DEVELOPMENT AND EVALUATION OF OUTER MEMBRANE PROTEIN VACCINE AGAINST DUCK PASTEURELLOSIS

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled **"Development and evaluation of outer membrane protein vaccine against duck pasteurellosis"** 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 ociety.

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CERTIFICATE

Certified that this thesis, entitled **"Development and evaluation of outer membrane protein vaccine against duck pasteurellosis"** is a record of research work done independently by **Dr. Ranjini. A. R,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to her.

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INTRODUCTION

Disease, of microbial origin is one of the major hurdles in the development of poultry industry. Apart from mortality, the production loss due to morbidity of the birds affects the poultry industry drastically. One such disease is pasteurellosis (Fowl cholera), caused by *Pasteurella multocida.* It is an acute contagious septicaemic disease of wild and domestic birds, especially chicken, turkeys, duck and geese (Chawak *et al.*, 2001).

Ducks are highly versatile in nature. They live happily under wide range of climatic conditions and are not affected by many of the diseases seen in chicken. Ducks occupy next place to chicken in production of table eggs. Duck population in India is 24 million and forms about seven per cent of the total world population. Kerala with a costal length of 580 kilometers has a sizeable population of ducks, around 6. 6 lakhs (Livestock census, 2003). Ducks are bred and reared on large scale in Alappuzha, Thrissur, Kottayam and Pathanamthitta districts of Kerala. Ducks, especially the indigenous varieties, are generally considered to be more hardy than chicken. However, disease outbreaks do appear in them from time to time. Presently the duck industry in Kerala is facing a major set back mainly due to the repeated out breaks of two diseases *viz.*, pasteurellosis and duck plague.

In 1986, the worldwide loss due to fowl cholera was estimated to be 200 million US dollars (Ratafia, 1988). Sources of infection include carrier birds, clinically diseased birds, their excretions and carcasses of poultry which have died of the infection. In per acute form, large number of birds in the flock will be found dead showing less premonitory signs. Marked depression, anorexia, mucous discharge from the nasal orifices and foetid diarrhea are the symptoms noticed in acute form of disease. In the chronic form, symptoms like conjunctivitis, dyspnoea, lameness and swelling of wattle may be seen (Gooderham, 1988). Diagnosis of the disease depends on identification of the causative bacterium,

from birds with signs and lesion consistent with the acute or per acute form of disease.

During outbreaks of pasteurellosis, administration of antibiotics remains the first measure of control of disease. But often, isolates exhibited difference in there antibiotic sensitivity patterns, which inturn was directly responsible for the treatment failure. So immunization is then sought as the next mode of approach.

In spite of immunizing poultry against fowl cholera with existing conventional vaccines, hundred per cent protection has never been achieved. Hence different subunits of *P. multocida* could be explored as possible immunogens in conferring hundred per cent protection to chicken against fowl cholera.

Iron is an essential element for most of the microorganisms due to its role in metabolic electron transport chain (Jain *et al.*, 2006). *P. multocida* while growing *in vivo* or *in vitro* in an iron restricted environment, expresses high molecular weight proteins called iron regulated outer membrane protein (IROMPs) that are highly immunogenic and hence could be used to produce subunit vaccine. Also they are found to be inducing high cross protective immunity (Kedrak and Opacka., 2002). Similarly *P. multocida* also expresses 18kDa fimbrial subunit proteins which possesses potential protective property and could be used as a potential immunogen in inducing cross protective immunity (Ruffolo *et al.*, 1997).

By immunizing ducks with outer membrane protein vaccine the mortality rate due to duck pasteurellosis can be minimized and the egg production and growth rate can be enhanced (Chawak *et al.*, 2001).

In this context, the present study is undertaken with the following objectives.

(1) To identify the immunogenic moieties of outer membrane protein (OMP) of *P. multocida* of avian origin.

(2) Preparation of OMP vaccine in iron sufficient media.

(3) Preparation of OMP vaccine in iron restricted media.

(4) Comparison of efficacy of outer membrane protein vaccine with other conventional vaccine of *P.multocida.*

Review of Literature

2. REVIEW OF LITERATURE

Fowl cholera (avian pasteurellosis) is a common and widely distributed disease of poultry and is of major economic importance. All species of birds, particularly turkeys are susceptible. The severity of disease is influenced by environmental stress, season and overcrowding (Chawak *et al.*, 2001).The carrier birds may act as reservoir (Hirsh *et al., 2004)*

2.1 ETIOLOGY OF PASTEURELLOSIS

The Gram negative facultative bacterium, *P. multocida* is an important veterinary pathogen with world wide distribution and causes fowl cholera among poultry. The causal organisms are usually of capsular type A and occasionally type D (Carter, 1955).

Tabatabai and Zehr, (2004) reported that fowl cholera (avian pasteurellosis) is caused by *P. multocida* serovars A:1, A:3 and A:4.

2.2 PREVALENCE OF AVIAN PASTEURELLOSIS

2.2.1 Prevalence in countries outside India

The earliest report on the occurrence of avian pasteurellosis was made by Androvandus in Italy in 1600. The disease occurred in Italy and France from 1770 to 1800 and Chabert in France, in the year 1780.

Fowl cholera in ducks was a serious problem in long island (USA), and it was diagnosed in 32 of 68 commercial duck farms (Dougherty, 1953).

Curtis (1979) reported the prevalence of turkey pasteurellosis in Britain.

Pasteurella multocida was isolated from quails, which suffered an acute condition with high mortality (Panigraphy and Glass, 1982).

Glisson *et al.* (1989) reported cases of *P. multocida* infection from quails in Georgia .

Sander and Glisson (1989) isolated *P. multocida* from chicken in the age group of 20 to 46 days in Georgia, while Waltman and Horne (1993) isolated *P. multocida* from chicken of four to eight weeks of age.

Bermudez *et al.* (1991) isolated *P. multocida* from Bob white quail.

Nakamine *et al.* (1992) isolated *P. multocida* subsp.m*ultocida* from Muscovy ducks for the first time in Japan.

Blackall *et al.*(1995) reported the outbreak of pasteurellosis from turkeys in Australia.

Morishita *et al.* (1996 a and b) reported the prevalence of fowl cholera in raports and psittacine birds, isolated *P. multocida* from both groups of birds.

Mariana and Hirst (2000) reported that fowl cholera was recognized in Indonesia in 1972 and there had been many subsequent outbreaks in ducks.

A higher prevalence rate of fowl cholera in ducks, caused by *P. multocida* was reported by Muhairwa *et al.* (2001) in Tanzania.

An investigation on poultry disease occurring in Bangladesh revealed that fowl cholera was implicated in 3.15 per cent of the cases (Talha *et al.*, 2001).

Dahl *et al.* (2002) opined that concurrent infections occurred with *P. multocida* and *Ascaridia galli* in free ranging chicken in Denmark.

Praveen *et al.* (2003) isolated *P.multocida* from a breeder flock in Lahore district in Pakistan.

2.2.2 Prevalence in India

Pasteurella infection in birds had been considered to be a serious economic problem to the Indian poultry industry.

The incidence of fowl cholera in ducks had been reported in India by Mulbagal *et al.* (1972).

Panda *et al.* (1981) isolated *P. multocida* from oedema fluid of wattle of the affected chicken in Orissa.

Pillai *et al.* (1993) isolated *P. anatipestifer* from fowl cholera outbreaks in ducks in Kerala.

Ramanatha and Gopal (1993) reported an outbreak of acute duck cholera in an organized duck farm in Karnataka state due to *P. multocida* A:1, with a morbidity of 40 per cent and mortality 20 per cent.

Chandran *et al.* (1995) reported an outbreak of pasteurellosis in Japanese quails in Madras.

An outbreak of duck cholera in Tripura caused by *P. multocida* A:1 with a mortality percentage of 30 to 35 per cent reported by Murugkar and Ghosh (1995).

(Jayakumar, 1998) isolated *P. multocida* from fowl cholera outbreak in Kerala.

Pasteurella multocida was isolated from oedema fluid of wattle of the affected duck in Andra Pradesh (Devi *et al.*, 2000).

Srilatha *et al.* (2003) reported an outbreak of fowl cholera in Japanese quail in Thirupathi, resulting in 33.7 per cent mortality.

Isolation of *P. multocida* was attempted by Antony (2004) from 157 suspected samples of avian pasteurellosis originating from ducks and fowl and 25 isolates were obtained from ducks and one from fowl.

Bhattacharya (2005) reported that eight outbreaks of duck cholera in ducks of Tripura. 77.77 per cent of *P. multocida* isolates were recovered from ducklings and 22.22 per cent of isolates were obtained from adult ducks.

2.3 ISOLATION AND CHARACTERIZATION OF *P. multocida.*

Isolation and characterization of *P. multocida* was based on colony characters, cellular morphology, staining and biochemical characters and biotyping.

Adlam and Rutter (1989) reported that in tissues, exudates and recently isolated cultures, the bacteria showed characteristic bipolar staining with connecting strand with Methylene blue or Leishman stain. Cells on repeated subcultures tend to become pleomorphic.

Quinn *et al.* (1994) reported that *P. multocida* produced round, moderate sized grayish non haemolytic and characteristic dew drop like colonies on blood agar after 24 h of incubation at 37° C. Usually *P. multocida* type A selectively produced large mucoid colonies due to their large capsular hyaluronic acid.

Pasteurella multocida are Gram negative, coccobacillary or short rod shaped, usually 0.2-0.4 x 0.6-2.5 μm in size and generally occur singly or in pairs and recently isolated organism show bipolar staining. Freshly isolated *P. multocida* produced smooth, grayish glistening transluscent colonies approximately one millimetre in diameter on blood agar after 24h incubation at 37° C and it does not grow on Mac Conkey agar. Old cultures, especially those grown on media devoid of blood may tend to produce smaller colonies (OIE, 2004).

2.3.1 Isolation

Pasteur (1880) first isolated the causative organism of fowl cholera in pure culture.

Generally visceral organs such as liver,spleen or heart blood of animals and birds, succumbed to acute form of disease and exudative lesions with chronic form of disease were used for isolation. Mouse served as biological medium to recover *P. multocida* from clinical specimens (Rhoades and Rimler, 1989).

Dextrose starch agar supplemented with six per cent chicken serum was used for studying the colony morphology of *P. multocida* by Chung *et al.* (2001).

2.3.2 Media

Ox or sheep blood agar could be used as routine medium for isolation of *Pasteurella* sp. (Quinn *et al.*, 1994).

Casein sucrose yeast agar (CYS) containing five per cent calf blood free of antibodies against *P. multocida* could also be used as medium for isolation (OIE, 2004).

2.3.2.1 *Selective Media For Isolation Of P. multocida*

A selective medium, Clindamycin Gentamicin Tellurite medium (CGM) by incorporating clindamycin (5µg/l), gentamicin (5µg/l), potassium tellurite (2.5µg/l) and amphotericin B (5 µg /ml) in five per cent horse blood agar had been developed by Knight *et al.* (1983).

Smith and Baskerville (1983) also developed a selective medium called 8HPG, which contained ferric citrate, lactose, phenol red, chloral hydrate, bacitracin, gentamicin, polymyxin and amphotericin B selectively to isolate *P. multocida* from nasal specimens of pig.

Warner (1996) developed a Transport Enrichment Medium (TEM) that will inhibit the growth of other bacterial and fungal contaminants. The medium consisted of brain heart infusion agar with amikacin, gentamicin, potassium tellurite and amphotericin B.

Lee *et al.* (2000) developed a selective medium containing sheep blood (10 per cent v/v), polymycin B (0.4µg /ml), crystal violet (1µg/ml) thallous acetate (120µg/ml), bacitracin (0.6 mg/ml) and cycloheximide (50 µg/ml) in dextrose starch agar for the selective isolation of *P. multocida* from chicken alimentary tract.

2.3.3 Biochemical Characterization

Primary biochemical tests that are used for identification of *P. multocida* are catalase, oxidase, production of acid from glucose and oxidative/fermentative utilization of glucose (Cowan, 1974).

All the isolates of *P. multocida* fermented glucose, sucrose, sorbitol and mannitol with the production of acid. None of them fermented lactose, trehalose and salicin (Wijewardana *et al.,* 1986).

According to Adlam (1989), the biochemical characters such as the ability to ferment maltose, dextrin and after several days inositol and the inability to produce indole by *P. haemolytica* made them easily distinguishable from *P. multocida.*

According to Kumar *et al.* (1996), the 43 *P. multocida* isolates of animal and avian origin from India fermented dextrose, mannose, fructose and all were negative for inulin, lactose, salicin, maltose rhamnose, inositol and dextrin.

Differentiation of *P. multocida* from *Riemeralla anatipestifer* was based on indole production, glucose and sucrose utilization and ornithine decarboxylase reactions, while the former was positive for all the three reactions, the latter gives a negative result for all the three tests (OIE, 2000).

Kielstein *et al.* (2001) opined that 107 Pasteurella isolates from porcine respiratory tract were characterized based on phenotypic criteria and biochemical characters.

Papova and Tzvetkov (2002) characterized a *P. multocida* isolate from pheasants based on phenotypic and biochemical properties.

2.3.4 Biotyping

Mutters *et al.* (1985) divided the taxon *P. multocida* into three sub species based on fermentation patterns of dulcitol and sorbitol. The sorbitol and dulcitol positive, variety became *Pasteurella multocida* subsp. *gallicida.* Positive for

sorbitol but negative for dulcitol were *Pasteurella multocida* subsp. *multocida*, and those strains negative for both became *Pasteurella multocida* subsp. *septica*.

Blackall *et al.* (1995) opined that twenty-two field isolates of *P. multocida* from turkeys were grouped into four distinct biochemical types called biovars. Three of these biovars matched the property of *P. multocida* subsp. *multocida*.

2.4 WHOLE CELL PROTEIN PROFILE BY SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Lugtenberg *et al.* (1986) differentiated the porcine isolates of *P. multocida* by the presence of major protein in 34 to 38 kDa region and named it as heavy ('H') protein.

Ireland *et al.* (1991) prepared soluble (boiled Ag) using glycerol, phenyl methyl sulphonyl fluoride and sodium dodecyl sulphate. The protein concentration was determined by using a protein assay kit. They demonstrated that the major difference, between SDS-PAGE profile of avian *P.multocida* serotype 1 isolates in the position of one of the major protein was in 34 to 38 kDa range.

Sonicated cells of *P.multocida* strain P52 on ammonium sulphate precipitation yielded a protein rich material comprising of at least 14 polypeptides in the molecular weight ranging from 30 to 91 kDa and of these the 65 kDa protein was identified as the major band (Srivastava, 1998).

Sulochana (1998) analyzed the SDS-PAGE profile of *P. multocida* and *Riemerella anatipestifer*. The *P. multocida* isolate had unique protein bands of 43, 23 and 10.5 kDa proteins, which were absent in *Riemerella anatipestifer*. Protein bands of 34, 33, 26, 9 and 4 kDa were unique for *Riemerella anatipestifer* but absent in the former strains. Ten other bands were similar to both the strains.

The whole cell protein profiles of the four duck isolates of *P. multocida* *viz.*, DP1, DP6, DP12 and DP25 and the fowl isolates FP1 and LKO when

analysed on SDS-PAGE revealed 20 to 26 protein bands, with molecular weights ranged from 102 kDa to 19 kDa Antony (2004).

2.5 OUTER MEMBRANE PROTEINS OF *P. multocida*

Lugtenberg (1986) and Benz (1988) opined that OMPs showed a low electrophoretic mobility in the form of multiple bands.

Jap and Walian (1990) reported that OMP functioned as molecular sieves to allow the diffusion of small hydrophilic solutes and also served as receptors for bacteriophages and bacteriocin.

According to Roy *et al.* (1994) porins are highly immunogenic and exposing the epitopes on the bacterial surface.

Pati *et al.* (1996) reported that approximately 50 per cent of dry matter of outer membrane of Gram negative bacteria consisted of more than 20 immunochemically distinct proteins. These proteins acted as interface between the bacteria and the host environment.

Some of the major outer membrane proteins (MOMPs) are called porins, porins are channel forming transmembrane protein found in the outer membrane and the outer membrane of Gram negative bacteria contain lipopolysaccharide (LPS), several minor proteins and limited number of major proteins (Jain *et al.*, 2006).

2.6 OUTER MEMBRANE PROTEIN GENES

According to Chevalier *et al.* (1993) OmpH is a homologue of P2 porin of *H. influenzae.* The monoclonal antibody against OmpH could passively protect mice against infection.

Kasten *et al.* (1995) cloned a gene encoding *P. multocida* homologue of P6 and showed it to be present in all sixteen somatic serotypes. The P6. OMP of Haemophilus *influenzae* had been shown to elicit protective immunity in animal model of infection.

Loosmore *et al.* (1997) reported that OMa87 showed high similarity to D15 protective outer membrane protein of *H. influenzae*. The rabbit antiserum against OMa 87 was able to passively protect mice against infection.

Luo *et al.* (1997) cloned the ompH gene and porin activity of ompH was demonstrated experimentally.

The gene encoding the OMa87 was cloned and characterized (Ruffolo and Adler, 1998).

Bosch *et al.* (2001) reported that treatment of bacterial culture with chelating agents such as 2,2' dipyridyl (DPD) induced expression of iron regulated genes.

2.7 EXTRACTION OF OUTER MEMBRANE PROTEINS

Several workers have developed various techniques for the extraction of OMPs of *P. multocida.*

Barenkamp *et al.* (1981) extracted the OMP of *H. influenzae* type b by sonication, followed by treatment with sodium lauryl sarcosinate. The protein concentration was determined by Lowry's method.

Lugtenberg *et al.* (1986) reported isolation of cell envelopes by differential centrifugation, disrupting the stationary phase cells by sonication. OMPs were extracted with Triton X-100 in the presence of 10 mM $Mgcl_2$.

Lu *et al.* (1988) extracted OMP from *P. multocida* by treating the cell extract with hyaluronidase, DNase and RNase after disrupting the cells by French pressure cell and finally subjecting them to sucrose gradient centrifugation.

Choi *et al.* (1989) extracted the OMP of *P. multocida* using two per cent sodium lauryl sarcosinate

Ireland *et al.* (1991) prepared soluble (boiled Ag) using glycerol, phenyl methyl sulphonyl fluoride and sodium dodecyl sulphate. The protein concentration was determined by using a protein assay kit.

Avian *P. multocida* cells were treated with proteinase K and phenyl methyl sulphonyl fluoride before passing through French pressure cell, then by adding sodium lauryl sarcosine and on centrifugation, the OMP could be recovered (Snipes *et al.*, 1988; Kennet *et al.*, 1993).

Confer *et al.* (1996) used sucrose density gradient centrifugation for sub cellular fractionation of *P. multocida* cultures.

Outer membrane proteins were prepared by mixing envelopes in sodium N lauryl sarcosine in 0.01M Tris buffer. The insoluble fraction was judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Confer *et al.*, 1996).

Morton *et al.* (1996) used two different techniques for separation of OMP, *viz.*, sucrose gradient ultra centrifugation and sarkosyl insoluble OMP separation. It was found that sarkosyl extraction was more effective and less laborious than sucrose gradient centrifugation method and proved that more OMP could be recovered using sarkosyl method of OMP extraction.

Ammonium sulphate precipitated proteins of *P. multocida* serotype 6:B was extracted by ion exchange chromatography. This method utilized DEAE sepharose CL-6B and sodium chloride gradients to elute the fractions (Srivastava , 1999).

Rajalakshmi (2001) prepared OMPs *P. multocida* from four avian isolates using the protocol of Barenkamp *et al.* 1981.

Outer membrane proteins from both *P. multocida* wild and fur strains were extracted (Bosch *et al.,* 2001) by using 0.1 M sodium acetate/0.2 M lithium chloride. The protein concentration of OMP was determined by Lowry's method.

Chawak *et al.* (2001) extracted OMP from *P. multocida* by growing cells in brain heart infusion agar (BHIA) added with 100 µM 2.2' dipyridyl and without 2.2' dipyridyl. Two per cent sodium lauryl sarcosine was used for the extraction of OMP.

Bosch *et al.* (2002) extracted OMPs of *P. multocida* serotype B:2 using sonication followed by two per cent sodium lauryl sarcosine.

Garrido *et al.* (2003) extracted OMP from *P. multocida* wild type and also the strain grown in presence of iron chelator 2.2' dipyridyl using 0.1M acetate buffer or 0.2 M lithium chloride.

Antony (2004) used 0.5 per cent sodium lauryl sarcosine for the extraction of OMP from *P. multocida* as per the method described by Davies and Donachie 1996.

Outer membrane proteins were prepared by growing *P. multocida* B:2 in brain heart infusion broth (BHIB), with or without 100 mg/ml of 2.2' dipyridyl and two per cent sodium lauryl sarcosine was used for the extraction of OMP (Wasnik *et al.*, 2004).

Filia *et al.* (2006) extracted OMP by growing *P. multocida* B:2 on BHIA as normal medium, BHIA supplemented with 2.2' dipyridyl (20µg/ml) as iron deplete medium and BHIA supplemented with 2.2' dipyridyl and ferric chloride ($FeCl_3$) 200µM as replete medium, as per the method described by Kim *et al.* 1991.

2.8 OUTER MEMBRANE PROTEIN PROFILES OF *P. multocida*

The outer membrane protein of Gram negative bacteria has a role in disease process as it act as interface between the host and pathogen. OMPs of *P. multocida* play a significant role in pathogenesis of pasteurellosis and have been identified as a potent immunogen.

Ikeda and Hirsh (1988) reported prominent iron regulated protein of 84 kDa in capsular type A isolate of rabbit. The poultry isolates revealed two iron regulated proteins of ,44 kDa and 98 kDa.

Using Barenkamp's procedure for extraction of OMPs, Kim *et al.* (1991) isolated OMPs from avian strains of *P. multocida*. They showed that culture medium did not have any significant difference on the OMP profile of the strains, but *in vivo* propagation had an appreciable effect on OMP profile composition, expressing several additional OMP bands at 27, 48, 56, 60, 80 and 94 kDa regions. All reference strains of the 16 serotypes of *P. multocida* except strains representing serotypes, A:1 and B:2 expressed major OMP in the 34.5 kDa and 38 kDa regions.

Ireland *et al.* (1991) reported that protein profiles of sonicated antigens of *P. multocida* serotype1 isolates from fowl cholera revealed quantitative difference in the molecular weight of protein present in the range of 34-38 kDa. Immunoblotting revealed a major immunogenic polypeptide in the 34 kDa region.

Outer membrane and associated proteins (OMAP) prepared from *P. multocida* grown in iron restricted conditions were superior immunogens compared to iron replete conditions. Coomasie Brilliant blue staining of SDS-PAGE gel showed one major OMAP band with a molecular mass of 35 kDa and three faintly staining OMAP bands at 30 kDa, 23 kDa and 17 kDa (Kennet *et al.*, 1993).

Kasten *et al.* (1995) developed an antibody specific for a 16 kDa OMP of rabbit strain of *P. multocida* and found that it reacted with all 16 somatic serotypes of *P. multocida* as well as vaccine strain CU and M9, indicating that the protein was expressed in all *P. multocida* strains.

Marandi and Mittal. (1995) opined that SDS-PAGE analysis of whole cell antigens of capsular serotype A, B:1, E and F indicated that molecular mass of OMPs were 37, 32, 38 and 36 kDa respectively. Capsular type D exhibited 32 kDa and 37 kDa.

Marandi and Mittal (1996) classified *P. multocida* serotype D strains in to two types (I and II), based on the electrophoretic mobility of MOMP's with MW of 32 or 37 kDa.

Pati *et al.* (1996) reported that OMP from *P. multocida* serotype B:2 under SDS PAGE revealed 10 major polypeptide bands ranging from 88 to 25 kDa. Immunoblotting idicated that three polypeptides of 44, 37 and 30 kDa were major immunogenes.

Mahasreshti *et al.* (1997) purified and characterized two major OMPS of *P. haemolytica*, designated as POMA and POMB with molecular weight (MW) of 35 and 32 kDa. They found out that POMA had significant N terminal sequence homology with OMPA protein of *E. coli.* No homology between the N-terminal amino acid sequence of POMB and those of other known bacterial proteins was found.

Chawak *et al.* (2001) reported one extra band with MW of 98 kDa among the avian strains of serotype A.

Kedrak and Opacka (2002) reported additional protein of 102 to 104 kDa and 110 to 112 kDa in bovine strains of serotype B:2 when harvested on medium with an iron chelator.

Tomer (2002) reported that in immunoblotting using rabbit hyper immune serum against P2 strain, the OMP of 37 kDa was the main antigenic protein in all field isolates as well as in vaccine strain.

Dabo *et al.* (2003) cloned and characterized *P.multocida* OMPA-like gene (Pm OMPA). The mature proteins, which had a molecular mass of 35,015 Da, showed significant similarity with *E coli* OMPA protein using immunoblotting

techniques. They showed that Pm OMPA was immunogenic. Their studies also indicated that protein might play a significant role in adherence to host cells.

Hornhuan *et al.* (2004) reported that the most prominent polypeptide bands had a MW of 30, 33 and 45 kDa.

In Iron restricted cultural condition, the buffalo isolates revealed additional iron regulated protein band of MW 102 kDa. (Jain *et al.*, 2006).

Filia *et. al.* (2006) reported that SDS PAGE analysis of OMPS of *P. multocida* serotype B:2 grown on Brain heart infusion agar (BHIA) and iron replete conditions revealed more than 12 protein bands, ranging from 26 to 100 k Da. Iron deplete condition revealed additional two protein bands of molecular MW 101 kDa and 104 kDa.

Arora *et al.* (2006) reported that 32 kDa protein band was immuno dominant along with 25 kDa in all the 17 *P multocida* isolates.

2.9 ELECTROPHORETIC CHARACTERIZATION

2.9.1 Western blot

This technique was first developed by Towbin *et al.* (1979) for transfer of ribosomal protein from polyacrylamide gel to nitrocellulose paper and the original band pattern was obtained with no loss of resolution. This technique is popularly known as Western blot. It can detect protein in nanogram quantities.

Employing Western blot analysis Lu *et al.* (1988) confirmed that the 27 k Da, 37.5, 49.5, 58 and 64.4 kDa proteins were the major outer immunogens of *P. multocida* in rabbits.

Kim *et al.* (1991) demonstrated that the *P. multocida* A:3 possessed iron regulating outer membrane protein 76, 84 and 94 kDa as revealed by immunoblotting using convalescent sera from turkey. These proteins were considered as protective.

Chawak *et al.* (2001) extracted OMP from *P. multocida* grown in iron sufficient media and iron restricted media were characterized by SDS- PAGE and immunoblotting. Immunoblot analysis revealed 17, 25.7, 32, 44.2, 49, 58.9 and 66.8 kDa polypeptide to immunogenic proteins, of which 17 and 25.7 kDa proteins were found to immuno dominant proteins.

Western blotting for hyperimmune serum against fowl isolate FP1, raised in rabbits, a 37.15 kDa outer membrane protein was found to be the major antigenic protein by Antony (2004).

2.10 PATHOGENICITY

Pasteurella septica is found in a wide variety of animals and birds and has its main habitat in the respiratory tract (Smith, 1955).

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

Carter *et al.* (1967) opined that strains of *P. multocida* from different sources varied in their virulence for experimental animals as well as natural hosts.

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.

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

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 of 10^8 organisms. Out of 22 isolates, eight killed mice in two to seven days and rest fail

to do so indicating that many of the isolates were normally avirulent for mice. The bacteria were re isolated from liver and spleen of dead mice.

According to Kumar *et al.* (1996) the *P. multocida* isolates were pathogenic to mice when injected intra peritoneally.

Dutta *et al.* (2001) conducted pathogenicity test in mice, by injecting 0.2 ml of 18 h broth culture. The strain was observed pathogenic to mice and the organisms were re isolated from heart blood and spleen.

Kapoor *et al.* (2004) tested the pathogenicity of *P. multocida* A:1 in four mice. They observed that the mice died with in 24-36 h which proved that all the isolates were pathogenic.

2.11 LETHAL DOSE $_{50}$

Fifty per cent lethal dose (LD_{50}) was described as a practical and reliable measurement of pathogenicity by Cruickshank *et al.* (1975).

Mukkur (1977) determined LD_{50} of *P. multocida* of bovine origin in mice as $5.0\pm2.1x10^4$ CFU by intranasal inoculation and 5.0±2.8 CFU by intra peritoneal inoculation.

Swamy (1994) reported that the LD_{50} of duck isolates estimated in mice and was found to be $10^{-7.83}$, which contained 14.96 viable cells per dose.

LD_{50} of *P. multocida* A:1 was estimated in mice and it was found to be 14.32+0.083 CFU (Ramanatha, 1994).

Jayakumar, (1998) determined that the LD_{50} of the *P. multocida* A:1 isolate in one month old ducklings and six month old ducks when inoculated subcutaneously to be 23 and 32 cells respectively.

Goto *et al.* (2001) estimated the LD_{50} both in quail and mice by intravenous inoculation with *P. multocida* A:1 and was found to be $4.3x10^4$ CFU and $3.9x10^2$ CFU respectively.

In hypocuprotic mice the LD_{50} of *P. multocida* (P52) strain was found to be 0.5 ml of 10^{10} dilution of an 18h broth culture while in control it was found to be 10^{-8} dilution (Soodan *et al.*, 2003)

Rajagopal (2007) determined the LD_{50} of DP1 in one month old ducklings, when inoculated intramuscularly were found to be 23 cells.

2.12 VACCINE STUDIES

Several types of vaccines ranging from modified live vaccines to subunit vaccines had been tried for pasteurellosis. The use of modern molecular techniques can produce new and improved vaccines.

Carter (1950) compared the immunity produced in mice by broth bacterins and chicken embryo vaccine made from *P. multocida* type 1 and showed that chicken embryo vaccine was superior over the broth bacterins and he insisted the use of virulent capsulated organisms for the preparation of fowl cholera vaccine.

Bacterin of *P. multocida* was prepared by pooling several isolates from various sources (Bolin *et al.*, 1952). These pooled bacteria were maintained in broth 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.

Iyer *et al.* (1955) reported that 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*.

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

Heddleston and Rebers (1972) found that fowl cholera bacterin prepared from infected embryonated turkey eggs when inoculated by oral/intramuscular

route induced immunity in turkey against infection with homlogous or heterologous serotypes of *P. multocida.*

Baba (1977) reported that 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. Further, he demonstrated that the ribonuclease treatment resulted in sixty per cent loss of immunological activity of the ribosomal preparation.

Stone *et al.* (1978) studied the influence of the composition of water in oil emulsion on their physical characteristics. In order to prepare vaccine, the aqueous phase of emulsion was mixed with nine volumes of mineral oil and one volume of oil phase, homogenized at 18,500 to 19,000 rpm for 30 seconds. Oil emulsion vaccine against New castle disease thus prepared had low viscosity, was stable for more than 12 weeks at 37° C and induced a marked immune response in chicken.

Comparative efficiency 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.

Kodama *et al.* (1982) reported that the saline extract antigen and LPS-protein antigen consistently conferred protection comparable to that of oil emulsion bacterin, whereas potassium thiocyanate and sodium salicylate extracted fractions showed less consistent immunogenicity in turkeys against *P. multocida.*

Lu *et al.* (1987) employed potassium thiocyanate extract as vaccine to *Pasteurella* infection in rabbits and protection was manifested by reduction in colonization of virulent *P. multocida,* lesion development and mortality. They suggested intra nasal vaccination as it elicited persisting Ig G and Ig A antibodies while intramuscular route elicited Ig G only.

Glisson *et al.* (1990) evaluated seven different injection sites in turkeys for *P. multocida* bacterin by measuring the immune response and local tissue reaction. Injections into ventral surface of the tail or subcutaneously along the dorsal mid line of the neck were the most suitable procedures. There were no differences in the immune response elicited irrespective of injection sites. The sites preferred when the ease of injection was concerned were the breast, tail and leg.

Heat inactivated crude capsular extract, potassium thiocyanate extract and sonicated antigens were used to immunize mice against *P. multocida.* The results indicated that irrespective of the type of antigen used, protection was conferred to vaccinated animals against homologous challenge, sonicated antigen conferred the maximum protection when compared to other immnogens (Manoharan *et al.*, 1997).

Kasten *et al.* (1997) reported that recombinant P6 like protein (rP61P) of *P. multocida* cloned in baculovirus expression system was used as vaccine via, subcutaneous route in turkeys and was found that parenterally administered recombinant protein vaccine was incapable of protecting turkeys from avian cholera as mortality observed was hundred per cent on contact challenging.

On comparing he saline extract vaccine was compared with *P. multocida* bacterin vaccine in mice and goat, Sharma *et al.* (1999) observed that the saline extract vaccine was found to be superior in terms of protection (83 per cent Vs 66 per cent) and PHA titre.

Marchart *et al.* (2003) produced bacterial ghosts, which were empty cells devoid of cytoplasmic and genomic material from *P. multocida,* by expression of phage Phi X 174 lysis gene E. These ghosts when used for immunization of rabbits and mice by subcutaneous route provided dose dependent protection ($1.15x10^8$ ghosts provided 100 per cent cross protection) against homologous challenge dose of up to 60 CFU (LD_{50}) and the results projected ghosts as new vaccine candidate.

Rajagopal (2007) compared the immunizing efficiency of oil adjuvanted ordinary bacterin, oil adjuvanated capsule enhanced bacterin and oil adjuvanated biofilm vaccine. The oil adjuvanated biofilm vaccine was found to be superior in terms of protection and PHA titre than others.

2.13 IMMUNOGENIC SUBUNITS OF *P. multocida* AS VACCINE

Confer *et al.* (1996) exhaustively reviewed the immunogenic antigens (immunogenes) of *P. multocida*, which included capsule, LPS and OMPs. Determination of the immunogenic role of non-LPS such as capsule, OMP and "*in vivo*" expressed Ag could be critical to understand immunity to fowl cholera.

Krishnamoorthy *et al.*(1996) extracted OMP, LPS and capsule of avian strain of *P. multocida and* compared the immunogenicity of these subunits with whole cell vaccine. It was concluded that the OMP was immunogenic, followed by LPS and capsule.

Sreevatsan *et al.* (1996) evaluated three experimental subunit vaccines, which included IROMP, LPS with Leukotoxin and capsular proteins against pneumonic pasteurellosis in cattle. This study demonstrated that the leukotoxin and IROMP present alone or in combination in vaccine preparation developed protective immune response in experimental pneumonic pasteurellosis.

Adler *et al.* (1999) described the vaccine antigens of *P. multocida*, which included OMPs, IROMPs, type IV fimbriae, capsule and iron transport protein. Whole cell bacterin could provide only shorter duration of protection against the homologous serotype. But the cross protective antigens expressed by "*in vivo*" conditions could induce protection against heterologous serotypes.

2.14 OMP VACCINE

Snipes *et al.* (1987) reported that the strains of *P. multocida* of avian origin expressed high molecular weight OMPs where in turkey plasma or in BHI broth containing iron chelator, 2,2' dipyridyl. Those OMPs were referred to as iron regulated OMPs. These IROMPs expressed in the medium containing the

2,2' dipyridyl (20 µg/ml) were similar to that of cross protective factors produced by the bacterium that were propagated in turkey plasma.

Kim *et al.* (1991) reported that *P. multocida* under iron restricted conditions expressed high molecular weight OMPs (94 and 88 kDa) called IROMP, which might be involved in inducing cross protective immunity against pasteurellosis.

Glisson *et al.* (1993) extensively studied the ability of expression of high molecular weight OMP (cross protective factor) of avian strains, of *P. multocida* in iron depleted medium. This cross protective factor induced heterologous serotype protection.

Kennet *et al.* (1993) found that the vaccination of cattle with IROMPs of serotype B:2 markedly increased protection against experimental infection with strain causing Haemorrhagic septicaemia.

Zaho *et al.* (1995) demonstrated the expression of iron regulated outer membrane proteins by porcine strains of *P. multocida* and found that the OMP profile differed significantly from IROMPs which contained two additional proteins of molecular weight (96 and 104 kDa).

Pati *et al.* (1996) and Srivastava, (1998) reported that OMPs of *P. multocida* were immunogenic and protective to buffalo calves and rabbits respectively.

Ruffolo *et al.* (1998) reported that the *P. multocida* in the iron limiting condition more often expressed a number of important virulent factors which included toxins, haemolysin and OMPs. It was reported that under iron limiting condition with 2,2' dipyridyl, three novel OMPs with apparent molecular weights of 94, 88 and 80 kDa, which were not present in the OMPs of *P. multocida* grown in iron-repelete conditions, were obtained and concluded that these OMPs expressed in iron limiting conditions were the protective fractions in mouse model. The antibodies directed against IROMPs involved in iron uptake might

block important receptors, preventing the uptake of essential iron, thus starving the bacteria for iron by rendering it from non available.

Srivastava (1999) reported that vaccine that was prepared from the *P. multocida* serotype B:2 grown under iron limiting condition was more immunogenic.

Chawak *et. al* (2001) extracted OMPS were from the organism group in iron restricted medium revealed as additional protein of 97.8 KDa which was found to be immunogenic. They also compared the efficacy of oil adjuvant whole cells (formalin killed) grown in iron sufficient or iron restricted conditions and OMPs extracted from them, assessed by determining the humoral and cellular response and resistance to challenge with homologous and heterologous strains. The antibody response was almost similar with all the four vaccines. The result indicated that 66.6 per cent protection rate against heterologus challenge in birds vaccinated with OMPS of *P. multocida* grown in iron restricted medium and 33.3 per cent protection conferred by whole cell grown in iron restricted medium.

Kedrak and Opacka (2002) evaluated subunit vaccines comprising IROMPs of *P. multocida* serotype A: 1 in calves and found that antibodies against IROMPs could be detected as early as seven days, which increased up to 14 days post vaccination.

Wasnik *et al.* (2004) in a comparative study of OMP vaccine and whole cell vaccine using *P.multocida* B:2 grown in with or without iron chelator, in rabbits found that OMP to be more immunogenic than other two vaccines and excellent cross protection was conferred by whole cell vaccine employing dipyridyl grown cells.

Hornhuan *et al.* (2004) reported that OMPs associated with virosome and ISCOMES gave protective index of 100 per cent than the whole cell vaccines.

Filia *et al.* (2006) prepared whole cell vaccine against Haemorrhagic septicaemia under three different growth conditions (normal, iron deplete, iron

replete) along with commercially available alum precipitated vaccine. The immune response was monitored by ELISA and micro agglutination test and showed continuous rise in antibody titre from day 0 to day 21. There was no significant difference in results between different vaccine groups in vaccinated rabbits.

2.15 PATHOLOGICAL LESIONS IN EXPERIMENTAL *PASTEURELLA* INFECTION IN DUCKLINGS/DUCKS

Schlink and Olson (1986) after challenging vaccinated birds by drinking water route observed lesions which predominated to be pneumonia, arthritis, sinusitis, pericarditis and septicaemia. They also observed that vaccinated birds revealed more of arthritic conditions, especially of hock joint, than the septicaemia in case of unvaccinated birds.

Rhoades and Rimler (1991) reported that during the course of pasteurellosis in poultry most of the postmortem lesions were associated with vascular disturbance which included subepicardial and subserosal haemorrhage in lungs, abdominal fat and intestinal mucosa and increased amount of pericardial and peritoneal fluid. The liver of affected birds revealed multiple small focal areas of coagulative necrosis.

Jayakumar (1998) observed postmortem lesions such as petechial haemorrhage throughout internal organs, whitish necrotic foci on liver and spleen and hydropericardium after experimental infection in one month old ducklings and six month old ducks.

Necropsy lesions in natural duck cholera infection included petechial haemorrhage on pericardium, enlarged liver with pin point necrotic foci on the surface and severe haemorrhages on the serosal surface of the proventriculus, intestine and inner surface of the abdominal wall (Pehlivanoglu *et al.*, 1999).

Arshad *et al.* (2003) described the following lesions after experimental *Pasteurella* infection in chicken *viz.*, pinhead necrotic foci in liver, pericarditis

and active inflammation at the injection sites. In survivors, air sacculitis, sternal bursits, erosive lesions, petechiation and discolouration of muscle were observed.

Shilpa *et al.* (2005) reported that challenge infection of vaccinated and control birds revealed gross lesions like petechiae on heart and thighs. Congestion of lungs, liver, spleen and kidneys, pericarditis and mottling of liver and spleen.

Filia *et al.* (2007) observed during the necropsy of buffalo calves of control group revealed petechial haemorrhages in the abdominal viscera, liver and epicardial muscles of heart. Heart blood revealed typical bipolar organisms on methylene blue staining.

2.16 ASSESSMENT OF IMMUNITY

Haemagglutination test was used for identification of serotypes of *P. multocida* and the technique involved saline extractions of capsular antigen followed by adsorption of the extract on to human "O" erythrocytes. Appropriate dilution of type specific serum produced agglutination of erythrocytes by (Carter, 1955).

Neter (1956) in his view on bacterial haemagglutination stated that passive haemagglutination test was considerably more sensitive than other serological tests since agglutinins undetected by other tests could be detected by this method.

Sawada *et al.* (1982) while performing indirect haemagglutination test (IHA) for detection of anti-pasteurella antibody used gluteraldehyde fixed sheep RBC (GA-SRBC) sensitized with crude capsular extract. This GA-SRBC was found to be stable for at least six months at 4°C and antigen-sensitized GA-SRBC could be used for at least one week without any loss of agglutinability by homologous serum.

Enzyme linked immuno sorbent assay (ELISA) was almost sensitive as that of IHA for measurement of antibody titres post vaccination with *P. multocida* and the measured antibody titres by both the methods correlated well. Following

and the measured antibody titres by both the methods correlated well. Following primary vaccination with CU strains antibody titres peaked around 20 days PV (Solano *et al.*, 1984).

Schlilnk and Olson (1986) noted a significant increase in titre one week after vaccination with CU and average titre remained higher than control until 13 and 15 weeks PV. The serum anti-pasteurella antibody titres were the lowest before vaccination with the average geometric mean titre (GMJ) being 1.58 and after one-week post vaccination GMT peaked to 3.79.

. Pati *et al.* (1996) opined that buffalo calves vaccinated with OMP vaccine or a commercial haemorrhagic septicaemia oil adjuvant vaccine developed highest mean $\log_{[10]}$ ELISA titres day 21 PV. The antibody titre detected using indirect haemagglutination assay were lower than ELISA titres. A passive mouse protection assay revealed the maximum protection.

Highest antibody titre of 128 was observed at day 20 PV with oil adjuvant vaccines and there was a decline in the antibody level to the lowest titre of eight, which was recorded on 80^{th} day (Jayakumar, 1998).

Humoral immune response as assessed by employing IHA and ELISA while the cellular immune response was studied using lymphocyte transformation assay. They observed good correlation between IHA and ELISA value and it was again correlated with protection rates. They employed sonicated antigen at a dilution rate of 1:4 for IHA test and cell mediated immune response was found only a limited role in anti-pasteurella immunity (Chawak *et al.*, 2001).

Islam *et al.* (2004) opined that passive haemagglutination antibody titres in ducks immunized with fowl cholera vaccine were found significantly increased post vaccination (PV) (124.6±52.83) and post challenge (263.11±99.8), in comparison to pre vaccination (5.60±1.96) values. Total leukocyte count (TLC) also significantly increased PV and post challenge.

in pooled sera increased from day 14 PV reacting a peak at day 28 or 35 followed by a decline at day 50 and further decline at day 90 PV.

Shilpa *et al.* (2005) carried out micro titre agglutination test to evaluate antibody titres on PV pooled serum. Formalin killed vaccine group showed an increase in titre from day zero till day 28 and highest titre observed was 80. Outer membrane protein and commercial vaccine group had low titre on 20 and 28 days PV. There was no strict correlation between levels of agglutinating antibodies and protection conferred.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Tarsons and Genei brand plasticware were used in this study. All chemicals used were of molecular biology grade, obtained from Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). Ready-made media were procured from Hi-Media Laboratories Private Limited, Mumbai, unless otherwise mentioned.

3.1 BACTERIA

The *Pasteurella multocida* A:1 strain (DP1) isolated from Niranam Duck farm (Pathanamthitta district), serotyped at IVRI, Izatnagar and maintained in the Department of Veterinary Microbiology, COVAS, Mannuthy was used for the entire study.

Purity of the isolate was checked based on morphology, cultural and biochemical characteristics as described by Barrow and Feltham (1993).

3.2 IDENTIFICATION

The bacterial isolate were identified based on morphology, cultural characteristics, tests for catalase and oxidase, growth on Mac Conkey's agar, haemolysis on blood agar, indole production, methyl red and Voges Proskauer reactions, urease activity, H_2S production, nitrate reduction, citrate utilization, lysine and ornithine decarboxylase activities, and production of acid from carbohydrates (glucose, galactose, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, dulcitol, sorbitol, trehalose, xylose and arabinose) as described by (Barrow and Feltham, 1993). The blood and tissue impression smears prepared from the ailing/dead birds were stained by Leishman's staining technique and examined under oil immersion objective of the microscope for the presence of bipolar organisms.

3.3 PATHOGENICITY TESTING OF ISOLATE

3.3.1 *Materials*

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

3.3.2 *Method*

Each mouse was inoculated intra-peritoneally with 0.1 ml of inoculum containing 3 x 10^8 organisms per ml in sterile normal saline. A control mouse was injected with 0.1 ml of sterile saline. A total of six mice were used. All the animals were observed for signs of infection. Blood smears were prepared from the dead mice and stained with Leishman's stain. Re-isolation of *P. multocida* from heart blood, lung, liver and spleen of the dead mice was carried out.

3.4 WHOLE CELL PROTEIN PROFILES OF *P. multocida*

3.4.1 Materials

The *Pasteurella multocida* A: 1 strain (DP1) isolated from Niranam Duck farm (Pathanamthitta district), serotyped at IVRI, Izatnagar and maintained in the Department of Veterinary Microbiology, COVAS, Mannuthy was used for the entire study.

3.4.2 Extraction of Whole Cell Proteins

3.4.2.1 *Materials*

Antigen preparation buffer

Tris (0.1M)	1.200 g
Phenyl methylsulphonyl fluoride	0.034 g
Glycerol	15.00 ml

Sodium dodecyl sulphate 2.000 g

pH was adjusted to 6.8 and the volume made up to 100 ml with distilled water.

3.4.2.2 *Method*

Whole cell proteins were prepared as per the method of Ireland *et al.* (1991). Pure cultures of *P. multocida* grown on bovine blood agar plates at 37°C for 18 h were harvested in antigen preparation buffer. The cell suspensions were mixed, placed in a boiling water bath for five minutes, centrifuged at 10,000 x g for three minutes and the supernatant collected. The protein concentration of the preparations was determined by modified Lowry's method using a bovine serum albumin (BSA) standard (Markwell *et al.*, 1978).

3.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.4.3.1 *Materials*

3.4.3.1.a Acrylamide-bisacrylamide stock (30 : 0.8)

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water to make	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C.

3.4.3.1.b 1.5 M Tris pH 8.8

Tris base	181.7 g
Distilled water to	1000 ml

Adjusted pH to 8.8 with 4N HCl. and stored at 4°C.

3.4.3.1.c 0.5 M Tris pH 6.8

Tris base	60.6 g.
Distilled water to	1000 ml

Adjusted pH to 6.8 with 4 N HCl. and stored at 4°C.

3.4.3.1.d Resolving gel (12.5 per cent)

Acrylamide : bisacrylamide (30: 0.8)	12.5 ml
Tris hydrochloride (1.5 M) pH 8.8	7.5 ml
Sodium dodecyl sulphate (ten per cent)	0.3 ml
Ammonium persulphate (ten per cent)	0.15 ml
N, N, N, N - tetra methyl ethylenediamine (TEMED)	0.01 ml
Distilled water	9.6 ml

3.4.3.1.e Stacking gel (four per cent)

Acrylamide stock (30.8 per cent)	0.67 ml
Tris hydrochloride (1.5 M) pH 6.8	1.25 ml
Sodium dodecyl sulphate (ten per cent)	0.05 ml
Ammonium persulphate (ten per cent)	25 µl
N, N, N, N - tetra methyl ethylenediamine (TEMED)	2.5 µl
Distilled water	3.00 ml

3.4.3.1.f Electrophoresis buffer

Tris base	3.0 g
Glycine	14.4 g

Sodium dodecyl sulphate	1.0 g
Distilled water to make	1000 ml

3.4.3.1.g Sample preparation buffer (2x)

0.5 M Tris hydrochloride, pH 6.8	2.5 ml
Glycerol	2.0 ml
Sodium dodecyl sulphate (ten per cent)	4.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.5 mg
Distilled water to make	10.0 ml

Distributed in small aliquots and stored at 4°C.

3.4.3.1h Destaining solution I

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water to	1000 ml

3.4.3.1i Destaining solution II

Glacial acetic acid	70 ml
Methanol	50 ml
Distilled water to	1000 ml

3.4.3.1j Coomassie brilliant blue (SRL) staining solution

Coomassie brilliant blue (R250)	0.5 g
Methanol	800 ml

Glacial acetic acid	140 ml
Distilled water to	2000 ml

3.4.3.2 *Method*

The whole cell protein extracts of *P. multocida* were analysed by discontinuous system of polyacrylamide gel electrophoresis (Laemmli, 1970)

Resolving gel solution of 12.5 per cent was prepared and degassed. Ten per cent ammonium persulphate and TEMED were added and poured between two glass plates so as to form a gel of one millimeter thickness and left for polymerization. Distilled water was layered on the top to ensure uniformity of the gel surface. The set up was left overnight at 4°C for complete polymerization. After polymerization, the distilled water was pipetted out and four per cent stacking gel was prepared and poured between the glass plates. The comb was then inserted and the apparatus left as such for complete polymerization for one and half hours. After polymerization, the comb was removed and the wells were washed thoroughly with running buffer. A small quantity of running buffer was added into each of these wells. Twenty micrograms of the sample was mixed with equal volume (10 µl) of sample preparation buffer and kept in boiling water bath for five minutes. The samples were loaded into individual wells under the column of buffer in each well. Standard high range molecular weight marker (GENEI) was loaded in one of the wells. The glass plates were fixed onto the vertical slab gel electrophoresis apparatus and electrophoresis buffer was carefully poured into the top and bottom reservoirs and electrophorosed at 10 mA constant current till the bromophenol blue marker reached near the bottom of the resolving gel. The gel was then removed from the glass plate, the stacking gel was snipped off and transferred to a Petri plate containing Coomassie brilliant blue staining solution for two to three hours. The gels were then destained till the background became clear and were viewed in white light and photographed.

3.4.3.3 Estimation of Molecular weight

The molecular weights of the different protein fractions were obtained by comparing the distance migrated by the different fractions with that of the standard marker proteins of known molecular weights. A standard graph was plotted with the $\log_{10}$ Da values of the marker protein on the Y-axis and the mobility of the known bands of the marker on the X-axis. The values of the distance migrated by the sample proteins were interpolated with the standard curve to determine the molecular weight of the polypeptide bands.

3.5 ANALYSIS OF OMP OF *P. multocida*

3.5.1. Extraction of OMP

3.5.1.1 Materials

3.5.1.1a Phosphate buffered saline (PBS) pH 7.2

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate dihydrate	1.06g
Potassium dihydrogen phosphate	0.2 g
Distilled water to	1000 ml

pH was adjusted to 7.2 and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.5.1.1b Tris-HCl 20 mM (pH 7.2)

Tris-HCl	3.15 g
Distilled water to	1000 ml

pH was adjusted to 7.2, autoclaved and stored at 4°C.

3.5.1.1c HEPES (N-2-hydroxyethyl Piperazine N-2 ethane sulphonic acid) buffer, 10mM , pH 7.4

HEPES buffer	0.238 g
Distilled water to	100 ml

pH was adjusted to 7.4, autoclaved and stored at 4°C.

3.5.1.1d Sodium N-lauryl sarcosine (0.5 per cent) (Sarkosyl, Sigma Aldrich, USA)

Five hundred milligram of sodium lauryl sarcosinate was dissolved in 100 ml of sterile 10 mM HEPES buffer (pH 7.4) just prior to use.

3.5.1.2 *Method*

The outer membrane protein (OMP) enriched extract from *P. multocida* was prepared as per the method described by Davies and Donachie, (1996).

The bacterial strains were sub cultured on Brain heart infusion agar containing five per cent defibrinated ovine/bovine blood. Pure culture was inoculated into 10 ml of sterile BHI broth and incubated over night at 37°C.

Two-litre flask containing 400 ml of BHI broth was pre-warmed overnight at 37°C. Overnight grown culture, approximately 0.4 ml was added to 400 ml of pre-warmed medium and incubated for twelve hour at 37°C. Same procedure was repeated using BHI broth added with 100 µM 2,2' Dipyridyl (Hi media). The cultures were placed on ice to stop the bacterial growth.

The bacteria were harvested by centrifuging at 8000 x g for 30 min at 4°C. The bacterial pellet was washed twice in sterile PBS (pH 7.2) and the final pellet was dissolved in seven milliliter of ice-cold HEPES buffer and kept for 20 min (pH 7.2). Bacterial cells were sonicated on ice for 5 min (Branson Sonifier 450) at 12 microns by placing the probe into the suspension to a depth of 1.0-1.5 cm to avoid frothing. The sonicated sample was poured into a fifteen millilitre

centrifuge tube, placed on ice for fifteen minutes and centrifuged at 4000 x g for twenty minutes at 4°C to remove the intact cells and debris. The supernatant was carefully transferred to ten millilitre polyallomer ultracentrifuge tubes and centrifuged at 1,00,000 x g for one hour at 4°C in a Beckman ultracentrifuge to pellet the cell envelopes.

The supernatant was discarded and the pellet was resuspended in seven millilitres of 0.5 per cent sodium N-lauryl sarcosine and kept at room temperature for twenty minutes. The sarkosyl insoluble outer membrane enriched fraction was pelleted by centrifugation at 1,00,000 x g for one hour at 4°C. The pellet was washed twice in distilled water and finally dissolved in 0.5 ml of Tris-HCl 20 mM pH 7.2. About 50 µl of the outer membrane protein was aliquoted into an Eppendorf tube for protein estimation. The remainder was stored at -70°C.

The protein content of the OMP-enriched extract of the isolate was determined by the modified Lowry's method of Markwell *et al.* (1978) using a bovine serum albumin standard.

3.5.2 Analysis of OMPs on SDS-PAGE

The outer membrane proteins of *P. multocida* were subjected to 100°C for ten minutes and analysed by discontinuous system of polyacrylamide gel electrophoresis, as in (3.4.3.2).

3.6 DETECTION OF ANTIGENIC OMPs OF *P. multocida*

3.6.1 Raising of Antiserum against *P. multocida* Whole Cell Proteins

3.6.1.1 *Materials*

3.6.1.1a Brain heart infusion broth

3.6.1.1b PBS pH 7.2 (3.5.1.1a)

3.6.1.1c 0.5 per cent formalin in PBS

Formaldehyde 0.5 ml

PBS pH 7.2 to 100 ml

3.6.1.1d Freund's complete and incomplete adjuvants (Sigma Aldrich, USA)

3.6.1.1e Rabbits

Adult male rabbits (New Zealand White) weighing about one kilogram were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy.

3.6.1.2 *Method*

A pure culture of DP1 was inoculated into 50 ml of BHI broth and incubated at 37°C for eighteen hours. The broth culture was centrifuged at 3000xg for 30 min. The supernatant was discarded and the pellet washed thrice in PBS, pH 7.2. The final pellet was resuspended to a concentration of 10^8 cell per ml in PBS, pH 7.2 containing 0.5 per cent formalin. Formalin was allowed to act for twenty four hours at 37°C.

3.6.1.2a Sterility test

The sterility of the formalinized suspension was checked by streaking 0.2 ml of the suspension on BHI agar and incubating at 37°C for seven days.

3.6.1.2b Preparation of antigen

The formalinized, sterility tested bacterial suspension was thoroughly emulsified with equal volume of Freund's complete adjuvant.

3.6.1.2c Inoculation of rabbits

One millilitre of the emulsified suspension was inoculated by subcutaneous route to each of two rabbits.

A booster dose was given on day fourteen, followed by another dose on day twenty one. The antigens used for booster doses were emulsified with Freund's incomplete adjuvant.

3.6.1.2d Test bleedings

On 28^{th} day the rabbits were bled from the ear vein, the serum separated and tested for presence of specific antibody by agar gel precipitation test.

3.6.3 Testing for Seroconversion by Agar Gel Precipitation Test (AGPT)

3.6.3.1 ***Materials***

3.6.3.1a Gel for AGPT

Agarose	1.0 g
Sodium chloride	0.85 g
Sodium azide	0.01 g
Distilled water to	100 ml

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

3.6.3.1b Agar coated slides

Clean glass slides were coated by smearing 0.5 per cent melted agarose in distilled water and drying in air by keeping the slides horizontally over glass rods.

3.6.3.2 ***Method***

Agar gel precipitation test was done as per the method of Pati *et al.* (1996) with minor modifications. Three millilitres of melted agarose was poured onto precoated glass slides and allowed to set. One central well and five peripheral wells, each with three millimetre diameter were punched in such a way that the distance between the central well and any peripheral well was three millimetres.

Distance between the adjacent peripheral wells was kept equal. The central well was filled with 20 µl each of sonicated *P. multocida*. The peripheral wells were filled with 20 µl of two-fold dilution of the sera samples. The slides were incubated at room temperature in a humid chamber for 24 h and were examined in diffuse light for the presence of precipitin lines.

3.6.4 Western Blotting

3.6.4.1 *Materials*

3.6.4.1a Nitrocellulose membrane (NCM)(Banglore GENEI)

3.6.4.1b Whatmann filter paper No.1

3.6.4.1c PBS pH 7.2 (3.5.1.1a)

3.6.4.1d PBS – Tween

This was prepared by adding 0.5 ml of Tween 20 to one litre of PBS, pH 7.2.

3.6.4.1e Two per cent BSA

Albumin bovine Fraction V (SRL) 2 g

PBS pH (7.2) to 100 ml

3.6.4.1f Blot buffer

Tris 0.1 M, pH 9.	1200 ml
Absolute methanol	80 ml
Distilled water	120 ml

3.6.4.1g Conjugate

Anti-immunoglobulin G horse radish peroxidase (Anti-IgG HRP) conjugate against rabbit raised in goat, obtained from Bangalore GENEI was diluted 1:2500 in PBS, pH 7.2.

3.6.3.1h Chromogen stock solution

50 mg of Diamino benzidine in 100 ml of PBS, pH 7.2

Stored at room temperature

3.6.4.1i Substrate solution

Three milliliter of chromogen stock solution in nine milliliter of PBS, pH 7.2

150 µl of one per cent hydrogen peroxide.

3.6.4.2 *Method*

The *P. multocida* outer membrane proteins were analysed by SDS-PAGE

Duplicate samples were loaded in the wells in the following fashion. Lane one-protein molecular weight marker and lane two, three and four DP1 respectively.

Electrophoresis was stopped when the bromophenol blue dye reached near the bottom of the gel. The gel was cut into two halves. The first half containing lanes one to four was stained in Coomassie brilliant blue staining solution for three hours, then transferred to destaining solution I for forty five minutes or till the background staining had cleared sufficiently. The gels were then transferred to destaining solution II and then photographed.

3.6.4.3 *Transfer of OMPs to NCM*

3.6.4.3a Western blotting

Western blotting was performed following the method of Towbin *et al.* (1979). The second portion of the gel containing lanes 1, 2, 3 and 4 was gently removed and placed on a moistened Whatmann filter paper (No.1).

A large staining glass tray was taken, in the centre of which was placed three micro-titration plates. Over these plates, a glass plate was placed. The glass tray was filled with 400 ml of blot buffer On the glass plate a Whatmann (No.1) filter paper of a size larger than the gel, wetted with blot buffer was placed, whose

ends were immersed in the blot buffer. The gel to be blotted was placed on the filter paper. The NCM soaked in blot buffer for five minutes was placed over the gel. Care was taken to ensure that the correct side of the NCM was facing the gel. Over the NCM was placed a small sheet of wet Whatman (No.1) filter paper. A ten millilitre glass pipette was then rolled over this to ensure that there were no air bubbles trapped between the gel and the NCM. Over the filter paper, stacks of filter paper were placed. On the top of this a glass plate was kept and on which a weight of two kilograms was placed. This set up was left as such overnight for complete transfer.

3.6.4.3b Identification of antigenic OMPs of *P. multocida*

Following complete transfer the NCM was washed in hundred millilitre of distilled water, followed by incubation in blot buffer for one hour at 37°C. This was followed by washing the NCM in PBS-T for one hour at 37°C. The NCM was then blocked by incubation in two per cent BSA in PBS, to prevent non-specific binding, for two hours at 37°C.

The NCM was then washed in PBS-T in a shallow Petri dish with four changes of fifty millilitre each of PBS-T over thirty minutes. The washed NCM was incubated with rabbit anti *P. multocida* serum (1:50 dilution in PBS) overnight at 4°C. This was followed by the washing step described earlier. Goat anti-rabbit HRPO conjugate (1:2500 dilution in PBS) was then added to the NCM and incubated at 37°C for one hour. The NCM was washed as described earlier and was then kept in substrate solution at room temperature with mild rocking until colour developed. After ten minutes the NCM was washed in distilled water and allowed to dry. The dried NCM was photographed.

3.7 LETHAL DOSE 50 (LD_{50}) TESTING IN DUCKLINGS/ADULT DUCKS

3.7.1 Experimental Birds

For the study, unvaccinated one month old ducklings were procured from University poultry and duck farm, Mannuthy.

Lethal dose 50 of *P. multocida* was determined in one month old ducklings as described by Jayakumar (1998).

Pasteurella multocida A:1 strain was passaged in mice to get a fully encapsulated virulent form. The virulent organisms isolated from mice were grown on blood agar at 37°C for twenty four hours. The growth on blood agar was harvested, washed thrice in PBS by centrifugation at 3000 × g for fifteen minutes and resuspended in the same buffer to contain 1.2×10^7 cells/ml. Then serial ten fold dilutions were made upto 1.2×10^0 cells/ml.

One month 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 bacteria with a dose of 0.1 millilitre per bird intramuscularly and the ninth group served as controls which were sham inoculated with 0.1 millilitre of sterile PBS (pH 7.4). Mortality was recorded and all the dead ducklings were examined for specific gross lesions caused by *P. multocida* and attempted re-isolation of the organism on blood agar from heart blood, liver and spleen. The method described by Reed and Muench (1938) was used for LD_{50} calculation.

3.8 PREPARATION OF VACCINE

3.8.1 Organism and growth conditions used

The *Pasteurella multocida* A: 1 strain (DP1) was used for the preparation of vaccine. The organism was grown in three different media *viz.*,

1, Organisms grown in Tryptone soya broth (TSB)

2, Organisms grown in Brain heart infusion broth (BHIB)

3, Organisms grown in BHIB supplemented with 100 µM 2,2' Dipyridyl.

3.8.2 Formalin inactivated oil adjuvanted *P. multocida* bacterins, OMP vaccine (Iron restricted & iron sufficient media)

3.8.2.1 *Formol saline (0.5 per cent)*

Formaldehyde (40 per cent)	5.0 ml
Sodium chloride	8.5 g

Dissolved the sodium chloride in one litre of distilled water and autoclaved at 121°C for fifteen minutes at 15 lbs pressure. Then the solution was cooled to room temperature and added five millilitre of formaldehyde.

3.8.2.2 *Blood agar*

Blood agar was prepared by adding 10 per cent sterile defribinated bovine blood to nutrient agar base at 45°C, following autoclaving at 121°C, 15 lbs pressure for fifteen minutes.

Organisms were grown in three different media *viz.*,TSB, BHIB and BHIB supplemented with 2,2'dipyridyl and incubated at 37°C for 24h and checked the purity by Gram's staining. The culture was harvested by centrifugation at 3000×g, washed thrice in the PBS (pH 7.4) and the OMP suspension and the bacterin were then resuspended in 0.5 per cent formol saline to a concentration of 3×10^9 cells/ml. The culture thus prepared was allowed to stand at room temperature for 48 h with occasional shaking, for the formalin to act.

The innocuity of all the bacterins was tested on blood agar at 37°C for 72h under five per cent carbon dioxide tension.

3.8.3 Liquid paraffin

Light liquid paraffin was pre-sterilized in hot air oven at 160°C for one hour.

3.8.4 Lanolin (Anhydrous) (Nav Niketan Pharmaceuticals, Mumbai)

Lanolin was pre-sterilized in hot air oven at 160°C for one hour.

Vaccines were prepared as per the method of Stone *et al.* (1978). Vaccine emulsions were prepared by combining aqueous and oil phase vaccine components in the presence of emulsifiers. The aqueous phase of emulsion consisted of 15 parts of formalin inactivated bacterin and oil phase was formed of nine parts of sterile light liquid paraffin and one part of sterile lanolin. The aqueous phase was added drop wise to constantly stirred oil phase and homogenized the mixture at 18,500 rpm for 30 sec. Drop test was employed to determine the emulsion type and then stored at 4°C.

3.9 STERILITY TESTING OF VACCINE

3.9.1 Blood Agar (3.8.2.2)

3.9.2 Tryptic Soya Agar

3.9.3 Modified Thioglycollate Medium

3.9.4 Sabouraud's Dextrose Agar

All the media were prepared as per manufacturer's instructions.

The sterility of the prepared vaccines was tested individually in blood agar and tryptic soya agar (TSA) for aerobic bacteria, modified thioglycolate medium for anaerobic bacteria and Sabouraud's dextrose agar (SDA) was used for detecting any fungal contaminant. The media were inoculated with 0.2 millilitre of the vaccine and incubated at five per cent carbon dioxide tension at 37°C for a period of seven days. Sabouraud's dextrose agar was also kept at room temperature and observed for seven days for any growth.

3.10 TOXICITY TESTING OF VACCINES

The toxicity of vaccines was assessed by injecting 0.5 millilitre and one millilitre of vaccine intramuscularly to three ducklings each separately for the vaccines. The injected birds were observed for a period of seven days for any untoward reaction or clinical manifestations.

3.11 VACCINATION PROGRAMME

A total of 120, one month old ducklings were divided into four groups with 30 birds in each group and the first three groups were vaccinated as described below.

3.11.1 Group I

Group I was vaccinated intramuscularly at the thigh region with 0.5 millilitre oil adjuvanated bacterin vaccine prepared from *P. multocida* grown in ordinary TSB.

3.11.2 Group II

Group II was vaccinated intramuscularly at the thigh region with 0.5 millilitre oil adjuvanated OMP vaccine prepared from *P. multocida* grown in BHIB alone (Iron sufficient media).

3.11.3 Group III

Group III was vaccinated intramuscularly at the thigh region with 0.5 millilitre oil adjuvanated OMP vaccine prepared from *P. multocida* grown in BHIB along with 2,2' Dipyridyl (Iron restricted media).

3.11.4 Group IV

Group IV was kept as control which was sham vaccinated with 0.5 millilitre of TSB alone at the thigh region.

3.12 COLLECTION OF SERUM SAMPLES PRE AND POST-VACCINATION

Blood was collected from all the 120 ducklings grouped into four separate groups, on 0, 7, 14, 21, 28, 42 and 60th day PV by cardiac puncture or by jugular venipuncture. The collected blood was allowed to clot and incubated at 37°C for 30 minutes. Serum was separated following overnight incubation at 4°C and stored at -20°C until use.

3.13 PASSIVE / INDIRECT HAEMAGGLUTINATION (PHA / IHA)

3.13.1 Sheep Blood For PHA

3.13.1.1 *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 graduated measuring cylinder and stirred until the chemicals dissolved completely. The volume was made up to one litre with distilled water and finally autoclaved at 121°C, 10 lbs pressure for 15 minutes. The solution was freshly prepared just before use.

Sheep blood required for PHA test was collected in Alsever's solution in the proportion of one part of sheep blood and two parts of Alsever's solution, following sterile precautions and stored at 4°C till further processing.

3.13.2 Whole Cell Ultrasonicated Antigen

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

The organisms grown on brain heart infusion agar (BHIA) were harvested in PBS (pH 7.4) and washed twice by centrifugation at 8000 x g for 20 min at 4°C in the same buffer. The washed cells were resuspended in PBS to contain 3 $\times 10^9$ cells/ml. The bacterial cell suspension held on an ice bath was then disrupted by sonication at 250 V for a total of five minutes with 10 × 30 sec bursts in a sonicator (Branson Sonifier 450) fitted with a 12 mm diameter titanium probe, by placing the probe 1.0-1.5 centimetre deep into the suspension to avoid frothing. The sonication was interrupted for 30 sec between each burst for cooling. The suspension so prepared was centrifuged at 8000 x g for 30 min, the supernatant was collected and sterility was tested by plating 0.2 millilitre on DSA plates and observed for 48 h. The sterile antigen was stored at -20°C until further use.

3.13.3 Fixation of Sheep Red Blood Cells (SRBC)

3.13.3.1 *Gluteraldehyde Solution (25 per cent) (Loba Chemie, Mumbai))*

3.13.3.2 *Gluteraldehyde Solution (1 per cent)*

25 per cent gluteraldehyde solution	40 ml
PBS (pH 7.4)	960 ml

Sheep red blood cells collected in Alsever's solution was fixed using gluteraldehyde. The SRBC in Alsever's solution was washed by centrifugation (650 × g for 20 min) six times with 0.85 per cent saline. After the last wash, the packed cells were resuspended in PBS 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 one per cent (v/v) with PBS and chilled to 4°C. The 10 per cent washed SRBC suspension was mixed with an equal volume of one per cent gluteraldehyde solution and the mixture was incubated at 4°C for 30 min with gentle stirring. The

gluteraldehyde solution was added only drop wise to the SRBC suspension in the ice bath. The mixture was then centrifuged at 650 × g for 10 min at 25°C. The pelleted, fixed cells were suspended in PBS, washed three times with PBS by centrifugation and re-suspended in PBS containing 0.1 per cent sodium azide to yield a 10 per cent suspension. The gluteraldehyde fixed-SRBC (GA-SRBC) was stored at 4°C.

3.13.4 Sensitization of GA-SRBC with whole cell ultrasonicated antigen

3.13.4.1 *Bovine Serum Albumin-PBS (BSA-PBS) With 0.1 per Cent Sodium Azide*

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate	
(Na_2HPO_4. 12 H_2O)	11.32 g
Potassium dihydrogen phosphate	
(KH_2PO_4)	2 g
BSA	2.5 g
Sodium azide	1 g
Distilled water	1000 ml

The solution was autoclaved at 121°C, 15 lbs pressure for 15 min.

A 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 one hour with occasional shaking. The sensitized cells were washed three times in PBS containing 0.25 per cent bovine serum albumin (BSA) and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (v/v).

3.13.5 Indirect/Passive Haemagglutination (IHA/PHA) test

Indirect haemagglutination test was performed to assess the immune status of the birds pre-vaccination and post vaccination. The procedure used was that of Sawada *et al.* (1982).

Serial two fold dilutions of antiserum were made in PBS and 25 µl of the sensitized SRBC was added to 25 µl of the antiserum dilution in U-bottom microtiter plates. The plates were shaken and allowed to stand for one to two hours at room temperature before the SRBC settling patterns were read. The IHA titer was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment), as compared with the pattern of the negative control (smooth dot in the center of the well). Controls consisted of unsensitized SRBC plus test serum and sensitized SRBC plus diluent.

3.14 HOMOLOGOUS CHALLENGE OF VACCINATED BIRDS

Ten birds from each vaccinated group were subjected to homologous challenge by intramuscular route with 0.1 millilitre of inoculum containing 100 LD_{50} of fully encapsulated virulent form of *Pasteurella multocida* serotype A: 1 on 42^{nd} and 60^{th} day PV respectively. The challenged birds were observed for a period of two weeks post challenge for mortality/clinical signs. All the dead birds were examined for specific lesions of pasteurellosis and re isolation of the organism on blood agar from heart blood, liver and spleen was attempted.

3.15 STATISTICAL ANALYSIS

Analysis of variance (one way) of the logarithm of IHA tires of sera collected on different days from the ducks was done to compare the different types of vaccines.

Results

4. RESULTS

Confirmation of the identity of the isolate DP1 was done based on morphology, cultural characteristics and biochemical characters. The isolate produced smooth, convex translucent, grayish white and non haemolytic colonies on blood agar under 5 per cent CO_2 tension. Gram's staining revealed Gram negative coccobacillary organisms arranged singly or in pairs (Fig. 1).

4.1 BIOCHEMICAL CHARACTERIZATION

4.1.1 Primary Characterization

The DP1 isolate was non motile, Gram negative coccobacilli. The isolate grew aerobically and anaerobically, did not grow on Mac Conkey's agar and were non haemolytic on blood agar. It was catalase and oxidase positive and fermented glucose (Table.1).

4.1.2 Secondary characterization

DP1 isolate tested was Indole positive, methyl red and Voges Proskauer negative, urease negative, did not produce H_2S, reduced nitrate, ornithine decarboxylase positive and citrate utilization negative.

With regard to the fermentation of sugars, isolate fermented glucose, galactose, mannose, maltose, trehalose, sorbitol and sucrose but could not utilize dulcitol, lactose, salicin and arabinose (Table. 2).

4.2 PATHOGENICITY TESTING IN MICE

The isolate was able to kill weaned mice, on inoculation with 0.1 ml of $0.3x10^8$ organisms intra peritoneally and subcutaneously. The mice died within eight hours when injected intra peritoneally and within 24 h when injected subcutaneously.

The gross lesions observed in the internal organs of the dead mice were petechiae in the pericardium and congestion of lung, liver and spleen. Fluid

Table.1 Primary Characterization of DP1

TEST	RESULTS
Gram's staining	-
Morphology	Cocco-bacilli
Motility	-
Oxidase	+
Catalase	+
Growth on Mac Conkey's agar	-
Growth anaerobically	+
O/F	+
Haemolysis	-

Table. 2 Secondary Characterization of DP1

TEST	RESULTS
Indole production	+
Methyl red	-
Voges Proskauer	-
H_2S production	-
Citrate	-
Nitrate	+
Ornithine decarboxylase	+
Urease	-
Sugar Fermentation	
Sorbitol	+
Glucose	+
Sucrose	+
Galasctose	+
Mannose	+
Lactose	-
Maltose	+
Arabinose	-
Salicin	-
Dulcitol	-
Trehalose	+

Fig.1 Pure culture of *P. multocida*
(Gram's stain 1000 x)

accumulation was also noticed in the peritoneal cavity of mice when inoculated intraperitoneally. Blood smear and impression smears from spleen and liver following Leishman's staining revealed large number of bipolar stained organisms. Re isolation of *P. multocida* in pure culture was done from the heart blood, lung, liver and spleen on bovine blood agar at 37°C under five per cent carbon dioxide tension.

4.3 WHOLE CELL PROTEIN PROFILE BY SDS-PAGE

Whole cell protein of DP1 isolate of *P. multocida* from ducks was extracted by method described by Ireland *et al.* (1991) and subjected to discontinuous system of SDS-PAGE along with standard protein marker (Fig. 2).

The standard protein marker (Genei, Bangalore) yielded five bands ranging from 97.4 kDa to 18.4 kDa. Examination of the whole cell protein profile of DP1 revealed 20 to 26 protein bands. Their molecular weights ranged from 102 kDa to 19 kDa, as determined by the calibration curve between Rf values and molecular weights of the standard protein markers. Five intensely stained major bands could be located at 85.90, 56.75, 52.23, 45.09 and 35.97 kDa.

4.4 ANALYSIS OF OMPs OF *P. multocida*

4.4.1 Protein concentration of OMPs

The outer membrane proteins of duck isolate of *P.multocida*(DP1) was extracted by the process of sonication and ultra centrifugation as per the method described by Davis and Donachie, (1996).

The protein concentration of the OMP was estimated by method of modified Lowry's method (Markwell *et al.*, 1978). The outer membrane protein concentration of DP1 was found to be 4 mg/ml.

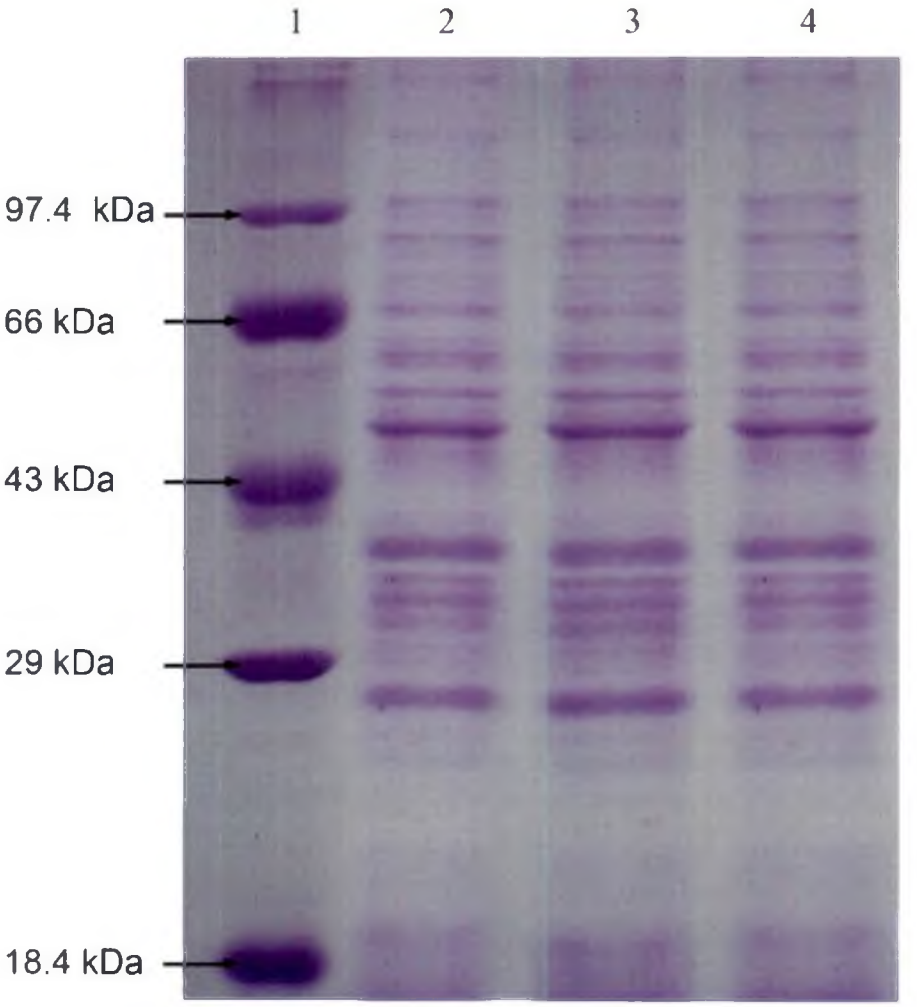


Fig.2 Whole cell protein profile of DP1
Lane 1 Medium range protein molecular weight marker
Lane 2-4 DP1

4.4.2 SDS-PAGE profile of OMP rich extract.

The outer membrane protein extracts and standard protein marker were subjected to SDS-PAGE.

The standard protein marker yielded five bands ranging in molecular weights from 97.4 kDa to 18.4 kDa. Outer membrane proteins extracted from the *P.multocida* grown in iron sufficient media revealed 10 protein bands with approximate molecular weight ranging from 91.84 to 19.02 kDa as determined by the calibration curve between Rf values and molecular weight of the standard protein markers. Molecular weights of the 10 different polypeptide bands were 91.84, 89.74, 79.43, 62.09, 51.05, 44.0, 37.15, 31.33, 26.36 and 19.02 kDa. Two protein bands with approximate molecular weight of 37.15 and 26.36 kDa were thicker than others, suggesting that they were the major outer membrane proteins. OMP extracted from organism grown in iron restricted condition revealed an extra protein band with molecular weight of 97.8 kDa. (Fig. 3).

4.5 DETECTION OF ANTIGENIC OMPS OF *P.multocida*

4.5.1 Agar Gel Immunodiffusion Test

On viewing the gel against diffuse light three precipitin lines, one thick and two faint bands, could be seen. Serum from rabbit number two in which precipitin lines could be seen upto 1:32 dilution was used for immunoblotting.

4.5.2 Western Blotting

Western blotting using rabbit hyperimmune sera showed a 37.15 kDa OMP. Apart from this two other proteins with molecular weights 31.33 kDa and 26.36 kDa, which stained less intensely than the previous one, could also be considered as major antigens. In addition to these three major antigens, protein with a molecular weight of 62.09 kDa that also stained less intensely (Fig. 4).

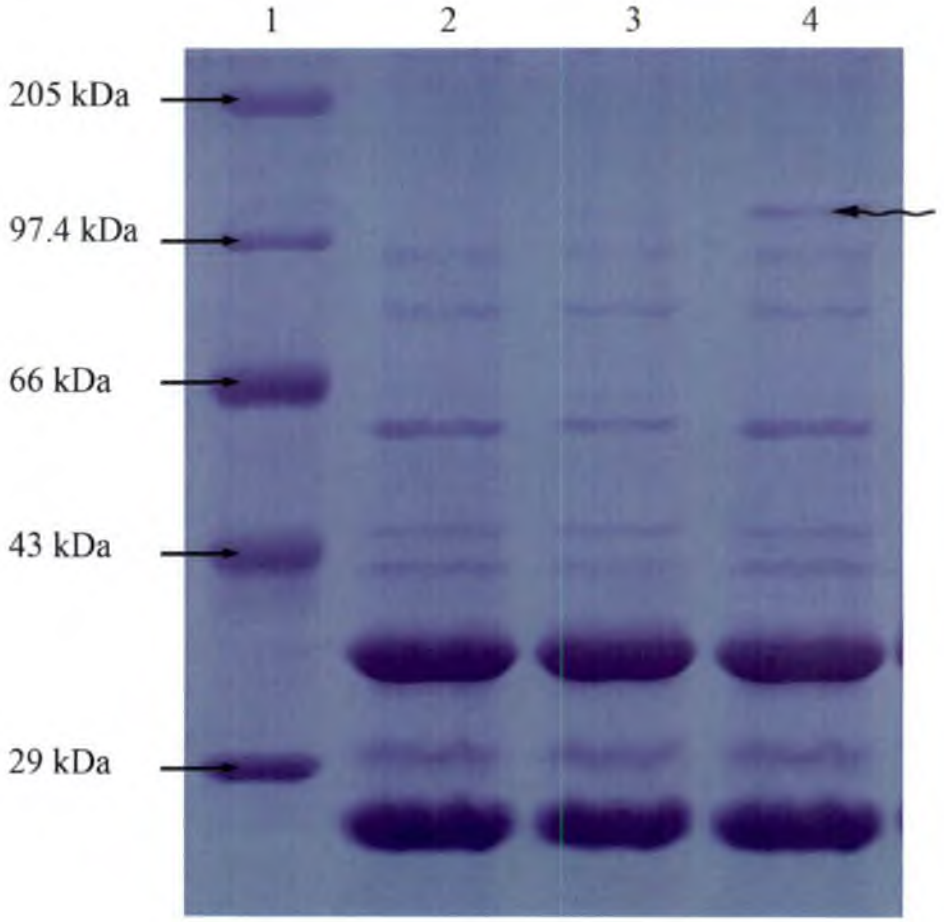


Fig. 3 SDS-PAGE Profile of OMP in iron sufficient and iron restricted media
Lane 1 Medium range protein molecular weight marker
Lane 2-3 DP1 in iron sufficient media
Lane 4 DP1 in iron restricted media

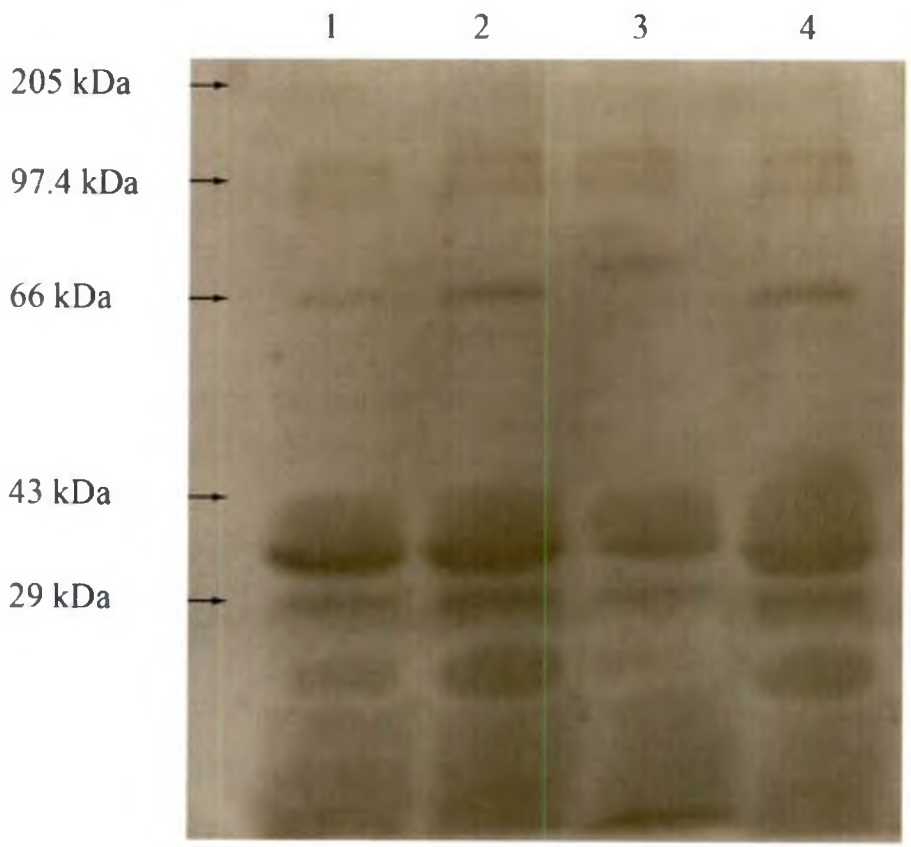


Fig.4 Immunoblots of *P. multocida*
Lane 1-4 DP1

4.6 LETHAL DOSE 50

4.6.1 LD_{50} in ducklings

The LD_{50} of the isolate when tested in one month old ducklings, the dilution giving 50 per cent end point with 0.1 ml subcutaneous dose was $10^{-7.13}$ The results of the experiment are furnished in Table. 3.

4.6.1.1 *Gross Pathological Lesions in Experimentally infected ducklings*

The gross lesions observed in experimentally infected ducklings were haemorrhages on epicardium, serous yellow fluid in pericardium (Fig. 5), pin point and diffuse patchy areas of necrosis on liver (Fig.6), pin point ecchymotic haemorrhages in intestinal serosa and mucosa (Fig. 7) and pulmonary oedema.

Bipolar organisms were detected from blood smears and organ impression smears by Leishman's staining. Colonies suggestive of *P. multocida* were isolated from heart blood, liver and spleen of all the succumbed ducklings on blood agar, following incubation at 37°C under five per cent carbon dioxide tension.

4.7 PREPARATION OF DIFFERENT TYPES OF VACCINES

All the vaccines prepared were homogenous suspensions which were easy for parenteral administration. When drop test was done using the prepared vaccines, the drop was intact and immiscible with water without any spreading.

4.7 STERILITY TEST OF THE VACCINES

All the three vaccines prepared were found to be sterile as no growth was observed in blood agar, Tryptic soya agar (TSA), modified thioglycollate medium and Sabouraud's dextrose agar (SDA), even after seven days of incubation under 37°C and five per cent carbon dioxide tension. Also no growth was detected in the inoculated SDA plates kept at room temperature for seven days.

Table. 3. LD_{50} in Ducklings

Dilution	Organisms present in 0.1 milliliter of inoculum	No. of birds inoculated	NO. died	No. alive	Cumulative value		Ratio +ve	% +ve
					+ve	-ve		
10^{-1}	1.2×10^{7}	6	6	0	39	0	39/39	100
10^{-2}	1.2×10^{6}	6	6	0	33	0	33/33	100
10^{-3}	1.2×10^{5}	6	6	0	27	0	27/27	100
10^{-4}	1.2×10^{4}	6	6	0	21	0	21/21	100
10^{-5}	1.2×10^{3}	6	6	0	15	0	15/15	100
10^{-6}	1.2×10^{2}	6	4	2	9	2	9/11	81.8
10^{-7}	1.2×10^{1}	6	4	2	5	4	5/9	55.5
10^{-8}	1.2×10^{0}	6	1	5	1	9	1/10	10

Proportionate distance = 55.5-50/ 55.5-10= 5.5/45.5= 0.1208

=13 cells

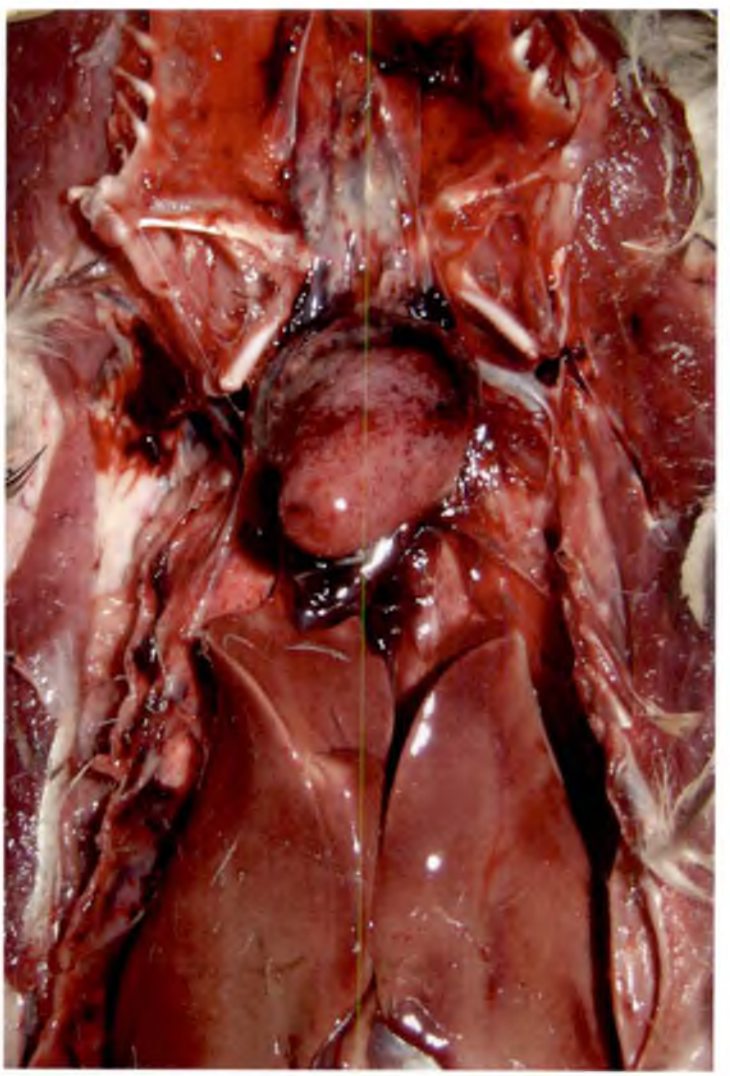


Fig.5 LD_{50} of ducklings - petechiae on epicardium

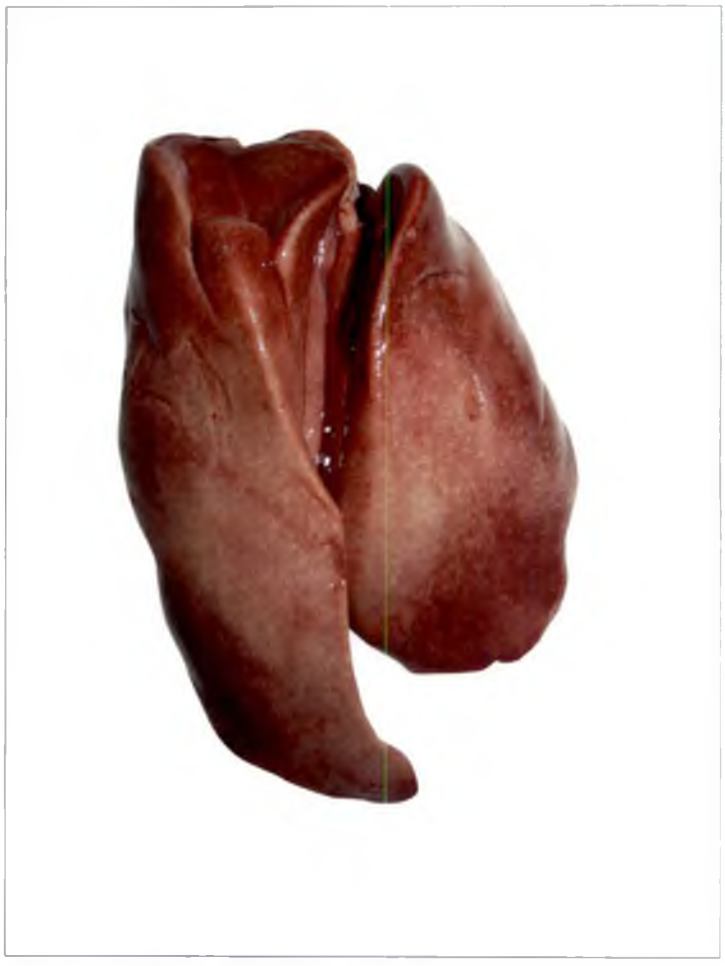


Fig.6 LD_{50} of ducklings - multiple pinpoint necrotic spots on liver

Fig.7 LD_{50} of ducklings - multiple haemorrhagic spots in the intestinal mucosa and serosa

4.8 TOXICITY TESTING OF VACCINES

The toxicity of vaccine was assessesd in ducklings by injecting the exact double dose (one milliliter) by intramuscular route in leg muscle. All the three types of vaccines did not cause any untoward effects except for transient lameness which subsided after one day post inoculation.

4.9 POTENCY TESTING OF THE VACCINE.

4.9.1 Passive /Indirect Haemagglutination (PHA/IHA)

The GA-SRBC could be stored at 4°C without haemolysis for the entire period of vaccine studies (two months) and the sonicated antigen extracted from DP 1, was found suitable for sensitization of fixed SRBC. The sensitized GA-SRBC was intact without loosing its agglutinability for more than 3 weeks under refrigerated conditions.

4.9.1.1 *PHA of Pre-immunization sera*

Some of the pre-immunization sera collected randomly from the birds have shown titres ranging from 1 to 2 $\log_2$.

4.9.1.2 *PHA of post-immunization sera*

Antibodies were detected in post vaccination sera as early as seven days in the first three vaccinated groups and titre increased during subsequent days, though the pattern differed on different days. PHA showing the representative titres of each group on day 14and 60 PV were shown in (fig.8 & 9)

4.9.1.2a Group 1

Titres of most sera on day 7, 14, 21, 28, 45 and 60 PV in ducks once vaccinated with oil adjuvanted ordinary bacterin ranged from 1 to 3 $\log_2$ on day seven and 1 to 4 $\log_2$ on days 14, 21, 28 and 45 PV and 1 to 3 log2 on 60 PV.

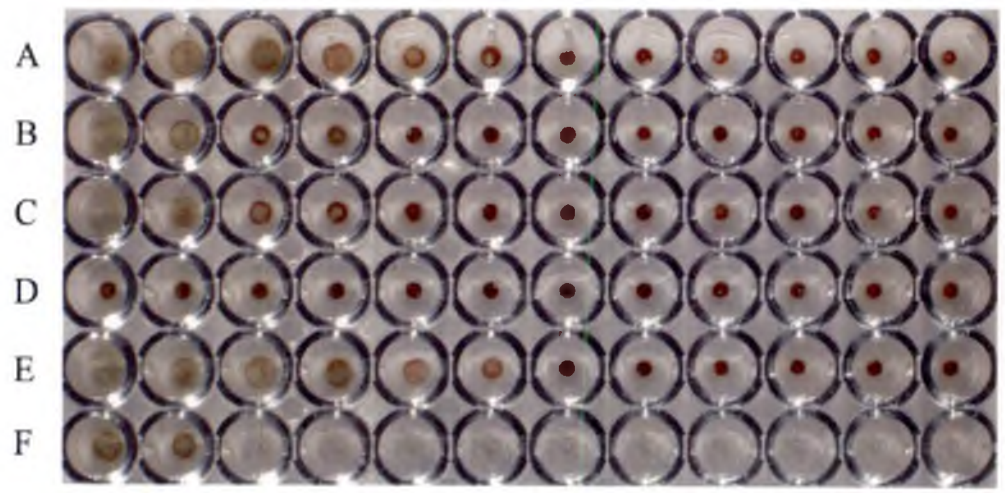


Fig.8 Passive haemagglutination test - day 14 PV

A : Group I

B & C : Group II

D : Negative control

E : Group III

F : Positive control

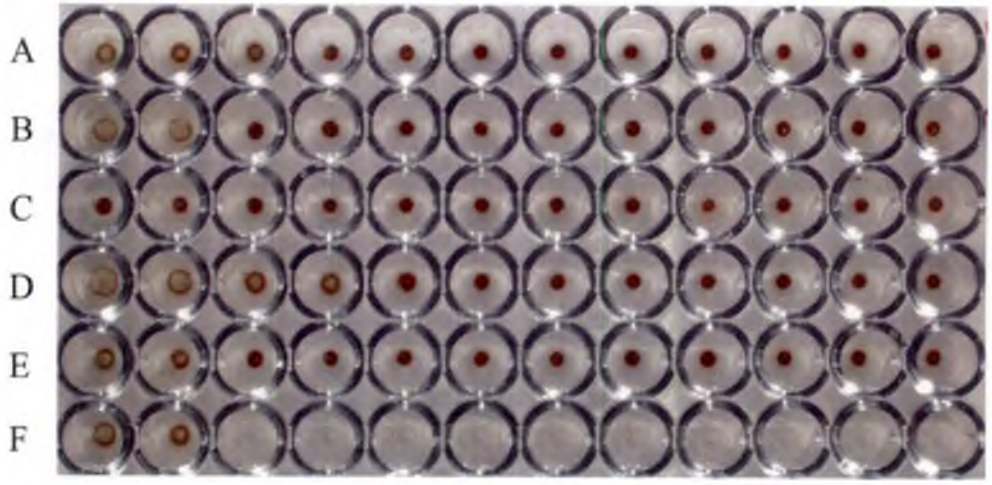


Fig.9 Passive haemagglutination test - day 45 PV

A : Group I

B & E : Group II

C : Negative control

D : Group III

F : Positive control

4.9.1.2b Group II

The IHA titres of group II vaccinated with oil adjuvanted OMP vaccine prepared under iron sufficient medium on days 7, 14, 21, 28, 45 and 60 PV ranged from 1 to 4 log_2, 1 to 5 log_2, 1 to 4 log_2, 1 to 4 log_2, 1 to 2 log_2 and 1 to 2 log_2 respectively.

4.9.1.2c Group III

Titres of the sera on day 7, 14, 21, 28, 45 and 60 PV in ducks once vaccinated with oil adjuvanted OMP vaccine prepared under iron restricted condition ranged from 1 to 6 log_2, 1 to 7 log_2, 1 to 7 log_2, 1 to 5 log_2, 1 to 4 log_2 and 1 to 4 log_2 respectively.

4.9.1.2d Group IV

The control group which was sham vaccinated with TSB did not reveal much antibody titres, with PHA values ranging from 1 to 3 log_2, irrespective of the days of collection. PHA titre of each group on 0, 7, 14,21, 28 ,45 and 60 PV were shown in (Table.4a-4d)

4.9.1.2e Statistical Analysis

Analysis of variance (one way) of the logarithm of IHA titres of sera collected on different days from the ducks was done. The mean logarithmic (ML) IHA titre of individual sera collected at days 7, 14, 21, 28, 45 and 60^{th} day PV are shown in (Table.5)

There was no significant difference in the mean titre among the groups during 0^{th} day. At 7^{th} day there was significant difference ($P \geq 0.05$) in group III and group I. On 14^{th} day there was significant difference in group III only. On 21^{st} day significant difference was found only in group I. Up to 21^{st} day OMP vaccine prepared under iron restricted condition was found to maintain the titre while the titres of group I decreased, on 7, 14 and 21^{st} day PV group III titre was significantly higher than group I, II and IV. At 21^{st} day PV group II and III were

Table. 4a Indirect Haemagglutination test of group I

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	4
Vaccinated once	7	8
Vaccinated once	14	16
Vaccinated once	21	16
Vaccinated once	28	16
Vaccinated once	45	16
Vaccinated once	60	8

Table. 4b Indirect Haemagglutination test of group II

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	4
Vaccinated once	7	16
Vaccinated once	14	32
Vaccinated once	21	16
Vaccinated once	28	16
Vaccinated once	45	4
Vaccinated once	60	4

Table. 4c Indirect Haemagglutination test of group III

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	4
Vaccinated once	7	64
Vaccinated once	14	128
Vaccinated once	21	128
Vaccinated once	28	32
Vaccinated once	45	16
Vaccinated once	60	16

Table. 4d Indirect Haemagglutination test of group IV

Vaccination status of ducks	No. of days post vaccination	IHA titre
Vaccinated once	0	4
Vaccinated once	7	8
Vaccinated once	14	8
Vaccinated once	21	4
Vaccinated once	28	4
Vaccinated once	45	4
Vaccinated once	60	4

Table 5. ANOVA table showing PHA Titre of serum samples of vaccinated birds on different days PV

Days	Group I	Group II	Group III	Group IV
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0^{th} day	0.308^{ns}±0.053	0.252^{ns}±0.051	0.268^{ns}±0.051	0.307^{ns}±0.056
7^{th} day	0.557^{c}±0.042	0.795^{b}±0.030	1.126^{a}±0.056	0.307^{d}±0.056
14^{th} day	0.961^{c}±0.042	1.245^{b}±0.051	1.410^{a}±0.059	0.307^{d}±0.056
21^{st} day	1.031^{b}±0.039	1.347^{a}±0.042	1.450^{a}±0.054	0.307^{c}±0.056
28^{th} day	0.879^{c}±0.028	1.139^{b}±0.031	1.351^{a}±0.059	0.307^{d}±0.056
45^{th} day	0.237^{b}±0.028	0.371^{b}±0.052	0.756^{a}±0.067	0.307^{b}±0.056
60^{th} day	0.237^{b}±0.028	0.371^{b}±0.052	0.756^{a}±0.067	0.307^{b}±0.056

*ns- non significant

The values with different superscripts in a row having significant difference ($p \geq 0.05$)

significantly comparable. All the vaccine groups had shown significant difference from the control group at all the stages of study. Groups I and II were showing significant difference in the mean titre during the entire study. The average PHA titre for the ducklings PV on different days are graphically represented. (fig.10 & 11)

4.9.2 Homologous Challenges of Vaccinated Birds

The vaccinated birds were challenged with virulent *P. multocida* A:1 at a dose of 100 LD_{50} on day 42 and 60 PV. The results of challenge experiments are presented in (Table. 6 &7).

The survivability of birds of group I, II and III challenged with 100 LD_{50} dose on day 42 PV was 70 per cent, 60 per cent and 60 per cent respectively. On 60^{th} day PV groups I, II and III showed 60 per cent, 60 per cent and 50 per cent protection.

When challenge test was done in the control group ten per cent of birds (One bird) survived on 42^{nd} day and on 60^{th} day 20 per cent (Two birds) survived.

The post mortem examination of dead birds of all the vaccine groups revealed less severe gross lesions than the control group. Blood smear and organ impression smears revealed bipolar organisms and the culture of heart blood and liver onto blood agar revealed typical colonies of *P. multocida,* even though the lesions were less severe.

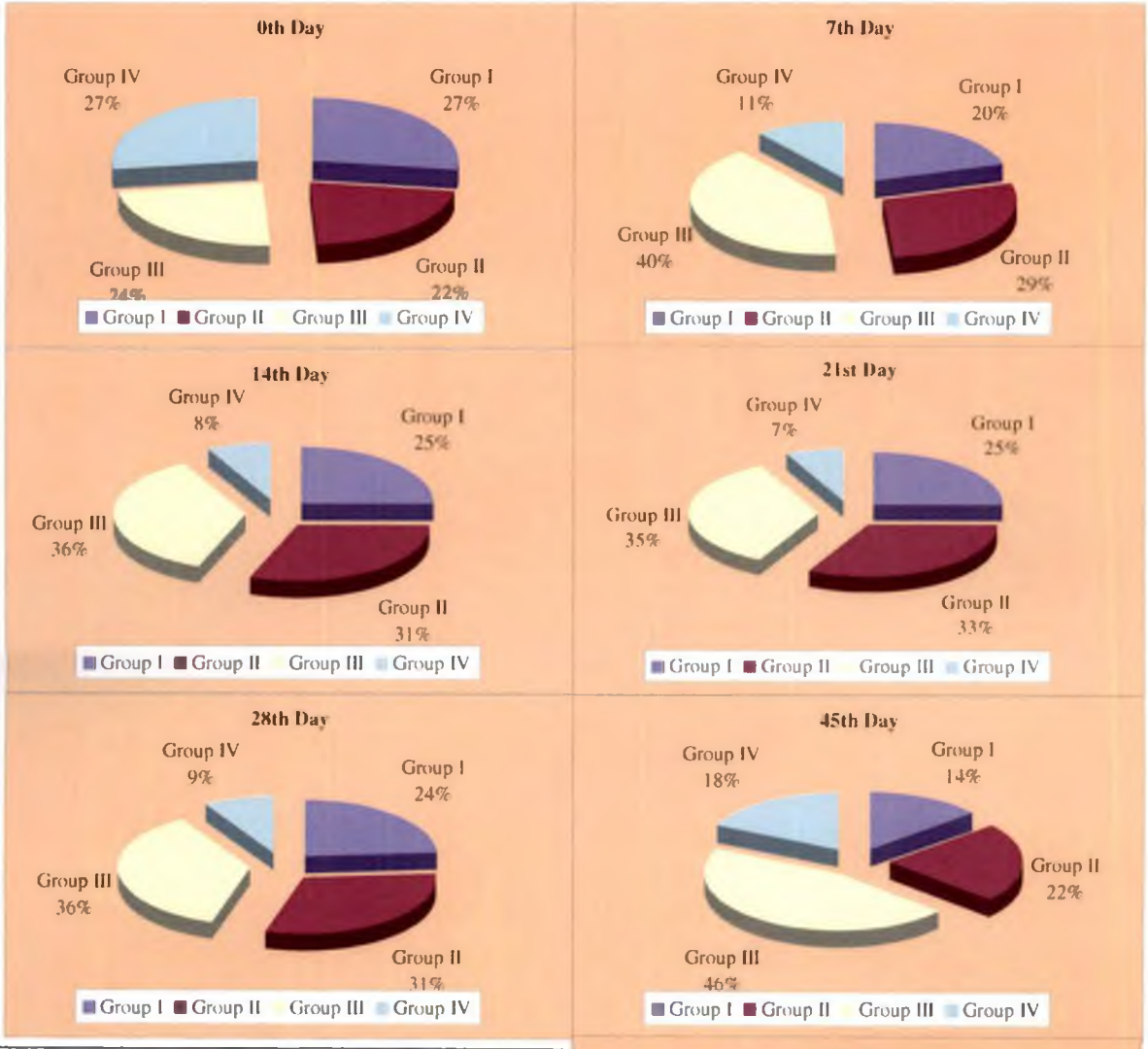
Table 6. Homologous challenge experiments on day 42 PV– challenge with 100 LD_{50}

Group	Vaccination status	No of days PV	No of birds challenged	No of birds survived	Percentage protection
I	Single vaccination	42	10	7	70
II	Single vaccination	42	10	6	60
III	Single vaccination	42	10	6	60
IV	Sham vaccination	42	10	1	10

Table 7.Homologous challenge experiments on day 60 PV– challenge with 100 LD_{50}

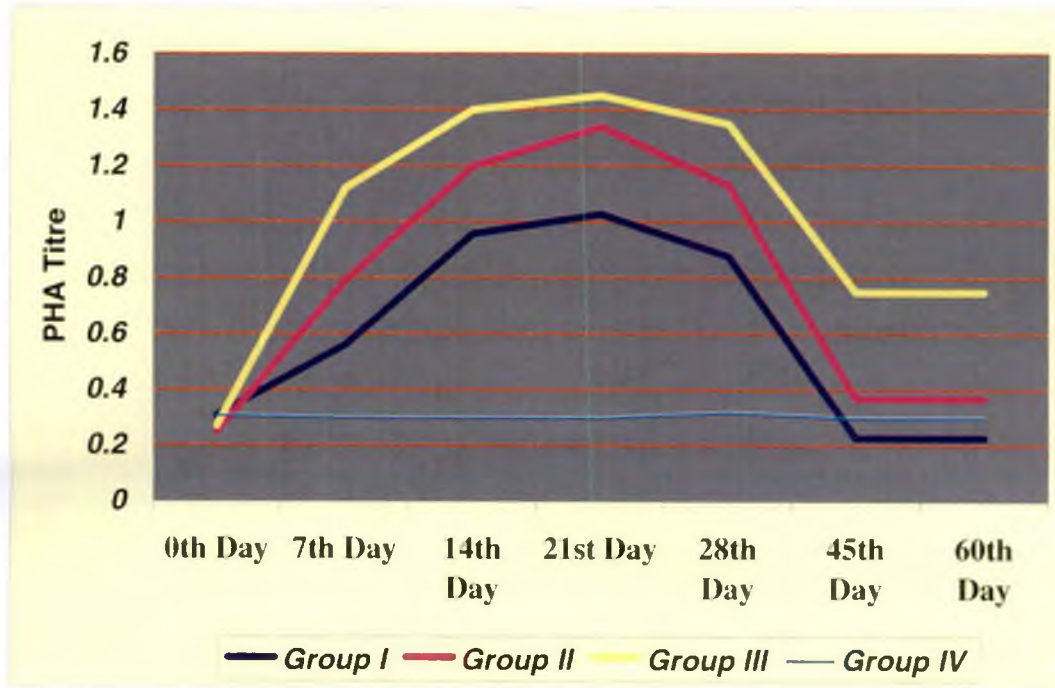
Group	Vaccination status	No of days PV	No of birds challenged	No of birds survived	Percentage protection
I	Single vaccination	60	10	6	60
II	Single vaccination	60	10	6	60
III	Single vaccination	60	10	5	50
IV	Single vaccination	60	10	2	20

Fig.10. Pie diagram - comparative PHA titre of ducklings pre and post vaccination



Group I - Ordinary Bacterin
Group II - OMP Vaccine Prepared
Under Iron Sufficient Condition
Group III - OMP Vaccine Prepared
Under Iron Restricted Condition
Group IV - Control

Fig.11 Comparative PHA titre of ducklings pre and post vaccination



Group I - Ordinary Bacterin

Group II - OMP Vaccine Prepared Under Iron Sufficient Condition

Group III - OMP Vaccine Prepared Under Iron Restricted Condition

Group IV - Control

Discussion

5. DISCUSSION

Avian pasteurellosis, also known as fowl cholera, is one of the economically important disease problems affecting poultry. The disease is caused by *P.multocida*, and inflicts high morbidity and mortality resulting from acute septicaemia, pneumonia or chronic disseminated infection (Chawak *et al.*, 2001).

In fatal and peracute nature of disease, vaccination is accepted as a principal means of controlling the outbreaks. Presently several vaccines are available such as alum-precipitated vaccine, aluminium hydroxide gel vaccine and oil adjuvant vaccines. Of these oil adjuvant vaccine is more potent but this will confer only a shorter duration of immunity. Several newer vaccine candidates are to be identified, characterized and used as subunit vaccines consisting of OMP (Pati *et al.*, 1996).

Iron is an essential element for most organisms due to its role in metabolic electron transport chains. Host iron is not largely available due to the presence of iron binding glycoproteins such as transferrin and lactoferrin. Pathogenic bacteria such as *P. multocida* overcome this *in vivo* by expressing a number of iron-regulated proteins and low molecular weight siderophores which sequester iron from iron binding host protein (Chawak *et al.*, 2001). Growth condition could influence the antigenic profile and surface epitopes of organisms. The bacteria isolated from tissue fluid and blood were shown to provide broader duration of immunity in terms of heterologous protection. (Heddleston and Rebers, 1972).

In the present study, *P. multocida* grown under three different growth conditions were employed for vaccine preparation. The immunopotency of the prepared oil adjuvanted vaccines was assessed by efficacy testing in one-month-old ducklings and the protection conferred was determined by homologous challenge.

The three different growth conditions were:

1. Organism grown in TSB
2. Organism grown in Brain heart infusion broth alone
3. Organism grown in Brain heart infusion broth added with 100 μM 2.2' dipyridyl.

5.1 PURITY CHECKING AND CHARACTERIZATION

DP1 isolate was streaked on blood agar, incubated at 37°C with mild CO_2 tension. These conditions were found to be ideal for the growth of *P. multocida.* These findings are in agreement with the earlier observations of Raja lakshmi, (2001) and Antony (2004)

The biochemical reactions for the characterization of *P. multocida* gave expected results, confirming the identity of the organism. Absence of haemolysis on blood agar, no growth in Mac Conkey's agar and negative urease activity were included as primary characters (Buxton and Fraser, 1977).

Heddleston (1976) reported that all the cultures of *P. multocida* isolate fermented glucose, sucrose and also reduced nitrate, did not fermented salicin or produce haemolysin. The reactions given by DP1 were in accordance with these findings.

Positive reactions to indole and ornithine decarboxylase have been described as the most suitable biochemical indication in the identification of *P. multocida. Riemerella anatipestifer* could be readily distinguished from *P. multocida* based on inability of the former to produce indole, non-fermentation of glucose, sucrose, maltose and a negative ornithine decorboxylase reaction (OIE, 2000).

On the basis of morphological, cultural and biochemical characteristics, the identity of DP1 was confirmed as *P. multocida.*

5.2 PATHOGENICITY STUDIES IN MICE

DP1 isolate was found to be pathogenic in mice. By intra peritoneal route, the mice died within eight hours post inoculation and by subcutaneous route mice died within 24 h.

Sambyal *et al.* (1988) and Jayakumar (1998) observed that *P. multocida* of duck origin killed mice within 24 h and 12 h post inoculation respectively. Gross lesions observed in inoculated mice were petechial haemorrhages in the epicardium and general congestion of all the visceral organs, particularly lung, liver and spleen. Fluid accumulation was also noticed in peritoneal cavity. On Leishman's staining, blood smear and organ impression smears revealed bipolar organism.

Woolcock and Collins (1976) observed an over whelming increase in the number of *P. multocida* organisms in visceral organs of mice, following experimental infection. Similar observations have also been made by Balakrishnan (1998) and Rajalakshmi (2001).

Kapoor *et al.*, (2004) observed same lesions in visceral organs and the mice died within 24 to 36 h.

5.3 WHOLE CELL PROTEIN PROFILE BY SDS -PAGE

Whole cell protein profile of DP1 isolate, when analysed by SDS-PAGE revealed 20 to 26 bands, molecular weight ranged from 102 kDa to 19 kDa.

The results are similar to findings of Lee *et al.* (1991), who had reported 26 bands in SDS-PAGE of whole cell protein of *P. multocida* isolate from chicken and turkeys with molecular weight ranging from 160 to 14 kDa.

Kedrak and Opacka (2002) analysed the whole cell protein profiles of 18 avian isolates of *P. multocida* serotype 1 and 3 on 10 per cent polyacrylamide gel and found that they contained 18 to 31 clearly visible protein fractions ranging in molecular weights from 140 to 24 kDa.

Ireland *et al.* (1991) obtained the protein profile for *P. multocida* serotype 1, isolated from a case of fowl cholera, the patterns obtained with Coomassie brilliant blue staining of whole cell proteins are similar. The major differences among isolates were seen in 38 to 34 kDa regions.

5.4 OUTER MEMBRANE PROTEINS OF *P. multocida*

The outer membrane protein of DP1, when analysed on SDS-PAGE revealed 10 protein bands with approximate molecular weights ranging from 91.84 to 19.02 kDa. Among these, two OMPs with molecular weights of 37.15 kDa and 26.36 kDa were considered as major OMPs, based on staining intensity and thickness.

Choi-Kim *et al.* (1989) reported that major OMPs of standard reference strain of *P. multocida* serotype 1 X-73 strain had a molecular weight of approximately 38 kDa. Two other proteins with approximate molecular weights of 29 and 37 kDa were also considered as major OMPs, which stained less intensely compared to 38 kDa protein. The results of the present study are in agreement with those of Kim et *al.* (1989).

Marandi and Mittal (1995) examined the OMPs of reference strains of five capsular serotypes of *P. multocida*. They found that the major OMPs when analysed by SDS-PAGE had a molecular mass of 37, 32, 38 and 36 kDa for the capsular serotypes A, B, E and F respectively. Capsular serotype D exhibited two OMP types, type I and type II with molecular weight of 32 and 37 kDa respectively.

Luo *et al.* (1997) have also reported that the major OMPs of *P. multocida* serotype A: 1, strain X-73 had a molecular mass of approximately 37 kDa.

Lu *et al.* (1988) and Chevalier *et al.* (1993) have also reported that a protein with a molecular mass of 37.5 kDa was the major OMP of *P. multocida.*

Confer *et al.* (1996) had observed that protein bands in the range of 100 to 16 kDa could be observed in the OMPs extracted from *P. multocida* serotype A: 3. The 35 to 36 kDa protein was considered to be the major OMP. Similar reports have also been made by Kedrak and Opacka (2002) in their study, who observed eleven protein bands with molecular weight ranging from 104 kDa to 20 kDa.

Rajalakshmi (2001) identified two major OMPs with molecular weight 27 and 32 to 33 kDa in the five isolates of *P. multocida* of avian origin. The major OMP was found to have a molecular weight of approximately 27 kDa.

Davies *et al.* (2003) isolated OMPs from 100 avian *P. multocida* isolates belonging to various capsular serotypes and identified two major OMPs with molecular weights ranging from 36.9 to 37.9 kDa and 33.1 to 38.3 kDa respectively. The 36.9 to 37.9 kDa protein could be correlated with the 37.15 kDa protein seen in avian isolate used in the present study.

Similar results have also been reported by Antony, (2004), who observed 10 protein bands with approximate molecular weight ranging from 91.84 to 19.02 kDa. Two protein bands with approximate molecular weight of 37.15 kDa and 26.36 kDa were thicker than others, suggesting that they were the major outer membrane proteins.

5.5 DETECTION OF ANTIGENIC OMPS OF *P. multocida*

By Western blotting using hyper immune serum against DP1 raised in rabbits, a 37.15 kDa outer membrane protein was found to be the major antigen protein, based on the thickness of the band. Two other proteins with molecular weights of 31.33 kDa and 26.36 kDa could also be considered as major antigens based on the staining intensity. These findings are in accordance with the studies of Lu *et al.* (1991) who identified a 37.5 kDa outer membrane protein of *P. multocida* serotype A: 3, as the major reactive antigen for a protective monoclonal antibody.

The results of the present study are also in agreement with those of Ireland *et al.* (1991), who opined that OMPs of molecular weights of 26, 31 and 34 kDa could be considered as major antigens.

Pati *et al.* (1996) have reported that the proteins with molecular weights of 30 kDa, 37 kDa and 44 kDa were the major immunogenes of *P. multocida.* However, the 44 kDa protein was not observed as antigenic by Western blotting although the protein was observed in SDS-PAGE stained gel.

Chawak *et al.* (2001) reported that two OMP with molecular weights of 17 kDa and 25.7 kDa were the major immunogens of avian strain of *P. multocida.* But in the present study, no protein with a molecular weight of 17 kDa was found to be antigenic. The 25.7 kDa protein might be equated to 26.36 kDa antigen identified in our study.

Western blotting revealed a 39 kDa protein to be a major immunogen of *P. multocida* X-73 serotype A:1 (Borrathybay *et al.*, 2003). This protein may be similar to the 37.15 kDa OMP identified as the major antigen.

5.6 LETHAL DOSE 50

P. multocida DP1 isolate was maintained in its virulent form during the period of study by repeated passaging in mice and subculturing on blood agar slants. Fifty per cent lethal dose (LD_{50}) was described as a practical and reliable measurement of pathogenicity by Cruickshank *et al.* (1975).

The median lethal dose of the isolate DP1 was determined in one-month-old duckling. The bacterial suspensions containing $1.2x10^8$ bacteria/ml was diluted using PBS (pH 7.2) to ten fold dilution to contain organism up to $1.2x10^0$/ml used for the determination of LD_{50} in ducklings. The result of LD_{50} was found to be $10^{-7.13}$, which contained 13 cells. Swamy, (1994) also got similar LD_{50} in ducklings and it was found to be $10^{-7.83}$, which contained 14.96 viable cells per dose.

The median lethal dose of *P. multocida* A: 1 was estimated in ducklings and it was found to be 14.32 ± 0.0833 colony-forming units (Ramanatha *et al.*, 1994).

The dead ducks had shown all the lesions observed in ducklings and additionally haemorrhages were noticed on peritoneum, which was in conjunction with Bhattacharya. (2005), who observed such lesions in ducks during a natural outbreak.

5.7 PREPARATION OF DIFFERENT TYPES OF VACCINES

Vaccination in the most attractive approach for controlling the duck pasteurellosis. Production of fowl cholera vaccine demands careful safety testing in the laboratory and potency testing.

Woolock and Collins (1976) found that protection with adjuvant treated *Pasteurella* preparations was always superior than non adjuvanated vaccines.

There are many adjuvants, which can be incorporated into fowl cholera vaccines. Lalrinliana *et al.* (1988) conducted studies to compare efficacy of different adjuvants employed in *P. multocida* vaccines and highest antibody titre was observed with oil adjuvant vaccine, while the alum precipitated and aluminum hydroxide gel vaccines induced low titres.

Iyer *et al.* (1955) used 10 parts of liquid paraffin and one part of lanolin to adjuvanate 15 parts of *P. multocida* bacterin. A similar combination was used in the present study. Very few vaccines are available against duck pasteurellosis in India and the avian vaccine usually necessitates booster, as the duration of immunity conferred will be for a short period.

In the present study, oil adjuvant inactivated vaccine was prepared under three different growth conditions. Tryptone soya broth was used as the medium for growth of ordinary bacterin, brain heart infusion broth alone was used as

medium for iron sufficient condition and brain heart infusion broth added with 2.2' dipyridyl was used as medium for iron restricted condition.

Formalinization was the most suitable method of inactivation of *P. anatipestifer* for vaccine preparation (Hary and Deb, 1979). The bacterin prepared in this study was successfully inactivated with 0.5 per cent formol saline after an incubation period of 72 h at room temperature. The outer membrane proteins were also effectively inactivated by formalin. A formalin treated antigen of *P. multocida* was found to be the most immunogenic in mice when compared to the antigens inactivated by other methods and even conferred a low degree of cross protection against heterologous challenge (Kim *et al.*, 1986).

The vaccine was prepared as per the method of Stone *et al.* (1978). The emulsion of vaccine was fixed according to Jayakumar. (1998). The composition of vaccine was tested for the emulsion type using drop test. The vaccine so prepared by homogenization at 18,500 rpm for seven minutes was not thick and was easier to administer. The oil phase of vaccine constituted by lanolin and liquid paraffin is cheaper and easily available.

Sterility of the three vaccines was done in different media. No growth could be detected even after seven days of incubation.

Since *P. multocida* is highly pathogenic to ducklings, toxicity testing of the prepared vaccine is very important. Injecting exact and double doses, which caused no ill effect both locally and systemically, constituted toxicity test.

5.8 POTENCY TESTING

Harry and Deb (1979) prescribed the dose of vaccine as $3x10^9$ cells for *P. anatipestifer*, when administered.

Many scientists doubted the potency of oil adjuvant whole cell vaccines. Dougherty (1953) came to the conclusion that inoculation with chemically killed broth culture bacterins conferred poor protection to ducklings from lethal challenge. Bierer and Deriux (1972) reported that oil based bacterin alone was

effective in preventing infection in turkeys but was not as good as avirulent live vaccine and the use of bacterin prior to the use of live vaccine proved more promising.

Conversely, many reports suggest that oil adjuvant bacterin elicit good immune response. Dua and Padurangaro (1978) observed that very high levels of antibodies were induced by oil adjuvanted bacterin when compared with live vaccine in turkeys.

Chawak *et al.* (2001) reported that OMP extracted from organisms grown in iron restricted medium might be responsible for cross protection. Cell mediated immune response did not appear to play any significant role in the immunity against. *P. multocida.*

According to Akand *et al.* (2004), intramuscular route of vaccination produced better result than subcutaneous route of vaccination. Since the intramuscular route of administration was easier and much faster to perform vaccination was done by this route.

Glisson *et al.* (1990) evaluated different injection sites in turkeys for *P. multocida* bacterin and there were no difference in the immune response elicited irrespective of the injection sites.

In the present study, the three types of vaccines were injected to one month old ducklings, by intramuscular route and blood samples were collected at 0, 7^{th}, 14^{th}, 21^{st}, 28^{th}, 45^{th} and 60^{th} day to perform PHA test.

5.9 PASSIVE HAEMAGGLUTINATION TEST

Blood samples from the birds of all the four groups were collected on different days and the antibody titre was assessed by passive haemagglutination test. The test was done as per Sawada *et al.* (1982) using GA-SRBC. It could be kept under refrigerated condition in accordance with Sawada *et al.* (1982), Ramanatha (1994) and Jayakumar (1998).

The IHA titre obtained for OMP vaccine prepared under iron restricted condition group (III) on day 14 PV was very much higher than that of other two groups and it was slightly reduced after 28th day PV, but was able to maintain a reasonable titre till day 60 PV. The antibody titre of group I and group II increased during the 7th day and it was decreased after 28 day PV. The titre obtained for Jayakumar (1998) was higher but the pattern was almost similar. Ramanatha (1994) got similar titre and was considerably increased by giving booster dose of vaccine at 24 days interval.

Kedrak and Opacka (2002) evaluated subunit vaccines comprising IROMPs of *P.multocida* serotype A: 1 in calves and found that antibodies against OMPs could be detected as early as seven days, which increased to 14 days PV.

Chawak *et al.* (2001) reported that the precipitating antibodies started appearing in 70 to 88 per cent birds of all the vaccinated groups at two weeks PV and 92 to 100 per cent birds were positive at two weeks PV and four to five weeks PV. The number of positive birds gradually declined.

From this it is evident that OMP are more immunogenic and elicited better immune response.

In the present study, analysis of variance (one way) of the logarithm of IHA titres of sera collected on different days from ducklings was done. There was no significant difference in mean titre among the groups during the 7th day. At day 14 PV, group III was significantly different from the rest of the two vaccine groups and control. The titre of group II and III were almost similar on day 21st. At day 45 and 60 PV, group III was found to maintain the titre while the titre of group I and II, decreased, making them significantly different. All the vaccine groups have shown significant difference from the control group to all stages of the study.

5.10 HOMOLOGUS CHALLENGE

The challenge results on 42nd day indicated that 70 per cent protection was afforded by group I, while group II and III had shown 60 per cent protection each. The control group showed 10 per cent protection. On 60th day, groups I and II had shown 60 per cent protection each and group III afforded only 50 per cent protection. In the control group two birds survived, indicating 20 per cent protection.

Even though, the mean antibody titre of group III on 45th and 60th day were higher when compared to group I and II, the percentage of protection afforded by group III was lesser than that of group I and equal to group II, when challenged on 42 day PV. On 60 day challenge the protection level afforded by group III as the lowest (50 per cent).

Chawak *et al.* (2001) reported that there was no correlation was observed between precipitating antibody and protection percentage.

Islam *et al.* (2004) vaccinated with alum precipitated formalin killed fowl cholera vaccine and observed that immunized ducks with PHA titre less than 1:64 died on virulent challenge exposure.

The antibody titre of group III birds ranged from 3 $\log_2$ to 7 $\log_2$ and so some other birds having titres less than 7 $\log_2$. This could be the reason for lower protection level shown by group III birds, because the birds were chosen at random and these birds selected might have had an antibody titre lower than 7 $\log_2$, that is the protection level as observed earlier by Islam *et al.* (2004).

Pati *et al.* (1996) reported that following challenge with virulent *P. multocida*, all the OMP vaccinated animals survived, whereas only two out of three HS oil adjuvant vaccinated animals withstood the challenge.

Srivastava (1999) also made similar observation wherein, vaccine prepared from *P.multocida* serotype B: 2 grown under iron limiting condition

were more immunogenic than that prepared from organism grown under iron sufficient condition.

Chawak *et al.* (2001) prepared two vaccines under two different conditions *viz.*, OMP vaccine prepared under iron restricted condition, whole cell vaccine under iron restricted condition. The antibody response was almost similar in two vaccines. The protection against homologous challenge was 100 per cent in two groups at four to six weeks and 66.6 per cent protection at 9 weeks PV. The result of the study is in contradictory to this result.

Wasnik *et al.* (2004) compared the efficacy of whole cell vaccine with 2.2' dipyridyl (DIP) and without DIP. The whole vaccine with DIP will evoke better immune response than the whole vaccine with out DIP. The whole cell DIP vaccine offered 50 per cent protection in rabbits.

When challenge test was done in control group, 10 per cent (one bird) survived on 42^{nd} day PV and twenty per cent (two birds) were survived on 60^{th} day. This could be due to individual variation among birds.

In conclusion, though the OMP vaccine prepared under iron restricted condition was found to provide a high mean antibody titre, the protection percentage afforded by it in comparison with ordinary bacterin and OMP vaccine prepared under iron sufficient media was low. Several workers have highlighted the enhanced immunogenicity of OMPs grown under iron restricted condition. Hence, this warrants for further investigation.

Summary

SUMMARY

Pasteurellosis and duck plague have been reported as the major cause of mortality in ducks in Kerala. Duck plague has been controlled to a great extent by the use of vaccines, but pasterellosis continue to be a major threat to duck farming in the state. In spite of immunizing poultry against fowl cholera with existing conventional vaccines, hundred per cent protection has never been achieved. Hence, different subunits of *P. multocida* should be explored as possible immunogens in conferring hundred per cent protection in birds against fowl cholera. The present study was under taken with this objective.

The purity checking of *P. multocida* A:1 strain (DP1) isolated from Niranam Duck Farm (Pathanamthitta District), serotyped at IVRI, Izatnagar and maintained in Department of Veterinary Microbiology, College of Veterinary & Animal Sciences, Mannuthy, was done as per standard procedures and the isolate gave all the specific reactions of *P. multocida.*

Pathogenicity testing of the isolates was done in six to eight weeks old mice by inoculum given via two different routes *viz.*, intra peritoneally and subcutaneously. The isolate killed the mice inoculated with $0.3x10^8$ organism per 0.1 ml. intra peritoneally within eight hours and within 24 h when injected by subcutaneous route, while the control mice were alive through out the observation period of seven days. The gross lesion observed were same in both the routes of inoculation and the organisms was re isolated from the dead mice.

Whole cell proteins of *P. multocida (DPI)* were extracted by growing it on bovine blood agar plates at 37^0C for 18 h and were then harvested in antigen preparation buffer and centrifuged at 10,000xg for three minutes. The whole cell proteins extracts were analysed by discontinuous system of SDS-PAGE. The DPI strain of *P. multocida* revealed 20 to 26 protein bands, molecular weight ranging from 104 kDa to 19 kDa.

Outer membrane proteins of the organism were extracted by growing it under two different conditions *viz.*, Iron sufficient (BHIB) and iron restricted media (BHIB along with 2,2'dipyridyl). OMPs were harvested using sodium N-lauryl sarcosine. Then, they were subjected to SDS-PAGE and western blotting, OMPs extracted from iron sufficient media revealed 10 protein bands, molecular weight ranging from 91.84 kDa to 19.02 kDa. OMPs from iron restricted condition revealed an extra protein band with molecular weight of 97.8 kDa. The protein concentration was estimated by modified Lowry method and was found to be 4mg/ml.

Western blotting revealed that 37.15 kDa protein is the major antigenic fraction of OMPs of avian isolate of *P. muloticida.* In addition to this have other proteins with molecular mass of 31.33 and 26.36 kDa were also found to be antigenic.

Median Lethal dose (LD_{50}) of *P. multocida* was determined in one month old ducklings.

The birds were inoculated with different dilution of fully encapsulated virulent bacteria at a dose rate of 0.1 millilitre per bird subcutaneously. Median Lethal dose of the isolate was found to be 13 cells and the dilution giving 50 per cent end point was $10^{-7.13}$. All the dead birds revealed gross lesions of pasteurollosis and the organism could be re-isolated from dead birds. The blood smear and the impression smear prepared from various organs revealed bipolar organisms.

Oil adjuvant inactivated vaccines were prepared from DPI grown under three different conditions *viz*;

1. Organism grown in TSB
2. Organism grown in BHIB
3. Organism grown in BHIB + 2,2' dipyridyl.

Vaccine emulsions were prepared by combining aqueous phase and oil phase. The aqueous phase of emulsion consisted of 15 parts of formalin inactivated bacterin and oil phase was formed of nine parts of sterile liquid paraffin and one part of sterile lanolin. The mixture was homogenized at 18, 500 rpm for 30 seconds. The sterility of prepared vaccine was tested in different media (SDA, TSA, Blood agar and thioglycolate broth). No growth was observed on any of the media used.

The toxicity of vaccine was assessed by injecting exact and double dose of vaccine intramuscularly to five ducklings, each separately for the vaccines. The birds did not show any untoward effects systemically and locally.

A total of 120 duckings of one month of age were divided into 4 groups, with 30 birds in each group and the first group was vaccinated with ordinary bacterin, 2^{nd} group with OMP vaccine prepared under iron sufficient condition, group III with OMP vaccine prepared under iron restricted condition and group IV served as control. The birds were vaccinated with 0.5 ml intra muscularly. The blood was collected from all ducks pre vaccination and at weekly interval upto 60^{th} day PV by cardiac puncture or by jugular puncture. Passive haemagglutination using GA-SRBC sensitized with sonicated antigen of DP1 was used to measure the humoral immune response. The IHA titres obtained for OMP vaccine groups on day 14 was very much higher than the other two groups. The highest titre obtained was 7 $\log_2$ for group III on 14^{th} day PV.

Ten birds from each vaccinated group were subjected to homologous challenge with 0.1 ml of inoculum containing 100 LD_{50} of fully encapsulated virulent from of *P. multocida* serotype A1. On 42 day PV Groups I, II and III had given 70, 60 and 60 per cent protection respectively, when challenged with 100 LD_{50} and on 60th day they gave 60, 60 and 50 per cent protection respectively.

Though the OMP vaccine prepared under iron restricted condition was found to provide high antibody titre, the protection percentage afforded by it in comparison with ordinary bacterin and OMP vaccine prepared under iron

sufficient media was low. Hence, the OMP vaccine prepared under iron restricted condition need to be subjected to further investigation.

References

REFERENCES

Adlam, C. 1989. The structure, function and properties of cellular and extra cellular components of *Pasteurella haemolytica. Pasteurella and Pasteurellosis* (eds. Adlam, C. and Rutter, J.M.) Academic Press, London, pp. 75-92

Adler, B., Bulach, D., Chung, J., Doughty, S., Hunt, M., Rajakumar, K., Serrano, M., Zanden, A., Zhang, Y. and Ruffolo,C. (1999). Candidate vaccine antigens and genes in *Pasteurella multocida. J. Biotech.* 73 (2-3): 83-90

Akand, M. S. I., Choudhury, K. A., Kabir, S. M. L., Sarkar, S. K. and Amin, K. M. R. 2004. Development of washed cell fowl cholera vaccine in Bangladesh. *Int. J. Poult. Sci.* 3: 534-537

Antony, P. X. 2004. Molecular characterization of *Pasteurella multocida* isolated from ducks in Kerala. Ph. D. thesis submitted to Kerala Agricultural University, Thrissur. p.126

Arora, A. K., Jand, S. K., Sandu K. S., Oberoi, M. S. N. 2006. Molecular characterization of *Pasteurella multocida* isolates from haemorrhagic septicaemia by ribotyping. *Indian J. Ani. Sci.* 74(2): 127-130

Arshad, M. J., Siddique, M. and Hussain, I. 2003. Somatic serotyping and pathogenicity testing of *P. multocida* strains isolated from layer chickens. *OnLine J. Biol. Sci.* 3: 376-379

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

Balakrishnan, G. 1998. Plasmid profile of avian strains of *Pasteurella multocida.* M.V.Sc. thesis submitted to Kerala Agricultural University, Thrissur. p 110

Barenkamp, S.J., Munson,R.S. and Granoff, D.M. 1981. Subtyping isolates of *Haemophilus influenzae* type B by outer membrane protein profiles. *J. Infect. Dis.* 143 : 668-676

Barrow, C. I. and Feltham, R. K. A. 1993. *Cowan and Steel's manual for identification of medical bacteria.* Third Edn. Cambridge University Press. p. 331

Benz. R. 1988. Structure and functions of porins from Gram negative bacteria. *Ann. Rev. Microbiol.* 42: 359

Bhattacharya, A. 2005. Isolation, characterization and antibiotic sensitivity of *Pasteurella multocida* from incidences of duck cholera in khaki Campbell and vigova super-M ducks in Tripura. *Indian Vet. J.* 82: 203-205

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

Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N. and Morrow, C.J. 1995. Characterisation of *Pasteurella multocida* isolated from fowl cholera outbreaks on turkey farms. *Aust. Vet. J.* 72: 135-138

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

Borrathybay, E., Sawada, T., Kataoka, Y., Okiyama, E., Kawamoto, E., Amao, H. 2003. capsule thickness and amounts of a 39 kDa capsular protein of avian *Pasteurella multocida* type A strains correlate with their pathogenicity for chickens. *Vet. Microbiol.* 97: 215-227

Bosch, M., Tarrago, R., Gurride, E., Campoy, S., Fernandez de Henestosa, A. R., Perz de Rozas, A. M., Badiola, I. and Barbe, J. 2001. Expression of the

Pasteurella multocida OMPH gene in negatively regulated by the Fur protein. *FEMS Microbiol. Lett.* 203: 35-40

Bosch, M., Gardio, E., Llagostera, M., Perez de Rozas, A. M., Badiola, I. and Barbi, J. 2002. *Pasteurella multocida* exbD, exbB and tonB gene are physically linked but independently transcribed. *FEMS Microbiol. Lett.* 210: 201-208

Bermudez, A. J., Munger, L. L and Ley, D. H. 1991. Pasteurellosis in bob white quails. *Avian Dis.* 35: 618-620

Buxton, A. and Fraser, G. 1977. *Animal Microbiology.* Vol.1. Blackwell Scientific Publication, Oxford, p. 586

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

Carter, G. R. 1955. Studies on *Pasteurella multocida.* I. A haemagglutination test for the identification of serological types. *Am. J. Vet. Res.* 16: 481-484

Carter, G.R. 1967. Pasteurellosis. *Pasteurella multocida and Pasteurella haemolytica. Advances in Veterinary Science,* Vol. 2 (eds. Brandly, C.A. and Cornelius, C.) Academic Press, New York, pp. 321-367

Chabert 1782. Cited by Rhoades, K.R. and Rimler, R.B. 1991. Pasteurellosis. *Disease of Poultry.* (eds. Calnek, B.W., Barnes, H.J., Bareand, C.W., Reid, W.M. and Yoder, H.W.) Ninth edition. Iowa State University Press, Ames, Iowa, pp 145-152

Chandran, N.D.J., Prabakar, T.G., Albert, A., David, B.P. and Venkatesan, R.A. 1995. Pasteurellosis in Japanese quail (*Coturnix coturnix japonica*). *Indian Vet. J.* 72: 876-877

Chawak, M. M., Verma, K. C. and Kataria, J. M. 2001. Characterization and immunizing properties of outer membrane proteins of *Pasteurella multocida* grown in iron sufficient and iron restricted media. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 22: 36-42

Chevalier, G., Duclohier, H., Thomas, D., Shechter, E. and Wroblewski, H. 1993. Purification and characterization of protein H, the major porin of *Pasteurella multocida. J. Bacteriol.* 175: 266-276

Choi, K.H., Maheswaran, S.K. and Felice, L.J. 1989. Characterization of outer membrane protein-enriched extracts of *Pasteurella multocida* isolated from turkeys. *Am. J. Vet. Res.* 50: 676-683

Choi –Kim, K., Maheswaran, S.K., Felice, L.J. and Molitor, T.W. 1991. Relationship between the iron–regulated outer membrane proteins and the outer membrane proteins of *in vivo* grown *Pasteurella multocida. Vet. Microbiol.* 28: 75-92

Chung, J. Y, Wilkie, I., Boyce, J. D., Townsend, K. M., Frost, A. J., Ghoddusi, M. and Adler, B. 2001. Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. *Infect. Immun.* 69: 2487-2492

Collins, F. M. 1976. Growth of *Pasteurella multocida* in vaccinated and normal mice. *Infect. Immun.* 8: 868-875

Confer, A. W., Nutt, S. H., Dabo, S. M., Panciera, R. J. and Murphy, G. L. 1996. Antibody responses of cattle to outer membrane proteins of *Pasteurella multocida* A: 3. *Am. J. Vet. Res.* 57: 1453-1457

Cowan, S.T. 1974. *Cowan and Steel's "Manual for the identification of medical bacteria.* 2nd edn. Cambridge University Press, Cambridge. p. 364

Cruickshank, R., Duguid, J. P., Marmion, B. P. and Swain, R. H. A. 1975. *Medical Microbiology,* 12th edn. Churchill Livingstone, Edinburg. p.1068

Curtis, P.E. 1979. Observations on avian pasteurellosis in Britain. *Vet. Rec.* 104: 471-474

Dabo, S.M., Confer, A.W. and Quijano, R.A. 2003. Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: evidence of its role in *P. multocida* interaction with extracellular matrix molecules. *Microbiol. Pathogenesis* 35: 147-157

Dahl, C., Permin, A., Christensen, J.P., Bisgaard, M., Muhaiwra, A.P., Petersen, K., Poulsen, J.S and Jensen, A.L. (2002) The effect of concurrent infections with *Pasteurella multocida* and *Ascaridia galli* on free range chickens. *Vet. Microbiol.* 4:313-324

Davies, R.L. and Donachie, W. 1996. Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. *Microbiology.* 142: 1895-1907

Davies, R.L., MacCorquodale, R. and Caffrey, B. 2003. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Vet. Microbiol.* 91: 169-182

Devi, V. R., Rao, T. S., Aruna, P. and Sujani, G. 2000. Incidence of duck cholera in Krishna district, Andhra Pradesh – a field report. *Indian Vet. J.* 79: 535-536

Dhanda, M.R., Das, M.S., Lal, J.M. and Seth, R.N. 1956. Immunological studies on *Pasteurella septica* 1, trials on adjuvant vaccines. *Indian J. Vet. Sci. Anim. Husb.* 26: 273

Dougherty, E. 1953. Disease problems confronting the duck industry. *Proc. Annu. Meet. Am. Vet. Med. Assoc.* pp 359-365

Dua, S. K. and Pandurangarao, C. C. 1978. Serological tests as indicators of immunity against *Pasteurella multocida* infection in sheep. *Can. J. Comp. Med.* 42: 489-495

Dutta, T.K. 2001. Molecular characterization of Indian isolates of *P. multocida* causing haemorrhagic septicaemia. PhD thesis submitted to Deemed University IVRI, Izatnagar. p. 86

Filia, G., J and, S. K., Arora, A. K. and Oberoi, M. S. 2006. Comparative analysis of outer membrane protein profile of *Pasteurella multocida* serotype B: 2 under iron regulated conditions. *Indian J. Anim. Sci.* 76: 897-899

Filia, G., J and, S. K., Arora, A. K. and Oberoi, M. S. 2007. Comparative efficacy of whole cell vaccines prepared from *Pasteurella multocida* grown under different growth condition, *Indian J. Ani. Sci.* 77:119-122

Garrido, M. E., Bosch, M., Medina, R., Bigas, A., Llagostera, M., Perez de Rozas, A. M., Badiola, I. and Barbe, J. 2003. Fur independent regulation of *P. multocida* HbPA gene encoding a haemin-binding protein, *Microbiology.* 149: 2273-2281

Glisson, J.R., Cheng, I.H.N., Rowland, G.N. and Stewart, R.G. 1989. *Pasteurella multocida* infection in Japanese quail (*Coturnix coturnix japonica*) – case report. *Avian Dis.* 32: 820-822

Glisson, J. R., Brown, J., Cheng, I. H. N. and Stewart, R. G. 1990. Alternative injection sites for a *Pasteurella multocida* bacterin. *Avian Dis.* 34: 214-217

Glisson, J.R., Contreas, M.D., Cheng, I.H.N. and Wang, C. 1993. Cross protection studies with *Pasteurella multocida* bacterins prepared from bacteria propagate in iron depleted medium. *Avian Dis.* 37: 1074-1079

Gooderham, K. R. 1988. Management and welfare of farm animals. *Ducks.* 3:23-253

Goto, Y., Nakura, R., Nasu, T., Sawada, T. and Shinjo, T. 2001. Isolation of *Pasteurella multocida* during an outbreak of infectious septicaemia in Japanese quail (*Coturnix coturnix japonica*). *J. Vet. Med. Sci.* 63: 1055-1056

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. *Res. Vet. Sci.* 27: 329-333

Hirsh, D. C., Mac Lachlanand Walker, R. L. 2004 Veterinary Microbiology- 2nd eds- Blackwell Publishing, Australia. Pp 84-90

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

Heddleston, K. L. 1976. Physiologic characteristics of 2,268 cultures of *Pasteurella multocida. Am. J. Vet. Res.* 37: 745-747

Hornhuan, A., Pathanasophan, P., Crommelin, D. J. A., Jiskot, W., Kersien, G. F. and Prakongpan, A. 2004. Characterization and protection in mice of outer membrane protein from *Pasteurella multocida* A:1 incorporated in lipid vaccine delivery systems. *Science Asia.* 30: 231-237

Ikeda, J. S. and Hirish, D. C. 1988. Antigenically related iron-regulated outer membrane protein produced by different somatic serotypes of *Pasteurella multocida. Infect. immun.* 56: 2499-2502

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

Islam, M. A., Samad, M. A. and Rahman, M. B. 2004. Evaluation of alum precipitated formalin killed fowl cholera vaccines with their immunologic responses in ducks. *Int. J. Poult. Sci.* 3: 140-143

Iyer, S. V. and Gopalakrishnan, K. S. 1955. Studies on haemorrhagic septicemia vaccines: The effect of adjuvants upon the immunizing value of formalin-killed *Pasteurella boviseptica* organism. *Indian Vet. J.* 31: 379-391

Jap, B.K. and Walian, P.J. 1990. Biophysics of the structure and function of porins. *Rev. Biophys.* 23:367-403

Jain, A., Roy, A., Joshi, C. G. and Purolut, J. H. 2006. Characterization of the *Pasteurella multocida* isolates by their outer membrane protein profiles. *Indian J. Comp. Microbial. Immunol. Infect. Dis.* 26:63-65

Jayakumar, P. S. 1998. Comparative efficacy of different vaccines against pasteurellosis in ducks. M.V.Sc thesis submitted to Kerala Agricultural University, Thrissur. p.126

Kapoor, V., katoch, R. C., Sharma, M. and srani, R. K. 2004. Pathogenicity test of *Pasteurella multocida* in mice A:1, *Indian J. Ani. Sci.* 74(5): 495-496

Kasten, R. W., Hansen, L. M., Hinojora, J., Bieber, D., Ruehl, W. W. and Hirsh, D.C. 1995. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *H. influenza. Avian Dis.* 41: 972-976

Kasten, R. W., Wakenell, P. S., Ahmad, S., Yilma, T. D. and Hirsh, D. C. 1997. Lack of protection against avian cholera by vaccination with recombinant p6-like protein from *Pasteurella multocida. Avian Dis.* 41; 972-976

Kawamoto, E., Sawada, T. and Maruyama, T. 1990. Prevalence and characterization of *Pasteurella multocida* in rabbits and their environment in Japan. *Jpn. J. Vet. Sci.* 52: 915-921

Kedrak, A. and Borkowska-Opacka, B. 2002. Immunological response to outer membrane proteins of *Pasteurella multocida* serotype A: 3 in calves. *Bull. Vet. Inst. Pulawy.* 47: 387-394

Kennett, L., Muniandy, N. and Mukkur, T.K.S. 1993. Comparative protective potential of non-living intact cells and purified outer membrane and associated proteins of *Pasteurella multocida* type 6: B grown under iron-regulated conditions. *Proceeding of International workshop on Pasteurellosis in production animals. Bali, Indonesia, 10-13 August, 1992.* pp. 144-148

Kielstein, P., Wuthe, H.H., Angon, O., Muttes, R. and Arrens, P. 2001. Phenotypic and genetic characterization of NAD-dependent Pasteurellaceae from respiratory tract of pigs and their possible pathogenetic importance. *Vet. Microbiol.* 81: 243-255

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

Knight, D.P., Paine, J.E. and Speller, D.C.E. 1983. A selective medium for *Pasteurella multocida* and its use with animal and human specimens. *J. Clin. Pathol.* 36: 591-594

Kodama, H., Matsumoto, M., Fuquay, J. I. and Syuto, B. 1982. Soluble fractions of *Pasteurella multocida*: their protective qualities against fowl cholera in turkeys. *Avian Dis.* 27: 283-291

Krishnamoorthy, V., Prasad, G. R., Piacent, A. N. and King, D. J. 1996. Pathogenesis of New castle disease in commercial and specific pathogen free turkeys experimentally infected with isolates of different virulent *Pasteurella multocida. Vet. Pathol.* 43: 168-178

Kumar, A.A., Harbola, P.C., Rimler, B.R. and Kumar, P.N. 1996. Studies on *Pasteurella multocida* isolates of animal and avian origin from India. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 17: 120-124

Lalrinliana, F., Sodhi, S. S., Baxi, K. K., Sambyal, D. S. and Soni, G. L. 1988. Efficacy of different adjuvants in *Pasteurella multocida* vaccine. *Indian. J. Comp. Microbiol. Immunol. Infect. Dis.* 9: 135-140

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685

Lee, M.D., Burch, F.T., Maurer, J.J., Henk, A. and Thayer, S. 2000a. DNA finger printing of plasmid-containing serotype A: 3,4 *Pasteurella multocida* isolated from cases of fowl cholera in chickens and turkeys. *Avian Dis.* 44: 201-204

Lee, C.W., Wilkie, I.W., Townsend, K.M. and Frost, A.J. 2000b. The demonstration of *Pasteurella multocida* in the alimentary tract of chickens after experimental oral infection. *Vet. Microbiol.* 72:47-55

Livestock census. 2003. Depatment of Animal Husbandry and dairying. Ministry of Agriculture, India. (http://www.dahd.nic.in)

Loosmore, S.M., Yang, Y.P., Coleman, D.C., Shortreed, J.M., England, D.M. and Klein, M.M. 1997. Outer membrane protein D 15 is conserved among *Haemophilus influenzae* species and may represent a universal protective antigen against invasive disease. *Infect. Immun.* 65: 3701-3707

Lu, Y., Pakes, S. P., Massey, L. and Stefanu, C. 1987. A potassium thiocyanate extract vaccine prepared from *Pasteurella multocida* A: 3 protects rabbits against homologous challenge. *Infect. Immun.* 55: 2967-2976

Lu, Y.S., Afendis, S.J. and Pakes, S.P. 1988. Identification of immunogenic outer membrane proteins of *Pasteurella multocida* 3:A in rabbits. *Infect. Immun.* 56: 1532-1537

Lugtenberg, B., Boxtel, R.V., Evenberg, D., Jong, M.D., Strom, P. and Frik, J. 1986. Biochemical and immounological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect. Immun.* 52:175-182.

Luo, Y., Glisson, J.R., Jackwood, M.W., Hancock, R.E., Bains, M., Cheng, I.N. and Wang, C. 1997. Cloning and characterization of the major outer membrane protein gene (*OmpH*) of *Pasteurella multocida* X-73. *J. Bacteriol.* 179: 7856-7864

Luo, Y., Zeng, Q., Glisson, J.R., Jackwood, M.W., Cheng, N and Wang, C. 1996. Sequence analysis of *Pasteurella multocida* major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *Vaccine.* 17: 821-831

Mahasreshti, P.J., Murphy, G.L., Wyckoff, J.H., Farmer, S., Hancock, R.E. and Confer, A.W. 1997. Purification and partial characterization of the OmpA Family of proteins of *Pasteurella haemolytica. Infect. Immun.* 65: 211-218

Manoharan, S., Jayaprakasan, V., Pillai, R. M. and Sulochana, S. 1997. Immunization of mice with antigens of *Pasteurella multocida* of rabbit origin. *Indian Vet. J.* 74: 1-4

Marandi, M.V. and Mittal, K.R. 1995. Identification and characterization of outer membrane proteins of *Pasteurella multocida* serotype D by using monoclonal antibodies. *J. Clin. Microbiol.* 33: 952-957

Marandi, M.V. and Mittal, K.R. 1996. Characterisation of an outer membrane protein of *Pasteurella multocida* belonging to the Omp A family. *Vet. Microbiol.* 53: 303-314

Marchart, J., Dropmann, G., Lechleitner, S., Schlapp, T., Wanner, G., Szostak, M. P. and Lubitz, W. 2003. *Pasteurella multocida* and *Pasteurella haemolytica*-ghosts: new vaccine candidates. *Vaccine.* 21: 3988-3997

Mariana, S. and Hirst, R. 2000. The immunogenicity of *Pasteurella multocida* isolated from poultry in Indonesia. *Vet. Microbiol.* 72: 27-36

Markwell, N. A. K., Haas, S. M., Biebes L. L. and Jobert, N. E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87: 206-210

Mittal, K. R., Jaiswal, T. N., Gupta, B. R. and Gupta, B. K. 1979. Studies on haemorrhagic septicaemia water in oil and multiple emulsion adjuvant vaccines. *Indian Vet. J.* 54: 773-778

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. 1996a. *Pasteurella multocida* in psittacines prevalence, pathology and characterization of isolates. *Avian Dis.* 40: 900-907

Morishita, T.Y., Lowenstine, L.J., Hirsh, D.C. and Brooks, D.L. 1996b. *Pasteurella multocida* in raptors: prevalence and characterization. *Avian Dis.* 40: 908-918

Morton, R. J., Panciera, R. J., Fulton, R. W., Frank, G. K., Ewing, S. A., Homer, J.T. and Confer, A.W. 1996. Vaccination of cattle with outer membrane

enriched fractions of *P. haemolytica* and resistance against experimental challenge. *Am. J. Vet. Res.* 56: 875

Muhairwa, A. P., Mtambo, M M. A., Christensen, J. P. and Bisgaard, M. 2001. Occurrence of *Pasteurella multocida* and related species in village free ranging chicken and their animal contacts in Tanzania. *Vet. Microbiol.* 78: 139-153

Murata, M., Moruchi, 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

Murugkar, H. V and Ghosh, S. S. 1995. *Pasteurella multocida* serotype A:1 in ducks in Tripura. *Indian J. Comp. Microbiol Immunol. Infect. Dis.* 16(3): 109-111

Mutters, R.S., Ihm, P., Pohl, S., Frederiksen, W. and Mannheim, W. 1985. Reclassification of the genus *Pasteurella trevisan* 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis* and *Pasteurella langaa. Intl. J. System. Bacterial.* 35: 309-322

Mukkur, T. K. S. 1977. Demonstration of cross protection between *P. multocida* type A and *P. haemolytica*, serotype 1. *Infect. Immun.* 18: 583-585

Mulbagal, A. N., Kulkarni, W. B. and Paranjape, V. L. 1972. Some observations on pasteurellosis in ducks. *Indian Vet. J.* 49:544-546

Nakamine, M., Ohshiro, M., Ameku, Y., Oshiro, K., Keruma, T., Sawada, T. and Ezaki, T. 1992. The first outbreak of fowl cholera in muscory ducks (*Carina moschata*) in Japan. *J. Vet. Med. Sci.* 54: 1225-1227

Neter, E. 1956. Bacterial haemagglutination and haemolysis. *Bact. Rev.* 20: 156-168

Office International Des Epizootics. 2000. *Manual of Standards Diagnostic Tests and Vaccines*. Fourth edition. Paris, France, p 582

Office International Des Epizootics. 2004. *Manual of Standards Diagnostic Tests and Vaccines for terrestrial animals*. Fourth edition. Paris, France, p 582

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

Pal, A., Srivastava, S.K. and Singh, V.P. 2002. Heat modifiability of outer membrane proteins of *Pasteurella multocida* srotype B:2. *Indian J. Exp. Biol.* 40: 106-108

Panda, S.N., Misra, B., Das, B.C. and Nayak, B.C. 1981. An outbreak of avian pasteurellosis in Orissa. *Indian Vet. J.* 58: 418-420

Panigraphy, B. and Glass, S. E. 1982. Outbreaks of fowl cholera in quail. *Avian Dis.* 26: 200-203

Papova, T.P. and Tzvetkov, Y.M. 2002. Fowl cholera in pheasants (*Phasianus colchicus*) - etiological investigation and effect of therapy with thiamphenicol. *Bulgarian J. Vet. Med.* 5: 23-28

Pasteur, L. 1880. De l'attenuation du virus du cholera des poules. *Campt. Renel. Acad. Sci.* 90: 673-696

Pati, U.S., Srivastava, S.K., Roy, S.C. and More, T. 1996. Immunogenecity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Vet. Microbiol.* 52: 301-311

Pehlivanoglu, F., Morishita, T. Y., Aye, P. P., Porter, R. E., Angrick, E. J., Harr, B. S. and Nersessian, B. 1999. The effect of route of inoculation on the virulence

of raptorial *Pasteurella multocida* isolates in pekin ducks (*Anas platyrhynchos*). *Avian Dis.* 43: 116-121

Pillai, R. M., James, P. C., Punnoose, K. T., Sulochana, S., Jayaprakashan, V and Nair, G. K. 1993. Out break of Pasteurellosis among duck population in Kerala. *Kerala J. Vet and anim.* 24: 34-39

Praveen, Z., Nasir, A.A., Tasleem, K and Shah, A. 2003. Fowl cholera in a breeder flock. *Pakistan Vet. J.* 23: 209-210

Quinn P. J., Carter M. E., Markey. B. and Caster, G. R. 1994. Clinical Veterinary Microbiology. Wolf publishing, Mosby year book Europe, Ltd. Pp. 254-258

Rajagopal, R. 2007. Production and evaluation of vaccines employing *Pasteurella multocida* A:1 grown under different growth conditions. M.V.Sc. thesis submitted to Kerala Agricultural University. Thrissur. p 146

Rajalakshmi, S. 2001. Nucleic acid and protein profiles of *Pasteurella multocida* of avian origin. M.V.Sc. thesis submitted to Kerala Agricultural University. Thrissur. p 115

Ramanatha, K.R. and Gopal, T. 1993. A note on the isolation and characterization of *Pasteurella multocida* from ducks. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 14: 32-33

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

Ratafia, M. 1988. Cited by Rhodes, K. R. and Rimler, R. B. 1989. In "Diseases of Poultry".

Reed, J. L. and Muench, H. 1938. A simple method of estimating fifty per cent end points. *Am. J. Hyg.* 27: 493-497

Rhoades, K.R. and Rimler, R.B. 1989. Fowl cholera. *Pasteurella and Pasteurellosis.* (eds. by Adlam, C and Rutter, J, M.). Academic Press, London, pp 95-113

Rhoades, K. R. and Rimler, R. B. 1991. Pasteurellosis. In: Calnek, B. W., Barnes, H. J., Bareand, C. W., Reid, W. M. and Yoder, H.W, (eds). *Diseases of Poultry.* 9th ed. Iowa State University Press, Ames, Iowa, p. 929

Roy, S., Das, A.B., Ghosh, A.N. and Biswas, T. 1994. Purification, pore-forming ability, and antigenic relatedness of major outer membrane protein of *Shigella dysenteriae* type 1. *Infect. Immun.* 62: 4333-4338

Ruffolo, C.G. and Adler, B. 1998. Cloning, sequence, expression and protective capacity of the *Oma 87* gene encloding the *Pasteurella multocida.* 87-Kda outer membrane antigen. *Infect. Immun.* 64: 3161-3167

Sambyal, D.S., Soni, G.L., Sodhi, S.S. and Baxi, K.K. 1988. Characterisation of *Pasteurella multocida* (serotype I) from an outbreak of fowl cholera in ducks. *Indian J. Anim. Sci.* 58: 1059-1060

Sander, J.E and Glisson, J.R. 1989. Fowl cholera in broilers.*Avian Dis.* 33: 816-819

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. Clin. Microbiol.* 15: 752-756

Schlink, G. T. and Olson, L. D. 1986. Fowl cholera vaccination of growing turkeys with CU strain via routes other than oral. *Avian Dis.* 31: 22-28

Sharma, R. K., Rahman, H. and Das, S. K. 1999. Saline extract vaccine of *Pasteurella multocida* and its comparison with conventional bacterin vaccine. *Indian Vet. J.* 76: 685-688

Shilpa., Verma, P.C. and Minakshi. 2005. Different vaccines against local fowl cholera isolate. *Indian J. Anim. Sci.* 75: 199-202

Smith, J.E. 1955. Studies on *Pasteurella septica* II. Some cultural and biochemical properties of strains from different host species. *J. Comp. Pathol. Therap.* 69 : 315-323

Smith, I. M and Baskerville, J. 1983. A selective medium for the isolation of *P. multocida* in nasal specimens from pigs. *Br. Vet. J.* 139: 476

Snipes, K. T., Hansen, L. M. and Hirsh, D. C. 1988. Plasma and iron regulated expression of higher molecular weight outer membrane proteins of *Pasteurella multocida. Am J. Vet. Res.* 49 (8): 1136-1138

Snipes, K. T., Gharikhanian, G. Y. and Hirsh, D. C. 1987. Fate of *Pasteurella multocida* in the blood vascular system of turkeys following intravenous inoculation: comparison of an encapsulated virulent strain with its avirulent, acapsular variant. *Avian Dis.* 31: 254-259

Solano, W., Giambrone, J. J. and Panangala, V. S. 1984. Comparison of enzyme linked immunosorbent assay and indirect haemagglutination test for quantitating antibody responses in chickens against *Pasteurella multocida. Avian Dis.* 27: 1034-1042

Soodan, J. S., Randhawa, S. S. and Sharma, N. K. 2003. LD_{50} of *Pasteurella mdtocida* infection in hypocuprotic and copper supplemented mice. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 24: 99-100

Sreevatsan, S., Ames, T. R., Warden, R. E., Yoo, H. S., Maheswary, S. K. 1996. Evaluation of three experimental subunit vaccine against pasteurellosis in cattle. Vaccine. 14: 147-154

Srilatha, C., Devi, V.R., Sujatha, K. and Ahmed, M.N. 2003. An outbreak of fowl cholera in Japanese quails. *Indian Vet. J.* 80: 6-7

Srivastava, S.K. 1998. Outer membrane protein of *Pasteurella multocida* serotype B:2 is immunogenic and antiphagolytic. *Indian J. Exp. Biol.* 36: 530-532

Srivastava, S.K. 1999. Immunogenecity of protein fractions of *Pasteurella multocida* in animals. *Indian J. Anim. Sci.* 69: 653-656

Stone, H. D., Brugh, M., Hopkins, S. R., Yoder, H. W. and Beard, C. W. 1978. Preparation of oil-emulsion vaccines with avian viral or mycoplasma antigens. *Avian Dis.* 22: 666-674

Sulochana. 1998. Final report of the ICAR scheme on "Development of cell culture duck plague vaccine for simultaneous vaccination with duck pasteurellosis", Department of microbiology, College of veterinary and Animal Sciences, Kerala Agril. University. Pp 28-30

Swamy, C. L. 1994. Evaluation of humoral immune response of ducks to *Pasteurella multocida* serotype A: 1. M. V. Sc thesis submitted to University of Agricultural Sciences, Bangalore. p.113

Tabatabai, L. B. and Zehr, E. S. 2004. Identification of five outer membrane proteins among cross protective factor protein of *Pasteurella multocida. Infect. Immun.* 72(2): 1195-1197

Talha, A.F., Hussain, S.M., Chowdhury, M.M., Bari, E.H., Islam, H.R. and Das, P.M. 2001. Poultry diseases occurring in Mymemsingh district of Bangladesh. *Bangladesh Vet. J.* 18:20-23

Tomer, P., Chatruvedi, G.C., Minakshi, P., Malik, P. and Monga, D.P. 2002. Comparative analysis of the outer membrane protein profiles of isolates of the *Pasteurella multocida* (B:2) associated with haemorrhagic septicaemia. *Vet. Res. Commun.* 26: 513-522

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad.Sci. USA.* 76: 4350-4354

Waltman, W. D and Horne, A. M. 1993. Characteristics of fowl cholera diagnosed in Georgia, 1989-91. *Avian Dis.* 37: 616-621

Warner, 1996. Cited by De Alwis, 1999.

Wasnik, R. V., Srivastava, S. K. and Paul, A. 2004. Cross-protective efficacy of whole cell and outer membrane protein vaccines of *Pasteurella multocida* serotype B: 2. *Indian J. Anim. Sci.* 74: 805-809

Wijewardana, T. G., De Alwis, M. C. L. and Bastianz, H. L. G. 1986. Cultural, biochemical, serological and pathogenicity studies of strains of *Pasteurella multocida* isolated from carrier animals and outbreaks of haemorrhagic septicaemia. *S. L. Vet. J.* 34: 43-57

Woolcock, J. B. and Collins, F. M. 1976. Immune mechanism in *Pasteurella multocida*- infected mice. *Infect. Immun.* 13: 949-958

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.* 153(7): 705-708

Zaho G., Pijoan, C., Choi, K., Maheswaran, S. K. and Trigo, E. 1995. Expression of iron regulated outer membrane proteins by passive strains of *Pasteurella multocida, Can. J. Vet. Res.* 59: 46:50

Zinkl, J., King, D. and Hurt, J. 1977. Aspergillosis in common crows in Nebraska. *Jounal of wildlife diseases.* 13 (2): 191-193

DEVELOPMENT AND EVALUATION OF OUTER MEMBRANE PROTEIN VACCINE AGAINST DUCK PASTEURELLOSIS

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ABSTRACT

A research work was undertaken to prepare effective vaccine *multocida* grown under different conditions and their immunop assessed in one month old ducklings.

The purity of the *P. multocida* A:1 strain (DP1) was confi standard procedure. Pathogenicity of the isolate was assessed in six to old mice. The isolate killed the mice with in 8 h intra peritoneally and when injected subcutaneously.

Whole cell protein of *P. multocida* (DP1) was extracted an subjected to discontinuous system of SDS- PAGE which revealed 20 protein bands of molecular weight ranging from 102 to 19 kDa. Afte *.multocida* in iron sufficient and iron restricted medium its OMPs we and they were analysed by SDS PAGE. In iron sufficient medium bands of MW which ranging from 91.84 to 19.02 kDa were revealed to the above a protein band with MW of 97.8 kDa were detected in ir medium.The protein concentration was estimated by Modified Lov and it was found to be 4mg/ml.

The Median Lethal Dose (LD_{50}) of *P. multocida* when determ month old ducklings was found to be $10^{-7.13}$ which contained 13 cells

Oil adjuvanted formalin inactivated vaccines were prepared grown under three different conditions *viz.*, TSB, BHIB and BHIB su with 100 μM 2,2'dipyridyl. Sterility, safety and potency test of the va done as per standard procedures.

A total of 120 one month old ducklings were divided in to with 30 birds in each group. The first three groups were vaccinated w bacterin, OMP vaccine prepared under iron sufficient condition and OMP vaccine prepared under iron restricted condition respectively and the fourth group served

as control.The birds were vaccinated with 0.5millilitre of vaccine intramuscularly. The blood was collected from all the ducks on day 0,7, 14, 21, 28, 45 and 60 day PV.

Passive haemagglutination was done and the increase in antibody titre was observed from day 7 PV onwards for groups I, II and III. The highest antibody titre was obtained at 14th day PV and 21st day PV for OMP vaccine prepared under iron restricted condition. All the vaccine groups had shown a significant difference from the control group at all stages of study. On homologous challenging OMP vaccine under iron restricted media on 42nd day PV afforded 60 per cent protection. On 60th day PV afforded 50 per cent protection.

Though the OMP vaccine prepared under iron restricted condition was found to provide high antibody titre, the protection percentage afforded by it in comparison with ordinary bacterin and OMP vaccine prepared under iron sufficient media was low. Hence, the OMP vaccine prepared under iron restricted condition need to be subjected to further investigation.