COMPARATIVE EFFICACY OF DIFFERENT VACCINES AGAINST PASTEURELLOSIS IN DUCKS

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**COMPARATIVE EFFICACY OF DIFFERENT VACCINES AGAINST PASTEURELLOSIS IN DUCKS**" is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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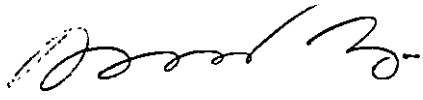
CERTIFICATE

Certified that the thesis entitled "**COMPARATIVE EFFICACY OF DIFFERENT VACCINES AGAINST PASTEURELLOSIS IN DUCKS**" is a record of research work done independently by **Sri. P.S. Jayakumar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.



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P.S. JAYAKUMAR

Dedicated To My

Beloved Parents

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

INTRODUCTION

Ducks occupy next place to chicken in production of table eggs. Duck population in India is 24 million and forms about 7 per cent of the total world population. Kerala with a costal length of 580 km has 8.5 lakhs of ducks which constitute about 3.5 percentage of the duck population in India.

Eventhough the duck production is confined to a limited area of the country it contributes substantially to the national income by providing employment to two to three lakhs of rural families.

Ducks are highly versatile in nature. They live happily under a wide range of climatic conditions and are not affected by many of the diseases seen in chicken. So any disease condition causing mortality and loss of production in ducks will cause a severe impact on the country's economy.

Presently the duck industry in Kerala is facing a major set back mainly due to the repeated outbreaks of two diseases viz., pasteurellosis and duck plague. Pasteurellosis due to *Pasteurella multocida* is one of the major bacterial diseases affecting ducks. It is a highly contagious septicaemic disease which occurs in acute or chronic form. Ducklings from four weeks of age are highly susceptible to the disease. As

the mortality rate is higher in productive and breeder stock, it causes heavy economic loss to the duck industry.

In Kerala, Pillai *et al.* (1993) reported an outbreak of Pasteurellosis in ducks for the first time in a flock which were vaccinated against duck plague. Since then in spite of the combined efforts by veterinary personnel and the farmers the duck mortality is continuing, either due to pasteurellosis or due to a combined infection by pasteurella and duck plague virus.

Eventhough a variety of drugs have been found effective in reducing the mortality their use is highly restrictive, expensive and not economically feasible. Continuous medication with prophylactic levels of antibiotic is costly and often ineffective when the flock is subjected to a severe challenge. For these reasons immunization against the disease by means of an effective vaccine will be of definite advantage.

Immunization against this disease dates back over 100 years to the experiments of Louis Pasteur, but even today the research continues to produce a more efficacious immunizing agent. Bacterins, cell fractions, cell extracts, live attenuated organisms and mutants were all had been on trial for immunization of ducks and each of them was reported with varying results. The lacuna in the success of immunization

trial was attributed to the presence of immunogenically distinct serotypes and their ability to cross infect divergent species. Cross protection between different serotypes of *P. multocida* even in the same host species is highly variable and protection is fully effective only when the autologous vaccine strain is used. Many different serotypes may exist simultaneously in an area and all may give rise to lethal outbreaks of Pasteurellosis.

Another problem being faced is the poor immunogenicity and short duration of immunity produced by the pasteurella antigens. Purified *P. multocida* capsule behaves as a hapten when injected in most animals and has not been extensively investigated as an immunogen.

Purified *P. multocida* lipopolysaccharide (LPS) is antigenic; however the extent of antibody response following immunization depends on animal species inoculated, LPS type used and route and method of inoculation (Rimler and Philips, 1986).

At present there is no vaccine available in the state of Kerala which can advocate protection of ducks against pasteurellosis. Since there are a number of antigenically different serotypes which do not confer any cross-immunity, the development of a vaccine incorporating the immunogen from local strains is of paramount importance.

Thus the present study was envisaged to assess and compare the immunogenicity of different vaccines prepared from local isolates of *P. multocida* by studying.

1. Survivability and maintenance of virulence of pasteurella isolates of duck origin.

2. Lethal dose 50 (LD_{50}) of the isolates in ducklings and ducks.

3. Immunization of ducklings.

4. Capacity to withstand challenge at various stages of immunization.

5 Assessment of antibody response by indirect haemagglutination test.

6. Duration of immunity.

7. Comparative efficacy of various immunogens.

Review of Literature

REVIEW OF LITERATURE

2.1 *Pasteurella multocida*: History and nomenclature

The generic name Pasteurella was proposed by Trevisan (1887) in recognition of Pasture's work on the causal agent of fowl cholera and his choice of the generic name for this organism was followed by several others (Lignieres, 1900; Bergey *et al.*, 1923; Topley and Wilson, 1929 and Rosenbusch and Merchant, 1939).

Since different isolates from divergent species had common characters, Lignieres (1900) proposed specific name for each organism according to the animal attacked, thus the name of the organism from fowls was *Pasteurella aviseptica*, from pigs, *Pasteurella suiseptica*, from cattle *Pasteurella boviseptica*, from sheep *Pasteurella oviseptica* and from rabbits *Pasteurella lepiseptica*.

As these organisms behaved as if they belonged to a single species, Topley and Wilson (1929) suggested that they should all be referred to by the name of *Pasteurella septica*.

Rosenbusch and Merchant (1939) named the typical haemorrhagic septicaemia organisms as *Pasteurella multocida* after comparing all the earlier names assigned to this bacteria and they proposed the grouping of an indole positive,

non-haemolytic haemorrhagic septicaemia organism in this species.

The name *P. multocida* was used in the sixth (Breed *et al.*, 1948), seventh (Breed *et al.*, 1957) and eighth (Buchnan and Gibbons, 1974) editions of Bergey's Manual of Determinative Bacteriology. In the above editions of Bergey's Manual, the genus Pasteurella was placed in the family Enterobacteriaceae as one of the genera of uncertain affiliations.

Hussaini (1975) published a review article on the taxonomy of *P. multocida.* In the nineth edition of Bergy's Manual of systematic Bacteriology genus Pasteurella has been placed in the family Pasteurellaceae among the different families under the section five (facultatively anaerobic gram negative rods) (Mannheim, 1984).

The family Pasteurellaceae included genera like Pasteurella, Haemophilus and Actinobacillus. The species included in the genus Pasteurella are *P. multocida*, *P. pneumotropica*, *P. haemolytica*, *P. ureae*, *P. aerogenes*, *P. gallinarum* and *P. anatipestifer*.

2.2 Isolation

Blood agar containing five per cent sheep/bovine blood can be used for isolation of Pasteurella (Carter, 1967). But blood agar is not satisfactory for identification of colonial variants. Carter (1967) found Brucella agar containing two per cent haemolysed rabbit serum satisfactory. Carter (1967) also used tryptic soy blood agar and tryptose blood agar containing five per cent sheep or bovine blood.

A solid medium called Yeast Proteose Cystine (YPC) was used to demonstrate colonial morphology by Namioka and Murata (1961).

Heddleston *et al.* (1964) reported that Dextrose starch agar can be used as a solid medium for the study of colonial morphology of *P. multocida*.

De Jong and Borst (1985) described a selective medium containing Tryptose soy agar with defibrinated sheep blood five per cent, gentamycin sulphate, potassium tellurite, amphotericin-B and bacitracin for the isolation of *P. multocida* and *Bordetella bronchiseptica*. This medium when compared with mouse inoculation and with modified Mac Conkey's medium was found to be more efficient in the isolation of toxigenic strains of *P. multocida* from nasal swab.

According to Smith and Phillips (1990) *P. multocida* grows at a temperature between 12 and 43°C with an optimal temperature of 37°C.

2.3 Identification

Dekruif (1921, 1922, 1923) and Webster and Burn (1926) demonstrated the existence of three colonial variants viz., (1) the smooth form which grows diffusely in broth and forms smooth and iridescent colonies on serum agar and is virulent for rabbits, (2) the rough form which gives a granular deposit in broth and form translucent bluish colonies on serum agar is completely avirulent for rabbits and (3) the mucoid form which is of intermediate virulence.

Cowan (1974) reported that the primary biochemical tests for the identification of *P. multocida* were the tests for catalase, oxidase, production of acid from glucose and oxidative/fermentative utilization of glucose.

The second stage table for the identification of *P. multocida* as described by Cowan (1974) are the growth on Mac Conkey's agar, growth in potassium cyanide; acid production from carohydrates such as arabinose, lactose, maltose, mannitol, raffinose, salicin, sorbital, sucrose, trehalose and xylose; beta galactosidase activity, ornithine

decarboxylase activity, nitrate reduction, nitrite reduction, production of indole and hydrogen sulphide.

According to Mannheim (1984) in the first edition of Bergey's Manual of Systematic Bacteriology Volume 4, the first stage table for the identification of *P. multocida* includes test for beta haemolysis, growth on Mac Conkey's Agar, indole production, urease activity, gas production from carbohydrate and acid production from lactose and mannitol.

P. multocida shows bipolar staining when taken from animal tissues or smooth colonies and tend to become bacillary in nature when taken from rough colonies (Smith and Phillips, 1990).

Smith and Phillips (1990) reported that all strains of *P. multocida* produced acid, but no gas in glucose and sucrose. Most strains fermented galactose, mannitol, mannose, sorbitol and xylose. Strains of *P. multocida* were without action on litmus milk and gelatin. They produced indole, reduced nitrate and formed a small quantity of hydrogen sulphide as detected by lead acetate paper. The MR and VP reactions were both negative. The catalase and oxidase reactions were both positive though rather weakly so. Methylene blue was reduced by *P. multocida*. This did not utilize citrate as the sole source of carbon. Urea was not decomposed by it. Most strains were positive for ornithine decarboxylase, but

negative for lysine, glutamic acid decarboxylase and arginine dihydrolase and were resistant to potassium cyanide.

2.4 Pathogenicity

Pasteurella multocida is found in a wide variety of animals and birds and has its main habitat in the respiratory tract (Smith, 1955). Strains of *P. multocida* from different sources vary in their virulence for experimental animals as well as natural hosts (Carter, 1967).

Smith (1958) observed that canine strains of *P. multocida* possessed low virulence for mice while cat strains possessed somewhat higher virulence which indicated that the strains of *P. multocida* differed considerably in their pathogenic potential to mouse, depending on the species of origin.

Muratta *et al.* (1964) tested the pathogenicity of serotypes 1:A and 5:A for chicken and mice and reported that serotype 1:A was not pathogenic for chicken while serotype 5:A was and that both serotypes were pathogenic for mice.

Sharma *et al.* (1974) reported that most prominent clinical symptoms in experimental avian pasteurellosis were hyperthermia, dullness, in co-ordination of movements, greenish yellow diarrhoea, laboured and painful breathing and unusual sitting postures. Chronic phase of the disease was

manifested by progressive emaciation, lameness, persistant diarrhoea and swollen joints.

Collins (1976) employed mice as the choice of animal for testing pathogenicity of *P. multocida*, and reported that an overwhelming increase in the number of *P. multocida* in visceral organs was the cause of death of mice when experimentally inoculated.

Krecov (1976) found that as few as 10 organisms can produce rapid death when given i/v and i/m, but infection was more difficult by other routes as five million organisms were needed to infect via stomach.

The pathogenicity of twenty strains of *P. multocida* isolated from rabbits were tested in mice by Okerman *et al.* (1979). Marked differences were observed in the subcutaneous LD_{50} and virulence for mouse depending on the type of colonies.

Hunter and Wobeser (1980) reported that Mallard ducks (Anas platyrhynchos) when challenged with two isolates of *P. multocida* developed either acute or chronic lesions, depending on the isolate of *P. multocida* and the dose of inoculum. Ducks that died of acute infection had lesions of haemorrhagic septicaemia with wide spread vascular damage and focal necrosis in liver, spleen and other organs. Ducks

surviving challenge developed chronic lesions in a variety of organs including brain, lung, air sacs, joints and eyes.

Mushin and Schoenbaum (1980) observed that strains of Heddleston's serotype 3:A were lethal for mice within 24 h while untypable strain differed in virulence.

Kawamoto *et al.* (1990) tested the pathogenicity of 22 isolates of *P. multocida* from rabbits in six week old female mice by inoculating 0.2 ml (10^8 organism) of 18 h nutrient broth culture. Out of 22 isolates eight isolates killed mice in two to seven days and the rest failed to do so indicating that many of the isolates were normally virulent for mice. The bacteria were recovered from liver and spleen of dead mice.

Pillai, *et al.* (1993) studied the pathogenicity of *P. anatipestifer* isolate from ducks in different experimental birds and animals viz., chicken, ducks, quail, mice, guinea pig and rabbit by different routes - oral, nasal, occular subcutaneous, intramuscular and intraperitoneal. All the experimental birds and animals except guinea pig succumbed to infection. They found that non parenteral routes of administration took more time for establishment of infection and death. They could also reisolate the organism from all the experimental animals and birds.

2.5 Antigens of *Pasteurella multocida*

2.5.1 Capsular antigens

Carter (1958) distinguished *Pasteurella multocida* strains from other varieties by the presence of hyaluronic acid in their capsular substance.

Penn and Naggy (1974) studied the capsular antigen obtained by saline and phenol water extraction from *P. multocida* and reported that it was type specific to provide immunity.

Kodama *et al.* (1981) isolated capsular antigens from *P. multocida* and their immunogenicity was tested in turkeys. Crude capsular antigens (CCA) provided eighty per cent to 100 per cent protection and purified polysaccharide antigen (PPA) failed to provide protection.

Encapsulated avian strain of *P. multocida* was shown to be resistant to bactericidal action of turkey serum, whereas non capsulated variants were not (Hansen and Hirsh, 1989). Removal of the capsule has resulted in the loss of resistance to the bactericidal effect of serum.

2.5.2 Lipopolysaccharides (LPS)

Heddleston *et al.* (1966) obtained particulate antigens having endotoxin properties from avirulent cells of two immunologically distinct strains of *P. multocida*, which when injected either intravenously in a saline suspension or subcutaneously as mineral oil emulsion provided 100 per cent protection in chicken.

A heat stable particulate LPS protein antigenic complex has been isolated from a virulent encapsulated strain of *P. multocida* by Rebers *et al.* (1967). Two injections of 15 μg of the antigen produced a high degree of immunity in mice without the development of any signs of toxicity.

Effects of *P. multocida* endotoxins on turkey poults indicated that LPS preparations from highly pathogenic strains and free endotoxin from one of these strains were all similar in lethal effect (Rhodes and Rimler, 1987).

2.6 Virulence and Experimental infection

Virulence of *P. multocida* can be determined by using mice as experimental animal (Roberts, 1947, Carter and Bigland, 1953, Collins, 1973 and Okerman *et al.*, 1979).

Yaw *et al.* (1956) reported that the colonial variants of three serotypes of *P. multocida* differed in their virulence to chicken and mice depending on the type of encapsulation.

Carter (1967) was of opinion that encapsulated strains recovered from acute or moderately acute natural infections were generally virulent.

Okerman *et al.* (1979) estimated the LD_{50} of 20 strains of *P. multocida* by infecting mice intraperitoneally with varying dose from 1×10^6 to 3×10^6 bacteria and observed the animals for 7 days. The results obtained showed marked difference in LD_{50} for different strains and the estimated LD_{50} ranged between 3×10^3 and 1×10^6 bacteria.

Mukkur (1979) determined the LD_{50} of *P. multocida* of bovine origin in mice as $5.0 \pm 2.1 \times 10^4$ cfu by intranasal inoculation and 5.0 ± 2.8 cfu by intraperitoneal inoculation.

By passaging the organism in mice at four weeks interval the virulence of fully encapsulated form of *P. multocida* of bovine origin could be maintained (Mukkur, 1979; Mukkur and Pyliotis, 1981).

Snipes and Hirsh (1986) observed the complement system as an important defensive mechanism against *P. multocida* in

turkeys and complement resistance as a virulence factor in avian strain of *P. multocida*.

In a virulence and toxigenicity study, Rhodes and Rimler (1989) demonstrated virulence of some capsular strains which caused 73 per cent mortality in poultry that received 900 organisms via intra air sac inoculation, indicating that the capsular strain should be considered potential cause of the fowl cholera.

Ramanatha *et al.* (1995) studied the virulence of *P. multocida* serotype A:1 isolate from ducks in mice. The isolate was found to be highly virulent for mice. The mouse LD_{50} in three replicate finals were 0.2 ml of $10^{-7.72}$, $10^{-8.63}$ and $10^{-9.58}$ dilutions of 18 h broth culture which contained 14.48, 14.28 and 14.16 colony forming units respectively with a mean value 14.32 + 0.0833.

2.7 Immunology

2.7.1 Killed whole cell vaccines (Bacterins)

Immunization against Pasteurellosis dates back over 100 years to the experiments of Louis Pasteur. Since then several other vaccines have been used to immunize animals and birds against this disease.

Mack and Records (1916) successfully used bacterins to produce resistance against fowl cholera and for them there was no apparent difference in the results whether homologous or heterologous strains of *P. multocida* were used in the preparation of bacterin.

Immunization of cattle against haemorrhagic septicaemia caused by *Pasteurella multocida* was attempted by several workers employing several immunizing agents such as attenuated organisms in glycerine broth (Hardenberg, 1916) aggressin obtained by candle filtered germ free inflammatory exudate (Gochenour, 1924) and 5 per cent saponin vaccine (Delpy, 1938).

Carter (1950) compared the immunity produced in mice by broth bacterins and chicken-embryo vaccine made from type 1 *P. multocida* and showed that chicken embryo vaccine was superior over the broth bacterins.

Three different immunogens such as formalinised broth vaccine, formalinised agar wash vaccine and heat treated agar wash vaccine were tried for vaccinating the buffalo calves and rabbits (Rau and Govil, 1950). Their results showed that agar wash vaccines when given in two doses with an interval of one month produced a higher grade immunity when compared to broth vaccine. It was also observed that the higher the

concentration of agar wash vaccine the stronger the immunity it conferred.

Bolin *et al.* (1952) prepared bacterin of *P. multocida* by pooling several isolates from various sources. These pooled bacteria were maintained in broth at 37°C until all organisms were dead and formalin was added to this bacterin as a preservative. The bacterin was shown to be highly immunogenic but the immunity was of short duration.

The advantage of incorporation of an oil adjuvant in preparation of an emulsified haemorrhagic septicaemia vaccine was reported by Bain and Jones (1955). Haemorrhagic septicaemia vaccine prepared with the addition of mineral oil and lanolin was shown to be highly protective in vaccinated calves when challenged with virulent strain of *P. multocida* (Iyer *et al.*, 1955).

Dhanda *et al.* (1956) reported the efficacy of oil adjuvant vaccine against haemorrhagic septicaemia and extensive laboratory and field trials were undertaken to assess its prophylactic value.

Bain and his associates were instrumental for developing a killed bacterial oil adjuvant vaccine which was able to confer solid immunity in cattle and buffaloe upto 12 months (Bain and Jones, 1958; Knox and Bain, 1960).

Heddleston and Hall (1958) compared the immunizing efficacy of oil adjuvant aqueous suspension, alum precipitated vaccine and chicken embryo vaccine and they were of the opinion that adjuvant vaccine was superior to others.

Heddleston and Reisinger (1959) reported that there was no significant difference in the ability of vaccines of different concentration to establish a high degree of immunity in chicken for a period of one year. They also suggested that when selecting strains to be incorporated in fowl cholera vaccines only those organisms isolated from acute fowl cholera and which will produce acute disease with at least 80 per cent mortality should be considered. The lowest concentration of a suspension of *P. multocida* that produced immunity when incorporated in an emulsified vaccine was shown to be 10 x McFarland I, when injected subcutaneously in the middle third of the neck. They further reported that a killed fowl cholera vaccine stimulated and maintained a high degree of immunity for one year in chicken under experimental condition.

Heddleston (1962) reported that two strains of *P. multocida* isolated from birds with fowl cholera were of different immunological types. Vaccine prepared with one type did not stimulate immunity against other. A bivalent emulsified killed vaccine of these two types stimulated and maintained a high level of immunity for 37 weeks against both

types. Serologically these two strains were of serotype 1 and serotype 2. However a bivalent aluminium hydroxide adsorbed vaccine did not maintain a high level of immunity.

Modified yeast extract agar was tried by Nangia *et al.* (1966) for growing *P. multocida* to be used in the preparation of oil adjuvant vaccine and was found to be economical in large scale production of vaccine. They have also tried the effect of temperature on the keeping quality of this vaccine and reported that the vaccine maintained its potency at 45°F for 814 days and at 37 to 42°C for 20 days. Further they employed rabbits for assaying the potency of the vaccine instead of bulls or bull calves and the duration of immunity reported as more than 840 days.

Bhasin and Biberstein (1968) compared the efficacy of two formalin killed and two heat inactivated bacterins belonging to different serotypes in turkeys. All four bacterins conferred significant immunity in turkeys to experimental fowl cholera. Inactivated bacterin containing *P. multocida* type A strain was superior over formalinised bacterins. They have also reported that no difference could be noted in the immunizing efficacies between oil adjuvant and aluminium hydroxide bacterins.

Cell fractions of *P. multocida* (P 1059) such as culture filtrate, cell wall cytoplasm and the combination of these

fractions prepared in three vehicles such as saline, alum (0.5 per cent) and Freund's complete adjuvant (50 per cent) were used as vaccines against fowl cholera in turkeys (Brown *et al.*, 1970). The combination of fractions appeared to be most promising over the protective effect of the commercial bacterins.

Mall and Nilakantan (1971) studied immunogenic efficacy of various *P. multocida* vaccines; (1) formalinized vaccine, (2) alum precipitated vaccine, (3) acetone dried cell vaccine, (4) acetone dried cell oil adjuvant vaccine, (5) oil adjuvant vaccine, (6) potassium thiocyanate extract oil adjuvant vaccine and sodium chloride extracted oil adjuvant. Of these oil adjuvant vaccine and potassium thiocyanate extract incorporated in oil adjuvants were found to be satisfactory.

Fowl cholera bacterins prepared as triturate of liver and blood from a turkey that died of acute fowl cholera induced immunity in turkeys against homologous and hetrologous serotype of *P. multocida*, but not by the bacterins prepared from bacteria grown on laboratory media (Heddleston and Rebers, 1972).

Heddleston and Rebers (1972) found that fowl cholera bacterins prepared from infected embryonating turkey eggs when inoculated by oral/intramuscular route induced immunity in

turkey against infection with homologous or heterologous serotypes of *P. multocida*.

Penn and Nagy (1976) observed that a saline extract of type B capsular antigen with aluminium hydroxide gel adjuvant was poorly immunogenic in rabbit, but the same vaccine elicited a dose dependent serological response in cattle, as evidenced by the passive mouse protection test.

Mittal *et al.* (1977) prepared a multiple emulsion vaccine by secondary emulsification of the oil adjuvant vaccine with Tween-80 and noted that both the oil adjuvant vaccine and multiple emulsion vaccine were equally immunogenic as assessed by direct challenge test and passive mouse protection test.

Potassium thiocyanate extract of *P. multocida* was used as immunogen by Gaunt *et al.* (1977) and showed that vaccinated chicken resisted infection on challenge with homologous as well as heterologous strains. Further they reported that KSCN extract obtained from *P. multocida* serotype 1 and 3 of avian origin presented antigenically identical components by gel diffusion tests.

Ribosomal fraction from *P. multocida* was shown to have intense protective antigenicity in mice and chicken when compared to lipopolysaccharide (endotoxin) and other bacterial cell fraction (Baba, 1977). Further he demonstrated that the

ribonuclease treatment resulted in 60 per cent loss of immunological activity of the ribosomal preparation.

Liow (1977) prepared a formalin killed emulsified vaccine and a formalin killed aqueous suspended vaccine from *P. multocida* serotype 1 and compared the efficacy in crossbred ducks. It was observed that a single dose of formalin killed aqueous suspended vaccine (FV) protected 67 and 39 per cent of the vaccinated ducks challenged at one week and four weeks post vaccination respectively. Two doses of FV given 6 weeks apart protected 92 per cent of the ducks against challenge. One dose of emulsified adjuvant vaccine (FAV) protected 92 per cent, 92 per cent and 83 per cent of ducks challenged at 1, 4 and 8 weeks post vaccination respectively. Ducks which survived homologous challenge were also found to be protected against rechallenge with heterologous *P. multocida* serotypes.

Vaccines prepared from broth cultures of *P. multocida* were studied for their immunogenicity against pasterellosis in turkeys (Matsumoto, 1977). The vaccine preparations differed in the culture medium used for growing the organism, method of inactivation and adjuvants added. Bacterin prepared with organism grown in BHI broth concentrated ten fold and inactivated with formalin was reported to be superior over the commercial broth vaccine in providing protection against

pasteurellosis when turkeys of 8-12 weeks age were vaccinated by the subcutaneous route.

A trivalent formalin inactivated bacterin containing serotypes 1, 2 and 5 of *Pasteurella anatipestifer* was developed by Sandhu (1979) and tested in ducks. A single dose of vaccine could not afford protection against heterologous challenge, whereas two doses of bacterin given at 10th and 17th days of age protected birds against challenge for two weeks post inoculation. A third dose given at 31 days of exposure to an active field infection during the period of protection extended protection period to market age (seven weeks). Aluminium hydroxide gel adsorbed bacterin gave no better result than bacterin without adjuvant. He had further observed that a single dose of multiple oil-emulsion bacterin gave absolute protection in birds upto market age, except for noticeable tissue reactions produced at the site of injection.

Rimler *et al.* (1979) reported that bacterin prepared as crude liver homogenate from turkeys infected with *Pasteurella multocida* serotype 1 or 3 induced cross protection. Further he reported that pasteurella harvested from blood of infected turkeys by a centrifugal technique was as immunogenic as liver homogenate.

According to Harry and Deb (1979), formalised vaccine was the most suitable over the other methods of inactivation

employed in the preparation of *Pasteurella anatipestifer* vaccines. They prescribed the dose of the vaccine as $3x10^9$ cells when administered intramuscularly.

Comparative efficacy of the oil-adjuvant and multi-emulsion oil-adjuvant vaccine against haemorrhagic septicaemia in cattle was assayed in calves, rabbits and mice. Both vaccines were shown to be safe and immunogenic as assessed by passive mouse protection test and rabbit challenge test (Mittal *et al.*, 1979; Yadav and Ahooja, 1983).

Rimler and Rhoades (1981) reported that treatment of freez-thawed suspension of *P. multocida* with DNA**se**, *hyaluronidase* lysozyme, EDTA and Triton-x 100 did not influence their ability to induce protection against homologous or heterologous serotype challenges. It was also noted that vaccines prepared from different serotypes of turkey grown *P. multocida* protected chicken and mice against homologous and heterologous serotype challenge exposures.

Baljer *et al.* (1982) compared the efficacy of vaccine prepared from different strains of *P. multocida* by formalin treatment and heat inactivation. The heat inactivated vaccine was also tried by different routes such as oral, intranasal and subcutaneous. The results obtained by them showed that the efficacy of vaccines were independent of the inactivation procedure to produce homologous protection.

Dense growth of *P. multocida* isolates were obtained in tryptic soy broth and modified tryptose broth (MTB), when the media were continuously shaken or aerated (Layton, 1984). Vaccines prepared by *P. multocida* grown in MTB when injected subcutaneously into ducklings at 2 and 3 weeks of age protected the ducklings against homologous challenge upto 6 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 8 weeks.

Sandhu and Layton (1985) tested a combined bacterin containing *E. coli* serotype 078 and *Pasteurella anatipestifer* as vaccine in White Pekin ducks in the laboratory and in the field. Vaccination done at 2nd and 3rd weeks of age provided significant protection against challenge with *E. coli* 078 and *P. anatipestifer* serotypes 1, 2 and 5. In field trials the combined vaccine produced significant reduction of mortality in commercial White Pekin ducks.

The immunogenicity of antigens of *P. multocida* prepared by various inactivation methods using heat, formalin, phenol, sodium azide and merthiolate was compared by Kim *et al.* (1986). It was reported that formalin treated antigen was most immunogenic in mice. These antigens were also shown to confer a low degree of cross protection against heterologous challenge.

Floren *et al.* (1988) reported that vaccination of 10 day old Pekin ducklings againt *P. anatipestifer* infection under laboratory conditions with a formol inactivated homologous monovalent oil emulsion vaccine induced solid immunity (95-100 per cent). An interval of 10 days between vaccination and challenge was required for protection. Vaccination of day old ducklings produced only 40 per cent protection.

Singh and Teo (1988) reported that a dense growth of *P. multocida* serovar A:1 could be obtained in aerated tryptose broth wherein the cell density exceeded McFarland tube No.10 and the viable bacterial count was more than 10^{10} colony forming units/ml. Broth vaccine prepared from the above culture when subcutaneously administered in one ml dose to seven days and 17 days old ducklings the immunity lasted upto 56 days.

Gao and Guo (1988) tested a formalin inactivated bacterin and a supersonic-treated formalin inactivated bacterin against *P. anatipestifer* in the laboratory. Two inoculations of the bacterin in White Pekin ducks at 1 and 2 or 2 and 3 weeks of age gave highly significant protection (90-100 per cent) against challenge with virulent organism at 3 or 4 weeks of age and both vaccines were found to be equally effective.

Inactivated *P. anatipestifer* vaccine which contained 10^9 cfu/0.5 ml along with aluminium hydroxide adjuvant (25 per cent final concentration) was tried on White Pekin ducklings

by Tim and Marshall (1989). A single subcutaneous inoculation (0.5 ml) of the above vaccine at 14 days provided protection against challenge exposure upto five weeks of age.

Giridhar *et al.* (1990) tested the efficacy of subcutaneous vaccinations in calves with different doses (3, 5 and 10 ml) of alum precipitated *P. multocida* vaccine by passive haemagglutination and complement fixation test. They observed that all the three vaccine could confer satisfactory immunity. CFT using killed whole cell antigen was better than PHA using capsular antigen for demonstration of antibody titre.

Azam *et al.* (1991) compared the immunogenicity of sonicated *P. multocida* vaccine with or without adjuvants and formalinized bacterin in rabbits by estimating the antibody using indirect haemagglutination test. The range of antibody titres observed in sonicated antigen with or without adjuvant was 8-64 while for formalin killed bacteria it was 4-16. Rabbits inoculated with the above vaccine uniformly showed a dose-dependent immune response.

The duration of immunity conferred by an alum precipitated haemorrhagic septicaemia vaccine in cattle was assessed by Venkatesh *et al.* (1991). A dose of 10 ml vaccine was injected subcutaneously, followed by one or more booster doses. Antibody titre was assessed by complement fixation

test and passive mouse protection test. Satisfactory immunity with persistant high antibody titre was found in the animals which received one or more booster injections.

Glisson *et al.* (1993) found that Strain x-73 (Serotype 1) and P-1059 (Serotype 3) of *P. multocida* of avian origin expressed additional membrane proteins, when grown in brain heart infusion broth containing iron chelator dipyridyl or when grown in BHI treated with iron chelator chelax 100. An inactivated oil emulsion vaccine prepared using bacterial cells expressing these membrane proteins was used to vaccinate chicken and turkeys. Birds were challenged two weeks after vaccination to determine whether the bacterin would induce heterologus immunity. It was found that the bacterin produced from culture medium which was low in iron content did not induce significant protection against heterologous challenge.

Three strains of *P. multocida* P-52 (7:B), standard strain used in conventional vaccines D-7 (1:A) a duck strain and NPg-6 a pig strain were used for preparation of three formalised vaccines using aluminium hydroxide gel as adjuvant by Singh *et al.* (1994). Three groups each of five buffalo calves were vaccinated subcutaneously with each vaccine containing 4 mg of bacterial protein per dose. When challenged 180 days after vaccination with P-52 strain, calves which were vaccinated with duck strain showed least protection (25 per

cent) P-52 moderate protection (75 per cent) and the pig strain showed 100 per cent protection which indicated the presence of some common antigens against the different serotypes of *P. multocida*.

Onet *et al.* (1994) vaccinated turkeys with different types of anti *P. multocida* vaccines (20 per cent aluminium hydroxide adjuvanated, 20 per cent calcium phosphate adjuvanated, 20 per cent oil adjuvanated and a food additive inactivated serotype-3 vaccine) and found that best protection was conferred by the inactivated 20 per cent oil adjuvanated vaccine. There was *no* strict correlation between level of agglutinating antibodies and the protection conferred by different vaccines.

Ramanatha (1994) studied the efficacy of formalin inactivated alum adsorbed broth bacterin of *P. multocida* A:1 from ducks in field and laboratory by immunizing four week old ducklings on 0 and 24th day. The humoral antibody levels were assessed by IHA test.

2.7.2 Live vaccines of *P. multocida*

2.7.2.1 Live attenuated vaccines

Wei and Carter (1978) mutagenised a wild strain of *P. multocida* into an avirulent streptomycin dependent strain

by treating the organisms with N-methyl-N-nitro-N-nitrosoguanidine. When this mutant strain was used as vaccine in mice and rabbits they were protected against homologous challenge with the wild strains.

A streptomycin dependent live *P. multocida* type-3 vaccine was used for prevention of fowl cholera in turkey poults (Chengappa *et al*, 1979). He vaccinated the birds giving two doses of vaccines three weeks apart by oral, intermuscular or subcutaneous route. The birds were shown to be protected at 6th week of age when challenged with virulent organism.

Hertman *et al*. (1979) developed a live fowl cholera vaccine from a virulent avian septicaemia strain by treating with N-methyl-N-nitro-N-nitroso guanidine. Mutants were selected that had either small colonies at 37°C or were sensitive to 41°C. All the mutants were virulent to turkeys. These mutant strains were used to vaccinate turkeys and it was found that the small colony mutant strains protected birds against both homologous and heterologous challenge.

The use of streptomycin dependent vaccine strain of *P. multocida* as vaccine was studied by Lu and Pakes (1981) and reported that vaccination protected rabbits against homologous challenge. They further observed that the vaccine prevented colonisation of the virulent challenge organism in lungs,

liver spleen, genital tract and blood but not in the nasal cavities.

Attenuation of *P. multocida* vaccine strain was attempted by treating with 2, 8 (3,6) diamino-10-methyl acridinium chloride and/or 2,8 (3,6) diaminoacridine (Kucera, 1981). They claimed that the chemically altered strain afforded protection in mice, calves, swine and sheep against challenge exposure with virulent *P. multocida* organism.

A virulent attenuated *P. multocida* serotype 6:B was tried as a vaccine in mice and pigs by Chen *et al.* (1984) and it was shown to induce a high level of immunity against virulent strain of homologous serotype but only low or no immunity against strains of heterologous serotypes. A similar work has been reported by Derieux (1984) wherein a virulent *P. multocida* gave protection in chicken upto 80 weeks.

Kadel *et al.* (1985) conducted a field trial evaluation of streptomycin dependent *P. multocida* and *P. haemolytica* vaccine for prevention of bovine pneumonia in calves. Vaccinal efficacy was defined in terms of great body weight gain, less severe clinical signs of pneumonia and lower death rates as compared with the same factors in non vaccinated calves. They have also observed that there was an economic advantage in administering a booster dose of the vaccine.

Percy *et al.* (1985) reported that rabbits immunized with streptomycin dependent mutants of *P. multocida* serotype 12:A were protected against homologous and heterologous (3:A) challenges. The vaccinated rabbits challenged with homologous strain showed a more rapid nasal clearance of the organism than the vaccinated group challenged in the heterologous strain.

The safety and efficacy of a freeze dried attenuated vaccine prepared from avian strain of *P. multocida* in rabbits was studied by Long *et al.* (1986). The vaccine was found to be effective in adult rabbits when subcutaneously injected with 10 to 40 hundred million bacteria, while the young rabbits were protected at comparatively lower dose, 5 to 20 hundred million bacteria.

Michael *et al.* (1986) reported that three aerosol application of M-3-G-mutant strain of *P. multocida* vaccine in turkeys elicited solid immunity in laboratory trials. In another trial one aerosol vaccination with M-3-G and one injection of an inactivated whole culture of *P. multocida* also conferred good immunity. When an oil-based emulsion bacteria was used after one aerosol application of M-3-G, immunity lasted for 11 weeks. It was also found to be effective under field trials.

Digiacomo *et al.* (1987) evaluated the safety and efficacy of a streptomycin dependent live *P. multocida* (12:A) vaccine

in rabbits. This vaccine strain could colonise in rabbit nares and was genetically stable in vivo. The vaccinated rabbits could withstand the challenge with a homologous pasteurella serotype and not against heterologous serotype.

The efficacy of a lyophilized live streptomycin dependent *P. multocida* vaccine against experimentally induced pneumonia was assessed by Chengappa *et al.* (1989) and reported that the control calves had significantly higher clinical scores and more severe gross lesions than the vaccinated calves although the vaccinated calves did not show a significant increase in the immunoglobulin M and G titres.

2.7.2.2 *Mutant avirulent strain*

Bierer and Derieux (1972) had studied the effect of live avirulent *P. multocida* strain P1059 in birds which were already vaccinated with oil base bacterin. When live avirulent bacteria were administered in drinking water at four weeks post vaccination the birds were protected upto 30 weeks.

Heddleston *et al.* (1975) showed that live fowl cholera vaccine administered in drinking water could protect the young turkeys against heterologous challenge. He has also demonstrated that serum from the vaccinated birds induced passive cross immunity in vaccinated chicks and turkeys.

Rice (1978) studied the immunogenic response of broiler chicks of 9 to 10 weeks of age against the Clemson University strain of *P. multocida*, when administered by the oral (drinking water) palatine cleft, ocular or subcutaneous route. All birds were challenged at 11 or 12 weeks of age with virulent X-73 strain of *P. multocida* by the palatine cleft method. Efficacy of the various vaccination routes differed among three experiments but the subcutaneous route was found to be better than the other two routes and conferred protection in 95-97 per cent of birds challenged.

Derieux (1984) inoculated broiler chicken once or twice with avirulent *P. multocida* by stick wing method. When these birds were challenged with pathogenic *P. multocida* after 20, 50 or 80 weeks post exposure they were found to be protected.

Sandhu (1991) studied the immonogenecity and safety of a live trivalent *P. anatipestifer* vaccine in white Pekin ducklings. Vaccination of one-day old ducklings by oral or aerosol administration provided protection for 42 days of age. They further reported that there was no untoward signs or mortality in one day old ducklings even when ten times the recommended dose was administered.

Materials and Methods

MATERIALS AND METHODS

3.1 Materials

3.1.1 Specimens for isolation of pasteurella organism

Bio-samples for isolation of Pasteurella were collected from ailing/dead ducks brought from Kuttanad area to the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy for Microbiological Investigation.

3.1.1.1 Sheep blood

The source of sheep blood was from the animals maintained in the University Goat Farm, Mannuthy as well as from sheep brought to the Municipal Slaughter House, Kuriachira.

3.1.1.2 Sheep blood for blood agar

The sheep blood for preparing blood agar was collected in a sterile round bottom flask containing glass beads following sterile precautions and the defibrinated blood was stored in 10 ml aliquotes at 4°C until used.

3.1.1.3 Sheep blood for indirect haemagglutination test

Sheep blood required for doing indirect haemagglutination test was collected in Alsever's solution following sterile

procedures in the proportion of one part of sheep blood and five parts of Alsever's solution and stored at 4°C until used.

3.1.1.4 Blood agar

Base medium for the preparation of sheep blood agar was dehydrated Trypticase Soy Agar (TSA) - (Hi-Media). It was prepared as per the manufacturer's instructions and then sterile defibrinated whole sheep blood was added at five per cent level to make it blood agar.

3.1.1.5 Nutrient broth (Hi-Media)

Prepared as per manufacturer's instructions.

3.1.2 Chemicals

Formaldehyde
Liquid paraffin and
Lanolin

3.1.3 Phosphate buffered saline (PBS) (pH 7.2, 0.15 M)

Composition

Sodium chloride	- 8.00 g
Potassium chloride	- 0.20 g
$Na_2 HPO_4$	- 1.15 g

$KH_2 PO_4$	- 0.20 g
Distilled water	- 1000 ml

3.1.4 Alsever's solution

Sodium chloride	- 4.2 g
Trisodium citrate	- 8.0 g
Citric acid	- 0.55 g
Glucose	- 20.5 g
Distilled water	- 1000 ml

Each ingredient in the above order was added to 500 ml of distilled water in a graduated cylinder and stirred until the chemicals dissolved completely. The volume was then made up to one litre with distilled water and finally steamed for 10 min. The solution was freshly prepared before use.

3.1.5 Laboratory animals and birds

3.1.5.1 Mice

Swiss albino mice six to eight weeks of age were procured from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy.

3.1.5.2 Ducks

Day old unsexed ducklings were purchased and reared under deep litter system. All the birds were fed with concentrate duck feed purchased from Meat Products of India, Koothattukulam. Feed and water were given *ad lib*. After the brooding stage (three weeks) the ducklings were grouped into different groups and reared in separate pens. All the ducklings were dewormed one week prior to the start of immunization trials. Ducklings were maintained upto an age of six months.

3.1.6 Bacterial cultures

Two isolates (DP1 and DP5) of *P. multocida* were employed in this study. The isolate DP1 was a culture maintained in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy, which was isolated, identified and maintained in the Department since 1993.

The isolate DP5 was the one isolated and identified during the period of this study.

3.1.7 Inoculum for LD_{50} determination

Pasteurella multocida organisms grown on TSA plates for 18 h were harvested and washed twice with PBS (pH 7.2). The

cells were then suspended in the same buffer to contain $3x10^8$ bacteria/ml (orginal suspension).

3.1.8 Inoculum for challenge

Pasteurella multocida isolates were passaged through mice to get a fully encapsulated virulent form. The virulent organisms isolated from mice were grown on blood agar plates at 37°C for 24 h. The growth on the blood agar plates were harvested and washed three times with PBS (pH 7.2). Packed bacterial cells were resuspended in PBS to contain $3x10^8$ bacteria/ml. Then serial ten fold dilutions were made to get bacterial suspension containing 100 LD 50 per 0.1 ml. The inoculum were separately prepared for DP1 and DP5 to be used as challenge dose in ducklings and also in adult ducks, based on LD50 calculated in each case.

3.1.9 Vaccines

Different vaccines were prepared from *Pasteurella multocida* isolates DP1 and DP5 separately. Organisms were passaged through mice to obtain fully encapsulated virulent form before the use of the organism for preparation of vaccines.

3.1.9.1 Bacterin

The pasteurella organisms grown on nutrient broth at 37°C for 18 h were seeded on to blood agar plates in 0.1 ml quantities. The bacterial colonies grown on blood agar for 24 h at 37°C were checked for its purity and the cultures without any contamination were harvested and washed three times with normal saline by centrifugation. The supernatant was discarded and 0.3 per cent formal saline was added to the packed bacteria and resuspended to get a population density of $3x10^9$ cells/ml. The vaccine thus prepared was kept at room temperature for 24 h for the formalin to act.

3.1.9.1.1 Sterility test

A quantity of 0.2 ml each of the three vaccines were separately inoculated on to blood agar plates as well as onto TSA plates and they were separately incubated in increased CO_2 tension and normal atmosphere at 37°C. The plates were observed for seven days for any growth or contamination.

3.1.9.1.2 Toxicity test

Six albino white mice were injected intraperitoneally with 0.5 ml each of the vaccine and four ducklings were inoculated subcutaneously with 1 ml each of the vaccine and

were observed for seven days for the development of any untoward reaction.

3.1.9.2 Bacterin with adjuvant

The bacterin prepared as above (3.1.9.1) was mixed after checking sterility and safety with adjuvants in the ratio of 15 parts of vaccine, 9 parts of liquid paraffin and 1 part of lanolin.

3.1.9.3 Whole cell ultrasonicated antigen with adjuvant

A modified method of procedure described by Ireland *et al.* (1991) was followed for the preparation of sonicated antigen.

The pasteurella organisms grown on blood agar plates were harvested in PBS and washed twice in the same buffer. The washed cells were resuspended in PBS to contain $3x10^9$ bacteria/ml. Bacterial cell suspension held on an ice bath was then disrupted by sonication at 250 V for a total of 5 min with 10x30 sec bursts in a sonicator fitted with a 12 mm diameter titanium probe (SONITRON, IMECO, Ultrasonics, Bombay).

The sonication was interrupted for 30 sec between each burst for cooling. Formalin was added to the sonicated

antigen to 0.1 per cent level and allowed to stand for 24 h at room temperature.

After checking sterility and safety as above the sonicated antigen was mixed with adjuvant in the ratio of 15 parts of antigen, 9 parts of liquid paraffin and one part of lanolin.

3.1.10 Gluteraldehyde fixed sheep RBC (GA-SRBC)

Sheep blood collected in Alsever's solution (3.1.4) was washed by centrifugation (650xg for 20 min) six times in 0.85 per cent sodium chloride solution. After the last wash the packed cells were suspended in PBS (pH 7.2) to yield a 10 per cent suspension (V/V) and chilled to 4°C in an ice bath. A 25 per cent solution of gluteraldehyde was diluted to 1 per cent (V/V) with PBS and chilled to 4°C. The 10 per cent suspension of washed SRBC was mixed with an equal volume of 1 per cent solution of gluteraldehyde and the mixture was incubated at 4°C for 30 minutes with gentle stirring. The mixture was then centrifuged at 650xg for 10 min at 25°C. The pelletted fixed cells were washed three times with PBS containing 0.1 per cent sodium azide.

3.1.11 Sonicated antigen for indirect haemagglutination test

The sonicated antigen prepared as above (3.1.9.3) was centrifuged at 8000xg for 30 min. The supernatent was collected and tested for its sterility by plating 0.2 ml on TSA plates and then stored at -60°C until further use.

3.1.12 GA-SRBC sensitized with sonicated antigen

The 10 per cent suspension of GA-SRBC was mixed with an equal volume of a two fold diluted antigen. The mixture was incubated at 37°C for 1 h with occasional shaking. The sensitized cells were washed three times 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).

3.2 Methods

3.2.1 Isolation of pasteurella organism

Samples of heart blood, liver and spleen collected aseptically from ducks died of suspected pasteurellosis were streaked onto blood agar plates and incubated at 37°C in a candle jar under increased carbon dioxide tension. The incubated plates were examined after 24 h. In negative cases the plates were again incubated for a further 24 h under the same conditions as above. Colonies suggestive of pasteurella

(round slightly convex colonies 2-3 mm in diameter, mucoid in consistency and sticky in nature) were stained with gram's staining technique to confirm its purity and morphological features.

3.2.2 Identification

The bacterial isolates were identified based on colony character, morphology and the various biochemical tests described by Cowan (1974).

3.2.3 Pathogenicity studies

Pasteurella multocida isolates DP1 and DP5 were tested for their pathogenicity in mice and ducks.

3.2.3.1 Pathogenicity in mice

The method described by Kawamoto *et al.* (1990) was followed. Four mice per isolate were used for this purpose. Each animal was injected intraperitoneally with 0.1 ml of inoculum containing 3 x 10^6 organism/ml. The control mice were injected with 0.1 ml sterile saline. All the injected animals were observed for seven days for the clinical symptoms of infection/death. Blood smears were prepared from experimentally inoculated animals at 24 h intervals and were stained with Wright's stain.

The reisolation of *P. multocida* from dead mice were attempted from liver, lung and heart blood.

3.2.3.2 Pathogenecity in ducks

Four ducks were used for each isolate. the birds were inoculated subcutaneously with 0.1 ml of the inoculum containing $3x10^8$ organism/ml. All the injected birds were observed for a period of seven days for clinical symptoms/death. All the dead birds were subjected to post-mortem examination and re-isolation of organism was tried from heart blood, liver, lungs and spleen.

3.2.4 Determination of LD50

The LD50 of each of the *P. multocida* isolates were determined in four week old ducklings and also in six month old ducks (adult).

The inoculum was diluted using PBS (pH 7.2) to ten fold dilutions to contain organism upto $3x10^1$/ml.

Four week old ducklings were randomly assigned to nine groups of six ducklings each and the first eight groups were separately inoculated with the different dilutions of the bacterial inoculum with a dose of 0.1 ml/bird subcutaneously and the nineth group served as control which were inoculated with 0.1 ml of sterile normal saline.

Mortality was recorded for 1 week post inoculation. All the dead ducklings were examined in detail for specific lesions of pasteurellosis and the organism was isolated and identified from each bird. LD50 was calculated by the Reed and Muench method.

The same procedure was followed for the determination of LD50 in adult ducks but the number of ducks used for each dilution were only 4.

3.2.5 Immunization programme

A total of 420 ducklings, 4 weeks old, which were serologically negative for *P. multocida* antibodies were divided into seven groups of 60 birds each and the first 6 groups was separately vaccinated as described below and the seventh group served as control.

3.2.5.1 Group I

Group I was vaccinated subcutaneously with one ml of formalin inactivated pasteurella bacterin prepared using DP1 isolate.

3.2.5.2 Group II

Group II was vaccinated subcutaneously with one ml of pasteurella bacterin with oil adjuvant prepared from DP1 isolate.

3.2.5.3 Group III

Group III was vaccinated subcutaneously with one ml of whole cell ultrasonicate antigen with oil adjuvant prepared from DP1.

3.2.5.4 Group IV

Group IV was vaccinated subcutaneously with one ml of formalin inactivated pasteurella bacterin prepared from DP5 isolate .

3.2.5.5 Group V

Group V was vaccinated subcutaneously with one ml of pasteurella bacterin with oil adjuvant prepared from DP5 isolate .

3.2.5.6 Group VI

Group VI was vaccinated subcutaneously with one ml of whole cell ultrasonicate antigen with oil adjuvant prepared from DP5 isolate.

A second dose of all the above vaccines were given to the birds 80 days after the first vaccination.

3.2.6 Challenge

Ten birds from each vaccination group were subjected to homologous challenge with 0.1 ml of inoculum containing 100 LD_{50} of fully encapsulated virulent form of each isolate at 20 days interval till 80th day and then at 90th and 120th day.

Blood was collected from the birds before vaccination and also at 20 days interval post vaccination to determine the antibody titre in the serum by indirect haemagglutination test.

3.2.7 Indirect haemagglutination test (IHA)

The test was carried out according to the method described by Sawada *et al*. (1982). The same procedure was followed for both the isolates.

IHA was carried out in U bottomed microtitre plates Serial two fold dilutions of antiserum in BSA-PBS were taken in 25 μl quantity in 24 wells. 25 μl of the sensitized SRBC was added to each well and the plates were shaken and allowed to stand for 24 h at 25°C before SRBC setting patterns were read. The IHA titre was expressed as the reciprocal of the highest dilution of serum showing a definite pattern (flat sediment) compared to the pattern in negative well (smooth dot in the centre of the well).

3.2.8 Statistical analysis

The data obtained were statistically analysed using two way classification and least significant difference test (LSDT) and studied the correlation between survivability percentage and antibody titre.

Results

RESULTS

4.1 Isolation

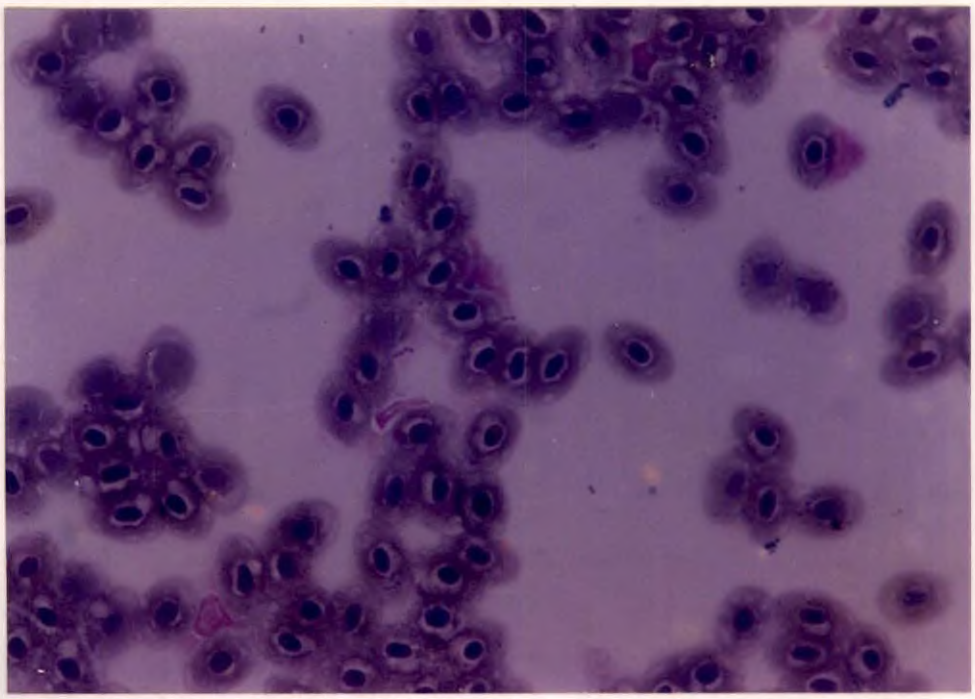
Ailing/dead ducks brought from Kuttanad area were used for the isolation of Pasteurella organism. The sick birds were showing listlessness, ocular and nasal discharge, greenish diarrhoea and paralysis of the legs and wings.

Post mortem examination of the dead ducks was conducted within six hours of death and bacterial isolation was tried from biomaterials collected from such birds. Gross pathological lesions observed were haemorrhages in most of the internal organs like proventriculus, intestine, lungs and myocardium. Accumulation of pericardial fluid was noticed in some birds. The liver and spleen were enlarged, congested and with white necrotic foci.

Heart blood smear and impression smears of liver, lungs and spleen stained with Wright's stain revealed bipolar organisms suggestive of Pasteurella by direct microscopical examination (Fig.1).

Inoculated blood agar plates presented bacterial colonies after 24 h incubation at 37°C under increased Co_2 tension. The colonies were smooth, convex and non-haemolytic.

Fig.1 Blood smear showing bipolar staining *Pasteurella multocida* organisms



Smears prepared from the above colonies stained by Gram's staining technique revealed Gram negative coccobacilli showing bipolar character.

4.2 Identification

Identification of the bacterial isolates at the first phase was based on the following tests. Gram's staining reaction, motility, growth on MacConkey's agar, Catalase activity, oxidase activity, growth under anaerobic conditions, carbohydrate breakdown (O/F) and haemolysis on blood agar.

The isolate was Gram negative, non motile, coccobacilli. They were oxidase and catalase positive, grew anaerobically, utilized glucose fermentatively and were negative for growth in Mac Conkey agar and haemolysis on blood agar.

The new bacterial isolate was subjected to second stage biochemical tests.

The results obtained for the isolate DP_5 was compared with that of the *P. multocida* isolate (DP_1) maintained in the laboratory and are presented in Tables 1 and 2.

Table 1. Primary tests of the *P. multocida* isolates

Tests	Isolates	
	DP1	DP5
Gram's reaction	G-ve	G-ve
Cell morphology	Cocco bacilli	Cocco bacilli
Motility	Non-motile	Non-motile
Oxidase	+	+
Catalase	+	+
Growth in MacConkey agar	-	-
Growth anaerobically	+	+
O/F	+	+
Haemolysis	-	-

Table 2. Second state biochemical tests of *P. multocida* isolates

Tests	Isolates	
	DP1	DP5
Indole	+	+
Gelatin liquifaction	-	-
Methyl red	-	-
Voges proskauer	-	-
H_2S production	-	-
Urease	-	-
Citrate	-	-
Sugar fermentation		
Sucrose	+	+
Galactose	+	+
Fructose	+	+
Mannose	+	+
Glucose	+	+
Maltose	+	+
Lactose	+	+
Xylose	+	+
Arabinose	+	-
Raffinose	-	+
Dulcitol	-	-
Trehalose	-	-

4.3 Pathogenecity studies

4.3.1 Mice

Two isolates of *P. multocida* viz., DP1 and DP5 were separately tested for their pathogenicity in mice. Each culture was injected into a group of four mice by intraperitoneal route. With DP1 isolate, when injected with 3 x 10^7 organisms two mice died within 24 h and the remaining two died within 48 h post inoculation.

Out of four mice inoculated with DP_5, three died within 12 h and the remaining one within 24 h.

Post mortem examination of dead mice revealed congestion of liver and spleen and no other lesions were observed in any of the internal organs. Blood smears prepared from heart blood and impression smears from liver, lungs and spleen, when stained with Wright's stain revealed bipolar organisms by direct microscopical examination. The bacteria could be isolated from liver, lungs and spleen.

4.3.2 Pathogenecity in ducks

The DP1 and DP5 isolates of *P. multocida* were also tested for their pathogenecity in ducks. Each culture was injected into a group of four ducks by subcutaneous route.

Out of the four ducks injected subcutaneously with an inoculum containing 3 x 10^7 organisms of DP1 isolate, one duck died within 48 h and the remaining three within 72 h.

Out of the four ducks inoculated with DP5 two died within 18 h and the remaining two within 24 h. The birds had developed symptoms of sneezing, cough, tremors of head, weakness and paralysis. The symptoms were apparent only one or two h before death.

Post mortem examination of the dead birds revealed petechial haemorrhage throughout the internal organs, especially on the myocardium (Fig.2). They further presented lesions like haemorrhagic enteritis, hydropericardium, enlarged liver with multiple small focal areas of necrosis and congestion of spleen (Fig.3&4).

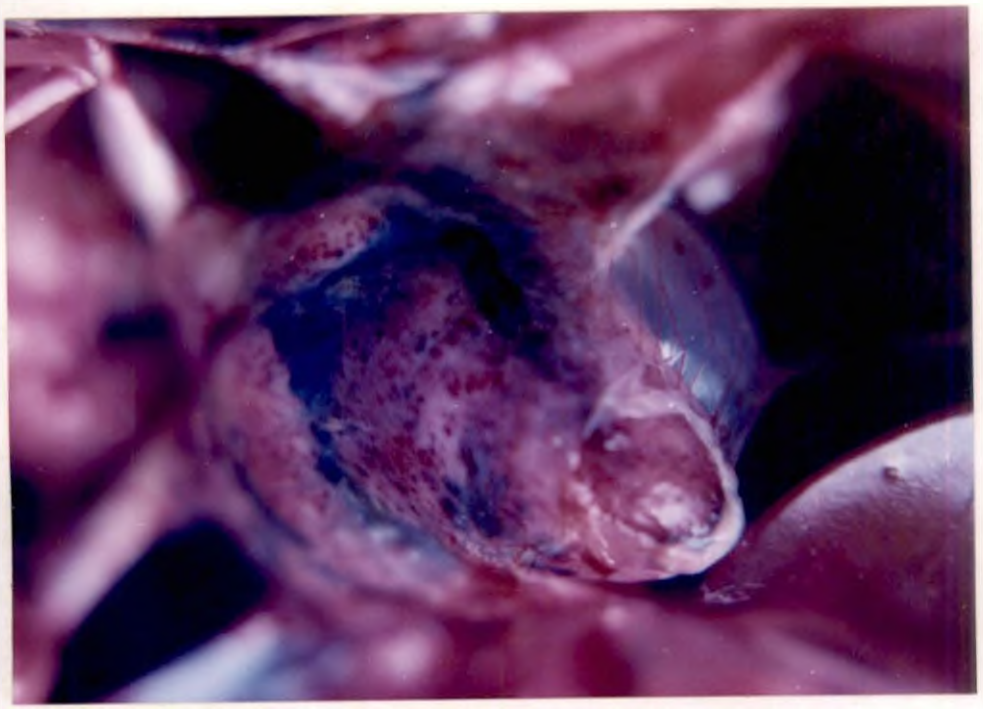
Pasteurella multocida organisms were demonstrated by microscopical examination of heart blood smear and were isolated from heart blood, liver, spleen and lungs.

4.4 Determination of LD_{50}

The LD_{50} of both isolates were determined in one month old ducklings and in 6 month old ducks. The results of the experiment are furnished in Tables 3 to 6.

Fig.2 Ducks - Experimental infection - postmortem lesions - Petechial haemorrhage on myocardium

Fig.3 Ducks - Experimental infection - postmortem lesions - Haemorrhagic enteritis



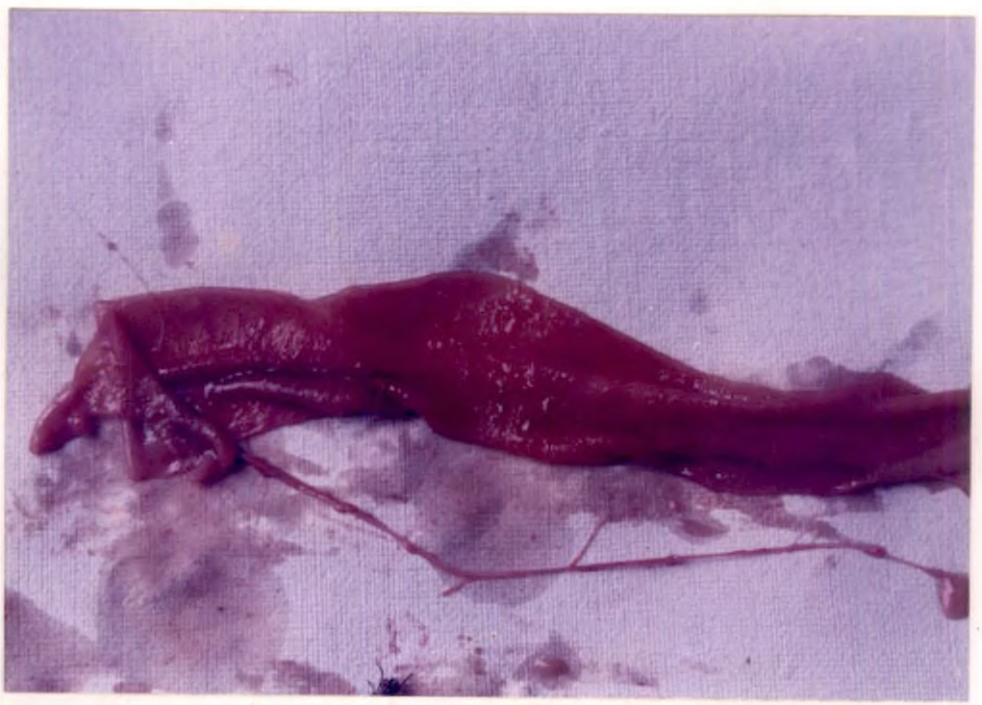


Fig.4 Ducks - Experimental infection - postmortem lesions - enlarged liver with multiple small focal areas of necrosis

The LD_{50} for DP5 isolate was 17 and 21 cells respectively for one month old ducklings and six month old ducks. The LD_{50} for DP1 isolate was 23 and 32 cells respectively for one month old ducklings and six month old ducks.

4.5 Preparation of vaccines

The *Pasteurella multocida* isolates DP1 and DP5 were separately used for the preparation of three vaccines, viz., bacterin, bacterin with oil adjuvant and adjuvanted sonicated antigen. All these vaccines prepared were homogenous suspensions which were easy for parentral administration.

4.6 Sterility test

The sterility of the above vaccines were tested individually in blood agar and tryptic soy agar. The plates were inoculated with 0.2 ml of the vaccine and incubated both at normal atmosphere and in increased CO_2 tension at 37°C for seven days.

The inoculated media were observed daily for any growth in the media. There was no growth in any of the above media indicating that vaccines prepared were sterile and without any contamination.

Table 3. LD_{50} in one month old ducklings using DP5 isolate

Bacterial Con. used	No.of birds	No. died	No. alive	Cumulative value		Ratio +ve	% +ve
				+ve	-ve		
$3x10^8$	6	6	0	40	0	40/40	100
$3x10^7$	6	6	0	34	0	34/34	100
$3x10^6$	6	6	0	28	0	28/28	100
$3x10^5$	6	6	0	22	0	22/22	100
$3x10^4$	6	6	0	16	0	16/16	100
$3x10^3$	6	5	1	10	1	10/11	90.9
$3x10^2$	6	4	2	5	3	5/8	62.5
$3x10^1$	6	1	5	1	8	1/9	11.11

$$\text{Proportionate distance} = \frac{62.5 - 50}{62.5 - 11.11} = 0.24$$

$$17.38 \text{ cells} = 17 \text{ cells}$$

Table 4. LD_{50} in 6 month old ducklings - DP5 isolate

Bacterial Con. used	No.of birds	No. died	No. alive	Cumulative value		Ratio +ve	% +ve
				+ve	-ve		
$3x10^8$	4	4	0	27	0	27/27	100
$3x10^7$	4	4	0	23	0	23/23	100
$3x10^6$	4	4	0	19	0	19/19	100
$3x10^5$	4	4	0	15	0	15/15	100
$3x10^4$	4	4	0	11	0	11/11	100
$3x10^3$	4	4	0	7	0	7/7	100
$3x10^2$	4	3	1	3	1	3/4	75
$3x10^1$	4	0	4	0	5	0/5	0

$$\text{Proportionate distance} = \frac{75-50}{75-0} = \frac{25}{75} = 0.33$$

$$= 21.38 = 21 \text{ cells}$$

Table 5. LD_{50} in one month old ducklings - DP1 isolate

Bacterial Con. used	No.of birds	No. died	No. alive	Cumulative value		Ratio +ve	% +ve
				+ve	-ve		
$3x10^8$	6	6	0	41	0	41/41	100
$3x10^7$	6	6	0	35	0	35/35	100
$3x10^6$	6	6	0	29	0	29/29	100
$3x10^5$	6	6	0	23	0	23/23	100
$3x10^4$	6	6	0	17	0	17/17	100
$3x10^3$	6	5	1	11	1	11/12	91.6
$3x10^2$	6	4	2	6	3	6/9	66.6
$3x10^1$	6	2	4	2	7	2/9	22.2

$$\text{Proportionate distance} = \frac{66.6 - 50}{66.6 - 22.2}$$

$$= \frac{16.6}{44.4} = 0.37$$

$$= 23.44 = 23 \text{ cells}$$

Table 6. LD_{50} in 6 month old ducklings - DP1 isolate

Bacterial Con. used	No.of birds	No. died	No. alive	Cumulative value		Ratio +ve	% +ve
				+ve	-ve		
$3x10^8$	4	4	0	24	0	24/24	100
$3x10^7$	4	4	0	20	0	20/20	100
$3x10^6$	4	4	0	16	0	16/16	100
$3x10^5$	4	4	0	12	0	12/12	100
$3x10^4$	4	4	0	8	0	8/8	100
$3x10^3$	4	3	1	4	1	4/5	80
$3x10^2$	4	1	3	1	4	1/5	20
$3x10^1$	4	0	4	0	8	0/8	0

$$\text{Proportionate distance} = \frac{80-50}{80-20} = \frac{30}{60} = 0.5$$

$$= 31.62 = 32 \text{ cells}$$

4.7 Toxicity test

The toxicity of the vaccines was assessed by infecting separately 0.5 ml of the vaccine intraperitoneally to six white mice and one ml subcutaneously to four ducklings of four weeks old. These inoculated mice and birds were observed for a period of seven days for any reaction or clinical manifestation.

All the three types of vaccines viz., bacterin, bacterin with oil adjuvant and whole cell ultrasonicated antigen with oil adjuvant did not produce any local or systemic reaction in any of the mice or ducklings.

4.8 Assessment of immunity

Four week old ducklings were used for the assessment of immunity. The ducklings were grouped into seven groups with sixty birds in each group and the first six groups were vaccinated separately using the different vaccines, the seventh group serving as the control. The birds received two doses of vaccine. The first dose was given at four weeks of age and the 2nd dose 80 days after the first. The birds were challenged with 0.1 ml of culture containing 100 LD_{50} of fully encapsulated virulent form of bacteria at 20 days interval till 80th day and then at 90th and 120th day after the first dose of vaccine.

The results obtained were statistically analysed and are presented in Tables 7 to 12 and Figures 5 and 6 depicts a graphical representation of percentage protection obtained in vaccinated birds challenged with DP1 and DP5 isolates respectively.

4.8.1 Group I

The birds in Group I were used to assess the immunity provided by a bacterin prepared using DP1 isolate. The baterin had protected 100 per cent, 70 per cent, 50 per cent and 20 per cent of the birds challenged with a virulent homologous strain at 20, 40, 60 and 80 days post vaccination respectively.

After the second dose of the vaccine, the protection obtained were 100 per cent and 70 per cent when challenged at 90th and 120th day respectively after the first vaccination.

All the control birds and some of the immunized birds died within 18 to 72 h post challenge exposure and the *P. multocida* organisms were recovered from these birds. The average survivability percentage obtained by statistical analysis were 62.523.

4.8.2 Group II

The efficacy of bacterin with oil adjuvant prepared using DP1 was assessed in this group. The percentage protection

Table 7. Results of challenge experiments - Group I

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Single vaccination	20	10	10	100
"	40	10	7	70
"	60	10	5	50
"	80	10	2	20
Vaccinated twice	90	10	10	100
"	120	10	7	70

Average survivability percentage = 62.523

Table 8. Results of challenge experiments - Group II

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Vaccinated once	20	10	10	100
"	40	10	10	100
"	60	10	10	100
"	80	10	7	70
Vaccinated twice	90	10	10	100
"	120	10	10	100

Average survivability percentage = 84.465

afforded when challenged with a virulent homologous strain at 20, 40, 60 and 80 days post vaccination were 100, 100, 100 and 70 respectively. The protection given after the second dose were 100 and 100 per cent respectively when challenged at 90th and 120th day.

The average survivability percentage as assessed by statistical analysis was 84.465.

4.8.3 Group III

A homologous protection of 100 per cent, 100 per cent, 80 per cent and 60 per cent was observed when birds immunized with an adjuvanted sonicated antigen prepared using DP1 isolate were challenged with a virulent homologous strain at 20, 40, 60 and 80 days post vaccination. The percentage protection afforded after the second dose was 100 per cent and 100 per cent respectively when challenged at 90th and 120th day after the first vaccination. The average survivability percentage was 79.035.

4.8.4 Group IV

A bacterin prepared using DP5 isolate was used to immunize the birds in this group. The protection given were 100, 70, 40 and 20 per cent respectively when challenged with a virulent homologous strain at 20, 40, 60 and 80 days post vaccination. After the booster dose the protectin were 100

Table 9. Results of challenge experiments - Group III

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Vaccinated once	20	10	10	100
"	40	10	10	100
"	60	10	8	80
"	80	10	6	60
Vaccinated twice	90	10	10	100
"	120	10	10	100

Average survivability percentage = 79.035

Table 10. Results of challenge experiments - Group IV

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Vaccinated once	20	10	10	100
"	40	10	7	70
"	60	10	4	40
"	80	10	2	20
Vaccinated twice	90	10	10	100
"	120	10	7	70

Average survivability percentage = 59.895

and 70 per cent respectively at 90 and 120 days after the first vaccination. The average survivability percentage was 59.895.

4.8.5 Group V

The birds in Group V were immunized with a bacterin with adjuvant prepared using DP5 isolate. The percentage protection afforded for a homologous challenge were 100, 100, 80 and 60 per cent respectively, when the birds were challenged at 20, 40, 60 and 80 days post vaccination. The birds which received a second dose at 80th day after first vaccination had shown 100 per cent and 100 per cent protection respectively when challenged at 90th and 120th day after the 1st vaccination. The average survivability percentage obtained was 79.035.

4.8.6 Group VI

The efficacy of a sonicated antigen with adjuvant prepared using DP5 isolate was tested in this group. The percentage protection given against a virulent homologous strain were 100, 80, 70 and 50 per cent respectively at 20, 40, 60 and 80 days post vaccination. The booster dose had provided 100 per cent and 100 per cent protection respectively at 90 and 120 days after the 1st vaccination. The average survivability percentage was 72.538.

Table 11. Results of challenge experiments - Group V

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Vaccinated once	20	10	10	100
"	40	10	10	100
"	60	10	8	80
"	80	10	6	60
Vaccinated twice	90	10	10	100
"	120	10	10	100

Average survivability percentage = 79.035

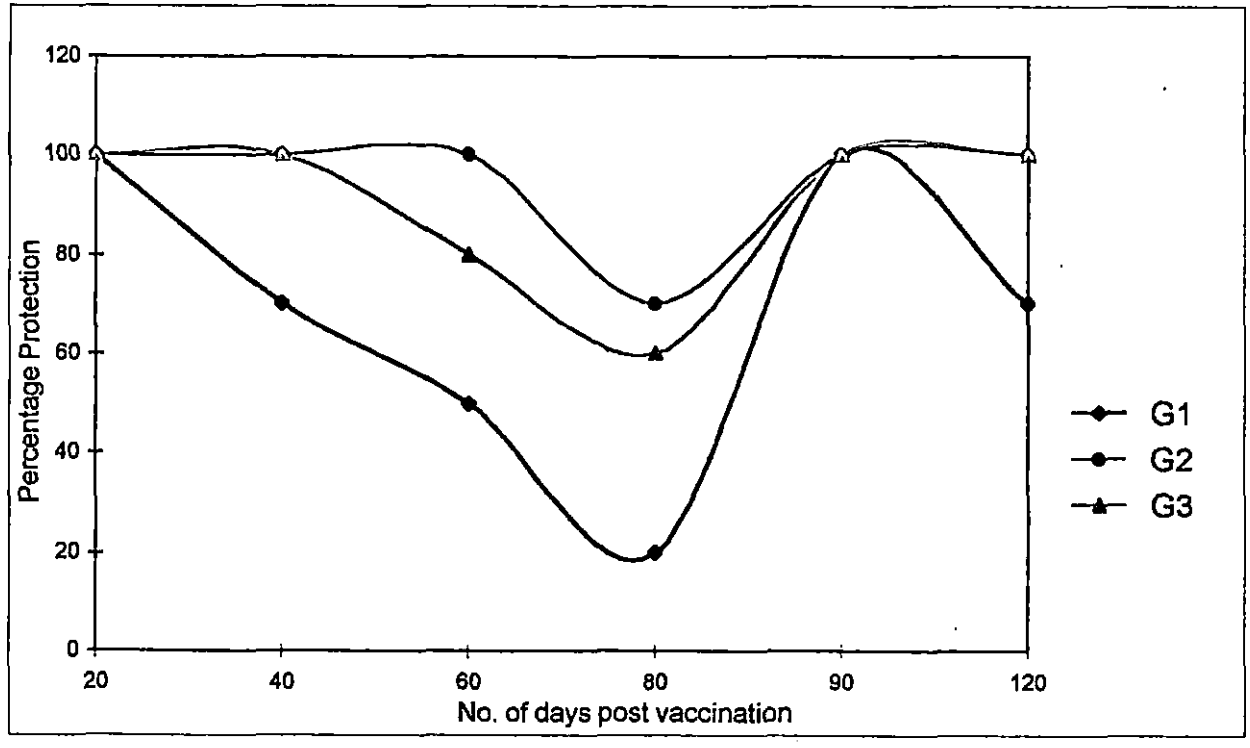
Table 12. Results of challenge experiments - Group VI

Vaccination status of birds	No. of days post vaccination	No. of birds challenged	No. birds survived	Percentage protection
Vaccinated once	20	10	10	100
"	40	10	8	80
"	60	10	7	70
"	80	10	5	50
Vaccinated twice	90	10	10	100
"	120	10	10	100

Average survivability percentage = 72.538

Fig. 5

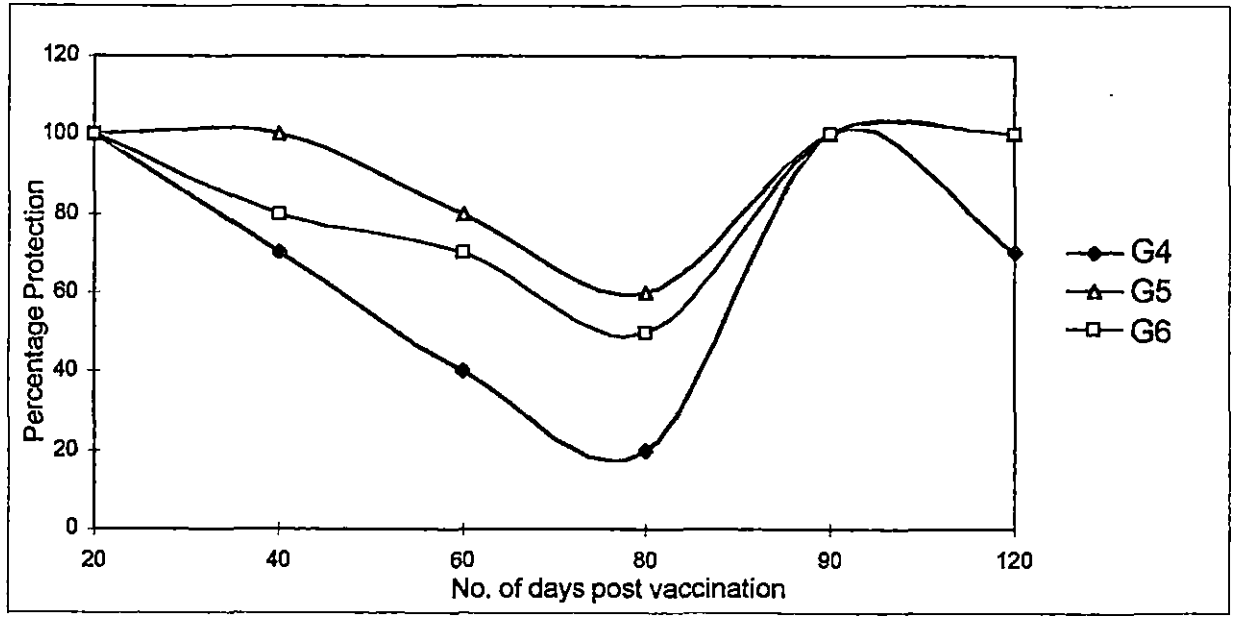
PERCENTAGE PROTECTION OF VACCINATED BIRDS CHALLENGED WITH - DP1 ISOLATE



G1 = Bacterin, G2 = Bacterin with oil adjuvant, G3 = Sonicated antigen with oil adjuvant prepared using DP1 isolate

Fig. 6

PERCENTAGE PROTECTION OF VACCINATED BIRDS CHALLENGED WITH - DP5 ISOLATE



G4 = Bacterin, G5 = Bacterin with oil adjuvant, G6 = Sonicated antigen with oil adjuvant prepared using DP5 isolate

4.9 Indirect haemagglutination test

The gluteraldehyde fixed sheep erythrocytes (GASRBC) sensitized with sonicated antigen were used for assessing the immune status of ducks. The sensitized SRBC were specifically agglutinated by the duck anti pasteurella serum. Controls of sensitized SRBC with diluent buffer (BSA-PBS) showed a definite negative reaction (Fig.7).

4.9.1 Group I

The serum samples collected from ducks vaccinated with a bacterin prepared using DP1 isolate showed an IHA titre of 0, 64, 128, 64, 32, 8, 128 and 64 respectively at days 0, 7, 20, 40, 60, 80, 90 and 120 days post vaccination (Table 13).

4.9.2 Group II

The IHA titre observed in the serum samples collected from birds in this group at days 0, 7, 20, 40, 60, 80, 90 and 120 post vaccination were 0, 64, 128, 128, 128, 64, 256 and 128 respectively (Table 14).

4.9.3 Group III

The serum samples collected from the birds in this group at day 0, 7, 20, 40, 60, 80, 90 and 120 post vaccination showed an IHA titre of 0, 64, 128, 128, 64, 64, 256 and 128 respectively (Table 15).

Table 13. Indirect haemagglutination inhibition test - Group I

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	64
"	60	32
"	80	8
Vaccinated twice	90	128
"	120	64

Table 14. Indirect haemagglutination inhibition test - Group II

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	128
"	60	128
"	80	64
Vaccinated twice	90	256
"	120	128

4.9.4 Group IV

The antibody titre in the serum of birds vaccinated with a bacterin prepared using DP5 isolate was assessed in this group. The IHA titres observed were 0, 64, 128, 64, 16, 8, 128 and 64 respectively at days 0, 7, 20, 40, 60, 80, 90 and 120 post vaccination (Table 16).

4.9.5 Group V

The birds vaccinated with a bacterin with adjuvant showed antibody titres of 0, 64, 128, 128, 64, 64, 256 and 128 when their serum samples are examined at days 0, 7, 20, 40, 60, 80, 90 and 120 days post vaccination (Table 17).

4.9.6 Group VI

The IHA titres observed in the serum samples of birds vaccinated with a sonicated antigen with adjuvant prepared using DP5 isolate were 0, 64, 128, 64, 64, 32, 128 and 128 respectively at days 0, 7, 20, 40, 60, 80, 90 and 120 days post vaccination (Table 18).

Comparative IHA titre obtained in post vaccinated sera of ducks vaccinated with different vaccines prepared using DP1 and DP5 isolates are presented grphically in Fig.8 and 9 respectively.

Table 15. Indirect haemagglutination inhibition test - Group III

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	128
"	60	64
"	80	64
Vaccinated twice	90	256
"	120	128

Table 16. Indirect haemagglutination inhibition test - Group IV

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	64
"	60	16
"	80	8
Vaccinated twice	90	128
"	120	64

Table 17. Indirect haemagglutination inhibition test - Group V

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	128
"	60	64
"	80	64
Vaccinated twice	90	256
"	120	128

Table 18. Indirect haemagglutination inhibition test - Group VI

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	0
"	7	64
"	20	128
"	40	64
"	60	64
"	80	32
Vaccinated twice	90	128
"	120	128

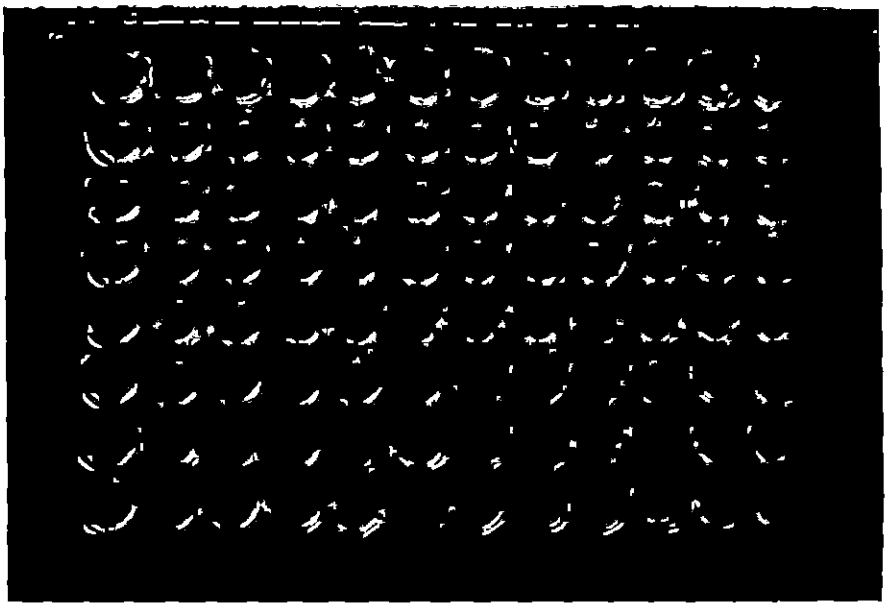

A
B
C
D
E

Fig.7 Indirect haemagglutination test

IHA Titre - Group I

A,B&C - IHA titre obtained in post vaccinal sera on days 20, 40 and 60 respectively

D - Positive control

E - Negative control

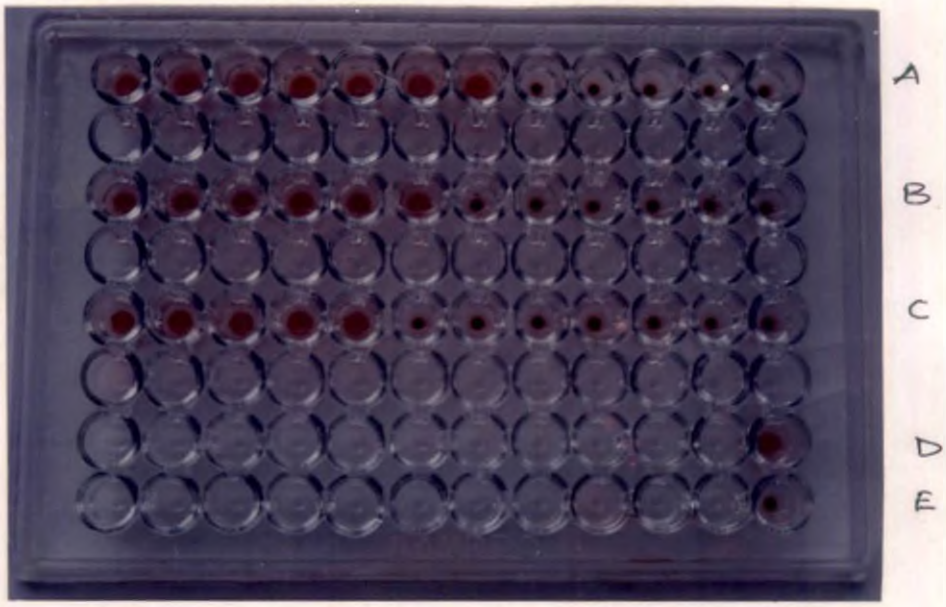
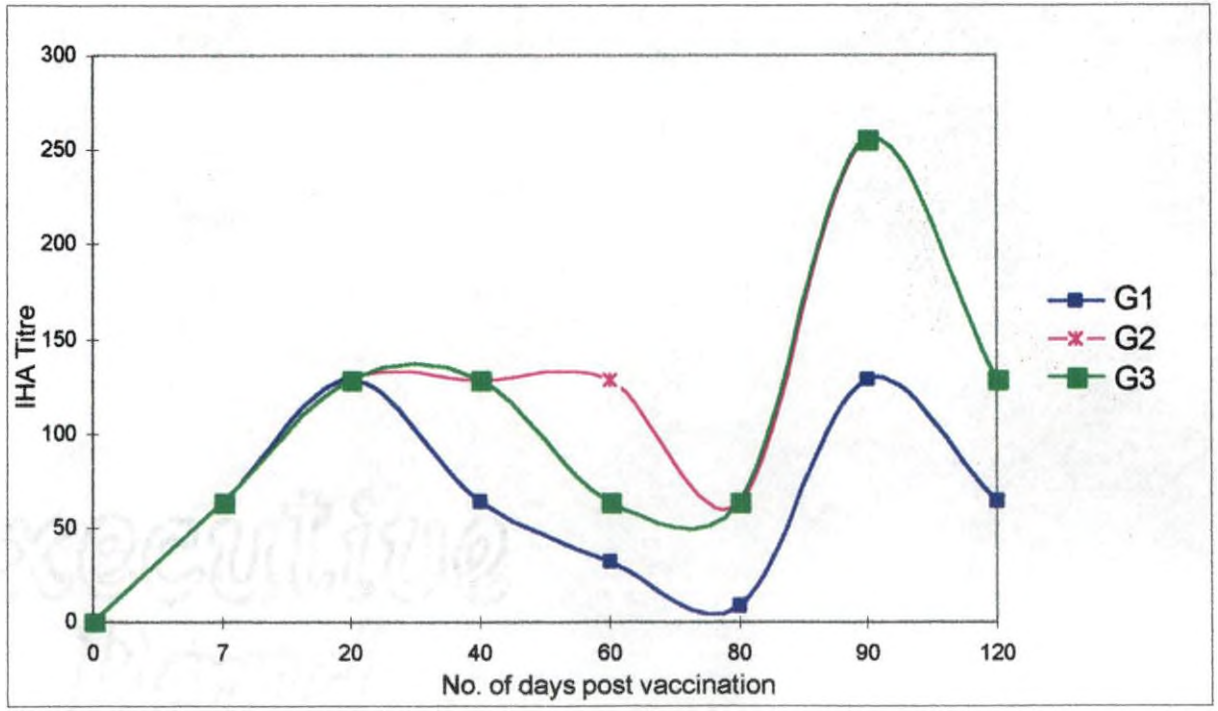

A
B
C
D
E

Fig. 8

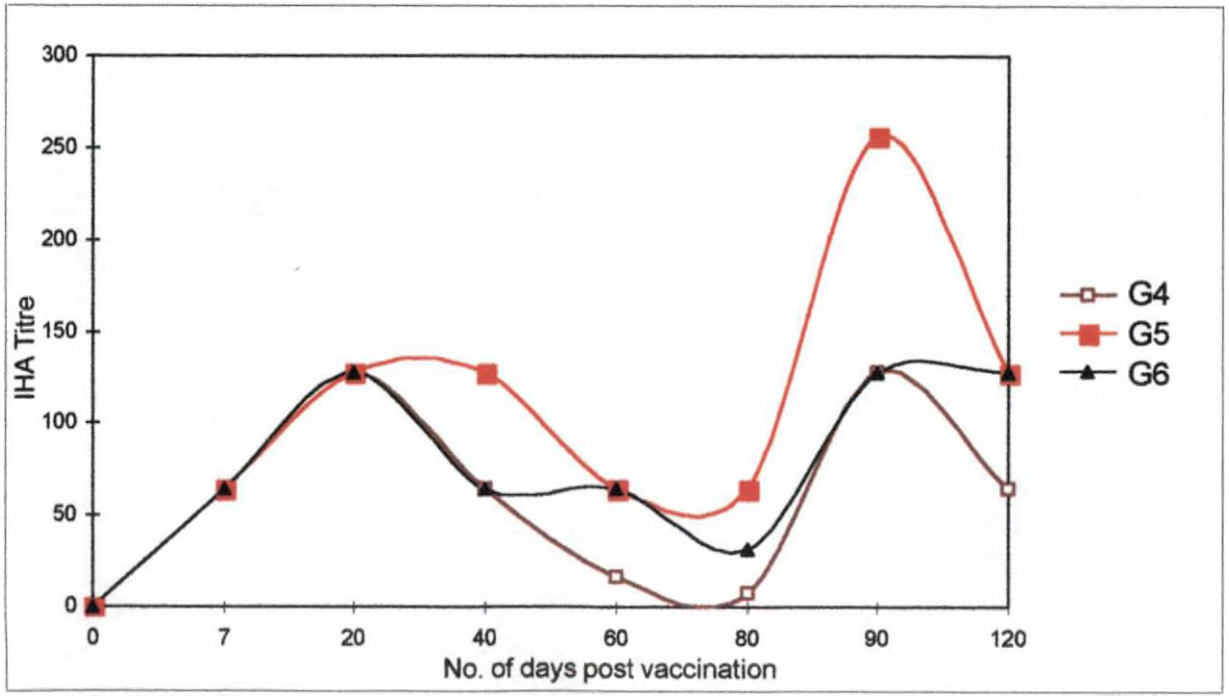
COMPARATIVE IHA TITRE IN POST VACCINATED SERA OF DUCKS - DP1 ISOLATE



G1 = Bacterin, G2 = Bacterin with oil adjuvant, G3 = Sonicated antigen with oil adjuvant prepared using DP1 isolate

Fig. 9

COMPARATIVE IHA TITRE IN POST VACCINATED SERA OF DUCKS - DP5 ISOLATE



G4 = Bacterin, G5 = Bacterin with oil adjuvant, G6 = Sonicated antigen with oil adjuvant prepared using DP5 isolate

Statistical analysis

The data obtained were statistically analysed using two way classification. Significant difference existed between different vaccines and different stages. Hence the means of different vaccines and different stages were compared using Least Significant Difference Test (LSDT) (Table 19).

In the case of vaccines the survivability percentage by giving bacterin prepared using DP1 and DP5 was significantly less when compared to bacterin with oil adjuvant prepared using DP1, adjuvanted sonicated antigen prepared using DP1 and oil adjuvant vaccine prepared using DP5. How ever no significant difference existed among bacterins prepared using DP1 and DP5 and adjuvanted sonicated antigen prepared using DP5.

It is found that for better survivability bacterin with oil adjuvant prepared using DP1, adjuvanted sonicated antigen prepared using DP1, oil adjuvant vaccine prepared using DP5 are preferable.

Correlation between survivability percentage and antibody titre was tested and it was observed that at all stages significantly high correlation existed as high titre ensured better survivability.

Table 19. Table of means

	Groups	Means	Total
Stage means	1	90.000	540.000
	2	74.503	447.020
	3	59.650	357.900
	4	44.408	266.450
	5	90.000	540.000
	6	78.930	473.580
Vaccine means	1	62.523	375.140
	2	84.465	506.790
	3	79.035	474.210
	4	59.895	359.370
	5	79.035	474.210
	6	72.538	435.230

Discussion

DISCUSSION

The duck industry of Kerala with a total duck population of 8.5 lakhs is facing a major economic loss mainly due to a bacterial disease known as Pasteurellosis which was first reported in Kerala by Pillai (1993) in ducks vaccinated against duck plague. Since then the duck mortality is continuing inspite of the combined efforts of veterinary personnel and duck farmers.

The protective antigen of *P. multocida* strains has not been conclusively identified so far and several of the antigenic fractions such as bacterins, cell fractions, cell extracts, live attenuated organisms and mutants are on trial for immunization of ducks. Since there are a number of antigenically different serotypes the protection is often effective only when the autologous vaccine strain is used.

In the present work three antigenic preparations viz., bacterin, bacterin with oil adjuvant and a whole cell ultrasonicated antigen with oil adjuvant prepared using local isolates P1 and P5 were studied for their immunogenicity in ducks.

5.1 Isolation

Blood agar containing five per cent sheep blood was used for the primary isolation of *P. multocida* from ducks.

Blood agar containing five per cent sheep/bovine blood is one of the recommended media for primary isolation of *P. multocida* eventhough it is described to be unsuitable for determining the colony morphology (Carter, 1967).

Yeast proteose cystine agar (Namioka and Murata, 1961) Dextrose starch agar (Heddleston *et al.*, 1964) and Brucella agar containing two per cent haemolysed rabbit serum (Carter, 1967) were also reported to be of use for primary isolation of *P. multocida* either alone or in combination.

Since only one medium i.e., blood agar containing five per cent sheep blood has been tried for primary isolation the efficiency of this medium can not be commented or compared.

5.2 Identification

The colonies produced by the isolates on blood agar plates after incubating for a period of 24 h at 37°C under increased CO_2 tension were smooth, convex, mucoid and nonhaemolytic.

Dekruif (1921, 1922, 1923) and Burn (1926) demonstrated the existence of three colonial variants viz. (1) the smooth form which grew diffusely in broth, formed smooth and iridescent colonies on serum agar and was virulent for rabbits; (2) the rough form which gave a granular deposit in broth, formed translucent bluish colonies on serum agar and was completely avirulent for rabbits and (3) the mucoid form which was of intermediate virulence. However, in the present study the colonies produced by DP1 and DP5 isolates were almost identical in their morphology.

Both the isolates grew well aerobically and under increased CO_2 tension at 37°C. The colonies under increased CO_2 tension were of more mucoid and flowing nature and were showing a tendency to grow along the direction of streak line. The mucoid nature of colony under increased CO_2 tension may be due to enhanced formation of capsular material.

Cowan (1974) described the primary tests for the identification of *P. multocida* as test for catalase, oxidase, production of acid from glucose and oxidative/fermentative utilization of glucose.

Both the isolates, DP1 and DP5 were gram negative, non motile, coccobacilli with some showing bipolar characters. They were oxidase and catalase positive, grew anaerobically

and utilized glucose fermentatively and were negative for growth on MacConkey's agar and haemolysis on blood agar.

These results were in accordance with the characters described for the species by Mannheim (1984) in the first edition of Bergy's Manual of systematic bacteriology.

Smith and Philips (1990) reported that *P. multocida* showed bipolar staining when taken from animal tissues or smooth colonies and tended to become bacillary in nature when taken from rough colonies. A similar observation was made in the present study also as the smears prepared from *in vitro* growth stained with Gram's stain were showing gram negative coccobacilli along with some organisms showing bipolar staining characters.

The second stage biochemical tests described for the identification of *P. multocida* by Cowan (1974) were nitrate reduction, nitrite reduction production of indole and hydrogen sulphide, growth in potassium cyanide, acid production from carbohydrates such as arabinose, lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose and xylose, gelatin liquefaction and ornithine decarboxylase activity.

Smith and Phillips (1990) reported that all strains of *P. multocida* produced acid but no gas in glucose and sucrose. Most strains fermented galactose, mannitol, mannose, sorbitol

and xylose. Strains of *P. multocida* were without action on litmus milk and gelatin. They produced indole, reduced nitrate and formed a small quantity of hydrogen sulphide as detected by lead acetate paper. The MR and VP reactions were both negative. The catalase and oxidase reactions were both positive, though rather weakly so. They did not utilise citrate as sole source of carbon. Most strains were positive for ornithine decarboxylase but negative for lysine, glutamic acid decarboxylase and arginine dihydrolase and were resistant to potassium cyanide.

The results of the second stage biochemical tests of DP1 and DP5 isolates were identical except for the acid production from Arabinose and Raffinose and in conformity with the characters described for the species.

5.3 Pathogenicity

Pathogenicity of the isolates DP1 and DP5 were tested separately in mice by inoculating $3x10^7$ organisms by intraperitoneal route. DP5 was found to be more pathogenic to mice as three of the inoculated mice died within 12 h. However all the inoculated mice died within 48 hours post inoculation.

Mushin and Schoenbaum (1980) observed that strains of Heddleston's serotype 3:A were lethal for mice within 24 h while untypable strains differed in their virulence.

Though the isolates under study were having the same colony morphology they differed in their degree of virulence in mice which indicated that colony morphology alone may not be used as a marker for virulence. This observation was in agreement with the findings of Okerman *et al.* (1979).

Except congestion of spleen and liver no other gross lesions were observed in the internal organs of any of the dead mice after experimental infection. However, the bacteria could be recovered from lungs, liver and heart blood in pure culture which indicated the cause of death. Collins (1976) observed that an overwhelming increase in the number of *P. multocida* in visceral organs was associated with death in mice when experimentally inoculated.

The DP1 and DP5 isolates of *P. multocida* were also tested for their pathogenicity in ducks. Four ducks were used for each culture. Ducks were inoculated subcutaneously with $3x10^7$ organisms. The birds had developed symptoms of sneezing, tremors of head and neck and paralysis.

Sharma *et al.* (1974) reported that the most prominent clinical symptoms in experimental avian pasteurellosis were

hyperthermia, dullness, in co-ordination of movements, greenish yellow diarrhoea, laboured and painful breathing and unusual sitting postures. Chronic phase of the disease was manifested by progressive emaciation, lameness, persistent diarrhoea and swollen joints.

Both DP1 and DP5 isolates were found to be pathogenic to ducks but the DP5 was found to be more virulent as all the inoculated ducks died within 24 h whereas it took 72 h for DP1 isolate.

Carter (1976) was of the opinion that encapsulated strains recovered from acute or moderately acute natural infections were generally virulent. This was true in the present study also as both the isolates were obtained from ducks died of acute natural outbreaks.

Hunter and Wobeser (1980) reported that Mallard ducks (Anas platyrhynchos) when challenged with two isolates of *P. multocida* developed either acute or chronic lesions, depending on the isolate of *P. multocida* and the dose of inoculum. Ducks that died of acute infection had lesions of haemorrhagic septicaemia with widespread vascular damage and focal necrosis in liver, spleen and other organs. Ducks surviving challenge developed chronic lesions in a variety of organs including brain, lung, air sacs, joints and eyes.

The postmortem examination of dead birds in this study revealed lesions such as petechial haemorrhage throughout the internal organs, whitish necrotic foci on the liver and spleen and hydropericardium which indicated the acute nature of the disease caused by these isolates.

5.4 LD50

The *P. multocida* isolates DP1 and DP5 were maintained in its virulent form during the period of study by repeated passaging in mice and subculturing on blood agar slants. When the stock culture was maintained at 4°C in air tight slants, the culture was to be subcultured once in four weeks. Mukkur and Pyliotis (1981) reported that virulence of *P. multocida* could be maintained only when subcultured on horse blood agar plates and passaged in mice on every 4th week.

The LD50 of the isolates DP1 and DP5 were determined both in one month old ducklings and 6 months old ducks. The bacterial suspension containing $3x10^8$ bacteria/ml was diluted using PBS (pH 7.2) to ten fold dilution to contain organisms upto $3x10^1$/ml and used for inoculation.

Four week old ducklings were randomly assigned to nine groups of six ducklings each and the first eight groups were separately inoculated with the different dilutions of bacterial inoculum with a dose of 0.1 ml/bird subcutaneously

and ninth group served as control which was inoculated with 0.1 ml of normal saline. Mortality were recorded for one week post inoculation.

The same procedure was followed for the determination of LD_{50} in adult ducks.

The LD_{50} of the isolates DP1 and DP5 were found to be different for young ones and adults and DP5 was found to be more virulent as the LD_{50} of DP5 for one month old ducks was 17 cells and for adult ducks LD_{50} was 21 cells where as LD_{50} of DP1 isolate for one month ducklings was determined as 23 and for adult ducks as 32.

Okerman *et al.* (1979) estimated the LD_{50} of 20 strains of *P. multocida* from rabbits by infecting mice intraperitoneally and the number of organisms ranged between $3x10^3$ and $3x10^6$ bacteria. Comparatively low LD_{50} of 14.32 ± 0.0833 colony forming units has been determined for *P. multocida* serotype A:1 isolate of duck origin in mice by Ramanatha *et al.* (1995).

The results of the study indicate that the isolates differ in their virulence and DP5 was more virulent to ducks. Also the LD_{50} for adult birds were higher than that for young birds. So the adult birds may be a bit more resistant to infection than young ones. In this study it was found that as

low as three organisms could cause the death of the birds indicating the highly virulent nature of the isolates.

Mukur (1979) determined a low LD50 of 5±2.8 colony forming units for *P. multocida* isolate of bovine origin in mice by intraperitoneal route of inoculation. Krecov (1976) found that as few as 10 organisms can produce rapid death when given i/v and i/m but infection was more difficult by other routes.

5.5 Vaccines

The different antigenic preparations viz. bacterin, bacterin with oil adjuvant and a whole cell ultrasonicate antigen with oil adjuvant were prepared separately using the DP1 and DP5 isolates of *P. multocida*. Virulence of the isolates were maintained by passaging through mice and fully encapsulated virulent form of the isolates were used for the preparation of vaccine.

Different antigens were prepared with the organisms that were grown on blood agar plates and after harvesting, the final bacterial concentration was adjusted to $3x10^9$ cells/ml. In the case of bacterin and bacterin with oil adjuvant vaccines 0.3 ml of formal saline was added for inactivation of bacteria, whereas in the case of whole cell ultrasonicated antigen, the organisms were disrupted by sonication at 250 V

for 5 minutes in a sonicator fitted with 12 mm diameter titanium probe and then formalin was added at 0.1 per cent level to ensure safety.

Three different immunogens such as formalinized broth vaccine, formalinized agar wash vaccine and heat treated agar wash vaccine were tried for vaccinating the buffalo calves and rabbits (Rau and Goil, 1950). Their results showed that agar wash vaccines when given in two doses with an interval of one month produced a high grade immunity when compared to broth vaccine. It was also observed that the higher the concentration of agar wash vaccine, the stronger the immunity it conferred. Similar was the results obtained in our study as high concentration of the organism was found to be required for providing protection against challenge and higher the concentration stronger was the immunity.

Harry and Deb (1979) were of the opinion that formalised vaccine would be the most suitable over other methods of inactivation employed in the preparation of *P. anatipestifer* vaccine and the dose prescribed by them was $3x10^9$ cells.

The three vaccines employed in the study were tested for their sterility and toxicity. Sterility was checked by inoculating vaccines individually onto blood agar plates and by incubating at normal atmosphere and at increased CO_2

tension. All the vaccines were found to be sterile and without any contamination.

Toxicity studies were carried out by inoculating each of these vaccines into ducklings and mice and all of them were found to be without any toxic effects, either local or systemic.

Two of the vaccines ie., bacterin and whole cell ultrasonicate antigen were then mixed with adjuvants in the ratio of 15 parts of vaccine, 9 parts of liquid paraffin and 1 part of lanolin.

The advantage of incorporation of oil adjuvant in preparation of an emulsified haemorrhagic septicaemia vaccine was reported by Bain and Jones (1955). Haemorrhagic septicaemia vaccine prepared with addition of mineral oil and lanolin was shown to be highly protective in vaccinated calves when challenged with virulent strain of *P. multocida* (Iyer *et al.*, 1955).

5.6 Assessment of immunity

The immunizing efficacy of the bacterin, bacterin with oil adjuvant and whole cell ultrasonicate antigen with oil adjuvant prepared separately using DP1 and DP5 isolates of *P. multocida* were studied by active immunization of ducks and

challenging them with virulent homologous strains at definite intervals.

The ducklings were immunized by giving two doses of the respective vaccines. The first dose was given at four weeks of age and the second dose was given 80 days after the first dose. The second dose was given because a sudden drop in immunity was observed by about 80 days post vaccination during the preliminary vaccination trials carried out using bacterins.

The immunized ducklings were challenged with 0.1 ml of inoculum containing 100 LD50 of fully encapsulated virulent form of bacteria at 20 days interval till 80th day and then at 90th and 120th day after the first dose of vaccine and simultaneously serum samples were collected on these days to assess the humoral antibody levels by indirect haemagglutination tests.

Similar experiment was carried out to study the efficacy of formalin inactivated, alum-adsorbed broth bacterin of *P. multocida* A:1 from ducks in field and laboratory by Ramanatha (1994) wherein four week old ducklings were immunized on 0 and 24th day and serum samples were collected on 0, 24, 58 and 94th day to assess the humoral antibody levels by IHA test.

In the present experiment none of the vaccinated birds died nor showed any clinical signs of illness during the period of observation. On challenge inoculation the rate of protection obtained varied with the type of vaccine as well as with the stages of challenge. The rate of protection varied from 20 to 100 per cent, depending on the type of vaccine and the time of post vaccination challenge whereas in non-vaccinated controls the survival rate was nil.

The rate of protection in ducks vaccinated with bacterin prepared using DP1 isolate was 100, 70, 50 and 20 per cent when challenged with virulent homologous strain at 20, 40, 60 and 80 days post vaccination respectively. But after the second dose of the vaccine the protection obtained were 100 and 70 per cent when challenged at 90th and 120th day respectively. On statistical analysis the average survivability percentage obtained were 62.523.

Similar was the results obtained when ducks were vaccinated with bacterin prepared using DP5 wherein the rate of protection obtained were 100, 70, 40 and 20 when challenged on 20, 40, 60 and 80th day post vaccination respectively and after the booster dose, the protection were 100 and 70 per cent on 90th and 120th day respectively with an average survivability percentage of 59.895.

So it was observed that eventhough the bacterin gives a high rate of protection ie. about 100 per cent on the 20th day post vaccination, a decline in immunity was observed as the days progressed and there was a sudden fall in immunity after 60th day. It was also observed that a 100 per cent immunity could be obtained on the 90th day after the booster dose of the vaccine but that too tended to decline as the immunity observed was only 70 per cent on 120th day.

Similar were the results obtained by Bolin *et al.* (1952) who reported that bacterin prepared using *P. multocida* isolates were highly immunogenic but the immunity was of short duration. Gao (1988) tested a formalin inactivated bacterin and a supersonic treated formalin inactivated bacterin against *P. anatipestifer* in the laboratory. Two inoculations of the bacterin in white pekin ducks at one and two or two and three weeks of age gave highly significant protection (90-100 per cent) against challenge with virulent organism at three or four weeks of age.

The results of the immunization study using bacterin with oil adjuvant prepared using DP1 isolate indicated that the vaccine gave 100, 100, 100 and 70 per cent homologous protection when challenged at 20, 40, 60 and 80th day post vaccination respectively and the booster dose at 80th day provided 100 per cent protection on the 90th and 120th day.

The average survivability percentage was 84.465. The bacterin with oil adjuvant prepared using DP5 isolate provided a homologous protection of 100, 100, 80 and 60 per cent at 20, 40, 60 and 80th day respectively and 100 per cent on the 90th and 120th day after the booster dose on 80th day with an average survivability percentage of 79.035.

Floren (1988) reported that vaccination of 10 day old pekin ducklings against *P. anatipestifer* infection under laboratory conditions with formal inactivated homologous monovalent oil emulsion vaccine induced solid immunity (95-100 per cent). A single injection of an oil-emulsified *P. multocida* bacterin in six week old duckling developed immunity that lasted for eight weeks (Layton, 1984).

The survivability rates observed when ducklings vaccinated with whole cell ultrasonicate antigen mixed with oil adjuvant prepared using DP1 isolate, subjected to a virulent homologous challenge, were 100, 100, 80 and 60 on 20, 40, 60 and 80th day respectively. After the booster dose the survivability rates were 100 per cent on 90th and 120th day and the average survivability percentage was 79.035. But the rates of protection obtained was comparatively lower in the case of whole cell ultrasonicated antigen prepared using DP5 isolate. Wherein the percentage protection were 100, 80, 70 and 50 on 20 40, 60 and 80 days respectively in comparison to

100, 100, 80 and 60 in the case of DP1 isolate vaccine. However after the booster dose 100 per cent protection was observed on 90th and 120th day.

No literature could be obtained regarding the use of whole cell ultrasonicated antigen with oil adjuvant as a vaccine against *P. multocida* infection in ducks. However the results obtained in this study indicated that the whole cell ultrasonicate antigen with oil adjuvant vaccine was better than bacterins and the results obtained were comparable to those of bacterin with oil adjuvant.

Gao (1988) found that a formalin inactivated bacterin and a supersonic treated formalin inactivated bacterin were equally effective against *P. anatipestifer* infection under laboratory studies. But the immunity observed was only for a short duration. The better survivability percentage and prolonged immunity observed in the present study may be due to the incorporation of oil adjuvant to the sonicated antigen.

On statistical analysis using two way classification and by comparing the means of different vaccines and different stages using least significant difference test, it was found that bacterin with oil adjuvant prepared using DP1 and DP5 isolate and whole cell ultrasonicate antigen prepared using DP1 isolate were better than other vaccines.

This is in confirmity with the results obtained by Mall and Nilakantan (1971) who studied the immunogenic efficacy of various *P. multocida* vaccines viz. (1) formalinized vaccine (2) alum precipitated vaccine (3) acetone dried cell vaccine (4) acetone dried cell oil adjuvant vaccine (5) oil adjuvant vaccine (6) potassium thiocyanate extract oil adjuvant vaccine and sodium chloride extracted oil adjuvant vaccine. Of these oil adjuvant and potassium thiocyanate extract incorporated in oil adjuvant were found to be satisfactory. Similarly Onet *et al.* (1994) found that when turkeys vaccinated with different types of *P. multocida* vaccines like 20 per cent aluminium hydroxide adjuvanted, 20 per cent calcium phosphate adjuvanted, 20 per cent oil adjuvanted and a food additive inactivated serotype 3 vaccine, the best protection was conferred by the inactivated 20 per cent oil adjuvanted vaccine.

5.7 Indirect haemagglutination test

In this study, along with challenge experiments, the humoral antibody levels of the vaccinated birds were also monitored using indirect haemagglutination test for which sheep red blood cells (SRBC) fixed and stabilized by one per cent gluteraldehyde (GA-SRBC) were employed. The GA-SRBC was found to be stable at 4°C without any deteriorating quality of SRBC during the six month period of the study. This saved

time in preparing the SRBC for every test and increased the reproducability of the results. This was in confirmity with Sawada *et al.* (1982) who found that stabilized SRBC could be stored for at least seven months at 4°C. Sonicated antigens adsorbed on to GA-SRBC were used to assess antibody titre in the serum. The serum samples were collected at regular intervals of 0, 7, 20, 40, 60, 80, 90 and 120 from all the vaccinated birds and titrated with the homologous antigens adsorbed on to GA-SRBC.

Ramanatha (1994) used crude capsular antigen adsorbed on to GA-SRBC to assess humoral antibody levels by IHA tests in ducks which received a single dose of the vaccine or two doses of the vaccine on day 0 and day 24 and also in unvaccinated ducks. The pre and post vaccinal sera were collected on day 0, 24, 58 and 94. He observed that the titres of both vaccinated groups were significantly higher than that of unvaccinated ducks and the titres of sera from twice vaccinated group was significantly higher than those from once vaccinated group. He also observed that the protection rate when challenged with virulent homologous strain was higher in birds with higher IHA titres.

In the present study the antibody titre obtained varied with the type of vaccine, and with different stages at which serum samples were collected to assess the antibody level. In

the case of bacterins prepared using DP1 and DP5 isolates the titre varied from 8 to 128. The highest antibody titre of 128 was observed at 20th day after the first vaccination. Then there was a decline in the antibody level and the lowest titre of 8 was recorded on 80th day. Again a high antibody titre of 128 was obtained after the booster dose.

The results obtained for bacterin with oil adjuvant prepared using DP1 and DP5 and sonicated antigen with oil adjuvant prepared using DP1 were comparable wherein the highest titre obtained after single vaccination were 128 at days 20, and 40 and then there was a decline in the antibody level and a comparatively low antibody titre of 64 was recorded on day 80 whereas in the case of sonicated antigen with oil adjuvant prepared using DP5 the titre obtained on day 80 was only 32.

After the booster dose oil adjuvant vaccines prepared using DP1 and DP5 and whole cell ultrasonicate antigen prepared using DP1 showed a high antibody titre of 256 on 90th day whereas in the case of whole cell ultrasonicate with oil adjuvant prepared using DP5 the tire obtained were 128.

The level of antibody and the survivability percentage were found to be highly correlated as high titre indicated high rate of protection.

Azam *et al*. (1991) reported that the range of antibody titre observed in rabbit after a single inoculation with sonicated adjuvanted vaccine ranged from 8-64 whereas the titre obtained for a formalin killed *P. multocida* bacterin ranged between 4-16 and the maximum IHA antibody titre was observed at 2nd and 3rd week.

After studying the results of challenge experiments and the antibody titre observed in the serum samples of all the different vaccines used in this study it was observed that oil adjuvant vaccines prepared using DP1 and DP5 and sonicated antigen with oil adjuvant prepared using DP1 were superior over other vaccines. Eventhough the results obtained for whole cell ultrasonicate antigen prepared using DP1 isolate were comparable with those of bacterins with oil adjuvant prepared using DP1 and DP5, by considering the ease of preparation the bacterin with oil adjuvant can be recommended for the use in the field. How ever more evaluation and field trials are required before advocating the vaccine for field use.

Summary

SUMMARY

The isolation of Pasteurella organisms was tried from ailing/dead ducks brought from Kuttanad area. Post mortem examination of dead ducks was conducted within six h of death and bacterial isolation was tried from biomaterials collected from such ducks. Gross pathological lesions observed in post mortem examinations were haemorrhages in the myocardium, intestine, congestion of spleen and white necrotic foci in the liver.

The results of the first stage and second stage biochemical tests obtained for the isolate obtained during the period of study (DP5) was compared with that of the *P. multocida* isolate (DP1) maintained in the laboratory. The isolates were Gram negative, coccobacilli, non motile and produced catalase. They were oxidase positive, grew anaerobically, utilized glucose fermentatively and were negative for growth in Mac Conkey agar and haemolysis on blood agar.

The results of the second stage biochemical tests of DP1 and DP5 isolates were identical and in confirmity with the character described for the species.

The DP1 and DP5 isolates were tested for their pathogenicity in mice and ducks. Intraperitoneal injection of $3x10^7$ organisms killed mice between 12-48 h post inoculation. When ducks were inoculated with 0.1 ml of inoculum containing $3x10^8$ organism/ml all the inoculated birds died within 24-72 h post inoculation. The organism could be re-isolated from dead birds and animals.

LD_{50} of the DPI isolate were 23 and 32 cells respectively for one month old ducklings and six month old ducks whereas the LD_{50} for DP5 isolate were 17 and 21 cells respectively for young and adult ducks.

The *Pasteurella multocida* isolates DP1 and DP5 were separately used for the preparation of three vaccines viz., bacterin, bacterin with oil adjuvant and oil adjuvanted sonicated antigen. All the vaccines were found to be sterile and without any toxic effects.

For the assessment of immunity four week old ducklings were used. The birds were injected with two doses of vaccine. The first dose was given at four weeks of age and second dose was given 80 days after the first dose. The vaccinated birds were challenged at 20 days interval till 80th day and then at 90th and 120th day after the first vaccination with 0.1 ml of culture containing 100 LD_{50} of fully encapsulated virulent form of bacteria.

The rates of protection in ducks vaccinated with bacterin prepared using DP1 isolate on challenging with a homologus strain were 100, 70, 50 and 20 per cent at 20, 40, 60 and 80 days respectively and after the second dose the protection obtained were 100 and 70 per cent when challenged at 90th and 120th day respectively.

The survival rates of 100, 100, 100 and 70 per cent were observed in ducks which were vaccinated with bacterin with oil adjuvant prepared using DP1 isolate, when challenged with virulent homologous strain at 20, 40, 60 and 80 days respectively. The results obtained after the second dose were 100 per cent, when challenged on 90th and 120th day.

A homologous protection of 100, 100, 80 and 60 per cent were observed when birds immunized with an oil adjuvanted sonicated antigen prepared using DP1 isolate was challenged at 20, 40, 60 and 80 days post vaccination respectively. Cent per cent protection was observed after the second dose when challenged at 90th and 120th day.

The percentage protection observed in ducks vaccinated with bacterin prepared using DP5 isolate were 100, 70, 40 and 20 per cent when challenged with a virulent homologous strain at 20, 40, 60 and 80 days respectively post vaccination. After the booster dose the protection rates were 100 and 70 per cent respectively at 90 and 120 days after the first vaccination.

The rate of protection observed in birds vaccinated with bacterin with oil adjuvant prepared using DP5 isolate were 100, 100, 80 and 60 per cent when challenged at 20, 40, 60 and 80 days respectively post vaccination and after the booster dose 100 per cent protection was observed at 90th and 120th day respectively. The percentage protection afforded for a homologous challenge in the case of ducks vaccinated with whole cell ultrasonicated antigen with oil adjuvant prepared using DP5 isolate were 100, 80, 70 and 50 per cent at 20, 40, 60 and 80 days respectively post vaccination. The booster dose had provided 100 per cent protection on 90 and 120 days respectively after the first vaccination.

For indirect haemagglutination test gluteraldehyde fixed sheep erythrocytes sensitized with sonicated antigen were used. The sensitized SRBC were specifically agglutinated by the duck antipasteurella serum. The serum samples were collected at regular intervals of 0, 7, 20, 40, 60, 80, 90 and 120 from all the vaccinated birds and titrated with the homologous antigens adsorbed on to GA-SRBC.

In the case of bacterins prepared using DP1 and DP5 isolates the titre varied from 8 to 128. The highest antibody titre of 128 was observed at 20th day after the first vaccination and at 90th day after the booster dose on 80th day. The lowest titre of 8 was recorded on the 80th day.

The antibody titre observed in the serum samples of ducks vaccinated with bacterin with oil adjuvant prepared using DP1 and DP5 isolate and sonicated antigen with oil adjuvant varied from 64 to 256. The highest titre of 256 was observed on day 90 after the booster dose on day 80 whereas the highest titre observed after a single vaccination were 128 observed on days 20 and 40 respectively post vaccination. The lowest titre of 64 was observed on the 80th day after the first vaccination. But the sonicated antigen with oil adjuvant prepared using DP5 isolate showed a titre varying from 32 to 128. The highest titre of 128 was observed on day 20 after the first vaccination and also on 90th and 120th days after the booster dose on 80th day. The lowest titre of 32 was observed on the 80th day after the first vaccination.

On statistical analysis it was found that the survivability percentage obtained in the case of ducks vaccinated with bacterin prepared using DP1 and DP5 isolates were significantly less when compared to bacterin with oil adjuvant prepared using DP1 and DP5 isolate and sonicated antigen with oil adjuvant prepared using DP1 isolate. Also a high correlation between survivability percentage and antibody titre was observed at all stages as high titre ensures better survivability.

It was found that for better survivability bacterin with oil adjuvant prepared using DP1 and DP5 isolate and whole cell ultrasonicated antigen with oil adjuvant prepared using DP1 isolate are preferable.

References

REFERENCES

Azam, M.W., Hussain, I., Ashfaque, M., Siddique, H., Mahmood, T. and Siddique, M.I. (1991). Comparative immunogenicity of sonicated *Pasteurella multocida* and formalised bacterin in rabbits. *Pat. Vet. J.* 11: 120-122.

Baba, T. (1977). Immunogenic activity of a ribosomal fraction obtained from *Pasteurella multocida*. *Infect. Immun.* 15: 1-6.

Bain, R.V.S. and Jones, R.F. (1955). Studies on haemorrhagic septicaemia of cattle. III. Production of adjuvant vaccine. *Br. Vet. J.* 3: 30.

Bain, R.V.S. and Jones, R.F. (1958). The production of dense cultures of *Pasteurella multocida*. *Br. Vet. J.* 114: 215-220.

Baljer, G., Chorherr, S. and Mayr, A. (1982). Efficacy and harmlessness of inactivated *Pasteurella multocida* vaccines on subcutaneous, oral and intranasal application in mice. *Zentralblatt fur Veterinarmedizin B.* 29: 275-283.

Bergey, D.H., Harrison, F.C., Breed, R.S., Hammer, B.W. and Huntoon, F.M. (1923). Cited by Hussaini, S.N. (1975).

Bhasin, J.L. and Biberstein, E.L. (1968). Fowl cholera in turkeys - The efficacy of adjuvanted bacterins. *Avian Dis.* 12: 159-168.

Bierer, B.W. and Derieux, W.T. (1972). Immunogenic response of turkeys to an avirulent. *P. multocida* vaccine in the drinking water. *Poult. Sci.* 51: 408-416.

*Bolin, F.M., Turn, J. and Eveleth, D.F. (1952). Immunity produced by *Pasteurella multocida* bacterins in chickens. *N. Dat. Agr. Expt. Sta. Bimo. Bul.* 14: 187.

Breed, R.S., Murray, E.G.D. and Hitchens, A.P. (1948). Cited by Hussaini, S.N. (1975).

Breed, R.S., Murray, E.G.D. and Smith, N.R. (1957). Bergey's Manual of determinative bacteriology, 7th edn. Williams and Wilkins, Baltimore. pp. 395-402.

Brown, J., Dawe, D.L., Davis, R.B., Foster, J.W. and Srivastava, K.K. (1970). Fowl cholera immunization in turkeys. I. Efficacy of various cell fractions of *P. multocida* as vaccines. *Appl. Microbiol.* 19: 837-841.

Buchman, R.E. and Gibbons, N.E. (1974). Bergey's Manual of determinative bacteriology, 8th edn. Williams and Wilkins, Baltimore. pp. 370-373.

Carter, G.R. (1950). Studies on *Pasteurella multocida* chicken embryo vaccines I. The comparative immunizing value of broth bacterins and chicken embryo vaccines in mice. *Am. J. Vet. Res.* 11: 252-255.

Carter, G.R. (1958). Some characteristics of Type A strains of *Pasteurella multocida*. *Br. Vet. J.* 114: 356-357.

Carter, G.R. (1967). Pasteurellosis: *Pasteurella multocida* and *Pasteurella haemolytica*. *Advan. Vet. Sci.* 11: 321-379.

Carter, G.R. and Bigland, C.M. (1953). Dissociation and virulence in strains of *Pasteurella multocida* isolated from a variety of lesions. *Can. J. Comp. Med.* 18: 473-479.

Chen, T.J., Li, H.J., Liang, Z.H., Zeng, M.Z., Qiu, Y.M. and Wang, Z.M. (1984). Serological classification of porcine strains of *Pasteurella multocida* from Guangdong province. II. Cross immunity between an attenuated vaccine strain. *Guangdong Agri. Sci.* (5): 38-40.

Chengappa, M.M., Carter, G.R. and Chang, T.S. (1979). A streptomycin dependent live *P. multocida* type-3 vaccine for in prevention of fowl cholra in turkeys. *Anim. Dis.* 23: 57-61.

Chengappa, M.M., Mc Laughlin, B.G., Kadel, W.L., Muddex, R.L. and Greer, S.C. (1989). Efficacy of a live *Pasteurella multocida* vaccine for the prevention of experimentally induced bovine pneumonic pasteurellosis. *Vet. Microbiol.* 21: 147-154.

Collins, F.M. (1973). Growth of *Pasteurella multocida* in vaccinated and normal mice. *Infect. Immun.* 8: 868-875.

Collins, F.M. and Woolcock, J.B. (1976). Immune responses to *Pasteurella multocida* in the mouse. *Jl. Reticuloendothelial Soc.* 19: 311-321.

Cowan, S.T. (1974). *Cowan and Steel's Manual for the Identification of Medical Bacteria.* 2nd ed. Cambridge University Press, Cambridge.

De Jong, M.F. and Borst, G.H.A. (1985). Selective medium for the isolation of *Pasteurella multocida* and *Bordetella bronchiseptica*. *Vet. Rec.* 116: 551.

De Kruif, P.H. (1921, 1922, 1923). Cited by Smith, G.R. and Phillips, J.E. (1990). Pasteurella. In. Topley and Wilson's *Principles of Bacteriology, Virology and Immunity Vol2*, Eds. Parker, M.T. and Duerden, B.I., 8th ed. pp. 384-395.

Delpy, L.P. (1938). Cited by Rau, K.G. and Govil, J.L. (1950).

Derieux, W.T. (1984). Response of broiler chickens to live *Pasteurella multocida* duration of immunity and minimum dose. *Avian Dis.* 28: 281-284.

Dhanda, M.R., Das, M.S., Lal, J.M. and Seth, R.N. (1956). Immunological studies on *Pasteurella septica*. I. Trials on adjuvant vaccines. *Indian J. Vet. Sci. Anim. Husb.* 26: 273.

Di Giacomo, R.F., Deeb, B.J., Bernard, B.L., Klassen, J.M. and Chengappa, M.M. (1987). Safety and efficacy of a streptomycin dependant live *Pasteurella multocida* vaccine in rabbits. *Lab. Anim. Sci.* 37: 187-190.

Floren, U., Storm, P.K., Kaleta, E.F. (1988). *Pasteurella anatipestifer* species incertae sedis in Pekin ducks: Pathogenicity tests and immunization with inactivated homologous monovalent (serotype 6/B) oil emulsion vaccine. *Deutsche Tierarztliche Wochenschrift* **95**: 210-214.

Gao, F. and Guo, Y.P. (1988). Studies of the vaccine against infectious serositis in ducklings. I. Serotyping of *P. anatipestifer* in China. 2. Preliminary studies of the inactivated bacterins against *P. anatipestifer*. In waterfowl production. Proceedings of the International Symposium, September 11-18. Beijing, China: 318-327.

Gaunt, G., Moffat, R. and Mukkur, T.K.S. (1977). Fowl cholera: Immunization of chickens with potassium thiocyanate (KSCN) extract of *Pasteurella multocida* serotype 3. *Avian Dis.* 21: 543-548.

Giridhar, P., Venkatesha, M.D., Agnihothri, G.J., Ananth, M. and Keshavamurthy, B.S. (1990). Merits of different tests in the evaluation of protection of calves given varying doses of alum precipitated haemorrhagic septicaemia vaccine. *Indian Vet. J.* **67**: 1002-1005.

Glisson, J.R., Contrevas, M.D., Cheng, H.N. and Wang, C. (1993). Cross protection studies with *P. multocida* bacterins prepared from bacteria propagated in iron-depleted medium. *Avian Dis.* **37**: 1074-1079.

Gochenour, W.S. (1924). Haemorrhagic septicaemia studies. I. The development of a potent immunizing agent (natural aggression) by the use of highly virulent strains of haemorrhagic septicaemia organisms. *J. Am. Vet. Med. Assoc.* 18: 433-445.

Hansen, L.M. and Hirsh, D.W. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. *Vet. Microbiol.* 21: 177-184.

Hardenbergh, J.B. (1916). Cited by Rau, K.G. and Govil, J.L. (1950).

Harry, E.G. and Deb, J.R. (1979). Laboratory and field trials on a formalin inactivated vaccine for the control of *Pasteurella anatipestifer* septicaemia in ducks. 27: 329-333.

Heddleston, K.L. (1962). Studies on Pasteurellosis. V. Two immunogenic types of *Pasteurella multocida* associated with fowl cholera. *Avian Dis.* 6: 315-321.

Heddleston., K.L. and Hall, W.J. (1958). Studies on Pasteurellosis II. Comparative efficiency of killed vaccines against fowl cholera in chickens. *Avian Dis.* 2: 322-335.

Heddleston, K.L. and Reisinger (1959). Studies on Pasteurellosis. III. Control of experimental fowl cholera in chickens and turkeys with an emulsified killed vaccine. *Avian Dis.* 3: 397-404.

Heddleston, K.L., Rebers, P.A. and Ritchie, A.E. (1966). Immunizing and toxic properties of particulate antigen from two immunogenic types of *Pasteurella multocida* of avian origin. *J. Immunol.* **96**: 124-133.

Heddleston, K.L. and Rebers, P.A. (1972). Fowl cholera: Cross immunity induced in turkeys with formalin killed in-vivo-propagated *P. multocida*. *Avian Dis.* **16**: 578-586.

Heddleston, K.L., Watko, L.P. and Rebers, P.A. (1964). Dissociation of fowl cholera strain of *Pasteurella multocida*. *Avian Dis.* **8**: 649-657.

Heddleston, K.L., Rebers, P.A. and Wessman, G. (1975). Fowl Cholera: immunogenic and serologic response in turkeys to live *Pasteurella multocida* vaccine administered in the drinking water. *Poult. Sci.* **54**: 217-221.

Hertman, I., Markenson, J., Michael, A. and Geier, E. (1979). Attenuated liver fowl cholera vaccine. I. Development of vaccine strain M3G of *P. multocida*. *Anim. Dis.* **23**: 863-869.

Hunter, B., and Wobeser, G. (1980). Pathology of avian cholera in mallard ducks. *Avian Dis.* **24**(2): 403-414.

Hussaini, S.N. (1975). A review on nomenclature and taxonomy of *Pasteurella multocida The Vet. Bullet.* **45**: (6): 130-132.

Ireland, L., Adler, B. and Milner, A.R. (1991). Proteins and antigens of *Pasteurella multocida* serotype 1 from fowl cholera. *Vet. Microbiol.* **27**: 175-185.

Iyer, S.V., Gopalakrishnan, K.S. and Ramani, K. (1955). The effects of adjuvant upon the immunizing value of formalin killed *Pasteurella multocida* organism. *Indian Vet. J.* **31**: 379-391.

Kadel, W.L., Chengappa, M.M. and Herren, C.E. (1985). Field trial evaluation of a Pasteurella vaccine in pre conditioned and non-preconditioned light weight calves. *Am. J. Vet. Res.* **46**: 1944-1948.

Kawamoto, E., Sawada, T. and Murayama, T. (1990). Prevalance and characterisation of *Pasteurella multocida* in rabbits and their environment in Japan. *Japan J. Vet. Sci.* **52**: 915-921.

Kim, J.Y., Park, J.M. and Kim, O.N. (1986). Studies on the immunogenicity of *Pasteurella multocida* isolated from swine in Korea. *Research Reports of the Rural Developing Administration* **28**: 77-93.

Knox, K.W. and Bain, R.V.S. (1960). The antigens of *Pasteurella multocida* Type 1. capsular lipopoly saccharides. *Immunol.* **3**: 352-362.

Kodama, H., Matsumoto, M. and Snow, L.M. (1981). Immunogenicity of capsular antigens of *Pasteurella multocida* in turkeys. *Am. J. Vet. Res.* **42**: 1838-1841.

Krecov, M.I. (1976). Examination of the role of the route of infection and nonspecific factors in the appearance of fowl cholera. (Summary of thesis). *Acta Veterinaria Yougoslavia* 26(1): 33-45.

Kucera, C.J., Wong, J.C.S. and Eis, R.L. (1981). Development of a chemically altered *Pasteurella multocida* vaccinal strain. *Am. J. Vet. Res.* 42: 1389-1394.

Layton, H.W. (1984). Efficacy of broth grown *Pasteurella multocida* bacterins in ducklings. *Avian Dis.* 28: 1086-1095.

Lignieres, J. (1900). Cited by Hussaini, S.N. (1975).

Liow, T.M. (1977). Study of fowl cholera bacterins in crossbred ducks. *Singapore Vet. J.* 1: 37-41.

*Long, G.Y., Liang, H.Z. and Liu, J.S. (1986). Immunization of rabbits against pasteurellosis. *Chinese J. Vet. Sci. Tech.* (2): 3-7.

Lu, Y.S. and Pakes, S.P. (1981). Protection of rabbits against experimental pasteurellosis by streptomycin dependent *Pasteurella multocida* serotype 3,A live mutant vaccine. *Infect. Immun.* 34: 1018-1024.

Mack, W.B. and Records, E. (1916). Cited by Heddleston, K.L. and Hall, W.J. (1958).

Mall, M.P. and Nilakantan, P.R. (1971). Evaluation of different vaccines in the control of avian pasteurellosis (fowl cholera). *Indian Vet. J.* 48: 331-335.

Mannheim, W. (1984). Pasteurellaceae. Bergey's Manual of Systematic Bacteriology. Vol.1. Eds. Krieg, N.R. and Holt, J.G., Williams and Wilkins. 1st ed. pp. 559-569.

Matsumoto, M. and Helfer, D.H. (1977). A bacterin against fowl cholera in turkeys: protective quality of various preparations originated from broth cultures. 21: 382-393.

Michael, A., Yarkon, E., Konshtok, R., Deleg, B.A. and Hornstein, K. (1986). Aerosol vaccination of turkeys against fowl cholera with the M-3-G mutant strain of *Pasteurella multocida* vaccine. *Israel J. Vet. Med.* 42: 79-83.

Mittal, K.R., Jaiswal, T.N., Gupta, B.R. and Gupta, B.K. (1977). Studies on the haemorrhagic septicaemia water in oil and multiple emulsion adjuvant vaccine I. Immunity trials in rabbits. *Indian Vet. J.* 54: 773-778.

Mittal, K.R., Jaiswal, T.N. and Gupta, B.K. (1979). Studies on haemorrhagic septicaemia oil adjuvant and multiple emulsion adjuvant vaccines II. Immunity trials in mice, rabbits and calves. *Indian Vet. J.* 56: 449-454.

Mukkur, T.K.S. (1979). Immunogenicity of chaotropically extracted protective antigens of *Pasteurella multocida* type A (Bovine origin) against experimental pasteurellosis in mice. *J. Gen. Microbiol.* 113: 37-43.

Mukkur, T.K.S. and Pyliotis, N.A. (1981). The potassium thiocyanate extract of *Pasteurella multocida*: electron microscopy and susceptibility of immunogenic activity to some physical, chemical enzymatic treatments. *J. Comp. Pathol.* **91**: 427-437.

Murata, M., Moriuchi, T. and Namioka, S. (1964). Studies on the pathogenicity of *Pasteurella multocida* for mice and chicken on the basis of O group. *Cornell Vet.* **54**: 293-307.

Mushin, R. and Schoenbaum, M. (1980). A strain of *Pasteurella multocida* associated with infection in rabbit colonies. *Lab. Anim.* **14**: 353-356.

Namioka, S. and Murata, M. (1961). Serological studies on *Pasteurella multocida* I. A simplified method for capsule typing of the organism. *Cornell. Vet.* 51: 498-507.

Nangia, S.S., Boxi, K.K. and Gulrajani, T.S. (1966). Haemorrhagic septicaemia oil adjuvant vaccine. Study of potency test in rabbits: duration of immunity and keeping quality. *Indian Vet. J.* **43**: 279-287.

Okerman, L., Spanoghe, L. and Debruycker, R.M. (1979). Experimental infection of mice with *Pasteurella multocida* strains isolated from rabbits. *J. Comp. Pathol.* **89**: 51-55.

Onet, E.G., Collins, N.F. and Richards, S. (1994). Observations on immunologic response of turkeys vaccinated against fowl cholera. *Studies and Researches in Vet. Med.* **2**: 97-101.

Penn, C.W. and Nagy, L.K. (1974). Capsular and somatic antigens of *Pasteurella multocida* type B and E. *Res. Vet. Sci.* **16**: 251-259.

Penn, C.W. and Nagy, L.K. (1976). Isolation of a protective, non-toxic capsular antigen from *Pasteurella multocida* type B and E. *Res. Vet. Sci.* **20**: 90-96.

Percy, D.H., Prescott, J.F. and Bhasin, J.L. (1985). *Pasteurella multocida* infection in the domestic rabbit: Immunization with a streptomycin dependent mutant. *Can. J. Comp. Med.* **49**: 227-230.

Pillai, R.M., James, P.C., Punnoose, K.T., Sulochana, S., Jayaprakasan, V. and Nair, G.K. (1993). Outbreak of pasteurellosis among duck population in Kerala. *J. Vet. Anim. Sci.* **24**: 34-39.

Ramanatha, K.R. (1994). Laboratory studies and preliminary field evaluation of vaccination trials against pasteurellosis in ducks. *Ind. J. Ani. Sci.* **64**: 1138-1142.

Ramanatha, K.R., Lakshminarayana, R. and Gopal, T. (1995). Virulence of duck isolate of *P. multocida* A:1 to mice. *Mysore J. Agric. Sci.* **29**: 266-268.

Rau, K.G. and Govil, J.L. (1950). An improved haemorrhagic septicaemic vaccine. *Indian Vet. J.* **27**: 105-113.

Rebers, P.A., Heddleston, K.L. and Rhodes, K.R. (1967). Isolation from *Pasteurella multocida* of a lipopolysaccharide antigen with immunizing and toxic properties. *J. Bacteriol.* **93**: 7-14.

Rhodes, K.R. and Rimler, R.B. (1987). Capsular groups of *P. multocida* isolated from avian hosts. *Avian Dis.* **31**: 895-898.

Rhodes, K.R., Rimler, R.B. (1989). Fowl cholera. In: Pasteurella and Pasteurellosis. Eds. Adlam, C. and Rutter, J.M., Academic Press, London. pp. 95-113.

Rice, J.T. (1978). Subcutaneous vaccination of broiler type chicken with a live avirulent *Pasteurella multocida* vaccine. *Poult. Sci.* **54**: 1514-1518.

Rimler, R.B., Rebers, P.A. and Rhoades, K.R. (1979). Fowl cholera: Cross protection induced by *P. multocida* separated from infected turkey blood. *Avian Dis.* **23**: 730-741.

Rimler, R.B. and Rhoades, K.R. (1981). Lysates of turkeys-grown *P. multocida*. Protection against homologous and heterologous serotype challenge expsoures. *Am. J. Vet. Res.* **42**: 2117-2121.

Rimler, R.B. and Philips, M. (1986). Fowl cholera: Protection against *Pasteurella multocida* by vitrosome-lipo-polysaccharide vaccine. *Avian Dis.* 30: 409-415.

Roberts, R.S. (1947). An immunological study of *Pasteurella septica*. *J. Comp. Pathol.* 57: 261-278.

Rosenbusch, C.T. and Merchant, I.A. (1939). A study of the haemorrhagic septicaemia pasteurellae. *J. Bacteriol.* 37: 69-89.

Sandhu, T. (1979). Immunization of White Pekin ducklings against *Pasteurella anatipestifer* infection. 23: 662-669.

Sandhu, T.S. and Layton, H.W. (1985). Laboratory and field trials with formalin inactivated *Escherichia coli* (078) - *Pasteurella anatipestifer* bacterin in White Pekin ducks. *Avian Dis.* 29: 128-135.

Sandhu, T.S. (1991). Immunogenicity and safety of a live *Pasteurella anatipestifer* vaccine in White Pekin ducklings: Laboratory and field trials. *Avian Pathol.* 20: 423-432.

Sawada, T., Rimler, R.B. and Rhoades, K.R. (1982). Indirect haemagglutination test that uses gluteraldehyde fixed sheep erythrocytes sensitized with extract antigens for detection of pasteurella antibody. *J. Cli. Microbiol.* 15: 752-756.

Sharma, R.N., Bhalla, N. P. and Lall, J.M. (1974). Observation of experimental infection of *Pasteurella septica* (avian) in domestic fowl. *Indian J. Anim. Health* 13(2): 105-109.

Singh, N., Sambyal, D.S., Soni, G.L. and Sodhi, S.S. (1994). Immunogenicity trials of *Pasteurella multocida* in buffalo calves. Buffalo Journal. 3: 221-227.

Singh, R. and Teo, T.P. (1988). Efficacy of an inactivated broth grown *Pasteurella multocida*. *Singapore J. Primary Industries* **16**: 24-33.

Smith, J.E. (1955). Cited by Smith, G.R. and Phillips, J.E. (1990).

Smith, G.R. (1958). Cited by Smith, G.R. and Phillips, J.E. (1990).

Smith, G.R. and Phillips, J.E. (1990). Pasteurella. *Topley and Wilson's Principles of Bacteriology, Virology and Immunity, Vol.2*. Eds. Parker, M.T. and Duerden, B.I. 8th Edn. pp. 335-351.

Snipes, K.P. and Hirsh, D.C. (1986). Association of complement sensitivity with virulence of *P. multocida* isolated from turkeys. *Avian Dis.* **30**: 500-504.

Timms, L.M. and Marshall, R.N. (1989). Laboratory assessment of protection given by experimental *P. anatipestifer* vaccine. *Br. Vet. J.* **145**: 483-493.

Topley, W.W.C. and Wilson, G.S. (1929). The principles of bacteriology and immunity. Vol.1, 1st edn. Edward Arnold, London.

Trevisan, V. (1887). Cited by Hussaini, S.N. (1975).

Venkatesh, M.D., Giridhar, P., Ananth, M. and Balakrishnan, B.S. (1991). Immune response in cattle to alum precipitated haemorrhagic septicaemia vaccine. *Indian Vet. J.* **68**: 812-817.

Webster, L.T. and Burn, C.G. (1926). Biology of Bacterium lepisepticum. III. Physical, cultural and growth characteristics of diffuse and mucoid types and their variants. *J. Exp. Med.* **44**: 343-358.

Wei, B.D. and Carter, G.R. (1978). Live streptomycin dependant *Pasteurella multocida* vaccine for the prevention of haemorrhagic septicaemia. *Am. J. Vet. Res.* 39: 1534-1537.

Yadav, M.S. and Ahooja, M.L. (1983). Immunity trials in mice, rabbits and calves with oil-adjuvant and multi emulsion oil-adjuvant vaccine against haemorrhagic septicaemia. *Indian J. Anim. Sci.* **53**: 705-708.

Yaw, K.E., Briefman, L. and Kakavas, J.L. (1956). A comparison of virulence for mice and chicken of different colonial variants of the three serological types of *P. multocida*. *Am. J. Vet. Res.* **17**: 157-159.

* Originals not seen

COMPARATIVE EFFICACY OF DIFFERENT VACCINES AGAINST PASTEURELLOSIS IN DUCKS

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ABSTRACT OF A THESIS

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ABSTRACT

Two isolates of *Pasteurella multocida* from ducks viz. DP1 and DP5 isolated from ailing/dead ducks from Kuttanad area and maintained in the virulent form were used separately for the preparation of different vaccines viz., bacterin, bacterin with oil adjuvant and sonicated antigen with oil adjuvant.

The biological and biochemical characters of both the isolates were compared and are in confirmity with the characters described for the species by earlier workers.

Both the isolates were pathogenic to ducks and mice as all the inoculated ducks and mice were killed with in 72 h. The LD_{50} for the DP1 isolate in one month old ducklings and six month old ducks were determined to be 23 and 32 cells respectively whereas the LD_{50} of DP5 isolate for one month old ducklings was determined to be 17 and for adult ducks LD_{50} was 21 cells.

Immunogenic potential of different vaccines prepared using DP1 and DP5 isolate were tested in ducks by giving two doses of vaccine. The first dose was given at four weeks of age and second dose was given 80 days after the first dose. The birds were challenged with 0.1 ml of culture containing 100 LD_{50} of fully encapsulated virulent form of bacteria at 20

days interval till 80th day and then at 90th and 120th day after the first dose of vaccine. A higher percentage of protection was conferred by oil adjuvant vaccines prepared using DP1 and DP5 isolate and sonicated antigen with oil adjuvant prepared using DP1 isolate.

The serum samples were collected from vaccinated birds at regular intervals of 0, 7, 20, 40, 60, 80, 90 and 120 for indirect haemagglutination test and the titres obtained were ranging from 8 to 256. More evaluation and elaborated field trials are required before advocating bacterin with oil adjuvant for field use.

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