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Vaccine Preperative Trial for Leptospirosis and their Pathological, Immunological Study by Serum Electrophoresis

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ABSTRACT

Leptospirosis is a fatal infectious disease caused by different serovars of Leptospira spirochetes affecting humans and animals. In the present study, the trials of the whole-cell killed formalin treated monovalent vaccine using *Leptospira icterohaemorrhagiae* and trivalent vaccine using *Leptospira icterohaemorrhagiae*, *Leptospira louisiana*, and *Leptospira hebdomadis* were studied. The serum electrophoresis studies were done after administration of the vaccine into the experimental albino mice along with the booster dose of the vaccinated serum by densitometric readings. Similarly, the pathological observations were made by dissecting the virulent mice, vaccinated mice, and comparing them with the control mice. The MAT titre was also studied after the booster dose administration of the vaccinated serum. The monovalent and trivalent whole-cell killed formalin treated vaccines shows significant raise in the total proteins, albumin, globulin, α 1 globulin, α 2 globulin, β globulin and γ globulins of the serum as well as increase in significant levels in the antibody levels after the administration of the booster dose at an interval of 14 days.

Keywords: Leptospira, Whole-cell killed formalin treated vaccine, Immunological study, Pathological study, Serum electrophoresis.

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INTRODUCTION:

Leptospirosis, a zoonotic disease caused by the spirochetal bacteria *Leptospira* is a common disease of livestock, pet animals with its prevalence worldwide [1]. The geographical distribution of leptospirosis has been overlapping over the decades and produce endemic and epidemic acute febrile diseases [2]. Sporadic outbreaks have been reported from the countries like USA, UK, Australia, New Zealand, parts of Europe, and among Asian countries. The recent incidence of leptospirosis is approximately 1.03 million cases with 58900 associated deaths that have been reported worldwide [3]. In India, Ayyar first isolated Leptospira interrogans serovar Icterohaemorrhagiae from dogs in Chennai, Tamilnadu, India, [4] and from then on the prevalence of leptospirosis have been reported in human and animal species [5-8]. The source of infection occurs through direct or indirect exposure to infected reservoir host animals which carry the leptospires in their renal tubules and shed it in their urine [9]. The brown rat (*Rattus norvegicus*) was considered an important source of infection although many wild and domestic animals can serve as reservoir hosts. Leptospirosis was initially known as Weil's syndrome [10, 11] and is mostly an occupational disease affecting humans who come in contact with rodents, pets, and polluted water [12-15]. The clinical features of leptospirosis depend on the host and serovar type which may be highly variable and nonspecific. Mostly asymptomatic or subclinical infections occur but when symptoms appear the onset is typically 2 to 30 days after exposure [16]. The broad spectrum symptoms of leptospirosis include pneumonitis [17, 18], meningitis [19], nephritis [20-23], hepatitis [24], pancreatitis [25] and erythema nodosum [26] and death [27, 28]. The whole-cell killed vaccines were developed and available in some countries for prevention during endemic and epidemic situations [29]. Apart from the whole-cell killed vaccines other vaccines like recombinant protein vaccines, lipopolysaccharide (LPS) vaccines, inactivated and attenuated vaccines, and DNA vaccines have been developed across worldwide [30]. The development of the whole-cell killed vaccine and their subsequent trials have been reviewed and published in the middle of the last century [31-34]. The targeting and identification of proteins among pathogenic leptospires have become one of the major

leptospirosis researches as it can provide cross-protection on various serovar groups [35-57]. In the present study, the whole-cell killed formalin treated vaccine preparative trials were conducted in albino mice and their immunological studies were done using serum electrophoresis by densitometric readings and SDS PAGE for determining their molecular weight along with the pathological observation of the dissected albino mice injected with virulent strains and the whole-cell killed formalin treated vaccine prepared compared to that of control mice.

MATERIALS AND METHODS:

Collection and maintenance of Virulent Leptopspiral serovars

The virulent serovars of *Leptospira icterohaemorrhagiae* (Fig 3), *Leptospira louisiana* (Fig 1), and *Leptospira hebdomadis* (Fig 2) were collected and maintained at Leptospirosis Research Cell, Institute of Microbiology, Madras Medical College, Chennai, Tamilnadu, India. The Virulent serovars were maintained in Ellinghausen McCullough Johnson & Harris (EMJH) medium [BD Difco] with a solution of albumin, polysorbate 80, and additional growth factors for the growth of Leptospira [58-65] and the final pH 7.5 ± 0.2 was maintained (Fig 4). The cultures were incubated for 5 to 7 days at 28-30^{0}C and they were checked for the growth and purity of leptospires under dark field microscopy (Nikon SMZ1500) to see that absence of any contamination or clumps or presence of enough quantity of growth. The turbidity of the cultures was checked and compared with 1.0 McFarland's standard for the level of growth as to obtain 2-3 x 10^8 leptospira /ml of media [66].

Monovalent and Trivalent vaccine preparation trial for Leptospirosis

The monovalent vaccine was prepared using the serovar *Leptospira icterohaemorrhagiae* and the trivalent vaccine was prepared using serovars *Leptospira icterohaemorrhagiae*, *Leptospira louisiana*, and *Leptospira hebdomadis* at 250 million/ml concentration (Fig 5). The cultures were killed with formalin (Merck) (0.5 ml formaldehyde) ± 36% in 100 ml culture. They were kept in room temperature for 30 minutes and the killed leptospires were then heated in a boiling water bath (95^{0}C) for 30 minutes. They were rotated every 15 minutes and then cooled at room temperature [67-70].

Experimental inoculation in albino mice

The albino mice were procured from Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Madavaram, Chennai, Tamilnadu, India. The mice weighed in the range of 18-20 g in weight. The procured albino mice were brought and maintained at Animal House at Jaya College of Arts & Science, Chennai, Tamilnadu, India. The mice were maintained separately with proper housing and ventilation. Box type cages were maintained as mice required shaded light with proper bedding materials like sawdust, paddy husk, sugarcane pith, and wood shavings and thoroughly changed at regular intervals. The sanitary condition of the cage is thoroughly checked and cleaned at regular intervals. The feeders and waterers are thoroughly disinfected using moist heat sterilization.

The prepared monovalent and trivalent vaccine was injected into the albino mice at 0.05 ml concentration intramuscularly along with Freund's complete adjuvant (Sigma-Aldrich) [71]. Before inoculation, the albino mice were tested for germ-free status without any infections.

Determination of Serum Globulin profile of inoculated albino mice

Cardiac puncture of albino mice was made by piercing the needle through skin and musculature of the left intercostal space between sixth and seventh ribs [72]. The heart was palpated before the introduction of the needle and the blood was drawn carefully with 1 ml tuberculin needle and the serum was separated by centrifugation (REMI – R8C). The separated serum from the monovalent and trivalent injected albino mice was compared with the serum of control mice by serum electrophoresis and densitometric readings.

Serum Electrophoresis

The procured serum from monovalent injected mice, trivalent injected mice, and control mice were subjected to serum electrophoresis. 1% Agarose was prepared in the tris-glycine buffer and 3.5 ml of agarose solution was carefully poured onto a clean glass slide using a pipette that was allowed to form a gel for 15-20 minutes. A few drops of undiluted serum were pipetted on to a clean glass slide and a pinch of bromophenol blue powder was added over it. Then the serum was mixed using a coverslip and stamped on to the gel at one end of the slide. The slide was then placed over the bridges of the apparatus and 75 -100 ml of Tris-glycine buffer was poured to each reservoir. The Whatmann -1 filter paper (3.0 x 2.5 cm) was wetted in the buffer and place on each side of the gel side and the other end of the paper wick was allowed to touch the buffer. The apparatus was connected to the power supply which was turned on for the electron mobility to run over the gel and the voltage was slowly increased to 100 volts. The gel was allowed to run for 1 to 2 hours till the blue color marker dye reaches the anodic end of the gel. Finally, the power supply was disconnected and the slides were removed and placed in a tray for staining purpose [73]. After staining the slides were removed and dried which were examined for bands on white light trans-illuminator (BIO-HELIX - BP001CU). The slides were finally subjected to densitometric readings.

Microscopic Agglutination Test (MAT)

The MAT is a qualitative and quantitative test having high diagnostic specificity and relatively low sensitivity. The microscopic agglutination test (MAT) is the reference test method for the sero-diagnosis of leptospirosis both in humans and in animals (World Health Organization (WHO), 2003; World Organization for Animal Health (OIE), 2008). This test detects antibodies to specific serovars using live leptospiral antigens and can be performed on serum from any species. The serum obtained from the vaccinated experimental mice was given subsequent booster doses at the time interval of 14 days in the range of 1st, 2nd & 3rd and the antibody level was determined by Microscopic Agglutination Test (MAT) [74].

Pathological Studies:

The pathological studies were performed by dissecting the mice [virulent mice, vaccinated mice, and control mice] and their physical internal body parts were observed and reported [75].

RESULTS:

The whole-cell heat-killed formalin treated monovalent vaccine was prepared using single serovar *Leptospira icterohaemorrhagiae* and a trivalent whole-cell heat-killed formalin treated vaccine was prepared using serovars *Leptospira icterohaemorrhagiae*, *Leptospira louisiana*, and *Leptospira hebdomadis*. The vaccines monovalent and trivalent were then injected into albino mice (Include

conditions) intramuscularly and their serum profile was observed by densitometric readings compared with the virulent injected albino mice serum and the control mice serum. The densitometric readings of the serum electrophoresis revealed the presence of total proteins, albumin, globulin, α 1 globulin, α 2 globulin, β globulin, and γ globulins in the monovalent, trivalent and virulent injected mice serum as well as in the control mice serum. The total proteins, albumin, globulin, α 1 globulin, α 2 globulin, β globulin and γ globulins showed a significant increase in monovalent vaccine injected serum (Fig 7; Graph 2) when compared to control mice serum (Fig 6; Graph 1) and a 2 fold increase of total proteins, albumin, globulin, α 1 globulin, α 2 globulin, β globulin, and γ globulins were observed in trivalent vaccine injected serum (Fig 8; Graph 3). The booster dose of the monovalent and trivalent vaccines along with Freund's adjuvant was given at an interval of every 14 days interval and the antibody level was determined. The microscopic agglutination titre (MAT) (Fig 9 & 10) revealed the significant increase in the antibody levels after the administration of the booster dose in the ratio 1:05 (1st booster dose), 1:20 (2nd booster dose) and 1:40 (3rd booster dose). The pathological studies revealed the enlargement of internal organs of the virulent strains injected albino mice such as spleen, liver, heart intestine, etc. which were absent when compared with the control and vaccinated albino mice.

DISCUSSION:

The whole-cell killed formalin treated vaccines from the present study showed a significant increase in the serum profile when compared to control mice serum especially 2 fold increase was observed with trivalent injected vaccine serum. Similarly, the antibody levels by microscopic agglutination test showed an increase in the antibody levels on every booster dose at an interval of 14 days. It is known that heat or chemical treatment of leptospires changes their effectiveness as vaccines. The phenol, Formalin, and thimerosal remove the outer envelope and damage the protoplasmic cylinder was reported during 1965 in an ultrastructure study [76]. It is known that Formalin treated antigens have lower sensitivity and greater cross-reactivity in the MAT [77]. The effectiveness of the killed vaccine is serovar specific which was documented in various studies [78, 79]. The variability of serovars in the locality of endemic leptospiral strains makes it difficult to construct a vaccine that can be used worldwide [80]. Studies revealed that heat-killed inactivated vaccine induces a strong antigen-specific proliferative response by peripheral blood mononuclear cells (PBMC) which was reported in vaccinated cattle after 2 months of the initial booster dose [81]. The whole-cell killed vaccine in the study illustrated that the antibody levels were low [1:05] during first booster dose and the significant increase in the antibody levels were observed in the 2nd booster dose (1:20) and 3rd booster dose (1:40) so this confirms an elevated immune response after successive booster doses (Graph 4) [82]. The pathological studies confirmed that the vaccinated mice (Fig 13) showed no significant change when compared to that of control mice (Fig 11) in the internal body organs whereas the combination of virulent serovars injected into the albino mice (Fig 12) showed severe enlargement of the organs such as spleen, liver, pancreas, etc. The results from the serum electrophoresis and densitometric readings, antibody elevation levels of the vaccinated animals confirmed from MAT titers as well as the pathological studies confirm and form the basis for the development of potent leptospiral vaccines for both epidemic and endemic cases of leptospirosis with new molecular biological tools and advance research systems.

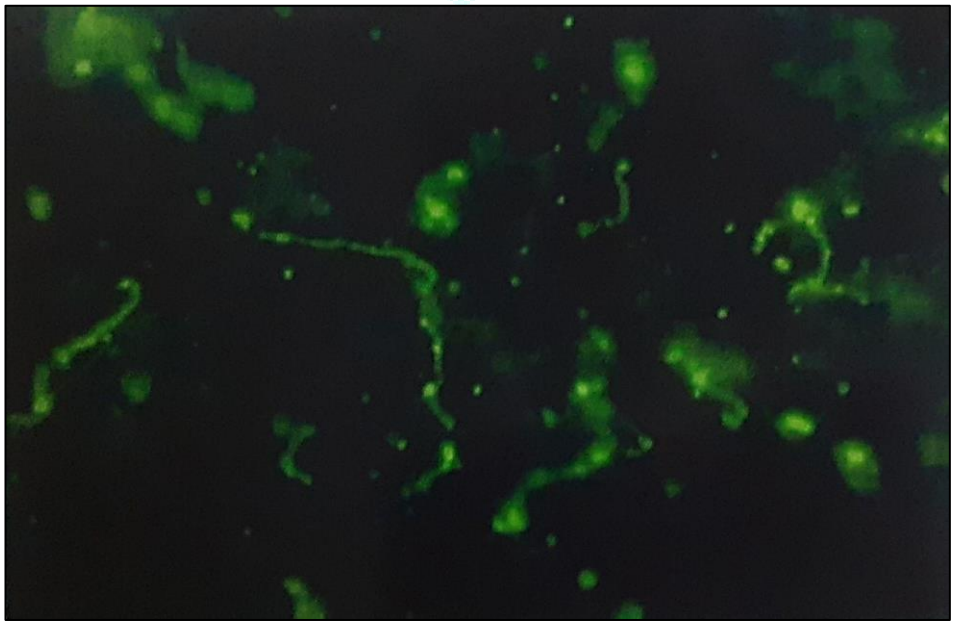


Figure 1: *Leptospira louisiana* under Fluorescence Microscopy

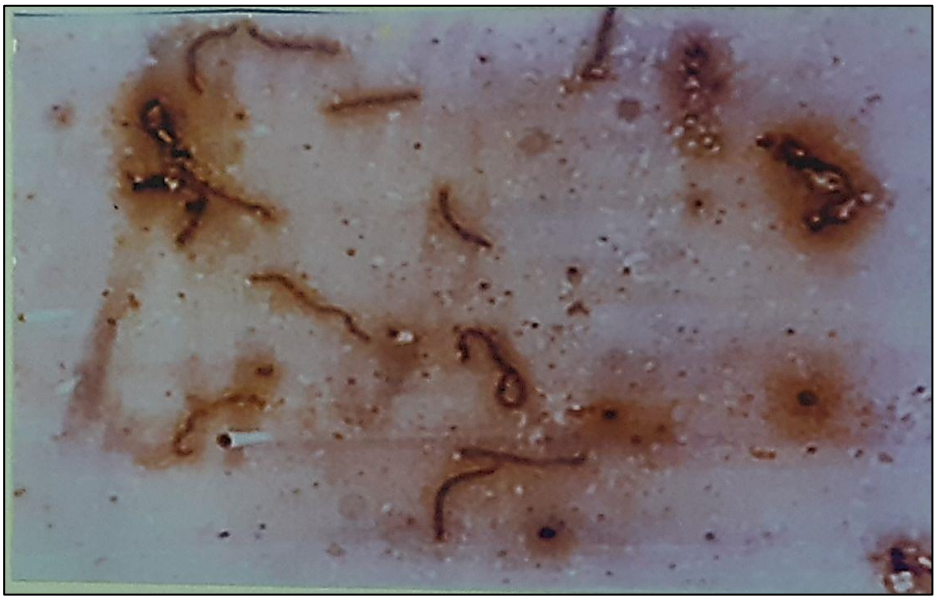


Figure 2: *Leptospira hebdomadis* under High Power Microscopy

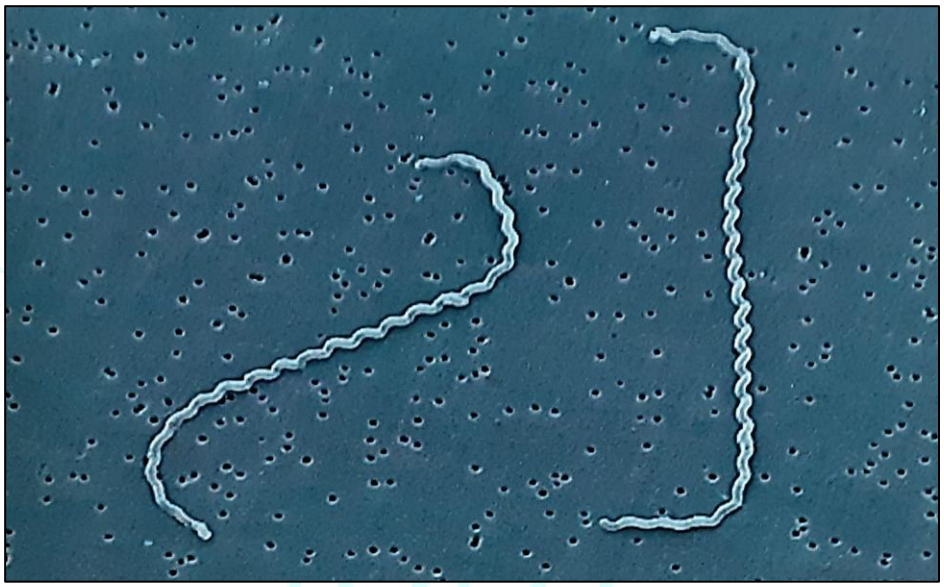


Figure 3: *Leptospira icterohaemorrhagiae* under Scanning Electron Microscopy

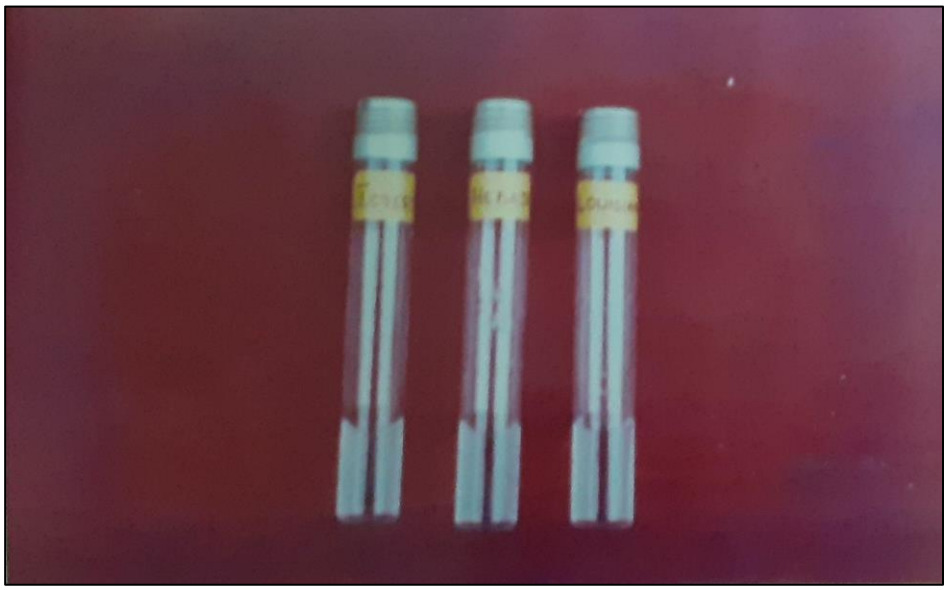


Figure 4: Virulent serovars of *Leptospira icterohaemorrhagiae*, *Leptospira louisiana*, and *Leptospira hebdomadis* in EMJH enriched media.

Figure 5: Whole-cell killed formaldehyde-treated monovalent and trivalent vaccine

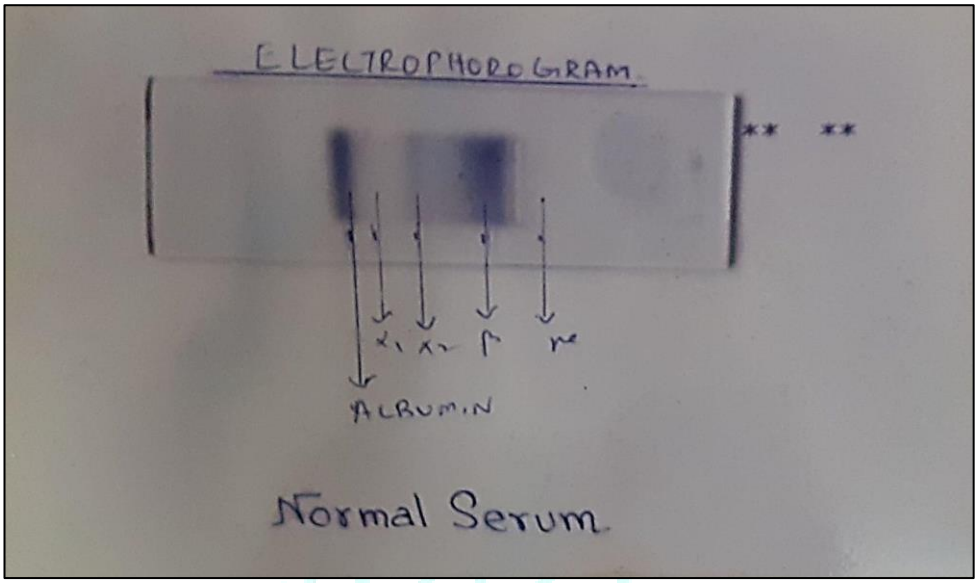


Figure 6: Electropherogram of control albino mice serum

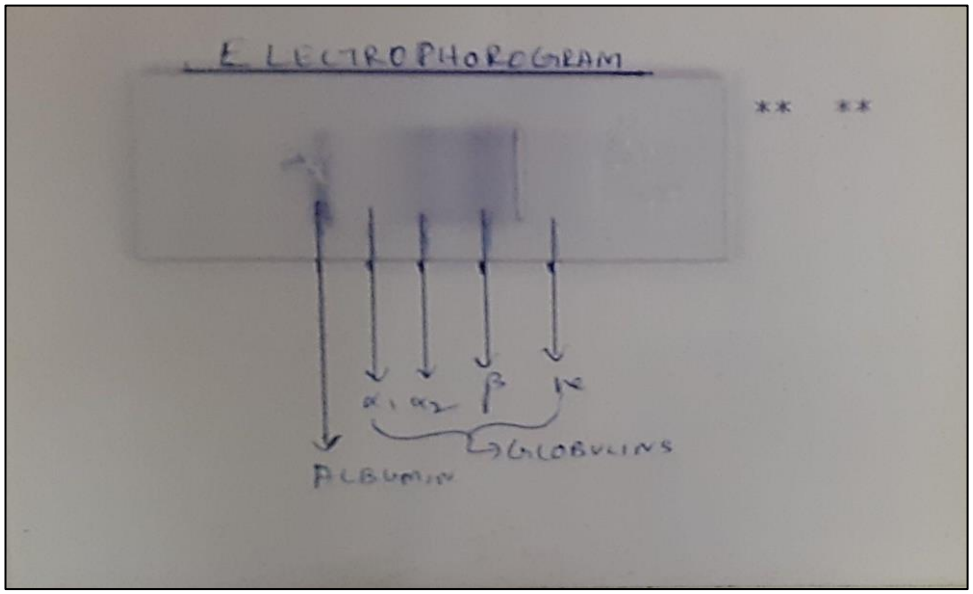


Figure 7: Electropherogram of monovalent albino mice serum

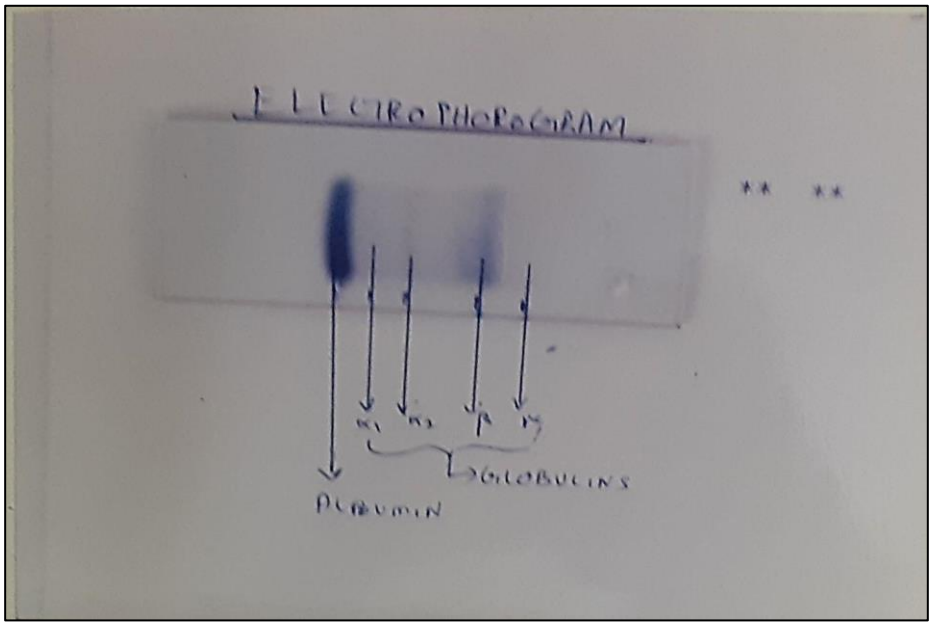
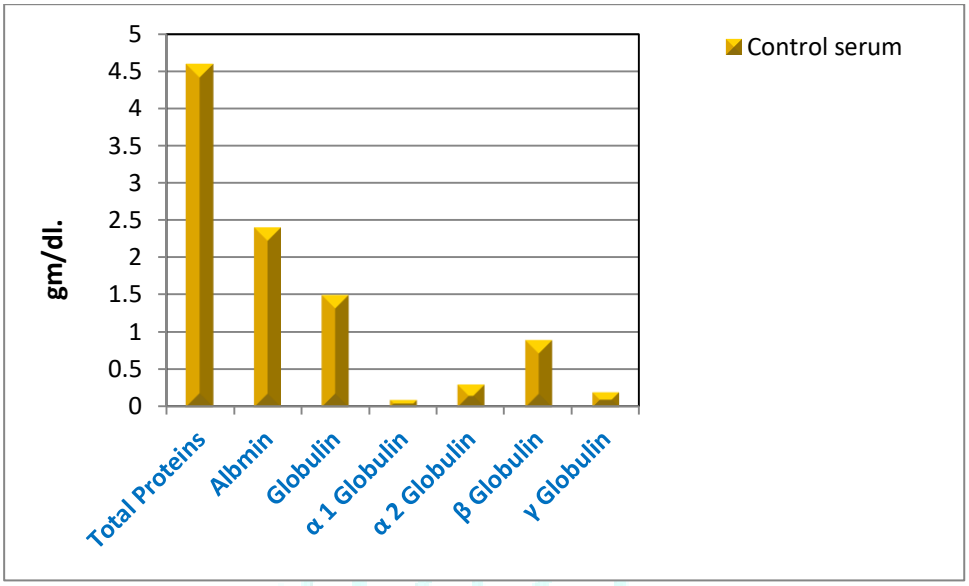
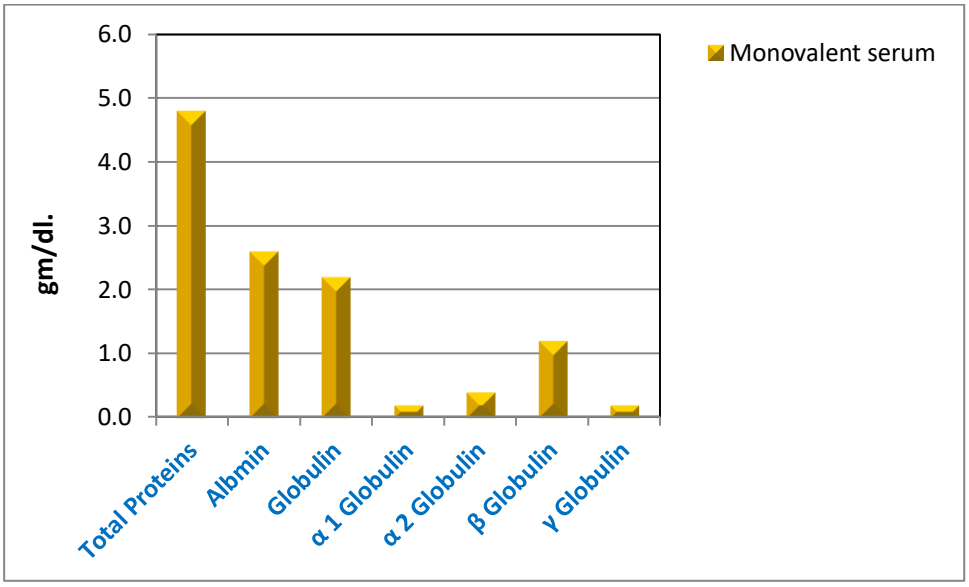


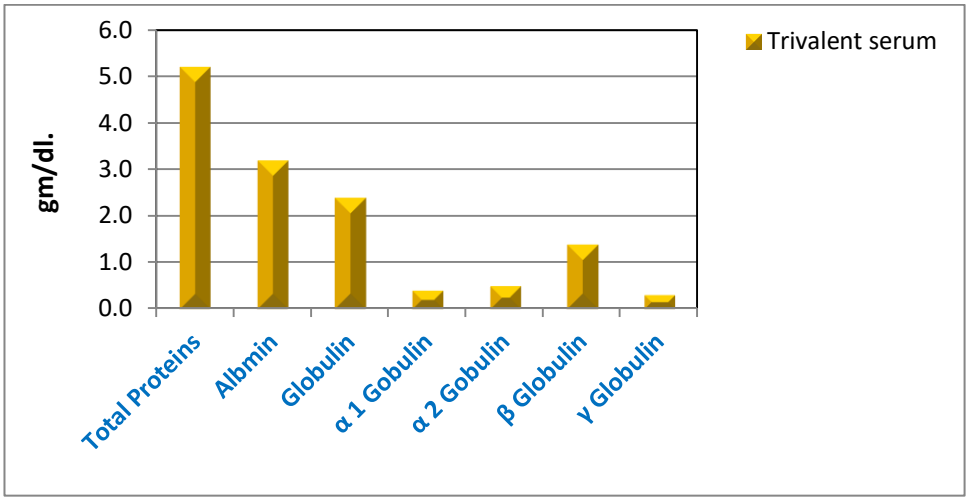
Figure 8: Electropherogram of trivalent albino mice serum



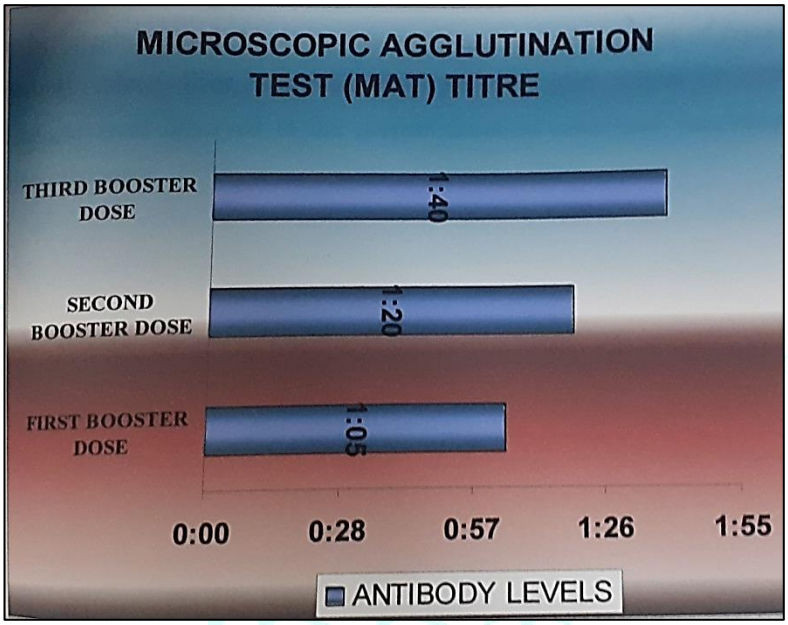
Graph 1: Serum profile of the control albino mice



Graph 2: Serum profile of the monovalent vaccinated albino mice



Graph 3: Serum profile of the trivalent vaccinated albino mice



Graph 4: Antibody levels of 3 booster doses with Freund's adjuvant [14 days interval]

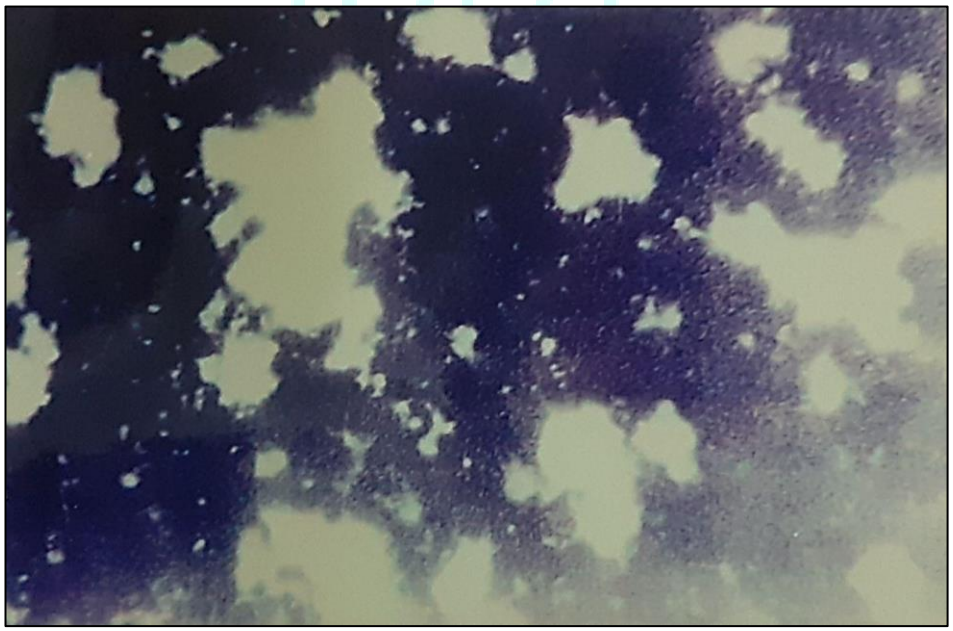


Figure 9: Positive reaction of microscopic agglutination test [MAT]

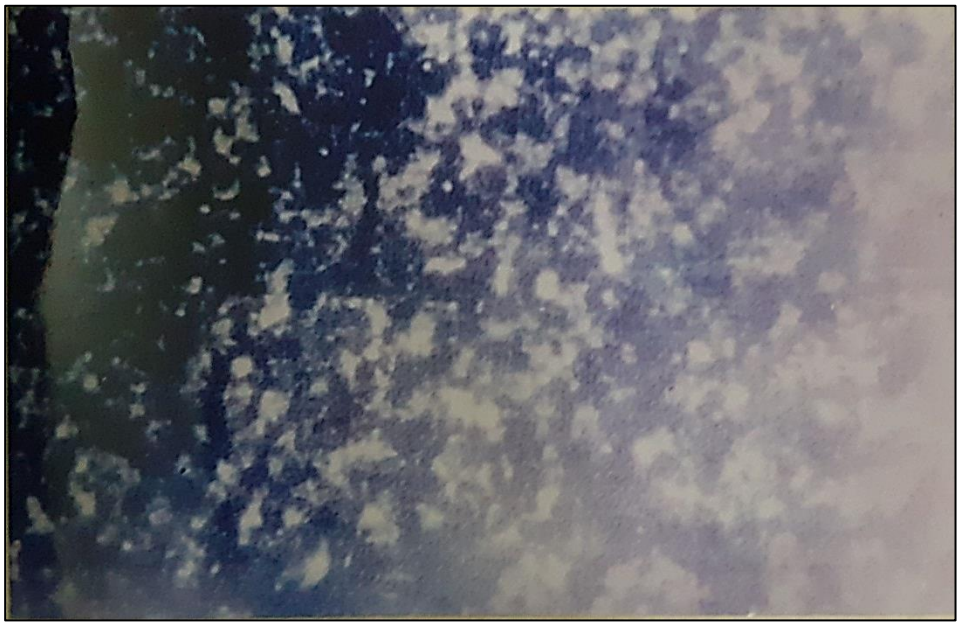


Figure 10: Negative reaction of microscopic agglutination test [MAT]

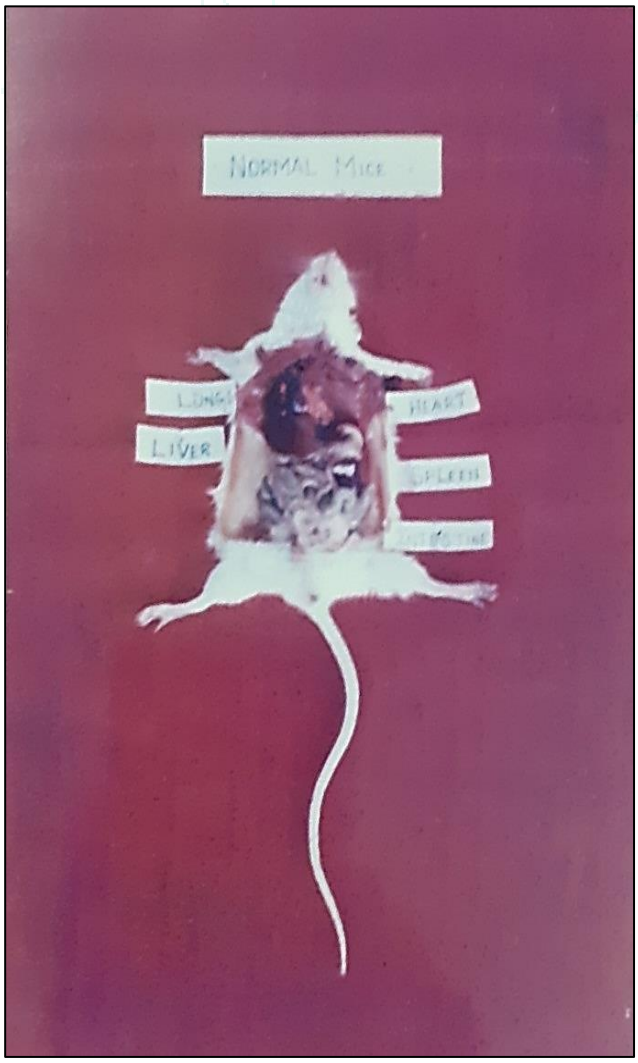


Figure 11: Pathological observation of the dissected control mice

Figure 12: Pathological observation of the dissected virulent mice

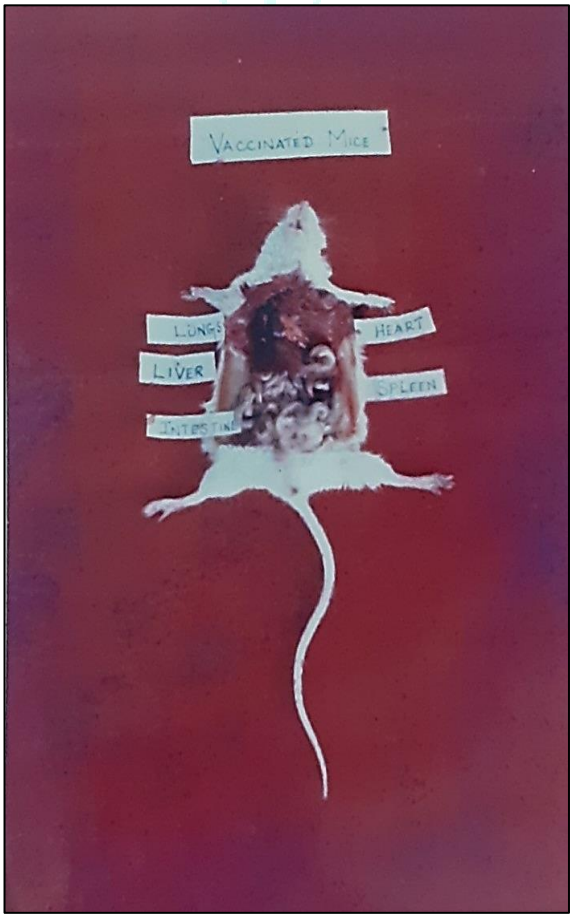


Figure 13: Pathological observation of the dissected Vaccinated mice

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AUTHORS CONTRIBUTION STATEMENT

Dr Vishnu Kiran Manam designed and performed the work whereas Dr G. Sumathi suggested the relevant changes during the course of the work and the proofreading of the manuscript.

CONFLICT OF INTEREST

Conflict of interest declared none.

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