 as food consumed divided by weight gain during the experimental period.

2.3.3 Blood sampling for hematology and biochemical test analysis.

Approximately 2 ml venous blood sample was obtained on day 0, 14, and 35 after complete vaccination and at day 60 after completion of experiment for serology testing (hematology, biochemistry, and antibody titer testing). Biochemical testing was performed on Cobas C311 machine using Roche standardized kits as per manufacturer's instructions. Moreover, adverse reactions (local and systemic) were also

observed after each shot of total 4 vaccination shot in all 54 subjects in experimental and control groups after each shot of total 4 vaccination shots (Figure 2.11)

Figure 2.11 Blood collection and serum separation: A blood sample of approximately 3 ml was collected from the marginal ear vein of rabbits on day 0 of vaccination, as well as on the 14th and 35th days following the initial dose. This was done using EDTA tubes to ensure optimal detection of plasma immunoglobulin levels. Samples were collected immediately prior to each subsequent vaccination, as well as on the 14th day and one week after completion of all doses, which occurred at a later time point (28 days).

Table 2.5 Biochemical Reference Ranges for Rabbits for clinical studies: (Alessandro Melillo, 2007 Rabbit Clinical Pathology)

AST (IU/L)		35-130	
ALT (IU/L)		45-80	
ALP (IU/L)		12-96	
CPK (IU/L)		140-372	
GGT (IU/L)	0-7.0 T. BIL (mg/dL)	0-0.7 Bile acids (mMol/L)	40
Amylase (U/L)		200-400	
Cholesterol (mg/dL)		10-80	
Urea (mg/dL)		20-45	
CREA (mg/dL)		0.5-2.5	
Ca (mg/dL)		11-14	
Phos (mg/dL)		4.0-6.5	
Na (mEq/L)		138-150	
K (mEq/L)		3.5-6.9	
Glucose (mg/dL)		75-155	
Total protein (g/dL)	5.4-7.5 Albumin (g/dL)	2.7-5.0	
Globulin (g/dL)		1.5-2.7	

AST aspartate aminotransferase, ALT alanine aminotransferase, ALP alkaline phosphatase, CPK creatine phosphokinase, GGT glutamyltransferase, T. BIL total bilirubin, CREA creatinine, CA calcium, Phos phosphorous, Na sodium, K potassium.

2.3.3.1 Estimation of Hematological parameters

The XN-550 (Sysmex), a compact hematology analyzer was used for complete hematological analysis of blood samples (Figure 2.12)

Principle and Procedure: The XN-550 is an automatic sampler with its improved workflow productivity and its rerun / reflex capability, continuous loading function, and instantaneous current detection method. The whole blood is introduced to the machine. The result is automatically displayed on the screen.

Figure 2.12 SYSMEX XN-550 Hematology Analyzer

2.3.3.2 Estimation of Biochemical parameters

The Cobas C311(Roche) analyzer was an automated, software program-controlled analyzer was used for medical chemistry evaluation.

Principle and Procedure: It is Spectrophotometric based procedure. Sample in clot tube is subjected to centrifugation for a duration of 10 minutes at 3000 revolutions per minute (rpm). The supernatant (plasma) is separated and put in another gadget cup. It takes 10 minutes to display the results on screen.

2.4 Microbiological Analysis of Fecal Samples for Bacterial Identification through Culture

The process of culture identification involved evaluating characteristics such as appearance, colony shape, and subculture features. Bacterial isolation was achieved using the streak plate method, employing a variety of culture media. Following this, bacteria were cultivated through the streaking technique. To ensure sterility, the Bunsen burner was employed for heating and sterilizing both the wire loop and the inoculation equipment. A loopful of the sample was then streaked onto each quadrant of the culture medium, ensuring sterility at each step. Subsequently, the inoculated medium was incubated at a temperature of 37°C ± 0.5°C for a duration of 18-24 hours. After the incubation period, a comprehensive assessment of the colony's characteristics was conducted, including size, shape, margin, and elevation.

Figure 2.13 The Cobas C311(Roche) chemistry analyzer.

2.4.1 Total Viable Count

The microbial population residing within the intestinal samples was collected using a sterile cotton swab. These swab samples were then subjected to culturing for an 18-hour incubation period at 37°C for Colony-Forming Units (CFUs), using 10 ml of Mueller Hinton Broth medium. Sequentially, fecal samples were individually collected, weighed, and transferred into sterile Stomacher bags. A buffered Peptone solution was introduced to the samples in a ratio of 1:9. Following this, homogenization was carried out using a Stomacher apparatus (BagMixer 400, Interscience). Through the utilization of buffered Peptone water, suitable 10-fold serial dilutions were formulated. Subsequently, 100 micro liter aliquots from each consecutive dilution were employed for the inoculation of selective media.

2.4.2 Preparation of Media for Different Bacterial Colony isolation and Identification

2.4.2.1 Plate Count Agar (PCA)

Plate Count Agar media (PCA) was used for total viable counting. It is frequently employed to quantify viable bacterial growth in samples, Plate Count Agar (PCA) or Standard Methods Agar (SMA) is a microbiological growth medium. Its primary purpose is to assess the total or viable bacterial content within a sample.

2.4.2.2 MacConkey Agar

MacConkey agar serves as an isolation medium for Gram-negative enteric bacteria. This medium differentiates between Gram-negative bacteria that ferment lactose and those that do not. It effectively separates coliforms and intestinal pathogens in various matrices, including water, dairy products, and organic samples.

2.4.2.3 Violet Red Bile Agar

A systematic approach for enumeration of coliform bacteria, Violet Red Bile Agar has historically been employed to quantify coliform organisms, including E. coli, in diverse products such as water, milk, dairy equipment, and various food items.

2.4.2.4 Blood Agar

Blood Agar is a widely utilized enriched medium for cultivating specific microorganisms and classifying them based on their hemolytic capabilities.

2.4.2.5 Mannitol Salt Agar (MSA)

For the formulation of Mannitol Salt Agar (MSA), dissolve 111 g of the powder in 1 liter of deionized or distilled water. Ensure a comprehensive blending of the solution, followed by its sterilization in an autoclave set to 121°C for a duration of 15 minutes. Prior to autoclaving, ensure complete dissolution by stirring the solution while briefly heating it to boiling.

2.4.2.6 Bismuth Sulphite Agar

Bismuth Sulphite Agar is a highly selective medium primarily used for the initial detection and separation of Salmonella. This medium is particularly effective for identifying Salmonella typhi and other salmonellae in sewage, pathological specimens, water supplies, and food items suspected to harbor these pathogens.

2.4.2.7 Agar Baird-Parker

Agar Baird-Parker, also known as Egg-Tellurite Glycine-Pyruvate Agar (GPA), is widely incorporated in recommended procedures for detecting the presence of Staphylococcus aureus in various substances, cosmetics, and even pool water. It can also facilitate the recovery of *S. aureus* from clinical specimens.

2.5 Vaccine and immunization strategy selection.

2.5.1 Test Vaccine for the experimental and control groups in the study:

The vaccine used in the experiment was a Cell Culture Rabies Vaccine (CCRV), Batch # RV-1619 and Expiry date April 2021 prepared by National institute of Health, Pakistan. The dosage form was injection, the specification was 0.5ml/ dose, total 5 doses are recommended for humans. Each dose contains purified inactivated Rabies virus >2.5IU/dose for (recommended for humans) as per WHO requirements. The vaccine has been well established product already been used in the market for control of Rabies in Pakistan (Figure 2.14).

Figure 2.14 Cell culture Rabies Vaccine (CCRV) used in experimental study.

2.5.2 Vaccine regimen and Immunization of Animals for improved antibody titer in less time

2.5.2.2 Recommended immunization regimens for rabies vaccine.

The World Health Organization (WHO) suggests rabies post-exposure prophylaxis (PEP) for individuals who are immunologically compromised and experience Category II exposures. It is strongly advised to thoroughly cleanse the bite site and promptly initiate vaccination using one of three specified regimens. When exposed to rabies virus, such as through a suspected dog bite (a primary cause of rabies) or any animal bite potentially transmitting rabies, it is crucial to immediately undergo vaccination using one of the subsequent three regimens to establish the required protective antibody level:

(a) The "Institut Pasteur Cambodia 2-2-2-0-0" regimen, involving 2-sites intradermal (ID) injections on days 0, 3, and 7, spanning a total of 7 days.

(b) The "Essen 1-1-1-1-0" regimen, consisting of a single-site intramuscular (IM) injection on days 0, 3, 7, and between day 14–28, lasting up to 14–28 days.

(c) The "Zagreb 2-0-1-0-1" regimen, comprising 2-sites IM injections on days 0 and a single-site IM injection on days 7 and 21, with a total duration of 21 days [1]. In cases of Category III injuries, additional administration of rabies immunoglobulin is recommended Beatriz P. *et al* 2019)

(d) ‘TRC intradermal immunization regimen “TRC ID” with 2-site (2-2-2-0-2) ID

The animals within the experimental groups received immunization utilizing the "Cell Culture Rabies Vaccine (CCRV)", a product developed by the Biological Production Division of NIH, Pakistan. Each dose of this vaccine contains purified inactivated Rabies virus with a potency exceeding 2.5 IU per dose, a level recommended for human administration. For the experimental protocol, the Thai-Red Cross "TRC" intradermal immunization regimen, commonly known as "TRC ID", was selected. This regimen involves administering 2-site shots (2-2-2-0-2) on days 0, 3, 7, 14, and 28. The rationale

behind this choice was to achieve a desirable antibody titer in a shorter span compared to the previously utilized intramuscular regimens, as illustrated in Figure 2.15.

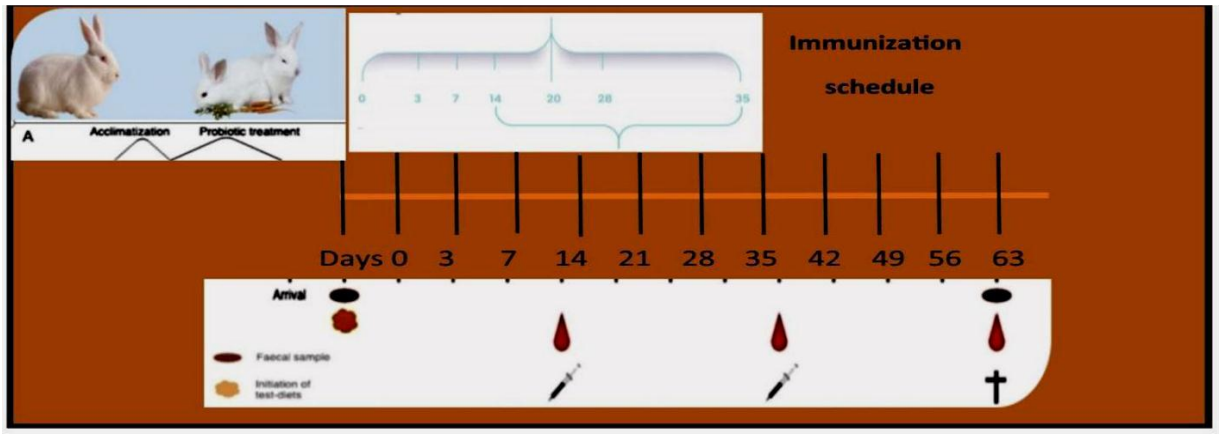


Figure 2.15 Schematic representation of the 63-day hyper-immunization protocol for poly-clonal antibody production in rabbits: 2-site injections / 3 blood samplings. After acclimatization dietary supplementation has been started 2 weeks prior to immunization. Initially TRC-ID regimen was followed at day 0, 3,7 & 28 followed by weekly boosters till day 63. Blood samples collected at days 0, 14, 35 & 63 for test analysis.

2.6 Safety studied and Reaction observation.

2.6.1 Local Reaction observation

Local reactions and incidences after rabies vaccination in experimental groups (Group A) with dietary interventions was observed in all 54 subjects in experimental and control groups after each shot of total 4 vaccination shots. After the insertion of 1st dose, subjects were monitored for 48 hours and none of the 6 subjects showed symptoms of pain, redness, pruritus, or any other reaction. Rabbits were observed for any symptoms for about 48 hours after the insertion of every dose and the result was the same as per experienced after the first dose.

2.6.2 Systemic Reactions:

Systemic reactions and incidences after vaccination and dietary interventions was studied in all animal groups. For evaluation of systemic reactions, temperature of the subjects was taken to observe any immediate reactions occurred within 30 minutes, 6, 24, and 48 hours observations before and after the insertion of all 4 shots were noted down.

2.7 Antibody determination by Platellia II^{TM} ELISA kit method (*in-vitro* test)

Blood specimens were procured using EDTA tubes, facilitating the extraction of optimal plasma antibody titers. The calculation of these titers was carried out using the Platellia II^{TM} ELISA kit (Bio-Rad), a recognized indirect immune-enzymatic assay specifically designed for the detection of antibodies targeting rabies virus glycoproteins. Notably, this standardized kit has been sanctioned by the Office International des Epizooties (OIE). The kit offers various advantages, including its rapidity in generating results within a span of 3 hours. It is characterized by its straightforward operational procedure, ensuring ease of use. Additionally, the kit promotes safety for those handling it and delivers both qualitative and quantitative outcomes. Moreover, the test has reported 99% sensitivity and 99.4% specificity. The

procedure for antibody detection utilizing the ELISA kit method adhered closely to the guidelines outlined by the manufacturer . Buffer solutions were meticulously prepared using chemicals of analytical reagent-grade quality, unless explicitly mentioned otherwise.

2.7.1. Reagent and supplies for ELISA test, (An indirect immune-enzymatic assay)

The evaluation of antibody titers was executed utilizing the Platellia II TM ELISA kit, procured from Bio-rad. This specific kit has been standardized and sanctioned by the Office International des Epizooties (OIE). Its function is to scrutinize animal sera for the detection of antibodies that specifically target the rabies virus.

2.7.2. Protocol for detection of antibodies by ELISA

The Platellia II kit ^{TM} was employed to detect rabies virus anti-glycoprotein antibodies in serum samples, adhering to the manufacturer's instructions. Notably, the utilization of ELISA test confers several advantages over NIH's previous employment of mouse neutralization test (MNT). These benefits encompass rapid results acquisition within a three-hour timeframe, as well as 99 % sensitivity and 99.4 % specificity. The ELISA test ensures safety for those handling it and provides both qualitative and quantitative outcomes (Norouzbabaei et al., 2022).

Serum samples were concisely diluted by combining 10 µl of sample with 990 µl of a dilution solution, followed by allocation of controls (both negative and positive), along with serum samples and quantification standards into micro-plates. Following an hour-long incubation at 37°C, three washing steps were executed to eliminate unbound antibodies and other proteins from the samples.

Each well received conjugate-protein A that was labeled with per-oxidase, followed by an hour-long incubation at 37°C and five washing cycles to remove unbound conjugate. Subsequently, peroxidase substrate and chromogen were introduced to each well before being kept at room temperature for thirty minutes. Afterward, a solution containing one normality H2SO4 was added to each well before measuring optical density using an

ELISA microplate reader (Diateck, China) set for wavelengths of both 450 nm and 620 nm.

2.7.3. Drawing the standard curve

To speed up manual data analysis, graph paper can be employed as a useful tool. This method involves plotting the mean values of OD readings for Quantification standards (S1 to S6) along the vertical axis (y), while marking corresponding concentrations in EU/ml on the horizontal axis (x). By connecting these six points with line segments, a series of lines is formed.

On the other hand, automated data reduction relies on a "point-to-point" function, following instructions provided with the kit. This function constructs a curve by utilizing OD readings obtained from standards. When dealing with an unknown sample, precise quantitative assessment of anti-rabies antibody titers can be achieved if its OD value falls within range of Optical Density mean values for S1 (0.125 EU/ml) and S6 (4 EU/ml). In such cases where this occurs, standard curve is utilized. By extrapolating value that corresponds to OD reading of sample onto y-axis of standard curve, we draw a horizontal line that intersects it at some point. The intersection point then links up to x-axis via vertical line which ultimately reveals antibody concentration expressed in Equivalent units (EU/ml).

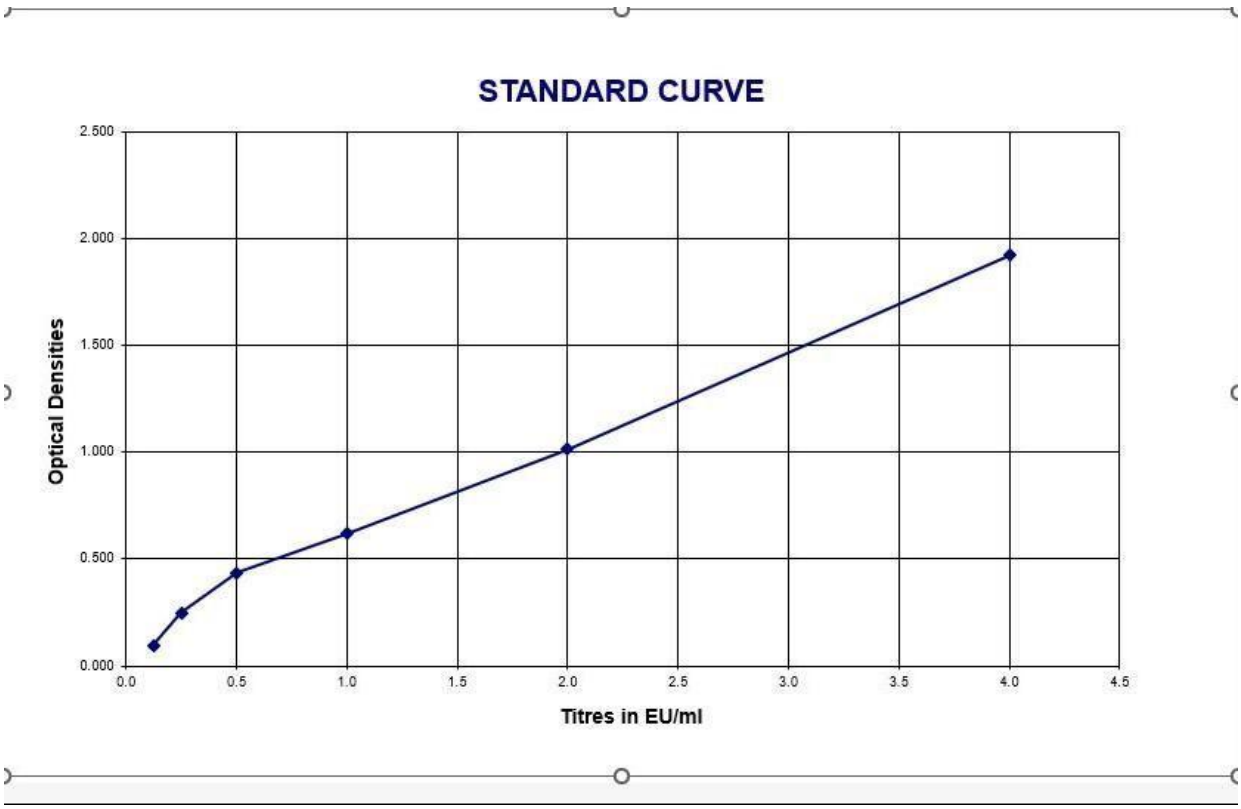


Figure 2.16 Standard curve for quantitative estimation of rabies antibody titre.

The standard curve was established by utilizing diverse dilutions of the quantification standards. Through comparison of the OD values derived from unknown samples against positive controls, it became possible to determine serum titer values. The direct reading taken from the standard curve corresponded accurately with quantified sera titer, represented as equivalent units per ml (EU/ml). Calculation of titer values employed a conversion software tool provided for this purpose. Results were then categorized into four groups: high sero-conversion, sufficient sero-conversion, insufficient sero-conversion and undetectable sero-conversion at respective titer values of 4, 0.5-4, 0.125-0.5 and 0.125 EU/ml respectively.

Table 2.6: Conditions of validation for quantitative determination.

Criteria	**Validation**
OD R3(i) < 0.05	The absorbance of each respective negative control should register below 0.05. **In case any value falls outside of this threshold, it is essential to repeat the test.**
0.300 ≤ OD R4a(i) ≤ 1.200	Each specific optical density value for the R4a positive control should fall within the range of 0.300 to 1.200. **If any of the R4a OD values deviate from this range, it is imperative to conduct a reiteration of the test.**
S1< S2< S3< S4< S5< S6	Compute the average optical density (OD) for S1 through S6 using the subsequent approach: For S1, calculate the mean of the two ODs associated with S1 (equivalent to 0.125 EU/ml). For S2, determine the mean of the two ODs linked to S2 (equivalent to 0.25 EU/ml), and so forth. It is important to note that the signal strengths of the standards should exhibit the following progression: S1 < S2 < S3 < S4 < S5 < S6.
0.7 ≤ S3/R4a ODs ≤ 1.3	The relationship between the mean of S3 standards (mean S3) and the mean of R4a (mean R4a) should register within the range of 0.7 and 1.3.

2.7.4. Interpretation of the results for quantitative determination:

The established threshold value is equivalent to the mean of the two optical densities for the S3 Quantification standard. This standard corresponds to the sero-conversion threshold value of 0.5 EU/ml, which serves as a reference point for measuring anti-rabies antibodies within a given sample. The measurement process involves comparing the optical density of the sample against a standardized curve and expressing serum titers in Equivalent units per ml (EU/ml), an internationally recognized unit that has

been established through sero-neutralization. An interpretation and conversion utility designed to translate OD values into titers is readily available on the CD accompanying this kit, which Bio-Rad readers can use as a resource (Rabies QT-ELISA Bio-Rad-Vers.200712.A.XLS).

2.7.5 Result Criteria for quantitative analysis:

The optical density measurements were acquired using an ELISA micro-plate reader (Diateck China) at wavelengths of 450 and 620 nm. The rabies antibodies were quantitatively determined by constructing a standard curve from standards S1-S6, prepared by serially diluting R4b calibrated positive controls and expressed in equivalent units per ml (EU/ml). The conversion of OD values into titer values was facilitated through the utilization of the provided conversion tool as outlined in the kit manual. The outcomes were then classified into distinct categories, including high sero-conversion level, sufficient sero-conversion, insufficient sero-conversion level, and undetectable sero-conversion. These categories correspond to titer ranges greater than 4, 0.5-4, 0.125-0.5, and below 0.125 EU/ml, respectively. Should the OD value of the unknown sample surpass the mean Optical Density value for the S6 standard, precise quantification becomes unfeasible. In order to ensure accurate quantification, it is necessary to prepare a further 1/10 dilution or even more of the sample. Subsequently, the assay should be repeated, aiming to attain an Optical Density that aligns within the range of the Standard curve (Table 2.7).

Table 2.7 General comprehension of the results, titer value calculations and result interpretation.

Outcomes in terms of Optical Density for the unidentified sample	**Titer value for the unfamiliar sample (X).**	**Interpretation of the result**
— OD sample > S6	X > 4 EU/ml	Elevated seroconversion level. For an accurate titer determination, it is essential to dilute the sample prior to conducting a repeated assay.
— — S3≤ OD sample ≤ S6	X in EU/ml (0.5 - 4 EU/ml)	Adequate seroconversion level.
— — S1≤ OD sample < S3	X in EU/ml (0.125 - 0.5 EU/ml)	Inadequate seroconversion level as per the PLATELIA™ RABIES II test.
— OD sample < S1	-	Non-detectable seroconversion.

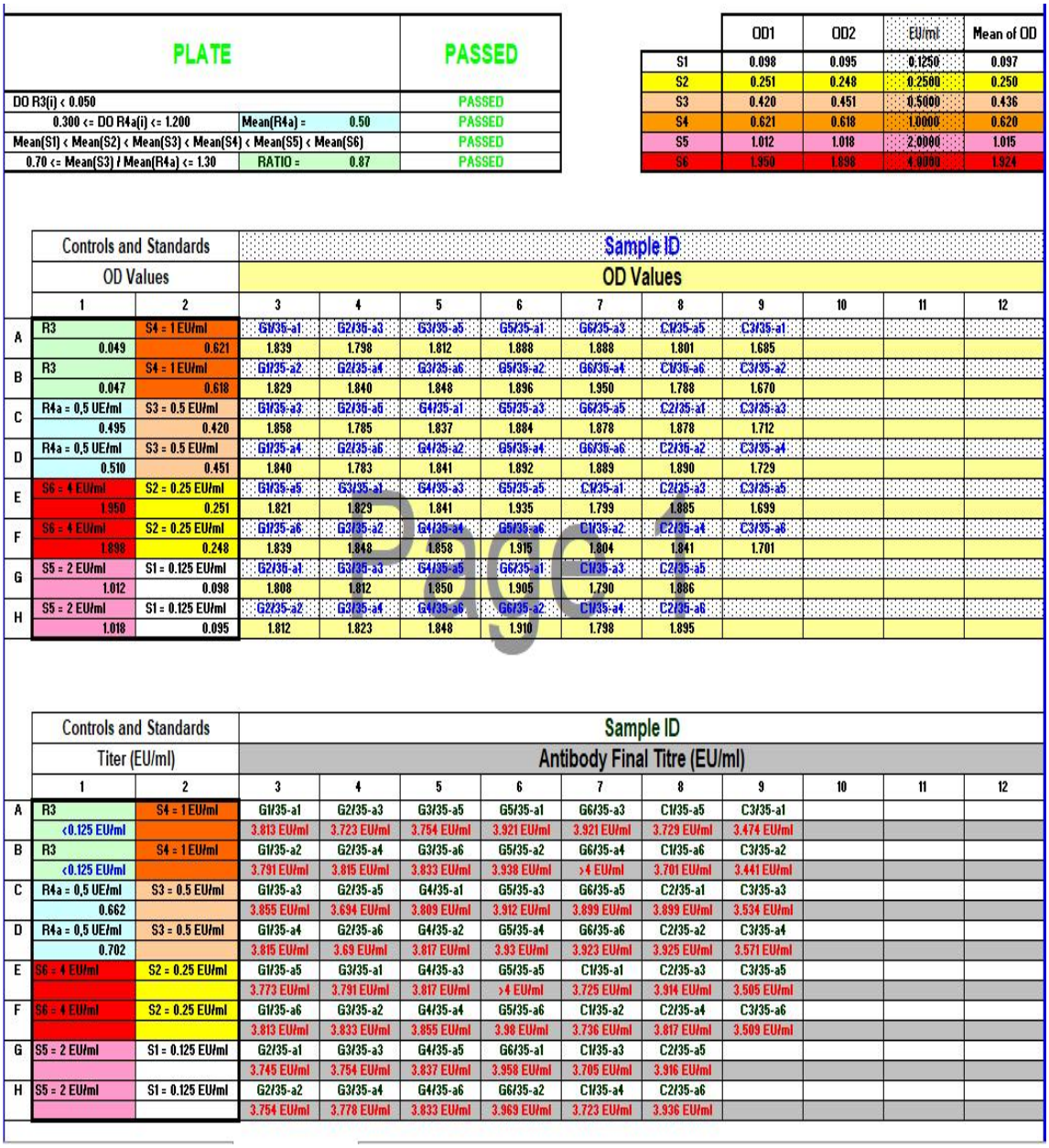


PLATE		PASSED
DO R3(i) < 0.050		PASSED
0.300 <= DO R4a(i) <= 1.200	Mean(R4a) = 0.50	PASSED
Mean(S1) < Mean(S2) < Mean(S3) < Mean(S4) < Mean(S5) < Mean(S6)		PASSED
0.70 <= Mean(S3) / Mean(R4a) <= 1.30	RATIO = 0.87	PASSED

	OD1	OD2	EU/ml	Mean of OD
S1	0.098	0.095	0.1250	0.097
S2	0.251	0.248	0.2500	0.250
S3	0.420	0.451	0.5000	0.436
S4	0.621	0.618	1.0000	0.620
S5	1.012	1.018	2.0000	1.015
S6	1.950	1.898	4.0000	1.924

Controls and Standards		Sample ID									
OD Values		OD Values									
1	2	3	4	5	6	7	8	9	10	11	12
A R3	S4 = 1 EU/ml	G1/35-a1	G2/35-a3	G3/35-a5	G5/35-a1	G6/35-a3	C1/35-a5	C3/35-a1			
0.049	0.621	1.839	1.798	1.812	1.888	1.888	1.801	1.685			
B R3	S4 = 1 EU/ml	G1/35-a2	G2/35-a4	G3/35-a6	G5/35-a2	G6/35-a4	C1/35-a6	C3/35-a2			
0.047	0.618	1.829	1.840	1.848	1.896	1.950	1.788	1.670			
C R4a = 0,5 UE/ml	S3 = 0.5 EU/ml	G1/35-a3	G2/35-a5	G4/35-a1	G5/35-a3	G6/35-a5	C2/35-a1	C3/35-a3			
0.495	0.420	1.858	1.785	1.837	1.884	1.878	1.878	1.712			
D R4a = 0,5 UE/ml	S3 = 0.5 EU/ml	G1/35-a4	G2/35-a6	G4/35-a2	G5/35-a4	G6/35-a6	C2/35-a2	C3/35-a4			
0.510	0.451	1.840	1.783	1.841	1.892	1.889	1.890	1.729			
E S6 = 4 EU/ml	S2 = 0.25 EU/ml	G1/35-a5	G3/35-a1	G4/35-a3	G5/35-a5	C1/35-a1	C2/35-a3	C3/35-a5			
1.950	0.251	1.821	1.829	1.841	1.935	1.799	1.885	1.699			
F S6 = 4 EU/ml	S2 = 0.25 EU/ml	G1/35-a6	G3/35-a2	G4/35-a4	G5/35-a6	C1/35-a2	C2/35-a4	C3/35-a6			
1.898	0.248	1.839	1.848	1.858	1.915	1.804	1.841	1.701			
G S5 = 2 EU/ml	S1 = 0.125 EU/ml	G2/35-a1	G3/35-a3	G4/35-a5	G6/35-a1	C1/35-a3	C2/35-a5				
1.012	0.098	1.808	1.812	1.850	1.905	1.790	1.886				
H S5 = 2 EU/ml	S1 = 0.125 EU/ml	G2/35-a2	G3/35-a4	G4/35-a6	G6/35-a2	C1/35-a4	C2/35-a6				
1.018	0.095	1.812	1.823	1.848	1.910	1.798	1.895				

Controls and Standards		Sample ID									
Titer (EU/ml)		Antibody Final Titre (EU/ml)									
1	2	3	4	5	6	7	8	9	10	11	12
A R3	S4 = 1 EU/ml	G1/35-a1	G2/35-a3	G3/35-a5	G5/35-a1	G6/35-a3	C1/35-a5	C3/35-a1			
<0.125 EU/ml		3.813 EU/ml	3.723 EU/ml	3.754 EU/ml	3.921 EU/ml	3.921 EU/ml	3.729 EU/ml	3.474 EU/ml			
B R3	S4 = 1 EU/ml	G1/35-a2	G2/35-a4	G3/35-a6	G5/35-a2	G6/35-a4	C1/35-a6	C3/35-a2			
<0.125 EU/ml		3.791 EU/ml	3.815 EU/ml	3.833 EU/ml	3.938 EU/ml	>4 EU/ml	3.701 EU/ml	3.441 EU/ml			
C R4a = 0,5 UE/ml	S3 = 0.5 EU/ml	G1/35-a3	G2/35-a5	G4/35-a1	G5/35-a3	G6/35-a5	C2/35-a1	C3/35-a3			
0.662		3.855 EU/ml	3.694 EU/ml	3.809 EU/ml	3.912 EU/ml	3.899 EU/ml	3.899 EU/ml	3.534 EU/ml			
D R4a = 0,5 UE/ml	S3 = 0.5 EU/ml	G1/35-a4	G2/35-a6	G4/35-a2	G5/35-a4	G6/35-a6	C2/35-a2	C3/35-a4			
0.702		3.815 EU/ml	3.69 EU/ml	3.817 EU/ml	3.93 EU/ml	3.923 EU/ml	3.925 EU/ml	3.571 EU/ml			
E S6 = 4 EU/ml	S2 = 0.25 EU/ml	G1/35-a5	G3/35-a1	G4/35-a3	G5/35-a5	C1/35-a1	C2/35-a3	C3/35-a5			
		3.773 EU/ml	3.791 EU/ml	3.817 EU/ml	>4 EU/ml	3.725 EU/ml	3.914 EU/ml	3.505 EU/ml			
F S6 = 4 EU/ml	S2 = 0.25 EU/ml	G1/35-a6	G3/35-a2	G4/35-a4	G5/35-a6	C1/35-a2	C2/35-a4	C3/35-a6			
		3.813 EU/ml	3.833 EU/ml	3.855 EU/ml	3.98 EU/ml	3.736 EU/ml	3.817 EU/ml	3.509 EU/ml			
G S5 = 2 EU/ml	S1 = 0.125 EU/ml	G2/35-a1	G3/35-a3	G4/35-a5	G6/35-a1	C1/35-a3	C2/35-a5				
		3.745 EU/ml	3.754 EU/ml	3.837 EU/ml	3.958 EU/ml	3.705 EU/ml	3.916 EU/ml				
H S5 = 2 EU/ml	S1 = 0.125 EU/ml	G2/35-a2	G3/35-a4	G4/35-a6	G6/35-a2	C1/35-a4	C2/35-a6				
		3.754 EU/ml	3.778 EU/ml	3.833 EU/ml	3.969 EU/ml	3.723 EU/ml	3.936 EU/ml				

Figure 2.17 Bio-Rad Platellia II Outcome Excel Spreadsheet Indirect enzyme- linked immuno-sorbent assay (ELISA) Excel spreadsheet for deriving endpoint titer outcomes, quantified in EU/mL (Equivalent Units), across all serum samples. Optical density (OD) values are to be provided (entered into the yellow fields) on the upper sheet, along with corresponding sample IDs (filled in gray fields). Through proprietary calculations, the spreadsheet evaluates the criteria defined by the kit standards and generates a standard curve. This curve serves as the basis for producing a quantitative titer result (EU/mL) for each individual sample.

2.8 Blood collection, plasma separation & pooling/ Evaluation of the Blood Parameters and

Fifty-four male New Zealand white rabbits grouped per different diets were immunized with cell culture rabies vaccine. After completing the immunization schedule, animals were bled, hyper-immune blood was collected, and plasma was separated after centrifugation at 2000 rpm for 15 min. The pool of plasma was stored at 2 to 8 °C until further processing. Downstream process flow for purification of poly-clonal antibodies from rabbit serum is described in Figure 2.5. Blood samples (approximately 2-3ml) were collected from each rabbit's marginal ear vein, adhering to established protocols, on four specific days: day 0, day 14, day 35, and day 60. The meticulously obtained blood was then divided into two distinct types of tubes, each containing appropriate anticoagulants (EDTA and heparin).Fifty-four male New Zealand white rabbits grouped per different diets were immunized with cell culture rabies vaccine. After completing the immunization schedule, animals were bled, hyper-immune blood was collected, and plasma was separated after centrifugation at 2000 rpm for 15 min. The pool of plasma was stored at 2 to 8 °C until further processing. Downstream process flow for purification of poly-clonal antibodies from rabbit serum is described in Figure 2.5.

2.8.1 Hematological Parameters evaluation

To assess primary hematological indicators including red blood cells (RBC), hemoglobin (HGB), hematocrit (Ht), white blood cells (WBC), lymphocytes (LYMPHO6=), monocytes (MONO6=), neutrophils (NEUT6=), eosinophils (EO6=), and basophils (BASO6=), blood samples were collected into EDTA (EthylDiamineTetrAcetate) tubes. Following collection, the tubes were carefully transferred to the laboratory for subsequent analysis, with measures taken to uphold the cold chain by placing them in a cooling box. The determination of the various hematological parameters was conducted utilizing an automated analyzer, the Shenzhen Mindray BC3000 plus.

2.8.3 Biochemical Parameters estimation

Biochemical assessments were executed to ascertain fundamental parameters

encompassing glucose (GLU), total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), total proteins (TP), albumin (ALB), urea, creatinine (CREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), iron (Fe), calcium (Ca), phosphorus (P), sodium (Na), and potassium (K). Blood samples were collected in Heparin tubes, subsequently transported in cooling boxes to a laboratory refrigerator for further handling. The collected blood samples were then subjected to centrifugation (1500 rpm/15 min) in order to extract plasma. The resulting plasma was carefully separated and stored at −20 °C until it was ready for subsequent analysis. The assessments were conducted using an automated analyzer, specifically the AUTOLAB AMS.

2.9 Downstream processing with Caprylic acid fractionation technique for further purification of antibodies from pooled plasma and rabies antiserum preparation

The caprylic acid fractionation of anti-sera was conducted following the method outlined by Rojas et al., 1994. The standardization of the caprylic acid fractionation approach was initially executed, focusing on three key variables: concentration, pH, and stirring time, carried out at the pilot scale. For the purification procedure, small portions of pooled plasma were employed. In essence, caprylic acid (Merck, Germany) was introduced into a reaction mixture containing 30 ml of pooled plasma from distinct groups, with a concentration of 5% (v/v). The mixture was subject to vigorous stirring utilizing a magnetic stirrer (Thermo Fisher Scientific; Waltham, MA, USA) at an ambient temperature of 22-25℃ for a duration of 60 minutes. Subsequently, the aliquots underwent filtration through Whatman filter paper (No.1), and the resulting filtrates were subjected to dialysis using a dialyzing membrane against distilled water. This step was performed to eliminate the added caprylic acid from the ultimate filtrate. The resultant IgG preparations were further concentrated via diafiltration. The purified anti-sera were formulated with 0.9% w/v NaCl and 0.25% w/v phenol, and the pH was adjusted to 7.0. The mixture was subsequently sterilized through filtration using a 0.22 mm pore membrane (Minisart, Sartorius, Germany) and dispensed into 10 ml sterile glass vials. These samples were stored in a refrigerator at 2-8°C for subsequent qualitative analysis (Figure 2.19, 2.20).

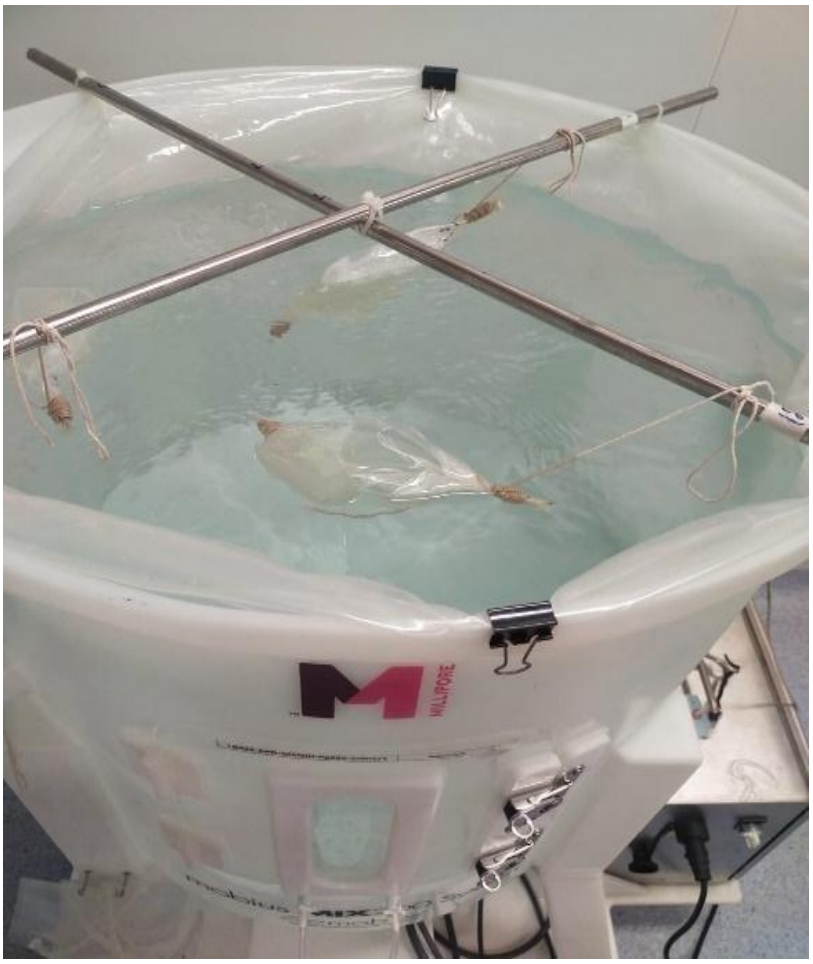


Figure 2.18 Dialysis process against W.F.I for removal of small molecules.

Dialysis is performed in large rotatory tanks in which water is rotating continuously. Sample preparation is poured into a molecular porous membrane and hung in rotatory tanks. The rotatory tanks continuously rotate for 48 hours, and smaller molecules like caprylic acid moves out of the membranes by the principle of a concentration gradient.

Figure 2.19 TFF for the purification and concentration of immunoglobulins at large scale.

TFF is an industrial machine that is used for the dia-filtration of immunoglobulins. It works in parallel to remove waste and contamination from the formulation and concentrates the immunoglobulins in the final formulation.

2.9.1. Caprylic acid fractionation of hyper-immune plasma

Caprylic acid fractionation of anti-sera was performed using the method described by Rojas *et al.* 1994. Standardization of caprylic acid fractionation method was initially carried out and 3 key variables were optimized: concentration, pH and stirring time at the pilot scale. Small aliquots of pooled plasma were used for the purification process. Briefly, caprylic acid (Merck, Germany) was added to 30 ml of pooled plasma from different groups to the reaction mixture with 5% (v/v) concentrations. The mixture was stirred vigorously using a magnetic stirrer (Thermo Fisher Scientific; Waltham, MA, USA) at ambient temperature 22-25℃ for 60 minutes. The aliquots were then filtered through Whatman filter paper (No.1), and the filtrates were dialysed using dialysing membrane against distilled water to remove added caprylic acid from the final filtrate. The IgG preparations obtained were concentrated by dia-filtration. Purified anti-sera were formulated with 0.9% w/v NaCl and 0.25% w/v phenol and pH adjusted at 7.0, mixture was sterile filtered through a 0.22 mm pore membrane (Minisart, Sartorius, Germany) and dispensed in 10 ml sterile glass vials. Samples were kept at 2-8°C in the refrigerator for further qualitative analysis.

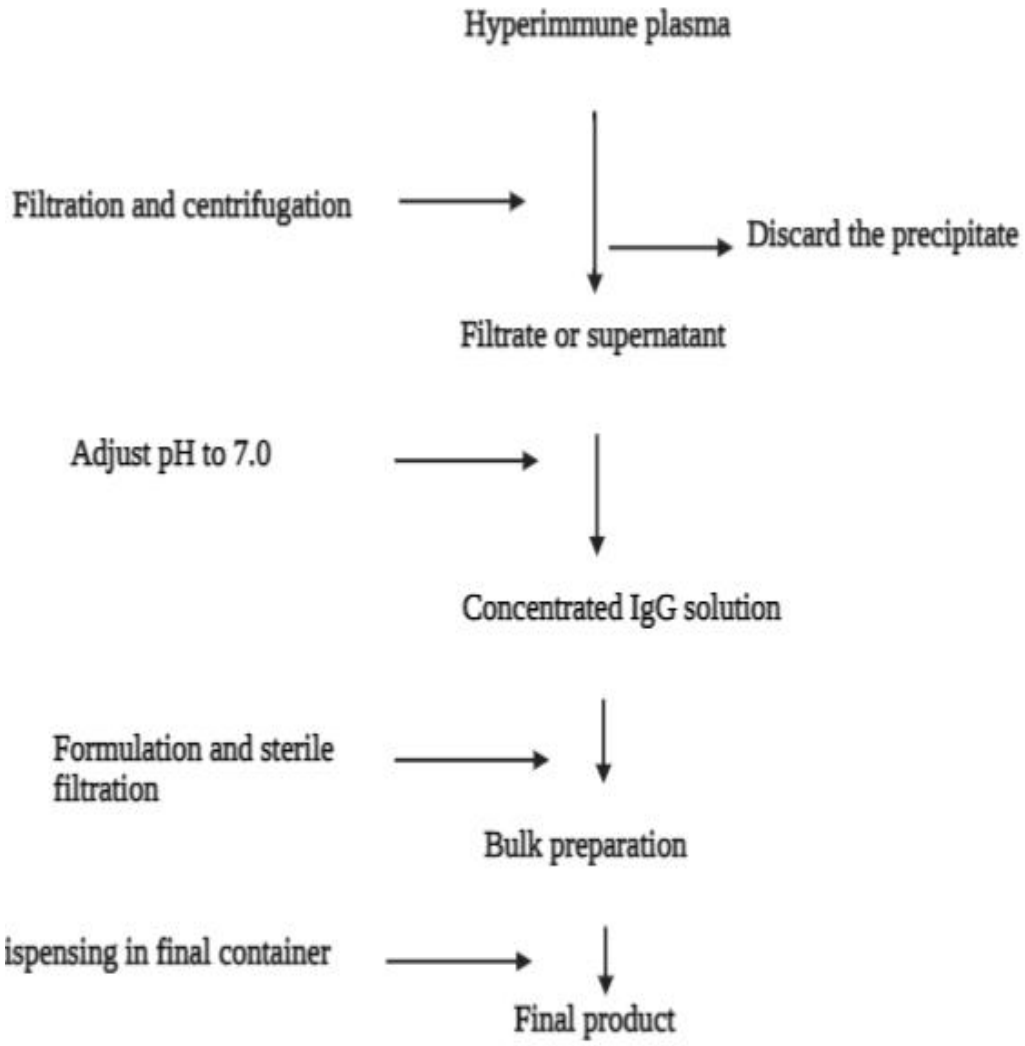


Figure 2.20 Caprylic acid removal methods using filters. The flowchart above shows the several filtering methods that were utilized to take caprylic acid out of the final formulation as well as the HPLC results of each method.

2.9.2 Optimization of physico-chemical parameters with caprylic acid fractionation technique

Pooling of plasma and further purification of RIGs with caprylic acid fractionation method was carried out. Optimization of physico-chemical parameters was done at small scale to get maximum product yield with relatively simple technique as compared to old purification method using salt precipitation. Ammonium sulphate precipitation has been applied since many decades but new Caprylic acid fractionation method has been suggested due to its several advantages, including cost efficacy, less time consuming, simper than multistep salt precipitation method etc.

2.9.2.1 Caprylic acid concentration optimization

To prepare aliquots of 100 ml of hyperimmune horse plasma, 1.76 N acetic acid was added to reach a pH of 5.8, as recommended by McKinney & Parkinson (1987). The final concentrations of caprylic acid were then adjusted to 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10% (v/v) by slowly adding it with constant stirring, followed by a 1-hour stir at room temperature. The mixtures were then filtered through Whatman-2 V paper and dialyzed for 48 hours against WFI. The immunoglobulins were then concentrated using TFF. The solutions were filtered through Whatman 2 V filter paper again after adding 0.15 M NaCl, and their pH was adjusted to 7.2 by adding 1 N NaOH (Rojas et al., 1994).

pH optimization

50 ml portions of hyperimmune plasma were prepare and their pH was adjusted using 1.76 N acetic acid to levels of 4.0, 5.0, 5.5, 5.8, 6.0, 6.5, and 7.0. The plasma was then fractionated by slowly adding caprylic acid to reach a final concentration of 5%.

Optimization of stirring time

Sample of 50 ml of hyper-immune plasma was obtained and its pH was altered to 5.8 through the addition of 1.76 N acetic acid. The plasma was then fortified with caprylic acid to reach a final concentration of 5%. The mixture was stirred intensively for 30 minutes, 45 minutes, and 60 minutes to establish a standardized stirring duration.

Optimization of stirring speed

To standardize the stirring intensity, samples were mixed for 60 minutes at four different speeds: 4/10, 6/10, 8/10, and 10/10. The remainder of the fractionation was completed as indicated.

2.10 Procedure for testing caprylic acid in anti-serum using reverse phase HPLC

The following are the typical steps for utilizing High-Performance Liquid Chromatography (HPLC) to evaluate the amount of caprylic acid in anti-serum: sample getting ready in order to prepare the stinger sample, it must be diluted in a suitable solvent (acetonitrile in this study) and filtered to eliminate any impurities.

Sample preparation:

The antivenom sample needs to be prepared by diluting it in a suitable solvent (acetonitrile in this research) and filtering it to remove any particulate matter.

Calibration: To generate a calibration curve, a series of standards for caprylic acid with known concentrations is prepared. The concentrated activity of caprylic acid in the anti-serum sample will be found using this curve**.**

Chromatographic separation**:** After the mixture's components were separated based on their chemical and physical properties, it was time to inject the extracted anti serum specimen and the caprylic acid (CA) standards into the HPLC system.

Caprylic acid can be found in a chromatogram using a UV-Visible detector, which is a suitable detector for the HPLC system.

Data analysis: The amount of the caprylic acid in the antivenom sample is determined by analysing the chromatogram that was produced by the HPLC analysis. The area beneath the peak corresponding to caprylic acid can be calculated by comparing the results of the chromatogram to the calibration spectrum.

Standard preparation

To start, a stock solution of caprylic acid was created at a concentration of 1000 µg/ml using a combination of Acetonitrile and distilled water in a 60:40 ratio. This stock solution developed several lower concentrations of caprylic acid standards, including 50, 25, 12.5, and 6.25 µg/ml.

S*ample preparation*

A solvent-solvent extraction method was used to prepare the serum samples for analysis. A total of 400 µl of the serum sample was combined with 600 µl of acetonitrile, followed by centrifugation for 5 minutes at 14000 rpm. The resulting clear supernatant was carefully gathered and set aside for subsequent analysis. It is imperative to bear in mind that the accuracy of the outcomes hinges on a variety of factors, encompassing sample preparation protocols, solvent choices, detector sensitivity, and the precision of the calibration curve. *A method was devised to use RP-HPLC to ascertain the presence and content of caprylic acid in samples of serum in order to conduct these research projects.*

Typical execution

A 1000 g/ml stock solution of caprylic acid was originally created using acetonitrile and distilled water in a 60:40 ratio. Standards for caprylic acid were created using this stock solution at lower levels of fifty, twenty-five 12,5, and 6.25 g/ml.

Methodology

The RP-HPLC analysis was conducted using Shimadzu's instrumentation, including the SCL-10AVP controller, DGU-14A degasser, FCV-10ALVP low-pressure mixer, LC-

10AT VP pump, and 3D-PDA detector (SPD-M10A VP). Software called LC Solution was used to examine the data. The Agilent zorbax C8 column with a size of 4.6 250 mm and a particle size of 5 m was employed in the analysis. The ratio of acetonitrile to distilled water in the mobile phase was 60:40, with a flow rate of 1.0 ml/min and an injection volume of 50 l. With a runtime of 10 minutes, the caprylic acid was discovered at a retention duration of 5.4 minutes and a wavelength of 210 nm.

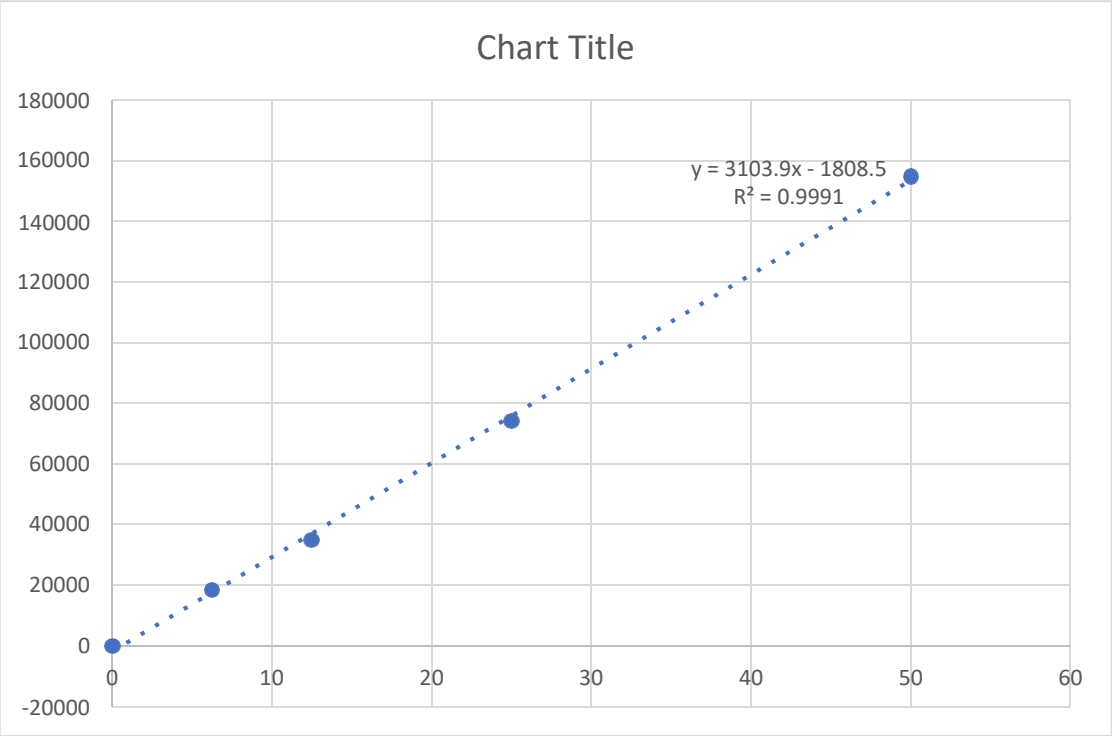


Figure 2.21. Measurement curve. Plotting several standard solution volumes against the area under the curve yields the calibration curve. The process's veracity is indicated by the value of R2 1.

2.11 Quality control test analysis of purified anti-sera after pre-filtration and dialysis

Following tests, analysis was performed on both samples to compare the fractionation methods. The evaluation of the purified anti-sera's quality was performed in accordance with the guidelines established by the institution for animal studies and following recommendations of WHO and British Pharmacopoeia Vol. II, Ed. 1999. Main tests include pH determination, total protein, albumin, total Nitrogen, total solids, general Safety test, and *in vivo* potency tests using mouse neutralisation test (MNT) as described for rabies immunoglobulins.

2.11.1 General Safety Test

This test is based on observations after intervention. The laboratory animals (mice and guinea pigs) were injected via intraperitoneal route, and the observed for 7 days as described n pharmacopoeia.

2.11.2 Total concentration of protein

The final formulation's concentration of all proteins is determined using the Biuret approach (Gornall et al., 1949). Using an ultraviolet (UV) spectrophotometer to measure the absorbing capacity of the anti-serum solution, the overall protein content of an anti-serum is determined. To do this, the antivenom is diluted in a Repa lysis buffering solution, and its absorption at 280 nm is measured after each dilution.

The turbidity and aggregates of protein measurement

To assess turbulence, the absorbance at 600 nm was determined using an UltraSPARC 2000 spectrophotometer (Rojas et al., 1994). The evaluation of protein aggregates was conducted by subjecting a 2 ml sample of each anti-sera to a Sepharose 6B column (83 x 2 cm). Prior to column usage, equilibration was achieved using a phosphate-buffered saline mixture with a pH of 7.2. Molecular weight standards, including bovine serum albumin (68,000 Da), bovine IgG (150,000 Da), and dextran blue (2,000,000 Da), were employed. Elution profiles were generated by measuring the

absorbance at 280 nm (Deyl, 1990).

2.11.3 Albumin concentration test

Anti-serum albumin concentration is assessed by SDS-PAGE. This method separate proteins according to their size and weight using an electric current. Larger proteins, like albumin, move more slowly and can be distinguished on the gel by distinct bands. By comparing the intensity of the band to established norms, the albumin concentration in the anti-serum can be determined.

2.11.4 Test for total solids content

Anti-serum's total solids content can be found by gravimetric analysis. To do this, weigh a sample of the anti-serum both before and after drying, then take the dried weight out of the equation. The total solids content of the anti-serum is represented by the difference between the two weights.

2.11.5 Abnormal toxicology test

To determine the efficacy of anti serum, investigators normally employ an array of tests that measure its toxicity, including chemical-based and biological assays, animal studies, and *in vitro* studies. These tests assess the number of different elements in the anti-serum, such as immune globulin and enzymes, as well as their potential to cause reactions that are undesirable.

2.11.6 Turbidity analysis

The quantity of light obstructed by particles cancelled in the anti-serum was measured using spectrophotometry at 600 nm in the present study's evaluation, while light obscuration measured represents the quantity of light blocked by particles that are too tiny to be determined using spectrophotometry.

2.11.7 Potency determination using MNT (Mouse neutralization *In-vivo* test)

Rabies neutralizing antibodies were also measured by using MNT protocols as per NIH method for detection of rabies virus specific neutralization antibodies and SOP for potency test determination of anti-rabies serum and immunoglobulins, as described in WHO reference book "Lab tech in Rabies" 4th Ed. 1996. The test comprises of 3 major steps.

a. Antigen preparation.
b. Serum-virus neutralization (Antigen-antibody nutarization).
c. Inoculation of serum-virus mixture into mice observation and interpretation of result.

a. Antigen preparation:

The CVS, which was stored at -80°C as a 20% [w/v] mouse brain homogenate, underwent rapid thawing in cold running tap water. Subsequently, two serial 10-fold dilutions (1/10 and 1/100) were performed utilizing an ice-cold diluent such as sterile PBS with 2% equine serum. Swiss albino mice were divided into three groups (n = 10), and each group received intracerebral inoculation of the diluted inoculum containing 30 µL; one control group was administered only diluent. The inoculated mice were observed daily for any indications of rabies over a period of fourteen to twenty-one days. Mice exhibiting symptoms of rabies were euthanized in an aseptic manner and their brains collected in sterile tubes before being centrifuged for further processing. A glass homogenizer was used to prepare the (20% w/v) brain tissue homogenate which was subsequently clarified by refrigerated centrifugation at 200 × g for ten minutes at 10 °C to minimize thermal virus inactivation. Aliquots of the clarified brain tissue homogenate were then stored at -80 °C in sterile cryovials. Finally, titration

experiments on mice determined the median mouse intracerebral lethal dose (MICLD50) for amplification of CVS-24 stock virus.

Serum-virus neutralization (Titration of RABV):

Brief methodology was described as follows: Serial two-fold dilution of test sera and standards were prepared using PBS buffer (pH7.3). To diluted serum added challenge virus standard (CVS) in equal quantity. LD50/0.03ml). the mixture was incubated for 90min in incubator at 37°C temp to speed up the neutralization process place Swiss albino mice in groups in properly labeled cages. The animals were immunized intra-cerebrally by trained technician of NIH. Observe the inoculated mice daily for 2 week (14 days) for any symptoms of rabies like paralysis of hind limbs etc. the mortality of mice before one week was not considered in the results as it may be cause by other reasons such as needle injury or by faulty techniques.

Thaw a vial of CVS 24 amplified by mouse brain passage expeditiously in cold running tap water. Execute eight consecutive 10-fold dilutions of challenge virus using ice-cold diluent. Employ calibrated pipettes and dedicated sterile tips for each aspiration, taking care to dispense the content without contacting the diluent. Assign nine groups of weanling Swiss albino mice (group size = 10) and inoculate them with an IC dose of inoculum containing various concentrations of RABV and diluent alone. Including a control group that receives only the diluent can help ascertain whether nonspecific death is caused by one or more components within it. Using a disposable syringe with a needle gauge ranging from 26-29 may facilitate precise dispensing of 30
µL of inoculum without causing excessive trauma to the subject mice. Observe the inoculated mice daily as previously mentioned, recording symptoms such as hind limb paralysis or sudden deaths; euthanasia upon first definitive signs is strongly advised. Calculate the titre value for RABV via determining its MICLD50 /30 µL using Reed-Muench method's fifty percent end/point calculation technique for accurate results interpretation purposes.

Neutralization of RABV by antibodies

1. Whole blood samples was maintained at ambient temperature to facilitate optimal clotting and serum separation while preventing hemolysis. Employ

centrifugation at 200 × g for 10 minutes at a controlled temperature of 10 °C to isolate the sera from clotted blood samples, followed by complement inactivation via incubation at 56 °C for a duration of 30 minutes.

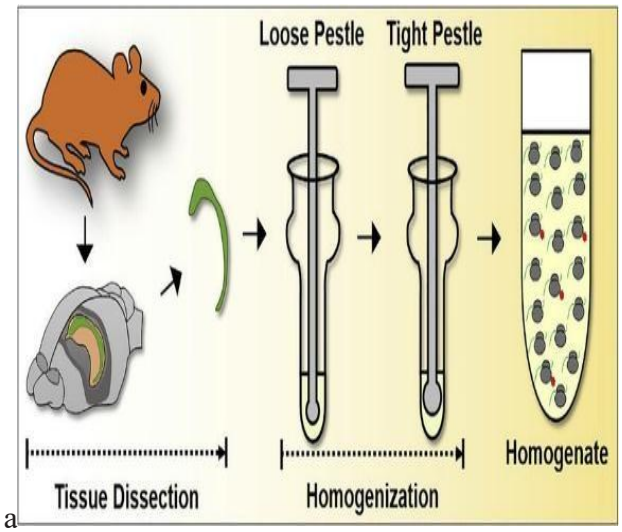
2. A series of five 5-fold dilutions was conducted on sera samples, starting from a ratio of 1:2.5, utilizing an ice-cold diluent and ensuring that the samples remain chilled on wet ice until utilized.

- To prepare a challenge virus of known titre, the virus must be thawed in cold running tap water and serial dilutions performed to achieve 100 MICLD50 per 30 µL. Diluting the stock 10–5.5 times will result in 1 MICLD50 per 30 µL, while diluting it 3162 times will result in 100 MICLD50 per 30 µL. Three serial 10-fold dilutions should be performed, followed by mixing the diluted virus with the diluent to prepare enough volume of working virus stock. Precision in dilution and calculation are critical for preparing the right dose of challenge virus, and the percentage mortality caused by back titrated virus will indicate accuracy of dose.

3. Combine Equivalent amounts of thinned sera (initiating dilution, 1:2.5) and challenge virus (100 MICLD50/30 µL). were combined and incubated at a temperature of 37°C for an hour and a half with periodic agitation to facilitate neutralization. Positive serum was incorporated with a recognized titre as a benchmark. The eventual dose of challenge virus will be 50 MICLD50 in every dilution, while the serum concentration will be diluted to 1:5.
4. A serial dilution was performed on the challenge virus with an initial concentration of 100 MICLD50 per 30 µL to obtain concentrations of 50 MICLD50, 10 MICLD50, 1 MICLD50, and 0.1 MICLD50. Following this, they were incubated the back-titrated challenge virus together with serum-virus mixtures at a temperature of 37 °C for a duration of 90 minutes while intermittently shaking during the neutralization process to ensure consistent treatment.
5. The serum–virus mixtures and diluted virus stocks were kept in cold immediately on wet ice until used for inoculation.
6. 15 sets of ten weaning Swiss albino mice as needed and administer 30 µL of

serum-virus mixtures were allocated, diluted virus stocks, and diluent alone to the corresponding group via the IC route. Preheating the inoculum to room temperature prior to IC inoculation can prevent cold-induced shock and subsequent mortality in mice. Utilize a specialized syringe and needle for administering each virus dilution per individual animal.

7. The inoculated mice were monitored, and signs were recorded, such as hind limb paralysis, as mentioned above. Nonspecific deaths may be due to trauma caused by needles, faulty technique, etc.
8. The percentage of mice which survived or became ill/died in various groups at the end of the observation period was determined.
9. iSigns or death of mice after 4 days of inoculation is usually considered as RABV-specific, which may be confirmed by DFAT as per the standard protocol. (Dean & Abelseth, 1973).



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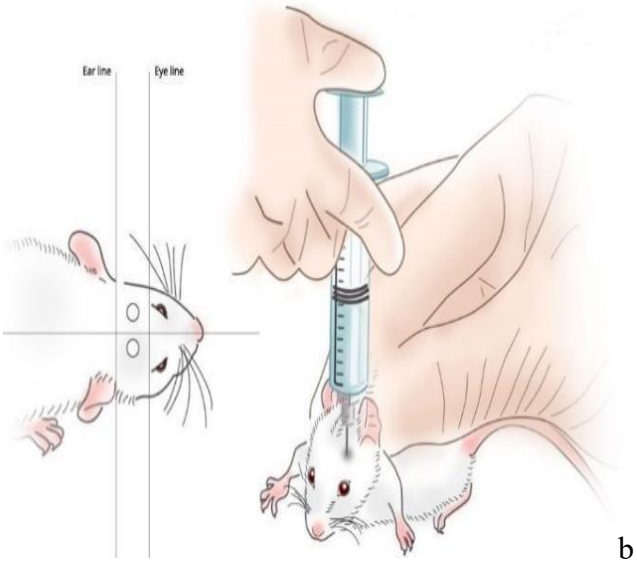


b

Figure 2.22 a) Antigen homogenate preparation procedure for MNT test, b)Intra-cerebral route for mice inoculation.

Analyses and interpretation of MNT results:

The immunogenicity study aimed to evaluate the impact of probiotics and supplementary dietary intake on rabies vaccine-specific humoral response. Statistical analysis was conducted to determine the results, with antibody titer calculated using the MNT method in accordance with Reed and Muench's (1935) classical statistical approach.

End point titration method, Reed and Muench method

Generally, the following formula is used to calculate "difference of logarithms" (difference of logarithms is also known as "proportionate distance" or "interpolated value"):

Difference of logarithms = [(mortality at dilution next above 50%)-50%]/[(mortality next above 50%)-(mortality next below 50%)].

Calculation of virus titer in mice using the Reed and Muench method:

Log10 virus dilution	Mice		Cumulative total			Percent mortality
	Died	Survived	Died	Survived	Total	
-1	10	0	57	0	57	57/57 × 100 = 100
-2	10	0	47	0	47	47/47 × 100 = 100
-3	10	0	37	0	37	37/37 × 100 = 100
-4	10	0	27	0	27	27/27 × 100 = 100
-5	10	0	17	0	17	17/17 × 100 = 100
-6	6	4	7	4	1	17/11 × 100 = 63
-7	1	9	1	13	14	1/14 × 100 = 7

Difference of logarithms = (63-50)/(63-7) = 0.23; log10 50% end point dilution = -6 - (0.23 × 1) = -6.23; 50% end point

Dilution = 10-6.23; the titre of the virus = 106.23 LD50/mL

The study attempts to evaluate the residual caprylic acid concentration in samples of purified immunoglobulins from three different separation processes, namely centrifugation followed by pre-filtration and dialysis and finally ultra-filtration using small scale tangential flow filtration apparatus (TFF).

2.12 Statistical Analysis

The data collected was analyzed using Graph Pad Prism (version 9) software. The effects of different treatments, including rabies vaccination, dietary supplementation with probiotics, and vitamin A, were evaluated through a one-way analysis of variance (ANOVA) in New Zealand white rabbits. Additionally, potential interactions between the treatments were examined. Tukey's post hoc test was then performed to identify any discrepancies between the control group and the various treatments. A probability level of $p=<0.001$ with a confidence interval of 95% was used to determine statistical significance for these effects.

CHAPTER 03 RESULTS

OBJECTIVE # 1

OVERALL GROWTH AND GENERAL HEALTH IMPROVEMENTS

3.1 Determination of physical and clinical parameters

Mortality and physical fitness observations: Animals were kept under observation and strict supervision of veterinary physician of animal house of NIH for mortality on daily basis or physical fitness during experiment. Detailed checkups were conducted on weekly basis on days 0,7,14 and 35 as per standard operating procedures (SOP) till completion of experiments. Animals were found active throughout the experiments.

3.1.1 Estimation of animal growth and Clinical parameters observations:

Basic laboratory parameters such as ALT, ALP, Urea and creatinine were also observed during experiment on days 0,14 and 35 keeping in view the safety aspects on health of animals. The experiments were run on Cobas C311 machine using Roche standardized kits as per manufacturer's instructions (Tables 3.1 & 3.2). After 14 days the bilirubin, ALT and ALP levels were found within the limits of reference range except two control groups C1A and C3A which showed slightly higher values. There was an increase trend in urea levels in all treatment and control groups from the reference. The range observed was between 37-128, while creatinine was within the limit in all the cases (Table 3.1). After 35 days data shows that all the clinical parameters were within the range and animals were healthy (Table 3.2).

3.1.2 Growth Performance Parameters &Weight Measurements

The live body weight of the rabbits was determined using digital weighing balance on weekly basis in the morning as described in earlier studies. Growth performance of rabbits were evaluated by measuring various parameters, including weight gain, specific growth rate, feed conversion ratio, and food conversion efficiency. The results are shown in table 3.3 as follows:

Weight gain for each group (Group 1, Group 2, Group 3, Group 4, Group 5, Group 6, and Control Group) ranged from 37.35 to 87.10, with Group 3 rabbits exhibiting the

highest weight gain. The specific growth rate for each group ranged from 8.70 to 20.4, with Group 3 rabbits exhibiting the highest specific growth rate. The feed conversion ratio for each group ranged from 7.8 to 17.54, with Group 1 rabbits exhibiting the highest feed conversion ratio. While the food conversion efficiency for each group ranged from 0.06 to 0.13, with Group 4 and Group 5 exhibiting the highest food conversion efficiency at 0.1. Overall, the study highlights the impact of dietary interventions on growth, general health of animals that results in better immunity and hence highlighting the importance of monitoring these growth performance parameters (Table 3.3; Figure 3.1).

3.1.3 Microbiological Analysis of Fecal Samples for Bacterial Identification through Culture (Total Viable Count calculations)

The total viable count for each group was measured on day 0, day 14, and day 35. At day 0, the total viable count of each group was found to be $2.5x10^6$, $4x10^4$, $3x10^4$, $4.2x10^4$, $5.8x10^4$, and $2.23x10^2$, for groups G-1, G-2, G-3, G-4, G-5, G-6, and Control Group, respectively. Group G-6 demonstrated the highest total viable counts, with a value of $5.8x10^4$ (Table 3.4 a).

On day 14, the total viable count for each group was measured again, and the values were found to be $2.8x10^3$, $5.2x10^4$, $5.3x10^4$, $3.8x10^4$, $4x10^4$, $5.6x10^4$, and $1.8x10^3$ for G-1, G-2, G-3, G-4, G-5, G-6, and the Control Group, respectively. Group G-6 demonstrated the highest number of total viable counts, with a value of $5.6x10^4$ (Table 3.4 b). Similarly at day 35, the total viable count was measured once again, and the values for each group were found to be $5.9x10^4$, $6.8x10^4$, $4.2x10^2$, $3.2x10^2$, $6x10^3$, $6.3x10^4$, and 5x104 for G-1, G-2, G-3, G-4, G-5, G-6, and Control group,respectively. Group G-2 demonstrated the highest total viable counts, with a value of $6.8x10^4$ (Table 3.4 c).

Table 3.1 Clinical pathology of experimental and control groups from pooled serum collected at day 14 in duplicate.

ANIMAL ID	BILIRUBIN (MG/DL)	ALT (IU/L)	ALP (IU/L)	UREA(MG/DL)	CREATI NINE (MG/DL)
Reference Range	**0-0.7**	**45-80**	**12-96**	**20-45**	**0.5-2.5**
C1A	0.15	47	103	37	0.65
C1B	0.15	50	50	74	0.67
C2A	0.15	75	37	50	0.73
C2B	0.15	78	50	64	0.76
C3A	0.15	67	104	32	0.80
C3B	0.15	61	49	55	0.81
G1A	0.15	53	54	65	0.89
G1B	0.15	61	66	55	0.85
G2A	0.15	79	29	60	0.88
G2B	0.15	80	61	92	0.91
G3A	0.15	54	80	75	0.70
G3B	0.15	62	74	74	0.76
G4A	0.15	66	38	74	0.83
G4B	0.15	61	57	128	0.84
G5A	0.15	76	28	67	0.62
G5B	0.15	79	29	74	0.66
G6A	0.15	68	62	75	0.70
G6B	0.17	66	43	281	0.73

Table 3.2 Clinical pathology of experimental and control groups from pooled serum collected at day 35 in duplicate.

ANIMAL ID	BILIRUBIN (MG/DL)	ALT(IU/L)	ALP(IU/L)	UREA(MG/DL)	CREATININE (MG/DL)
Reference range	**0-0.7**	**45-80**	**12-96**	**20-45**	**0.5-2.5**
C1A	0.14	36	51	36	0.62
C1B	0.14	28	48	34	0.57
C2A	0.13	77	46	48	0.83
C2B	0.15	79	48	43	0.86
C3A	0.14	65	42	31	0.87
C3B	0.14	60	49	33	0.81
G1A	0.14	73	61	42	0.90
G1B	0.14	76	63	39	0.85
G2A	0.14	65	57	38	0.88
G2B	0.14	63	60	36	0.91
G3A	0.13	50	53	33	0.90
G3B	0.14	54	55	31	0.86
G4A	0.14	62	36	30	0.91
G4B	0.13	58	35	36	0.71
G5A	0.14	73	27	35	0.60
G5B	0.15	76	28	33	0.63
G6A	0.14	65	61	32	0.68
G6B	0.16	61	57	36	0.52

Table 3.3 Rabbits growth performance and food conversion ratio comparison.

S.N	Parameters	G1	G2	G3	G4	G5	G6	Control G 7
1.	Weight Gain (%)	37.35	70.50	87.10	75.00	63.20	47.50	39.45
2.	Specific Growth Rate (%)	8.70	15.12	17.36	15.70	13.70	10.92	20.4
3.	Feed Conversion Ratio	17.54	9.5	7.8	9.01	9.7	13.16	7.7
4.	Feed Conversion Efficiency	0.06	0.11	0.13	0.1	0.1	0.08	0.12

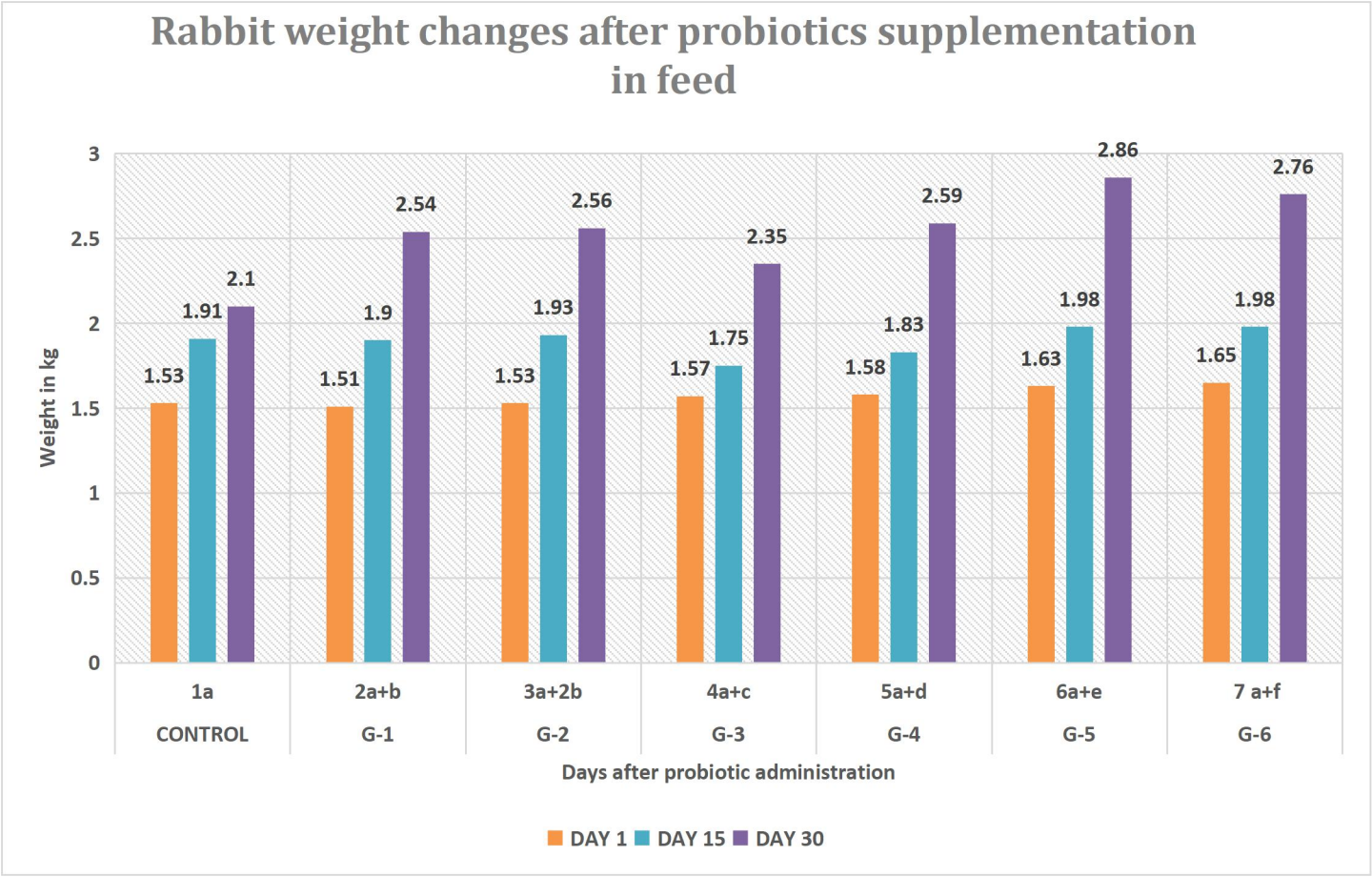


Figure 3.1 Improvement in growth was observed gradually from day 1 after starting supplementation till day 30th. a=Basal diet, a+b=Basal diet + Probiotic-1 (EQ), a+2b=Basal diet + Probiotic-2(PPZ),a+c=Basal diet + Probiotic-3 (TF,1 Packet/Day), a+d=Basal diet + Lab strain (LB),a+e=Basal diet + commercial Strain with O.C ,a+f=Basal diet + Local strain with O.C(organic carrots).

Table 3.4 Fecal analysis & Enumeration of bacteria in feces of rabbit before dietary interventions **at day 0.**

Samples	E.coli	Salmonella	Staphylococci	Entero-bacter	Coli-form	TVC
G-1	$2.5x10^6$	Nil	$4x10^3$	$3.7x10^5$	$5.3x10^4$	$2.5x10^4$
G-2	$1.5x10^2$	Nil	$1.8x10^4$	$2.5x10^2$	2.2×10^1	$4x10^4$
G-3	$5.3x10^2$	Nil	$2x10^1$	$4.7x10^5$	$1.9x10^1$	$3x10^4$
G-4	$4x10^4$	Nil	2.5×10^4	$2.6x10^3$	$1x10^2$	$4x10^4$
G-5	$2x10^4$	Nil	$7x10^4$	$5.1x10^5$	$3.5x10^1$	$4.2x10^4$
G-6	$3.2x10^4$	Nil	$4x10^4$	NIL	$1x10^2$	$5.8x10^4$
CONTROL GROUP	$1.1x10^3$	NIL	$1.3x10^2$	$3.3x10^2$	1x10	$2.23x10^2$
Day 14.						
G-1	$3.2x10^3$	Nil	$3.8x10^2$	$2.3x10^2$	$2.2x10^2$	$2.8x10^3$
G-2	$4.1x10^4$	Nil	$2.9x10^4$	$4.2x10^3$	$1.9x10^4$	$5.2x10^4$
G-3	$5x10^3$	Nil	$2.5x10^4$	$5x10^2$	$3.5x10^4$	$5.3x10^4$
G-4	$3.3x10^2$	Nil	$4x10^2$	$4.2x10^2$	$1x10^3$	$3.8x10^4$
G-5	$2.4x10^3$	Nil	$3.3x10^4$	$1.7x10^3$	$4.2x10^3$	4x104
G-6	$5.2x10^2$	Nil	$5x10^3$	$5.2x10^2$	$5x10^3$	$5.6x10^4$
CONTROL GROUP	$1.9x10^3$	NIL	$1.6x10^6$	$1.5x10^5$	$1.4x10^4$	$1.8x10^3$
Day 35.						
G-1	$2.6x10^2$	Nil	$3.2x10^3$	$3.5x10^2$	$2.7x10^5$	$5.9x10^4$
G-2	$5.6x10_4$	Nil	$4.3x10^4$	$4x10^2$	$3.6x10^3$	$6.8x10^4$
G-3	$4.2x10^3$	Nil	$2.4x10^3$	$2.3x10^4$	$4.1x10^3$	$4.2x10^2$
G-4	$2.4x10^4$	Nil	$4.4x10^3$	$5.2x10^2$	$5.4x10^2$	$3.2x10^2$
G-5	$3x10^2$	Nil	5x10^6	$3.4x10^5$	$2.8x10^6$	$6x10^3$
G-6	$4x10^2$	Nil	2.8x10^6	$2.5x10^8$	5x10^4	$6.3x10^4$
CONTROL GROUP	$2.1x10^3$	NIL	$2.3x10^3$	$1.8x10^3$	$2.2x10^4$	$5x10^4$

3.2 Blood sample collection and serology studies

The blood samples (2-3 ml) were drawn from all the rabbits from ear's marginal vein following standard protocols on day 0, 14, 35, and 60. The blood, carefully sampled, was distributed into two different types of tubes that contain anticoagulants (EDTA, heparin).

3.2.1 Evaluation of hematological Parameters

For the determination of main hematological parameters such as red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), white blood cells (WBC), lymphocytes (LYMPHO6); monocytes (MONO6), neutrophils (NEUT6), eosinophils (EO6) and basophils (BASO6), EDTA (Ethyl Diamine Tetra Acetate) tubes were used for blood sampling. The tubes were subsequently transported to the laboratory for further analysis placed in a cooling box to maintain the cold chain. The values of the various hematological parameters were estimated by an automatic analyzer Shenzhen Mindray BC3000 plus.

The hematological parameters were observed for all experimental groups over 35 days. Initially at day 0, the values of WBC, RBC, HGB, PLT, HCT, MCV, MCH, MCHC, NEUT, LYMPH, and MONO were found to be 7.9, 7.18, 9.614, 373.67, 66.934, 61.24, 54.12, 39.3, 5.43, 3.52, and 1.354, respectively (Table 3.4). Similarly on day 14, WBC, RBC, HGB, PLT, HCT, MCV, MCH, MCHC, NEUT, LYMPH, and MONO were found to be 7.86, 7.87, 15.50, 483.66, 48.53, 65.26, 21.01, 31.96, 3.59, 3.59, and 0.66, respectively (Table 3.5).

However, on day 35, significant changes in hematological parameters were observed in the experimental groups, with the values of WBC, RBC, HGB, PLT, HCT, MCV, MCH, MCHC, NEUT, LYMPH, and MONO being 8.05, 7.90, 16.13, 490.66, 49.23, 65.50, 21.66, 32.93, 3.51, 3.56, and 0.540, respectively. Notably, the control group exhibited lower values of all studied hematological parameters than the experimental groups as shown in Tables 3.5.

3.3 Selection of Immunization regimens and animal grouping regimens.

The study examined the immune response of rabbits to rabies vaccine given through intramuscular (IM) and intradermal (ID) routes. The ID regimen was found to be more effective in terms of immunogenicity and vaccine volume consumption compared to the IM route.The three ID regimes, either 1- week 2 sites (IPC, R-2), 28-day 2-site (TRC-R-3), or 4-site one-week regimen (Alternate, R-4 regimen), resulted in significantly higher mean titer values compared to the control IM regimen group R-1 ($p<0.001$).

The ELISA method with PLATELIA II kit was used to measure the immunologic response of groups R1, R2, R3, and R4 after 14 days of immunization. The mean optical densities showed an increase in OD between groups R1 with R2, R3, and R4 indicating a higher sero-conversion and immune response from ID dose in groups R2 and R3 compared to the control group (R1). The increase in OD between groups R1 and R2 presents a significant titer from ID compared to IM. After 28 days of immunization, the results showed a significantly higher OD values for ID as compared to IM. A comparatively high difference in titer was obtained between control and group R2.

The increase in OD observed in the R1 group between 14 to 35 days amounted to a mere 11.43%. In contrast, groups R2, R3 and R4 exhibited a significantly higher response to ID vaccination with OD increases ranging from 19-22% ($p<0.01$) (Table 3.6, Figure 3.4). The sero-conversion rate was defined as the proportion of vaccines achieving RVNA titers ≥0.5 IU/ml.

Table 3.5: Hematological parameters of animals at day zero, day 14 and day 35.

ID	WBC	RBC	HGB	PLT	HCT (%)	MCV (fL)	MCH (pg)	MCHC	NEUT	LYMPH	MONO
	At day Zero										
G1	6.18±0.34	6.5±0.42	6.46±0.45	373.67±12	62.72±0.3 9	60.25±0.8 9	35.23±0.4 5	30.3±0.27	2.794±0.6 5	2.92±0.34	0.42±0.41
G2	7.88±0.11	7.03±0.15	9.614±0.0 4	326.33±22	66.23±1.7 0	59.7±1.13	54.12±0.9 3	29.85±0.3 0	5.4±0.18	2.73±0.21	1.354±0.1 1
G3	7.90±1.63	7.18±0.49	8.80±0.52	367.334±6	64.76±1.9 1	61.24±3.1 5	43.634±0. 96	30.1±0.29	3.92±0.45	3.25±0.13	1.04±0.15
G4	5.941±0.25	6.86±0.47	6.99±0.05	365.66±17	66.934±1. 89	62.37±0.5 3	42.46±0.9 3	30.4±0.27	2.86±0.07	3.39±0.26	0.67±0.07
G5	5.98±0.13	6.97±0.41	6.44±0.24	357.33±5. 55	64.23±1.3 2	59.22±0.7 7	39.83±0.8 2	30.2±0.30	3.46±0.29	3.14±0.33	0.47±0.11
G6	5.94±0.29	7.12±0.15	6.68±0.44	363.33±9. 53	61.53±1.4 1	61.23±0.5 4	32.50±0.5 0	39.3±0.28	2.93±0.06	3.52±0.04	0.33±0.11
Control	6.42±0.14	6.24±0.24	6.22±0.17	360.65±10	62.38±1.1 4	59.11±0.3 5	37.25±1.5 0	29.31±0.2 8	3.02±0.40	2.80±0.16	0.49±0.14
	At day 14										
G1	7.43±0.27	7.87±0.02	15.13±0.69	466.33±12. 11	48.53±1.42	63.36±0.50	20.13±0.23	31.3±0.46	3.59±0.09	3.59±0.08	0.66±0.09
G2	7.05 0.01	6.95±0.03	13.93±0.08	483.66±4.1 7	45.56±1.13	62.8± 0.36	19.7±0.41	31.86±0.31	3.21±0.01	3.35±0.06	0.34±0.02
G3	7.28±0.40	7.18±0.42	15.50±0.95	454.33±19. 80	43.76±1.36	65.26±1.48	20.8±0.20	31.86±0.24	2.60±0.14	3.35±0.03	0.45±0.08
G4	6.96±0.62	7.31±0.37	14.50±0.66	469.33±1.7 6	43.73±1.04	63.63±0.54	20.66±0.26	31.96±0.42	2.48±0.08	2.94±0.18	0.38+0.03
G5	6.88±0.49	6.91±0.16	15.00±0.36	466.33±6.0 6	44.3±1.22	63.03±0.16	21.01±0.40	31.26±0.84	2.36±0.05	2.84±0.19	0.41±0.06
G6	7.86±0.37	7.01±0.51	14.40±0.75	463.66±13. 54	41.70±1.10	64.83±1.23	21.00±0.40	31.56±0.44	3.09±0.37	2.273333	0.626667
Control	7.7±0.16	6.45±0.16	14.50±0.56	462.04±4.4 8	42.70±0.47	66.90±0.85	38.27±0.82	3.04±0.32	2.65±0.10	0.45±0.03	0.40±0.11

	At day 35										
G1	7.30±0.34	7.90±0.30	15.63±0.55	474±7.02	49.23±0.88	63.83±0.71	20.83±0.80	31.16±0.64	3.51±0.13	3.56±0.09	0.54±0.17
G2	7.06±0.17	7.001±0.04	15.37±0.32	490±1.45	46.5±0.77	62.34±1.64	20.83±0.88	31.53±0.61	3.19±0.01	3.44±0.12	0.33±0.008
G3	7.21±0.37	7.23±0.42	15.39±0.33	467.66±3.7 1	44.8±0.36	65.50±1.92	20.96±0.12	31.30±0.90	2.61±0.12	3.32±0.06	0.46±0.086
G4	7.02±0.63	7.31±0.38	16.13±0.42	464.67±3.4 2	45.43±0.80	63.96+1 24	20.03±0.85	32.93±0.80	2.52±0.10	2.91±0.21	0.42+0.02
G5	6.90±0.47	7.10±0.19	15.33±0.40	469.66±6.8 8	43.3±0.47	64.16±0.95	21.1±40.61	31.7±1.06	2.63±0.21	2.90±0.24	0.38±0.05
G6	8.05±0.26	7.10+0.53	15.9±0.05	469.66±4.1 7	44.41±0.81	64.56+1.10	21.66±0.87	32.33±0.70	3.21±0.39	2.39±0.08	0.51±0.21
Control	7.72+0.20	6.55+0.17	15.834±0.18	468.80+3.1 9	45.874±0.9 2	65.62±41.12	37.924±0.36	83.99+0.34	2.76+0.12	0.46+0.01	0.40+0.11
Reference range	5.5-12.5	5.46-7.94	10.4-17.4	304-656	41.70-57.0	58.5-66.5	33-50	33-50	33-50	38-54	4-12

Table 3.6. Selection of Immunization regimens and animal grouping regimen details.

Group ID	**Vaccination regimen applied**	**Immunization days 0-3-7-14-28**	**Sites of injection**	**Total vaccine Vials used/animal**
R-1 (Control	Essen IM Single dose 0.5 ml.	1-1-1-1-1 28 days	1-site	5 vials
R-2	IPC (ID) 0.1ml/site	2-2-2-0-0 7 days	2-site	1.1 vials
R-3	TRC (ID) 0.1ml/site	2-2-2-0-2 28 days	2-site	1.1 vials
R-4	Alternate (ID) 0.1ml/site	4-4-4-0-0 7 days	4-site	3 vials
Booster vaccine doses	Weekly single dose immunization			

3.4 Safety studies and Reaction observation after Immunization:

3.4.1 Local Reaction observation studies

Local reactions and incidences after rabies vaccination in experimental groups (A) and control C groups was observed in all 54 subjects in experimental and control groups after each shot of total 4 vaccination shots. After the insertion of 1st dose, subjects were monitored for 48 hours and none of the 6 subjects showed symptoms of pain, redness, pruritus, or any other reaction. Rabbits were observed for any symptoms for about 48 hours after the insertion of every dose and the result was the same as per experienced after the first dose shown in Table 3.7.

Figure 3.2. Clinical examination of New Zealand White rabbit.

3.4.2 Systemic Reactions studies:

Systemic reactions and incidences after vaccination and dietary interventions was studied in all animal groups. For evaluation of systemic reactions, temperature of the subjects was taken to observe any immediate reactions occurred within 30 minutes, 6, 24, and 48-hours Observations before and after the insertion of all 4 shots were noted down as shown in Table 3.8.

3.5 Antibody determination by Platellia II ELISA Kit method (In-vitro test)

3.5.1 Qualitative analysis /sero conversion level determination

The results were demonstrated as high, sufficient, insufficient and undetectable sero-conversion level having greater than 4, 0.5-4, 0.125-0.5 and below 0.125 EU/ml titer, respectively as shown in Table-3.9.

Table 3.7 Safety observation for Adverse Reactions including local reactions and incidences after rabies vaccination in experimental groups (A) & (C) control groups.

Groups	Shot	Animals/ Group n=6	Pain		Pruritus		Others		Total	
			n	%	n	%	n	%	n	%
A (G1-G6)	1st	36	Nil	0	Nil	0	Nil	0	Nil	0
	2nd	36	Nil	0	Nil	0	Nil	0	Nil	0
	3rd	36	Nil	0	Nil	0	Nil	0	Nil	0
	4th	36	Nil	0	Nil	0	Nil	0	Nil	0
Total		n=36	Nil	0	Nil	0	Nil	0	Nil	0
C (C1,C2,C3)	1st	18	Nil	0	Nil	0	Nil	0	Nil	0
	2nd	18	Nil	0	Nil	0	Nil	0	Nil	0
	3rd	18	Nil	0	Nil	0	Nil	0	Nil	0
Total		n=18	Nil	0	Nil	0	Nil	0	Nil	0

Remarks: No adverse local reactions observed in experimental animals after immunization shots during study period.

Table 3.8: Results of safety observation of Adverse Reactions: Systemic reactions observations after rabies vaccination in experimental groups (A) & (C) control groups.

Groups	**Shot**	**Animals/ Group n=6**	**Mild fever**		**Moderate fever**		**Severe/ Others**		**Total**	
			n	%	n	%	n	%	n	%
A (G1-G6)	1st	36	Nil	0	Nil	0	Nil	0	Nil	0
	2nd	36	Nil	0	Nil	0	Nil	0	Nil	0
	3rd	36	Nil	0	Nil	0	Nil	0	Nil	0
	4th	36	Nil	0	Nil	0	Nil	0	Nil	0
Total		n=36	Nil	0	Nil	0	Nil	0	Nil	0
C C1, C2, C3	1st	18	Nil	0	Nil	0	Nil	0	Nil	0
	2nd	18	Nil	0	Nil	0	Nil	0	Nil	0
	3rd	18	Nil	0	Nil	0	Nil	0	Nil	0
Total		n=18	Nil	0	Nil	0	Nil	0	Nil	0

Remarks: No adverse systemic reactions observed in experimental animals after immunization shots during study period.

Table 3.9 Sero-conversion rate in rabbits against rabies vaccination after administration of first dose.

Dietary groups	Probiotic alone (N = 12)	Probiotic With Vit A (N = 12)	Probiotics with Carrots (N = 12)	Vit A alone (N = 6)	Carrots alone (N=6)	Control without supplements (N = 6)
Group ID	G1 + G2	G3+G4	G5+G6	C1	C2	C3
Seroconversion at day 14	SSC	SSC	SSC	SSC	SSC	SSC
Seroconversion at day 35	SSC	SSC	SSC	SSC	SSC	SSC
Seroconversion at day 60	SSC	SSC	HSC	SSC	HSC	SSC

Table 3.10 Criteria used for result interpretation of Qualitative analysis.

Criteria	**Conditions of validation**
OD R3 (i) < 0.05	The absorbance value for each individual negative control should be less than 0.05. The test should be repeated even if one value is out of this limit.
0.300 ≤ OD R4a(i) ≤ 1.200	OD value of the R4a positive control should be between 0.300 and 1.200. The test must be repeated if at least one of the R4a OD value is outside of this range.
1.500 ≤ OD R4b (i) ≤3.500	Optical densities (OD) values of R4b positive control must be between 1.500 and 3.500. However, a maximum of one individual deviant value can be eradicated if OD is less than 1.500 or higher than 3.500. The test must be repeated if the two R4b positive control OD values are out of this range.

3.5.2 Standard curve for quantitative analysis

The optical density (OD) values were measured using ELISA micro-plate reader (Diateck China) at 450 and 620 nm. Anti-rabies antibodies were quantitatively determined by constructing a standard curve from standards S1-S6, which was prepared by serially diluting R4b calibrated positive controls. The titer values were obtained from OD by using conversion tool provided with the kit manual and expressed in equivalent units per ml (EU/ml).(Figure 3.3)

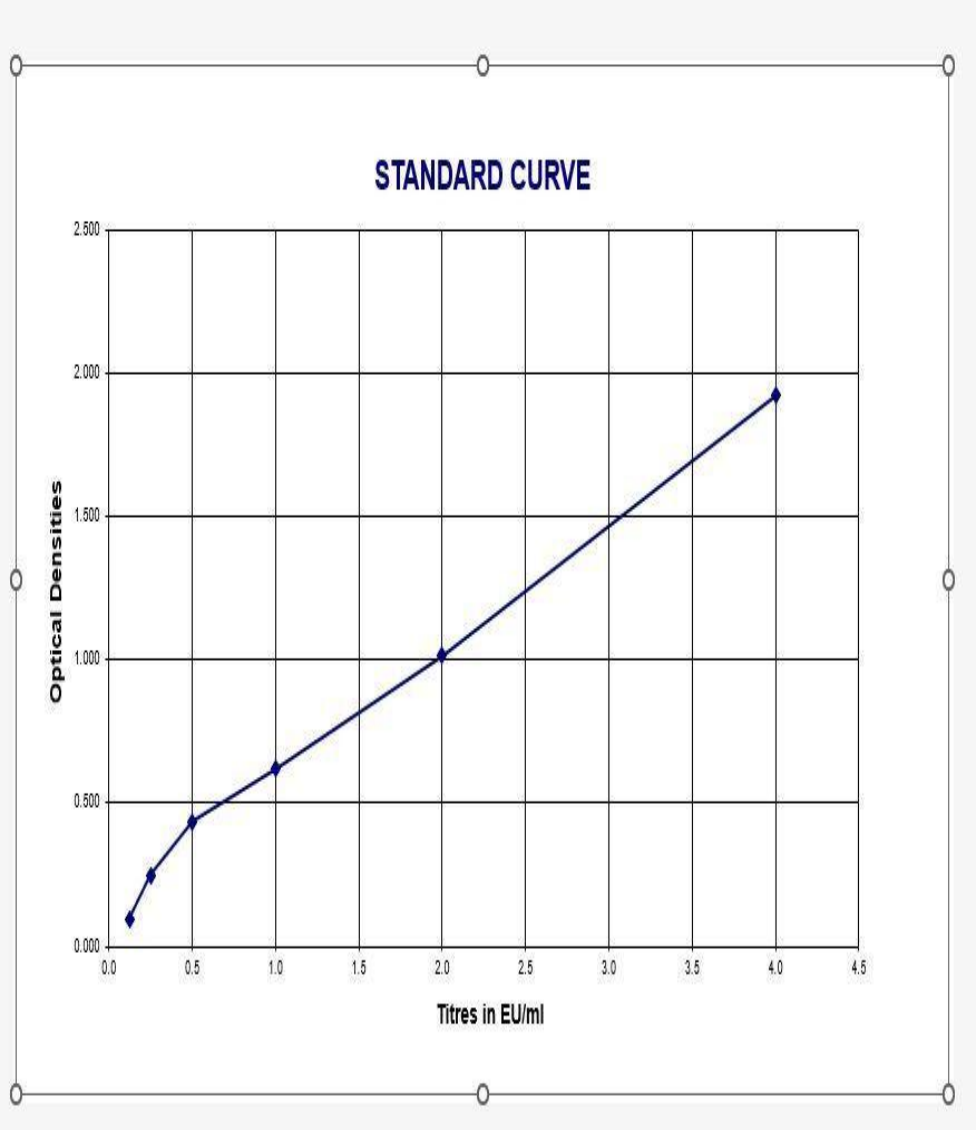


Figure 3.3 Standard curve is utilized to compute titer values based on optical density (OD). The OD of an unknown sample is compared to positive controls in order to determine the titer values. The quantified sera titer can be directly determined by reference to the standard curve, and it is expressed in equivalent units per milliliter (EU/ml).

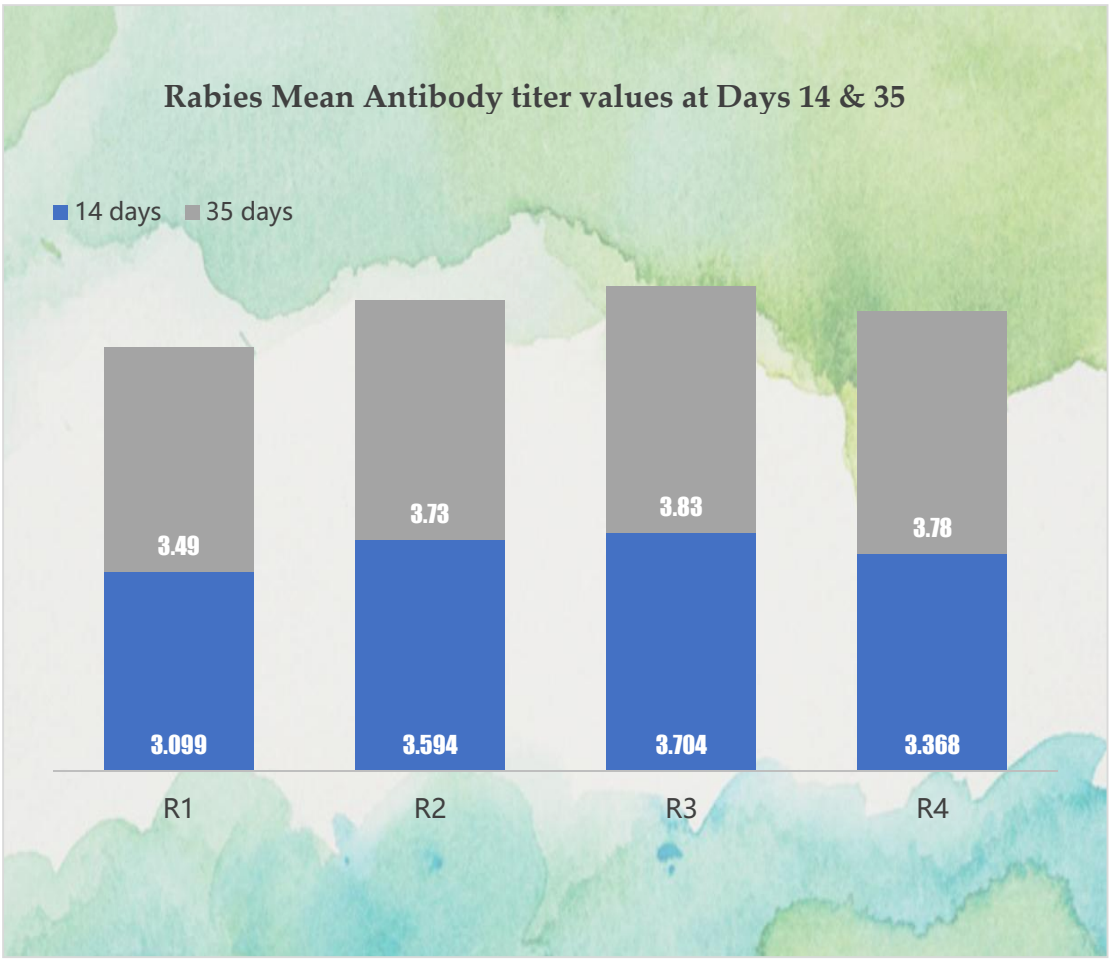


Figure 3.4. Mean Rabies neutralizing antibody titre at day 14 and 35 among regimen selection groups immunized with different rabies vaccine schedules. Analysis of variance (ANOVA) was employed to compare immune outcomes across these groups. Additionally, Turkey's post hoc test was utilized to identify disparities between treatments and the control group; significant effects were determined at a probability level of p=<0.001, Cl 95%.

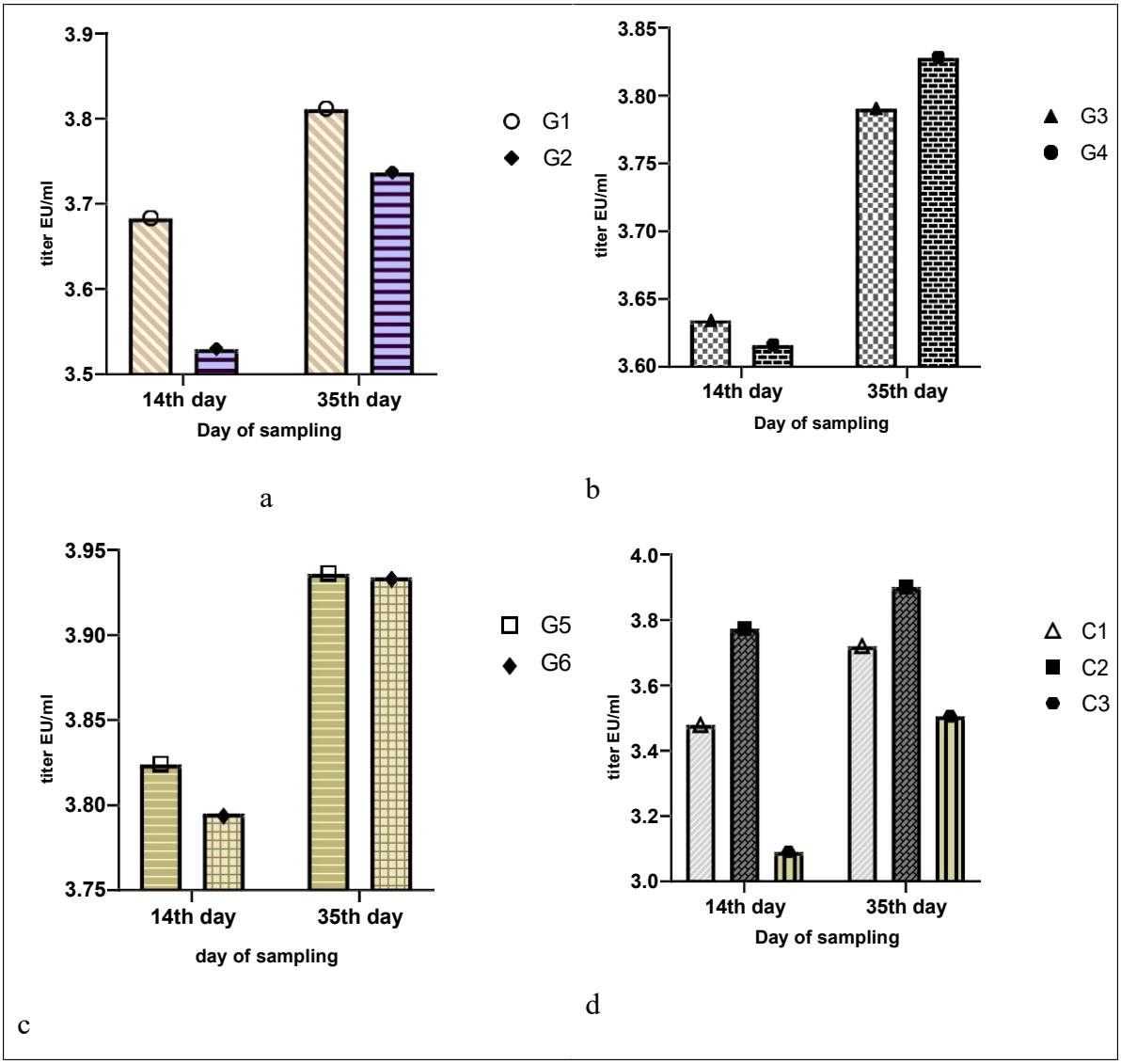


Figure 3.5 Mean antibody titer values obtained by concomitant dietary interventions and rabies immunization at days 14th and 35th . Mean titer value s (± standard deviation) of rabies antibodies of NZW rabbits after first time-vaccination. Animals were administered different feed supplementation as per following sequence: a) G1), (G2), b) (G3, G4), c) (G5,G6), d) C1,C2 & C3 on daily basis for 6 weeks. Results of Two way ANOVA show significant difference among groups difference (p=<0.001,CI 95%) for both sampling days (day 14 and 35). The values of antibody titer continuously increased until 35 days with same increasing trend in experimental groups (G1 - G6) than control group C3 (at day 35, mean titer range in experimental groups was between 3.7 to 3.9 EU/ml than mean value 3.5 EU/ml of control.

3.6 Humoral response after immunization with cell culture rabies vaccine

The World Health Organization (WHO) recommended a minimal protective antibody titer of 0.50 UI/ml for effective prevention against rabies, which was also considered in our study. On day 0, the results of the rabies neutralizing antibody titers obtained from the serum of New Zealand white rabbits subjected to different experimental treatments did not show reactivity. This indicated that none of the animals had previously been exposed to or immunized against rabies virus. Thus, differences in rabies antibody titer values observed after 14 and 35 days were elicited by both rabies vaccination and dietary treatments provided to the animals. The overall results of our experiment demonstrated that mean rabies antibody titers across all experimental groups were significantly higher than those observed in the control group (Figure 3.5). Therefore, dietary treatments with probiotics and vitamin A exerted a better effect on humoral immune response against rabies vaccine immunization compared to simple immunization alone among rabbits.

3.6.1 Probiotic supplementation effects on humoral immune response against rabies immunization in experimental groups G1 and G2

Two distinct probiotics, PBA and PBB, were administered to animal groups G1 and G2 for a period of six weeks in conjunction with rabies vaccination. The titers obtained after 14 and 35 days are presented in Fig 3.5 & 3.6 (a). Both types of probiotics exhibited enhanced effects on the humoral response to vaccination. A significant difference was observed after day 14 which gradually increased by day 35, thus demonstrating a noteworthy increase in the humoral response due to dietary supplementation across all animals within both probiotic groups. Comparison via Two-way ANOVA of antibody titers between both study groups and control groups C3 revealed a significant difference ($p=<0.001$, CI95%) as shown in the figures 3.6 and 3.9.

3.6.2 Effect of Probiotics and vitamin A supplementation on humoral immune response against rabies immunization in experimental groups G3 & G4 .

Vitamin A was administered to animals in groups G3 and G4, alongside PBA and PBB respectively. On day 14th, a noticeable increase in antibody titer enhancement was

observed in both groups. This continued to rise until day 35 for all animals as shown in Table 3.9. The results are presented in Fig-3.5, 3.6 (b). The impact of both commercial probiotic brands on the humoral response of vaccination was significant for all animals within G3 & G4 groups. When compared with control group C3's antibody titer, a two- way ANOVA comparison showed a significant difference ($p=<0.001$, CI 95%) in the antibody titers of both study groups.

3.6.3 Effect of probiotics and organic carrots feeding on humoral immune response against rabies immunization in G5 & G6 experimental groups

The diet of animals in groups G5 and G6 consisted of probiotic A (PBA) and probiotic B (PBB), respectively, along with fresh organic carrots. The titer began to develop after one week, and sufficient sero-conversion and antibody concentration were measured for each individual study animal on the 14th day (Table 3.12). The mean titer values of G5 & G6 are shown in Fig 3.5 c. The recorded results indicated a range of values from 3.6 to 3.9 in experimental groups, which are significantly higher than all other experimental groups as well as control group C3. Likewise, antibody titers reached their highest value on the 35th day for all study groups, demonstrating high sero-conversion rates. Two-way ANOVA comparison revealed a significant difference ($p=<0.001$, CI95%) between the antibody titers observed in both study groups compared to control group C3.

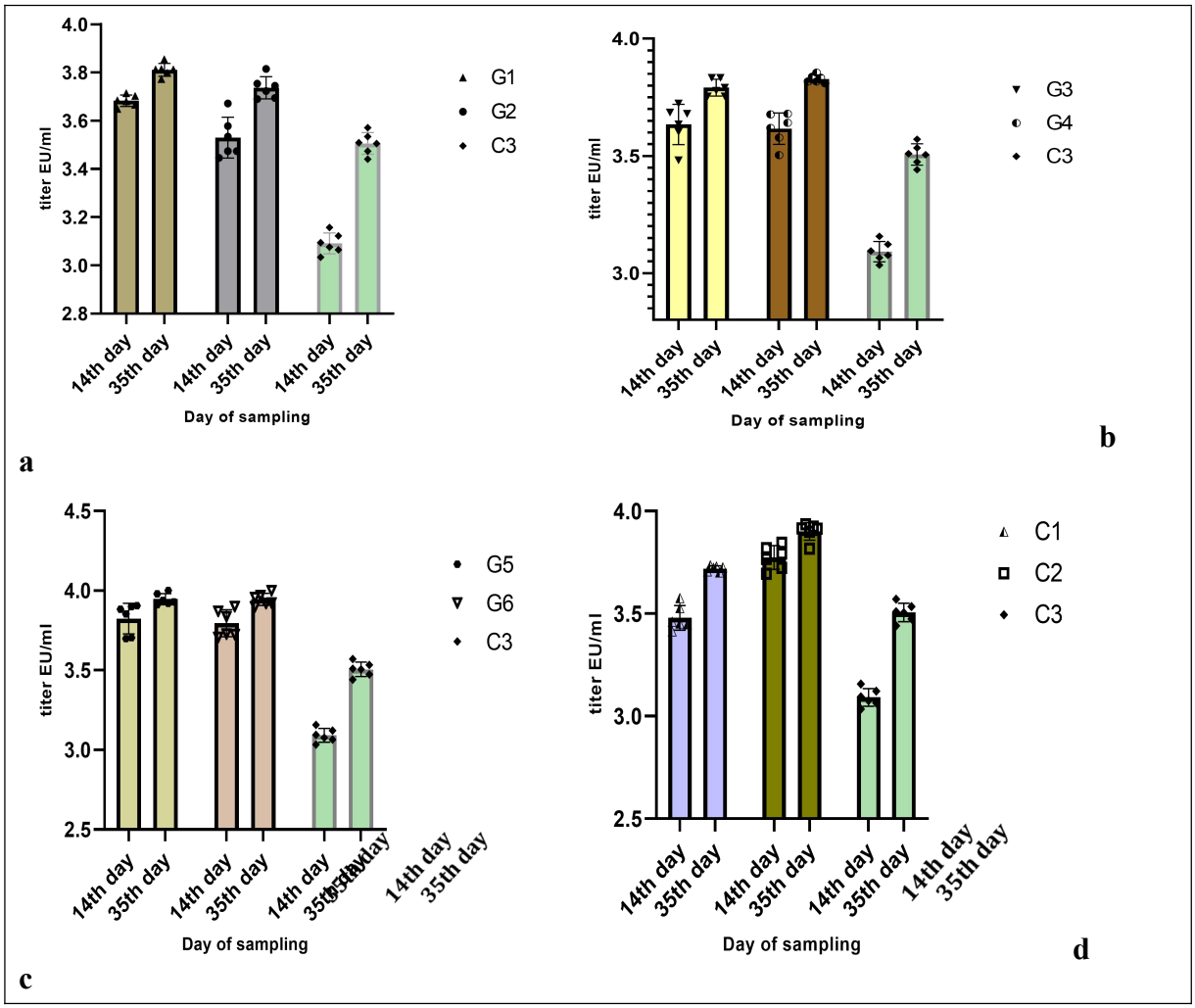


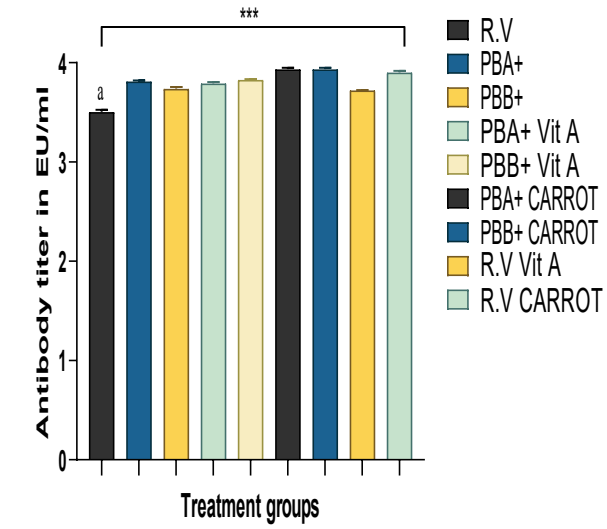
Figure 3.6: Enhancement of antibody response with Probiotics supplementation in experimental groups as compared to control group C3: All animals in experimental groups G1 to G6 (n=6 per group) were administered with cell culture rabies vaccine (CCRV), along with supplementation of probiotics and vitamin A-rich food. The intervention groups were divided into three categories: a) G1 & G2 (probiotics only), b) G3 & G4 (probiotics and vitamin A), c) G5 & G6 (probiotics with organic carrots). Serum samples were collected on days 14th, 35th, and 60 to monitor changes in antibody titer values. At day 14th, all animals within each intervention group displayed an increase in rabies-specific antibodies titer compared to control group C3 as demonstrated in Figures (a-c). Similarly, the comparison between C3 control and dietary subgroups without probiotics (C1& C2) also exhibited enhancement of titer values as seen in Figure (d). Adequate sero-conversion level was attained after receiving 0,3,7 vaccine shots on day 14th that exceeded the protective titer level of 0.5 EU/ml. The titer values continued to gradually enhance until reaching maximum sero- conversion at day 60. Two-way ANOVA comparison of antibody titers showed significant differences among all study groups when compared with control group C3($p=<0.001$).

3.6.4 Humoral response studies in sub-controls and control group (C1, C2 and C3).

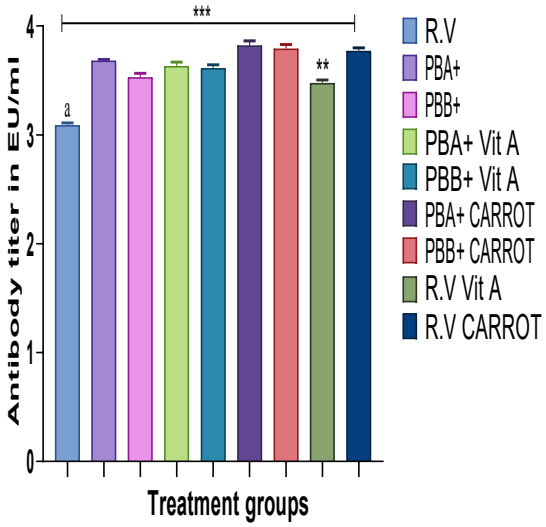
The study aimed to investigate the impact of dietary modifications on animals. Two sub-control groups were selected for observation, with one group receiving only a dose of vitamin A and rabies vaccination, while the other group's diet was enriched with fresh organic carrots as a natural source of vitamin A. All animal groups showed gradual improvement in their mean antibody titer values from day 14 to day 35, with each group demonstrating significant enhancement at both time points. The primary control group that received no supplementation displayed the lowest concentration of antibodies among all study groups, demonstrating a comparatively sluggish and feeble sero-conversion even after one week or one month of immunization when compared with experimental groups.

3.6.5 Overall humoral response studies on mean antibody titer values (IgG) after 14th and 35th days in all 9-experimental groups.

The study assessed anti-rabies antibody titer levels in vaccinated animals using mean absorbance at 490 nm and a cutoff value of >0.5 EU/ml. All vaccinated animals had antigen-specific antibodies above the protective titer in their serum. Animals receiving dietary interventions such as probiotics, vitamin A, or organic carrots in conjunction with the vaccine had significantly higher antibody titers measured by ELISA test on day 14 after one week of immunization compared to the control group. The mean values ranged from 3.5 EU/ml to 3.9 EU/ml compared to the control group's mean titer of only 3.09 EU/ml.Titer values increased prominently for all treatment groups on day 35 and surpassed the generally accepted protective threshold of 0.5EU/ml required for further antiserum production processing following rabies vaccination protocol standards.



a



b

Figure 3.7 Overall mean antibody titer comparison in serum samples of different rabbit treatment groups a) after 14 days, b) after 35 days post vaccination, (n=6/group). "a" represents a control group with simply rabies vaccination (R.V), Groups PBA & PBB were supplemented with two different commercial probiotics, Groups PBA + Vit A and PBB + Vit A with administered Vitamin supplement, PBA+Carrots & PBB + Carrots groups were fed with organic carrots and both probiotic brands respectively. Data was analyzed using Graph Pad Prism (version 9) computer.

software. Differences among experimental treatments were tested using Tukey's post hoc test after ANOVA. A statistically significant difference between groups was determined ($p < 0.001$, CI 95%) , as compared to control group RV.

Table 3.11 Distribution of subject animals in various sub-groups based on different dietary treatments (vitamin A & organic carrots) with Probiotics and rabies vaccination.

Group ID	Intervention	No. of animals	Mean antibody titers measured by ELISA (EU/ml)		Total vaccine doses utilized for each animal
			Day 14	Day 35	
G-1	RV + PBA	n=6	3.683	3.811	4 x 0.2ml
G-2	RV + PBB	n=6	3.529	3.737	4 x 0.2ml
G-3	RV + PBA + Vit A	n=6	3.634	3.790	4 x 0.2ml
G-4	RV + PBB + Vit A	n=6	3.616	3.828	4 x 0.2ml
G-5	RV + PBA + O.carrot	n=6	3.824	3.936	4 x 0.2ml
G-6	RV + PBB + O.carrot	n=6	3.795	3.934	4 x 0.2ml
C-1	RV + Vit A (sub control)	n=6	3.479	3.719	4 x 0.2ml
C-2	RV + O. carrots (sub control)	n=6	3.773	3.901	4 x 0.2ml
C-3	RV only* (Control group)	n=6	3.091	3.506	4 x 0.2ml

Table 3.12 Antibody titer of individual animals included in study groups at 14th and 35th day. The comparison of antibody titer values of all groups from G1 to G6,C1,C2 with C3 control. After initial one week of immunization at days 0,3,7 , sufficient sero-conversion level was achieved at day 14 ,which is well above protective titer 0.5 EU/ml. The values gradually enhanced further till day 35th with high sero-conversion levels in all individual animals of dietary intervention groups as compared to Control group (C3). Two-way ANOVA comparison of antibody titers in all study animals with control group C3 show significant difference (p=<0.001,CI 95%) in antibody titer values.

Groups	Day of Sampling	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6
G1	14th	3.666	3.65	3.681	3.685	3.703	3.714
	35th	3.813	3.799	3.855	3.815	3.773	3.813
G2	14th	3.474	3.446	3.672	3.534	3.578	3.474
	35th	3.745	3.754	3.723	3.815	3.694	3.69
G3	14th	3.481	3.723	3.602	3.685	3.635	3.679
	35th	3.791	3.833	3.754	3.778	3.754	3.833
G4	14th	3.679	3.503	3.677	3.641	3.578	3.619
	35th	3.809	3.817	3.817	3.855	3.837	3.833
G5	14th	3.699	3.705	3.905	3.853	3.899	3.883
	35th	3.921	3.938	3.912	3.930	>4	3.98
G6	14th	3.707	3.729	3.901	3.872	3.725	3.837
	35th	3.958	3.969	3.921	>4	3.899	3.923
C1	14th	3.413	3.448	3.452	3.460	3.527	3.575
	35th	3.725	3.736	3.705	3.723	3.729	3.701
C2	14th	3.694	3.846	3.723	3.767	3.793	3.820
	35th	3.899	3.925	3.914	3.817	3.916	3.936
C3	14th	3.076	3.065	3.094	3.034	3.157	3.122
	35th	3.474	3.441	3.534	3.571	3.505	3.509

OBJECTIVE # 2

Optimization strategies and downstream processing to get higher yield of Rabies antiserum and its characterization.

Rationale/Summary :

To improve vaccine administration in developing countries, cost-effective strategies like using a multi-site intradermal route is recommended by WHO. Therefore three different intradermal (ID) regimens were compared with traditional IM route in initial regimen selection experiment , to achieve an enhanced immune response in a shorter time frame and with reduced vaccine requirements for animal immunization. The experiment was performed to check non-inferiority of ID regimen. Finally Thai-Red cross intradermal regimen (TRC-ID) was selected for further immunization , keeping in view better antibody titer as shown in (Figure-3.8)

Mean Rabies neutralizing antibody titre at day 14 and 35 among regimen selection groups immunized with different rabies vaccine schedules. R-1 control group was immunized with intramuscular (Essen IM, Single dose 0.5 ml). Groups R-2 ,R-3 & R-4 were vaccinated with intradermal (ID) routes ,IPC (ID) 0.1ml/site, TRC (ID) 0.1ml/site and Alternate (ID) 0.1ml/site respectively. Antibody titer is represented as EU/ml as determined by ELISA kit method. The obtained results were subjected to statistical evaluation using the Graph Pad Prism (version 9) software in order to ascertain the significance of differences between groups with control group . Analysis of variance (ANOVA) was employed to compare immune outcomes across these groups. Additionally, Turkey's post hoc test was utilized to identify disparities between treatments and the control group; significant effects were determined at a probability level of $p=<0.001$, Cl 95%. (Figure 3.8,Table-3.13)

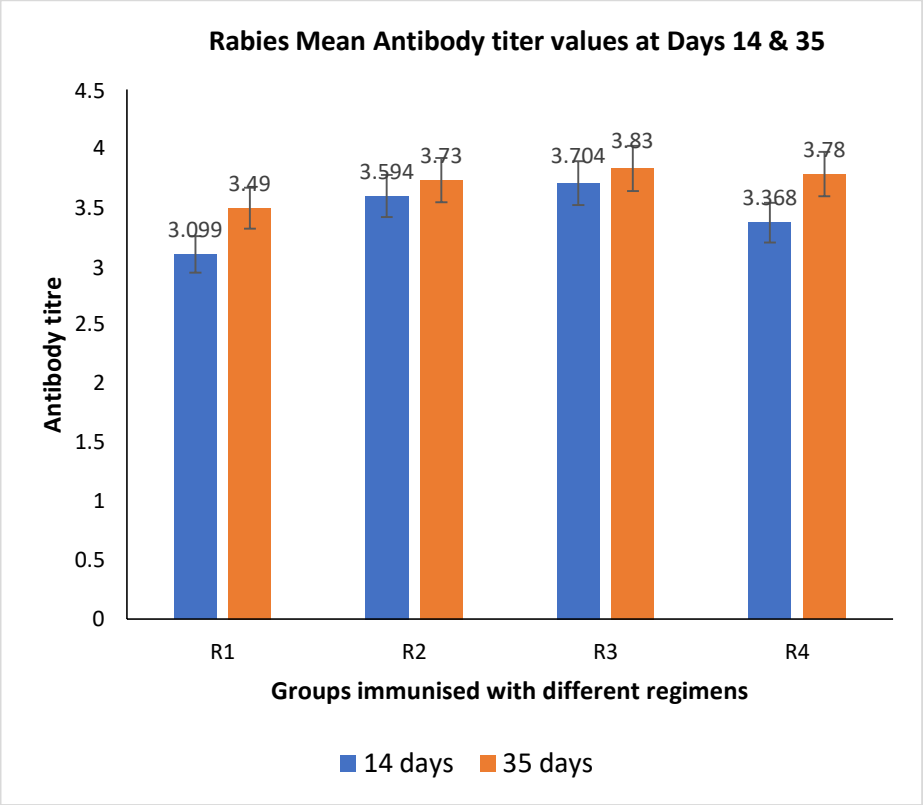


Figure 3.8 Mean Rabies neutralizing antibody titer at day 14 and 35 among regimen selection groups immunized with different rabies vaccine schedules. R-1 control group was immunized with intramuscular (IM) route while R-2, R-3 & R-4 were vaccinated with intradermal (ID) routes. Antibody titer is represented as EU/ml as determined by ELISA kit method.

Table 3.13. Comparison of serum OD mean values by ELISA for regimen selection study groups (R2, R3, R4 and R1 Control).

Day	Regimens	Mean OD	Variance	t-value	p-value (two-tailed)
14	Control	1.5046	3.63E-05	4.3027	0.0006
	R2	1.6983	0.0001		
	Control	1.5046	3.63E-05	4.3027	0.0002
	R3	1.7813	0.0001		
	Control	1.5046	3.63E-05	4.3027	0.0027
	R4	1.6376	3.63E-05		
35	control	1.687	0.0001	4.3027	0.0016
	R2	1.7976	6.33E-06		
	control	1.687	0.0001	4.3027	0.0031
	R3	1.839	3.6E-05		
	control	1.687	0.0001	4.3027	0.0314
	R4	1.802	0.0007		

3.7 Strategies for enhancement of poly-clonal antibodies (PAb) production in animals (Hyper-immunization)

All experimental animals were hyperimmunized to get higher antibody yield after 28 days of routine immunization. The results showed gradual increase in titer values till day 60. Maximum antibody titer was achieved at day 60 as shown in Figures 3.9-3.11 in G1- G6 respectively.

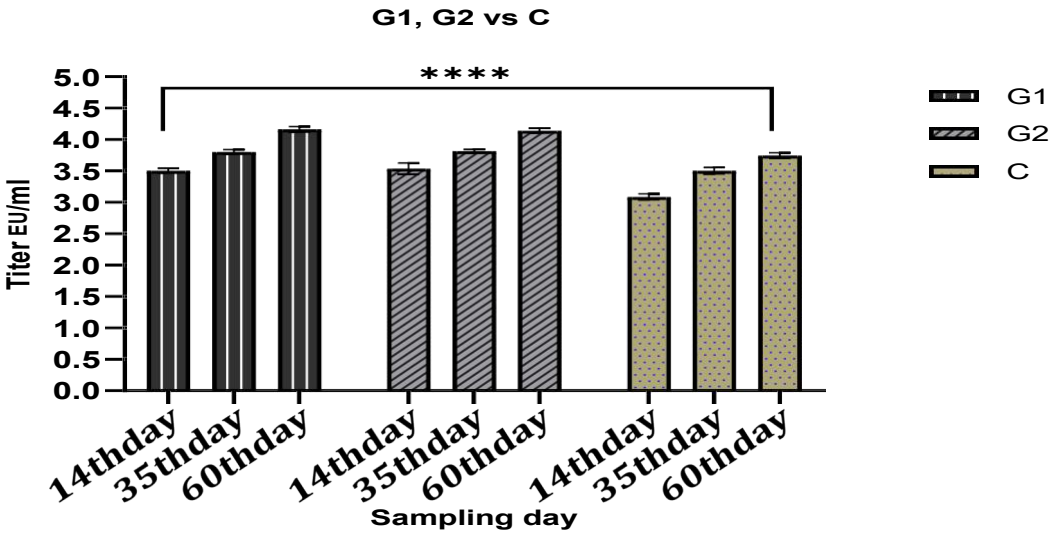


Figure 3.9 Antibody titer of animals included in study groups G1, G2 and C at 14th, 35th day and 60th day after hyper immunization.

Antibody titer values of G1 and G2 animals are compared with C control (without supplements). The sufficient seroconversion level has been achieved at day 14 after 0,3,7 vaccine shots well above protective titer 0.5 EU/ml. The values gradually enhanced till day 60^{th} with approximately high sero-conversion levels in all intervention groups as compared to Control group (C). Two-way ANOVA comparison of antibody titers in both study groups with control group C antibody titer show significant difference ($p=<0.001$, CI = 95%).

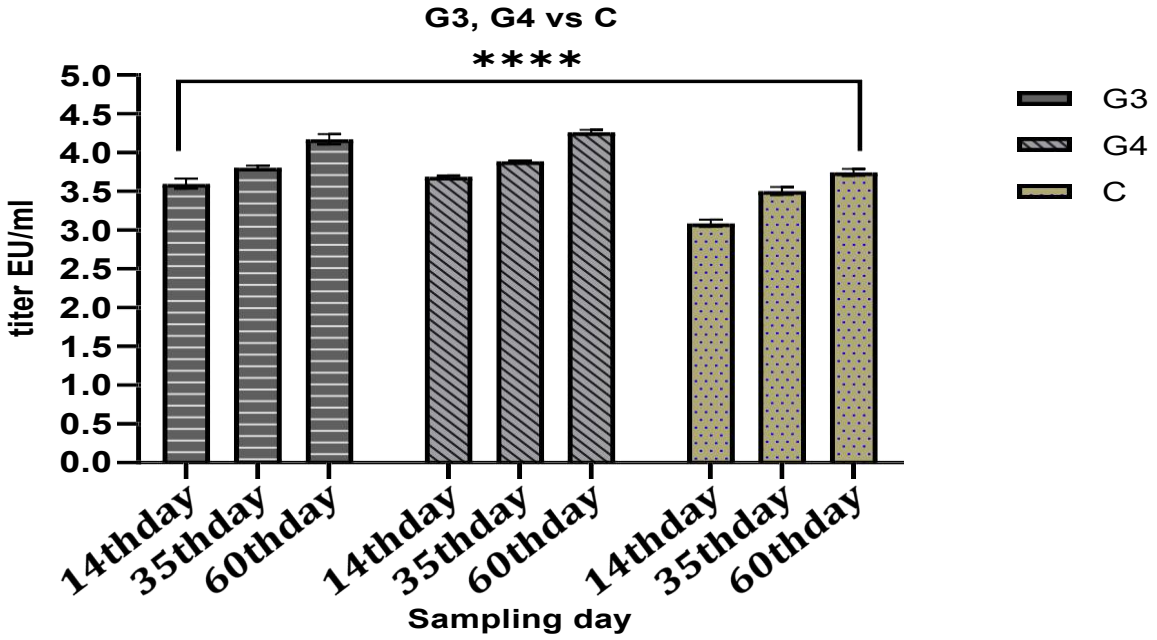


Figure 3.10 Antibody titer of animals included in study groups G3, G4 and C at 14th, 35th and 60th day after hyper-immunization of animals.

Two-way ANOVA comparison of antibody titers in both study groups (G3 and G4) with control group C antibody titer show significant difference ($p = < 0.001$, CI 95%). The seroconversion level has been achieved at day 14 after one week of immunization (day 0, 3, 7), well above recommended protective titer 0.5 EU/ml. The values gradually enhanced till day 35th and 60th with approximately high sero-conversion levels in all intervention groups as compared to Control group (C).

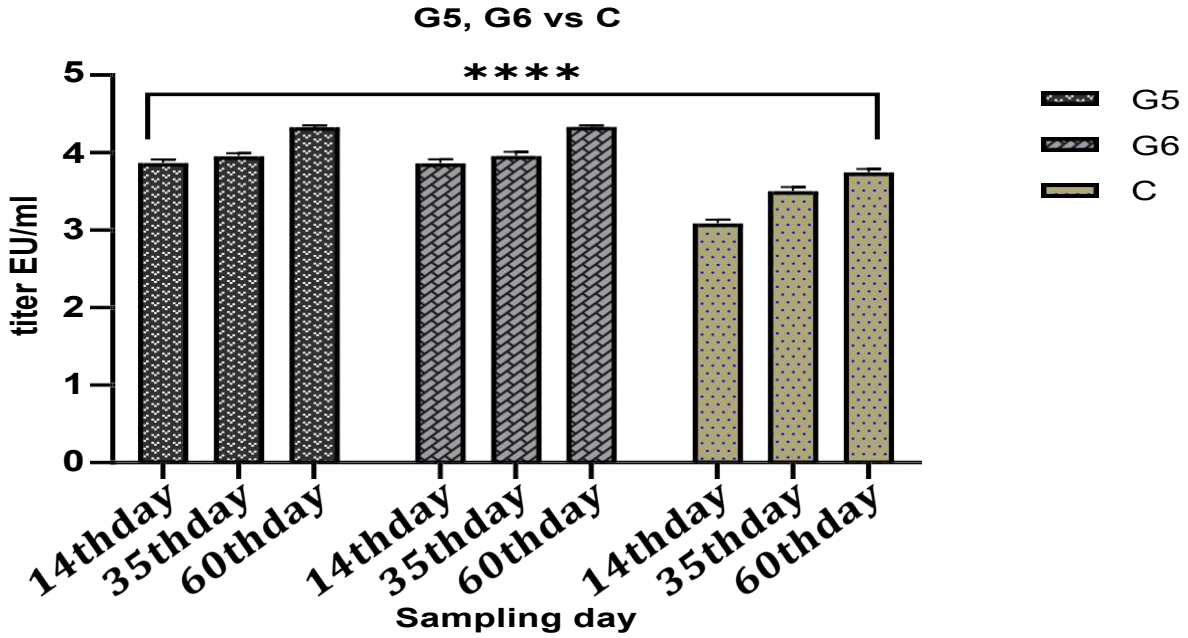


Figure 3.11 Comparing Antibody titer of animals of G5 & G6 groups at 14th, 35th and 60th day after hyper-immunization of animals.

Titer values of G5 and G6 animals fed with fresh organic carrots in feed along with probiotics. The values gradually enhanced from day 14th till day 60th with approximately high sero-conversion levels in all intervention groups as compared to control group (C) and other probiotic groups. Two-way ANOVA comparison of antibody titers in all study groups with control group C antibody titer show significant difference ($p=<0.001$, with 95% Cl value).

3.7.1 Overall humoral response on rabies specific antibody titer values (IgG) in 6-experimental and controls groups after 14th, 35th and 60th day by ELISA after applying hyper-immunization strategy.

The anti-rabies antibody level was determined by calculating the mean absorbance at 490 nm and comparing it to the cutoff value (>0.5 EU/ml). Serum samples above this threshold were considered positive. In response to vaccination, all vaccinated animals exhibited antigen-specific antibodies in their serum that exceeded protective titer levels. According to ELISA results, animals that received dietary interventions such as probiotics, zinc or organic carrots alongside vaccination had notably higher antibody titers at 14 days after one week of immunization (days 0,3,7), with mean values ranging from 3.5 EU/ml to 3.9 EU/ml compared to control group C which only underwent vaccination (with a recorded titer of 3.09 EU/ml). These significantly higher values are well above the generally accepted protective threshold of 0.5 EU/ml for rabies vaccination and figure 3.10 shows continued increase in antibody titer until day 60 post-vaccination with similar patterns observed across experimental groups G1-G6 when compared against control group C's pattern of increasing titers over time (Figure 3.12).

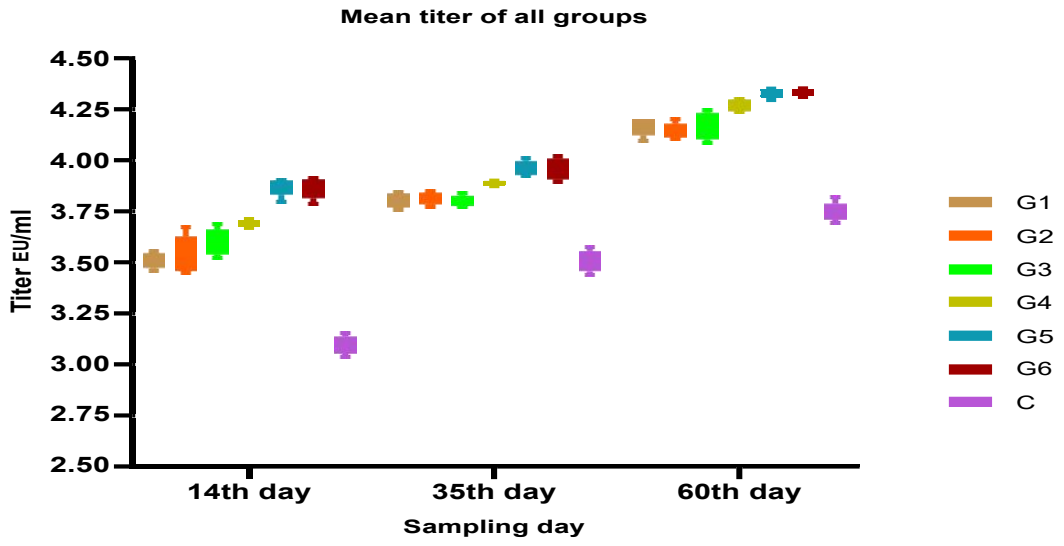


Figure 3.12 Antibody titer base comparison of all study groups after hyper-immunization strategy to get maximum antibodies for production of rabies antiserum.

3.8 Downstream processing with Caprylic acid fractionation technique for further purification of antibodies from pooled plasma.

Pooled plasma was processed further for purification of RIGs with caprylic acid fractionation method. Optimization of physic-ochemical parameters was done at small scale to get maximum product yield with relatively simple technique as compared to old purification method using salt precipitation.

3.8.1 Optimization of Caprylic acid method parameters

To prepare aliquots of 100 ml of hyper-immune horse plasma, 1.76 N acetic acid was added to reach a pH of 5.8, as recommended by McKinney & Parkinson (1987). The final concentrations of caprylic acid were then adjusted to 5%(v/v)m pH value 7.0 , 60 minutes of stirring time and high stirring speed as previously optimized in the lab and reported by (Rojas et al., 1994).

3.8.2 Caprylic acid concentration optimization

The results are shown in Figure 3.13.The best values of total protein concentration were obtained using 5% C.A concentration, therefore 5% concentration was chosen for further experimentation.

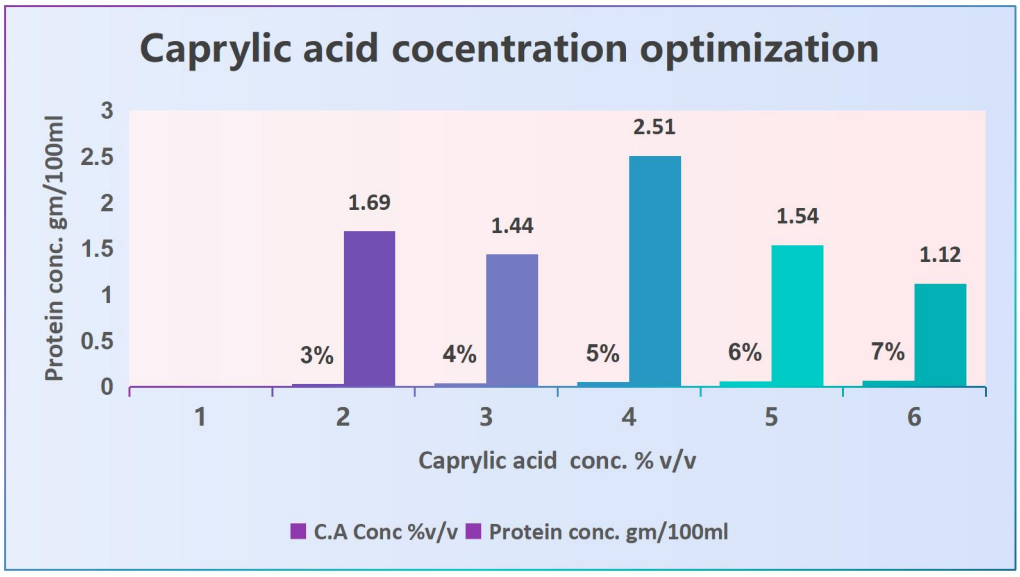


Figure 3.13: Optimization of Caprylic Acid (C.A) Concentration. Various concentrations of caprylic acid were meticulously adjusted to 1%, 2%, 3%, 4%, 5%, 6% and 7% (v/v) with constant stirring to determine the optimal conditions. The protein concentration was calculated, and it was determined that the best values were obtained at a 5% C.A concentration.

3.8.3 pH optimization

Different pH was adjusted using 1.76 N acetic acid to levels of 4.0, 5.0, 5.5, 5.8, 6.0, keeping the C.A concentration of 5% . Protein concentration values were calculated to find best results as shown in Figure 3.14.

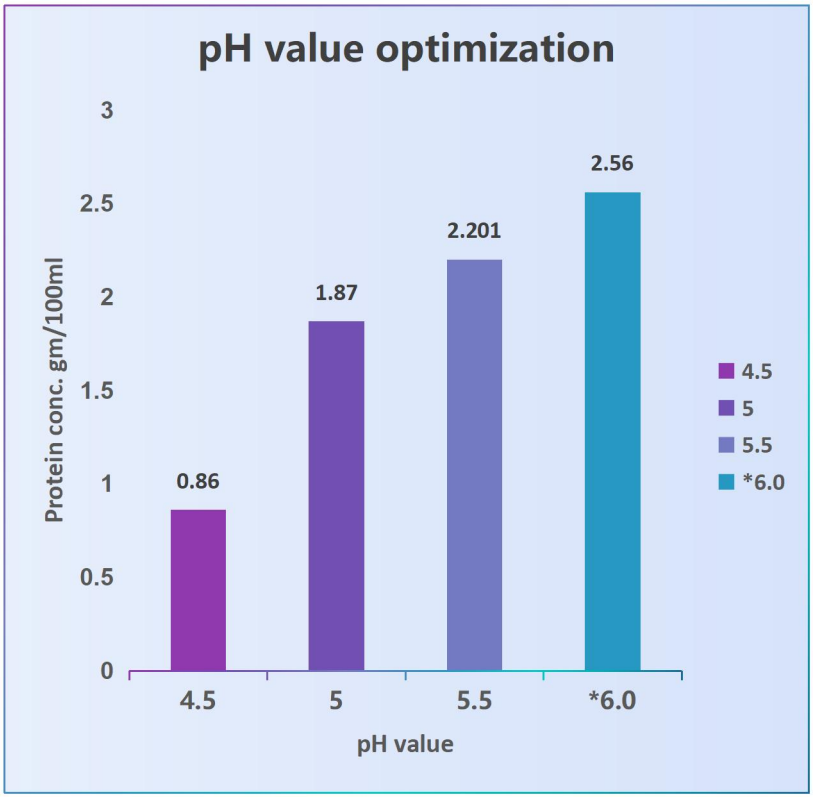


Figure 3.14: Optimization of pH value. Protein concentrations were determined at varying pH levels of 4.5, 5.0, 5.5, and 6.0 with continuous agitation to establish the optimal conditions. The highest protein concentration values were achieved at a pH of 6.0 while maintaining a constant concentration of 5% C.A.

3.8.4 Optimization of stirring time and speed (RPM)

The plasma mixture was fortified with 5% caprylic acid ,pH 6.0 and was stirred intensively for 30 and 60 minutes to establish a standardized stirring duration and speed.To standardize the stirring intensity, samples were mixed for 30 and 60 minutes at three different speeds ,300 ,150 and 75 RPM. Results shown in Figure 3.15 demonstrate that maximum yield of protein concentration was obtained at maximum speed 300 and are approx similar for both timings.

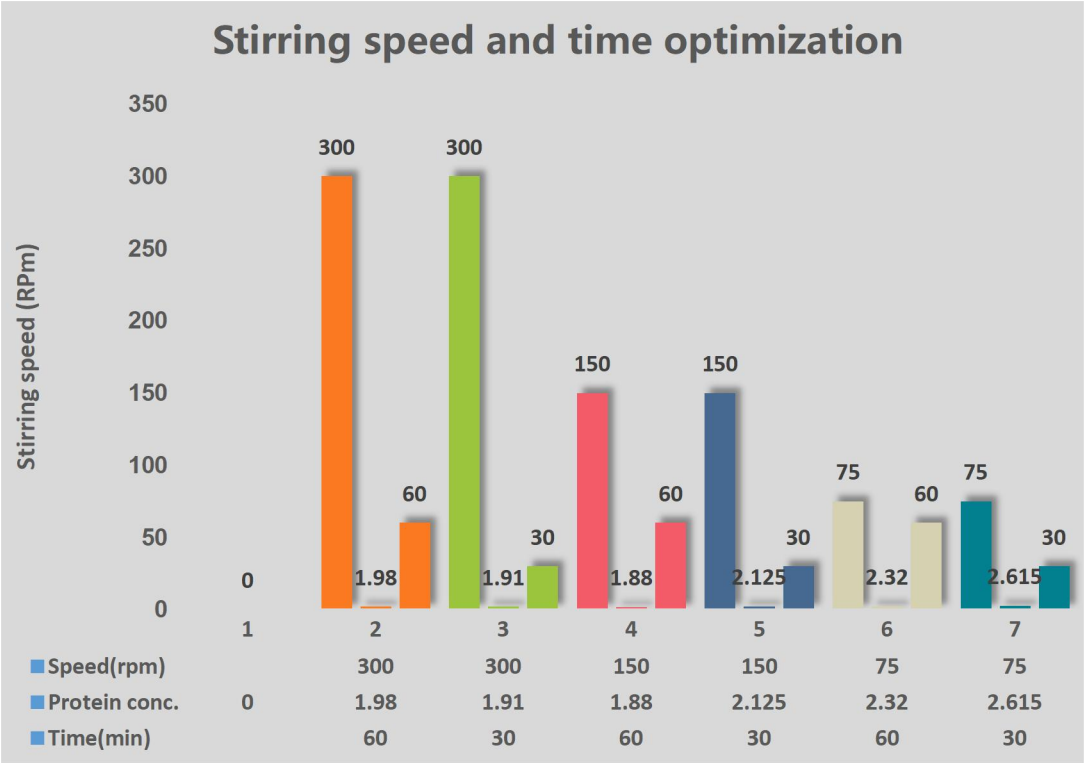


Figure 3.15 Optimization of stirring time and speed (RPM). Plasma with 5% caprylic acid and pH 6.0 was stirred for 30 and 60 minutes at different speeds (300, 150, and 75 RPM). Maximum protein concentration yield was obtained at the highest speed of 300 RPM for both timings.

3.9 Quality control test analysis of purified anti-sera after pre-filtration and dialysis

All essential tests such as Sterility, abnormal toxicity, endotoxin level detection and Potency (calculated by MNT) for final preparation were found to be satisfactory. The abnormal toxicity test observations for any abnormal behavior or clinical signs related to toxicity in animals was done on daily basis till 7 days. Body weight, and consumption of water and food was recorded regularly over the observation period of our experiment. The main characteristics of the final preparations are summarized in Table 3.14. The final product obtained was clear solution, without any turbidity. Osmolality was close to physiological value. Mean protein concentration was 3.76 g/L, with traces of albumin.

The results in Table 3.15 showed that all animals remained healthy and active throughout our experiment, and slight increased body weight showed the beneficial effects of dietary supplements on animals. The clear product was obtained after filtration. The main

characteristics of the final anti-serum preparation are observed. Osmolality was close to physiological value. Mean protein concentration was 3.76 g/L, with only traces of albumin (less than 1%). All essential tests such as Sterility, abnormal toxicity, endotoxin level detection and Potency (calculated by MNT) for final preparation was satisfactory.

All the animals remained healthy and fit during the experiment with 100% survival rate. Safety study results showed no local or systemic reactions. All the basic hematological and biochemical parameters of all animals were well within acceptance criteria.

3.9.1 QC/Physiochemical analysis of Antiserum

After the antiserum is manufactured, it must go through the quality control analysis for a thorough drug quality and safety testing of the final purified anti serum product. This treatment involved numerous testing procedures. The findings of these necessary chemical experiments at varied doses of caprylic acid are listed in table 3.14.

Table 3.14 Test result summary of the purified IgG-enriched plasma fraction.

Parameters	Results	Acceptance criteria	Test Method
Appearance	Clear, no visible particles	Clear to slightly opalescent pale yellow	Visual observation
pH	6.3	6.0 -7.0	pH meter
Osmolality,	295 +/- 15	> 240 mosm/kg	Osmometer
Total proteins, g/L	3.76 %	Upto 10 %	Biuret
Total Nitrogen	0.6 %	Up to 2.7 %	Kit method
Total solids	4 %	Up to 19 %	Gravimeter analysis
Albumin, g/L	0.53%	Less than 1%	Bromocresol green
Caprylic acid, µg/mL	< 750	< 750	HPLC
Endotoxin, EU/ml	< 0.5	< 0.5	LAL test
Sterility	Pass	No growth during incubation	Sterility test (growth medium)
Abnormal toxicity	Pass	No paralytic symptoms	Rat test
Potency	206 IU/ml	NLT 150 IU/ml	MNT

Table3.15 Baseline characteristics of the study animals.

Basic Characteristics	Probiotic alone (= 12)	Probiotic with Vit A (N = 12)	Probiotics with carrots (N = 12)	Vit A alone (N = 6)	Carrots alone (N=6)	Control without supplements (N = 6)	Total (N= 54) Mean values	Remarks
Group ID	G1+G2	G3+G4	G5+G6	C1	C2	C3		Range
Age of animals	5.1 months	5.1 m	5.1 m	5.2 m	5.2 m	5.1 m	5.15 m	5-6 months
General health								
(%) survival ratio	100% S	100% S	100% S	100% S	100 % S	100% S	100 % S	No death recorded
Weight (kg)	1.8	1.7	1.5	1.6	1.6	1.5	1.7 kg	1.5 to 2.0kgs
Physical fitness	Fit	Fit	Fit	Fit	Fit	Fit	Fit	Physically fit
Safety studies								
Local reactions	Nil	Nil	Nil	Nil	Nil	Nil	Nil	No adverse reactions observed
Systemic reactions	Nil	Nil	Nil	Nil	Nil	Nil	Nil	
Hematology parameters	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Well within range
Biochemistry	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Well within range

3.9.2 Potency determination using MNT (Mouse neutralization In-Vivo Test): rabies specific antibody titter values in 9-experimental groups after 35 days of vaccination:

The rabies specific antibody titers from serum samples preparations were determined with mouse neutralization test method (MNT). The neutralizing potency is expressed in international units (IU)/ml in reference to “In house reference standard for Anti-Rabies Immunoglobulin", obtained from National Institute of Health (NIH). Mean antibody titre were between 75.6-76.4 in groups G1 too G4, while mean titre of 103 and 194.6 were recorded in G5 and G6. Control group1 and C3 were having lower average titre as compared to experimental groups only C2 was having good titre (Figure 3.13).

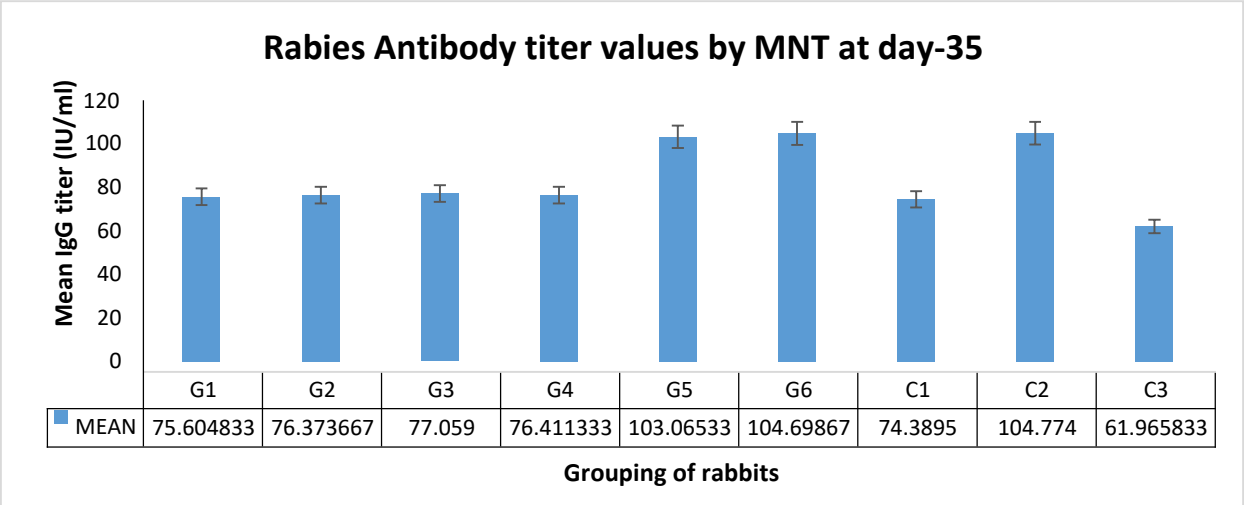


Figure 3.16 :Mean titer value calculations by MNT method after complete vaccination at 35th day in all 9 study groups. Maximum potency calculated shows highest results with organic carrots in G5 & G6. All experimental groups showed better potency calculated in IU/ml bu Reed and Meunch method than C3 control.

3.9.3 RP-HPLC for Caprylic Acid Determination

The HPLC was used to quantify the residual concentration of caprylic acid in purified immunoglobulins samples of three different separation techniques, i.e., Dialysis, TFF, and Centrifugation followed by filtration. The sample treated according to the procedure mentioned above is processed through HPLC. The chromatogram showed a series of peaks corresponding to the sample's various components. If a peak is present at the expected retention time for caprylic acid, then it is likely that caprylic acid is present in the sample. The height of the peak is proportional to the amount of caprylic acid present. It quantifies the amount of caprylic acid

by comparing the peak height to a standard curve obtained from known concentrations of caprylic acid. To be certain that a peak is due to caprylic acid, The retention time and UV spectra of the peak is compared to those of a standard solution of caprylic acid.

The resulting peaks demonstrate that TFF contains less caprylic acid, 6.2 g/ml C.A, than Dialysis, which includes 20.4 g/ml C.A. In contrast, HPLC examination of centrifuged samples revealed the entire absence of C.A as shown in Figure 3.1. The resultant chromatogram showed the absence of respective peaks for caprylic acid when the consequent peaks and their UV spectrum were compared and analysed with the reference UV spectrum. Complete removal of caprylic acid by the procedure mentioned above indicates that the additional centrifugation step should be added to manufacturing immunoglobulins from equine serum through the caprylic acid-based purification method.

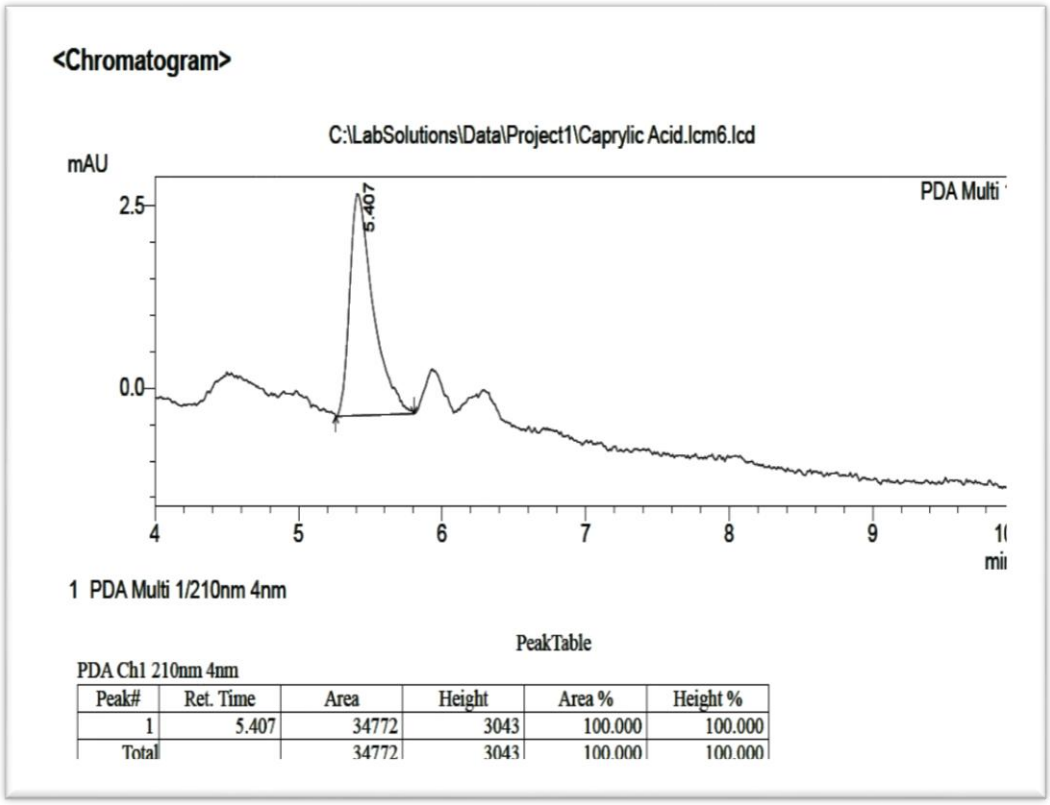


Figure 3.17 Chromatogram of Octanoic acid (caprylic acid) reference standard at 12.5 ppm. A chromatogram of the standard solution of caprylic acid is obtained by mixing the pure sample in a mobile phase, as mentioned by (Herrera, M, 2009), with slight modifications; the peak of caprylic acid is obtained at Rt 5.414 min. It is confirmed by comparing the UV spectrum, chromatogram, and 3d graph of the standard sample with the spectrum of Caprylic acid reported earlier (Ertel & Carstensen, 1987).

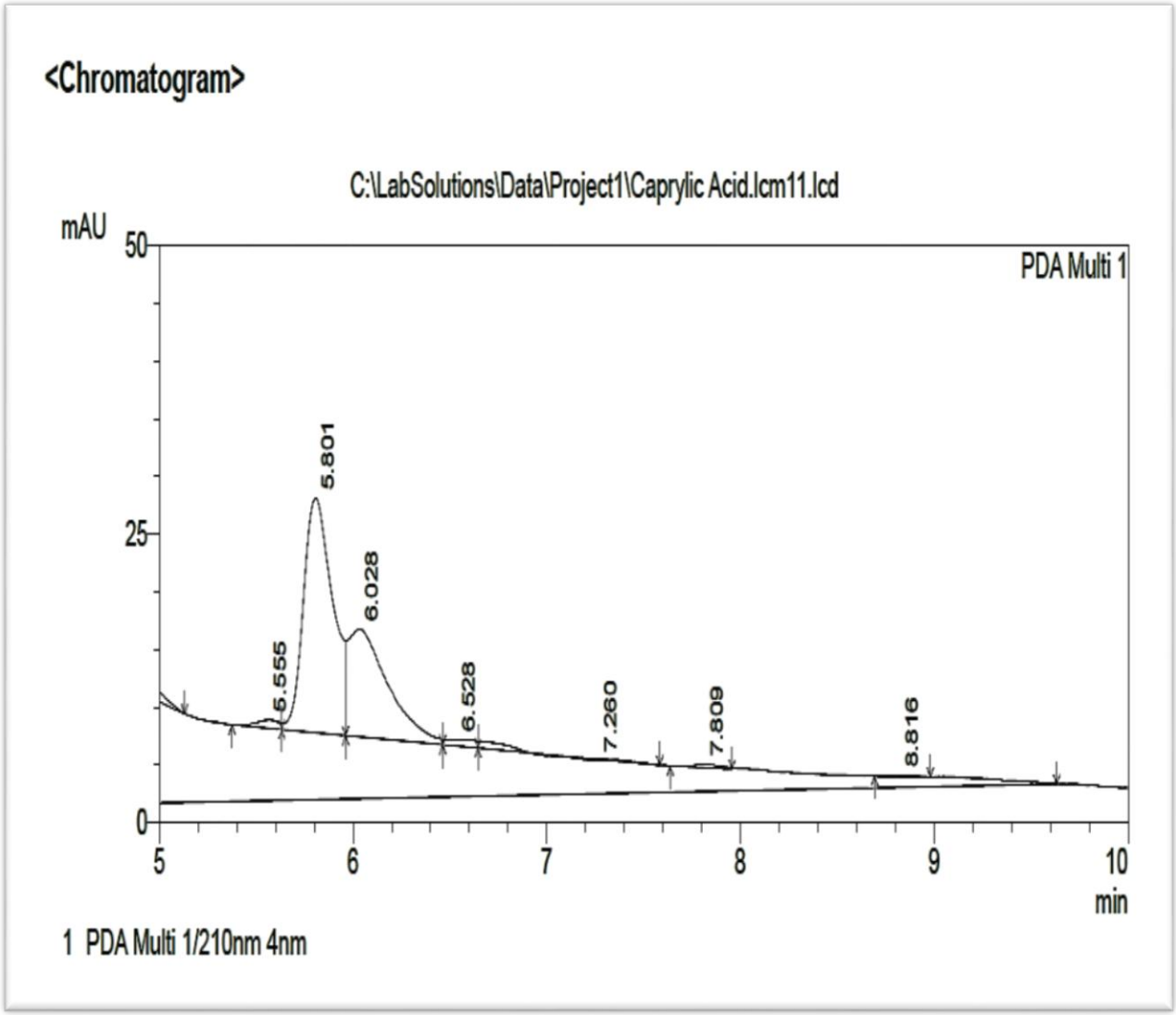


Figure 3.18 Chromatogram of Centrifuged sample (Final product)

Chapter 4

DISCUSSIONS

Dog-mediated rabies causes tens of thousands of mortalities annually. However, such miserable deaths can be prevented through timely post-exposure prophylaxis (PEP). The availability of rabies vaccine and purified immunoglobulins remain poor in many rabies-endemic countries due to the high costs of cell culture vaccines, human-derived anti-sera, poor access, and supply issues. Rabies is included in the list of neglected tropical diseases and 100% fatal zoonotic disease. Fortunately, it is a vaccine preventable disease with timely provision of effective post exposure prophylaxis (PEP). The vaccine efficacy issue has gained the attention of scientists in recent years for the development of effective adaptive immune responses in host organisms due to various biological and other factors (Siddiqui et al., 2012). High costs of rabies vaccines and specific antiserum is still a barrier to provide cost effective treatments to poor patients in low- and middle-income countries like Pakistan. Therefore, simple, and affordable strategies are required by the manufacturers to get better immunization outcomes, in order to reduce the mortalities associated with rabies. This study aims to explore the simple dietary strategies to enhance the rabies vaccine specific antibody titers to produce animal derived cost effective (RIGs) and strengthen local production of poly-clonal antibodies in Pakistan.

4.1 Overall improvements in general health and immunity with probiotics and vitamin A rich diet in the feed of animals.

Primary objective of our study was overall growth, health & immunity improvements, and specific humoral response enhancement against Rabies vaccine with simple cost-effective dietary interventions like probiotics, Vitamin A and organic carrots. Total 54 animals were assigned equally in the six study groups and 3 control groups (n=6). Animals received either probiotics, vitamin A supplements or organic carrots, 4 doses of rabies vaccine, and obtained serum samples thrice at days 0,14 and 35 for serology testing and blood collection was done at day 63 post vaccination for further plasma processing. There were no apparent differences between the group animals about age, sex, housing conditions.

4.2 Rabies vaccine immunogenicity and enhancement of antibody response with Probiotics supplementation in G1 & G2 experimental groups.

On day 0, the serum samples of all animals in the treatment groups failed to exhibit any rabies neutralizing antibodies titer, indicating that none of the animals had been exposed to or vaccinated against rabies. The disparity in rabies antibody titers between probiotic intervention groups G1 and G2 was noted on day 14. All animals were sero-positive following initial vaccine doses. Among those who completed the four-dose vaccination regimen and received probiotics A and B, a high rate of sero-conversion to rabies vaccine occurred gradually at days 35 and 60 post-vaccination ($p > 0.001$, 95% CI). Thus, variations in rabies antibody titers observed across both G1 & G2 treatment groups regardless of brand name were induced by both the administration of rabies vaccinations as well as probiotic therapy.

Probiotics are gram-positive microorganisms that serve as "functional foods" or nutritional supplements and play a vital role in the overall health of their hosts. They possess the unique ability to modify, alter, and restore the host's pre-existing intestinal flora, thereby facilitating smooth functioning of the intestinal environment. The most utilized probiotics include *Lactobacilli, Bifidobacterium, S. Boulardii,* and *B. Coagulans*. Lactic acid bacteria (LAB) represent one of the most extensively studied groups of gram-positive bacteria including *Lactobacilli, Streptococci* and *Enterococci.* These beneficial microorganisms are classified as GRAS (Generally Recognized as Safe) by intentional health authorities. Over the last two decades they have been widely employed as an important research tool particularly in developing mucosal vaccines. The mucosal immune system is considered an active immunological site that plays a crucial role in defending against pathogens; numerous studies indicate that probiotics can enhance both innate and adaptive immunity mechanisms in this regard. A recent study on fish demonstrated how oral administration of probiotics can influence both systemic and local immunity responses within host animals (Simon et al., 2021).

The Potential Role of Probiotics as Vaccine Adjuvants. Recently, probiotics have been extensively researched as innovative vaccine adjuvants with the potential to augment the humoral response of existing vaccines. Jayawardena et al. (2020) reported on the synergistic effects of probiotics and vitamin A supplementation with vaccination on the humoral response

to rabies vaccine, evaluated in New Zealand White rabbits. Our study employed various experimental groups that were categorized based on dietary interventions, as previously delineated in numerous studies. Different brands of multi-strain commercial probiotics and vitamin A supplements were utilized in these groups. The results demonstrate that all animals in both the probiotic treatment groups alone or combined with a vitamin A-rich diet (G1 to G6) evince superior antibody titers against rabies vaccine compared to the control group, as evidenced by Figure-Two-way ANOVA analysis indicating significant differences among groups on both sampling days 14th and 35th ($p=<0.001$, CI 95%).

Our findings are verified by similar results in pre-clinical studies conducted on multiple animal models, including pigs, mice, chickens, and fish. These studies suggest that certain probiotic strains play an immuno-modulatory role in vaccine responses of host animals (Arczewska-Włosek et al., 2022). Parreno et al. 2022 demonstrated the immune-stimulating effect of probiotic IgG as a vaccine adjuvant with the human Rota virus vaccine in pig models. Similarly, Moradi K. et al., 2021 described the significance of probiotic-based mucosal vaccines as a promising protective strategy against respiratory and non-respiratory viruses observed during the recent COVID-19 pandemic. This study also highlighted advances made in modulating immune responses via genetically modified probiotics (Licciardi and Tang 2011; Peroni and Morelli 2021). Our experiments demonstrate that simple dietary interventions along with vaccination resulted in higher immune response compared to control group C3 across all experimental groups. A visible increase was observed in titer values after initial three doses on day 14th (from approximately 8% to15% sero-conversion) for both probiotic groups A and B (G1 and G2) versus control group C3, $p = 0.001$. This increase was more pronounced following completion of four doses over a total immunization period of 28 days. All treatment groups showed significant improvement from minimum18% to maximum 25.93%, one month post-vaccination when compared with C3 control group.

The influence of probiotics and vitamins on gut immunity:

The results therefore support the fact that probiotics and vitamins influence the gut microbiota which in turn activates innate and adaptive immunity by activating the pathways controlling it thereby influencing an overall increase in the humoral immune response against the rabies vaccine. Thus, our results are in correlation with previous findings with other

vaccines on animal models as well as human trials. Probiotic supplementation with vaccination, therefore, enhances overall immunity, especially the adaptive immune response. The mucosal immune system plays a crucial role in the defense mechanism against pathogens and therefore considered as an active immunological site for several interactions. As reported previously in many studies, probiotics effect both innate and adaptive immune responses. A previously published study on fish using probiotics supplementation, has demonstrated that probiotic administration through the oral route can influence both the systemic and the local immunity of the host animals (Guo et al. 2020; Simon et al. 2021). Previously many studies on animal models have pointed out that specific probiotic strains have immuno-modulatory effects on vaccine responses that can be applied to human subjects as well (Praharaj et al., 2015). Zimmermann et al., 2018 systematically reviewed prospective randomized placebo-controlled studies in humans investigating the effect of probiotics on humoral vaccine responses in 26 studies indicating beneficial effect of probiotics in about half of the studies. Similarly, a randomized double-blind placebo-controlled pilot study showing protective titter in 84% of those receiving *Lactobacillus* GG; having potential to act as an adjuvant to enhance immunogenicity of influenza vaccine (Davidson LE et al. 2011).

The gut microbiota is largely being considered an attractive target to enhance vaccine effectiveness in vulnerable populations of lower- and middle-income countries by various interactions. Idrees et al., 2022 also reported that probiotic bacterial strains produce many effector molecules that interact and modulate the host immune system, host gut epithelial barrier functions, gut microbiota composition, also affect the central nervous system and the host's metabolic responses. Probiotics communicate with their hosts through molecular signaling. In the ever-progressing field of vaccinology adjuvants play a key role, that helps to provoke potent and very specific immune responses for protection against diseases.

Vaccines and vaccine adjuvant are stated as biological response modifiers. Biological response modifiers (BRM) are molecules that can regulate or restore immune responsiveness. They are being used to manipulate the immune system for the treatment of autoimmune, infectious, or neoplastic diseases. BRMs include a variety of naturally occurring and synthetic molecules (Speil and Rzepka 2011). Many studies highlighting the possible role of probiotics as vaccine adjuvants have been carried out on oral vaccines such as (Rota vaccines) or mucosal

vaccines like influenza vaccine (Sahoo et al. 2020). However considerable variation has also been reported regarding the choice of probiotic dose, strain viability, purity, duration, and timing of probiotic administration. French P, Penny R. 2009 described the use of probiotic bacteria as an adjuvant for an influenza vaccine. A randomized, placebo-controlled study conducted on 47 participants revealed that the daily intake of a probiotic strain known as *Lactobacillus ferentum* VRI 003, in conjunction with an intramuscular injection of influenza vaccine, demonstrated promising results. The findings suggest that oral consumption of this probiotic bacterium could function as an effective and affordable adjuvant for influenza vaccines while posing minimal risk to patients. For the last few decades role of probiotics in immuno-modulation and their possible health benefits on humans has been a widely studied and is of topic for research interest by nutritional and health researchers. The potential adjuvant roles of probiotics has been demonstrated in review articles and clinical studies on humans stating the possible benefits of probiotics such as safety profiles, low cost intervention, and ease of administration as compared to other vaccine adjuvants.

During the Covid-19, probiotics had been highlighted due to their potential adjuvant role on the inflammatory immune responses in viral infections and have shown promising results, thus demonstrating the effectiveness of probiotics in respiratory viral infections (Lehtoranta et al. 2020, Alagawany et al., (2021). This immuno- modulatory role of probiotics has also been stated to be due to complex interactions in the gut and the release of cytokines that regulates the immune responses. It also involves various immune system components such as interleukins (ILs), interferons (IFNs), transforming growth factors (TGF), tumor necrosis factors (TNFs),and chemokines which are released from immune cells like dendritic cells (DCs), lymphocytes, granulocytes, macrophages, mast cells, epithelial cells.

Probiotic bacteria are reported to communicate with their hosts through molecular signaling. Probiotic bacteria thus act as immunological adjuvants that generate immuno- stimulatory effects mediated by cytokines (Lehtoranta et al., 2020). However, the interactions of the immune system and probiotic bacteria under *in vitro* conditions may differ from *in vivo, therefore further* detailed studies and clinical trials may be required for future perspectives on probiotic research (Daliri et al. 2021). Oresanya, et al., 2022 reported that administering bi-annual high dose vitamin A supplements to children aged 6–59 months can considerably

decrease child mortality rates. However, in Nigeria, the coverage of vitamin A supplementation (VAS) remains low. To enhance child survival, the World Health Organization advises integrating VAS into other public health programs. In this regard, a study was conducted on co-implementing vitamin A supplementation with seasonal malaria chemo prevention in Sokoto State, Nigeria. Poor populations of developing world are often facing vitamin A and other nutrient deficiencies, especially vulnerable populations including children and the elderly having the weak immune system. Therefore vitamin-rich diet may also be associated with the modulation of the gut microbiota of the host animals as previously stated in many studies and trials (Correa et al. 2022).

4.3 Rabies vaccine immunogenicity and humoral immune response with Probiotics and vitamin A supplementation in G3 & G4 groups

The influence of vitamin A as a supplement on vaccine humoral response has also been investigated in this study. With the similar dietary protocol, addition of vitamin A supplementation also showed improved results than control group without supplementation. Twelve animals out of the 54 in groups G 3 and G4 which received vitamin A supplementation with probiotics and n= 6 who received only vitamin A without probiotics (C1) were sero-converted sufficiently to rabies vaccine at day 14 post 3 doses ($p > 0.001$, 95% CI). At fifth week after initial dose, similar gradually improved antibody response was observed at day 35 and 60 in all 18 animals of G3, G4 and C1 sub-control were highly sero-positive for rabies IgGs .

4.4 Rabies vaccine immunogenicity and humoral immune response with Probiotics supplementation and organic carrots feeding in G5 & G6 groups.

Similarly, animals of G5 & G6 receiving probiotics supplementation and organic carrot rich diet and n=6 animals of C2 sub- group (only fed with carrots without probiotics) showed significant increase in rabies antibody titre values as compared to C3 control without any supplementation. All animals from groups G5 and G6 had a significant increase ($p<0.001$) in the production of rabies antibodies after 14, 35 and 60-days Table 3. ($p > 0.001$, 95% CI determined by two-way ANOVA). In general, after 3 or 4 doses of rabies vaccination, all the rabbits had shown good rabies antibody titres, irrespective of whether they had received probiotic alone or added vitamin A as natural or supplementation to the diet mixture. In this way, dietary supplementation caused a persistent immune-stimulant effect and gradual

enhancement from day 0 to day 60 on the antibody titre values of all intervention groups as compared to control C3 group having no supplementation. A significant increase in specific antibody titre against rabies vaccine was observed after the initial 3 doses of rabies vaccine as observed in G3, G4, G5 and G6, either with vitamin A capsules or natural organic food source as organic carrots. This enhancement appears to be associated with vitamin A supplementation (VAS) which has also shown health benefits when administered with different vaccines as previously reported in some studies. VAS is also recommended with immunization programs for children in developing countries. Our results are in correlation with a previously published study on the role of VAS on rabies vaccine immunogenicity in a small clinical trial involving 40 human volunteers, conducted at the National Institute of Health, Pakistan (Siddiqui et al. 2001).

In our study based on animal models, we have incorporated probiotics with vitamin A, keeping for the purpose of Poly-clonal antibody production using simple dietary interventions. The findings are quite encouraging toward the implementation of such alternate strategies to see the effects of natural products as Adjuvants on animal models. Such simple natural products acting as safe nutraceutical that can be added in animal feed for improvement in growth, overall well-being and better immunity. Thus using this approach with rabies vaccine may be an attractive strategy for antiserum production, thereby reducing fatalities caused by rabies in poor setups. Carrots are considered one of the healthiest vegetables because of their high phytochemical content including phenolic, ascorbic acid, carotenoids and polyacetylenes which exert anti-inflammatory activities. Many benefits of carotenoids have previously been reported (Eggersdorfer and Wyss 2018). Roselli and co- workers investigated that use and health benefits of organic carrots, that induced some changes in lymphocyte population (increase in regulatory T cells) hence exerting a positive effect on immune outcomes of hot. This is first evidence of increase in regulatory T cells induced by organic food consumption (Roselli et al. 2012). Similarly with organic carrots in groups G5, G6 and sub-control group C2 also provide evidence of previously reported health benefits of carrot's natural components on immunity. Thus probiotic, vitamin A and carrot rich diets appeared to be associated with the modulation of gut microbiota of the host animals as previously stated in many clinical trials. Keeping in view all previous studies and comparing our results, it can be concluded that that adding probiotics and vitamin A supplements in animal feed exerts a better effect on general health of animals and immunity which ultimately resulted effect on humoral immune response against vaccination.

Secondary objective of the study was to adopt cost effective strategies to enhance humoral response in donor animals for production of Poly-clonal antibodies (PAb). These strategies include selection of suitable vaccination regimen to get improved titer in less time, antibody titer determination using in vitro test such as Platellia II^{TM} ELISA kit (Bio-Rad) instead of classical mouse neutralization (MNT) that is time consuming and requires large number of animals. Similarly, enhancement of PAb production in animals with weekly hyper-immunization strategy and further purification with new Caprylic acid fractionation technique to get highly purified rabies antiserum as the final product. The conventional intramuscular (IM) vaccine administration is a barrier in most developing countries due to more vaccine requirements and a one-month immunization schedule. Therefore, cost-effective strategies such as using a multi-site intradermal (ID) route are essentially required for animal humoral response development for antiserum production. In second part of study, three different ID regimens were compared with the traditional IM route of immunization in New Zealand white rabbits, using cell culture rabies vaccine (CCRV) for immune response enhancement after 14 and 35 days. The experiment focused on animal models used for producing rabies immunoglobulins (RIGs) to investigate the non-inferiority of ID regimen over IM regimen for anti-sera production. ELISA method was used initially to measure rabies specific antibody titers.First strategy to get was to select suitable immunization regimen recommended for rabies vaccine to get improved antibody titer level in less time. Thus, comparing intradermal (ID) rabies vaccination with conventional intramuscular (IM) regimen on humoral response of New Zealand White rabbits to produce animal derived poly-clonal antibodies (PAbs). The experiment was carried out to evaluate the sero-conversion against rabies vaccine with shorter (3 to 4 dose) intradermal (ID) vaccination regimens. These three regimens are based on the 2-site ID Thai Red Cross (TRC) and were compared to the routinely used “Essen” regimen (5-dose intramuscular IM). The conventional intramuscular (IM) vaccine administration is a barrier in most developing countries due to more vaccine requirements and a one-month immunization schedule.

Immune response in terms of antibody titer was calculated from OD values with conversion tool provided with the kit. For the four groups (R1, R2, R3 and R4) antibody titer values were 3.099, 3.594, 3.704 and 3.368 IU/ml respectively, after 14 days (Table 3.13). All groups showed sero-conversion above the recommended threshold of protective titer values at day 14 Table 3.13. R3 showed highest titer compared to other groups. Similarly experimental groups immunized through ID doses showed higher antibody titer than R1 control group. All the groups reached 'protective' rabies virus neutralizing antibody titer >0.5 IU/ml, at day 14. After 35 days, antibody titer for R1, R2, R3 and R4 were 3.49, 3.73, 3.83 and 3.78 IU/ml respectively. The antibody titers indicate a significant increase in regimen immunized via intradermal (R2, R3 and R4), all having titer above 3 IU/ml. However, groups immunized within one week (R2 and R3) presented the highest titer >3.5 IU/ml at day 14, where R3 showed the titer 3.7 IU/ml. Moreover, at day 35, antibody titers among all the groups reached > 3.4 IU/ml. A similar trend of increased antibody titer among the three groups (R2, R3 and R4) was observed even after 35 days, suggesting ID routes non-inferior over IM. Although, the titer at day 35 was more than the titer observed at day 14 but high sero-conversion was attained since day 14. This could also be due to the provision of dietary supplements and probiotics. The results suggest a significant immune response in less course of time among rabbit groups immunized intradermally (ID at multi-sites) than control group R-1(5-dose, single site IM immunization for one month). Among all animals' rabbits of R2 and R3 show significant antibody titers after one-week immunization course. However, results shown by R-2 (IPC 1 week, 2 site regimen is crucial as it gives antibody titer of 3.59 IU/ml after 14 days upon consumption of the least amount of dose experimented i.e., 0.6ml. The use of less dosage would make it more cost-effective method for better yield of final product (RIG) from donor rabbits.

WHO has revised its recommendations for rabies prophylaxis (PEP) and has emphasized to adopt the most immunogenic yet cost-effective method of vaccination to achieve the most significant benefit for vaccination campaigns in humans. (World Health Organization 2018; Quiambao et al. 2022). Several studies addressed the respective immunogenicity of the ID regimens due to the advantage of using fewer doses and less consumption of vaccine in comparison with previously used intramuscular (IM) rabies vaccine regimens that require more vaccine and longer duration to achieve desired RIG titers (Gongal and Sampath 2019,

Quiambao et al. 2019). There is an increasing body of evidence indicating that reducing Post-Exposure Prophylaxis (PEP) to just three visits in a single week does not have any negative impact on sero-conversion rates or immune memory. Quiambao et al. (2022) conducted a randomized non-inferiority trial comparing the efficacy of a one-week, four-site PEP regimen with the updated Thai Red Cross regimen using purified Vero cell rabies vaccine. Therefore, cost effective strategies such as using a multi- site intradermal (ID) route are essentially required for animal humoral response development for antiserum production. In this study, three different ID regimens were compared with the traditional IM route of immunization in New Zealand white (NZW) rabbits, using cell culture rabies vaccine (CCRV) for immune response enhancement after 14 and 35 days. Our results demonstrated better response to the ID regimen than the IM route in terms of immunogenicity and volume consumption of the vaccine. The three ID regimes, either 1-week 2 sites (IPC, R-2), 28-day 2-site (TRC-R-3), or 4-site one-week regimen (Alternate, R-4 regimen), resulted in significantly higher mean titer values compared to the control IM regimen group R-1 ($p<0.001$). Overall, intradermally vaccine administration is considered more immunogenic and also shows safety, efficacy and cost-effectiveness (Gongal and Sampath 2019; Denis et al. 2019). Therefore, application of this method for Poly-clonal antibody production would help cope with vaccine and RIG shortage, thus providing effective post-exposure prophylaxis (PEP) for better control of rabies in developing countries like Pakistan.

4.5 Hyper-immunization strategy for enhancement of PAb production in animals and optimization of caprylic acid fractionation technique for purification of final product Rabies immunoglobulins / antiserum (Objective # 4)

Immunoglobulins (IgGs) are the essential plasma derived products, usually manufactured from bulk plasma pools of donor animals in highly regulated facilities using current good manufacturing practices (GMP) production technologies on large industrial scale. However, these products are usually produced with low capacity and cause short supply in lower middle-income countries (LMICs). It is therefore critical to develop small-scale, easy-to-use technologies that can be adapted to process the plasma cost effective products. poly-clonal anti- sera against the rabies virus glycoprotein antigen are either produced in small animals like rabbits or larger animals such as sheep, horses, and camels. The purified anti-sera are

diluted in phosphate-buffered saline (PBS) and pH set between 6.0 -7.0. Hyper-immunization of animals for rabies antiserum production is a technique developed initially by at the Queen Saovabha Memorial Institute (QMSI) Bangkok, Thailand by immunizing horses with a purified Vero cell rabies (PVR) vaccine. The animals are given a series of injections of the vaccine in increasing volumes subcutaneously into the lateral aspect of the neck. The total immunization period may be extended to maximum 105 days before first bleeding to get hyper-immune plasma. Similar strategy was adopted in our experimental study with hyper-immunization of NZW rabbits and provided excellent outcomes in terms of rabies antibody titer values required for manufacturing of animal derived rabies antiserum.

Moreover, our experiment has shown simple ways to get purified IgG-enriched plasma fraction with caprylic acid-based fractionation process. The second part of our experiment is based on caprylic acid precipitation of non-IgG molecules from plasma mixture to get purified concentrated RIGs. This method is already used in the commercial manufacturing of Anti-sera products especially horse derived antivenom immunoglobulins (Rojas et al. 1994). In our experiment, product yield i.e. 50-60% containing IgG fraction was obtained. Residual proteins such as albumin was low (below 1%) in compliance with requirements for commercial intravenous IgG. These experiments were aimed to optimize the production of antiserum using caprylic acid purification. The study evaluated various factors such as yield, filtration time, turbidity, protein aggregates, protein and albumin concentrations, and electrophoretic profile. Optimal conditions were found to be adjusting plasma pH to 5.5 with 1.76 N acetic acid, adding 5% concentrated caprylic acid, and agitating for 1 hour before filtration. The process resulted in a highly enriched preparation of IgG with a good neutralizing ability and a yield of 60%. The method was compared to the traditional ammonium sulfate method, with results indicating that the caprylic acid method was faster, had higher yield, and lower protein and albumin levels, potentially reducing the risk of allergic reactions in humans. The RIG batch prepared here showed good potency and antibody titer value was approximately 200 IU/ml, well above least recommended 150 IU/ml criteria. Moreover, the preparation had shown the levels of essential tests well within British Pharmacopoeia limits for anti-sera products. The disposable commercial hemodialyzer are reported to be effective in removing caprylic acid to undetectable levels and further concentration of IgGs. Mixture is further subjected to filtration, sterile filtration and dispensed into sterile container. IgG concentration and dialysis process usually

done with this method is faster and yields a clear solution free of particles. Using caprylic acid along with low pH treatment is known for viral safety measure for effective viral load reduction in both human (Brodsky Y et al. 2012) and horse derived IgGs. Our study confirms that the optimization of different parameter such as pH, concentration of caprylic acid, time and stirring speed as optimized in previous experiments were quite effective in getting more purified product with better product recovery. Better IgG recovery (approx. 50–60%) was achieved, consistent with previously reported recovery of anti-rabies immunoglobulins from horse plasma (Temponi et al., 1989, Mariani et al., 1991, Brodsky et al., 2012).

The purification of IgG from equine serum after precipitation with caprylic acid involves various techniques for removing the residual caprylic acid from the final product. These techniques range from affinity columns, ion exchange chromatography, and precipitation with different agents to conventional filtration, diafiltration, and dialysis. The residual caprylic acid concentration has not been reported after these methods. This study aimed to determine the best purification techniques for removing caprylic acid from the final antivenom formulation. Three techniques were evaluated: dialysis, TFF, and centrifugation. Results showed that TFF had the lowest residual concentration of caprylic acid compared to dialysis. Centrifugation was found to be the most effective in removing caprylic acid, as it was not detected in the final formulation. These results have suggested that incorporating centrifugation into the industrial production process could potentially reduce the likelihood of allergic reactions caused by antivenoms. The choice of purification technique will depend on various factors such as the scale of operation, desired purity, and yield.

Conclusions:

Rabies is a fatal zoonotic disease with 100% mortality, but preventable with the timely and correct use of post-exposure prophylaxis (PEP) that includes vaccination and rabies immunoglobulins (RIGs) in case of severe bites. In developing countries, a shortage of vaccines, anti-sera and overall high total treatment costs leave most dog bite victims untreated, thus rabies prevention remains a challenge for successful control in developing countries like Pakistan. Simple and practical measures are required to improve the efficacy of vaccines for the vulnerable population of lower middle-income countries with weak immunity. Moreover, enhancement of IgG production in donor animals is necessary to provide better and faster access to needy patients regarding immunoglobulin therapy. There is a dire need to strengthen the local manufacturing of rabies vaccines and animal derived rabies anti-sera for underprivileged populations (Afzal, et al., 2022)

Our study aims to develop a cost-effective vaccine strategy for the deadly disease "rabies" which significantly impacts the lives of poor populations in developing countries and needs attention to control this disease. Primary objective of this study was to enhance rabies vaccine-specific immune outcomes with simple dietary strategies based on probiotics and vitamin A to enhance the humoral immune response and hence the antibody titer in animals. Previous studies have investigated different approaches to improve vaccine efficacy using vaccine adjuvants. However, we have used dietary interventions like probiotics as vaccine adjuvants and vitamins in this study which are safe, easy to adopt and provide a more robust immune response with fewer vaccine dose requirements. Therefore, concomitant vaccines and probiotics/vitamins administration would be an extremely cost-effective approach for enhancing specific humoral response against vaccination. Our study concludes that probiotics and dietary supplementation with vitamin A offer a relatively cost-effective and safe intervention to improve rabies vaccine efficacy and humoral immune response as well as the duration of protection and reduce the requirement of extra doses, thus compensating for the vaccine shortage issues in developing countries like Pakistan. Further studies are still needed on optimizing probiotic strains, dosage, and administration time with the rabies vaccination schedule. Moreover, immuno-modulatory interactions of probiotics with gut microbiota may further be investigated in future. Concomitant vaccines and probiotics/vitamins administration

would be an extremely cost-effective approach that could substantially reduce the rabies disease burden with global efforts to eliminate dog-mediated rabies till 2030. Further studies are still needed on the optimization of strains, dosage, and duration of probiotics administration on large donor animals to produce animal derived anti-sera for better immune outcomes, especially for production of animal derived hyper-immune plasma.

Secondary objective of the study was to adopt cost effective downstream processing strategies for production of animal derived Rabies immunoglobulins / antiserum (RIGs). Hyper-immunization strategy was adopted for enhancement of PAb production in animals. After plasma collection and pooling further optimization of purification methods using caprylic acid fractionation technique was applied for purification of final product. The caprylic acid fractionation method is an excellent alternative to classical salt precipitation techniques as it produces purified immunoglobulin fraction with better yield in a short period. The cost-effective strategies used could be of interest to commercial manufacturers to produce large-scale rabies immunoglobulins from animal plasma in developing countries. Previous studies have employed caprylic acid as a means of separating IgG from serum and fluid (Chanutin et al., 1960; Perosa et al., 1990; Steinbuch et al., 1969). The exact mechanism underlying this fractionation remains unclear, but it has been observed to cause precipitation of non-IgG proteins, resulting in an enriched IgG solution (Rojas et al., 1994). While this method has primarily been used for research purposes, (Dos Santos et al., 1989) utilized caprylic acid in conjunction with pepsin digestion to purify antivenom fragments. The need for more efficient fractionation processes in antivenom production to minimize the incidence of allergic reactions in snake bite patients remains an ongoing area of investigation. Recent studies have established the most optimal conditions for purifying animal derived immunoglobulins through caprylic acid precipitation from hyper-immune plasma, which has been found to be more effective than conventional ammonium sulphate fractionation. Unlike previous research by (McKinney et al., 1987) that recommended dilution of serum with acetate buffer, the current studies have established that excellent results can be achieved by adjusting the pH of undiluted plasma to 5.8 with 1.76 N acetic acid, followed by direct administration of caprylic acid. This approach eliminates the need for a concentration step, making it more practical. If the neutralizing titre

decreases after precipitation and dialysis, immunoglobulins can then be concentrated through ultra-filtration or ammonium sulfate precipitation.

The implementation of the previously established optimal conditions for purifying horse IgG using caprylic acid precipitation from hyper-immune plasma resulted in a highly enriched preparation of IgG with exceptional neutralizing ability and a yield of approximately 60%. These findings were in contrast to the ideal results previously reported by other researchers who fractionated sera from various sources(McKinney et al., 1987; Steinbuch et al., 1969) indicating the need for determining suitable laboratory conditions specific to each product. Subsequently, these conditions were determined through research and development trials, and the process was scaled up at NIH in Islamabad, where plasma quantities of 10 litters were used. The results obtained were comparable to those of small-scale investigations, demonstrating the robustness and reproducibility of the process at larger scale. Second conclusion of our study is that by adopting different immunization strategies for donor animals, improving downstream process methodology using cost effective methods will overall reduce the cost of anti-sera and overcome the shortage of vaccines and improve vaccine efficacy issues.

Future Prospects:

The human gut microbiota is crucial in regulating human metabolism and immune function. Recent studies have shown that the gut microbiota can function as an endocrine organ, affecting numerous physiological processes in the human body. Probiotics are live microorganisms that confer health benefits when consumed adequately, is an essential component of the gut microbiota that promotes immune responses and contributes to overall health. Given the importance of gut mucosal immunity, further exploring probiotics' potential effectiveness in vaccine clinical trials and examining their role in human immunological pathways is essential.

More research is needed to establish the safety and durability of immune responses elicited by probiotics, particularly in vulnerable populations such as infants, older people, and immune-compromised individuals. To ensure the safety and efficacy of probiotics, it is essential to develop robust methods for evaluating their functionality and security in both in vitro and in vivo settings. Furthermore, a regulatory framework must be established to allow for specific health claims on probiotic food labels in cases with strong scientific evidence

supporting their health benefits. By promoting the adoption of clear guidelines and regulations for probiotics, we can help to maximize the potential benefits of these beneficial microorganisms for human health.

Prospective studies

Our research studies have focused on the protocol optimization of the caprylic acid-based purification method from animal serum. Quality control physiochemical parameters are also tested and passed for the final formulation of antivenom. HPLC analysis has shown that the caprylic acid is successfully eliminated in the purification techniques involved in the research project. But further research analysis is required for the immunoglobulin's purity profile analysis.

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LIST OF PUBLICATIONS

1. **Najam, A., Ahmad, S., Abid, R., Ali, H., Husnain, M., Aziz, T., Adeel, S.S., Muhammad, N. and Ghazanfar, S.(2023).** Immune-adjuvant effect of vitamin A and probiotics supplementation on humoral response to cell culture rabies vaccine in rabbits. 3 Biotech, 13(7), p.232.

Under Review :

2.Enhancement of Rabies Vaccine Humoral Response in Rabbits with Probiotics and Purification of Immunoglobulins For Poly-Clonal Anti Rabies Serum Production **(PLOS-ONE).**

3. Comparing intra-dermal (ID) rabies vaccination with conventional IM regimen on humoral response of New Zealand white rabbits for the production of animal derived Poly clonal antibodies in Pakistan (submitted).

Turnitin Originality Report

Study on Modulation of Vaccine Immune Response with Probiotic Supplementation in Animal Models by Amina Najam .

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