COMPARATIVE EFFICACY OF DIFFERENT ANTIGENIC PREPARATIONS FROM *Pasteurella multocida* FOR DETECTION OF ANTIBODIES BY ENZYME IMMUNO ASSAY

By

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THESIS

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DEDICATED TO ARUN

DECLARATION

I hereby declare that the thesis entitled "**COMPARATIVE EFFICACY OF DIFFERENT ANTIGENIC PREPARATIONS FROM *Pasteurella multocida* FOR DETECTION OF ANTIBODIES BY ENZYME IMMUNO ASSAY**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or any other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled **"COMPARATIVE EFFICACY OF DIFFERENT ANTIGENIC PREPARATIONS FROM *Pasteurella multocida* FOR DETECTION OF ANTIBODIES BY ENZYME IMMUNO ASSAY"** is a record research work done independently by **Mrs. RINITA SINGH** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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CONTENTS

List of Tables

LIST OF FIGUERS

INTRODUCTION

INTRODUCTION

Ducks, one of the hardy birds of Anatidae family, at a population of 24.0 million is second to chicken in the production of eggs and meat in India. The duck population is mostly concentrated in southern and eastern states of this country. Kerala has one third of the total duck population which is mostly centered in Alleppey, Kottayam, Kuttanad, Pathanamthitta and Trichur districts.

The marshy and wet lands of these areas provides conducive condition for duck rearing. Now a days duck farming in Kerala has become more enterprising and popular among farmers because of hike in demands for duck egg and meat.

The management and feed cost of the duck is rated low compared to poultry industry in this area, as they can thrive on forage and vegetation and does not require a pen for housing. Though the ducks are comparatively quite hardy and resistant, few infectious diseases cause considerable economic loss to this industry. The occurrence of two important diseases, duck pasteurellosis and duck plague have been foreshadowing the interest and growth of duck farming in Kerala in the recent past, considerably affecting the economy of the state.

Duck pasteurellosis, caused by Pasteurella multocida (P. multocida) is a contagious disease which usually appears as a septicaemic disease associated with high morbidity and mortality.

Pasteurellosis causes economic loss due to high mortality, morbidity and also by high culling and condemnation rates at slaughter. The rapid onset and spread of this disease makes medication often impracticable and ineffective. It is also known that chemotherapy of duck pasteurellosis cannot cure the birds completely from all the ill effects of this disease and also the cost of treatment is comparatively very high. Thus prevention of the disease by immunization turns out to be the only alternatative to save the birds from this malaise.

As such there is no recommended fool proof vaccine against duck pasteurellosis. The homologous / heterologous serotypes of P. multocida in killed, or product forms are being tried as vaccine in ducks but with varying results. None of the above vaccines were reported to confer long lasting immunity to birds against homologous / heterologous challenge.

On experimental inoculation, a number of antigenic extracts of P. multocida are shown to stimulate protective immunity to poultry. These include potassium thiocyanate extract (KSCN extract), capsular extraction with sodium chloride, lipopolysaccharide protein complex, formalized cells, cytotoxin and ultra sonicated cell lysates. These antigens are also used in various serological tests for detection of specific antibody.

Various immunological tests viz., gel diffusion test (GDT), indirect haemagglutination (IHA), microtitre agglutination, counter immuno electrophoresis (CIE) and complement fixation test (CFT) measuring antibodies against P. multocida have been employed to

evaluate the effectiveness of vaccination and for diagnosis of the disease.

However, most of the above serological tests, with the exception of IHA have shown poor correlation between P. multocida antibody and protection against challenge (Alexander and Soltys, 1973).

Since the development of Enzyme Linked Immuno Sorbent Assay (ELISA) (Engvall and Perlmann, 1972) which was considered more sensitive and convenient to perform than conventional serological tests, this assay has been used to quantify humoral antibodies against P. multocida in birds. The disadvantages reported for ELISA included the potential alteration of antigen conformation upon binding to plastic, non specific adherence of some antibodies to the plates and variable capacities of different antigens present in a complex mixture to bind to microtitre plate wells.

The Dot Immunobinding Assay (DIA), an alternative to ELISA for detection of antibodies to soluble antigens, has been known to possess higher specificity than ELISA. The DIA is considered as a test of field application in terms of its high specificity, convenience and easiness.

Several researchers have developed ELISA and DIA for detection of antibodies against P. multocida for both chicken and turkeys (Marshall et al., 1981, Avakian and Dick, 1985), but so far not for ducks.

Thus this study was designed to:

a. develop ELISA and DIA for ducks to determine humoral antibody against different vaccines prepared from P. multocida.
b. determine if these antigenic extracts of P. multocida viz., crude capsular extract, KSCN extract and ultra sonicated cell lysate are suitable for use in ELISA and DIA antigen.
c. compare sensitivity of ELISA with that of IHA.

2. REVIEW OF LITERATURE

Avian pasteurellosis is a contagious disease which usually appears as a septicaemic condition associated with high morbidity and mortality. This septicaemic disease in poultry was first studied by Chabert (1782). The term fowl cholera was coined by Mailet (1836).

Pasteur (1880) could isolate the causative organism from typical cases of fowl cholera and he was the first to show that these organisms lost their virulence when repeatedly cultivated in vitro. He further showed that these attenuated bacteria could be safely used to immunize chicken.

The generic name Pasteurella was proposed by Trevisan (1887) in recognition to Pasteur's work on the causal agent of fowl cholera. Rosenbusch and Merchant (1939) suggested the species name for the organism as P. multocida and this name is repeatedly used in Bergey's manual of determinative bacteriology since 1948.

2.1 Duck Pasteurellosis

Hilbert and Tax (1938) reported pasteurellosis in ducks with higher mortality which could be controlled by autogenous phenol killed vaccine.

Mulbagal et al. (1972) reported that outbreaks of pasteurellosis among ducks and ducklings have been recorded in India as early as 1947 from erstwhile states of Hyderabad and Bengal.

Occurrence of this disease was then reported from Assam (Halder, 1972), Maharashtra (Mulbagal et al., 1972) and Orrisa (Pande, 1981).

Pillai et al. (1993) isolated Pasteurella organisms from ducks in Kerala during an outbreak of septicaemic disease with high mortality.

Most reported outbreaks of fowl cholera affected chicken turkeys ducks and geese.(Rhoades and Rimler,1991)

Fowl cholera in ducks was a serious problem in Long Island where it was diagnosed on 32 of 68 commercial duck farms. Losses usually occurred in ducks over four weeks of age and mortality reached 50 per cent (Dougherty, 1953).

Masound and Konsh (1963) reported several outbreaks of fowl cholera in ducks and chicken in Alexendria city.

Montgomery et al. (1979) reported 90 per cent mortality among diving ducks due to pasteurellosis in and around Chesapeak bay during March and April 1978.

Schimmel (1990) reported heavy losses due to pasteurellosis in a large flock of muscovy ducks which was controlled by immunizing ducklings at 10-24 days of age with autogenous vaccine.

Bhaumik and Dutta (1995) reported heavy mortality among 20-60 days old ducklings in Tripura due to P. multocida infection.

2.2 Antigens of Pasteurella multocida

2.2.1 Capsular Antigen

Carter (1952) identified three different groups of P. multocida by performing precipitation test utilizing the capsular antigen.

Based on passive haemagglutination of erythrocytes sensitized by capsular antigens of P. multocida Carter (1955) could differentiate it to four groups as A,B,C and D.

Briefman and Yaw (1958) separated monosaccharides from capsular substance of type 1 and 3 of P. multocida and they identified pentose as ribose and hexoes as galactose.

Carter (1958) reported the presence of hyaluronic acid in the capsular material of mucoid strain of P. multocida which was easily disintegrated by hyaluronidase.

Bain and Knox (1961) recovered a fraction containing protein with some polysaccharide and lipopolysaccharide from Robert's capsular type 1 strain.

The efficiency of slide agglutination test was compared with haemagglutination test to detect the capsular types of P. multocida using formalinized antigen by Namioka and Murata (1961 a) and they reported that the results of both tests were comparable.

Carter and Rappy (1963) extracted lipopolysaccharides from P. multocida belonging to types A, B and D by phenol water method and used them in indirect haemagglutination test for detection and measurement of antibody against lipopolysaccharide.

Maheswaran et al. (1973) isolated capsular polysaccharide from P. multocida of turkey origin. All the isolated strains contained hyaluronic acid.

Penn and Nagy (1974) identified two major antigenic components of P. multocida types B and E as capsular antigen and endotoxin, by subjecting the organism to saline and phenol water extraction respectively. For characterization of these antigens they had employed immunodiffusion, immunoelectrophoresis and tube agglutination test.

The saline extract of P. multocida was purified and analysed by chromatography and electrophoretic method by Syuto and Matsumoto (1982). The purified antigen had a carbohydrate and protein ratio of 1:5. On SDS - PAGE the purified antigen showed three protein band and one carbohydrate band which was protective at dose between 10 and 50 μg of protein, when two doses were given at 14 days interval to 10 to 20 week old ducklings.

Chemical and immunogenic characters of the saline extract of type 1 P. multocida of turkey origin was studied by Kajikawa and Matsumoto (1984). The crude saline extract contained protein of about 650 μg per ml and carbohydrate 152 μg per ml. SDS - PAGE analysis

revealed four identical components with molecular weights of 44,000, 31,000, 25,000 and 20,000 Da respectively.

Lin et al. (1984) extracted crude polysaccharide capsular antigen from fully virulent strain of P. multocida with 2.5 per cent sodium chloride, which was found to be useful in vaccination.

The physico-chemical nature of the capsular antigen was studied by Lin et al. (1988) and they estimated the protein content as 55.5 per cent, carbohydrate 47.6 per cent and phospholipid 4.7 per cent.

Pabello and Smith (1993) used salt gradient paper chromatography for detection of hyaluronic acid from capsular material of P. multocida and suggested that the capsular material of serotype A and D contained hyaluronic acid or a substance similar to hyaluronic acid which migrated to the same level as the purified hyaluronic acid .

The presence of hyaluronic acid in P. multocida type A was demonstrated by Pandit and Smith (1993) by sodium chloride gradient chromatography, followed by alcian blue staining and also by turbidometric method using acidified horse serum.

Conard et al. (1996) extracted lipopolysaccharides from freeze dried cells of P. multocida strain P - 1581 by the phenol chloroform petroleum ether method. Biochemical analysis showed that the primary neutral sugars were glucose, galactose and heptose; 3 deoxy D manno 2 octulosonic acid was present at a low concentration.

They further identified the primary fatty acids present were as 3-hydroxy tetradecanoite and tetradecanoate in the ratio of 2:1.

2.2.2 Somatic Antigens

The somatic antigens of P. mulltocida were studied by Namioka and Murata (1961 b). They utilized various antigens viz., formalinized, heat, alcohol and hydrochloric acid treated cells as somatic (O) antigen, for agglutination and adsorption with antisera raised against O antigen. They reported that the O antigen which was prepared by hydrochloric acid treatment alone represented the true O antigen. This O antigen was further differentiated as common and specific antigen.

Six somatic groups of P. multocida were identified employing agglutination and adsorption test by Namioka and Murata (1961 c). The serotypes were designated by numbers, for the specific somatic antigen, followed by the capital letter standing for the specific capsular antigen.

Heddleston et al. (1972) were the first to show that the gel diffusion test could be used to type somatic antigen of P. multocida of avian origin. They could identify 16 different types of 'O' antigen from P. multocida isolates using supernatents of cell suspensions heated at 100° C for one hour, as antigen. This O antigen presumbly included the 11 'O' group which were described previously by Namioka and Murata. (1961 c).

Nineteen cell envelope and 55 cytoplasmic antigens were reported by Bhasin and shaw (1980) by crossed immunoelectrophoresis from cytoplasm of P. multocida serotype I.

Wu and Qian (1987) employed agar gel precipitation test to determine the somatic antigen of P. multocida from divergent species of animals and showed that the somatic antigen content of bacteria was shared between isolates from different species.

2.2.3 Outer Membrane Protein (OMP)

Lugtenberg et al. (1986) studied the biochemical properties of P. multocida cell envelope protein. The protein H was found to share properties with pore protein of the Enterobacteriaceae family and was considered as a vaccine candidate.

Snipes et al. (1988) reported that the strain of P. multocida of poultry origin expressed high molecular weight OMP when grown in turkey plasma or in BHI broth containing chelator dipyridyl.

Lu et al. (1988) identified, by immunoprecipitation and western blot analysis, several immunogenic OMP fractions of P. multocida isolates from rabbits as 27 KDa, 37.5 KDa, 49.5 KDa, 58.7 KDa and 64.4 KDa. The OMP was shown to be immunogenic and was able to detect antibodies in lung lavage of immunised rabbits.

Choi et al. (1989) examined the OMP enriched extract of avian strains of P. multocida by using sodium dodecyl sulphate

polyacrylamide gel electrophoresis and observed that strain specific markers could differentiate vaccine strain from field and reference strains.

Abdullahi et al. (1990) separated OMP by sarkosyl method from 30 strains of P. multocida , isolated from cattle. Based on the protein profiles of OMP detected by SDS- PAGE the strains could be grouped into 12. Further they immunized mice with heat treated bacterin and tried to correlate the difference in the antigen pattern with protection, but with a negative result.

Lu et al. (1991) reported that vaccine prepared from outer membrane of P. multocida 3 : A could provide protective immunity to mice against the homologous challenge following vaccination.

Based on structural and functional data Chevalier et al. (1993) opined that protein H of P. multocida was a pore forming protein related to the superfamily of non specific bacterial porins.

The N-terminal amino acid sequence of the 35 KDa major OMP of P. multocida was found to share a strong homology with those of homotrimeric non specific porins of gram negative bacteria (Lubke et al., 1994). They further reported that the capacity of OMP preparation of P. multocida to bind to respiratory mucosal surface preparation could be inhibited significantly by using a polyclonal anti P-35 antiserum.

2.2.4 Potassium thiocyanate extract (KSCN extract)

A protein complex fraction from P. septica type I was isolated as KSCN extract by Bain (1955) and successfully used to immunize mice. This finding was later confirmed by successfully vaccinating hill cattle by Mukkur and Nilakantan (1969). The crude KSCN extract and sodium chloride extract of P. multocida were composed of protein and carbohydrate.

Mukkur and Pyliotis (1981) reported that the immunogenic activity of KSCN extract of P. multocida type A was associated with the glycoprotein component. Further chemical analysis of the extracted antigen revealed to contain protein, LPS, DNA, RNA and hyaluronic acid. Electronmicroscopical study of the extract also revealed the presence of some fragments of membrane like material which could be of cell wall origin.

Mckinney and Rebers (1982) subjected KSCN extract of P. multocida to ultracentrifugation which got separated into particulate and soluble fractions consisting of protein and carbohydrate. They further showed that these fractions were antigenically different by electrofocusing and SDS- PAGE analysis.

Mukkur et al. (1982) reported that the major immunogenic determinants in the KSCN extract of P. multocida were protein in nature and that the polysaccharides were synergestic in conjunction with the protein fractions.

Twenty five different components were demonstrated when KSCN extract of P. multocida was subjected to cross immunoelectrophoresis by Ryu and Kaeberel (1986). They also compared the antigenic content of KSCN extract obtained from strains P - 2383 and P - 1062 and found that most antigenic components were common to both except for two strain specific antigenic components. They further reported that the KSCN extract fraction contained 12 per cent carbohydrate and 27 per cent proteins.

Lu et al. (1987) did the chemical analysis of KSCN extract of P. multocida 3: A of rabbit origins. The extract contained protein of about 2 mg per ml., carbohydrate 462.5 µg per mg of protein DNA 105 µg per mg of protein, RNA 100 µg per mg of proteins and lipopolysaccharides 256 µg per mg of protein.

2.2.5 Sonicated Antigen

Rahman et al. (1987) evaluated the use of sonicated antigen from P. multocida to sensitize erythrocytes and performed radial hemolysis test to measure antibody titre against P. multocida of bovine origin.

Ireland et al. (1991) studied the soluble proteins in the sonicated antigen from chicken isolates of P. multocida serotype I. The major differences in the isolates were in the position of major protein at 34-38 KDa region as detected by SDS - PAGE.

Procedure for the preparation of sonicated antigens and

their use as plate antigen in ELISA to detect specific antibodies against P. multocida has been given by many workers. (Solano et al., 1983, Briggs and Skeels, 1984 Avakian et al., 1986 and Choi et al., 1990)

2.3 VACCINE

Pasteur (1880) was the first to introduce live attenuated vaccine for prevention of fowl cholera. In field use, his method did not prove practical because uniform attenuation could not be obtained. Since then numerous attempts were made to produce efficient vaccine against pasteurellosis.

2.3.1 Killed Vaccine

Hilbert and Tax (1938) prepared autogenous Pasteurella vaccine from ailing ducks by treating the organism with 0.5 per cent phenol which reported to provide immunity to ducks as well as chicken against pasteurellosis.

Jacotot (1940) mixed bacterial suspension in immune serum or toxin obtained by lysing the culture with distilled water at 48°C for 26 hr. He reported that this vaccine could prevent pasteurellosis in cattle and that the endotoxin conferred protection in rabbit when challenged with virulent organism.

The efficiency of the oil adjuvant aqueous suspension, alum precipitated and chicken embryo vaccine were compared by Heddleston

and Hall (1960). They found that the adjuvant vaccine was superior over the other two.

A multiple emulsion vaccine by secondary emulsification of the oil adjuvant vaccine with Tween 80 was prepared by Mittal et al. (1977). They reported that both the vaccines were equally immunogenic, as assessed by direct challenge test and passive mouse protection tests, three weeks post vaccination.

A formalin killed emulsified vaccine and a formalin killed aqueous suspension vaccine from P. multocida serotype 1 were compared for their efficacy in cross bred ducks by Liow (1977). A single dose of such vaccine could protect 67 per cent and 39 per cent of the vaccinated ducks challenged at one week and four week post vaccination respectively. Two doses of this vaccine given six weeks apart protected 92 per cent of the ducks against the challenge. One dose of emulsified adjuvant vaccine protected 92 per cent, 92 per cent and 83 per cent of the ducks challenged at one, four and eight weeks post vaccination, respectively. Further he reported the survival of birds following rechallenge with the heterologous serotypes.

Mukkur (1978) observed that the KSCN extract of P. multocida was non toxic to calves and that the antibody response which was determined by agglutination test and bactericidal test was significantly higher in calves inoculated with KSCN extract in Freund's adjuvant than in those inoculated with the extract in saline tris buffer.

A formalinized whole cell vaccine and polysaccharide

vaccine of P. multocida of duck origin were compared by Lipipun et al. (1983) and they reported that the polysaccharide vaccine was superior over formalinized whole cell vaccine in the production and maintenance of antibodies for a period of 132 days in rabbit.

Layton (1984) produced a dense growth of P. multocida in tryptic soya broth and modified typtose broth for the preparation of formalin killed bacterin. These vaccines when given to two to three weeks old ducklings they with stood challenge upto six weeks of age. He also reported that a single injection of an oil emulsified P. multocida bacterin in six week old ducklings developed immunity that lasted for eight weeks.

The immunogenicity and cross protectivity of KSCN extract from P. multocida type A was observed in mice by Ryu and Kaeberle (1986). The KSCN extract could protect against challenge with homologous bacteria, but there was no consistent reciprocal protection between different strains of serotype, indicating lack of correlation between serotype and cross immunogenicity.

Azam (1987) compared the immunogenicity of the sonicated P. multocida vaccine with or without adjuvant and formalinised bacteria in rabbit, by estimating the antibodies using indirect haemagglutination test. The range of antibody titre observed in sonicated antigen with or without adjuvant was 8-64 while for formalin killed bacteria it was 4-16.

Bushueva et al. (1987) observed that for preparation of

vaccine, inactivation of P. multocida by sublethal concentration of formaldehyde and ultra violet light was superior over conventional method of formaldehyde in providing immunity in fowl.

Formalin inactivated alum adsorbed broth bacterin of P. multocida, type A : 1 from duck origin was tested in laboratory and field by Ramanatha (1994). An indirect haemagglutination test using glutaraldehyde fixed sheep erythrocytes sensitised with crude capsular polysaccharide was conducted on duck sera for monitoring humoral antibody levels. The highest ranges of IHA titres of sera from once or twice immunized ducks were 80-640 and 1280-10240 respectively as against 0-40 from unvaccinated ducks.

2.3.2 Live Vaccines from P. multocida

Bierer and Derieux (1972) observed that immunity in birds vaccinated with an oil based bacterial vaccine could be prolonged to 30 weeks by giving injection of avirulent strain of P. multocida P-1059 fourth week post vaccination.

Olson (1977) reported that the Clemson University (CU) strain of live cholera vaccine given orally was very much effective to protect turkeys against the pulmonary, arthritic and cranial form of fowl cholera.

Dua and Maheswaran (1978) observed that vaccination of turkeys by administering the CU strains of P. multocida in drinking water induced local antibodies in the tracheal secretion, demonstrated

by the indirect immunofluorescence technique. Local antibodies were induced by tenth day and persisted upto 42 days post vaccination.

Wei (1978) used P. multocida type B for the development of streptomycin dependent vaccine. P. multocida strain R-473 was altered by exposure to N-methyl N-nitro N-nitrosoguanidine to increase the likelihood of encountering a streptomycin dependent mutant and was plated on agar containing 400 µg of streptomycin per ml. Mice and rabbits when vaccinated with such strain could withstand challenge with wild type R-423, 28 days post vaccination.

Hertman et al .(1979) developed a live fowl cholera vaccine from virulent strain of fowl cholera by treating with N-methyl N-nitro N-nitrosoguanidine. Mutants were selected that were either small colonies or those sensitive to temperature (41 $^{\circ}$ C). All the mutants were avirulent to turkeys. These strains when used to vaccinate turkey could protect vaccinated flock against both homologous and heterologous challenge.

Derieux and Dick (1980) inoculated avirulent P. multocida at 14, 22 and 34 weeks of age in broiler breeder flock. Challenge with pathogenic P. multocida serotype I at 68 weeks indicated that inoculation with avirulent P. multocida two to three times provided better protection than single exposure.

Gazikhanian et al. (1983) exposed turkey breeder to P. multocida (CU strain) by both oral and wing web stick routes. Significant protection lasting to 25-30 weeks after vaccination was

reported from such a vaccination schedule.

Ducks were given a commercial killed P. multocida bacterin at fourth week of age, followed by avirulent vaccine administration in drinking water at either fourth week or four weeks and six weeks of age. The best protection was presented by the ducks which received the avirulent bacteria given twice, when challenged with the heterologous strain (Chang, 1984).

The safety and efficacy of a streptomycin dependent live P. multocida serotype (12:A) vaccine in rabbits was evaluated by DiGiacomo et al. (1987). This vaccine strain could colonize in rabbit nares and was genetically stable in vivo. The vaccinated rabbit could withstand challenge with homologous strain alone.

Hafaore et al (1989) reported that mutagenesis of the CU vaccine strain of P. multocida with N-methyl N-nitro N-nitrosoguanidine produced temperature mutant strain that grow at 37° C but not at 42° C. Of the seven mutants tested for immunogenicity in turkeys two protected them against challenge with virulent serotype 3, P. multocida strain (P-1059).

Ficken et al. (1991) vaccinated turkeys with cell free culture filtrate of P. multocida strain R44/6, mixed with incomplete Freund's adjuvant by different routes viz., oral, via air sac and subcutaneous and compared with controls, vaccinated with a commercial bacterin. At 13 weeks of age antibody against P. multocida could be detected in both groups but birds which received cell free

extract via air sac were best protected against the heterologous challenge followed by the commercial bacterin.

2.4 DIAGNOSIS

Presumptive diagnosis of pasteurellosis is made by microscopic demonstration of bipolar stained bacteria in blood smear or tissue impression smear stained with Wright's stain. From acute cases of pasteurellosis, organisms are isolated from blood, liver, spleen and lungs, while in chronic cases from localised lesion and blood. In birds without evidence of apparent infection, bone marrow is considered as the tissue of choice for isolation. Pasteurella infection in animals and birds are diagnosed by cultural isolation of the organism from the diseased, followed by identification by routine tests and serological typing (Rhoades and Rimler, 1989).

2.4.1 Agglutination Test

Mushin et al. (1976) successfully employed serum agglutination test for diagnosis of fowl cholera and also for assessment of specific antibody in unvaccinated flock.

Rimler (1978) identified two serogroups of P. multocida associated with haemorrhagic septicaemia in cattle by coagglutination test with antibodies coated with Staphylococci.

Schlink (1979) developed a microtitre agglutination test for assaying antibodies to P. multocida in turkeys. When compared with

tube agglutination test, microagglutination test required less antigen, serum and was easy to perform.

2.4.2 Indirect Haemagglution Test (IHA)

Carter (1972) compared IHA test with rapid plate test for detection of antibodies against P. multocida in chicken. He reported that the former could identify specific capsular antigen while the latter identified somatic antigen.

Five serological tests viz., single tube agglutination, double dilution tube agglutination, agar agglutination, passive haemagglutination and passive mouse protection test were evaluated for their efficacy in predicting the fate of vaccinated and unvaccinated sheep challenged with an ovine strain of P. multocida by Dua and Panduranga rao (1978). They observed that passive haemagglutination predicted the fate of vaccinated sheep while agar agglutination test indicated the immune status.

Sawada et al. (1982) performed IHA test using glutaraldehyde fixed sheep erythrocytes sensitized with KSCN extract and heat extract antigen of P. multocida for detection of antibody. They observed that IHA was capsular group specific with heat extract antigen.

Sanchis et al. (1988) reported that passive haemagglutination, performed with glutaraldehyde fixed sheep RBC showed specificity as good as that of ELISA or immunodiffusion for detection of antibodies against P. haemolytica.

Schimmel et al. (1988) reported that indirect haemagglutination could be applied successfully for detection of antibodies against P. multocida and P. haemolytica in the serum and colostrum of adult cattle and calves.

2.4.3 Couter Immunoelectrophoresis (CIE)

Carter and Chengappa (1981) recognized P. multocida type B and E as the primary cause of haemorrhagic septicaemia in cattle and buffalo by performing CIE. They further suggested that either serum or tissue could be used as a source of antigen.

2.4.4 Complement Fixation Test (CFT)

Giridhar et al. (1990) reported that CFT using killed whole cell antigen was better than passive haemagglutination test using capsular antigen for measuring protective antibody titre against pasteurella vaccine.

2.4.5 Enzyme Immuno Assays (EIA)

Since the development of Enzyme Linked Immuno Sorbent Assay (ELISA) by Engvall and Perlmann (1972) this test has been adopted for the quantification of infectious agents and antibodies. A decade later a modified form of ELISA was first reported for detection of antibodies/enzymes in nitrocellulose membrane (Hawkes and Gordon, 1982., Huet and Forma, 1982).

2.4.5a EIA for Detection of Antigen

Dawkins et al. (1990) developed an ELISA to identify P. multocida in cattle by employing polystyrene microtitre plates coated with PBS containing 2 μg per ml of known anti P. multocida 0019BA immunoglobulin fraction.The assay was found to have specificity of 99 per cent and sensitivity at least 86 per cent.

2.4.5b EIA For Detection of Antibody

Burrells et al. (1979) used capsular extract of P. haemolytica biotype 1 as antigen for detection of antibodies by employing ELISA. They observed that reaction time required for antigen and antibody was two hours and for conjugate incubation, three hours. After reaction of the enzyme, substrate colour intensities in individual wells were measured using a flow through system resulting in accurate, objective assessment.

Marshall et al. (1981) compared ELISA and microtitre agglutination test for detection of antibodies in turkeys vaccinated orally against fowl cholera. ELISA could measure antibody titre as high as 1: 4409 whereas the highest titre measured by microtitre agglutination was 1:128. They reported good correlation between antibody titre and protection following challenge.

Sonicated antigen was employed for ELISA and IHA and these were compared to measure antibody response in chicken vaccinated against P. multocida by Solano et al. (1983). Antibody titre

measured by ELISA and IHA were of high correlation but ELISA was observed to be twice as sensitive as IHA.

Donachie et al. (1983) used phenol water extract of P. haemolytica as coating antigen for antibody detection from sera of lambs vaccinated with sodium salicylate extract of P. haemolytica serotype A1 or A9. The specificity of this antigen was demonstrated by marked reduction of reactivity between serum from SPF lambs vaccinated with sodium salicylate extract of serotype A1 or A9.

Briggs and Skeels (1984) monitored serum antibody response in chicken vaccinated against P. multocida by ELISA. The positive/negative ratio method of analysis was used to determine the antibody titre of vaccinated chicken. After a $\log_e$ transformation of the ELISA titre, a linear relationship was confirmed between ELISA titre and positive/negative ratio. Regression analysis was used to construct a standard curve and derive an equation from this relationship. Using this equation, only one dilution was needed to determine the antibody titre of any unknown serum sample.

Dick and Johnson (1984) determined the immunity in broiler against P. multocida by ELISA. Mean ELISA titre as $\log_{10}$ values and survival rate of vaccinated birds after virulent challenge were 5.75 and 48 percentage, respectively.

Avakian and Dick (1985) assessed the antibody titre of birds by performing ELISA with sample of blood collected on filter paper

strips and serum separated after clotting. There was no significant differences in antibodies estimated between these two samples and thus they concluded that eluates of blood derived from whole blood dried on filter paper strip could be used as an alternative to serum in ELISA for measuring antibodies against P. multocida.

Probability of survival based on ELISA titre following challenge of vaccinated chicken with X-73 strain of P. multocida was predicted by Briggs et al. (1985). They reported that the probability of survival was less than 25 percentage for ELISA titre of 0-265, 25-50 per cent for titre of 266 to 370, 50 - 75 per cent for titres of 371-475 and more than 75 percentage for titres above 475.

Three antigenic preparations of P. multocida viz., lipopolysaccharide, boiled cell extract and boiled whole bacterium antigen were compared for their suitability by ELISA, to detect IgG antibodies against P. multocida by Klaassen et al. (1985). They reported that while all the three antigens gave high titre, lipopolysaccharide could detect only against homologous sera. They further reported that whole cell antigen was least sensitive. The boiled cell antigen was chosen as the best antigen preparation to use in ELISA.

Opuda _ Asibo et al. (1985) performed an indirect ELISA to determine the relative quantities of class specific antibodies to P. haemolytica in serum and bronchioalveolar washing of calves and this test was observed superior over indirect bacterial agglutination test.

Avakian et al. (1986) studied the suitability of the different

antigenic preparations such as potassium thiocyanate (KSCN), capsular antigen (CAP), lipopolysaccharide protein antigen and heat stable salt soluble antigen of P. multocida as plate coating antigen in ELISA. When antibody titres were correlated with survival after challenge, the KSCN and CAP antigens placed more non-survivors into low antibody titre ranges and more survivors into high antibody titre ranges than the other plate antigens.

By using agar gel diffusion test (AGDT) Harvey et al. (1985) assessed serum antibody against P. multocida in naturally infected rabbits and the results were correlated with nasal culture examination. The data obtained indicated that application of ELISA might prove efficacious in identifying apparently healthy, consistently nasal culture negative rabbits as clinical carriers of P. multocida.

Sacks et al. (1986) developed and evaluated a two stage strategy for serum antibody titre against P. haemolytica by using ELISA and a series of dilutions. The procedure described claimed to save considerable time and materials when a large number of assays were to be performed.

Lukas et al. (1987) tested the efficacy of ELISA for detecting serum immunoglobulin against P. multocida in naturally and experimentally infected rabbits and observed that there was no ELISA negative, culture positive animal.

A dot immunobinding assay was developed to detect serum

IgG specific for lipopolysaccharides of rabbit isolates of P. multocida by Manning et al. (1987). It was suggested to be more reliable than nasal culture. The test was also claimed to be sensitive, specific, easy to perform, cost effective and also provided a permanent record.

Townsend et al. (1987) performed ELISA for detection of IgG to P. haemolytica capsular polysaccharide. The capsular polysaccharide of P. haemolytica was covalently coupled to poly l lysin and then used to adsorb onto the microtitre plates.

Gillette et al. (1989) observed ELISA more sensitive than IHA in detecting colostrum IgG to P. haemolytica in new born calves.

Choi et al. (1990) developed DIA for detecting antibodies against P. multocida in turkey serum. Different antigenic preparations viz., whole cell antigen, sonicated cell lysate, crude capsular extract, formalin extract and heat stable antigen were tested and compared for their use as coating antigen in plate ELISA and DIA. Whole cell and

Zaoutis et al. (1991) considered ELISA as the most suitable test for screening rabbit colonies for detecting antibodies against P. multocida.

Using common salt extraction as plate coating antigen Erler et al. (1991) developed an ELISA for determining antibodies to P. multocida and P. haemolytica in serum of calves and pigs, lung lavage of piglets and cow colostrum. They could establish a correlation between serological titre and protection against infection.

sonicated cell lysate antigens showed higher sensitivity whereas formalin extract and heat stable antigens were more specific coating antigens in both assays. The specificity of DIA was reported to be greater than ELISA in distinguishing better heterologous serodiagnosis of P. multocida and the test was recommended because of its distinct advantages of high specificity and ease to perform.

Natalia and Patten (1993) compared ELISA with PMPT as alternative method for investigating the response of cattle to vaccination with P. multocida. He concluded that both PMPT and the HS-antibody ELISA were not specific to HS causing strains of P. multocida but might only indicate previous exposure to Pasteurella antigen.

Kawamoto et al. (1994) compared IHA test, GDPT and ELISA for detection of serum antibodies to P. multocida in naturally and experimentally infected rabbits. There was 98 per cent correlation between ELISA and positive culture method, 86.3 per cent between ELISA and GDPT and 25.5 per cent correlation between IHA and positive nasal culture. They considered ELISA superior in serodiagnosis of rabbit pasteurellosis.

The optimum test condition for ELISA was investigated by Jung et al. (1995). They determined the ratio of OD values of positive control serum to negative control serum. The best results were obtained with a 1:5 dilution of sonicated antigen, a 1:400 dilution of test serum and 1:1000 dilution of commercial goat antiduck horse radish peroxidase conjugate. The optimum antigen antibody reaction time and a substrate enzyme reaction time reported were 30 min and 45 min, respectively.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Buffers and Reagents

3.1.1.1 Alsever's Solution

Glucose	-	20.5g
Sodium Chloride	-	4.2g
tri-sodium citrate	-	8.0g
Citric acid	-	20.5g
Distilled water	-	1000 ml

The ingredients were dissolved thoroughly in one litre of distilled water and finally steamed for 10 min.

3.1.1.2 Ammonium Sulphate Solution (ASS)

3.1.1.2a Saturated Ammonium Sulphate (SAS) Solution

This was prepared by adding 760g of ammonium sulphate to one litre of double distilled water. This was heated at 56°C for 30 min. in a water bath with continuous stirring. The solution was filtered to remove insoluble impurities and then cooled at room temperature. The pH of the solution was adjusted to 7.0 with ammonia solution just prior to use.

3.1.1.2b Working Ammonium Sulphate Solution

Solution of 66 per cent strength was prepared (V/V), freshly from stock ASS.

3.1.1.3a Borate Buffer

Boric acid	-	6.184g
Borax	-	9.536g
Sodium chloride	-	4.384g
Distilled water	-	1000 ml

3.1.1.3b Borate Buffered Saline

Borate buffer	-	5 ml
Normal saline	-	95 ml

The pH of the solution was adjusted to 8.5 using 1N Na OH.

3.1.1.4 Carbonate Bicarbonate Buffer

Sodium carborate	-	1.59 gm.
Sodium bicarbonate	-	2.93 gm
Distilled water	-	1000 ml

The pH was adjusted to 9.6 using 1N NaOH

3.1.1.5 Decolourizer for AGID and IEP

3.1.1.5a Decolouriser I

Methanol	-	120 ml
Acetic acid	-	30 ml
Distilled water	-	30 ml.

3.1.5.1b Decolouriser II

Absolute alcohol	-	140 ml
Acetic acid	-	20 ml
Distilled water	-	40 ml

3.1.1.6 Gel for AGID

Agarose	-	0.8g
Sodium chloride	-	0.85 g
Phenol	-	1 drop
Distilled water	-	100 ml

To dissolve agarose in saline the solution was boiled for five min.

3.1.1.7 Gel for IEP

Agarose	-	0.8g
Trisbarbitol buffer	-	100 ml

The solution was given heat treatment till agarose got dissolved completely.

3.1.1.8 Phosphate Buffered Saline (PBS)

3.1.1.8a Phosphate buffered saline -10 x

Sodium chloride	-	80.00 gm
Potassium chloride	-	2.00 gm
di Sodium hydrogen phosphate ($Na_2HPO_4 12H_2O$)	-	11.33 gm
Potassium dihydrogen phosphate (KH_2PO_4)	-	2.00 gm
Distilled water	-	1000 ml

3.1.1.8b PBS working solution

PBS 10 x (3.1.1.8a)	-	200 ml
Distilled water	-	800 ml

The pH was adjusted to 7.2 using 1N Hcl.

3.1.1.9 PBSTween - 20 (PBST)

Tween 20	-	500 µl
PBS (3.1.1.8b)	-	100 ml

3.1.1.10 PBST with Bovine Serum Albumin (2 per cent)

Bovine serum albumin	-	2 g
PBST (3.1.1.9)	-	100 ml

3.1.1.11 Potassium Thiocyanate (KSCN) Solution

Potassium thiocyanate	-	4.865 gm
Sodium chloride	-	4.685 gm
Distilled water	-	100 ml

The pH of the solution was adjusted to 6.3 using 1N NaOH.

3.1.1.12 Protein Reagent

Hundred microgram of coomassie brilliant blue G-250 was dissolved in 50 ml of 95 per cent ethanol. In the above solution 50 ml of 85 per cent phosphoric acid was added and diluted to one litre with distilled water.

3.1.1.13 Protein Solution

Bovine serum albumin was dissolved in 0.15 M sodium chloride to contain 500 µg to 7000 µg per millilitre of the solution.

3.1.1.14 Stain for AGID

Amido black 10B	-	0.1 g
Sodium chloride	-	0.9g
Distilled water	-	100 ml

3.1.1.15 **Stain for IEP**

Amido black	-	0.1g
Sodium acetate-		
acetic acid buffer	-	100 ml
(0.2M, pH 3.2)		

3.1.1.16 **Substrate Solution for DIA**

3-3' Diaminobenzidinetetrachloride	-	5 mg
Hydrogen peroxide (30 per cent)	-	30 µl
PBST (3.1.1.9)	-	10 ml

3.1.1.17 Substrate Solution for Plate ELISA

2-2' Azinodiethylbenzithiazoline 6 -		
Sulfonic acid (ABTS)	-	11 mg
Sodium citrate buffer	-	50 ml
Hydrogen peroxide	-	25 µl

3.1.1.18 0.005 per cent Tannic acid

Tannic acid	-	0.005g
PBS (3.1.1.8b)	-	100 ml

3.1.1.19 **Tris barbitol buffer**

Barbitone sodium	-	9.9g
Tris (hydroxymethyl)-		
aminoethane	-	17.7gm
Sodium azide	-	0.3 gm
Distilled water	-	2000 ml

The pH of the solution was adjusted to 8.6 using 1N Hcl

3.2 BIOLOGICALS

3.2.1 **Bacteria**

Pasteurella multocida isolated and identified from the cases of duck pasteurellosis whose virulence was maintained by regular mouse passage, every six weeks, was used for antigen and vaccine preparation.

3.2.2 Antigenic Preparations

3.2.2a Crude capsular extract (CCE)

This was prepared by following the procedure described by Lin et al. (1984) with some modifications. Pasteurella multocida cultured in Brain Heart Infusion Agar (BHIA) at 37°C for 18 hr was harvested in PBS (3.1.1.8b). The bacterial suspension was washed thrice in PBS by centrifugation at 3000 x g for 15 min. The washed cells were resuspended in 2.5 per cent sodium chloride to yield 7.5 $x10^9$ cells per ml. The suspension was shaken for one hour at 56° C in a shaker water bath.

The bacterial suspension was centrifuged for half an hour at 17,000 x g. The supernatant was collected and dialysed for 48 hr against normal saline with frequent changes. Sterility in BHIA was checked and the sterile preparation was stored at -20° C in small aliquots, until further use.

3.2.2b Potassium thiocyanate extract (KSCN extract)

This was prepared by following the method described by Mukkur (1978) with some modifications.

Eighteen hour old culture of P.multocida from BHIA plate was harvested and washed thrice in PBS (3.1.1.8b) by centrifugation at 3000 x g for 15min. After washing, cells were suspended in KSCN solution

(3.1.1.11) to have a concentration of 7.5 x 10^9 cells per ml. The suspension was held at 4° C for 72 hr and then shaken in a shaker water bath for five hr at 37° C. The cells were sedimented by centrifugation at 18,000 x g for 30 min and discarded. Supernatant was collected and dialysed against frequent changes of PBS for 72 hr. Sterility of the preparation was checked and this preparation was stored at -20° C, until further use.

3.2.2c. Sonicated antigen

A modified method of procedure described by Manoharan and Jayaprakasan (1995) was followed for the preparation of sonicated antigen. Colonies of P.multocida grown in BHIA for 18 hr at 37° C were harvested in PBS and washed in the same buffer for three times by centrifugation at 3000 x g for 15min. The cells were suspended in PBS to have 7.5 x 10^9 cells per ml. This suspension was held on an ice bath, then disrupted by sonication for a total of 15 min with 30 x 30 sec burst in a sonicator (BRANSON Model - 250 sonifier using 1/8 inches diametre tappered tip). The sonication was interrupted for 30 Sec between each burst for cooling. Temperature of the suspension was maintained below 5° C . The sonicate was centrifuged at 8000 x g for 30 min, supernatant was collected and after sterility checking it was stored at -20° C until used.

3.2.2d Sonicated antigen for coating ELISA plate

The sonicated antigen to be used as plate antigen in ELISA was prepared from organisms which were suspended in carbonate bicarbonate buffer (3.1.1.4) and then subjected to sonication by following the procedure described in 3.2.2c.

The protein content of the antigenic preparations were estimated by following the procedure described by Bradford (1976) and by employing

phenol - sulphuric acid method(Dubois,et al,1956) carbohydrate estimation was made.

3.2.3 Duck Serum

Blood was collected from ten normal healthy ducks and allowed to clot. Serum was separated and clarified by centrifugation this serum was stored at -20° C until used.

3.2.4 Antiduck Serum

Three healthy rabbits aged four months were used to raise antiduck serum. Blood serum was collected from all the rabbits as pre immunization sample.

One ml of duck serum was homogenized with one ml of Freund's complete adjuvant and injected intramuscularly into each rabbit. Three booster doses of 0.5 ml serum each without adjuvant were given intramuscularly at weekly interval. Ten days following the last injection, serum was collected and subjected to agar gel immunodiffusion test (3.3.1) and immunoelectrophoresis (3.3.2) to determine the antibody response.

3.2.5 Duck Gamma Gloubulin

Fifty millilitre of 66 per cent ammonium sulphate solution (3.1.1.2b) was added drop by drop to 50 ml serum sample with constant stirring. The mixture was then allowed to stand overnight at 4° C. The suspension was centrifuged in a refrigerated centrifuge at 3000 x g for 30 min. The precipitate obtained was then dissolved in borate buffered saline (3.1.1.3b) to give a final volume of 20 ml. The ammonium sulphate was

removed from this precipate by dialysing it against borate buffered saline at 4^0 C, changing saline in every 6 hr until there was no ammonium sulphate in the dialysate as evidenced by absence of turbidity on testing with 10 per cent barium chloride solution.

The concentration of protein in the globulin fraction was determined by Biuret method described by Inchiosa (1964). Purity of the separated globulin was checked by performing agar gel immunodiffusion (3.3.1) and immunoelectrophoresis (3.3.2).

3.2.6 Antiduck Gamma Globulin

Six healthy rabbits were used to raise antiduck gamma globulin. The procedure described for raising of antiduck serum was followed. One millilitre of globulin with protein concentration of 10-15 mg constituted a single dose of antigen.

3.2.7 Conjugate

The procedure described by Avrameas (1969) was followed with some modifications.

The antiduck gamma globulin was reconstituted in borate buffered saline (3.1.1.3b) to obtain 5 - 7 mg of protein per ml of which the pH was adjusted to 6.9 by addition of 0.1M solution of potassium phosphate. Ten or fifteen millligram of horse radish peroxidase (HRPO - SRL) was added to every millilitre of globulin solution and was gently shaken until the enzyme dissolved completely. Then 0.05 ml of one per cent glutaraldehyde (freshly prepared) was added to this and mixture was shaken for 2 hr at room temperature by end to end rotation. This globulin enzyme conjugate was then

dialysed overnight at 4° C against repeated changes of physiological saline, pH 7.4. After dialysing the conjugate was clarified by centrifugation at 1500 x g for 15 min and the supernatant was stored in small aliquotes at -20° C until use.

3.2.8 Sensitized Sheep Red Blood Cells

The procedure described by Sawada et al. (1982) was followed for sensitization of sheep RBC.

3.2.8a Fixation of Sheep RBC

One part of fresh sheep blood was collected in five parts of Alsever's solution(3.1.1.1) and the erythrocytes were washed six times with physiological saline by centrifugation at 600 x g for ten min. The washed cells were suspended in PBS 7.2 (3.1.1.8b) which was previously chilled to 4° C, to yield ten per cent suspension (v/v), and was mixed with an equal amount of one percentage glutaraldehyde and kept at 4° C for 30 min with intermittent gentle stirring. The mixture was then centrifuged at 650 x g for 10 min at 25° C and the pelleted cells were resuspended in PBS washed thrice with PBS. Finally the fixed cells (GA-SRBC) were resuspended in PBS containing 0.1 per cent sodium azide to yield a ten per cent suspension and was stored at 4° C until used.

3.2.8b Tanned GA- SRBC(TG-SRBC)

A ten per cent suspension of GA-SRBC was mixed with an equal volume of PBS containing 0.005 per cent tannic acid (w/v) and the mixture was incubated at 37° C for 30 min with occasional shaking. The tanned GA-RBC (TG-SRBC) were pelleted by centrifugation at 650 x g for 10 min at 25° C and washed thrice with PBS. After last wash, the TGA-SRBC were

resuspended in PBS to yield a ten per cent suspension.

3.2.8c Sensitization of GA - SRBC with sonicated / crude capsular extract antigen

A ten per cent suspension of GA-SRBC was separately mixed with an equal volume of a serial two fold dilution of sonicated antigen(3.2.2c) crude capsular extract antigen (3.2.2a) .The mixture was then incubated at 37^{0} C for one hr with occasional shaking. The sensitized cells were washed three times in PBS by centrifugation at 650 x g for 10 min and resuspended in PBS containing 0.25 per cent bovine serum albumin (BSA-PBS) and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (v/v). The IHA was performed using this sensitized SRBC after further dilution of one in 16 with BSA-PBS.

3.2.8d Sensitization of T-GA-SRBC with KSCN extract antigen

A ten per cent suspension of TGA-SRBC was mixed with equal volume of serial two-fold dilution of KSCN extract antigen (3.2.2b). The mixture was incubated at 37^{0} C for 30min with occasional shaking. The sensitized cells were washed three times with BSA-PBS by centrifugation at 650 x g for ten min and suspended in PBS containing 0.1 per cent sodium azide to yield 0.5 per cent suspension (v/v), until used. The IHA was performed using this sensitized SRBC after further dilution of one in 16 with BSA-PBS.

3.2.9 VACCINES

3.2.9.a Bacterin

Pasteurella multocida was grown in BHIA for 24 hr at 37^{0} C. The cells were harvested and washed three times in normal saline by

centrifugation at 3000 x g for 15 min. The washed cells were resuspended in 0.3 per cent formol saline to have a final concentration of 7.5 x 10^9 cells per ml. The bacterial suspension in formol saline was kept at room temperature for 48 hr with occasional shaking. The sterility of the formalinized vaccine was checked by placing 0.2 ml of vaccine on BHIA. This bacterin formed the vaccine and was stored at 4^0 C till further use .

3.2.9.b Bacterin with Adjuvant

Fifteen parts of bacterin, nine parts of sterile liquid paraffin and one part of sterile lanolin were mixed thoroughly to give a uniform suspension and formed the bacterin with adjuvant vaccine. After sterility checking this vaccine was stored at 4^0 C till further use.

3.2.9.c Sonicated Vaccine

Sonicated antigen was used as sonicated vaccine. Fifteen parts of the sonicated antigen, nine parts of sterile liquid paraffin and one part of sterile lanolin were mixed thoroughly to give a uniform suspension and formed the sonicate adjuvanated vaccine.

3.2.10 Test Serum

Pre immunization and post immunization sera collected from healthy vaccinated and unvaccinated ducks formed the test serum. Fourty ducklings of one month age were grouped into four comprising ten birds in each group.

First second and third groups of birds were vaccinated with one ml each of formalin killed bacterin, formalin killed bacterin with adjuvant and sonicated antigen with adjuvant respectively.

Fourth group birds were kept as unvaccinated controls, and one ml of normal saline per bird was injected to this group.

Blood serum was collected from all the birds on 0, 21st ,30th and 35th days post vaccination to monitor specific antibody.

Dot immunobinding assay, plate ELISA and indirect haemagglutination were separately carried out to monitor the antibody response in vaccinated / control birds (group one, two, three and four) employing the different antigenic preparations such as crude capsular, KSCN and sonicated antigen.

3.3 METHODS

3.3.1 Agar Gel Immuno Diffusion (AGID)

Agar gel immunodiffusion was done as per the method described by Heddleston et al. (1972). Melted agarose (3.1.1.16) was poured onto glass slides and then wells were cut at equidistance so as to get one central and two peripheral wells. The purity of duck gamma globulin fraction separated was checked by charging the wells separately with duck serum, duck gamma globulin and antiduck whole serum. The slides were then kept in humid chamber at room temperature for 48 hr and examined for the development of precipitin lines.

The slides were then washed for 24 hr in normal saline with repeated changes and subsequently in distilled water for another 24 hr. They were then dried at 37° C after keeping a wet filter paper strip on its surface and then dried. The dried slides were stained with amido black (3.1.1.14) for 15 min. The stained slides were cleared by changing over to decolourizing solution I(3.1.1.5a) and II (3.1.1.5b) and examined.

The specificity of antiduck -rabbit gamma globulin was checked by charging the wells separately with duck serum, duck gamma globulin and antiduck -rabbit gamma globulin.

3.3.2 Immunoelectrophoresis (IEP)

The purity of duck gamma globulin and the specificity of antiduck gamma globulin was checked separately by performing IEP (Nair, 1990). Melted 0.8 per cent buffered agarose (3.1.1.7) was poured onto slides kept in levelled surface. A central trough and two peripheral wells at one third distance from one end were cut on the agarose gel. Before electrophoresis, agarose from the wells were removed and seperately filled with duck serum or duck gamma globulin. A drop of bromophenol blue dye was added to the anode side of the well as an indicator . The slides were then subjected to electrophoresis by passing current at the rate of 4 mA/slides until the indicator dye reached a centimetre distance from the anode end of the slides. After electrophoresis gel from the trough was removed and filled with antiduck whole serum or antiduck globulin rabbit serum. The slides were kept in a humid chamber at room temperature for 24 hr. The slides were then examined against light for the presence of precipitin lines, washed dried and stained as done in AGID (3.3.1)

3.3.3 Carbohydrate estimation

The carbohydrate content of the antigen was determined by using phenol-sulphuric acid method with glucose, galactose and ribose as standard (Dubois et al., 1956).

Two millilitre of sugar solution containing 20-260 μg of glucose/galactose/ribose were pipetted into 12 test tubes and 0.05 ml of 80

per cent phenol was added to each tube separately. Then five ml of concentrated sulphuric acid was added rapidly in order to obtain good mixing. The tubes were allowed to stand for ten min, shaken well and then placed in a water bath at 30^0 C for 30 min. Colour absorbance was measured at 490 nm in UV–VIS spectrophotometre (ELICO SL - 159) using blanks prepared by substituting distilled water for sugar solution. A standard curve was prepared by plotting the amount of carbohydrate against the corresponding absorbance. The estimation was done for three times and the average was taken.

3.3.4 Protein Assay

Standard bovine serum albumin solution in 0.1 ml quantity was pipetted into12 X 100mm test tube and five ml of protein reagent (3.1.1.12) was added to it. After thorough mixing the colour absorbance at 595 nm was measured in UV - VIS spectrophotometer (ELICO SL- 159) after two min but before one hour against a reagent blank prepared from 0.1 ml of saline and five ml of protein reagent. A standard curve was plotted with the amount of protein against the corresponding absorbance.

The amount of protein in the antigen was determined by referring the standard curve constructed as above. The estimation was done for three times and the average was taken.

3.3.5 Indirect Haemagglutination (IHA)

Indirect haemagglutination was performed by following the method described by Sawada et al. (1982).

Serial two fold dilutions of test serum (3.2.10) in BSA PBS were

taken in, 25 µl of quantities in 24 wells of a U bottomed microtitre plates. Twenty five microliters of the sensitized SRBC (3.2.8) was added to each well and the plates were shaken and allowed to stand for 2 hr at 25° C.

Three control wells were set in the experiment, one as positive with 25 µl of sensitized GA-SRBC (3.2.8c) / TGA-SRBC (3.2.8d) plus 25 µl of test serum, second negative control was 25 µl of serum and 25 µl of PBS-BSA. Third control was set by adding 25 µl of sensitized SRBC plus 25 µl of BSA-PBS.

The IHA titre was expressed as the reciprocal of the highest dilution of serum having a definite positive pattern (flat sediment) compared to the pattern in negative control well (smooth dot in the centre of the well).

3.3.6 Plate ELISA

Plate ELISA was performed as per the procedure described by Soloano et al. (1983) with some modifications. Each well of the U bottomed 96 well microtitre plate was coated with one in ten dilutions of antigens diluted in carbonate bicarbonate buffer (3.1.1.5) in 100 µl volume.

The plates were incubated at 4° C for 12 hr. At the end of the incubation plates were washed in PBST (3.1.1.9) three times. Unreacted sites were blocked by addition of one per cent Bovine serum albumin (3.1.1.10) in 100 µl to each well and incubated for one hr at 37° C.

The plates were washed three times in PBST and dried by tapping against the filter paper. The serial two fold diluted test serum (3.2.10) starting from 160 upto 10, 240 was added to all the wells.

Plates were incubated for one hr at 37° C and then washed thrice with PBST. Conjugate (3.2.10) (1:500) of 100 µl quantity was added to all the wells except in the substrate control and incubated for one hr. at 37° C. After washing three times with PBST freshly prepared substrate (3.1.1.17) 100 µl quantity was added to all wells but not in the conjugate control.

After 30 min incubation reading was taken in a multiscan ELISA reader (state fax 2100) at 405 nm.

3.3.7 Dot Immunobinding Assay (DIA)

By following the method described by choi et al. (1990) DIA was performed. One microlitre of pasteurella antigen(3.2.2) was dotted onto a nitrocellulose membrane. After air drying the antigen was fixed to the membrane by baking at 80° C for 30 min. The unreacted sites were blocked by incubating the membrane at 37°c in five per cent solution of skim milk powder for 30 min.

The membranes were then seperately incubated in 1:100 dilution of known positive, known negative and test serum(3.2.10) at 37° C for 45 min and then washed thrice in PBST (3.1.1.9). Diluted (1:100) labelled antiduck gammaglobulin (3.2.7) was then flooded on the membrane, and then incubated at 37° C for 30 min then washed thrice in PBST. Finally the membranes were treated with freshly prepared substrate solution(3.1.1.16) for 10 min and reaction was immediately stopped by rinsing the membrane in tap water.

3.3.8 Statistical Analysis of Data

The comparative efficacy of different antigens employed in IHA

and plate ELISA was analysed by subjecting the data to statistical analysis. Two way analysis of variance was done following the method of Snedecor and Cochran (1967). The sensitivity and specificity of IHA, plate ELISA and DIA was calculated by chi-square described by Pearson (1900).

RESULTS

4. RESULTS

4.1 Duck serum

The protein concentration of the pooled duck serum obtained from ten healthy ducks was 82 mg per ml.

4.2 Antiduck serum

Antiduck serum which was produced in rabbits when tested against duck serum by immunoelectrophoresis developed nine precipitin arcs extending to both cathode and anode sides of the antigen well. The precipitin arcs seen towards the cathode side of the well were identified as globulins considering their nature and position. (Fig. 1)

4.3 Duck gamma globulin

The duck gamma globulin was precipitated from the duck whole serum using 33 per cent ammonium sulphate concentration. Protein concentration of duck gamma globulin obtained was 14 mg per ml by Biuret method.

The purity of the gamma globulin was checked and compared with duck serum by subjecting it to AGID and IEP against anti duck serum.

In AGID three distinct precipitin lines were produced between anti duck serum and duck serum while the duck gamma globulin presented only two identical precipitin lines (Fig. 2)

Figure 1
Immunoelectrophresis

A - Duck serum
B - Antiduck serum

Figure 2.
Agar gel immunodiffusion

A - Antiduck serum
B - Duck globulin
C - Duck serum

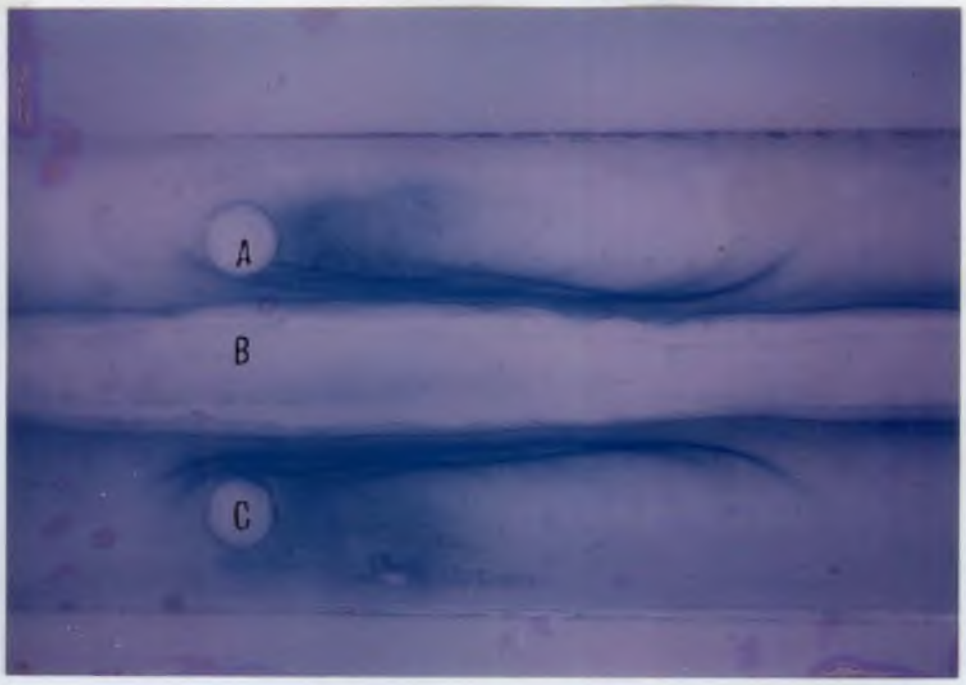
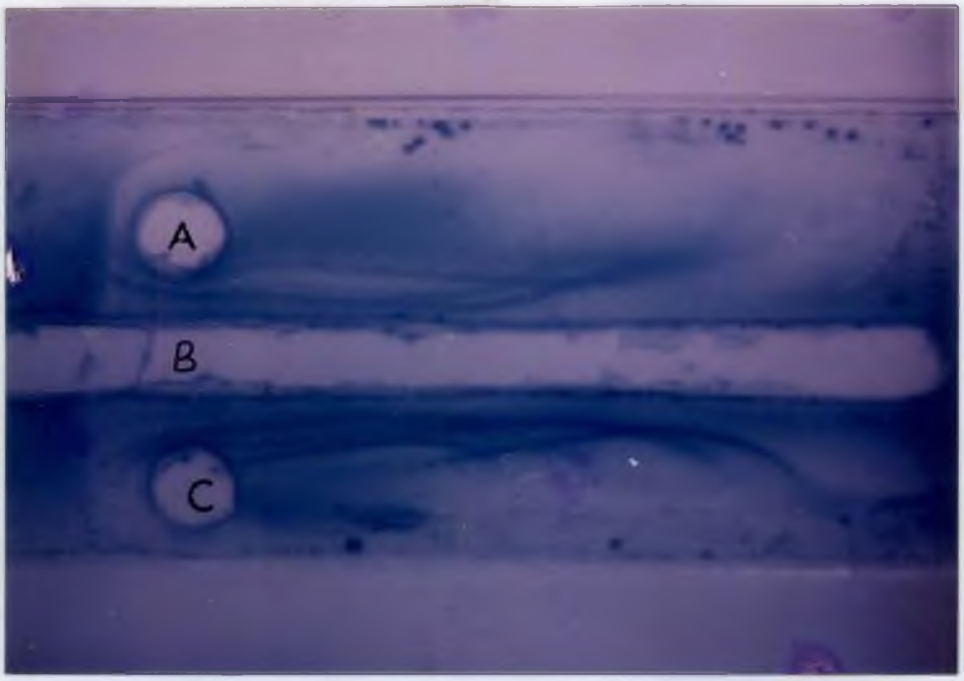


Figure 3
Immunoelectrophoresis

A - Duck gamma globulin
B - Antiduck serum
C - Duck serum

Figure 4
Agar gel immunodiffusion

A - Duck gamma globulin
B - Antiduck rabbit gamma globulin
C - Duck serum



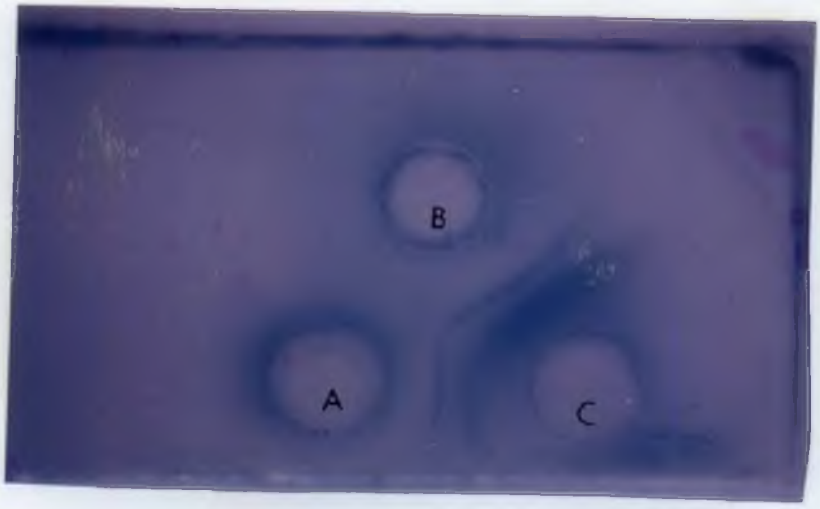


Figure 5.
Immunoelectrophoresis

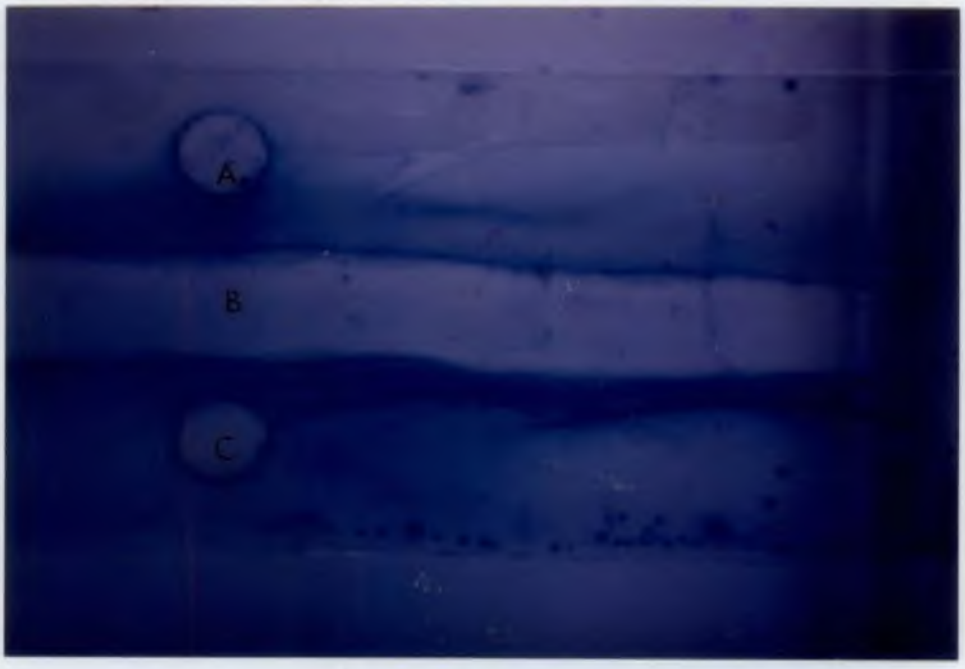
A - Duck gamma globulin.
B - Antiduck- rabbit gamma globulin.
C - Duck serum.

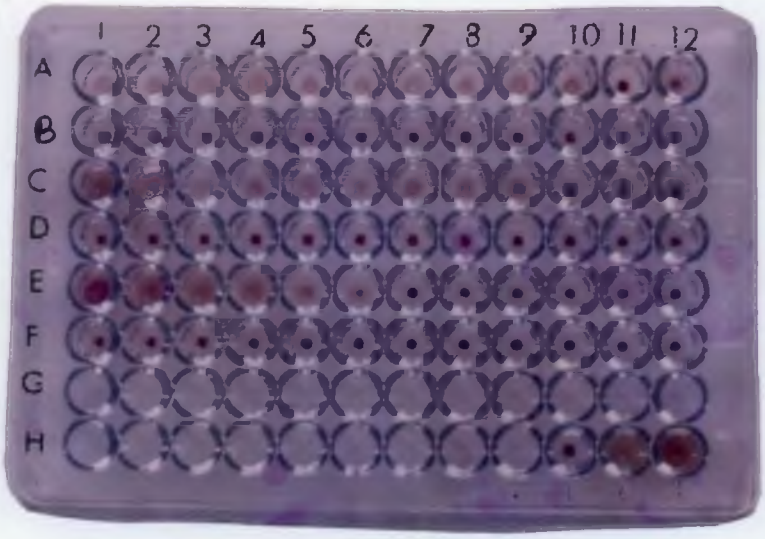
Figure 6.
Indirect haemagglutination test

A & B - Test subjected against SRBC Sensitized with sonicated antigen.

C&D - Test sera subjected against SRBC sensitized with KSCN extract.

E&F - Test sera subjected against SRBC sensitized with CCE.
H -10 - Negative control
11 - Serum control.
12 - Positive control.





Two distinct precipitin arcs were produced in IEP by duck gamma globulin and five precipitin arcs in case of duck serum, when reacted against anti duck serum. The position of the precipitin arcs produced were near to the antigen well confirming the purity of the globulin separated. (Fig. 3)

4.4 Anti duck gamma globulin

The procedure followed for raising antisera against duck gamma globulin in rabbit gave sufficient antibody in the serum of rabbit, when tested by AGID on 31st day post injection. The specificity of the antiduck gamma globulin was evidenced when it was reacted and compared with duck gamma globulin and duck serum. The gamma globulin fraction of the rabbit serum was separated by precipitation with 33 per cent ammonium sulphate and its purity and specificity was checked by both AGID and IEP (Fig. 4 and Fig. 5).
The protein concentration of the anti duck gamma globulin ranged between 12-14 mg per ml.

4.5 Conjugate

The procedure followed in the present experiment produced stable enzyme complex with anti duck gamma globulin. The protein concentration of anti duck gamma globulin used for preparation of enzyme conjugate was 5-7 mg per ml.

4.6 Antigenic preparations of P. multocida

The virulence and pathogenicity of Pasteurella multocida

was maintained by repeated mouse passage at six week intervals. The culture could be maintained in the fully encapsulated form by the passage procedure followed.

4.6a Crude capsular extract

Capsular antigen separated by 2.5 per cent sodium chloride, heat extract was estimated to have protein concentration of 3.8 mg per ml by Bardford method.

Galactose, glucose and ribose content in this preparation were estimated as indication of its carbohydrate content and concentrations were 450, 600 and 200 μg per ml, respectively. (Table 1)

4.6b Potassium thiocyanate extract

Potassium thiocyanate extract obtained was estimated to have a protein concentration of 3.76 mg per ml by Bradford method.

Galactose, glucose and ribose content in the extract were estimated as an indication of carbohydrate content and concentration were 50 μg per ml, 850 μg per ml and 200 μg per ml respectively. (Table 1)

4.6c Sonicated antigen

Protein concentration of the preparation was estimated as 3.85 mg per ml. Galactose, glucose and ribose content in this preparation were estimated as indication of its carbohydrate content and concentrations were 400 μg per ml, 600 μg per ml and 300 μg per ml, respectively. (Table 1)

Table 1. Carbohydrate estimate of the antigenic preparations

Antigenic Preparations	Carbohydrate		
	Galactose	Glucose	Ribose
Crude Capsular Extract	450	600	200
Potassium thiocyanate Extract	50	850	200
Sonicated Antigen	400	600	300

4.7 Vaccine

All the three forms of vaccines viz., bacterin, bacterin with adjuvant and sonicated antigen with adjuvant were prepared using a uniform concentration (7.5 x 10^9 cells per ml) of virulent encapsulated form of P. multocida. The vaccines produced and used were sterile and non-toxic to ducklings at a dose of one ml per bird.

4.8 Test serum

Neither the vaccinated nor the control groups of birds developed any illness/symptoms during the period of observation. Serum was collected from every bird on zero, 21st, 28th and 35th day post vaccination and was subjected to IHA, ELISA and DIA.

4.9 Indirect haemagglutination

Irrespective of the type of antigen used for sensitization of SRBC, IHA titre of 1:16 and above was considered as positive for the presence of specific antibody.

4.9a Birds vaccinated with bacterin

Prevaccination sera gave an average IHA titre below 1:16 when tested against SRBC sensitized with CCE, KSCN extract and sonicated antigen. All the test serum samples were positive by IHA performed, using SRBC sensitized with different antigens. The average titre ranged between 1:90 and 1:256 during 21st to 35th day post vaccination. The highest individual serum titre (1:512) was detected by SRBC sensitized with sonicated antigen. (Table 2).

Table 2. Indirect Haemagglutination Titres of the Birds Vaccinated with Bacterin

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:2	1:4	1:4	1:64	1:64	1:128	1:64	1:64	1:128	1:64	1:64	1:128
2.	1:2	1:4	1:4	1:64	1:64	1:128	1:64	1:64	1:128	1:64	1:64	1:128
3.	1:2	1:4	1:8	1:64	1:64	1:128	1:64	1:64	1:256	1:64	1:64	1:128
4.	1:2	1:4	1:8	1:64	1:64	1:128	1:128	1:128	1:256	1:64	1:128	1:256
5.	1:4	1:4	1:8	1:64	1:64	1:128	1:128	1:128	1:256	1:64	1:128	1:256
6.	1:4	1:4	1:8	1:64	1:128	1:128	1:128	1:128	1:256	1:128	1:128	1:256
7.	1:4	1:8	1:8	1:128	1:128	1:256	1:128	1:128	1:256	1:128	1:128	1:256
8.	1:4	1:8	1:16	1:128	1:128	1:256	1:128	1:256	1:256	1:128	1:128	1:256
9.	1:4	1:8	1:16	1:128	1:128	1:256	1:128	1:256	1:256	1:128	1:256	1:256
10	1:8	1:16	1:16	1:128	1:256	1:512	1:256	1:256	1:512	1:128	1:256	1:512
AVERAGE	1:4	1:6	1:10	1:90	1:109	1:205	1:122	1:147	1:256	1:96	1:134	1:243

Table 3. Indirect Haemagglutination Titres of the Birds Vaccinated with Bacterin with Adjuvant

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:2	1:4	1:4	1:8	1:32	1:64	1:32	1:32	1:64	1:64	1:64	1:128
2.	1:2	1:4	1:4	1:8	1:32	1:64	1:32	1:32	1:128	1:128	1:128	1:128
3.	1:2	1:4	1:8	1:32	1:64	1:128	1:64	1:64	1:128	1:128	1:128	1:128
4.	1:4	1:4	1:8	1:32	1:64	1:128	1:64	1:64	1:128	1:128	1:128	1:128
5.	1:4	1:4	1:8	1:64	1:64	1:128	1:64	1:128	1:128	1:128	1:128	1:256
6.	1:4	1:4	1:8	1:64	1:64	1:128	1:64	1:128	1:128	1:128	1:128	1:256
7.	1:4	1:8	1:8	1:64	1:128	1:128	1:128	1:128	1:256	1:128	1:256	1:256
8.	1:8	1:8	1:8	1:128	1:128	1:128	1:128	1:128	1:256	1:128	1:256	1:256
9.	1:4	1:8	1:8	1:128	1:128	1:128	1:128	1:128	1:256	1:128	1:256	1:256
10	1:8	1:8	1:16	1:128	1:128	1:256	1:128	1:256	1:256	1:256	1:256	1:512
AVERAGE	1:4	1:6	1:8	1:66	1:83	1:128	1:83	1:108	1:1728	1:134	1:172	1:2304

Fig 7

GRAPHICAL PRESENTATION OF IHA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH BACTERIN

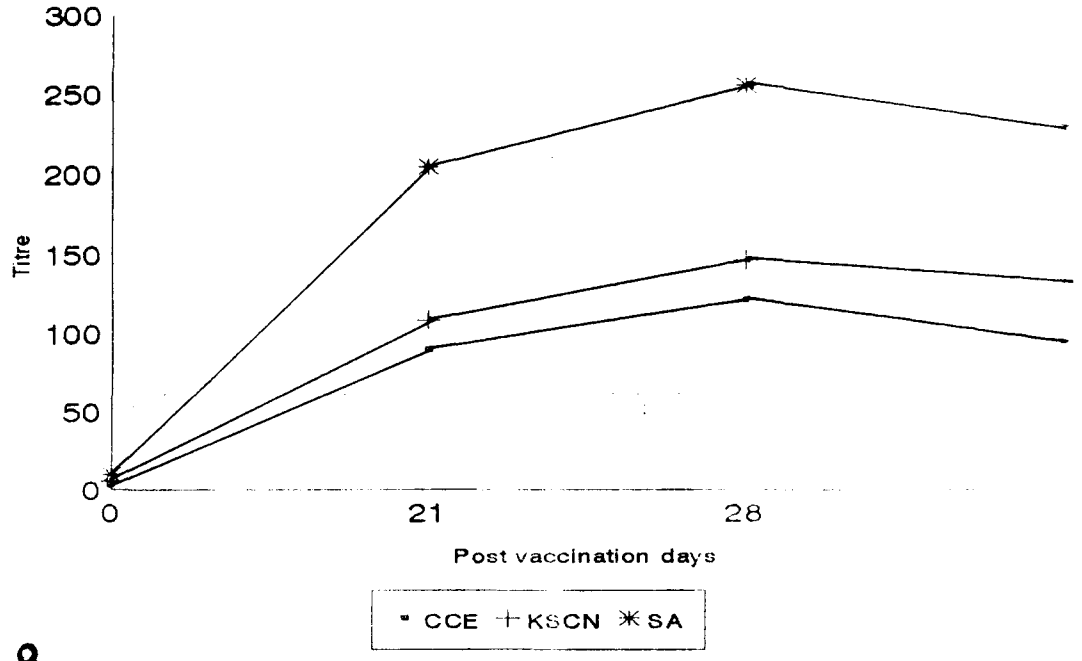
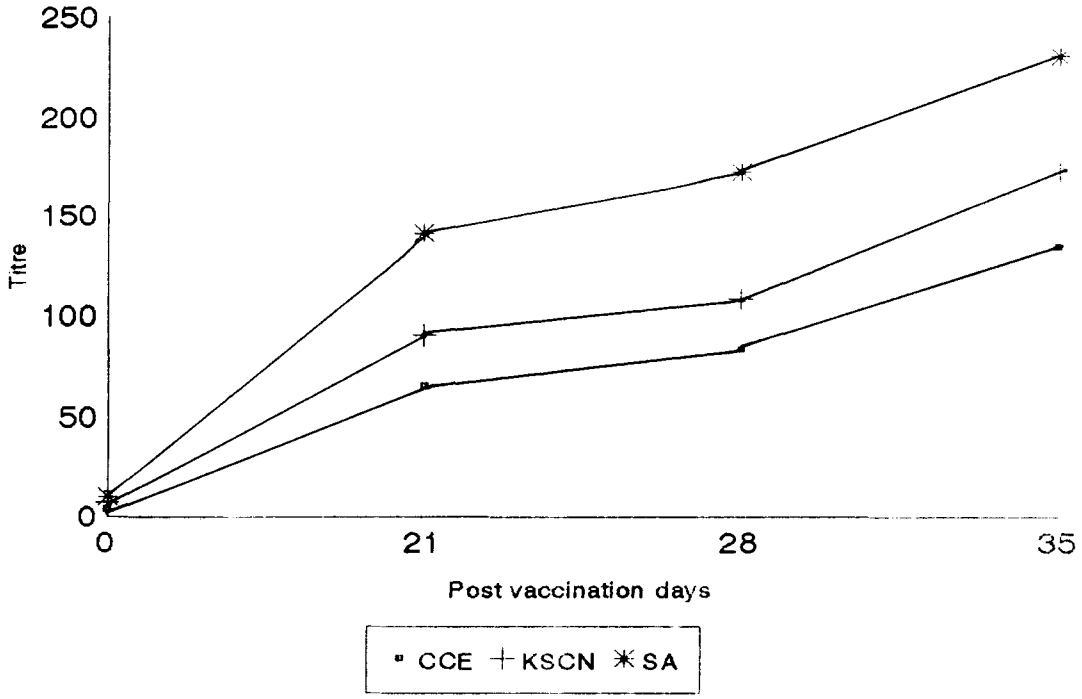


Fig 8

GRAPHICAL PRESENTATION OF IHA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH BACERIN WITH ADJUVANT



Average serum antibody titres detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 7.

4.9b Birds vaccinated with bacterin mixed with adjuvant

An average IHA titre below 1:16 was obtained when pre vaccination sera were tested against SRBC sensitized with CCE, KSCN extract and sonicated antigen. All the test sera samples were positive by IHA performed using SRBC sensitized with different antigens. The average titre ranged between 1:66 and 1:230, 21st to 35th day post vaccination. The highest individual serum titre (1:512) was detected by SRBC sensitized with sonicated antigen. (Table 3).

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 8.

4.9c Birds vaccinated with sonicate adjuvanated vaccine.

Pre vaccination sera gave an average IHA titre below 1:16 when tested against SRBC sensitized with CCE, KSCN extract and sonicated antigen. All the test sera were positive by IHA performed using SRBC sensitized with different antigens.

The average IHA titre ranged between 1:32 and 1:320, 21st to 35th day post vaccination, when IHA was performed using CCE, KSCN extract and sonicated antigen. The highest individual serum titre (1:1024) was detected by SRBC sensitized with sonicated antigen. (Table 4).

Table 4. Indirect Haemagglutination Titres of the Birds Vaccinated with Sonicate Adjuvanated Vaccine

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:2	1:4	1:4	1:8	1:32	1:64	1:32	1:64	1:128	1:32	1:32	1:128
2.	1:2	1:4	1:4	1:8	1:32	1:64	1:32	1:64	1:128	1:32	1:64	1:128
3.	1:2	1:4	1:8	1:8	1:32	1:128	1:32	1:64	1:128	1:32	1:64	1:128
4.	1:2	1:4	1:8	1:8	1:32	1:128	1:64	1:64	1:256	1:64	1:64	1:256
5.	1:4	1:8	1:8	1:32	1:32	1:128	1:64	1:128	1:256	1:64	1:128	1:256
6.	1:4	1:8	1:8	1:32	1:64	1:128	1:64	1:128	1:256	1:64	1:128	1:256
7.	1:4	1:8	1:8	1:32	1:64	1:128	1:64	1:128	1:256	1:128	1:128	1:256
8.	1:4	1:8	1:8	1:64	1:64	1:256	1:64	1:128	1:256	1:128	1:128	1:256
9.	1:4	1:8	1:16	1:64	1:64	1:256	1:128	1:128	1:512	1:128	1:128	1:512
10	1:4	1:16	1:16	1:64	1:128	1:256	1:128	1:128	1:512	1:128	1:256	1:1024
AVERAGE	1:3	1:7	1:9	1:32	1:54	1:154	1: 67	1:102	1:269	1:80	1:112	1:3200

Table 5. Indirect Haemagglutination Titres of the Control Birds

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:2	1:2	1:8	1:2	1:2	1:4	1:2	1:2	1:2	1:2	1:4	1:4
2.	1:2	1:2	1:8	1:2	1:2	1:4	1:2	1:2	1:4	1:2	1:4	1:8
3.	1:2	1:4	1:8	1:2	1:2	1:8	1:2	1:4	1:8	1:2	1:8	1:8
4.	1:2	1:4	1:8	1:2	1:4	1:8	1:4	1:4	1:8	1:4	1:8	1:8
5.	1:2	1:4	1:8	1:4	1:4	1:8	1:4	1:4	1:8	1:4	1:8	1:8
6.	1:2	1:4	1:8	1:4	1:4	1:8	1:4	1:4	1:8	1:4	1:8	1:8
7.	1:4	1:8	1:16	1:4	1:4	1:8	1:4	1:8	1:8	1:4	1:8	1:16
8.	1:4	1:8	1:16	1:4	1:4	1:8	1:4	1:8	1:8	1:8	1:8	1:16
9.	1:4	1:8	1:16	1:4	1:16	1:16	1:4	1:8	1:16	1:8	1:16	1:16
10	1:4	1:8	1:16	1:4	1:16	1:16	1:4	1:8	1:16	1:8	1:16	1:16
AVERAGE	1:3	1:7	1:11	1:3	1:5	1: 9	1:3	1:5	1:9	1:5	1:9	1:11

Fig 9

GRAPHICAL PRESENTATION OF IHA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH SONICATE ADJUVANATED VACCINE

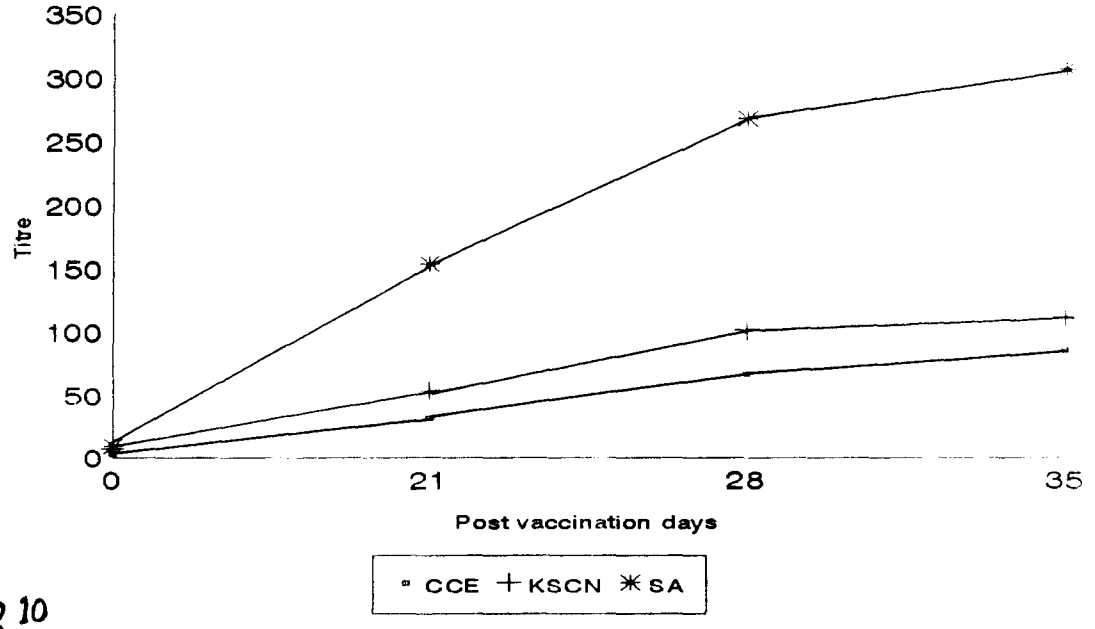


Fig 10

GRAPHICAL PRESENTATION OF IHA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF CONTROL BIRDS

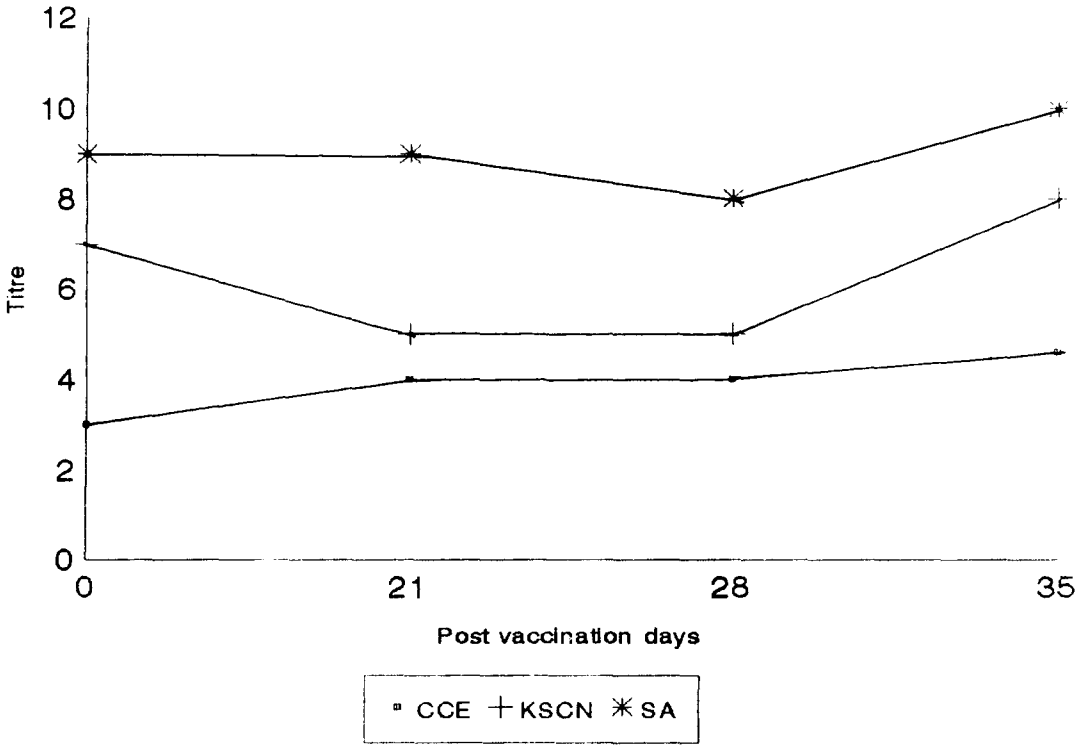


Table 6. ELISA Titre of the Birds Vaccinated with Bacterin

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:20	1:40	1:40	1:640	1:640	1:1280	1:640	1:640	1:1280	1:640	1:640	1:640
2.	1:20	1:40	1:80	1:640	1:640	1:1280	1:640	1:640	1:1280	1:640	1:640	1:1280
3.	1:20	1:40	1:80	1:640	1:640	1:1280	1:640	1:640	1:1280	1:640	1:1280	1:1280
4.	1:40	1:80	1:80	1:640	1:640	1:1280	1:1280	1:1280	1:2560	1:640	1:1280	1:1280
5.	1:40	1:80	1:80	1:640	1:1280	1:1280	1:1280	1:1280	1:2560	1:640	1:1280	1:2560
6.	1:40	1:80	1:80	1:12801	1:1280	1:2560	1:1280	1:2560	1:2560	1:640	1:1280	1:2560
7.	1:40	1:80	1:80	1:12801	1:1280	1:2560	1:1280	1:2560	1:2560	1:1280	1:1280	1:2560
8.	1:40	1:80	1:80	1:12801	1:2560	1:2560	1:2560	1:2560	1:5120	1:1280	1:2560	1:2560
9.	1:40	1:80	1:160	1:25601	1:2560	1:5120	1:2560	1:2560	1:5120	1:2560	1:2560	1:5120
10	1:40	1:160	1:160	1:25601	1:2560	1:5120	1:2560	1:2560	1:5120	1:2560	1:2560	1:5120
AVERAGE	1:34	1:84	1:92	1:12161	1:1408	1:2432	1:1472	1:1664	1:2944	1:1152	1:1536	1:2496

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 9.

4.9d Control birds

Pre and post vaccination sera from the control birds gave an average IHA titre below 1:16. (Table 5)

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 10

4.10 Enzyme linked immunosorbent assay

An ELISA titre of 1:160 and above was considered as positive for the presence of specific antibody.

4.10a Birds vaccinated with bacterin

Pre vaccination sera gave an average ELISA titre below 1:160 when tested against CCE, KSCN extract and sonicated antigen. All the test sera samples were positive by ELISA employing different plate coating antigens. The average titre ranged between 1:1216 and 1:2944, 21st to 35th day post vaccination. The highest individual serum titre (1:5120) was detected for sonicated antigen.(Table 6).

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 11.

Table 7. ELISA Titre of the Birds Vaccinated with Bacterin with Adjuvant

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:20	1:40	1:40	1:320	1:640	1:640	1:640	1:640	1:640	1:640	1:640	1:1280
2.	1:20	1:40	1:80	1:320	1:640	1:640	1:640	1:640	1:640	1:640	1:640	1:1280
3.	1:20	1:40	1:80	1:320	1:640	1:1280	1:640	1:640	1:1280	1:640	1:640	1:1280
4.	1:20	1:40	1:80	1:640	1:640	1:1280	1:640	1:640	1:1280	1:640	1:1280	1:2560
5.	1:20	1:80	1:80	1:640	1:640	1:1280	1:640	1:640	1:1280	1:1280	1:1280	1:2560
6.	1:20	1:80	1:80	1:640	1:640	1:2560	1:640	1:1280	1:2560	1:1280	1:1280	1:2560
7.	1:20	1:80	1:80	1:640	1:640	1:2560	1:1280	1:1280	1:2560	1:1280	1:1280	1:2560
8.	1:20	1:80	1:160	1:640	1:1280	1:2560	1:1280	1:1280	1:2560	1:1280	1:1280	1:2560
9.	1:40	1:80	1:160	1:12801	1:1280	1:2560	1:1280	1:1280	1:2560	1:2560	1:2560	1:2560
10	1:40	1:160	1:160	1:12801	1:1280	1:2560	1:2560	1:2560	1:5120	1:2560	1:2560	1:5120
AVERAGE	1:24	1:72	1:100	1:672	1:832	1:1920	1:896	1:1088	1:2042	1:1220	1:1349	1:2560

Fig 11

GRAPHICAL PRESENTATION OF ELISA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH BACTERIN

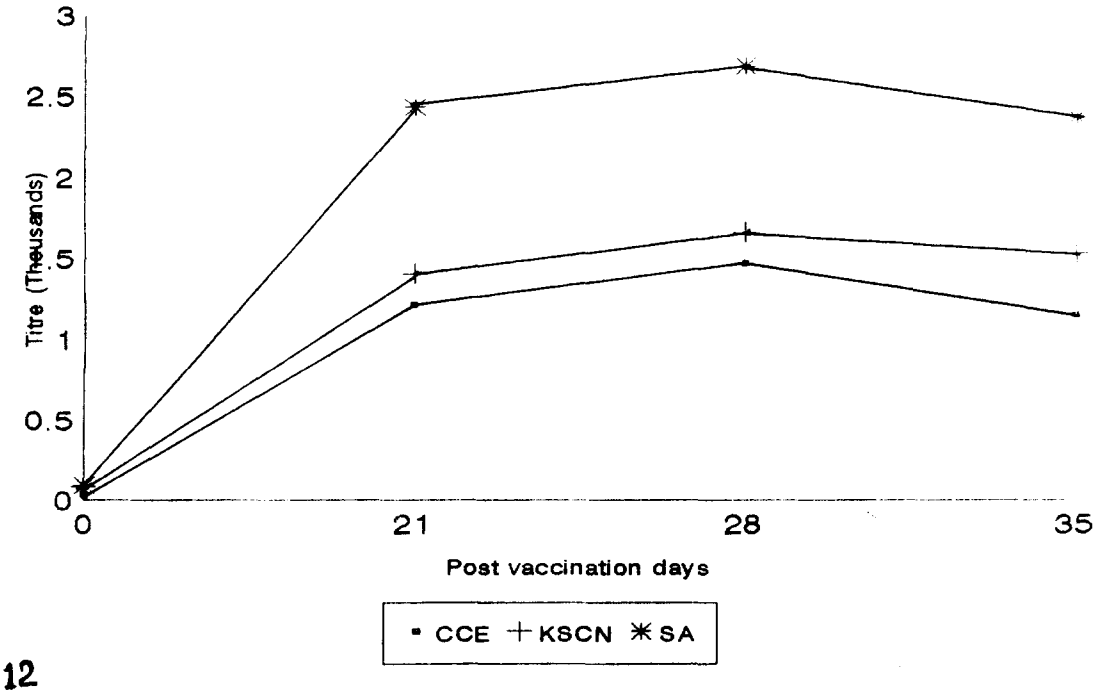


Fig 12

GRAPHICAL PRESENTATION OF ELISA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH BACTERIN WITH ADJUVANT

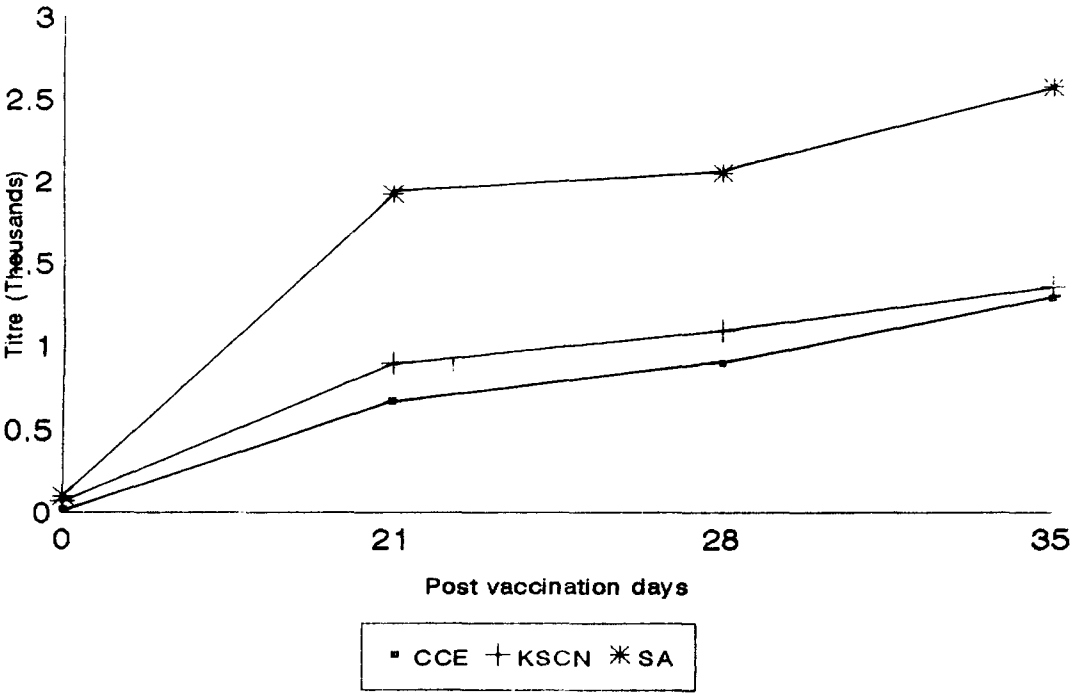


Table 8. ELISA Titre of the Birds Vaccinated with Sonicate Adjuvanated Vaccine.

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:20	1:40	1:40	1:160	1:320	1:1280	1:320	1:640	1:1280	1:320	1:640	1:1280
2.	1:20	1:40	1:40	1:320	1:320	1:1280	1:640	1:640	1:1280	1:640	1:640	1:2560
3.	1:20	1:40	1:80	1:320	1:320	1:1280	1:640	1:640	1:2560	1:640	1:640	1:2560
4.	1:40	1:80	1:80	1:320	1:640	1:1280	1:640	1:640	1:2560	1:640	1:640	1:2560
5.	1:40	1:80	1:80	1:320	1:640	1:2560	1:640	1:1280	1:2560	1:640	1:640	1:2560
6.	1:40	1:80	1:80	1:320	1:640	1:2560	1:640	1:1280	1:2560	1:640	1:1280	1:2560
7.	1:40	1:80	1:80	1:640	1:640	1:2560	1:640	1:1280	1:2560	1:1280	1:1280	1:2560
8.	1:40	1:80	1:80	1:640	1:640	1:2560	1:640	1:1280	1:5120	1:1280	1:2560	1:5120
9.	1:40	1:80	1:160	1:640	1:640	1:2560	1:1280	1:1280	1:5120	1:2560	1:2560	1:5120
10	1:40	1:160	1:160	1:640	1:1280	1:2560	1:1280	1:2560	1:5120	1:2560	1:5120	1:1024
AVERAGE	1:32	1:76	1:88	1:432	1:608	1:2048	1:736	1:1152	1:3072	1:1184	1:1600	1:3712

4.10b Birds vaccinated with bacterin mixed with adjuvant

Pre vaccination sera gave an average ELISA titre below 1:160 when tested against different plate coating antigens viz., CCE, KSCN extract and sonicated antigen.

The average titre ranged betwen 1:672 and 1:2560, 21st to 35th day post vaccination. The highest serum titre 5120 was obtained when sonicated antigen was used to coat the plate. (Table 7).

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 12.

4.10c Birds vaccinated with sonicate adjuvanated vaccine

Pre vaccination sera gave an average ELISA titre below 1:160 when tested against CCE, KSCN extract and sonicated antigen. The average ELISA titre ranged between 1:432 and 1:3712, 21st to 35th day post vaccination. The highest ELISA titre for individual serum was 1:10240 when it was tested against sonicated antigen. (Table 8).

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 13.

Table 9. ELISA Titre of the Control Birds.

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	1:20	1:20	1:20	1:20	1:20	1:40	1:20	1:20	1:20	1:20	1:20	1:20
2.	1:20	1:20	1:40	1:20	1:40	1:40	1:20	1:40	1:40	1:20	1:20	1:40
3.	1:20	1:40	1:40	1:20	1:40	1:40	1:40	1:40	1:80	1:40	1:40	1:80
4.	1:40	1:40	1:80	1:40	1:40	1:80	1:40	1:40	1:80	1:40	1:40	1:80
5.	1:40	1:80	1:80	1:40	1:80	1:80	1:40	1:40	1:80	1:40	1:40	1:80
6.	1:40	1:80	1:80	1:40	1:80	1:80	1:40	1:80	1:80	1:40	1:40	1:80
7.	1:40	1:80	1:80	1:40	1:80	1:80	1:40	1:80	1:80	1:80	1:80	1:80
8.	1:40	1:80	1:160	1:80	1:80	1:80	1:40	1:80	1:80	1:80	1:80	1:80
9.	1:80	1:80	1:160	1:80	1:80	1:160	1:80	1:80	1:160	1:80	1:80	1:80
10	1:80	1:160	1:160	1:80	1:160	1:320	1:80	1:80	1:160	1:80	1:80	1:160
AVERAGE	1:42	1:60	1:90	1:46	1:70	1:100	1:40	1:58	1:86	1:52	1:52	1:78

Fig 13

GRAPHICAL PRESENTATION OF ELISA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF BIRDS VACCINATED WITH SONICATE ADJUVANATED VACCINE

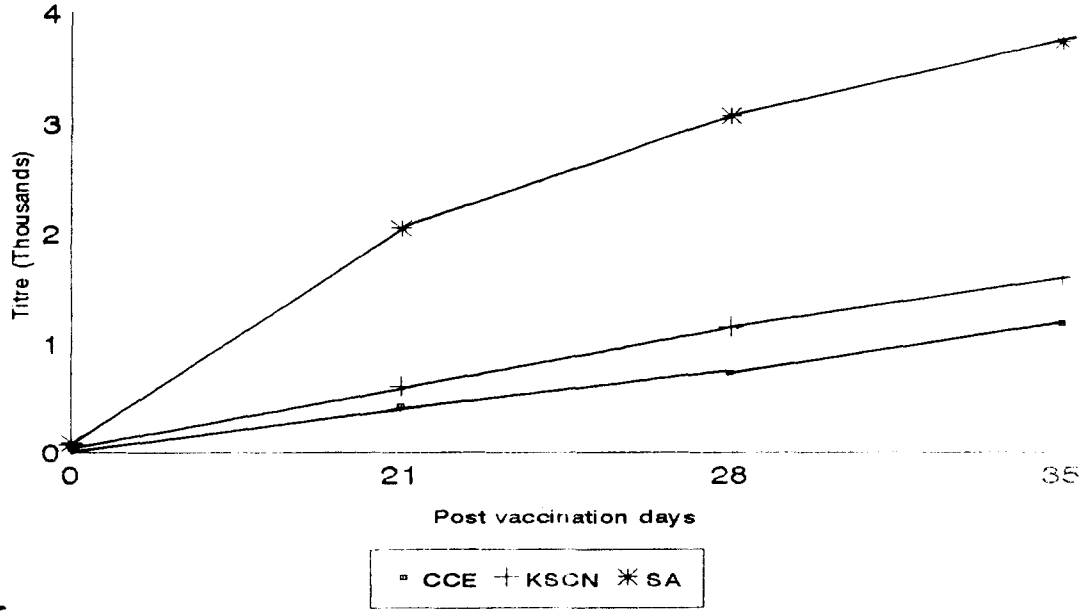


Fig 14

GRAPHICAL PRESENTATION OF ELISA TITRE USING DIFFERENT ANTIGEN PREPARATIONS IN SERA OF CONTROL BIRDS

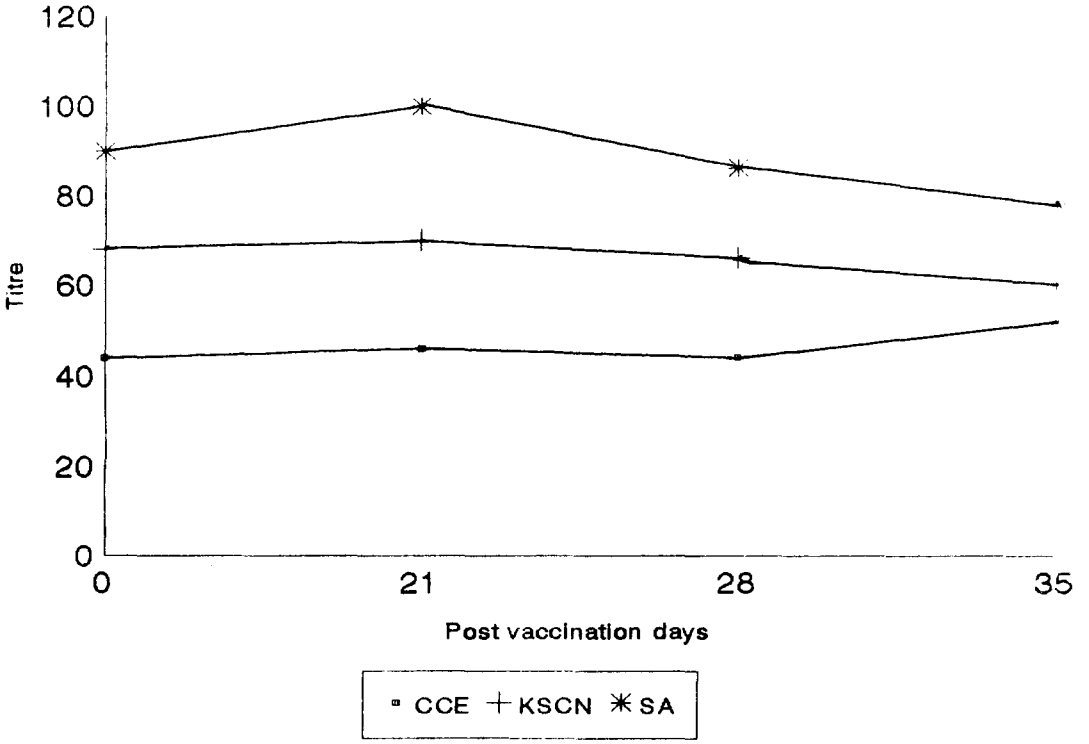


Figure 15
Dot immunobinding reactions

A - Positive reaction.
B - Negative control.

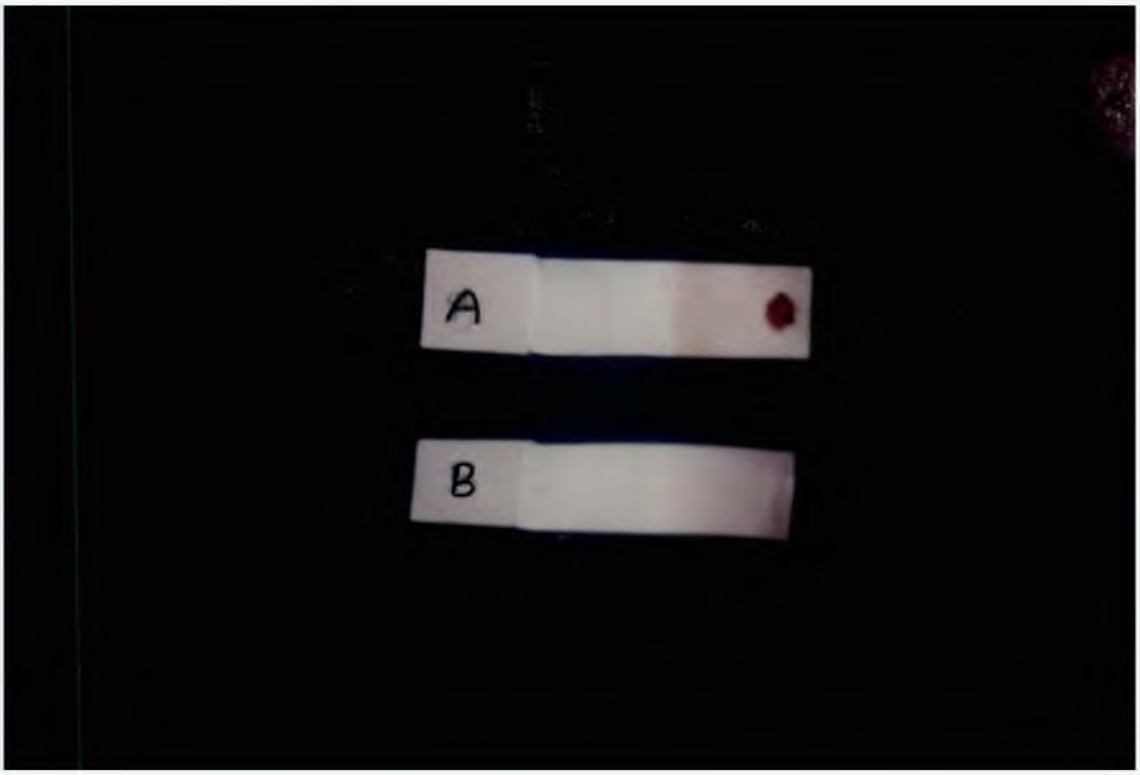


Table 10. DIA Reaction Shown by Birds Vaccinated with Bacterin

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	-	-	+	+	+	+	+	+	+	+	+	+
2.	-	-	+	+	+	+	+	+	+	+	+	+
3.	-	-	-	+	+	+	+	+	+	+	+	+
4.	-	-	-	+	+	+	+	+	+	+	+	+
5.	-	-	-	+	+	+	+	+	+	+	+	+
6.	-	-	-	+	+	+	+	+	+	+	+	+
7.	-	-	-	+	+	+	+	+	+	+	+	+
8.	-	-	-	+	+	+	+	+	+	+	+	+
9.	-	-	-	+	+	+	+	+	+	+	+	+
10	-	-	-	+	+	+	+	+	+	+	+	+

4.10d Control birds

All the birds gave an average ELISA titre below 1:160 at all the four intervals when tested with CCE, KSCN extract and sonicated antigen. (Table 9)

Average serum antibody titre detected by different antigenic preparations at specified intervals are presented for comparison in Fig. 14.

4.11 Dot Immunobinding assay

A brown colour spot on nitrocellulose membrane, developed at the site of antigen application, was considered as positive for the presence of specific antibody.

4.11a.Birds vaccinated with bacterin

Irrespective of the antigens used for coating NCM prevaccination sera from all the birds gave negative reactions, except one with sonicated antigen.

Post vaccination sera from all the birds gave positive reactions. (Table 10).

4.11b Birds vaccinated with bacterin with adjuvant

Prevaccination sera subjected to NCM coated with CCE, KSCN extract and sonicated antigen all gave the negative reaction, except one positive with sonicated antigen.

Table 11. DIA Reaction Shown by Birds Vaccinated with Bacterin with Adjuvant

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	-	-	+	-	+	+	+	+	+	+	+	+
2.	-	-	-	-	+	+	+	+	+	+	+	+
3.	-	-	-	+	+	+	+	+	+	+	+	+
4.	-	-	-	+	+	+	+	+	+	+	+	+
5.	-	-	-	+	+	+	+	+	+	+	+	+
6.	-	-	-	+	+	+	+	+	+	+	+	+
7.	-	-	-	+	+	+	+	+	+	+	+	+
8.	-	-	-	+	+	+	+	+	+	+	+	+
9.	-	-	-	+	+	+	+	+	+	+	+	+
10	-	-	-	+	+	+	+	+	+	+	+	+

Table 12. DIA Reaction Shown by Birds Vaccinated with Sonicate Adjuvanated Vaccine

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	-	-	+	-	-	+	+	+	+	+	+	+
2.	-	-	-	-	+	+	+	+	+	+	+	+
3.	-	-	-	-	+	+	+	+	+	+	+	+
4.	-	-	-	+	+	+	+	+	+	+	+	+
5.	-	-	-	+	+	+	+	+	+	+	+	+
6.	-	-	-	+	+	+	+	+	+	+	+	+
7.	-	-	-	+	+	+	+	+	+	+	+	+
8.	-	-	-	+	+	+	+	+	+	+	+	+
9.	-	-	-	+	+	+	+	+	+	+	+	+
10	-	-	-	+	+	+	+	+	+	+	+	+

Table 13. DIA Reaction Shown by Control Birds

Sl. No.	0 Day			21st Day Post vaccination			28th Day Post vaccination			35th Day Post vaccination		
	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen	CCE	KSCN	Sonicated Antigen
1	-	-	+	-	+	-	-	+	-	-	-	+
2.	-	-	-	-	-	-	-	-	-	-	-	-
3.	-	-	-	-	-	-	-	-	-	-	-	-
4.	-	-	-	-	-	-	-	-	-	-	-	-
5.	-	-	-	-	-	-	-	-	-	-	-	-
6.	-	-	-	-	-	-	-	-	-	-	-	-
7.	-	-	-	-	-	-	-	-	-	-	-	-
8.	-	-	-	-	-	-	-	-	-	-	-	-
9.	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-

Irrespective of the antigen used all the post vaccination sera gave positive reaction except two false negative when tested against sonicated antigen. (Table 11)

4.11c Birds vaccinated with sonicate adjuvanated vaccine

Prevaccination sera when subjected to NCM coated with CCE, KSCN and sonicated antigen all gave negative reaction except two positive when tested against sonicated antigen.

Postvaccination sera from all the birds gave positive reaction when tested against sonicated antigen whereas two false negative reactions were obtained when subjected to CCE and one false negative when tested against KSCN extract. (Table 12).

4.11d Control birds

Sera from all the birds at various intervals gave negative reactions when tested against CCE whereas sonicated antigen gave three false positive and KSCN extract gave two false positive reactions. (Table 13).

4.12 Statistical analysis

4.12a Analysis of variance

On statistical analysis highly significant differences were observed between the four groups. (control and three vaccinated groups) The four groups were compared pairwise to find out the group which gave the maximum mean titre, using least significant difference test.

Table.14 **ANALYSIS OF VARIANCE TABLE**

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Replication	9	6249489.983	694387.776	3.6228	0.0002
Factor A(Groups)	3	38958598.200	12986199.400	67.7525**	0.0000
Factor B(Antigens)	2	24921464.033	12460732.017	65.0110**	0.0000
AB	6	13964072.900	2327345.483	12.1424	0.0000
Factor C(Tests)	1	70902836.267	70902836.267	369.9191**	0.0000
AC	3	22778218.533	7592739.511	39.6134	0.0000
BC	2	16962736.033	8481368.017	44.2496	0.0000
ABC	6	9775339.567	1629223.261	8.5001	0.0000
Factor D(Intervals)	3	37698284.067	12566094.689	65.5607**	0.0000
AD	9	18149326.200	2016591.800	10.5211	0.0000
BD	6	10405304.633	1734217.439	9.0479	0.0000
ABD	18	10200498.700	566694.372	2.9566	0.0000
CD	3	21593212.400	7197737.467	37.5525	0.0000
ACD	9	11538592.533	1282065.837	6.6889	0.0000
BCD	6	8116359.300	1352726.550	7.0575	0.0000
ABCD	18	8287209.367	460400.520	2.4020	0.0009
Error	855	163878870.017	191671.193		
Total	959	494380412.733			

(**) Highly Significant

Table 15 Sensitivity and specificity of IHA, ELISA and DIA

Antigenic Preparations	IHA		ELISA		DIA	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Crude Capsular Extract	93	100	100	100	94	100
Potassium thiocyanate Extract	100	87	100	92	97	98
Sonicated Antigen	100	76	100	79	100	91

Group one (birds vaccinated with bacterin) had the highest mean value, followed by group three (birds vaccinated with sonicate adjuvanated vaccine) and group two (birds vaccinated with bacterin mixed with adjuvant). Control group gave the minimum mean value.

Significant difference was observed in the case of antigens also. Of the three antigens no significant difference was observed between crude capsular extract and KSCN extract. Sonicated antigen gave the significantly higher mean titre value than the other two antigens viz. crude capsular extract and KSCN extract, but KSCN extract had a mean value greater than that of crude capsular extract. (Table 14).

4.12b Specificity and sensitivity of IHA, ELISA and DIA

The sensitivity and specificity of IHA, ELISA and DIA were calculated based on the results obtained with sera samples from control birds and pre immunization sera (40 samples from the control birds and 30 samples as pre immunization sera) in comparison to post immunization sera samples. (30 each from the different vaccinated groups. ie. 90 sera samples) (Table 15).

DISCUSSION

5. DISCUSSION

Duck pasteurellosis caused by P. multocida is one of the major causes of economic loss to duck industry. (Mulbagal et al., 1972, Montgomery et al., 1978, Bhaumik and Dutta, 1995). This disease is considered as a major threat to duck farming in Kerala as frequent outbreaks of duck pasteurellosis is being reported from several parts of the state. (Pillai et al., 1993).

Though the importance of the disease is well understood, reports on successful vaccination and assessment of immunity in vaccinated birds are scanty. The success of any vaccination programme depends on how best and quick monitoring of immune response is made in a herd/flock. Though several serological tests ~~are~~ have been employed in detecting antibodies against P. multocida, IHA and ELISA are considered to be of high sensitivity in chicken (Solano et al., 1983). Thus there is a need to develop, a rapid, sensitive reproducible test to detect and measure antibody response to P. multocida in ducks either for diagnosis or seromonitoring.

Considering the above aspects, the present study was undertaken. The technical programme of this study included vaccination of ducklings with three different vaccines such as bacterin, bacterin mixed with adjuvant, sonicate adjuvanated vaccine and monitoring antibody using IHA, ELISA and DIA, employing the three antigenic preparations viz., crude capsular extract (CCE), potassium thiocyanate extract (KSCN) and sonicated antigen.

5.1 Anti duck serum

In the present study the antisera raised against duck serum produced nine distinct precipitin arcs by IEP. In a similar study Nair (1990) could get 13 precipitin arcs when used a higher dose of serum and lengthy immunization protocol to raise anti duck serum.

5.2 Duck gamma globulin

Duck globulin was salted out with 33 per cent saturation of ammonium sulphate from pooled duck serum containing 82 mg of protein per ml and the globulin recovery was 14 mg of protein per ml. The purity of globulin, when checked by AGID and IEP, was satisfactory as evidenced by the precipitation pattern provided by globulin and whole duck serum against anti duck serum. Nair (1990) was of the opinion that precipitation with 33 per cent saturation of ammonium sulphate was superior over 40 per cent saturation of ammonium sulphate in getting pure gamma globulin from duck serum. The present observations are in confirmity with that of Nair (1990).

5.3 Anti duck gamm globulin

Duck globulin having 10 - 15 mg protein per ml when repeatedly injected into rabbit at weekly interval produced sufficient level of anti duck globulin by 31st day. The gamma globulin fraction of the rabbit serum was separated with 33 per cent ammonium sulphate concentration and purity and specificity was evidenced by precipitation reaction when subjected to AGID and IEP against duck gamma globulin and duck serum.

5.4 Conjugate

Anti duck gamma globulin with the protein concentration of five to seven mg per ml was labelled with horse radish peroxidase by adopting the method described by Avrameas (1969). Such glutaraldehyde fixed enzyme conjugated preparation was stable upto four months of storage at -20°C, without any decrease in enzyme activity or immune reactivity. Engvall and Perlmann (1972) have reported that glutaraldehyde fixed enzyme conjugate was comparatively more stable over the conjugate prepared without glutaraldehyde fixation.

5.5 Antigenic preparations of P. multocida

Three different antigenic preparations such as CCE, KSCN extract and sonicated antigen were prepared from the virulent, encapsulated P. multocida. The virulence and pathogenicity of the organism was maintained by repeated mouse passage at six week intervals. Mukkur (1978) observed that P. multocida can be maintained in its encapsulated and virulent form when mouse passage was done every four weeks.

5.5a Protein in the antigenic preparations

The average protein content in CCE, KSCN extract and sonicated antigen were 3.8, 3.76 and 3.85 mg per ml respectively. The antigens were sterile and stable upto six months of the study period when stored at -20°C. The use of these antigens either for sensitization

of RBC used in IHA (Sawada et al.,1982, Giridhar et al., 1990) or for coating the ELISA plates (Solano et al., 1983, Avakian et al., 1986) were reported earlier.

Kajikawa and Matsumoto (1984) quantified the protein content of antigen extracted with saline and purified by gel chromatography from P. multocida of turkey origin and reported that it contained 650 μg of protein per ml of antigen. In another experiment Lu et al. (1987) studied the different components of KSCN extract antigen of P. multocida type 3: A and was found to contain two mg of protein per ml.

In the present study all the antigenic preparations had a higher protein content. The reason for this could be the higher concentration of bacteria used for the preparation.

5.5b Carbohydrate content

Carbohydrate content of the three antigenic preparations was estimated as galactose, glucose and ribose by phenol-sulphuric acid method. Sonicated antigen presented the maximum carbohydrate content followed by capsular extract. (Table 1).

Mukkur (1969) reported the presence of glucose but not galactose in the KSCN extract of P. multocida. Kajikawa and Matsumoto (1984) quantified the carbohydrate content in the antigen extracted with saline and purified by gel chromatography from P. multocida of turkey origin and reported to contain 152 μg of carbohydrate per ml of antigen.

Lu et al. (1987) estimated the different components of KSCN extract antigen of P. multocida type 3: A and found that it contained 462 μg of carbohydrate per mg of protein. Lin et al. (1988) described the qualitative features of capsular material prepared from P. multocida and reported that it contained 52.5 per cent protein, 47 per cent carbohydrate, 10 per cent of phospholipids, traces of nucleic acids and O-acetyl group.

In the present method employed for the preparations of different forms of antigens, the initial bacterial suspensions were uniformly adjusted to contain $7.5x10^9$ bacteria per ml and at the end of each procedure the antigenic preparation was obtained as soluble supernatent.

The reason for the high content of protein and carbohydrate in the sonicated antigen could be that it contained the cell lysates/disintegrates while the other two antigens were only the extracts which ought to contain only fractions of the organisms.

5.6 Vaccines

The three different vaccines viz., bacterin, bacterin with adjuvant and sonicate adjuvanated vaccines were prepared from fully encapsulated, virulent P. multocida isolate of duck origin, whose virulence was periodically monitored by pathogenicity in mouse.

5.7 Sensitization of sheep red blood cells

Sheep RBC were fixed with glutaraldehyde before they were

sensitized with different antigens and such fixed SRBC could be stored at 4°C upto six months. According to Sawada et al. (1982) the use of glutraldehyde fixed SRBC saved time spent in preparing SRBC suspension for every test. Moreover, it was reported to increase the reproducibility of the test as the same RBC lot could be used in the entire seromonitoring experiment. GA-SRBC were directly sensitized with either CCE or sonicated antigen while tanned GA-SRBC was used for KSCN extract antigen. Irrespective of the sensitization procedure employed, sensitized GA-SRBC/TGA-SRBC could be used for atleast one week for IHA test without any loss of agglutinability by specific antisera.

Tannic acid treatment of GA-SRBC was shown to be required for sensitization with KSCN extract antigen of P. multocida (Mukkur, 1978, Sawada et al., 1982). Untreated GA-SRBC (as opposed to tannic acid treatment) sensitized with KSCN extract could detect only low titres of antibody. Sawada et al. (1982) further showed that protein antigens of P. multocida were better adsorbed only to TGA-SRBC when compared to other antigens rich in polysaccharide moiety.

Ever since Pasteur introduced live attenuated vaccine for the prevention of fowl cholera, numerous attempts were made to produce efficient vaccine against pasteurellosis. Various homologous and heterologous serotypes of P. multocida in killed or product forms have been tried as vaccine in ducks. Duck pasteurellosis could be controlled by autogenous phenol killed bacterin (Hilbert and Tax 1938, Schimmel, 1990) Other than bacterin, bacterin with oil adjuvant had also given good results. (Heddleston and Hall 1960, Liow 1977, Mittal et al., 1977).

Formalin inactivated alum adsorbed broth bacterin of P. multocida (type A: 1 from duck origin) had given IHA titre of 80-640 and 1280-10240 from once and twice vaccinated ducks. (Ramanatha, 1994)

Live vaccine had also been tried and was found to give good protection to birds. (Olson, 1977, Dua and Maheswaran, 1978)

Streptomycin dependent mutant of P. multocida was reported to provide immunity from homologous as well as heterologous strains. (Hertman et al., 1979, DiGiacomo et al., 1987).

It was found that when bacterin was given which was followed by avirulent vaccine administered orally, can prolong the immunity (Chang, 1984).

In the present study, three different vaccines namely bacterin, bacterin with adjuvant and adjuvanated sonicate vaccines were prepared by taking an initial concentration of bacteria as 7.5×10^9 cells per ml. The vaccines prepared were safe and non toxic to ducklings at a dose of one ml per bird. All the vaccinated birds were found to have specific antibody detected from 21st day post vaccination onwards.

5.8 Evaluation of immunological response

Ducklings of four weeks of age were vaccinated separately with bacterin, bacterin with adjuvant and sonicate adjuvanated

vaccine. Serum samples of each vaccinated and control groups showed the specific antibody activity, detected by IHA/ELISA/DIA at 0, 21, 28 and 35th day post vaccination. The specific antibody activity in the serum was detected by IHA/ELISA/DIA employing the different antigenic preparations such as CCE, KSCN and sonicated antigens were compared.

5.8a Indirect haemagglutination

The three different antigenic preparations were separately used to sensitize SRBC and to detect antibody in the sera of birds.

All post immunization sera samples were positive from 21st day to 35th day. Successful use of various antigenic preparations viz. potassium thiocyanate extract (Sawada et al., 1982), heat stable antigen (Sawada et al., 1982), sonicated antigen (Solano et al., 1983) and crude capsular extract (Giridhar et al., 1990) for sensitization of SRBC were reported earlier.

The results of the present study showed that SRBC coated with sonicated antigen could detect significantly higher titre in the sera of birds vaccinated with bacterin. (Table 2).

On comparison, IHA employing KSCN extract or sonicated antigen showed cent per cent sensitivity over CCE (93 per cent). On the other hand the IHA performed with CCE antigen presented cent per cent specificity, followed by KSCN extract (87 per cent) and sonicated antigen (76 per cent).

In a similar experiment where in immune response of rabbits against P. multocida was studied, Manoharan and Jayaprakasan (1995), have shown that sonicated antigen was superior over CCE and KSCN extract as sensitizing antigens of RBC in IHA.

Sawada et al. (1982) observed that IHA test was capsular group specific when antigen and KSCN extract of P. multocida were adsorbed onto GA-SRBC but not to TGA-SRBC. Giridhar et al. (1990) reported that the saline extract of P. multocida, as capsular extract antigen, influenced the sensitivity of IHA, giving a poor correlation with direct potency test.

5.8b Enzyme linked immunosorbent assay

ELISA, which was introduced and developed by Engvall and Perlmann, (1972) was considered as the most sensitive and rapid assay. (Burrells et al., 1979, Marshall et al., 1981)

ELISA has been recommended as a routine serological assay because of its accuracy in quantifing antibody response and higher sensitivity over IHA (Solano et al., 1983) This test is more advantageous in reporting exact antibody titre when reading is based on the colour change , which is directly proportional to antibody present. (Briggs and Skeels, 1984).

Although an automated plate reading is used for reading result, this level of sophistication is not essential as positive reaction shows a marked colour change. (Dawkin et al., 1990).

The present study demonstrated the usefullness of the different antigenic preparations from P. multocida viz., CCE, KSCN extract and sonicated antigens as plate coating antigen in ELISA to quantify the specific antibody in the serum of vaccinated ducks. Various antigenic preparations which have been successfully employed as plate coating antigens in ELISA includes, sodium salicylate extract (Burrells et al., 1979), phenol water extract (Donachie et al., 1983), heated whole cell extract (Klaassen et al., 1985), lipopolysaccharides (Avakian et al., 1986), formalinized cells (Opuda-Asibo et al., 1986), 2.5 per cent sodium chloride extract (Hwang et al., 1986) and potassium thiocyanate extract (Lukas et al., 1987).

The three different antigenic preparations employed in this study had more or less similar concentration of protein ranging from 3.76 to 3.85 mg per ml and carbohydrate content ranging from 1100 to 1300 μg per ml. These antigens were diluted one in ten with carbonate-bicarbonate buffer. This concentration was found to give optimum result as the plate coating antigen, by checker board titration. The diluted antigens (1:10) were separately used to coat polystyrene plates by incubating them at 4°C over night. Such antigen coated plates were employed to detect specific antibody against P. multocida in the sera of vaccinated birds. The result of the present experiment indicated that the concentration of antigen and procedure employed in the plate ELISA were able to detect specific antibody against P. multocida.

The protein concentration of antigenic preparations from P. multocida used as coating antigens reported earlier were one microgram (Lukas et al., 1987), eight micrograms (Avakian et al., 1986)

and ten microgram per ml (Dick and Johnson, 1984).

The present result shows that a comparatively higher protein content in the antigenic preparations was required in detecting the specific antibody against P. multocida in duck serum as higher dilutions of antigens failed to do so.

Irrespective of the antigens used for coating plate, the birds vaccinated with bacterin gave a higher mean titre value, followed by those birds vaccinated with adjuvanated sonicate and bacterin with adjuvant vaccine.

Plate ELISA employing sonicated antigen detected significantly higher titre in the vaccinated birds, irrespective of the types of vaccine used for immunization.

All the three plate coating antigens viz., CCE, KSCN extract and sonicated antigen showed cent per cent sensitivity while least specificity (79 per cent) was noted with sonicated antigen. (Table 15)

The results were comparable to the result obtained by Choi et al. (1990) wherein they reported higher sensitivity of ELISA when sonicated antigen was used, but with a low specificity. The reason, they attributed for the low specificity was, as bacterial cells when disrupted by ultrasonication both internal and external antigenic substances could be solubilized to bind non-specifically to antibodies in the serum.

Though there were apparent differences in the titre of

antibodies detected by CCE and KSCN extract, the difference was not statistically significant but KSCN was found to have a higher mean titre value than CCE. Harvey et al. (1986) reported that ELISA was efficacious in identifying apparently healthy, consistently nasal culture negative rabbits as subclinical carriers of P. multocida, when capsular extract was used for coating microtitre plates.

Avakian et al. (1986) reported that the capsular and KSCN extracts were more suitable as ELISA plate coating antigen than the standard sonicated antigen. They further reported that antigen recovered through milder extraction processes (especially KSCN extract and capsular extract) contained a high ratio of antigen that detected protective antibody.

As per the result of this study though the sonicated antigen could detect significantly high titre of antibody in the sera of vaccinated birds, its specificity was shown to be far low compared to CCE and KSCN extract.

An immunological test of high specificity is the one always preferred for diagnosis of infectious diseases rather than its sensitivity. CCE could provide both cent per cent sensitivity and specificity in detecting specific antibodies against P. multocida in ducks, although it failed to detect equally high titre of antibody in the serum as detected by sonicated antigen.

5.8c Dot immunobinding assay

Dot immunobinding assay which is an alternative of ELISA

for detection of antibodies to soluble antigens has been known to possess high specificity and field application in terms of its convenience and easiness to perform. The plate ELISA which is a routine laboratory test of high sensitivity is reported to have its own disadvantages such as potential alteration of the antigen conformation upon binding to plastic, non specific adherence of some antibodies to plates and variable capacities of different antigens present in a complex mixture to bind microtitre plate wells. (Choi et al., 1990)

The disadvantages of plate ELISA were reported to be circumvented by dotting antigen to diazobenzloxymethyl membrane (Hawkes and Gordon 1982) or nitrocellulose membrane (Huet and Forma, 1982) as adsorption of antigens on to membrane is more uniform for longer time than on microtitre plates.

Choi et al. (1990) suggested that DIA could be of better utility over plate ELISA in detecting antibody against pasteurellosis of birds of unknown, clinical/vaccination status.

In the present study nitrocellulose membranes were coated with CCE, KSCN extract and sonicated antigen, separately for detection of antibodies from the vaccinated birds.

Irrespective of the antigen used, birds vaccinated with bacterin gave all positive reaction. Birds vaccinated with adjuvanated bacterin gave all the positive reactions except two negative when NCM was coated with CCE. Birds vaccinated with adjuvanated sonicated vaccine gave all positive reaction except three when NCM was coated with CCE.

The specificity and sensitivity of DIA ranged from 91 per cent to cent per cent. This observation indicated that irrespective of the antigen employed DIA is of higher value in terms of specificity and sensitivity in detecting specific antibodies against P. multocida in duck sera, in agreement to the observation of Choi et al. (1990).

In the present study, the sensitivity and specificity of IHA employing different antigen were compared with that of plate ELISA and DIA in detecting specific antibody against P. multocida in ducks. The results indicated that the sensitivity of plate ELISA, irrespective of the types of antigens used for coating of microtitre plates, was cent per cent followed by IHA with a sensitivity ranging between 93 per cent to 100 per cent.

On the other hand irrespective of the antigen used for adsorbing NCM, the specificity of DIA (91 to 100 per cent) was higher over IHA (76 to 100 per cent) and plate ELISA (79 to 100 per cent) see table 15.

Solano et al. (1983) observed that ELISA was twice as sensitive as IHA when sonicated antigen was employed.

The result of this study was comparable with Choi et al. (1990) wherein they reported significantly higher specificity of DIA than that of ELISA.

SUMMARY

6. SUMMARY

Outbreaks of duck pasteurellosis are frequently reported from various parts of Kerala. The acute nature of the disease makes medication impracticable. The only alternative is to prevent the disease by vaccination. This study was undertaken to monitor antibody from ducks vaccinated with three different type of vaccines (bacterin, bacterin with adjuvant and sonicate adjuvanated vaccine) by IHA, plate ELISA and DIA, employing three different antigens.

Antispecies serum was raised in rabbit using duck serum with a protein concentration of 82 mg per ml. Four injections were given at weekly interval. The production of antibody was checked by AGID, when found satisfactory rabbits were bled, serum was separated and stored at -20°C .

Duck globulin was separated from duck serum by 33 per cent saturation of ammonium sulphate. Purity of the globulin was checked by AGID and IEP against duck serum and antiduck serum. Such duck globulin with protein concentration of 14 mg per ml was used to raise hyper immune sera in rabbits. Production of antibody was checked by subjecting the sera to AGID and then the globulin fraction was precipitated by 33 per cent concentration of ammonium sulphate. The purity was checked by IEP.

This antiduck-rabbit gamma globulin with a protein concentration of five to seven mg per ml was used for labelling with HRPO by following two step conjugation which utilized glutraldehyde. Such conjugates were stored at -20° C , till used.

For coating the microtitre plates/NCM for EIA or sensitization of SRBC, different antigenic preparations of P. multocida viz., CCE, KSCN extract and sonicated antigens were prepared.

The protein concentration of the extract by Bradfrod method were 3.8, 3.76, and 3.85 mg per ml respectively. The carbohydrate estimate was 1250, 1100 and 1300 for CCE, KSCN extract and sonicated antigen respectively as estimated by phenol - sulphuric acid method.

Three different vaccines were prepared from P. multocida viz., bacterin, bacterin with adjuvant and sonicate adjuvanated bacterin. Bacterin was prepared by suspending 7.5 X 10^9 cells per ml in formol saline, adjuvanated bacterin by mixing one part of lanolin and nine parts of liquid paraffin in fifteen parts of bacterin. Sonicate adjuvanated vaccine was prepared by mixing fifteen parts of sonicated antigen, nine parts of liquid paraffin and one part of lanolin.

Forty ducklings of four weeks age were used for immunization. They were divided into four groups, each group comprising of ten ducklings. Group one, two and three were vaccinated with one ml of bacterin, bacterin with adjuvant and sonicate adjuvanated vaccine, respectively. Fourth group was injected with one ml of normal saline which served as control.

The antibody was monitored from the vaccinated ducks by IHA, plate ELISA and DIA, employing three different antigens, on zero 21st,28th and 35th day post vaccination.

Group one (birds vaccinated with bacterin) had the highest mean titre value followed by group three (birds vaccinated with sonicate adjuvanated vaccine) and group two (birds vaccinated with bacterin with adjuvant).

Of the three antigens sonicated antigens gave significantly higher mean titre value than the other two antigens. Though there were no significant differences between KSCN extract and CCE, the mean titre value was higher in the case of KSCN extract.

Irrespective of the antigens used for adsorbing NCM, the specificity of DIA (91 to cent per cent) was of higher rate over IHA (76 to cent per cent) and plate ELISA (79 to cent per cent).

The plate ELISA, irrespective of the types of antigens used for coating microtitre plates, gave the highest sensitivity (93 to cent per cent) followed by IHA and DIA.

Capsular extract when employed as antigen presented cent per cent specificity while the other two antigens gave a low percentage of specificity. The observations of this study suggest that capsular antigen is superior over KSCN extract and sonicated antigen in IHA, plate ELISA and DIA in the detection of specific antibodies against P. multocida in ducks.

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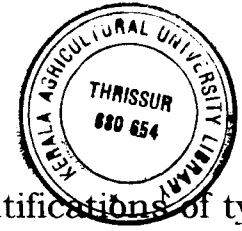

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COMPARATIVE EFFICACY OF DIFFERENT ANTIGENIC PREPARATIONS FROM *Pasteurella multocida* FOR DETECTION OF ANTIBODIES BY ENZYME IMMUNO ASSAY

By

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ABSTRACT OF A THESIS

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ABSTRACT

In this study IHA, plate ELISA and DIA were employed to monitor antibody from ducks vaccinated with three different types of vaccine (bacterin, bacterin with adjuvant and sonicate adjuvanated vaccine) prepared from P . multocida. Three different type of antigens viz., Crude capsular extract (CCE), Potassium thiocyanate extract (KSCN) and sonicated antigen were used to coat NCM/ microtitre plate or for sensitization of SRBC.

Forty ducklings of four week age were used for immunization. They were divided into four groups each group comprising of ten ducklings. Groups one, two and three were vaccinated with bacterin, bacterin with adjuvant and sonicate adjuvanated vaccine, respectively. Antibody was monitored upto 35th day post vaccination by IHA, Plate ELISA and DIA, employing CCE, KSCN and sonicated antigen.

Group one (vaccinated with bacterin) gave a higher mean titre value followed by IHA and plate ELISA, irrespective of the type of antigen employed, followed by group three (birds vaccinated with sonicate adjuvanated vaccine) and group two (birds vaccinated with bacterin with adjuvant). Irrespective of the antigens employed in the tests, plate ELISA gave the highest sensitivity (cent per cent) followed by IHA and DIA, whereas the highest specificity was observed by DIA, over IHA and plate ELISA.

When the comparison was made between antigens a high mean titre value was obtained with sonicated antigen, followed by KSCN extract and CCE. Crude capsular extract antigen gave cent per

cent specificity by all the tests, while the other two antigens gave a low percentage of specificity. As the immunological test with highest specificity is the one preferred, CCE was shown to be superior over KSCN extract and sonicated antigen in IHA, plate ELISA and DIA for the detection of specific antibodies against P. multocida in ducks.