STUDIES
ON
CONGLUTINATING COMPLEMENT ABSORPTION TEST FOR THE DIAGNOSIS AND DETECTION OF IMMUNE STATUS OF CATTLE AGAINST RINDERPEST

THESIS

SUBMITTED TO THE FACULTY OF VETERINARY SCIENCE
RAJENDRA AGRICULTURAL UNIVERSITY, BIHAR IN
PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE (VETERINARY)

BY

RAGHUNANDAN PRASAD SINGH

BIHAR VETERINARY COLLEGE

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DEDICATED

TO

MY PARENTS

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Dated, the 29th July, 1976.

This is to certify that the work embodied in this Thesis entitled "STUDIES ON THE CONGLUTINATING COMPLEMENT ABSORPTION TEST FOR THE DIAGNOSIS AND DETECTION OF IMMUNE STATUS OF CATTLE AGAINST RINDERPEST" is the bonafide work of Shree Raghunandan Prasad Singh and was carried out under my guidance and supervision.

29/7/76

(S.S. MISHRA).

C E R T I F I C A T E

Certified that the research work encorporated in this Thesis has not been published in part or in full in any of the journal.

R P Singh
29/7/16.

(Raghunandan Prasad Singh).

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R P S 29/5/76

(R. P. SINGH).

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C O N T E N T S

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INTRODUCTION

INTRODUCTION

Rinderpest (R.P.), a disease of antiquity was once the most dreadful disease of all ruminants especially cattle and wart pigs all over the world. Since the adoption of All India R.P. Control Programme in 1956, there was miraculous fall in the number of outbreaks from 8000 in 1956 to 295 in 1968-69 (Nilakantan, 1970). Though it is a significant achievement, this should not create false sense of security and slackening of stringent measures being taken against rinderpest. Edifice of such an apprehension is based on the following facts and observations.

Firstly, rinderpest is still present enzootically in equitorial and north east Africa and parts of Indian subcontinent (Scott, 1964; Nilakantan, 1970). Secondly the recent national efforts to upgrade cattle through the use of exotic germ plasms have given rise to populations that are truely at risk, a fact which becomes evident by recent outbreaks of rinderpest in Bangalore City in corss bred animals. Thirdly, the susceptibility of small ruminants and pigs and prevalance of disease among wild ruminants pose great problem for the eradication of the disease due to the possibility of such animals playing role in maintaining the virus and its transmission to susceptible cattle population. Fourthly, animals have been reported to suffer from mild or cutaneous forms of R.P. in mass vaccinated area. Lastly, the cattle have been

reported to suffer from vague clinical signs simulating to rinderpest, such as, diarrhoea of short duration, often associated with mucosal and skin lesions.

Considering the facts that animals suffer from vague clinical signs simulating to R.P. or from true R.P. but with aberrant clinical signs or with classical symptoms, it became imperative to diagnose the cases of R.P. with full certainty and differentiate it from other allied diseases so that R. P. eradication and follow up programme could be implemented effectively.

Although there exists several tests for diagnosis of R.P.,none is far from certain defects. Animal inoculation test is simple and reliable but it is time consuming and costlier since large number of animals are required and the susceptibility of animals is not guaranteed. In general, Neutralization test is a reliable indicator of immune status of the animals but this test also requires a lot of animals as well as an equipped laboratory including tissue culture facilities. The agar gel diffusion test, a simple and reliable tool, unfortunately, suffers from the defects that its results are meaningful only when the reactions are positive since negative results do not confound the provisional diagnosis. Fluorescent antibody technique is sensitive in detecting R.P. virus in the tissues or blood leucocytes but its application as diagnostic tool has been frustrated by the problem of background fluorescent in the cells (Liess and Plowright, 1963b; Liess, 1965). Measles haemagglutination

inhibition (MHI) test has increasingly been used in the recent years for the diagnosis of rinderpest (Waterson et al., 1963; Ramachandran, 1970) but measles antigen is not available in India and at the same time it is difficult to get steady supply of monkey erythrocytes for this test. Complement fixation test has been useful in detecting both R.P. antigen in the infected tissues and circulating antibody in the serum but it is meaningful only in positive cases.

After having foreseen the difficulties of the common diagnostic tests, employed against rinderpest, attempt has been made to find out the suitability of conglutinating complement absorption test (CCAT) as a diagnostic tool against rinderpest. CCAT has been reported to be more sensitive and delicate than the conventional CFT test but has rarely been used in practice for the diagnosis of rinderpest. This test gives consistent results with high titre of antibody, while complement fixation test gives variable results and antibodies to rinderpest are often not detected (Singh, 1970).

Therefore, the present investigation has been undertaken with a view to study the utility of CCAT for the serological diagnosis of rinderpest in cattle and buffaloes under experimental conditions. Efforts have been made to prepare and standardise the antigens, in particular, and other ingredients in the light of various factors involved in extraction, for better yield of antigen. The temperature and duration of fixation of complement during the actual test were also studied. An endeavour has also been made to study the dynamics of rise and

fall of conglutinating R.P. antibody in buffaloe calves inoculated with Freeze dried goat tissue virus vaccine and tissue culture rinderpest vaccine.

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REVIEW OF LITERATURE.

REVIEW OF LITERATURE

Animal inoculation test.

In past, for isolation and identification of rinderpest virus animal inoculation technique was in general use. In this test the virus containing suspected materials were inoculated to susceptible and immune, calves, buffaloe calves, goats, sheep, pigs and rabbits. The appearance of clinical signs in susceptible indicator host and failure in the immune host was observed.

Scott and Brown (1961) discussed the utility of this method and indicated that the method was simple and reliable, subject to the condition that susceptibility of the test animal was guaranteed. White (1962) commented that due to wide spread use of vaccines the application of this test was limited.

Nakamura (1963) was of opinion that the greatest disadvantage of this technique was large number of animals required with their strict isolation.

Agar diffusion test.

White (1958b) successfully employed Oudin's technique of agar gel precipitation reaction for the detection of rinderpest precipitating antigens in the lymph nodes of R.P. infected cattle. He along with Scott (1960) improved the sensitivity of the technique by favouring tube agar gel

diffusion instead of plate technique.

Several other workers utilized agar gel diffusion test for the diagnosis of the disease or for the study of rinderpest virus components (Stone, 1960; Brown and Scott, 1960a; White and Scott, 1960; Scott, 1962a; White, 1962; Provost et al., 1963). Stone (1960) studied R.P. virus using gel diffusion test and reported two types of antigenic components in the virus. Same year Brown and Scott, conducted the test by using lymph nodes obtained through biopsy. The positive samples were obtained one to 8 days after on set of fever, the highest percentage being observed on 3rd to 5th day. Ishii et al. (1964) however reported three antigenic components in R.P. virus using this technique.

Most of the workers were of the opinion that agar gel diffusion tests could be meaningful if they were positive since negative result did not refute the provisional diagnosis of the disease.

Neutralization test.

Walker et al. (1946) were the first to develop neutralization test to detect R.P. antibody. They inoculated a mixture of virulent virus with immune serum into the susceptible calves which served as indicator host. Later on Jenkin and Walker (1946) modified the technique by using spleens of cattle infected with lapinized virus as a source of virus and rabbit as a indicator host. Various other susceptible hosts namely rabbit (Nakamura and Wagatuma, 1940),

Chickens (Baker and Greig, 1946) and embryonated hens egg (Nakamura et al., 1955), were utilized in the neutralization test as an experimental host to detect rinderpest antibodies. These workers were of the view that positive correlation existed between the presence of circulating antibodies and resistance of the animals, such findings were later on confirmed by several other workers.

Neutralizing antibodies were detected as early as 5th day post infection (Scott and Brown, 1958), and significant level were usually evident a week after onset of illness and peak titre was noticed two to four weeks following onset of illness (MacOwan, 1956; Plowright, 1959; Johnson, 1962b). Brown and Raschid (1958) detected significant level of neutralizing antibodies in the sera of cattle vaccinated with caprinized strain which were kept isolated on island for 13 years. The cattle when challenged was found to be solidly immune.

Plowright and Ferris (1957) adopted rinderpest virus in bovine kidney monolayers and recognised specific cytopathic effect (CPE) in the infected cells. C.P.E. consisted of multinucleated gaint cells with intracytoplasmic inclusions. These workers later in the year 1962 developed the technique of neutralization test in tissue culture system which made the test much economical and became the method of choice for detecting rinderpest neutralizing antibodies.

Most of the workers considered neutralization test to be the most reliable and useful among all the tests against

rinderpest. However, the cost involved with the use of animals and the modern laboratory facilities required for the test limited its application on large scale.

Cytopathology.

Plowright and Ferris (1957) were the first to adopt virulent Kabat 'O' strain of rinderpest virus in plasma clot in monolayer culture of bovine kidney testis cells. The authors in the year 1959 reported that the cytopathic changes in tissue cell culture infected with rinderpest virus were characteristic. The virus was identified by the appearance of cytopathic changes which were characterized by multinucleated syncytium formation and intracytoplasmic and intranuclear inclusion bodies (Plowright and Ferris, 1962b).

Provost and Villemott (1961) recognised two types of multinucleated cells, the first type comprised of aggregates of nuclei without intranuclear inclusions in vaculated eosinophilic mass while the second type consisted of slightly vaculated eosinophilic mass ringed with nuclei containing inclusions.

Provost et al. (1965) observed that rinderpest virus multiply in the cytoplasm but not in the mitochondria and the virus was released by budding of cellular surface.

Histopathology.

Thiery (1956) was the first who described intracytoplasmic and intranuclear inclusions in the impression smears of the tissue of epithelium lining the tonsilar crypts

of cattle infected with rinderpest virus. The inclusions were found to be black whereas the background was red and pink. This laid to a provisional diagnosis of rinderpest.

Thiery (1956b) and Nakamura (1965) prepared sections of lymph nodes and affected alimentary tract mucosa and stained the section by Haematoxylin and Eosin. They observed syncytia and inclusions in the stained sections. However, the presence of syncytium was found related to the severity of the disease and the strain of virus involved.

Allergic test.

Yashchinski (1960) reported positive intra dermal reaction in R.P. immune cattle injected intradermally with living caprinized and lapinized viruses. However, he failed to get positive reaction in the dead virus strains. He also observed positive intradermal reaction in susceptible animal following intradermal injection of antigen and 24 hours hater injecting with hyperimmune serum at the same site.

Haemagglutination test.

Haemagglutination test has been demonstrated in two different ways, viz. direct and indirect methods. In the direct method fowl and sheep R.B.C. were found to be agglutinated by lapinized R.P. virus. Brotherson (1951), Singh (1962) also conducted this test using infected goat spleenic tissues as an antigen and R.B.C. from goats, buffaloes, horse, sheep and guineapigs and opined that some unidentified factors

in the splenic tissues interfered the agglutination of R.B.C. Results with R.B.C. of dogs and fowls were negative.

The indirect haemagglutination test was done by coating inert insoluble particles or R.B.C. with R.P.antibody followed by addition of R.P. antigen which resulted in agglutination of R.B.C. or the coated particles. Segre (1957), Huygelen (1960), Provst et al. (1964) applied the method to detect R.P. antibody.

Ishii and Watanabe (1971) detected R.P. antibody by indirect haemagglutination test by using goat R.B.C. treated with tanic acid and boiled rinderpest antigen, Singh (1972) and Singh et al. (1972) detected R.P. antigen in the infected lymph nodes and spleen using sheep erythrocytes coated with rinderpest antibody through diazolinkage with tetrazotized benzidine.

Measles haemagglutination inhibition (MHI) test.

Waterson et al. (1963) while studying the components of measles virus and their relation to the components of rinderpest and canine distemper viruses discovered that the haemagglutination of monkey erythrocytes by measles haemagglutinin was inhibited by rinderpest antisera. It was also noted that the reaction was both specific and sensitive. This finding was confirmed by Bogel et al. (1964) who concluded that the test was an ideal aid for rinderpest diagnosis. They advocated that the bovine sera should be treated with M/90 sodium periodate to remove the nonspecific

agglutinins for monkey erythrocytes normally present in bovine sera.

Enders - Ruckle (1965) reported that the treatment of measles virus with Tween 80 and ether increased the sensitivity of measles antigen for haemagglutination than untreated measles virus.

Maurice et al. (1969) and Provost et al. (1969) studied the possibilities and limitation of the MHI test against rinderpest. They concluded that the test lacks the sensitivity necessary to verify the immune status of vaccinated animals but was useful for the retrospective diagnosis of the rinderpest convalescent animals.

Ramachandran (1970) while evaluating preliminary MHI test against R.P. developed a simple antibody test "Kit" which was designed for use by field veterinarian without having prior laboratory experience. He concluded that the MHI test was simple and easy to perform, the reagents were stable and the results could easily be interpreted.

Ramachandran and Scott (1972) studied the utility of MHI test for evaluation of the immune status of the vaccinated animals. It was observed that the titre of haemagglutination inhibition antibodies in the sera of cattle vaccinated with goat tissue vaccine was higher than that in cattle immunized with cell culture vaccine. The percentage of incidence of development of MHI antibodies was found to be 55% and 31% in G.T.V. and T.C.R.P. vaccinated animals respectively.

The MHI antibodies were detected upto 1277 days in cattle immunized with G.T.V. Colostrum derived antibodies were detected in the sera of cow-calves and buffaloe calves within 12 hours after birth and upto 12 weeks of age.

Fluorescent antibody technique.

The fluorescent antibody technique is the most sensitive test available so far for the study of development of R.P. virus in the cells in vitro but its application as a diagnostic aid has failed so far due to background fluorescence in smear of animal tissues (Liess and Plowright, 1963b; Liess, 1965).

Complement fixation test (C.F.T.).

Petroff (1922) was perhaps the first to detect complement fixing rinderpest antibody in the sera of cattle recovered from experimental infection. Subsequently the presence of complement fixing antibodies was detected in the sera of cattle recently recovered from rinderpest infection by several other workers (Cooper, 1946; Walker et al., 1946; Nakamura et al., 1955; Nakamura, 1958; Moulton and Stone, 1961; Scott, 1964; Singh and Gulrajani, 1968 and Singh, 1970).

These workers while studying the appearance of complement fixing antibody in sera of animals naturally infected or vaccinated against rinderpest, observed that the antibodies appeared in the sera about a week after the onset of illness, reached their peak levels in 2-3 weeks and thereafter declined rapidly. Persistence of the antibody was found to be

remarkably variable. It was noted that the titre of these antibodies declined early, as such was not capable of giving consistent results.

Nakamura (1929) successfully applied C.F.T. test to study rinderpest virus using this test. Complement fixing antigens were further detected in the intestinal lymph node, spleen, lungs, thymus, tonsils, abomasal, caecal and duodenal mucosa by several other workers (Sasaki, 1931; Fukusho et al., 1953; Nakamura and Wagatuma, 1937; Boulanger, 1957a,b; Nakamura, 1958; Taylor, 1959; Stone and Moulton, 1961; Scott, 1964, 1967).

Majority of these workers, however, observed direct relationship between complement fixing antigens and the intensity of clinical and thermal reactions. Peak titre was attained within 48 hours, maintained for further 48 to 72 hours after which it declined with the decrease of the fever and was undetectable in unfebrile and diarrhoeic stages. The antigens were not detected if samples were collected from dead animals or from animals killed in the terminal stages of the illness.

Conglutinating complement absorption test (CCAT).

Bordet and Streng (1909) observed aggregation of sensitized sheep erythrocytes in the presence of fresh horse serum complement by a substance called conglutinin present in heated beef sera. This phenomenon which required participation of complement was called conglutination reaction to distinguish it from agglutination which was independent of

complement. Streng (1909) demonstrated that heated beef serum supplied two essential factors : (i) antierythrocytes antibody and (ii) a clumbing substance called conglutinin.

Conglutinin is a normal serum protein being naturally present in ruminants, particularly cattle and in some other bovidae. Kujumgiev (1943) found African buffaloes serum to be rich in conglutinin. Coombs et al. (1961) observed that the conglutinin titre in ox serum varied between 80 to 1280. It was also found that conglutination occurred not only with horse serum complement but with cat and pig sera as well. Human and dog sera were also used but were less active.

Coombs et al. (1961) extensively studied various serological aspects of CCAT in relation to diagnosis of different diseases. They defined immunoconglutinin as a group of antibody produced in response to C^3 and C^4 components of the fixed complement i.e. immunoconglutinin is immunoglubulin to fixed C^3 and C^4 and reacts in same way as normally occurring conglutinin is bovine sera.

The properties of conglutinin have been reviewed by Coombs (1947), Coombs et al. (1961), Sage et al. (1963), and Lachmann (1967) which could be presented as follows :

1. It caused the clumping or conglutination of particles or antigen antibody complexes which had been coated with non hemolytic complement.
2. It was beta glubulin with a sedimentation coefficient of 7S and molecular weight of 750,000.

3. It reacted specifically with the cell bound complement components, C^3.
4. It was Euglobulin and was precipitated by 33 % saturated ammonium sulphate. It was soluble at physiological ionic strength and pH to a concentration of 5 mg/ml.
5. It was more readily soluble in the presence of ethylene diamine tetraacetate solution.
6. It resisted heating at 56°C for 30 minutes and treatment with 0.1 M Ammonia which destroyed C^3 and C^4.

The mechanism of conglutination and its distinguishing features as compared to conventional CFT according to Carpenter (1967) are tabulated below :

Comparison of the principles of the conglutinating complement absorption and hemolytic complement fixation tests.

Reagents	Conglutinating complement absorption test	Hemolytic complement fixation test.
	Reagents	
Antigen	Antigen.	Antigen.
Antibody	Inactivated antiserum (56°C for 30 minutes)	Inactivated antiserum (56°C for 30 minutes).
Complement	Horse serum	Guineapig serum.
Indicator system.	Sheep RBC + inactivated (56°C for 30 minutes) beef serum (which contains sheep antibody and conglutinin).	Sheep RBC + Rabbit anti-sheep amocaptor (Hemolysin).

Reagents	Conglutinating complement absorption test	Hemolytic complement fixation test.

Results

Inference:

Complement fixed by antigen-antiserum system (positive test)	Homogeneous suspension of R.B.C.	Homogenous suspension of R.B.C.
Complement not fixed by antigen-antiserum system but free to act on indicator system (Negative test).	Clumping (Conglutination of R.B.C.).	Hemolysis of R.B.C.

The CCAT has not yet received wide application although the test is reported more sensitive than homolytic CFT. Hole and Coombs (1947) were the first to detect antibody in the serum of pony convalescent from glanders by using this test. The example was followed by Wolfe and Kornfeld (1948), Rice and Avery (1950) and others for the diagnosis of Brucellosis of cattle and rickettsial and viral diseases including Psittacosis, lymphogranuloma, Q fever, vaccinia and influenza. However, this test remained dormant for about a decade as regards its applicability for the diagnosis of R.P. It was not until 1968 that Singh took up studies on rinderpest wherein this test was applied extensively alongwith C.F.T. to detect rinderpest antibodies.

Singh and Gulrajani (1968) demonstrated rinderpest antibody using antigen extracted from lymph nodes of infected

goats by CCAT as early as 5-7 days after vaccination or infection till 40th day. Higher titres of antibodies were detected after challenge. Subsequently Singh (1969) reported a procedure for the preparation of R.P. antigen reactive to CCAT from infected lymph nodes.

Singh (1970) compared CFT and CCAT tests for detection of R.P. infection and appearance of antibody in serological reactions following vaccination. The serum samples were obtained from animals vaccinated with caprinized virus and inoculated simultaneously with virulent rinderpest virus and antirinderpest serum respectively. The CCAT gave consistent result with high titre of antibody but CFT gave variable results and antibodies were often not detected.

Singh (1972) detected conglutinating complement absorbing antibodies in buffaloe bulls following simultaneous inoculation with virulent rinderpest virus and antirinderpest sera. Peak antibody titre was detected 24 to 40 days post infection after which there was a decline in the titre although antibody persisted upto 65 days. Animals showing better thermal reactions, developed high titres of antibodies than those with little or no such reaction.

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MATERIALS AND METHODS

MATERIALS AND METHODS

GLASSWARES, REAGENTS AND DILUENTS:

Veronal buffer diluent: - Veronal buffer diluent was prepared on the lines of Cruickshank (1965) having the following ingredients : -

Nacl	-	8.5 gm.
Barbitone (diethyl-barbituric acid)	-	0.575 gm.
Sodium barbitone	-	0.20 gm.
$MgCl_2$, $6H_2O$	-	0.168 gm.
$Cacl_2$	-	0.028 gm
Distilled water	-	1 litre

In practice, a stock solution (5x) containing 5 times of each ingredient in a litre of distilled water was prepared. At the time of use, this stock solution was diluted 5 times in distilled water.

The modified Alsever's solution : - This was prepared on the lines of Muschel and Lowe (1955) as follows :

Glucose	-	2.05 gm
Sodium citrate	-	0.8 gm
Sodium chloride	-	0.42 gm
Citric acid	-	0.055 gm
Distilled water	-	100 ml

The solution was sterilized by autoclaving at 10 lb steam pressure for 20 minutes and stored at 4°C.

N/50 Sodium Hydroxide solution (N/50 NaoH) : - This solution

was prepared by titrating it with standard N/50 oxalic acid solution.

Normal saline solution (N.S.S.) :- Physiological saline solution, pH 7.2 was prepared by dissolving 8.5 gm of sodium chloride in 1000 ml of distilled water, filtered and autoclaved at 15 lbs pressure for 20 minutes.

VIRUS STRAINS :

Caprinized rinderpest virus strain : - This strain of virus was obtained from Bilological Products Section, Institute of Animal Health and Production (IAHP), Patna-14. This virus strain was mainly used for preparation of rinderpest antigen from infected goats tissues (mesentric lymph nodes and spleen) for standardization of CCA test and other serological works.

Virulent rinderpest bull virus strain : - This virus strain was procured from the Division of Virology, Indian Veterinary Research Institute, Mukteshwar and was used for the challenge test in connection with the potency testing of different batches of freeze dried goat tissue vaccine (F.D.G.T.V.) at Biological Products Section, Patna. This strain was utilised for the preparation of antigen from infected buffaloe calves for the purpose of comparison at some stages of the work.

Lapinised rinderpest virus strain : - This was also procured from the above source and maintained by passage through rabbits. This strain was used for the preparation of anti-rinderpest rabbit serum.

PREPARATION AND STANDARDIZATION OF INGREDIENTS OF CCAT:

A. Preparation of R.P. antigen :

During the investigation two kinds of R.P. antigen, namely, caprinized virus antigen and virulent R.P. bull virus antigen from the tissues of infected goats and buffaloe calves were prepared to standardize the CCAT for the diagnosis of R.P.

(i) Caprinized virus antigens :

Inoculation of animals and collection of tissues : - Healthy goats of about 1½ years age, inoculated subcutaneously with 1 ml of 1:100 dilution of caprinized virus for the production of F.D.G.T.V. vaccine at IAHP, Patna were utilized for the preparation of this antigen. Only those infected goats which exhibited thermal reaction of 104°F and above on the 3rd to 4th day, were selected as donors. The tissues collected for the preparation of antigen consisted of mesentric lymph nodes and spleen. The tissues were collected under aseptic conditions. The fat and fascia were removed thereafter, the tissues were thoroughly minced and made into a paste with the help of mortor and pestle and processed separately for drying (dessication).

Dessication : - The pulp was spread in a thin layer in a sterilized glass petridish and was loaded in a primary freeze drying unit at -40°C. Drying was done for 22 hours. The materials were unloaded next day and powdered with the help of mortor and pestle. The powder was then sieved through

a sieve with mesh of 1/100/sq. inch. The powder was ampouled at the rate of 0.2 gm in each sterilized glass ampoule. The ampoules were necked in oxy-acetylene flame. Secondary drying was done over P_2O_5 for 18-20 hours. The ampoules were sealed under vaccum and kept at room temperature for varying intervals of time.

For the preparation of normal antigen for control, mesentric lymph nodes and spleens were collected from uninfected, healthy goats, processed and dried in a similar manner as for infected tissues and stored likewise.

(ii) Virulent rinderpest bull virus antigen :

The lymph nodes and spleen from the buffaloe calves infected subcutaneously with 1 ml, 1:100 dilution of virulent virus were collected on the height of temperature usually 6-8 day post inoculation. The animals were used as susceptible controls during the potency testing of F.D.G.T.V. vaccines. The tissues were processed in the same way as for caprinized antigen except that the tissues were dried over P_2O_5 under high vaccum in L5 drier machine and stored likewise.

Extraction of antigen :

Dried tissueswere weighed in 0.2 gm quantities in pyrex glass test tube and suspended in 3 ml of N/50 NaoH and the resultant glue was thoroughly mixed with a glass rod. Then it was kept at variable intervals of time and temperature to study the maximum yield of the antigen.

After treatment with N/50 NaoH, normal saline was

added to the gelatinous mass slowly while the mixture was transformed into heavy suspension and the final suspension was made to 10 ml volume. The suspension was kept at varying pH, time intervals and temperature to study optimal requirements for better yield of antigen through extraction.

Finally the suspension was centrifused at 3500 r.p.m. for 40 minutes, the supernatant was separated which formed the stock of antigens. Merthiolate was added to this antigen in a final concentration of 1:10,000 to serve as a preservative.

B. Preparation of antirinderpest serum :

Hyper immune hill bull sera : - Rinderpest hyper immune hill bull serum employed for the standarization of antigens was obtained from Indian Veterinary Research Institute, Mukteshwar in ampoules containing 2 ml amount in freeze dried state. The serum was stored at -20°C before use.

Hyper immune rabbit serum : - This immune serum was used at some stages to check and compare the results. Rinderpest hyper immune rabbit serum was prepared as follows :

Six young healthy rabbits, each weighing about one kilogram were used for raising homologous R.P. hyper immune sera. Each rabbit was first given 1 ml injection of inactivated lapinized virs (1:10) intravenously. Virus was inactivated by ultraviolet irridiation for 45 minutes at room temperature. This was followed by repeated I/v injections of lapinized live virus in the form of infected rabbit spleens

and lymph glands (1:100) as per schedule given below :

Day	Amount and route of injection
1st day	1 ml of inactivated virus I/v.
10th day	1 ml of 1:100 live virus I/v.
17th day	1 ml of 1:100 live virus I/v.
24th day	1.5 ml of 1:100 live virus I/v.
31st day	1.5 ml of 1:100 live virus I/v.

The rabbits were test bled during successive injections to ascertain the titre of antibody. Finally the rabbits were bled on the 7th day following last injection and tested for antibody level. The sera found suitable were pooled, distributed in small amounts and stored at -20°C.

For the purpose of collection of normal sera to serve as control, healthy buffaloe calves and rabbits, free from rinderpest were obtained and the animals were bled in small flasks, allowed to stand in slanting position at room temperature for two hours and then kept at 4°C in refrigerator overnight. The clear serum was collected next day, filled in small amounts and stored at 4°C.

Test sera from vaccinated animals : - The test sera from vaccinated animals comprised of sera samples collected at varying times from buffaloe calves vaccinated with Freeze dried goat tissue and tissue culture rinderpest vaccines separately for the purpose of assessing response of conglutinating antibody in relation to its first appearance and dynamics of rise and fall at increasing time intervals and other sera

collected for the assessment of antibody.

Vaccination of animals : - Buffaloe calves about one to two years of age and free from rinderpest were used for vaccination with F.D.G.T.V. and T.C.R.P. vaccines for the study of presence of conglutinating antibody in their sera. The buffaloe calves were observed clinically for two-three days before taking them under the experiment. During the period, body temperature was recorded both morning and evening. Animals having normal body temperature were considered suitable for the experiment. Sera samples were also collected and tested for the presence of conglutinating antibody, if any before vaccination.

Buffaloe calves, were divided into two groups each consisting of three animals. The first group of animals received field dose of F.D.G.T.V. vaccine (1 ml subcutaneously). The second group of animals were inoculated with TCRP vaccine (one ml subcutaneously). Clinical observations were made after vaccination. The animals which did not exhibit any recognizable clinical symptoms except slight rise of body temperature upto 2°F were selected for bleeding.

Bleeding schedule : - The vaccinated animals were bled serially on 5th, 7th, 10th, 14th, 17th, 21st, 24th and 28th day post vaccination and thereafter at weekly intervals.

Blood samples were collected in sterilized test tubes, kept in slanting position for 2 hours at room temperature and then transferred to refrigerator overnight for better collection of sera. Next day clear serum was collected

separately with the help of sterile pipettes. Sometimes, the serum was clarified by light centrifugation, ampouled and stored at 4°C for further test. Usually sera samples for antibody assessment were tested within 5 weeks of their collection. The sera samples for antibody assessment were heat inactivated at 56°C in a water bath for 30 minutes.

C. Preparation of indicator system :

Collection of sheep erythrocytes (SREC) : - A healthy sheep belonging to Biological Products Section, Institute of Animal Health and Production, Patna and tested for nonfragility of red blood cells was used for this purpose. The sheep was bled from jugular vein and the blood was collected in sterilized flask in equal volume of Alsever's solution. The SRBC remained undeteriorated for a period of 8 days when kept at 4°C after collection of blood in this solution. Sheep blood was centrifused at 1500 r.p.m. in graduated centrifuse tube for 5 minutes and the supernatant alongwith the buffy coat was discarded. The SRBC were washed thrice with normal saline. Finally the cells were resuspended in normal saline and centrifused at the same speed for 10 minutes to prepare the packed SRBC. A 0.25% to 0.5% suspension of SRBC was prepared in normal saline solution from packed erythrocytes for use in the test.

Sensitization of SREC : - Sensitization was brought about by mixing 0.25% SRBC suspension with equal volume of heat inactivated cattle serum (1:20 dilution) which contained 4 to 8

minimum conglutinating dose (vide infra) and was incubated at 37°C in a water bath for 30 minutes. This constituted the indicator system. Fresh indicator system was prepared each day before the test.

D. Standardization of conglutinin :

Collection of bovine sera for conglutinin : - Young cattle were utilized for the collection of sera for conglutinin. Animals in good health having no history of rinderpest immunization or infection were selected for this purpose. Blood samples were collected from several cattle in small bottle which were left at room temperature for 2 hours and then kept at 4°C overnight. The sera were separated out and then inactivated at 56°C for 30 minutes in a water bath. Each of the sera samples was titrated for minimal conglutinating activity and also tested for the presence of nonspecific activity or any antibody. From the results of titration, only those sera were selected which had no nonspecific activities and contained 4 to 8 MCD at 1:20 dilution of the serum.

The animals whose serum was found suitable were bled in large amounts and the serum was separated and heat inactivated as described earlier, ampouled in 1 ml amounts and stored in deep freeze at -20°C.

Titration of conglutinin : - Each of the serum samples was titrated for conglutinin by the method of Coombs et al. (1961). Serial two fold dilutions of each serum sample were made in N.S.S. in separate test tube. Then 0.1 ml of each

dilution was taken in a set of seven test tubes to which 0.1 ml of 0.25% SRBC was added as indicated in the protocol of Table 1. It was incubated in a water bath at 37°C for 20 minutes. Horse complement (1:20) was then added in 0.1 ml amounts in the test system. N.S.S. was added in 0.2 ml volume to each tube and the mixture was incubated again in a water bath at 37°C for 30 minutes. Appropriate controls for complement, conglutinin and SRBC were kept as indicated in the protocol.

The tubes were then centrifused at 1500 r.p.m.for 1 minute in a centrifuge. The result was read by resuspension technique as suggested by Coombs et al (1961). Test tube was shaken by holding it between the thumb and fore finger and shaken by 10 rapid horizental movements of the fore arm each terminating with a flick of the wrist. A homogenous stable cell suspension was observed in abscence of conglutination whereas conglutinated cells appeared as irregular clumps which rapidly sedimented leaving the supernatent fluid clear.

E. Standardization of horse complement:

Collection of serum : - A healthy young horse with satisfactory complementary activity was selected and maintained in the Post Graduate Department, Bihar Veterinary College,Patna. Blood sample was collected in aseptic conditions in a pre-chilled flask. The flask was kept in slanting position for 20 minutes to allow clotting of the blood and then the flask was kept overnight at 4°C. The clear serum was collected next day with the help of a pipette in a pre-

TABLE - 1.

Showing protocol for titration of conglutinin.

Reagents in ml	Dilution of conglutinin							Controls		
	5	10	20	40	80	160	320	Conglutinin 1:5	Conglutinin 1:10	Cells
Conglutinin (Bovine serum inactivated)	.1	.1	.1	.1	.1	.1	.1	.1	.1	-
Sheep RBC .25%	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
	Incubated at 37°C for 20 minutes.									
Horse complement (1:20 dilution)	.1	.1	.1	.1	.1	.1	.1	-	-	.1
N.S.S.	.2	.2	.2	.2	.2	.2	.2	.3	.3	.3
	Incubated at 37°C for 30 minutes.									
Total volume	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Tube No.	1	2	3	4	5	6	7	8	9	10

chilled sterile vials in 1 ml quantities and kept in deep freeze at -20°C and thawed only once before use.

Titration of complement : - The complement was titrated alone as well as in the presence of constant antigen (1:4) or serum (1:20) in separate set of three series of tubes as per protocol given in Table 2. The titration in presence of antigen or antibody was done to ascertain any possible variation in the titre of complement. From the above titration, the dose or amount of complement to be used in the test was assayed. The smallest amount or the highest dilution of complement which caused complete conglutination of sensitized RB C was taken as minimum complement dose (MCD).

F. Balancing of non-specific properties of antigens :

Both specific and normal antigens were used simultaneously for balancing their non-specific activities, if any, usually observed in the reaction with certain sera. This was carried out by using antigens diluted in 2 fold steps and sera (1:20), in a relatively low concentration of complement usually 1.4 MCD. The protocol of the method is presented in Table 3.

The test was repeated with a higher concentration of complement (2 MCD) in case the sera demonstrated non-specific activity in order to obtain precise comparison of results for different dilutions of antigens. Appropriate controls comprising of R.P. positive and normal antigens, known negative serum devoid of non-specific effect, known positive serum and complement were included in the test.

TABLE 2.

Showing protocol for titration of horse complement.

Reagent in ml	Dilution of complement										Controls			
	10	20	30	40	50	60	70	80	90	100	Complement 1:10	Complement 1:20	Conglutinin	Cell
A.Complement.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	-	-
N.S.S.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.3	.3	.3	.4
	Incubated 30 minutes at 18-20°C.													
Indicator system.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	-	-	.2	-
Control SRBC ·25%	-	-	-	-	-	-	-	-	-	-	.1	.1	-	.1
	Incubated 30 minutes at 37°C													
Tube No.	1	2	3	4	5	6	7	8	9	10	31	32	33	34
B.Complement.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1				
N.S.S.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1				
Antigen 1:4 dilution.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1				
	Incubated 30 minutes at 18-20°C.													
Indicator system.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2				
	Incubated 30 minutes at 37°C													
Tube No.	11	12	13	14	15	16	17	18	19	20				
C.Complement.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1				
N.S.S.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1				
Serum 1:20 dilution.	.1	.1	.1.	.1	.1	.1	.1	.1	.1	.1				
	Incubated 30 minutes at 18-20°C.													
Indicator system.	.2	.2	.2.	.2	.2	.2	.2	.2	.2	.2				
	Incubated 30 minutes at 37°C.													
Tube No.	21	22	23	24	25	26	27	28	29	30				

TABLE - 3.

Protocol showing balacing of non-specific properties of antigens.

Reagents in ml	Dilution of antigens										Controls										
	2	4	8	16	32	2	4	8	16	32	R.P. antigen			Control antigen			Serum		Comp-lement	Cong-luti-nin	Cell
											1:2	1:4	1:2	1:2	1:4	1:2					
R.P. antigen.	.1	.1	.1	.1	.1						.1	.1	.1								
Normal antigen.						.1	.1	.1	.1	.1				.1	.1	.1					
Serum 1:20 dilution.	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1							.1	.1			
Complement 1-2(MCD)	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	-	.1	.1	-	.1	-	.1		
N.S.S.											.1	.1	.2	.1	.1	.2	.1	.2	.2	.3	.4
	Incubated at 18-20°C for 30 minutes																				
Indicator system.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	-
SRBC .25%																					.1
	Incubated at 37°C for 30 minutes																				
Total volume.	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

The infected antigens which maintained the highest level of specific activity were utilized in the test proper.

G. Determination of optimal dilution of antigen in presence of hyper immune hill cattle sera :

The titration of antigen and hyper immune serum was done in chess board manner. Two folds dilutions of hyper immune serum starting from 1:10 to 1:640 were prepared. Each dilution in 0.1 ml amount was added to each of 0.1 ml amount of antigen dilutions in two fold steps from 1:4 to 1:64. To this serum virus mixture 0.1 ml amount of horse complement diluted to contain 1.5 MCD was then added. The tubes were incubated at 18° to 20°C for 30 minutes and then indicator system in 0.2 ml amount was added to each tube. The tubes were then incubated at 37°C in a water bath for 30 minutes and the results were recorded as described in the test proper (vide infra). Appropriate controls were included in the test as per protocol presented in Table 4.

The optimally reactive dilution of antigen is the highest dilution exhibiting complete absorption of complement with the highest dilution of serum (Table 4).

Conglutinating complement absorption test proper :

After standardization of each constituent ingredient of conglutinating complement absorption test, the final method adopted for this test was as follows :

Serial two fold dilutions of sera sample to be tested were made with veronal buffer as diluent. The dilutions

TABLE - 4.

Showing chessboard titration of rinderpest antigen with standard hill bull antiserum.

Antigen dilution	Antiserum dilution							Antigen control
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	
1:2	4	4	4	3	2	2	0	0
1:4	4	4	4	4	2	0	0	0
1:8	4	4	4	4	0	0	0	0
1:16	4	4	3	0	0	0	0	0
1:32	2	0	0	0	0	0	0	0

ranged from 1:20 to 1:320 or more. To 0.1 ml volume of each serum dilutions was added predetermined optimal dilution of antigen (0.1 ml) and complement, usually 1.5 MCD or higher dose (upto 2 MCD) if needed. The unit volume of each reagent was kept as 0.1 ml. The tests tubes were incubated at 18-20°C for 30 minutes to allow absorption of complement. Afterwards the indicator system in 0.2 ml volume was added making the total volume per tube upto 0.5 ml. The complete test was then incubated at 37°C in a water bath for 30 minutes.

Antigen and serum controls were always included in each series of the test. V.B.S. was added to replace any components not required in the controls. The protocol for CCAT proper including complete set of controls is presented in Table 5.

Reading of results : - The test was read by a resuspension technique. After final incubation, the tubes were centrifused at 1500 r.p.m. for 1 minute to deposit the cells. The tubes were then shaken lightly to resuspend the cells, allowed to remain up right in the rack at room temperature for 3 to 5 minutes and then shaken again and read for conglutination as follows :

100% conglutination	= 0
75% conglutination	= 1+
50% conglutination	= 2+
25% conglutination	= 3+
0% no conglutination	= 4+

The tubes showing a reading of 3+ or above were considered positive.

Showing protocol of CCAT proper.

Reagents in ml	Dilution of test serum											
	20	40	80	160	320	640	20	40	80	160	320	640
Test serum	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
R.P.antigen 1:4 dilution.	.1	.1	.1	.1	.1	.1						
Normal antigen 1:4 dilution.							.1	.1	.1	.1	.1	.1
Complement 1.5 MCD	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
	Incubated at 18-20°C for 30 minutes.											
Indicator system.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2
	Incubated at 37°C for 30 minutes.											
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12

continued

CONTROLS

Reagents in ml	Dilution of test serum						Antigen		Known sera						Comp-lement	Cong-glutinin	Cell
	20	40	80	160	320	640	1:4		Positive 1:20		Negative 1:20						
Test serum	.1	.1	.1	.1	.1	.1											1
Known positive serum.									.1	.1	.1	.1					55
Known negative serum.													.1	.1	.1	.1	1
R.P.antigen.							.1	.1	-	-	.1	-	-	-	.1	-	-	-			
Normal antigen.									.1	.1	-	.1	-	-	-	.1	-	-			
Complement 1.5 MCD	-	.1	.1	.1	.1	.1	.1	-	.1	-	.1	.1	.1	-	.1	.1	.1	-	.1		
V.B.S.	.2	.1	.1	.1	.1	.1	.1	.2	.1	.2	-	-	.1	.2	-	-	.1	.2	.2	.3	.4
	Incubated at 18-20°C for 30 minutes.																				
Indicator system.	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	-
S.R.B.C. .25%																					.1
Tube No.	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33

Assay of titre of rinderpest antigen or antibody with CCAT:

After the standardization of the test the titre of different batches of rinderpest antigens prepared during this experiment and the titre of conglutinating antibody in the sera of animals either inoculated with rinderpest vaccines or having no information regarding vaccination was assayed.

A. Assay of rinderpest antigen titre : - Titre of different batches of rinderpest antigens from the animals inoculated with rinderpest caprinized and virulent bull virus strains were assayed. Altogether 12 batches of R.P. antigen were titrated. For assaying antigen of unknown titre, a two fold serial dilutions were made in veronal buffer and 0.1 ml of each dilution was taken for titration using hyper immune hill bull serum (1:20) dilution, complement and indicator system and appropriate controls as per protocol depected in Table 5.

B. Assay of conglutinating antibody in vaccinated animals: The response of antibody in sera of animals vaccinated with FDGTV and TCRP vaccines at varying interval of times was assayed. For the assay, two fold dilutions of test serum were made in veronal buffer to which predetermined optimal dilution of rinderpest known positive antigen, 1:4 dilution, complement, indicator system and appropriate controls were added as per protocol depected in Table 5. Usually serum samples were tested for the presence of antibody within 5 weeks of collection of sera. Each sera sample was assayed separately keeping both pre and post immunization sera in parallel.

C. Assay of antibody in animals taken at random : - Serum samples were obtained from 60 cattle which were vaccinated with FDGTV vaccine about a month earlier from the adjoining villages. The samples were processed for the presence of antibody in a similar manner used for vaccinated animals.

Serum samples from 80 animals were also obtained from animals belonging to adjoining villages and from the animals brought to hospital which had no information as regards their vaccination status against rinderpest. The sera samples were assayed for conglutinating complement absorbing antibody as described above.

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R E S U L T S

R E S U L T S

A. Study on complement and conglutinin :

Generally a non-specific absorption of complement by a few normal antigens and sera during conglutinating complement absorption test was observed when the dose of complement employed was less than 1.5 MCD. This non-specific effect observed during its reaction with antigen and antibody was removed when the dose of complement was increased to 2 MCD. In practice, therefore more than one dilution of complement was employed while doing the actual test. A satisfactory result of CCAT was obtained when preliminary incubation with complement was done at 18°C to 20°C for ½ hour, longer incubation (1 hour) at lower temperature (4°C) did not yield better result.

Few of the bovine sera collected for the purpose of conglutinin, indicated anticomplementary activity, while titrating, such sera were not included in the test.

B. Study on different factors influencing production of R.P. antigen :

(i) Influence of virus strains and types of tissues : - Two virus strains, caprinized and virulent rinderpest bull viruses inoculated in goats and buffaloe calves respectively were used for the preparation of rinderpest antigens, using infected lymph glands and spleens. The antigens were extracted with N/50 NaoH. The results of assays of their titre have

been presented in Table 6.

It would be evident from the result that antigens derived from lymph nodes of goats infected with caprinized strain gave better yield as compared to antigen prepared from infected spleens. The highest titre observed with one case in the former was 1:32 while in the latter the highest titre was only 1:8.

Rinderpest antigens prepared from lymph nodes of buffaloes infected with virulent strain exhibited an antigen titre 1:16 in one case while the titre of antigen from infected spleen did not increase above 1:8.

It was also apparent that antigens at lower dilution (1:2) demonstrated some non-specific effect. However, this effect was not observed at 1:4 and above dilutions.

It would be apparent that caprinized virus antigen showed higher titre (1:32) while the maximum titre observed in case of virulent virus was only 1:16.

(ii) Effect of sieving of tissues powder : - The results obtained after studying the influence of sieving during the process of antigen preparation on the non-specific activities have been summarized in Table 7. It was observed that the unsieved antigen exhibited non-specific activity upto 1:4 dilution which was largely eliminated at 1:2 dilution if the powdered tissues were sieved before proceeding further. It was also observed that coarser powder did not yield homogenous suspension while the sieved powder did so.

(iii) Effect of keeping dry powder at varying time and temperature : - The data of the experiments on maintaining the dried powder for varying intervals of time and temperature in order to eliminate the non-specific effect have been presented in Table 8. It was apparent that the non-specific effect of antigen assessed immediately after drying was to its maximum dilution (1:8). This effect was gradually eliminated (1:4 dilution) when the interval increased upto one month and was almost eliminated in two months interval. It was also observed that non-specific activities remained same when the dried antigen was kept in deep freeze at -20°C.

Effect of temperature during NaoH extraction : - The experiments were conducted by maintaining the NaoH extraction at varying temperature (4°C, 18°C and 30°C) but for a fixed time (2 hours) in order to get optimal yield of antigen. The results of this experiment revealed better yield of antigen at 4°C as well as at 18°C (Table 9). On the contrary slight reduction in antigen titre was observed when extraction was done at 30°C.

Effect of pH of antigen suspension : - The results of study on the effect of varying the pH (7, 7.6 and 8) of suspension during antigen extraction for its better yield have been presented in Table 10. A better yield of antigen titre (1:16) was observed when the suspension was maintained at pH 7.6. Neither increase pH 8 nor decrease in the pH (7) of suspending fluid had beneficial effect, rather each variation resulted in decrease of antigen titre (1:8).

Effect of time and temperature on antigen suspension : - Antigen suspension (pH 7.6) was kept at 4°C and 30°C for 2, 4 and 18 hours to observe the maximum extraction of the antigen. Result has been presented in Table 11. When the suspension was maintained for 18 hours at 4°C an increased antigen titre (1:16) was observed while reduction in the titre of antigen (1:8) was noted at 30°C.

(C) Study on the assay of rinderpest conglutinating antibody Titre.

Comparative response of FDGTV and TCRP vaccines :

None of the buffaloe calves (male) employed for the study of antibody response after vaccination exhibited antibody in their sera before the test. After vaccination, animals revealed only a slight rise of body temperature (1 - 2°F) and no other untowards clinical symptoms were noted. The data on antibody titre in the sera of animals assessed at varying intervals of time in each animal have been summarized in Table 12. The observations with individual animal have also been described below.

F.D.G.T.V. vaccinated animals : - Buff calf No. 1 : - The antibody appeared (1:20) on the 10th day post vaccination (PV) reached its peak titre (1:80) on 18th day and thereafter gradually declined until 42nd day after which no trace of antibody was detected.

Buff calf No. 2 : - The antibody was first detected (1:20) on the 10th day, reached highest level (1:80) on 18th

day then declined until 49th day (1:20), after which antibody disappeared.

Buff calf No. 3 : - Antibody response (1:20) was detected earlier on 7th day which reached at its maximum height (1:80) on the 14th day, the titre began to decline until 42nd day and thereafter it became undetectable.

Vaccination with TCRP : - Buff calf No. 1 : - The onset of antibody response (1:20) was detected on 14th day, reached its peak titre (1:80) on 24th to 28th day which was followed by decrease and was detected till 42nd day (1:20).

Buff calf No. 2 : - Detectable antibody titre (1:20) was observed on 10th day reaching its height (1:40) on 18th day thereafter declined (1:20) till 35 day. Antibody was not detectable on 42nd day.

Buff calf No. 3 : - Antibody response was delayed but was detected (1:20) on 14th day and increased to the maximum (1:40) between 21st to 28th day. The titre declined and was detectable upto 42nd day.

Assessment of antibody in animals with a history of vaccination : - Altogether 60 sera samples were collected from cattle having history of vaccination about a month ago before assessing of the sera for the presence of conglutinating antibody. Out of these animals, 35 or 58% exhibited detectable antibody titres (1:20) and in the rest 25 or 42% animals antibody was not detected.

Assessment of antibody in animals taken at random :- A

trial of 80 sera samples were collected from the animals taken at random from the adjoining areas and hospital on which no information whether vaccinated or not was available. A total of 76 sera samples i.e. 95% did not reveal presence of conglutinating antibody while three i.e. 3.7% demonstrated its presence and one animal i.e. 1.2% had a doubtful titre (1:10).

TABLE - 6.

Titre of NaoH extracted rinderpest antigen from different sources.

Animal and types of tissues from which antigens were prepared	Dilution of antigen				Dilution of hyper immune bull serum used
	1:4	1:8	1:16	1:32	
Goat lymphnodes					
1/75 (ILN)	4+	4+	4+	2+	
Normal lymphnodes	0	0	0	0	
2/75 - (ILN)	4+	4+	3+	0	
(NLN)	0	0	0	0	
3/75 - (ILN)	4+	4+	4+	4+	1:20
(NLN)	0	0	0	0	
4/75 - (ILN)	4+	4+	4+	0	
(NLN)	0	0	0	0	
5/75 - (ILN)	4+	4+	3+	0	
(NLN)	0	0	0	0	
Goat spleen					
1/75 - (IS)	4+	4+	2+	0	
(NS)	0	0	0	0	
2/75 - (IS)	4+	4+	2+	0	1:20
(NS)	0	0	0	0	
Buff calf lymphnode					
1/75 - (ILN)	4+	4+	1+	0	
(NLN)	0	0	0	0	
2/75 - (ILN)	4+	4+	4+	0	1:20
(NLN)	0	0	0	0	
3/75 - (ILN)	4+	4+	2+	0	
(NLN)	0	0	0	0	
Buff calf spleen					
1/75 - (IS)	4+	2+	0	0	
(NS)	0	0	0	0	
2/75 - (IS)	4+	4+	0	0	1:20
(NS)	0	0	0	0	

ILN - Infected lymphnode antigen.
NLN - Normal lymphnode antigen.
IS - Infected spleen antigen.
NS - Normal spleen antigen.

Lymphnodes and spleens were taken from the same animals.

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TABLE - 7.

Influence of sieving on the non-specific activities of antigens.

Batch no. of antigens prepared from goat lymph nodes	Antigen dilutions			
	1:2	1:4	1:8	1:16
1/75 - Unsieved.	4+	4+	0	0
Sieved.	2+	0	0	0
2/75 - Unsieved.	4+	4+	0	0
Sieved.	3	0	0	0
3/75 - Unsieved.	4+	4+	0	0
Sieved.	2+	0	0	0
4/75 - Unsieved.	4+	1+	0	0
Sieved.	0	0	0	0
5/75 - Unsieved.	4+	4+	1+	0
Sieved.	2+	0	0	0

TABLE - 8.

Effect of different intervals of time on the non-specific activities of antigens tested against normal cattle and bovine serum.

Time intervals and antigen batch no.	Antigen dilution						Normal antigen control
	1:14		1:8		1:16		
	BS	CS	BS	CS	BS	CS	
Immediately after antigen preparation.							
1/75	4+	4+	3+	2+	1+	0	0
2/75	4+	4+	3+	3+	0	0	0
3/75	3+	3+	2+	2+	0	0	0
4/75	3+	3+	3+	1+	0	0	0
5/75	4+	4+	3+	2+	0	0	0
One month after preparation.							
1/75	3+	2+	1+	0	0	0	0
2/75	3+	2+	0	0	0	0	0
3/75	2+	2+	0	0	0	0	0
4/75	2+	1+	0	0	0	0	0
5/75	4+	2+	1+	0	0	0	0
Two months after preparation.							
1/75	2+	0	0	0	0	0	0
2/75	1+	1+	0	0	0	0	0
3/75	0	0	0	0	0	0	0
4/75	1+	1+	0	0	0	0	0
5/75	2+	1+	0	0	0	0	0
Antigen kept at -20°C for two months.							
1/75	4+	4+	3+	2+	0	0	0
3/75	3+	3+	2+	2+	0	0	0

BS = Bovine normal serum. CS = Cattle normal serum.

TABLE - 9.

Effect of varying temperature of extraction on the yield of antigen.

Batch no. of antigen	Time 2 hours	Temperature		
		4°C	18°C	30°C
1/75		1:16	1:8	1:8
2/75		1:16	1:16	1:8
3/75	2 hours	1:32	1:16	1:8
4/75		1:16	1:16	1:4
5/75		1:16	1:16	1:8

TABLE - 10.

Effect of varying pH of suspension on the yield of antigen.

Batch no.of antigen	Time intervals	Temperature	pH of the antigen suspension		
			7	7.6	8
1/75			1:8	1:16	1:8
2/75			1:8	1:16	1:8
3/75	18 hours	4°C	1:16	1:32	1:8
4/75			1:16	1:16	1:8
5/75			1:8	1:16	1:8

TABLE - 11.

Effect of maintaining the antigenic suspension at varying temperature and times on the yield of antigen.

Batch no.of antigen	Time intervals in hrs.	pH of suspension	Temperature		Hyper immune serum.
			4°C	30°C	
3/75	2	7.6	1:8	1:8	1:20
	4		1:16	1:8	
	18		1:16	1:8	

TABLE - 12.

Comparison of conglutinating complement absorbing antibody titre in buffaloe calves vaccinated with FDGTV and TCRP vaccines assayed at varying intervals.

Days post-vaccination	Vaccination with FDGTV			Vaccination with TCRP		
	BC1	BC2	BC3	BC1	BC2	BC3
5th day	-	-	-	-	-	-
7th day	-	-	1:20	-	-	-
10th day	1:20	1:20	1:40	-	1:20	-
14th day	1:40	1:40	1:80	1:20	1:20	1:20
18th day	1:80	1:80	1:80	1:20	1:40	1:20
21st day	1:80	1:80	1:80	1:40	1:40	1:40
24th day	1:40	1:40	1:40	1:80	1:40	1:40
28th day	1:40	1:40	1:20	1:80	1:40	1:40
35th day	1:40	1:40	1:20	1:20	1:20	1:20
42nd day	1:20	1:20	1:20	1:20	-	1:20
49th day	-	1:20	-	-	-	-

BC = Buff calf.

DISCUSSION

D I S C U S S I O N

The problem facing eradication of rinderpest still continues to dominate the time and minds of scientists engaged over this problem in India and abroad. The epidemiology of the disease in relation to infection in wild and small ruminants as well as in wart pigs, and the possibility that these animals may act as reservoir of the virus and facilitate transmission of the same to the susceptible cattle is still not clearly understood. In India, the foci of rinderpest infection still persists, since reports of outbreak in cross bred animals and small ruminants are still coming which pose a great threat to a large population of cross bred new susceptible cattle for high risk of infection.

It was the conventional procedure until 1956 to diagnose rinderpest provisionally on the basis of history, clinical symptoms and post mortem findings (Scott, 1964). However, prevalance of certain diseases exhibiting clinical manifestations simulating to rinderpest and other rinderpest like diseases have made the problem complicated warranting confirmation of provisional diagnosis obligatory. So, far, the confirmation of rinderpest is based on some serological tests which can be conducted only in well equipped laboratories, moreover, they are costlier and time consuming (Scott and Brown, 1958; Plowright and Ferris, 1957; Nakamura, 1958).

On the contrary the conglutinating complement absorption test has been attributed since long to be much more sensitive test for serological diagnosis of diseases but has remained out of practice for a considerable period of time. Recently it has been reported that conglutinating complement absorption test was capable to detect rinderpest antibodies more consistently as compared to conventional complement fixation test (Singh, 1970).

The requirement and necessity for confirmation of rinderpest through serological reaction has not been diminished, rather increasingly being felt, in the light of the circumstances mentioned above to diagnose the disease with certainty and differentiate it from rinderpest like diseases.

Studies on various factors influencing production of rinderpest antigen :

For the detection of conglutinating antibody in suspected sera, known rinderpest antigens are employed which ought to be free from non-specific and anticomplementary effects, so that correct results may be obtained.

In the past, a number of different techniques were employed to prepare rinderpest antigens from infected tissues for use in complement fixation and conglutinating complement absorption tests by different workers (Boulanger, 1957b; Nakamura, 1958; Stone and Moulton, 1961; Singh, 1969).

Boulanger (1957b) used infected cattle spleen, emulsified it with chilled acetone, centrifused and

resuspended in acetone. Finally extraction was done with anhydrous ether which was evaporated, dry power was suspended in N.S.S. overnight and lightly centrifused. The supernatant consisted the antigen.

Stone and Moulton (1961) prepared antigen from infected cattle lymph nodes, minced it with equal volume of phosphate buffer, homogenized adjusted to pH 6.8 and finally extracted the antigen by differential centrifugation methods.

Nakamura (1958) used fresh lymph nodes of cattle, ground, with buffered saline to give 10% suspension, frozen overnight, heated and again frozen overnight. The supernatant after centrifugation constituted the antigen.

The antigens prepared by these workers were, however, only partially successful since these antigens demonstrated non-specific as well as anticomplementary activity.

Nakamura (1958) used lymph nodes ground in the buffer and dried the infected bovine tissues in calcium chloride dissicator at room temperature under vaccum for one week and at 37°C for 3 weeks. Using this method Nakamura was able to eliminate non-specific effect of tissues to a great extent.

In the present study antigen was prepared from pooled lymph nodes of goats infected with caprinized strain, minced, freeze dried, powdered and finally sieved. The sieved powder was kept for 2 months at room temperature. Then

antigen was extracted in N/50 NaoH, suspension was kept at pH 7.6 and extraction was done overnight at 4°C. The supernatant after centrifugation constituted the antigen. It is evident from the Table 6 that higher yield of antigen (1:32) was obtained when caprinized strain was used instead of virulent virus, that too, infected goat lymph tissues evidenced better yield in comparison to infected spleen. This finding could be explained by the fact that caprinized strain have become completely fixed in goat through repeated passages, as such, produced severe thermal reaction, a pre-requisite to get a better antigen. Similarly greater avidity of lymph nodes for the caprinized strain may possibly explain higher titre of antigen obtained from these tissues Table 6.

It is worthwhile to note that sieving of freeze dried tissue powder removed to a large extent the non-specific effect of the antigen as indicated in Table 7. Unsieved powder showed non-specific effect at 1:4 dilution of the antigen which was eliminated completely by sieving. It may be pointed out here that 1:4 dilution of the antigen is the optimal dilution used in the actual test, as such, removal of non-specific effect upto this dilution is a significant finding. The plausible explanation for this effect would be that the coarser particles mainly consisted of capsules and connective tissues and contained such substances which interfered with the reaction or hindered optimal extraction of the antigen.

Another important observation to be mentioned

here is the further removal of non-specific effect of the antigen by maintaining the freeze dried powder for 2 months at 30°C (Table 8).

The non-specific activities were removed upto 1:4 dilution of the antigen, a titre which is used in the actual test. Again, the mechanism of such effect is not clear, but it is expected that by keeping the sieved powder at 30°C for 2 months, the substances responsible for non-specific effect might become inactivated or destroyed while by keeping the tissues at -20°C for 2 months interfering substances are not inactivated.

The results of effect of varying pH, intervals of time, temperature during antigen extraction to get optimal conditions have been presented in Table 9 to 11. Interestingly a pH of 7.6 was observed to be the most congenial to engender good yield of antigen devoid of adverse effect. This observation is in partial agreement to the finding of Nakamura (1958) who maintained the pH varying from 7.2 to 7.6 during extraction of complement fixing antigen but is almost in full agreement with Singh (1969). N/50 NaoH extraction proved to be a satisfactory method (Table 9) which is also in agreement with Nakamura (1958) and Singh (1969).

Studies on the assessment of conglutinating complement absorbing antibodies :

Having standardized the antigen to be used in CCAT, it was then endeavoured to study CCA antibody response

in animals vaccinated with FDGTV and TCRP vaccines. The assay of antibody response was also made in animals having recorded history of vaccination as well as in animals having no information, what soever about vaccination.

The pattern of immune response in buffaloe calves to FDGTV and TCRP vaccination at varying intervals of time are indicated in Table 12. It was observed that the titre of conglutinating complement absorbing antibody in the sera of buffaloe calves vaccinated with FDGTV was higher than with TCRP vaccinated animals. In one animal which was vaccinated with TCRP the peak titre was as high (1:80) as that of FDGTV. The onset of antibody response appeared as early as 7th day in the former which persisted until 49th day (Table 12). Antibody in case of latter appeared a bit latrr usually between 10 to 14th days which persisted for a little lesser time until the 42nd day. The pattern of antibody response in FDGTV vaccinated animals was, its first detection on 7th to 10th day, reached its maximum titre between 14 to 18th day and remained detectable until 49th day. In case of animals vaccinated with TCRP, the antibody was first detected between 10th to 14th day, highest titre on 21st to 24th day which was maintained for about a week but antibody was not detected after the 42nd day.

Appearance of rinderpest antibody in vaccinated or recovered animals have been confirmed by several different methods like complement fixation test, gel diffusion, neutralization test, and measles haemagglutination inhibition test.

Conventional complement fixation test has been applied to detect antibody in sera of animals naturally infected or vaccinated against rinderpest and the workers have reported that antibody appear in the sera about a week after the onset of illness, increased to its peak titre in 2-3 weeks and then declined (Cooper, 1946; Nakamura, 1958; Moulton and Stone, 1961; Scott, 1964).

Persistence of antibody was found to be variable with inconsistent results. Singh (1970) compared complement fixation test and conglutinating complement absorption test for the detection of rinderpest infection and appearance of antibody following vaccination. The CCAT was reported to give consistent results with high titre of antibody but CFT gave variable results and antibodies were not often detected.

Scott (1964) extensively reviewed the pertinent literature of the application of Agar gel diffusion test for the detection of rinderpest antibody. He observed that rinderpest antibodies were not detected consistently, since positive precipitating lines were not observed in some of the confirmed rinderpest cases.

Serum neutralization test has been reported to detect neutralizing antibodies most accurately as early as the 5th day after infection (Scott and Brown, 1958), significant levels achieved about a week after the onset of illness and peak titre was attained two to four weeks after the onset of illness (Macowan, 1956; Plowright, 1959; Johnson, 1962b). But they informed that the test required large

numbers of susceptible and immune animals and well equipped tissue culture laboratories as such this test has the limitation that they could not be performed quickly in an ordinary laboratory, in a routine way.

Measles haemagglutination inhibition test has been reported to detect rinderpest antibodies accurately (Ramachandran, and Scott, 1972) and was detected upto 1277 days in cattle after vaccination with FDGTV. Colostrum derived antibodies were detected in the sera of cow-calves and buffaloe calves within 12 hours after birth and upto 12 weeks of age. Though the test is reported to be sensitive in detecting antibodies against rinderpest but it suffers from the defects that measles haemagglutination inhibition antigen is not yet prepared in India as well as it is difficult to get steady supply of Monkey erythrocytes.

In the present study, the antibody was detected from 7th to 49th day in case of FDGTV vaccinated animals without any exception. The CCAT can be applied as a diagnostic tool to confirm rinderpest antibodies in recovered animals. It may also be applied to assess the immune response and the immunizing efficiency engendered by different vaccines presently used in India. The CCAT proved to be reliable and satisfactory method for detecting rinderpest antibody which is inagreement with the finding of Singh (1969, 1970).

The results obtained on the assessment of antibody level in 60 vaccinated animals with freeze dried goat

tissue vaccine out of which 35 animals exhibited presence of antibody in their sera. Similarly out of 80 sera samples collected from animals having no information whatsoever, three evinced conglutinating antibody to a detectable level (1:20). This finding aids to the significance of CCAT which may satisfactorily be applied for assaying immune response in vaccinated animals but only for a very short period.

It would be worth mentioning that the conglutinating complement absorption test requires less time provided all the standardized ingredients are available, besides the cost involved in carrying out this test is negligible since the common ingredients like horse complement, bovine sera conglutinin and sheep erythrocytes could be obtained easily without incurring much expense.

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S U M M A R Y

S U M M A R Y

Attempt has been made to apply conglutinating complement absorption test as a diagnostic tool to confirm rinderpest antibodies. Various ingredients namely conglutinin, complement, indicator system, antigen and antibody have been standardized.

A method of antigen preparation used in the test has been described in relation to various conditions like effect of virus strains, sieving, intervals of time, temperature and pH in order to get maximum yield of antigen free from non-specific effects. Caprinized strain engendered high titre of antigen (1:32) as compared to virulent virus (1:16). Simultaneously infected lymph nodes evinced a greater yield of antigen (1:32) whereas infected spleen gave antigen titre of (1:16) only. Sieving of freeze dried tissue powder and maintaining the powder at 30°C for 2 months were able to remove the non-specific effect upto 1:4 dilution of the antigen during its extraction. Keeping the pH at 7.6 during extraction, an optimal yield of antigen was obtained.

Cattle sera were collected as a source of conglutinin. The non-specific effect was observed rarely with a few sera which gave anticomplomentary activities. Such sera were not included in the study.

Horse sera was employed as source of complement and was titrated to determine the minimum complementary dose. Some non-specific effect was observed where a low dose of

complement (1.2 to 1.4 MCD) was employed in the test. The effect was however, eliminated when higher complement dose (1.5 to 2 MCD) was used in the test.

Sheep erythrocytes 0.25% to 0.5% was used as the indicator system for conglutination which was found to be satisfactory for conglutinating complement absorption test.

Conglutinating antibody in buff calves vaccinated with FDGTV was detected as early as 7th day which persisted till 49th day. Antibody in TCRP vaccinated animals appeared between 10th to 14th day and persisted till 42th day.

Conglutinating antibody was assessed in 60 cattle with a recorded history of vaccination, of these animals 35 or 58% evinced detectable titre (1:20) while 25 or 42% was negative for it. Out of 80 animals taken at random for detecting conglutinating complement absorbing antibody in their sera, three or 3.7% revealed the presence of antibody while 76 were negative for it and one animal demonstrated a doubtful titre.

The test is less time consuming, sensitive, reliable and cheaper. It gives consistent results of conglutinating antibodies. The ingredients required namely horse complement, bovine conglutinin sheep erythrocytes can easily be procured and the antigen is stable which can be maintained in the laboratory.

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