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DEVELOPMENT AND EVALUATION OF DIFFERENT VACCINES 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 DIFFERENT VACCINES 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 Society.

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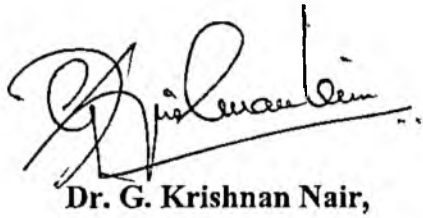
13-04-2010

CERTIFICATE

Certified that this thesis, entitled **"DEVELOPMENT AND EVALUATION OF DIFFERENT VACCINES AGAINST DUCK PASTEURELLOSIS"** is a record of research work done independently by **Jesto George,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, associateship or fellowship to him.



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Jesto George

Dedicated to my family.

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LIST OF ACRONYMS USED

APV	Alum precipitated vaccine
BHI	Brain heart infusion
BSA	Bovine serum albumin
CBD	Calgary biofilm device
CFU	Colony forming units
DP1	*Pasteurella multocida* serotype a: 1 isolate dp1
EHEC	Entero haemorrhagic *escherichia coli*
EPS	Exopolysaccharide
GA-SRBC	Glutaraldehyde fixed- sheep red blood corpuscles
GMT	Geometric mean titre
h	Hours
Ig	Immunoglobulin
IHA	Indirect haemagglutination
LD_{50}	Lethal dose 50
$LD_{50(11w)}$	Lethal dose 50 of *p. Multocida* at 11 weeks of age
$LD_{50(21w)}$	Lethal dose 50 of *p. Multocida* 21 weeks of age
LPS	Lipopolysaccharides
MDT	Mean death time
MHC	Major histocompatibility complex
MIHAT	Mean indirect haemagglutination titre expressed in $\log_2$
OMP	Outer membrane proteins
PBS	Phosphate buffered saline
PBV	Post booster vaccination
PCR	Polymerase chain reaction
PPV	Post primary vaccination
SDA	Sabouraud dextrose agar
TSA	Tryptose soya agar
TSB	Tryptone soya broth
W	Week

Introduction

1. INTRODUCTION

Poultry industry occupies a prominent position in agricultural sector all over the world and India is now the world's fifth largest egg producer and the eighteenth largest producer of broilers (FAO, 2003).

Among the domesticated avian species, ducks occupy the second position in India and are easy to rear in terms of feeding, housing and management. India has a 5700 kilometers long coastline and extensive water shed which are highly supportive for duck farming. Even with these promoting factors, present duck population in India is around 24 million, which comprise only 10 per cent of its poultry population. Hence it is clear that in India we are not at all are exploiting our resources and potential to support duck farming.

Generally ducks are more resistant to diseases compared to chicken. But ducks being water birds are more prone to spread of diseases like duck plague and duck pasteurellosis which pose a serious threat to duck farming. Pasteurellosis caused by *Pasteurella multocida* (*P. multocida*) usually occur as a septicaemic disease with high morbidity and mortality (Glisson *et al.*, 2003). It can occur either as primary disease or as secondary complication to viral diseases, stress and other managemental faults. When duck pasteurellosis occurs in association duck plague, mortality rate spikes, thereby sweeping the majority of the birds in a flock.

Duck plague is effectively prevented by vaccination. With regard to duck pasteurellosis present day vaccines used are formalin inactivated oil adjuvanted bacterins. They give protection only for a short duration of time and the immunity often wanes, resulting in disease outbreaks. Thus booster vaccination becomes a requirement to produce a solid long lasting immunity. But perusal of available literature indicated a lack of information on booster immune response of ducks

against *P. multocida* bacterin. Hence attempts should be made to fill up this gap in scientific knowledge.

Biofilms are communities of microorganisms attached to a surface (Otoole *et al.*, 2000). Formation of biofilms by bacterial species may be regarded as a strategy for survival and reproductive success rather than maximizing or increasing the biomass. *Pasteurella multocida* also could form biofilm at 42°C and such a growth pattern had resulted in production of novel immunogenic proteins. Hence biofilms could be recommended as candidate antigens for vaccines against fowl cholera (Hugar, 2004). Therefore an investigation into primary and secondary immune responses of ducks to vaccines developed from *P. multocida* biofilm antigens seems fruitful in the prevention of duck pasteurellosis.

Adjuvants are important components of vaccines as they help to elicit an early, high and long-lasting immune response with less antigenic dose. Aluminium compounds are the most widely used adjuvants with routine human and veterinary vaccines. The use of saponin compounds improved the immunogenicity of the aluminium adjuvant (Rurangirwa *et al.*, 1987). Hence it is quite logical to think that a combination of both may offer better protection than either of them used alone.

Bacterial ghosts have inherent adjuvant properties and the most important advantage in using them as immunogens is that no inactivation procedures that denature relevant immunogenic determinants are employed in their production. Thus ghost vaccine carries the advantages of live vaccine and at the same time is free from the risk associated with live vaccines (Lubitz *et al.*, 1999). Hence an attempt to produce a recombinant ghost system against *P. multocida* of duck origin will greatly help in formulating a ghost vaccine in future.

Considering all the above mentioned facts, the present study was undertaken with the following objectives.

a) Development of biofilm vaccines against duck pasteurellosis using oil, saponin and aluminium hydroxide as adjuvants.
b) Experimental evaluation of the immunogenicity of different biofilm vaccines in ducks, in comparison with oil adjuvanted bacterin.
c) To attempt development of a recombinant ghost system by expression of PhiX174 gene *E* in *Pasteurella multocida (P. multocida)* of duck origin.

Review of Literature

2. REVIEW OF LITERATURE

2.1. FOWL CHOLERA (AVIAN PASTEURELLOSIS)

Fowl cholera (avian pasteurellosis), caused by *Pasteurella multocida* (*P. multocida*) is a sporadic or enzootic bacterial disease of major economic importance among poultry population of the world (Rimler and Glisson, 1997). It is a contagious disease affecting both domesticated and wild birds. The disease usually appears to be septicaemic with high morbidity and mortality, although chronic or benign conditions also occur (Glisson *et al.*, 2003).

After the first report of *P. multocida* in 1881, this Gram-negative bacterium has been identified as the causative agent of many other economically important diseases in a wide range of hosts (Harper *et al.*, 2006).

2.1.1 Pasteurella multocida – Taxonomy and Nomenclature

Mutters *et al.* (1985) re-classified of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology and it clearly separated genus *Pasteurella* from the *Actinobacillus* group. According to this new taxonomy genus *Pasteurella* consisted of the following 11 species: *Pasteurella multocida, P. dagmatis, P. gallinarum, P. canis, P. stomatis, P. avium, P. volantium, P. anatis, P. langaa* and two new species, which were provisionally designated as *Pasteurella* species A and B.

Based on fermentation patterns of dulcitol and sorbitol, the taxon *P. multocida* could be divided into three subspecies. The sorbitol and dulcitol positive variety were classified under *P. multocida* subsp. *gallicida*; those strains which were negative for both were classified under *P. multocida* subsp. *septica* and

those positive for sorbitol but negative for dulcitol were *P. multocida* subsp. *multocida* (Mutters *et al.*, 1985).

Genus *Pasteurella* is classified under the family *Pasteurellaceae* along with other genera *Actinobacillus* and *Haemophilus*. *Pasteurella multocida* is the most common pathogen of the genus causing severe disease in poultry, leading to heavy economic loss (Bottone, 1998).

2.1.2 Incidence and Prevalence

2.1.2.1 Prevalence of the Disease Outside India

Fowl cholera outbreak in a flock of turkeys in USA produced a loss of over 68 per cent within six days (Alberts and Graham, 1948).

An epornitic of avian cholera had been reported in the spring, 1975 among waterfowl in Nebraska, America and the causative organism was identified to be *P. multocida* serotype 1 (Zinkl *et al.*, 1977).

Carpenter *et al.* (1989) recovered 49 isolates of *Pasteurella multocida* from 11 cases of fowl cholera out breaks in turkeys and failed to isolate the organism from healthy birds, even though isolation trials were conducted.

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

Pasteurella multocida had been implicated in sinusitis reported in Khaki Campbell ducks (*Anas platyrhynchos*) in Thailand (Songserm *et al.*, 2003).

Mbuthia *et al.* (2008) isolated *P. multocida* from 25.9 per cent healthy-looking ducks when they were screened for bacterial diseases, indicating that carrier state was also present in ducks.

2.1.2.2 Occurrence in India

In India the earliest documented report of fowl cholera was in 1947 (Mulbagal *et al.,* 1972). After that, there were intermittent case reports of fowl cholera from different parts of the country.

In 1988 Kulkarni *et al.* (1988) reported an outbreak of fowl cholera in six-day-old chicks in an organized poultry farm in Parbhani, Maharashtra. In the same year *P. multocida* was isolated and characterized from an outbreak of fowl cholera in ducks from Srinagar, Jammu and Kashmir (Sambyal *et al.*, 1988).

Later Ramanath and Gopal (1993) reported outbreak of pasteurellosis among ducks maintained in an organized duck farm in Karnataka state. The causative strain was found to be *P. multocida* A: 1. Murugkar and Ghosh (1995) reported similar outbreak among ducks of Tripura, caused by the same strain.

Isolates of *P. multocida* were obtained on bovine blood agar from ducks showing disease symptoms in Kuttanad area of Kerala (Jayakumar, 1998). Investigation on 20 outbreaks of duck cholera in Andhra Pradesh by Devi *et al.* (2000) revealed that most of the outbreaks occurred during early monsoon period and the age group most affected was four to twelve months.

Twenty seven duck isolates and two poultry isolates had been obtained from Kerala in 2004. Two biotypes were observed among the duck isolates and they were *P. multocida* subsp. *septica* and *P. multocida* subsp. *multocida* (Antony, 2004).

Bhattacharya (2005) reported mortality ranging from 11 to 20 per cent after investigating eight outbreaks of duck cholera in ducks of Tripura. A high percentage (77.77 per cent) of *P. multocida* isolates were recovered from ducklings up to six weeks of age, while only 22.22 per cent of the isolates were from adult ducks.

Incidence of avian pasteurellosis in wild geese in captivity has also been reported from Tamil Nadu (Purushothaman *et al.*, 2008). Among migratory birds of India, a very high indirect haemagglutination (IHA) titre of 64 and above was observed among six per cent of total birds screened. This high antibody titre reflected recent infections of pasteurellosis in the migratory bird population (George *et al.*, 2009).

2.1.3 Susceptibility

The environmental factors such as overcrowding, climatic variation, nutrition and concurrent disease were the main factors that affected the incidence and severity of the disease (Alberts and Graham, 1948).

The immune status of the bird gave protection against the strain of *P. multocida* organism with which they had previous contact, but birds were often susceptible to other strains of *Pasteurella*. Although all species of birds are susceptible to pasteurellosis, turkeys are considered to be the most susceptible species. Ducks are less susceptible and chicken seem to be the least susceptible of the three (Jordan and Pattison, 1996).

The adult birds have been reported to be more susceptible than younger stock (Jordan and Pattison, 1996; Petersen *et al.*, 2001).

2.1.4 Source of infection

Hunter and Wobeser, (1980) commented that mallard ducks that survived acute pasteurellosis sequestered bacteria, thereby acting as potential carrier birds. Asymptomatic carriers of *P. multocida* acting as reservoir of infection, thereby transmitting disease by direct or indirect contact had also been reported by Carter and Dealwis (1989).

The carrier birds, clinically diseased birds and their excretions, carcasses of birds which had died of infection and rodents could act as source of infection (Jordan and Pattison, 1996). In recent years, molecular typing methods have been applied to epidemiological studies on avian strains of *P. multocida* of different origin. The results obtained indicated that, wild birds might be a source of infection to commercial poultry (Christensen and Bisgard, 2000).

Chronically infected birds appeared to be the major source of infection and transmission seldom occurred through eggs (Glisson *et al.*, 2003). Most strains of *P. multocida* isolated from related outbreaks showed genetic relatedness on molecular epidemiological investigation, suggesting that the consecutive outbreaks were mostly due to recurrences rather than reinfection (Kardos and Kiss, 2005). Thus the pathogen might survive among carrier birds, causing disease outbreaks among the flock, when immunity waned.

2.1.5. Clinical Signs and Lesions

2.1.5.1 Clinical Signs

The clinical signs were varied in different forms of the disease. In per acute form, birds were found to be dead in good bodily condition without any premonitory symptoms. In acute forms depression, anorexia, mucous discharge from orifices, cyanosis and fetid diarrhoea were the typical signs observed. Chronic form was characterized by depression, conjunctivitis and dyspnea, with more survivability (Jordan and Pattison, 1996).

2.1.5.2 Gross Lesions

Mallard Ducks that died of avian cholera had lesions of septicaemia with widespread vascular damage and focal necrosis in liver, spleen and other organs.

Ducks that survived challenge developed chronic lesions in a variety of organs, including brain, lung, air sacs, joints, and eyes (Hunter and Wobeser, 1980).

Pneumonia was the common lesion in turkeys infected with *P. multocida.* The other pathological changes observed were swollen liver with multiple small areas of coagulative necrosis and necrotic foci of spleen. Localized lesions were observed in chronic cases and the tissues involved were sinuses, pneumatic bones, lungs, air sac, wattles, sternal bursa, foot pad, hock joints, peritoneal cavities, oviduct, meninges and conjunctivae (Rimler and Rhoades, 1989).

The gross pathology of pasteurellosis in experimental mice was characterized by splenomegaly, lymphadenopathy and petechial haemorrhage in internal organs, similar to that observed in cattle and buffalo. Thus mouse would seem to provide an ideal tool to study pasteurellosis (Ramdani *et al.*, 1990).

.Kwon and Kang (2003) found that the consistent clinical lesions in fowl cholera outbreak in Baikal teals (*Anas formosa*) were enlarged liver with multifocal necrotic changes, petechial or ecchymotic haemorrhages on the heart and mucoid exudates in the duodenal mucosa.

2.1.5.3 Microscopic Lesions.

2.1.5.3.1 Histopathology of Spleen

2.1.5.3.1.1 Histopathology of Spleen in Ducks

Necrosis of lymphoid follicles of spleen was the main lesion observed in both experimental fowl cholera (Hunter and Wobeser, 1980) and natural fowl cholera outbreaks in ducks (Fujihara *et al.*, 1986) . Nakamine *et al.* (1992) observed decrease in lymphocytes and marked increase in reticulocytes as the main changes in the spleen of Muscovy ducks infected with *P. multocida.*

In an outbreak of pasteurellosis in ducks massive necrosis in the lymphoid follicles of spleen and pneumonia were observed by Hegde *et al.* (1996).

2.1.5.3.1.2 Histopathology of Spleen in Other Species

Prantner *et al.* (1990) noted that there was degeneration of peri-arteriolar reticular cells in spleen and degenerated cells progressed to coalescing coagulative splenic necrosis with extracellular bacterial colonies in experimental pasteurellosis in turkeys.

In fowl cholera affected psittacine the evident splenic lesions were multiple randomly distributed foci of coagulation necrosis containing degenerating mononuclear cells and numerous clusters of bacteria, lymphoid hyperplasia and plasmacytosis (Morishita *et al.* 1996).

Multiple foci of acute necrosis containing degenerated heterophils and clusters of bacteria surrounded by multinucleated giant cells were the lesions observed in spleen of fowl cholera affected raptors by Morishita *et al.* (1997).

Splenic reticular cell hyperplasia was observed in sub acute to chronic fowl cholera in quails (Miguel *et al.*, 1998). Fibrin thrombi were present throughout the splenic white pulp in broilers in experimental pasteurellosis (Fisher *et al.*, 1998).

Splenic changes reported by Shilpa *et al.* (2005) in experimental pasteurellosis in layers were lymphoid depletion, reticuloendothelial cell hyperplasia and secondary lymphoid nodules.

2.1.5.3.2 Histopathology of Liver

2.1.5.3.2.1 Histopathology of Liver in Ducks

Congestion and multiple areas of focal necrosis in liver were the main lesions observed in natural outbreaks of pasteurellosis in mallard ducks (Hunter and Wobeser, 1980) and Muscovy ducks (Fujihara *et al.,* 1986; Nakamine *et al.*, 1992).

A large number of bacteria were also observed in these necrotic areas and within the sinusoids. In addition to these microscopic lesions, Hegde *et al.* (1996) observed interlobular connective tissue proliferation and biliary hyperplasia in liver.

2.1.5.3.2.2 Histopathology of Liver in Other Species of Birds

Multifocal necrotic areas and inflammatory cell infiltration were observed in outbreaks of pasteurellosis in liver of other bird species like turkeys (Jeffrey *et al.*, 1993; Prantner *et al.*, 1990; Morishita *et al.*, 1990), chicken (Sahoo *et al.*, 1994; Kwon and Kang, 2003; Shivachandra *et al.*, 2005), psittacine birds (Morishita *et al.*, 1996), raptors (Morishita *et al.*, 1997) and quails (Roy *et al.*, 2004).

Periportal lymphocytic and heterophilic infiltration was also found in liver in a case of chicken pasteurellosis (Gustafson *et al.*, 1998). Mild hepatic amyloidosis was observed in a case of sub acute to chronic fowl cholera in quails (Miguel *et al.*, 1998). Lymphocytic infiltration was seen in hepatic portal triads in broilers in experimental pasteurellosis (Fisher *et al.*, 1998).

2.1.5.3.3 Histopathology of Caecal Tonsil

In birds the wall of caecum contains a lymphoid tissue called caecal tonsil and is well developed in the proximal part of caecum. In water fowls the organ occur as annular bands (Mc Lelland, 1975; Shivaprasad, 1998). Lymphoid depletion had been reported as a lesion in cecal tonsil in pigeons in circo virus infections (Shivaprasad, 1998).

2.1.5.3.4 Histopathology of Bursa of Fabricius

Fisher *et al.* (1998) noted mild lymphoid depletion and foci of necrosis in bursa and thymus of broilers experimentally inoculated with *P. multocida.*

The bursa of Fabricius of chicken is a unique organ of the poultry for the production of B- lymphocytes. It is a blind, globular shaped, sac-like, dorsal diverticulum of the proctodeal wall of the cloacae, attached to the dorsal aspect of the proctodeum (Akter *et al.*, 2006).

2.1.6 Pathogenesis and Virulence

2.1.6.1. Virulence Determinants of Pasteurella multocida

The loss of ability of a virulent strain of *P. multocida* to produce the capsule resulted in loss of virulence (Heddleston *et al.,* 1964).

The loosely bound endotoxins of *P. multocida* were found to be composed of nitrogen containing lipopolysaccharides which had the capability of inducing symptoms of acute fowl cholera, when injected in sufficient quantity to fowl. The serologic specificity of the organism was associated with lipopolysaccharide and free (loosely bound) endotoxin. Endotoxins were also able to induce active immunity (Heddleston and Rebers , 1975).

In Gram negative bacteria, the capsule lies outside the outer membrane and is composed of highly hydrated polyanionic polysaccharides. These molecules can mediate a number of biological processes, including invasive infections (Roberts, 1996).

Acapsular *P. multocida* organisms were readily taken up by murine peritoneal macrophages and were removed from blood, spleen and liver, while wild-type capsulated bacteria were significantly resistant to phagocytosis and multiplied in the body, following intra peritoneal challenge in mice (Boyce and Adler, 2000).

Harper *et al.* (2006) reviewed that, key virulence factors identified till date included capsule and lipopolysaccharide. The capsule was clearly involved in

bacterial avoidance of phagocytosis and resistance to complement, while complete lipopolysaccharide was critical for bacterial survival in the host. Other virulence factors identified were *P. multocida* toxin (PMT), putative surface adhesins and iron acquisition proteins. They opined that many key virulence factors were yet to be identified, including those required for initial attachment and invasion of host cells and for persistence in a relatively nutrient poor and hostile environment.

Therefore it is unequivocally accepted that the presence of the capsule is the crucial virulence determinant for *P. multocida,* although other factors could also be demonstrated.

2.1.6.2 Mean Death Time (MDT) of Pasteurellosis and Pathogenicity Testing

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

When doses as few as 20 colony forming units (CFU) of *P. multocida* M1404, the type strain for Carter group B and the serotype responsible for Asian haemorrhagic septicaemia, were injected intra peritoneally into BALB/c mice, an overwhelming septicaemia was produced in mice within 30 h. Thus kinetics of infection demonstrated a very rapid *in vivo* spread of the organism with no evidence of inhibition of bacterial cell growth by natural host defense mechanisms, even with the very small dose of bacterium (Ramdani *et al.*, 1990).

Murugkar and Ghosh (1995) tested the pathogenicity of *P. multocida* serotype A: 1 isolated from ducks in Tripura, in different hosts such as pigeon, duck, mice and rabbit by intraperitoneal route and found that the isolate killed all the hosts, though at varying time intervals. In their study the mice died within the least time (12 h) compared to others. Thus the duck isolates may be considered to be pathogenic to mice also.

When *P. multocida* isolates obtained from wild geese in captivity were subjected to mouse bioassay, Purushothaman *et al.* (2008) observed them to be virulent with a MDT between 12 and 18 h.

2.1.6.3 Serum Resistance to Pasteurella multocida.

In experimental studies, the less virulent strains of *P. multocida* were found to have a lower survival rate in turkey serum than highly virulent strains. Thus serum-resistant strains may have a survival advantage in the body of host, which allows them to proliferate and produce disease (Taylor, 1983; Morishita *et al.*, 1990). The inhibitory effect of avian serum on growth of various isolates of *P. multocida* had been demonstrated by other workers also. Separate studies on outbreak strains revealed that 60 per cent of the strains were resistant to turkey serum (Lee *et al.*, 1999) and 88 per cent were resistant to chicken serum (Diallo & Frost, 2000). The growth of around 20 per cent of *P. multocida* strains obtained from fowl cholera outbreaks was inhibited in presence of duck sera (Muhairwa *et al.*, 2002).

2.1.7 Isolation and Identification

2.1.7.1 Isolation

Pasteurella multocida can be easily isolated from tissues such as liver, spleen, lungs and from the heart blood of birds during the acute phase of the disease and from localized lesions in chronically affected birds. *Pasteurella multocida* is somewhat fastidious and isolation from clinical specimen is usually made on media containing five per cent sterile serum or blood of bovine, ovine or equine origin (Rimler and Rhoades, 1989).

The organism was found to grow well in ovine blood agar in the temperature range between 12°C to 43°C, with an optimal temperature of 37°C (Smith and Philips, 1990). Most workers made primary isolation of *P. multocida* from ailing

ducks by inoculating the clinical material into blood agar and incubating at 37^0C for 24 h under microaerophilic conditions. Following incubation on blood agar the orgamism produced mucoid, convex, greyish-white and non-haemolytic colonies (Rajini *et al.,* 1995; Jayakumar, 1998; Muhairwa *et al.,* 2001 and Antony, 2004).

Favorable temperature for the growth of *P. multocida* was found to be at 35±5°C, whereas poor growth was observed at below 25±5°C and the organism did not grow at 50°C or more. Shaking up to 500 rpm was also found to have a positive effect on growth (Shah *et al.*, 2008).

2.1.7.2 Identification

2.1.7.2.1 Morphology and Staining

According to Rimler and Rhodes (1989) the *Pasteurella* cells usually measure 0.2-0.4 X 0.6 – 2.5 μm. They also commented that growth under unfavorable condition or repeated sub culturing could induce pleomorphism. Old laboratory cultures tend to consist predominantly of filamentous forms.

Tissue impression smear or blood smear from septicaemic cases, stained by Giemsa or Leishman's methods, reveal large number of bipolar organisms (Quinn *et al.*, 2002).

2.1.7.2.2 Cultural Characters

Colonies of *P. multocida* were grayish, shiny and non hemolytic on blood agar. Colonies of some pathogenic strains were mucoid due to production of thick capsules. The colonies had a subtle, but characteristic sweetish odour. Most pathogenic *Pasteurella* sp. did not grow on Mac Conkey agar (Quinn *et al.,* 2002).

On primary isolation from birds with fowl cholera, *Pasteurella* colonies were found to be iridescent, sectored with various intensities of iridescence, or blue with

little or no iridescence, when observed under obliquely transmitted light. Iridescence was related to presence of capsule (Glisson *et al.*, 2003).

2.1.7.2.3 Biochemical Characterization

Out of 42 *P. multocida* isolates, none fermented lactose, trehalose, or salicin in sugar fermentation tests (Shigidi and Mustafa, 1979). De-carboxylation of ornithine, production of acid from mannitol and indole production could be used for the sub species classification of *P. multocida* (Bisgaard *et al.*, 1991). Biochemical studies conducted on 43 *P. multocida* isolates of animal and avian origin from India revealed that all the isolates fermented dextrose, mannose and fructose and all were negative for fermenting inulin, lactose, salicin, maltose, rhamnose, inositol and dextrin (Kumar *et al.*, 1996) .

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

2.1.7.2.4 Serotyping and Serogrouping

Carter system of classification for *P. multocida* is based on passive haemagglutination of erythrocytes by capsular antigen. Five capsular serogroups, namely, A, B, D, E and F had been reported under this system (Carter, 1955). Heddleston system of serotyping of *Pasteurella multocida* based on gel diffusion precipitin tests, employing heat extracted somatic antigen and anti *Pasteurella* sera prepared in chicken, classified *P. multocida* into sixteen somatic serotypes named 1 to 16 (Heddleston *et al.*, 1972). The Carter and Heddleston systems may be combined and used to designate serotype, so that a serotype would be designated by its capsular type, followed by its somatic type as determined by the agar gel

precipitin test (Carter and Chengappa, 1981).Thus current serotyping methods classify *P. multocida* into five capsular serogroups (A, B, D, E, and F) and 16 somatic serotypes (1 to 16). The multiplex polymerase chain reaction based on cap specific primers is a rapid alternative to the conventional capsular serotyping system in the definitive classification of *P. multocida* capsular types. The method can also clarify the distinction between closely related serogroups A and F (Townsend *et al.*, 2001).

A polymerase chain reaction (PCR) targeting the hyaC-hyaD gene, to identify strains of *P. multocida,* belonging to serogroup A, had been developed later. A set of serogroup specific PCR primers amplified a 564 bp product from genomic DNA. This method detected as low as 10 nanogram of bacterial DNA and had a specificity of 100 per cent for *P. multocida* serogroup A (Gautam *et al.*, 2004).

Shivachandra *et al.* (2005) detected multiple strains of *P. multocida* in fowl cholera outbreaks by PCR based typing. Their investigations of fowl cholera outbreaks in a poultry farm indicated that molecular methods of detection and typing were rapid in comparison with conventional methods for epidemiological investigations, although both methods were efficient in the identification and characterization of *P. multocida* strains.

2.1.7.2.5. Polymerase Chain Reaction

Lee *et al.* (1999) modified *Pasteurella multocida* specific PCR for detection of the bacteria from chicken alimentary tract, even when only ten organisms were present in the sample.

Townsend *et al.* (1998) developed a *P. multocida* specific PCR (PM-PCR) that identified all subspecies of *P. multocida viz.*, subsp. *multocida*, subsp. *gallicida* and subsp. *septica*, through specific amplification of an approximately 460 bp DNA

fragment within the KMT1 gene using species specific primers KMTISP6 and KmT177. This PM-PCR was later used for the diagnosis of *P. multocida* antigen in suspected avian samples (Antony, 2004; Shivachandra *et al.*, 2005 and Leotta *et al.*, 2006).

2.1.8 Antibiotic Sensitivity

A single disc method based on the measurement of diameter of inhibitory zones for testing antibiotic susceptibility of bacterial isolates was developed by Bauer *et al.* (1966).

A high sensitivity of *Pasteurella* isolates to penicillin has been reported (Morris *et al.*, 1989; Williams and Horne, 1993).*Pasteurella multocida* isolates of duck origin were found to be sensitive to chloramphenicol, chlortetracycline, oxytetracycline, co-trimoxazole, nalidixic acid, gentamicin, nitrofurantoin, streptomycin, kanamycin and neomycin and to a lesser extent against polymyxin-B, penicillin G, amoxicillin, cloxacillin, lincomycin and vancomycin (Rammanath and Gopal, 1993).

Antibiogram studies by Diallo *et al.* (1995) on 45 avian strains of *P. multocida* showed that all the strains were resistant to streptomycin, lincomycin and trimethoprim and susceptible to ampicillin, penicillin, gentamicin, erythromycin, trimethoprim, nitrofurantoin and sulfanilamide.

Per acute fowl cholera is rapid and hence antibiotic treatment is rarely of value. In less acute forms, a number of drugs have proved to be effective. In order to eradicate infection from premises, it is necessary to depopulate, cleanse and disinfect buildings and equipment (Jordan and Pattison, 1996).

All the 27 *P. multocida* isolates of avian origin from Kerala were found to be uniformly sensitive to enrofloxacin, chloramphenicol and pefloxacin. Seven isolates

representing 25.92 per cent of total number were resistant to co-trimoxazole. *Pasteurella multocida* DP1 strain isolated from an outbreak at Niranam (Kerala) was found to be sensitive to ciprofloxacin, bacitracin, enrofloxacin, pefloxacin, gentamicin, tetracycline, nitrofurantoxin, chloramphenicol, co-trimoxazole and resistant to ampicillin, cloxacillin, penicillin, streptomycin, metronidazole, and furazolidone (Antony, 2004).

2.1.9 Immunity to *Pasteurella multocida*

2.1.9.1 Humoral Immune Response of Duck

Higgins and Warr (1993) reviewed that four types of immunoglobulins (Ig) had been identified in ducks, *viz.,* IgM, a secretory Ig resembling IgM, a 7.8S IgG, and a 5.7S IgG. Structurally and antigenically the 5.7S IgG resembles a F(ab')2 fragment of the 7.8S IgG. When ducks mount serum antibody responses, the sequence of Ig involvement is IgM →7.8S IgG →5.7S IgG.

Immunoglobulin (Ig) Y is the major antibody produced by chickens (*Gallus domesticus*), and is continually synthesized, excreted into the blood and transferred to the egg yolk, where it is accumulated. It provides their offspring with an effective humoral immunity against the commonest avian pathogens until full maturation of their own immune system (Silva, and Tambourgi, 2010).

2.1.9.2. Passive (Maternal) Immunity

Immunoglobulin G is the immunoglobulin class which is active in yolk-sac transmission of maternal antibodies in ducks and the levels of maternally derived 7.8S IgG in duckling sera decreased after 5 days of age, reaching minimum levels at about 14 days of age. Increases in the serum levels of 7.8S IgG, 5.7S IgG and IgM occurred after 20 days of age, reflecting *de novo* synthesis by the duckling and the adult serum profile was achieved by 71 days of age (Liu and Higgins, 1990).

An investigation on the relationship between decrease in egg yolk antibody and serum antibody to *P. multocida* following booster vaccination conducted by Ling *et al.* (1998) revealed that they were directly linked. They also observed that the egg yolk antibody and serum antibody declined six and three days respectively following booster vaccination and the decline was found to be proportional to titre before booster vaccination.

2.1.9.3 Active Immunity

2.1.9.3 .1 Protective Ability of Specific Opsonins

In the absence of specific opsonins, more than 90 per cent of the challenge organism remained in the extracellular growth phase throughout the challenge period. The unvaccinated mice died nine to twelve hours after intravenous challenge of 10^3 to 10^4 viable organisms due to this uncontrolled growth of the organism in all tissues tested (Collins, 1973). When unimmunized mice were inoculated with *P. multocida* opsonized with specific anti *Pasteurella* antibodies, they were inactivated rapidly by normal mouse peritoneal macrophages. At the same time the bacteria were slowly inactivated by normal mouse alveolar macrophages when the non opsonized organisms were given (Collins *et al.*, 1983).

2.1.9.3 .2 Antibody Response Against Vaccination

In a serological study conducted among 400 waterfowls by Donahue and Olson (1969) although they are able to detect antibodies for *P. multocida,* no organisms were isolated from nasal pharynx from any of them.

Mice vaccinated with two doses of 10^8 CFU of heat-killed (60°C for 60 min) *P. multocida* organisms survived 100 to 1,000 lethal challenge doses of the same organism. The maximum resistance to challenge was observed 21 to 28 days after the booster dose of antigen, and this correlated with an 8 to 16 fold increase in

specific agglutinin titres over the same time (Collins, 1973). The protective nature of hyperimmune mouse serum to a challenge of 500 to 5,000 organisms of *P. multocida* was also proven by Woolcock and Collins (1976).

When encapsulated organisms were used for vaccination, Chae *et al.* (1990) could not find any significant differences in opsonizing capacity of sera from vaccinated groups of mice, compared to non vaccinated groups. He also noted a difference between two groups when de-capsulated organisms were used as vaccines, thereby emphasizing the disability of serum anti-capsular antibodies in exerting a complement mediated bactericidal effect. This was supported by the study conducted in experimental mice by Lu *et al.* (1991) which demonstrated that, it was the antibodies against *P. multocida* outer membrane proteins (OMP), but not lipopolysaccharides (LPS), which most likely protected vaccinated animals against pasteurellosis.

The IHA titres of anti *Pasteurella* antibodies in sera of one month old ducklings procured for experimental purpose were reported to be zero and when they were immunized with bacterin prepared from *P. multocida* DP1 strain, showed mean titres varying from 8 to 128 (Jayakumar, 1998). But when Akand *et al.* (2004) assessed the mean IHA titres of anti *Pasteurella* antibodies in sera samples of healthy non vaccinated fayoumi birds; titres were found to be ≤ 4.0.

2.1.10. Outer Membrane Proteins (OMPs)

Outer membrane proteins are at the interface of bacterium and host and are likely to play important roles in host specificity and disease. *Pasteurella multocida* show adaptation to specific host niches and this is evident from the fact that *P. multocida* isolates associated with different diseases and host species expressed different iron-uptake proteins, or regulated expression of proteins (Wheeler, 2009).

2.1.11. Lethal Dose 50 (LD 50) of *Pasteurella multocida*

Before vaccination trials, it is necessary to determine the LD_{50} of the challenge organisms to establish the efficacy and specificity of the vaccine. Use of highly virulent strains of *P. multocida* for vaccination experiments were reported earlier.

A potassium thiocyanate (KSCN) extract based vaccine prepared from organisms with a LD_{50} as low as 4×10^2 cells per mouse was shown to be effective (Smith *et al.*, 1981).

The LD_{50} was found to be very low for isolates from ducks, when compared to isolates from mammals in case of *P. multocida* and results obtained by different workers lie in close range. When the LD_{50} of *P. multocida* isolated from duck was estimated in mice, it was found to be 15 viable cells per dose (Swamy, 1994). The LD_{50} of *P. multocida* A: 1 was estimated in mice and it was found to be 14 CFU by 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, were 23 and 32 cells respectively.

According to Basagoudanavar *et al.* (2006), LD_{50} of *P. multocida* was found to be 25 CFU in mice.

Rajagopal (2007) inoculated serial dilutions of *P. multocida* A: 1 DP1 strain intramuscularly to one month old ducklings and determined the LD_{50} to be 23 cells.

Ranjini (2007) and Indu (2008) estimated the same as 13 cells and 32 viable cells respectively when tested on ducks under similar experimental conditions.

2.2 BIOFILMS

2.2.1. Introduction

From Louis Pasteur onwards, traditional bacteriological research was based on isolation and maintenance of organism by pure culture in well defined bacteriological media. Further studies were conducted by growing bacteria on these nutrient rich unrestricted conditions, where they grew as individuals. Later a different mode of growth of bacteria was discovered, which actually mimicked the community life of higher life forms. Scientists called such growing communities of bacteria as biofilms.

Biofilms are physiologically distinct from planktonic (free swimming) bacteria of the same species (Gilbert *et al.*, 1997). They are communities of microorganisms attached to a surface (Otoole *et al.*, 2000) and their formation is a ubiquitous phenomenon on surfaces in contact with liquids (Muruga *et al.*, 2001).

Prakash *et al.* (2003) defined biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment.

Formation of biofilms by bacterial species may be regarded as a strategy for survival and reproductive success rather than maximizing or increasing the biomass. Although bacterial infections are widely reported in animals, their association with biofilms is rarely discussed even though they are of significant importance to both medical and veterinary science (Clutterbuck *et al.*, 2007).

2.2.2. Biofilms and Diseases

Biofilms are believed to be involved in many animal diseases like mastitis (*Streptococcus agalactiae, Staphylococcus aureus*), pneumonia (*Mannhaemia haemolytica, P. multocida*), liver abscess (*Fusobacterium necrophorum*), lymphadenitis (*Corynebacterium pseudotuberculosis, Streptococcus* sp.), enteritis (*Escherichia coli, Salmonella* sp.) and wound infections (*Staphylococcus aureus, Pseudomonas aeruginosa*) (Costerton *et al* ., 1999).

2.2.3. Multi species biofilms and Mono species biofilms

Generally multi species biofilms dominate in many natural habitats, whereas mono species biofilms are preferably found in organs of higher animals subjected to infection (Otoole *et al.*, 2000). In natural conditions, mono species biofilms are relatively rare; thus most biofilms are composed of mixtures of micro-organisms. This adds to the interspecies and intra species interactions and to the general complexity of the biofilms (Sutherland, 2001). According to Greenberg (2003) biofilms are formed as either mono- or multi species communities, in which the cells interact with each other to adapt them to varying environmental conditions.

2.2.4. Biofilm structure

The surface-attached communities of bacterial cells in a bacterial biofilm are embedded in an extracellular matrix of biopolymeric substances (Costerton *et al.*, 1995). These polysaccharides interact with a wide range of other molecular species, including lectins, proteins and lipids, thereby forming various types of tertiary structures within a biofilm. In these tertiary structures, cells and cell products are also trapped (Sutherland, 2001).

According to Eighmy *et al.* (1983) in biofilm presence of an extensive glycocalyx network was seen to form a matrix around the bacteria and anchor the biofilm to the stub surface.

Confocal scanning laser microscopic studies on *Serretia marcescens* biofilms by Rice *et al.* (2005) indicated that in them the structure of biofilm depended on the nutrient availability and under high nutrient conditions a flat undifferentiated biofilm was formed and under low nutrient conditions a classic differentiated biofilm was formed.

2.2.4.1 Studies on Biofilm Structure

2.2.4.1.1 Light Microscopic Studies on Biofilm Structure

Eighmy *et al.* (1983) carried light microscopic studies on biofilm formations and observed that in biofilm formations of waste water mostly the attached bacteria were Gram negative and the overlying, loosely bound component contained Gram-negative filaments.

2.2.4.1.2 Scanning Electron Microscopic Studies on Biofilm Structure

Eighmy *et al.* (1983) observed a variety of bacterial morphologies in scanning electron micrograph of biofilm samples. Bacillary, coccal, spiral, and helical morphologies were seen. The bacteria comprising the biofilm appeared to be more clumped rather than being a confluent monolayer of cells. Many micro colonies were seen embedded in a common capsule. By 144 h of biofilm formation, filaments as long as 30 micrometer was seen as a component of the biofilm and these filaments were tangled among the underlying, firmly bound component.

In scanning electron microscopic studies on biofilm formed by a poultry isolate of *Pasteurella* in TSB on Calgary biofilm device, a confluent monolayer of cells like structures were visualised (Olson *et al.*, 2002).

2.2.5. Changes during Biofilm Formation

Microorganisms undergo wide variety of changes during their transition from planktonic organisms to biofilm mode of growth and review of scientific literature indicates that the planktonic to biofilm transition is a complex and highly regulated process (Otoole *et al.*, 2000).

Dunne (2002) reviewed that at microscopic level biofilms might look like an underwater coral reef with pyramid or mushroom shaped projections extending away from the surface and channels and caverns running throughout.

The biofilm cells can increase their resistance to antibiotics a hundredfold compared to free-living ones and this ability gives them a significant role in medical microbiology (Greenberg, 2003).

There was sufficient evidence to show that expression of a lot of genes was up regulated in biofilm mode of growth. The significantly up-regulated genes were those encoding binding proteins, proteins involved in the synthesis of murein and glucosaminoglycan polysaccharide, intercellular adhesin and other enzymes involved in cell envelope synthesis and function, formate fermentation, urease activity, the response to oxidative stress and as a consequence thereof, acid and ammonium production. These factors might contribute to survival, persistence, and growth in a biofilm environment. Under planktonic growth conditions the main up-regulated genes were those encoding for toxins and proteases (Genetik and Morgenstelle, 2005).

2.2.6. Factors Leading to Biofilm Formation

The amount of exopolysaccharide (EPS) synthesis by bacteria will depend greatly on the availability of carbon substrates (both inside and outside the cell) and on the balance between carbon and other limiting nutrients. The presence of excess

available carbon substrate and limitations in other nutrients, such as nitrogen, potassium or phosphate, will promote the synthesis of EPS (Sutherland, 2001).

Nutrient limitation was also attributed to cause enhancement of capsule (Zaragoza and Casadevall, 2004). Rezende *et al.* (2005) were of the opinion that the capsular polysaccharides played an important role in the formation of biofilms.

The F9 fimbriae of *E. coli* have the ability to mediate strong biofilm growth (Ulett *et al.*, 2007).

2.2.7 Substrates for Biofilms

Incubation of *Vibrio cholerae* with chitin particles resulted in adsorption of vibrios onto chitin and this chitin-adsorbed *Vibrio cholerae* survived exposure to acid better than non adsorbed vibrios. *Vibrio cholerae* multiplied in dialyzed chitin suspended in 4.2 per cent NaCl, and mean *Vibrio* counts changed little after incubation with chitin at 19°C for 24 h, but fell sharply in the absence of chitin, suggesting chitin as a good substrate for adherence and multiplication of the bacteria (Nalin *et al.*, 1979).

In providing a surface of attachment for *Vibrio cholerae* biofilm, chitin was distinct from the others substrates like silicates and cellulose because many *Vibrio* species were able to use it as a sole carbon and nitrogen source (Colwell and Spira, 1992).

A lot of other materials had been used as attachment substrate for biofilm production and some of them are polystyrene or PVC plates (Wakimoto *et al.*, 2004; Parizzi *et al.*, 2004), flow cells (Filoche *et al.*, 2004), stainless steel (Giaouris and Nychas, 2006), glass surfaces (Rezende *et al.*, 2005) and bentonite clay (Vadakel, 2001)

2.2.8. Resistance of Biofilm

Biofilms are considered to be highly resistant to antimicrobial agents when compared to planktonic cells. Biofilm cells are comparatively more resistant to antibiotics, anti bacterial antibodies and phagocytic cells. Different workers have imputed different reasons for this; thereby it is evident that the causes for these antibiotic resistances are multifactorial, some of which are explicated below.

Jensen *et al.* (1990) showed that biofilms induced an oxidative burst response by polymorphonuclear leukocytes which was slow and only 25 per cent of the response to planktonic bacteria. The findings of Yasuda *et al.* (1994) also showed that adherent bacteria had increased resistance to killing by phagocytes.

Several mechanisms have been proposed to explain the high resistance of biofilms to antibiotics, including restricted penetration of bactericidal antibiotics due to the diffusion barrier (Lewis, 2001), slow growth owing to nutrient limitation, expression of genes involved in the general stress response and emergence of a resistant phenotype (Resch *et al.*, 2005). Bacteria produce a virulence-related polysaccharide exocellular slime (the glycocalyx), which preferentially adheres to the surfaces of biomaterials and compromised tissues. This biofilm resists antibiotic penetration and provides a degree of protection from antibodies and macrophages (Leid *et al.*, 2002).

Paradoxically Olson *et al.* (2002) reported similar antibiotic sensitivity profiles for both planktonic and biofilm cells of *P. multocida* except for the trimethoprim/Sulfadoxine response. This might be one of the reasons for positive response of most antibiotics, during different types of *Pasteurella* infections.

Staphylococcus epidermidis cells grown in a biofilm mode showed resistance to opsonic killing by antibody mediated phagocytosis when compared to planktonic

cells and this was not due to poor penetration of antibody, complement, or phagocytes into the biofilm, but because of high levels of antigen within the biofilm that prevented bacterial opsonization by the antibody (Cerca *et al.*, 2006).

As the biofilms represent microbial societies with their' own defense and communication systems, mechanism of biofilm-associated antimicrobial resistance seems to be multi-factorial and may vary from organism to organism. The organisms within biofilms are notorious for their resistance towards the host immune response and antibacterial agents compared to their free-living planktonic counterparts (Clutterbuck *et al.*, 2007).

Our modern view of biofilm infections leads to the realization that their effective control will require a concerted effort to develop therapeutic agents that target the biofilm phenotype and prophylactic agents that inhibit community signaling between microbes, thereby preventing the formation of biofilms.

2.2.9. Biofilm Formation by *Pasteurella multocida and its Yield*

Tryptone soya broth was recommended as the medium of growth for inducing slime production in case of *Staphylococcus epidermidis* by Christensen *et al.* (1982).

Biofilm formation by *P. multocida* and its yield has been attempted by various workers. In tryptone soya broth (TSB) *P. multocida* A: 1 strain produced maximum biofilm at a concentration of 0.32 per cent supplemented with bentonite clay at 0.3 per cent level under 37 ^{0}C. Under such conditions the concentration of biofilm cells peaked on day three with a count of 4.2×10^{10} CFU/g of bentonite clay and persisted even after 50 days of incubation with a count of 1.3×10^{8} CFU/g. The planktons declined rapidly after peaking on day one, proving the increased *in vitro* persistency of biofilm cells (Vadakel, 2001).

When avian strain of *P. multocida* were grown in biofilm mode on Calgary Biofilm Device (CBD) using TSB supplemented with 2 per cent foetal calf serum and 10 per cent carbon dioxide as growth medium, within a period of 5 h the biofilm concentration reached 1.5 X 10^5 CFU / peg of CBD, compared to planktonic cells concentration of 5.5 X 10^9 CFU / ml, in media surrounding the peg (Olson *et al.*, 2002). They also commented that *P. multocida* required special growth conditions, such as the addition of fetal bovine serum, incubation under 10 per cent carbon dioxide, and a longer culture time to form a biofilm on CBD.

Pasteurella multocida also formed biofilm at a high temperature of 42°C under nutrient restricted conditions provided by 0.32 per cent TSB supplemented with bentonite clay at 0.3 per cent level. Such a growth pattern resulted in production of novel immunogenic heat shock proteins and hence was recommended as candidate antigen for biofilm vaccines against fowl cholera (Hugar, 2004).

2.3 ADJUVANTS, VACCINES AND ASSESSMENT OF IMMUNE RESPONSE.

2.3.1 Adjuvants

2.3.1.1 Importance of Adjuvants

Protection with adjuvant-treated preparations of antigens is always superior to that seen with non-adjuvant treated vaccines (Woolcock and Collins, 1976). Adjuvants are important components of vaccines as they help to elicit an early, high and long-lasting immune response with less antigenic dose. Thus they reduce the antigenic exposure and consequently the vaccine production costs. With the use of adjuvants the immune response can be selectively modulated to major histocompatibility complex (MHC) class I or MHC class II and T helper 1 (Th1) or T helper 2 (Th2) types, which is very important for protection against diseases (Gupta and Siber, 1995).

In the opinion of Hunter (2002), vaccine adjuvants are usually agents that increase the intensity of immune responses and influence the balance between antibody and cell-mediated immunity at the cost of toxicity. Likely it seems that adjuvant research will be increasingly important as the science of vaccines advances.

2.3.1.2 Saponin Adjuvanation

2.3.1.2.1 Saponin Source

Saponins are structurally diverse class of compounds occurring in many plant species, which are characterized by a skeleton derived of 30-carbon precursor, oxidosqualene, to which glycosyl residues are attached. Traditionally, they are subdivided into triterpenoid and steroid glycosides (Vincken *et al.*, 2007).

The most widely used saponin based adjuvants are Quil A and its derivative QS-21, isolated from the bark of *Quillaja saponaria molina*, the adjuvant property of which, have been evaluated in numerous clinical trials. Their unique capacity to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocytes (CTLs) against exogenous antigens make them ideal for the use in subunit vaccines, vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines (Sun *et al.*, 2009).

2.3.1.2.2 Advantages of Saponin as a Vaccine Adjuvant

It was demonstrated that a purified Quillaja component designated QH-C had a strong adjuvant activity and little or no toxicity in the doses tested. The mice tolerated up to 400 microgram of free QH-C dissolved in phosphate buffered saline (PBS), when inoculated subcutaneously (Ronnberg *et al.*, 1995).

The saponin-adjuvanted bacterin (Quil A at the rate of 1 mg/ml) prepared from a virulent local isolate of *P. multocida* had efficiently protected the vaccinated

rabbits against challenge, as well as, it offered a good protection against spontaneous pasteurellosis among rabbits in Al Ahasa, Saudi Arabia. Hence, saponin was used as an adjuvant to pass over some of the drawbacks of the aluminium hydroxide adjuvant in the available commercial vaccines (Hatem and Kotb, 1998). When a recombinant *Taenia ovis* vaccine, formulated with saponin as adjuvant, was given to sheep, a significant protection was achieved after one month against challenge infection. Satisfactory protection (79 per cent) was still present when sheep were challenged after 6 months of immunization. The optimum dose of saponin that produced peak antibody titre without any tissue reaction was found to be 10 mg. Dialyzed saponin also retained its adjuvant properties and saponin content could be increased up to 30 mg per dose without site reaction, resulting in a still higher peak antibody titre (Harrison *et al.*, 1999).

Saponin was found to have an equally effective mucosal adjuvant activity in raising serum IgG antibody levels comparable to that of sodium fluoride, when chicken were orally immunized using bovine serum albumin as antigen (Hoshi *et al.*, 1999).

2.3.1.2.3 Disadvantages of Saponin as a Vaccine Adjuvant

Ticks from dogs immunised with Freund's adjuvant, produced only lower mean egg mass weight than ticks from dogs inoculated with gut extract and saponin. This suggested that Freund's adjuvant was better in adjuvant activity compared to saponin (Szabo and Bechara, 1997).

Although saponins are well recognised as potent immune stimulators, their applicability as vaccine adjuvants has been limited due to associated toxicity (Skene and Sutton, 2006). The *Quillaja* saponins have serious drawbacks such as high toxicity, undesirable haemolytic effect and instability in water soluble form, which limits their use as adjuvant in vaccination (Sun *et al.*, 2009).

2.3.1.3 Aluminium Hydroxide as Adjuvant

2.3.1.3.1 Uses of Aluminium Hydroxide as Vaccine Adjuvant.

Aluminium compounds are the most widely used adjuvants with routine human and veterinary vaccines, even though the search for alternate adjuvants is going on. They have good track record of safety, low cost and adjuvanticity with a variety of antigens. For infections where humoral immunity plays important role in offering protection, aluminium adjuvants formulated under optimal conditions are the adjuvants of choice (Gupta, 1998). Experimentally it had been shown that aluminium hydroxide adjuvant had a significantly stronger effect than aluminium phosphate adjuvant. Both compounds significantly increased the expression of CD86 on dendritic cells of mouse, but only aluminium hydroxide adjuvant induced moderate expression of CD80 (Sokolovska *et al.*, 2007).

2.3.1.3.2 Mechanism of Adjuvanticity of Aluminium Hydroxide and its Advantages.

When bacterial lipopolysaccharides were given intramuscularly along with aluminium hydroxide gel, a remarkable reduction in lethality in mice was observed, which in turn, was preceded by reduced serum endotoxin levels (Norimatsu *et al.*, 1995).

The mechanism of adjuvanticity of aluminium compounds includes formation of a depot; efficient uptake of aluminium adsorbed antigen particles by antigen presenting cells due to their particulate nature and optimal size and stimulation of immune competent cells of the body through activation of complement, induction of eosinophilia and activation of macrophages (Gupta, 1998).

There is experimental evidence that aluminium hydroxide directly stimulates monocytes to produce proinflammatory cytokines activating T cells. Activated Th2

cells release interleukin (IL)-4, which in turn can induce an increase in the expression of MHC class II molecules on monocytes, leading to its enhanced accessory functions (Ulanova *et al.*, 2001). Aluminium hydroxide stimulated macrophages displayed potent ability to induce MHC-II-restricted antigen specific memory responses, at the same time maintaining the macrophage morphology and this might be one of the reasons for the long lasting immunity induced by aluminium hydroxide adjuvanted vaccines (Rimaniol *et al.*, 2004).

When *Streptococcus pneumoniae* antigens were adjuvanted with aluminium hydroxide, the elicited antibody responses in mice were found to be higher than formulations prepared with aluminium phosphate or non-adjuvanted antigens (Levesque and Alwis, 2005).

The aluminium adjuvants (aluminium hydroxide and aluminium phosphate) were also able to increase macrophage's capacity to potentiate autologous memory T lymphocyte proliferation. Although non-aluminium mineral adjuvants also have this capacity, only aluminium adjuvants induced CD83 expression and increased CD86 on macrophages, thereby suggesting that aluminum and non-aluminum adjuvants exerted their immuno-activities by distinct mechanisms on macrophages (Rimaniol *et al.*, 2007).

According to Sokolovska *et al.* (2007) the exact mechanism by which these adjuvants enhanced the immune response and predominantly stimulated a Th2 humoral immune response was not well understood. Aluminium-containing adjuvants activated dendritic cells of mouse and influenced their ability to direct Th 1 and Th 2 responses through the secretion of IL-1 and IL-18.

The manner by which aluminium containing adjuvants potentiate the immune response is related to the structure, properties of the adjuvant and adsorption mechanism. Immuno-potentiation occurs through the following sequential steps:

inflammation and recruitment of antigen-presenting cells, retention of antigen at the injection site, uptake of antigen, dendritic cell maturation, T-cell activation and T-cell differentiation (Hem and Hogenesch , 2007).

Thus aluminium compounds have been shown to enhance the immune response by different mechanisms like depot formation, direct or indirect stimulation of dendritic cells and monocytes, activation of complement, memory T lymphocyte proliferation and by inducing the release of chemokines. The relative significance of these mechanisms remains to be determined.

2.3.1.3.3 Limitations of Aluminium Adjuvants

Limitations of aluminium adjuvants included local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses (Gupta, 1998).

When Aluminium hydroxide gel, were used as vaccine adjuvants a granulomatous inflammatory reaction was elicited, consisting mainly of macrophages with foamy cytoplasm, small lymphocytes and giant cells at the injection sites. These changes persisted up to eight weeks or longer. At a dose of three mg per millilitre aluminium hydroxide induced very low levels of antibody against tetanus toxoid antigen (Goto *et al.*, 1997).

Based on studies on Cynomolgus monkey, Verdier *et al.* (2005) concluded that aluminium adjuvanted vaccines administered by the intramuscular route triggered histopathological changes restricted to the area around the injection site. These lesions persisted for several months, but were not associated with abnormal clinical signs.

2.3.1.3.4 Advantages of Combined Adjuvants

The use of quill-A improved the immunogenicity of the aluminium adjuvant and helped to avoid the use of oil components (Rurangirwa *et al.*, 1987). With this combined vaccine, antibodies peaked at a time, similar to that found when a vaccine containing single adjuvant was administered (Buonavoglia *et al.*, 1998; Foggie *et al.*, 1971).

But aluminium adjuvants have little stimulatory effect on the cell mediated immune response (Tizard, 2004). Saponins were found to have the unique capacity to stimulate Th1 or cell mediated immune response (Sun *et al.*, 2009).

2.3.1.4 Emulsion Adjuvants

2.3.1.4.1 Mechanism of Action of Emulsion adjuvants

Emulsion adjuvants are depot adjuvants that cause tissue irritation and destruction. The tissue damage produces inflammation and cell death. Products of the inflammation stimulate dendritic cells and macrophages which ultimately stimulate immune response. The inflammation also increases the persistence of antigen at the site of injection and thereby prolongs the immune response (Tizard, 2004).

It has been experimentally proven that emulsion adjuvants will stimulate production of considerable level of reactive oxygen species and thereby vaccine induced cell death in nearby tissue when inoculated intramuscularly. They also induce actin polymerization and macro-pinocytosis, thereby enhancing antigen internalization by antigen presenting cells. Thus it is suggested that emulsion adjuvants not only have the roles of adjuvant-induced cell death in antigen delivery but also has immunomodulatory effect (Yang and Shen, 2007).

2.3.1.4.2 Oil-in-water Emulsion Adjuvants

Freund *et al.* (1948) opined that antibody formation was enhanced and sustained when the typhoid bacillus antigen was incorporated into a water-in-oil emulsion prepared with paraffin oil and anhydrous lanolin. They also compared effect of other materials and combinations like Tween 80 and paraffin oil; "lecithin" and paraffin oil; peanut oil and anhydrous lanolin, but found the adjuvant effect of paraffin oil and anhydrous lanolin to be superior to others.

The use of oil adjuvant vaccine is quite inconvenient because of the difficulty in passing the thick emulsion through the syringe and sixteen gauge needle (Mukkur and Nilakantan, 1968).

Stone *et al.* (1978) studied the composition, physical properties and adjuvanticity of oil adjuvanted vaccines. They found that, in case of oil adjuvanted vaccines the viscosity of the vaccine decreased as the concentration of oil increased and emulsions containing both aqueous and oil phase emulsifiers had lower viscosity than those containing oil phase emulsifiers alone. In order to prepare vaccine, the 15 volumes of aqueous phase of emulsion was mixed with nine volumes mineral oil plus one volume of oil phase emulsifier and homogenized at 18,500 to 19,000 rpm for 30 seconds. The vaccines thus prepared were stable for more than 12 weeks at 37°C and induced a marked primary antibody response in chickens.

Lalrinliana *et al.* (1988) conducted studies to compare efficacy of different adjuvants employed in *P. multocida* vaccines and the highest antibody titre was observed with oil adjuvant vaccines while the alum precipitated and aluminum hydroxide gel vaccines induced low titres.

Suli *et al.* (2004) prepared experimental lipoid adjuvant of the oil-in-water (O/W) type, using squalene at the level of 5 per cent (w/v) and detergents Poloxamer

105 in the concentration of 4 per cent (w/v) and Abil-Care in the concentration of 2 per cent as emulsifiers. He compared its adjuvant activity and found that the oil in water type lipoid adjuvant had 1.8-fold more immunogenic activity compared to aluminium hydroxide in eliciting antibody response to rabies vaccine.

Field trials conducted by Muneer *et al.* (2005) also justified replacement of alum precipitated bacterin with a newly developed oil adjuvanted bacterin. They prepared the vaccine by mixing mineral oil (SEPPIC, France), lanolin and bacterial suspension in the ratio of 45:5:50, respectively. Mineral oil and lanolin were mixed at low speed of 13000 rpm in a Hamilton Beach Drink mixer and then at a high speed of 18000 rpm for 15 min.

2.3.2 Vaccination against *Pasteurella* sp.

Leonchuk and Tsimokh (1977) reported that vaccination against *Pasteurella* by intra muscular route gave stronger and more lasting immunity than sub cutaneous route.

Wu *et al.* (1986) compared primary and secondary immune responses in chickens vaccinated with fowl cholera attenuated vaccine prepared from *P. multocida* strain 807 and established the importance of double vaccination in antibody raise. Onet *et al.* (1994) found that increment on second vaccination was at least 3 times in case of pasteurellosis. Hence booster vaccination definitely had added advantageous effect on protection and is a must, to prevent losses.

In general the *P. multocida* vaccines currently used are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live culture vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccination (PV) sequelae such as pneumonitis and arthritis. Multivalent

vaccines typically incorporate somatic serotypes 1, 3, and 4 as they are the commonly isolated avian serotypes. The strain of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic (OIE, 2004).

2.3.2 .1. Age of Vaccination Among Ducks and Immune Response

Layton (1984) observed that when ducklings, immunized subcutaneously with oil adjuvanted *P. multocida* bacterin at one week of age, were challenged at six weeks of age, only low immunity was there. When immunized at two and three weeks of age, they had high levels of immunity at four and five week of age (eighty per cent survival), but by six weeks of age, protection had begun to decline (survival rate fifty five per cent). When ducklings were immunized at six weeks of age, a strong immunity was induced which lasted for eight weeks. He commented that at younger age they might be immunologically immature and unable to respond to antigenic stimuli, even though it was presented in an adjuvanted form.

Pasteurella multocida bacterin is usually administered as two doses at two to four week intervals. As with most killed vaccines, full immunity cannot be expected until approximately two weeks after the second dose of a primary vaccination course (OIE, 2004).

2.3.2 .2 Vaccines and Cross Protection between Isolates

Heddleston and Rebers (1975) noted that cross protection was not induced in turkey and mice by bacterin prepared from agar grown culture, but 90 per cent protection was conferred by *P. multocida* bacterin prepared from organisms obtained from tissue fluid, heart blood and liver of turkeys that died of acute fowl cholera. Fifty per cent protection was given by *P. multocida* grown in blood at 42°C

suggesting that temperature of incubation might be a deciding factor in the production of *P. multocida* immunogens.

On heterologous challenging of immunized ducks, the survival rates were lesser and lesions scored were higher than homologous challenged groups (Layton, 1984).

There is lack of cross-protection between different serotypes of *P. multocida* and commercial vaccines prepared from organisms grown *in vitro* fail to protect birds against heterologous field challenge. This is the main factor that hampers vaccine development against pasteurellosis (Rimler and Rhoades, 1989).

An oil emulsion bacterin vaccine prepared from *P. multocida* organisms grown in the chorioallantoic sac of embryonated chicken eggs was found to be slightly more efficacious in 12 week aged chicken than the commercial vaccine tried. This conferred cross protection, implying that *P. multocida* when grown *in vivo* could express protective antigens common to several serotypes (Mariana and Hirst, 2000).

2.3.2 .3. Live Vaccines

Olson and Schlink (1986) conducted challenge studies among turkeys vaccinated with live Clemson University (CU) strain of *P. multocida*. They observed that the immunity continued for only thirteen weeks after last vaccination, in turkeys vaccinated twice at three weeks apart, and for only eight weeks in those vaccinated only once.

Avakian *et al.* (1989) showed that when broiler mini breeder hens, vaccinated against fowl cholera by both live and polyvalent oil based vaccines, were challenged at 42 weeks of age, all the birds that received live vaccine survived while the survival rate was 86 per cent among birds that received killed vaccine.

Hofacre *et al.* (1989) vaccinated turkeys by administering *P. multocida* mutant PM#1 or PM#3, either by the oculo-nasal method or oral route in the drinking water. The induced level of protection was comparable to vaccination with the CU strain or the M-9 vaccine. Their study made it apparent that the less-virulent mutant organisms and the M-9 vaccine required a higher concentration of organism per vaccine dose than the CU strain to provide similar protection. Hence virulent organisms are recommended for selection of vaccine strain.

Empirically derived, live, attenuated vaccines often offer good protection against heterologous serotypes, but as the basis for attenuation is undefined, there is the risk of reversion of virulence (Adler *et al.*, 1999).

2.3.2 .4 Inactivated Vaccines

2.3.2 .4.1 Formalin Inactivated Alum Precipitated Vaccines

Laboratory and field trials were conducted by Ramanatha (1994) with formalin inactivated, alum adsorbed broth bacterin of *P. multocida* strain A: 1 in ducks. The birds were vaccinated at 4 weeks of age and a booster dose was given after an interval of 24 days. Direct challenge was done with homologous strain, 70 days after second vaccination. The survival rates in twice vaccinated birds were 100 per cent and the protection rates correlated well with indirect haemagglutination test (IHA) titres. Following a duck cholera outbreak, Murugkar and Ghosh (1995) vaccinated the ducks with formalin inactivated bacterin vaccine as a measure of prophylaxis. The vaccine prepared from the same isolate was found to be highly effective in controlling the outbreak. Islam *et al.* (2004) evaluated the efficacy of two locally prepared alum precipitated, formalin killed fowl cholera vaccines in ducks. The birds were vaccinated at the age of eight weeks, followed by a booster dose after two weeks of primary vaccination. Results showed that vaccines induced sufficient

cellular and humoral immune responses which resulted in satisfactory level of protection of 90 to 95 per cent, respectively.

2.3.2 .4.2 Aluminium Hydroxide Gel Adjuvanted Pasteurella multocida Vaccines

Filia *et al.* (2006) studied the efficacy of different types of formalin killed whole cell vaccines against pasteurellosis. The antigen used for them were *P. multocida* P52 strain (B:2), grown under three different conditions, *i.e.*, grown on brain heart infusion (BHI) agar, iron deplete and iron replete conditions, using aluminium hydroxide gel as adjuvant, along with commercial haemorrhagic septicaemia vaccine (APV) in rabbits. Results showed a gradual increase in the antibody titre within each group on days 7, 14 and 21, but there was no significant variation in the titre between groups.

2.3.2 .4.3 Oil Adjuvanted P. multocida Vaccines

Mukkur and Nilakantan (1968) formulated oil adjuvanted haemorrhagic septicaemia vaccines by emulsifying formalin-treated encapsulated cells of *P. multocida* (9 X 10^6 bacterial cells per ml) emulsified in liquid paraffin-lanolin mixture taken in the ratio 10:1. They compared the adjuvanticity of oil emulsion with that of sodium alginate and concluded that sodium alginate was inferior to the oil adjuvant in the enhancement of antibody production.

Immuno potency testing of *P. multocida* strain A:1 oil adjuvant vaccine in one month old ducklings gave 100, 100, 100 and 70 per cent homologous protection when challenged at day 20, 40, 60 and 80 PV respectively, with an average survivability percentage of 84.46 (Jayakumar, 1998).

When efficiency of oil adjuvanted vaccine prepared from *P. multocida* serotype B6 was compared with that of conventionally used alum precipitated vaccine (APV) under field conditions for induction of immune response in cattle and

buffaloes, comparatively higher antibody titres were observed in animals of all age groups vaccinated with oil adjuvant vaccine throughout the trial period. But the titres in animals vaccinated with APV declined 3 months after vaccination and reached at minimal level at 180 days following vaccination. These results justified the replacement of aluminium based APV with oil adjuvanted vaccine for the control of haemorrhagic septicaemia in animals (Muneer *et al.*, 2005).

Laboratory trials conducted by Ruzauskas (2005) against rabbit pasteurellosis, revealed that when rabbits were vaccinated subcutaneously with one ml of formalin inactivated oil-in-water adjuvanted bacterin containing not less than 4 $\times 10^9$ bacterial cells, the percentage of survivals was 100 per cent to *Pasteurella multocida* infection. The vaccine was also found to be safe without any side effects on rabbits, except slight swelling at the site of injection.

Rajagopal (2007) observed that oil adjuvanted biofilm vaccine was found to be superior in terms of protection and IHA titre than oil adjuvanted ordinary bacterin and oil adjuvanted capsule enhanced bacterin in conferring protection against *P. multocida* in ducks.

Oil adjuvanted ordinary bacterin was found to be superior in terms of protection than OMP vaccine when the immunopotency of both were tested in ducks (Ranjini, 2007).

2.3.2 .4.4 Biofilm Vaccines

A preliminary vaccination trial carried out in chicks revealed that killed biofilm oral vaccine against *E. coli* gave a better protection of 83.3 per cent when compared to 33.3 per cent protection of killed free cell oral vaccine and 16.6 per cent protection of unvaccinated group of chickens (Shivaraj and Krishnappa, 2002).

According to Arun and Krishnappa (2004) fowl cholera was a biofilm associated disease and hence the use of biofilm form of pathogen for vaccines could be an alternative strategy for evolving effective immunoprophylaxis against fowl cholera. They also observed that biofilm cells of two *Pasteurella* strains were demonstrably and profoundly different from their free cell counterparts in their outer membrane protein profile, when analysed by SDS PAGE. On western blotting it could be shown that some unique proteins of biofilm cells were highly immunogenic.

2.3.3 Assessment of Immune Response against *P. multocida* Vaccination

2.3.3 .1 Indirect Hemagglutination Test

An Indirect hemagglutination (IHA) procedure was developed by Carter (1955) for the identification of different capsular antigens of *P. multocida* and on the basis of these differences, four different types or groups, *viz*., A, B, C, and D were designated . An Indirect hemagglutination test was considerably more sensitive than other serological tests since agglutinins undetected by other tests could be detected by this method (Neter, 1956). Later Carter improved IHA test for the detection of Type A strains of *P. multocida*. The mucoid cultures were treated with testicular hyaluronidase so that, the hydrolysis of the capsular hyaluronic acid presumably released the specific antigen and thereby facilitated easy adsorption of antigen on erythrocytes (Carter and Rundell, 1975).

Dua and Pandurangarao (1978) recommended IHA test for assessing the immune status of unvaccinated sheep, while agar agglutination test was found to be most reliable in predicting fate of vaccinated sheep during challenge test.

An IHA test, that uses glutaraldehyde fixed- sheep red blood corpuscles (GA-SRBC) sensitized with heat extracted antigens could be used for detection of *Pasteurella* antibody. Glutaraldehyde fixed- sheep red blood corpuscles were found

to be stable for at least six months. Heat extracted or potassium thiocyanate extracted antigens of *Pasteurella* strains could be adsorbed onto GA-SRBC or tanned SRBC, respectively. The indirect hemagglutination test reaction was capsular group specific with heat extracted antigen-sensitized SRBC, but no such specificity was observed with potassium thiocyanate extract antigen-sensitized tanned SRBC (Sawada *et al.*, 1982).

When eight week old chicken were vaccinated with oil adjuvanted *P. multocida* (CU strain) bacterin, the mean indirect haemagglutination titre expressed in $\log_2$ (MIHAT) obtained were 1.2 , 2.5, 3.8 and 3.5 on days 7, 14,21,35 post vaccination (PV) respectively. When booster dose was given on day 35 PV the MIHAT obtained were 5, 4 and 3.6 on days 7, 14 and 21 post booster vaccination (PBV) respectively (Selano *et al.*, 1983).

Olson and Schlink (1986) observed a significant increase in titre, one week after vaccination with CU strain of *P. multocida* and average titre remained higher than control until 13 and 15 weeks P V. The serum anti-*Pasteurella* antibody titres were lowest before vaccination, with the average geometric mean titre (GMT) being 1.58 and after one week PV, GMT peaked to 3.79.

Swamy (1994) found that the optimum amount of purified capsular polysaccharide of *P. multocida* required to sensitize one millilitre of 2.5 per cent GA-SRBC for PHA was 60 µg. Further, PV titres in once vaccinated ducks on days 24, 44, 64 and 84 ranged from 20 to 40, 20 to 80, 80 to 160 and 80 to 160 respectively. The titre of twice vaccinated ducks after 84 days ranged 160 to 640, the minimum protective titre assessed being 320.

The survival rate of birds vaccinated against *P. multocida* were found to be well correlated with that of IHA titres, when challenged with vaccine strain of bacterium (Ramanatha., 1994) .

Cytophilic and opsonin adhering antibodies appeared from day seven PV with *P. multocida* oil adjuvanted vaccine and peaked on day 21 PV. Opsonin adhering antibodies were found to be higher and challenging with virulent organisms showed a continuous rise of both types of antibodies from the very first day to 10 days P V, indicating an intense and accelerated memory response (Maurya and Jaiswal, 1996).

In an immunization trial with multiple emulsion vaccine against pasteurellosis in calves performed by Verma and Jaiswal (1997), a fall in mean antibody titres during 24 and 48 h post-challenge infection was recorded, whereas a steady increase in the titre after 72 h up to 10 days was noticed. The pre-challenge mean titre in animals correlated with survival of animals.

2.4 ESTIMATION OF PROTEIN

Lowry devised a sensitive method for estimation of proteins in biological samples. Lowry's protein assay relies on the formation of protein copper complex and reduction of phosphor molybdate phosphor tungstate reagent (folin ciocaltau phenol reagent) by tyrosine and tryptophan residues of protein Lowry *et al.* (1951).

2.5 RECOMBINANT GHOST SYSTEM

2.5.1 Development of Bacterial Ghosts

Bacteriophage ΦX174 encodes a gene for lysis of *Escherichia coli* called gene *E*. The activation of gene *E* results in the formation of a membrane protein called protein E which has the ability to fuse the outer and inner cell membranes, resulting in a transmembrane tunnel (of 40-80 nm diameter) formation throughout the entire cell envelope of *E. coli* (Witte *et al.*, 1990).

The lysis gene *E* of bacteriophage phiX174 is entirely embedded in gene *D*. Expression studies of genes *D* and *E* in *E. coli* minicelis and lysis times obtained in

the presence or absence of gene *D* translation showed that unlike other overlapping gene pairs, gene *E* expression was independent from the upstream translation of gene *D* (Blasi *et al.*, 1990).

Lee and Henk (1996) had identified versatile, commonly available, shuttle vectors which were stably maintained in *P. multocida.* Their study indicated that recombinant proteins could be cloned in *P. multocida* after prior construction in *E. coli.* The cloning vector could be easily transferred by conjugation to *Pasteurella* by RSF1010-based shuttle vector.

Marchart *et al.* (2002) produced *P. multocida* bacterial ghosts by the expression of phage ΦX174 lysis gene *E.*

Haidinger *et al.* (2003) accomplished production of bacterial ghosts from *E. coli* by the controlled expression of phage phiX174 lysis gene *E.*

2.5.2 Use of Bacterial Ghosts as Vaccines

In studies conducted by Mader *et al.* (1997) no differences were observed between the immune responses of the rabbits, when equivalent doses of bacterial ghosts and antibiotic-treated whole cells were administered, indicating that the bacterial ghosts exhibited all the antigenic properties of the living cells.

Eko *et al.* (1999) reviewed that ghosts did not need the addition of adjuvants to induce immunity in experimental animals and they could also be used as carriers or targeting vehicles or as adjuvants in combination with subunit vaccines. They also mentioned that as no inactivation procedures that denature relevant immunogenic determinants were employed in the production of ghosts, they were superior to other inactivated vaccines. As carriers of foreign antigens there was no limitation in the size of foreign antigens to be inserted as the capacity of all spaces including that of membranes, periplasm and internal lumen of the ghosts could be fully utilized.

According to Haslberger and Hensel (1999) bacterial ghosts had been shown to be innovative systems to prepare vaccines of various bacteria with all features of the intact bacterial cell envelopes, especially all antigenic epitopes. They studied the uptake of bacterial ghosts in dendritic porcine cells and raw macrophages and found that bacterial ghosts effectively stimulated monocytes and macrophages for the induction of Th1 response. Their results also confirmed that bacterial ghosts had no toxic effects in rabbits after intravenous administration in doses stimulating significant humoral responses.

Jalava *et al.* (2003) reviewed that bacterial ghosts might be utilised as a new strategy to develop effective vaccines in modern veterinary medicine due to their simplicity of the production method, safety, independence from the cold chain and versatility, as a combination vaccine.

Mayr *et al.* (2005) observed that oral administration of mice with entero haemorrhagic *Escherichia coli* (EHEC) ghosts without the addition of any adjuvant induced both cellular and humoral immunity. Immunized mice challenged at day 55 showed 86 per cent protection against lethal challenge with a heterologous EHEC strain after single-dose oral immunization. A protection of 93.3 per cent was obtained after one booster at day 28, whereas the controls showed 26.7 per cent and 30 per cent survival, respectively. These results indicated that it was possible to develop an efficacious single-dose oral EHEC bacterial-ghost vaccine.

2.5.3 *Pasteurella multocida* Bacterial Ghosts

Marchart *et al.* (2002) produced *P. multocida* bacterial ghosts by the expression of phage phiX174 lysis gene *E.* They were empty cells devoid of cytoplasm and genomic material. When immunization of cattle was induced by adjuvanted *P. multocida* ghosts, the level of protection obtained against homologous challenge was similar to that of a commercially available vaccine.

Materials and Methods

3. MATERIALS AND METHODS

Borosil brand of glass ware and plastic ware of Tarson brand were used in the study. Chemicals used were of molecular biology grade, obtained from M/S Sigma-Aldrich, Loba Chemie and Sisco Research Laboratories (SRL) Private Limited. Ready-made media were procured from Hi-Media Laboratories Private Limited (Mumbai), unless otherwise mentioned.

3.1 BACTERIAL STRAIN USED

The *P. multocida* serotype A: 1 (DP1) isolated from Niranam duck farm (Pathanamthitta district, Kerala state, India), serotyped at IVRI, Izatnagar and maintained in freeze dried form in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy was used for the entire study.

Two hundred and fifty microlitres of sterile phosphate buffered saline (PBS) was injected into the freeze dried vial and shaken till the contents got dissolved. A loopful of the inoculum obtained was streaked on sheep blood agar and incubated at 37 ^{0} C for 24 hours in a candle jar. The colonies obtained were maintained by subculturing on tryptose soya agar (TSA).

Purity of the isolate was checked based on morphology, cultural and biochemical characteristics as described by Barrow and Feltham (1993). The biotyping of the organism was done as described by Mutters *et al.* (1985).

3.2 POLYMERASE CHAIN REACTION FOR DETECTION OF *P. multocida* (PM-PCR)

3.2.1 Materials

3.2.1.1 PCR Reaction Buffer (10x)

This included 500 mM KCl, 100 mM Tris-HCl pH 9.0 and 15 mM $MgCl_2$.

3.2.1.2 Taq DNA Polymerase (Sigma)

Taq DNA polymerase enzyme with a concentration of 3 U/µl.

3.2.1.3 Magnesium Chloride

Magnesium chloride with strength of 25 mM

3.2.1.4 Deoxy Ribonucleotide Triphosphate (Sigma)

Deoxy ribonucleotide triphosphate (dNTP) mix

2.5 mM (10 mM each of dGTP, dCTP, dATP and dTTP in equal volume)

3.2.1.5 Primers for PM-PCR

Specific primers to detect the *Pasteurella multocida* (species specific) designed by Townsend *et al.* (1998) were used. The sequences of the primers were as follows:

KMTISP6: 5'-GCT GTA AAC GAA CTC GCC AC-3'

KMTIT7: 5'-ATC CGC TAT TTA CCC AGT GG-3'

The primers were custom synthesized by M/s Sigma.

3.2.2 Method

3.2.2.1 Preparation of Template DNA for PCR

A pure colony of the bacteria was inoculated into five milliliters of Brain heart infusion (BHI) broth and incubated at 37°C for 18 h. One and a half milliliters of this broth culture was transferred to a micro centrifuge tube (AXYGEN) and

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centrifuged at 3000 x g for 10 min. The pellet was washed twice in PBS and the final pellet was re-suspended in 100 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000 x g for five minutes. The supernatant was stored at - 20°C for further use as template DNA for PCR.

3.2.2.2 Polymerase Chain Reaction

Polymerase Chain Reaction was conducted for the detection of *P. multocida* by the method described by Townsend *et al.* (1998) using the primer pair KMTISP6-KMTIT7.

A 25 µl reaction mixture was prepared in 0.2 ml thin walled PCR tube. The reaction mixture consisted of the following.

Template DNA	5 µl
Primers	20 pmol of each primer
10 x PCR buffer	2.5 µl
Taq DNA polymerase	1.0 unit
dNTP mix	1 µl
Triple distilled water to	25 µl

The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

Initial denaturation		95°C for 4 min
30 cycles	denaturation	95°C for 45 sec
	annealing at	55°C for 45 sec
	extension	72°C for 45 sec
Final extension		72°C for 6 min

3.2.3 Detection of PCR Product by Submarine Agarose Gel Electrophoresis

3.2.3.1 Materials

3.2.3.1.1 0.5 M EDTA (pH 8.0)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 70 ml of triple distilled water and the pH was adjusted to 8.0 with 1N NaOH. The volume was made up to 100ml, filtered, autoclaved and stored at room temperature.

3.2.3.1.2 TAE (Tris Acetate EDTA) Buffer (50x) pH 8.0

Tris base	48.40 g
Glacial acetic acid	11.42 ml
0.5 M EDTA pH 8.0	20.00 ml
Distilled water to	1000 ml

TAE (1x) was prepared by mixing one part of TAE (50x) with forty nine parts of triple distilled water. Autoclaved and stored at room temperature.

3.2.3.1.3 Agarose Gel (1.5 per cent)

Agarose low EEO (Bangalore Genei)	1.5g
TAE buffer	100 ml

3.2.3.1.4 Gel Loading Buffer (6x)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g

Sucrose	40.00 g
Distilled water to	100 ml

Stored at 4°C.

3.2.3.1.5 Ethidium Bromide (1 per cent solution)

Ethidium bromide (SRL)	100 mg
Distilled water	10 ml

Stored at 4°C in amber coloured bottles

3.2.3.1.6 DNA Molecular Size Marker

100 base pair DNA ladder (Sigma)

3.2.3.2 Method (Electrophoresis)

The PCR product was detected by electrophoresis in 1.5 per cent agarose gel in Tris acetate EDTA (TAE) buffer (1x). Agarose was dissolved in TAE buffer (1x) by heating. When the mixture cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed well. Agarose was then poured into clean, dry, gel platform, fixed on a gel cast and the comb was kept in proper position. Once the gel was set, the comb was removed gently and the tray containing the gel was removed from the gel cast and was placed in the buffer tank. Buffer (TAE 1x) was poured till the gel was completely covered.

Five microlitres of the amplified PCR product was mixed with one microlitre of 6x gel loading buffer and the samples were loaded in the wells. The 100 base pair DNA ladder was used as DNA molecular size marker.

Electrophoresis was carried out at a level of 5V/cm of gel until the bromophenol blue dye migrated more than two-third of the length of the gel.

The gel was visualized and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.3 PATHOGENICITY AND LETHAL DOSE 50 (LD_{50}) TESTING OF *P. multocida* A:1 (DP1)

3.3.1 PATHOGENICITY TESTING

3.3.1.1 Laboratory Animals

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.

3.3.1.2 Phosphate Buffered Saline

3.3.1.2.1. Phosphate Buffered Saline 10x

NaCl	80.00 g
KCl	2.00 g
$Na_2 HPO_4$	11.33 g
$KH_2 PO_4$	2.00 g
Distilled water to	1000 ml

3.3.1.2.2. Phosphate Buffered Saline 1x

PBS 10x	100 ml
DW	900 ml

3.3.1.3 Inoculation of Mouse

An 18 h of *P. multocida* suspended in PBS, containing approximately 3×10^8 organisms/ml, was inoculated (0.1 millilitre) intra peritoneally to each of four mice. Another four mice were kept as controls which were mock inoculated with sterile PBS (pH 7.4). All the mice were observed for seven days post inoculation.

Blood smears and impression smears of spleen and liver 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 attempted on blood agar and TSA by incubating at 37°C under five per cent carbon dioxide tension for 24 h.

3.3.2 LETHAL DOSE 50 (LD_{50}) TESTING IN DUCKS

3.3.2.1 Experimental Birds

A total of 440 unvaccinated one month old ducklings were procured from a private breeder at Haripad, Alleppy district, Kerala. The ducks were quarantined for a period of 14 days in NATP experimental animal house of department of poultry science, college of veterinary and animal sciences, Mannuthy and was maintained there itself until used for experiments. Out of these 440 birds, 90 birds were utilised for determination of LD_{50} and rest 350 birds were used for vaccine trials.

Lethal dose 50 of *P. multocida* was determined twice, first at 11 weeks of age ($LD_{50(11w)}$) and then at 21 weeks of age ($LD_{50(21w)}$) as described by Jayakumar (1998). Forty two birds were used for determination of $LD_{50(11w)}$ and 36 birds were used for determination of $LD_{50(21w)}$. Six birds each were kept as controls in ($LD_{50(11w)}$) and ($LD_{50(21w)}$) determination. Pathogenicity testing was conducted at experimental animal shed of department of veterinary pathology of college of veterinary and animal sciences, Mannuthy.

3.3.2.2 Determination of Lethal Dose 50 in Ducklings of 11 Weeks of Age

3.3.2.2.1 Preparation of Inoculum

Pasteurella multocida A: 1 (DP1) was passaged in mice to get a fully encapsulated virulent form. The virulent organisms isolated from mice were grown on Tryptose Soya Agar (TSA) at 37°C for 24 h. The growth on TSA was harvested, washed thrice in PBS by centrifugation at 3000 × g for 15 min at 4 ° C and re-suspended in the same buffer. Plate count was done to assess the number of bacteria and the volume of bacterial suspension was adjusted so as to contain 3×10^7 cells/ml. Then serial ten fold dilutions were made up to 3×10^1 cells/ml.

3.3.2.2.2 Method

Eleven week old ducklings were randomly assigned to eight groups of six ducklings each. The first seven groups were separately inoculated with 0.1 millilitre each of different doses of the bacteria ranging from 3×10^6 cells to 3×10^0 cells. The inoculation was done subcutaneously at wing web. The eighth group, which served as controls was sham inoculated with 0.1 millilitre of sterile PBS (pH 7.4).

Number of death in each group and the time of death of each bird were recorded for a period of 15 days post inoculation. 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. Further confirmation of the bacterial species was done by PM-PCR.

The method described by Reed and Muench (1938) was used for LD_{50} calculation. The time required for death of each bird was noted and mean death time (MDT) was calculated for each group.

3.3.2.3 Determination of Lethal Dose 50 in Ducks of 21 Weeks Age

3.3.2.3.1 Preparation of Inoculum

The inoculum was prepared as given in 3.3.2.2.1 and volume of bacterial suspension was adjusted so as to contain 3×10^{11} cells/ml. Then serial tenfold dilution was made up to 3×10^{6} cells/ml present in each dilution retrospectively.

3.3.2.3.2. Method

Ducklings of 21 weeks of age were randomly assigned to 7 groups of 6 ducklings each. The first six groups were separately inoculated with 0.1 millilitre each of different doses of the bacteria ranging from 3×10^{10} cells to 3×10^{5} cells. The inoculation was done subcutaneously at wing web. The seventh group served as controls which was sham inoculated with 0.1 millilitre of sterile PBS (pH 7.4).

The rest of the procedure was followed as given in 3.3.2.2.2.

3.4 PRODUCTION OF BIOFILM ANTIGEN

3.4.1 Materials

3.4.1.1Tryptone Soya Broth (TSB) (Merck)

3.4.1.2 Chitin Flakes (Loba Chemie, Mumbai)

3.4.1.3 Nutrient Agar (Merck)

3.4.1.4 Phosphate Buffered Saline (pH 7.4)

Prepared as described in 3.3.1.2

3.4.2 Method

3.4.2.1 Preparation of Inoculum for Biofilm Production

Stock culture of *P. multocida* was revived by mouse inoculation method as described above and sub cultured on blood agar. The purity of the isolate was ensured as described in section 3.1. For preparation of the inoculum, a colony of *P. multocida* from blood agar was suspended in TSB and incubated for 24 h at 37°C. An 18 h broth culture of *P. multocida* containing approximately 3×10^8 organisms/ml was used as inoculum for biofilm production.

3.4.2.2 Medium for Biofilm antigen Production

Tryptone soya broth at a concentration of 0.32 per cent was used for biofilm production in the study. Chitin flakes at 0.5 per cent level were added as the inert substrate for providing surface of attachment or colonization to trigger biofilm formation. After addition of chitin flakes the media were sterilised by autoclaving.

3.4.2.3 Production of Biofilm Antigen.

a) Tryptone soya broth and chitin flakes were suspended in 1000 ml of double distilled water in a 2000 ml conical flask and sterilized by autoclaving at 121°C for 15 min and kept at 4°C till inoculation.
b) The sterility of the broth was assured by inoculating 0.1 milliliter of the broth to blood agar.
c) The flask was inoculated with 10 ml of DP1 inoculum prepared as above (3.4.2.1) and incubated at 42°C for 72 hours.
d) A loopful of broth was inoculated on sheep blood agar and TSA.
e) If more than one type colonies were obtained on sheep blood agar and / or TSA the broth was discarded and prepared fresh,

f) If only one type colony was obtained on TSA the purity of the isolate was checked based on morphology, cultural and biochemical characteristics (Barrow and Feltham, 1993) and by carrying out PM-PCR as described by Townsend *et al.* (1998).

g) The flask was rotated for fifty times and the chitin flakes were separated by filtration using a double layered muslin cloth as sieve.

h) The filtered broth was centrifuged at 8000 x g for 20 min at 4 $^{\circ}$ C in a refrigerated centrifuge.

i) The supernatant was discarded and sedimented cells were resuspended in PBS (pH-7.4).

j) The cells were washed three times in PBS by centrifuging at 8000 x g for 20 min at 4 $^{\circ}$ C and re-suspending in PBS in each cycle. In the final re-suspension the biofilm cells were concentrated to two per cent of the original broth volume.

k) The concentrated biofilm cells were subjected to total viable count.

l) The bacterial cell suspension was diluted with PBS to contain 3 X 10 ^{9} cells per ml and used as biofilm antigen for vaccine production.

3.5 PRODUCTION OF PLANKTONIC ANTIGEN

3.5.1. Media and Conditions for Planktonic Antigen Production

Tryptone soya broth at a concentration of 3. 2 per cent was used as the medium for production of planktonic cell antigen.

3.5.2. Method

a) The reference isolate DP1 was revived by mouse inoculation method as described above and first subculture was made on to blood agar and purity of the isolate was checked as described above (*3.4.2.3.*).

b) A colony of *P. multocida* from blood agar was suspended in TSB and incubated at 37°C for 24 h.

c) Purity check of the broth was done as described in 3.4.2.3.

d) The broth was centrifuged at 8000 x g for 20 min at 4 ° C in a refrigerated centrifuge using 50 ml centrifuge tubes (M/s Axygen).

e) The supernatant was discarded and sedimented cells were resuspended in PBS (pH-7.4).

f) The cells were washed three times in PBS by centrifuging at 8000 x g for 20 min at 4 ° C and resuspending in PBS in each cycle.

g) During the final re-suspension the volume of the planktonic cell suspension was adjusted to two per cent of the original broth volume.

h) The concentrated planktonic cells were subjected to total viable count.

i) The cell suspension was diluted with PBS to contain 3 X 10^9 cells per ml was used as planktonic antigen for vaccine production.

3.6 MORPHOLOGICAL STUDIES ON *P. multocida* BIOFILM AND PLANKTONIC CELLS

3.6.1 Light Microscopic Study

Gram's staining of both planktonic and biofilm smears of *P. multocida* strain DP1 was done and the stained smears were checked for the presence of Gram negative coccobacillary organisms under oil immersion objective of the microscope.

3.6.2 Scanning Electron Microscopic Studies

3.6.2.1 Preparation of Mini Slides

Both planktonic and biofilm cell suspension of *P. multocida* A:1 strain (DP1) containing 3 X 10^9 organisms per ml were prepared. Glass slides were cut in to small pieces (mini slides) having a length of 0.5 cm on each side. The bacterial cell suspension was smeared over the glass piece and heat fixed.

3.6.2.2. Platinum Coating of Mini Smear

The platinum coating of mini smear was done by sputtering method using auto fine coater (Jeol-JFC-1600).

3.6.2.3. Scanning Electron Microscopy

Scanning electron microscope (Jeol -6390- LA) was used to conduct morphological studies. Images of both planktonic and biofilm cells of DP1 were captured at 1500x, 2500x, 5000x, 8000x and 15000x magnification and compared.

3.7. FORMULATION OF VACCINES

3.7.1 Formalin Inactivation of Biofilm and planktonic antigen.

3.7.1.1 Formal 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 15 min at 15 lbs pressure. Then the solution was cooled to room temperature and added five millilitre of formaldehyde.

3.7.1.2 Inactivation of Antigen

a) The cell suspension containing 3 X 10^9 cells per ml was centrifuged at 8000 x g for 20 min at 4 ° C in a refrigerated centrifuge using 50 ml centrifuge tubes (M/s Axygen).

b) The supernatant was discarded and pelleted cells were resuspended in formal saline (0.5 per cent).

c) Incubated the formalinised cell suspension at 37°C for 24 h, with intermittent shaking.

d) The viability of the bacterial cells in the suspension was checked by inoculating a loopful of it on sheep blood agar and Sabouraud dextrose agar (SDA) and incubating at 37°C for 7 days.

e) If no growth was observed on both media, the antigen was used for formulation of vaccine.

3.7.2 Preparation of Vaccine

3.7.2.1 Reagents

3.7.2.1.1 Liquid Paraffin (Merck)

Light liquid paraffin was pre-sterilized in hot air oven at 160°C for 1h.

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

Lanolin was pre-sterilized in hot air oven at 160°C for 1h.

3.7.2.1.3 Saponin Solution (0.15 per cent)

Purified Saponin (Sigma)	15 mg
Normal saline	10 ml

Mixed well in a magnetic stirrer and sterilized by filtration using 0.22 μ membrane filter (Whatmann).

3.7.2.1.4 Aluminium Hydroxide Solution (0.4 per cent)

Aluminium hydroxide	40 mg
Normal saline	10 ml

Sterilized by autoclaving at 121 ^{0} C for 15 min.

3.7.2.1.5 Formalinised Biofilm Antigen

3.7.2.1.6 Formalinised Planktonic Antigen

3.7.2.1.7 Normal Saline

3.7.2.2 Method

3.7.2.2.1 Oil Adjuvanted Ordinary Bacterin (OV)

Vaccine was 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 planktonic antigen 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 15 min. Drop test was employed to determine the emulsion type and then stored at 4°C.

3.7.2.2.2 Oil Adjuvanted Biofilm Vaccine (OBV)

Vaccine was prepared as per the method of Stone *et al.* (1978) as described in 3.7.2.2.1 except that here formalin inactivated biofilm antigen was used instead of formalin inactivated planktonic antigen.

3.7.2.2.3. Saponin Adjuvanted Biofilm Vaccine (SV)

Formalinised biofilm antigen	30 ml
Saponin solution (0.15 per cent)	10 ml
Normal saline	10 ml

Mixed well in a magnetic stirrer for 15 min and stored at 4° C.

3.7.2.2.4. Saponin and Aluminium Hydroxide Adjuvanted Biofilm Vaccine (SAV)

Formalinised biofilm antigen	30 ml
Saponin solution (0.15 per cent)	10 ml
Aluminium hydroxide solution (0.4 per cent)	10 ml

Mixed well in a magnetic stirrer overnight at 4° C and stored at 4° C.

3.7.3 Sterility Testing of Vaccines

3.7.3.1 Materials

3.7.3.1.1 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 15 min.

3.7.3.1.2 Tryptose Soya Agar (Merck)

3.7.3.1.3 Sabourand's Dextrose Agar (Merck)

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

3.7.3.2 Method

The sterility of the prepared vaccines was tested individually in blood agar and TSA for aerobic bacteria, modified thioglycollate 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.

3.7.4 Safety Testing of Vaccines

The safety of vaccines was assessed by injecting 0.5 millilitres and one millilitre *i.e.* double dose 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.

3.8 PASSIVE / INDIRECT HAEMAGGLUTINATION (PHA / IHA)

The IHA test was done as described by Sawada *et al.* (1982).

3.8.1 Materials

3.8.1.1. Sheep blood

The sheep maintained in university sheep and goat farm of college of veterinary and animal sciences, Mannuthy was used as a source of sheep blood.

3.8.1.2. Sensitised Glutaraldehyde Treated Sheep Red Blood Corpuscles (GA-SRBC)

3.8.1.2.1. Preparation of Sheep Red Blood Corpuscles (SRBC) Suspension.

3.8.1.2.1.1. Alsever's Solution

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

Autoclaved at 121°C, 10 lbs pressure for 15 min and stored at stored at 4°C till use.

3.8.1.2.1.2 Collection of SRBC

Alsever's solution was taken to half the volume of a 15 ml test tube. Sheep blood required for PHA test was collected by jugular venipuncture using an 18 G sterile needle. Blood was allowed to flow into the test tube drop by drop up to three fourth the volume, following sterile precautions (*i.e* one part of sheep blood collected in two parts of Alsever's solution). The collected blood was stored at 4°C till further processing.

3.8.1.2.2. Fixation of Sheep Red Blood Cells (SRBC)

3.8.1.2.2.1. Materials for fixation of SRBC

3.8.1.2.2.1.1 Glutaraldehyde Solution (25 per cent) (Merck)

3.8.1.2.2.1.2 One Per Cent Glutaraldehyde Solution

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

3.8.1.2.2.2 Method for Fixation of SRBC

Sheep red blood cells collected in Alsever's solution were fixed using glutaraldehyde. 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 final wash, the sedimented cells were resuspended in PBS (pH 7.4) to yield a 10 per cent suspension (v/v) and chilled to 4°C in an ice bath. A 25 per cent solution of glutaraldehyde was diluted to one per cent (v/v) with PBS (pH 7.4) and chilled to 4°C. Equal volume of one per cent glutaraldehyde solution was added drop by drop to the 10 per cent washed SRBC suspension, with intermittent stirring. The SRBC suspension was mixed and incubated at 4°C for 30 min with gentle stirring. The mixture was then centrifuged at 650 × g for 10 min at room temperature. The sedimented, fixed cells were resuspended in PBS. It was then washed three times with PBS by centrifugation (650 × g for 10 min at room temperature) and re-suspended in PBS containing 0.1 per cent sodium azide to yield a 10 per cent suspension. The glutaraldehyde fixed-SRBC (GA-SRBC) was stored at 4°C.

3.8.1.2.3 Whole Cell Ultrasonicated Antigen

3.8.1.2.3.1 Preparation

Preparation of whole cell ultrasonicated antigen was done as described by Ireland *et al.* (1991), with slight modifications. *Pasteurella multocida A:1* (DP1) maintained at department of veterinary microbiology, college of veterinary and animal sciences, Mannuthy was used as the source of antigen. The bacterial colonies grown on brain heart infusion agar (BHIA) were harvested in PBS (pH 7.4) , washed twice by centrifuging at 8000 x g for 20 min at 4°C and were resuspended in PBS (pH 7.4). The concentration of washed cells was adjusted so as to contain 3 $\times 10^9$ cells/ml. The bacterial cell suspension was taken in a glass beaker kept on ice. The prepared suspension was sonified in a sonicator (Branson Sonifier 450) fitted with a 12 mm diameter titanium probe. At a horn frequency of 20 kHz, 0.1 second long bursts were given at the rate of one pulse per second for a total of five minutes, after placing the probe 1.0-1.5 centimeter deep into the suspension to avoid frothing. The sonified suspension thus obtained was centrifuged at 8000 x *g* for 30 minutes and supernatant was collected. Sterility of the sonified antigen was tested by plating 0.2 milliliters of the supernatant on blood agar plate. The plates were incubated at 37 ^{0} C for 48 h and were observed for the development of bacterial colonies. The sterile antigen preparation was stored at -20°C until further use.

3.8.1.2.3.2 Estimation of Protein in Ultrasonicated Antigen

The protein content of the whole cell ultrasonicated antigen was estimated as described by Lowry *et al.* (1951).

3.8.1.2.3.2.1 Reagents

Solution A

Sodium potassium tartarate	-	1 ml (2 per cent)
Copper sulfate	-	1 ml (1 per cent)

Sodium carbonate - 98 ml (2 per cent in 0.1 N NaOH)

Solution B

Folin ciocaltau phenol reagent - 1 Normal

3.8.1.2.3.2.2 Procedure

Twenty microlitres of sample and different concentrations of standard bovine serum albumin (BSA) were made up to 1.2 ml in distilled water. To this, six millilitres of solution A was added and then incubated at room temperature for 10 min. Three hundred microlitres of solution B was then added to the vortexed reaction mixture and incubated at room temperature for another 30 min. Optical density was read at 750 nm (red filter). Standard curve was plotted based on absorbance of known BSA concentrations from which the protein concentration of the sample was deduced.

3.8.1.2.4. Sensitization of GA-SRBC with Whole Cell Ultrasonicated Antigen

3.8.1.2.4.1. Bovine Serum Albumin-PBS (BSA-PBS) with 0.1 per cent Sodium Azide

Sodium chloride	-	80 g
Potassium chloride	-	2 g
Disodium hydrogen phosphate ($N a_2HPO_4$. 12 H_2O)	-	11.32 g
Potassium dihydrogen phosphate (KH_2PO_4)	-	2 g
BSA	-	2.5 g
Sodium azide	-	1 g
Distilled water to	-	1000 ml

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

3.8.1.2.4.2. Method

A 10 per cent suspension (v/v) of glutaraldehyde treated SRBC was mixed with equal amount of two fold diluted sonicated antigen (DP1) and 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 and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (v/v). The sensitized SRBC thus obtained was stored at 4 ° C until use.

3.8.2 Procedure for Indirect Haemagglutination 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 antisera were made in PBS and 25 µl of the sensitized SRBC was added to 25 µl of the antiserum dilution in U-bottom microtitre 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.

3.9 VACCINATION PROGRAMME

3.9.1 Grouping of Birds

A total of 350 unvaccinated six week old ducklings were divided into five groups with 70 birds in each group. All the birds were tagged by wing banding.

Blood was collected from all the birds, serum separated (zero day serum sample) and they were subjected to IHA test.

3.9.2 Vaccination of Birds

The first four groups were vaccinated with 0.5 ml of different vaccines as described below and the fifth group was sham vaccinated with 0.5 ml of normal saline.

Group	Inoculum
I	OV
II	OBV
III	SV
IV	SAV
V	Normal saline

3.9.3. Post Primary Vaccination (PPV) Serum Collection

Blood was collected from all the birds by jugular venipuncture on day 7, 14, 21, 35, 49 and 70. The collected blood was allowed to clot and incubated at 37°C for 30 min. Serum was separated following overnight incubation at 4°C. Serum was centrifuged at 650 x g for 20 min at 4 °C to separate cellular components. The supernatant was transferred into 2 ml micro centrifuge tubes (M/s Axygen) stored at -20°C until further use. Antibody titre was determined by IHA test.

3.9.4. Booster Vaccination of Birds

Vaccines were prepared once again as described earlier (3.7). On day 70 PPV, booster vaccination was done on four vaccine groups with same vaccine at same dosage (0.5 ml) as that of primary vaccination. Similarly the control group was sham vaccinated with 0.5 ml of normal saline.

3.9.5 Post Booster Vaccination Serum Collection

Blood was collected from both experimental and control groups on day 77, 84 and 133. The collected blood was allowed to clot and incubated at 37°C for 30 min. Serum was separated as described in 3.9.3.

3.10 ASSESSMENT OF IMMUNE RESPONSE

The comparative efficacy of the different vaccines was determined based on IHA titre and challenge test.

3.10.1 Assessment of Antibody Response

The humoral immune response to *P. multocida* was assessed by indirect haemagglutination test as described earlier (3.8.2). The IHA titre was expressed in $\log_2$. The entire data was tabulated and MIHAT calculated using the software Microsoft excel 2007. The tabulated data were analysed statistically using the software statistical package for social sciences (version 10). As the vaccinated and control birds were subjected to four challenge tests, the number of observations was not uniform throughout the experiment. Based on the uniformity in the number of observations the total number of observations were divided to three sets *viz.*, set I with $\log_2$ IHA titre values recorded from day 0 to day 70, set II with $\log_2$ IHA titre values recorded from day 70 to day84 and set III with $\log_2$ IHA titre values recorded on day 133. Analysis of variance (one way) of $\log_2$ IHA titres of sera collected on day 0, 7, 14, 21, 35, 49, 70, 77, 84, and 133 PV from the ducks was done.

3.10.2 Challenge Test

Challenge test was conducted four times using the inoculum prepared as described in section 3.3.2.2.1.

Ten birds each from each group were subjected to challenge with 0.1 ml of inoculum containing 100 $LD_{50(11W)}$ of fully encapsulated virulent isolate on days 49 and 84 PPV. On day 98 PPV (day 28 PBV), another ten birds each from every group

was subjected to challenge with 200 $LD_{50(11W)}$ of fully encapsulated virulent isolate.

On day133 PPV (day 63 PBV) ten birds each from every group was subjected to challenge with 0.1 ml of inoculum containing 100 $LD_{50\ (21w)}$ of fully encapsulated virulent isolate.

Mortality of birds in each group after the administration of the challenge dose of *P. multocida* was recorded till 15th day of inoculation. The birds were routinely observed for any clinical signs. The time required for death of each challenged bird was also noted, tabulated and the MDT was calculated.

The organisms in the heart blood were demonstrated using Leishman's staining. Attempts were made to re-isolate *P. multocida* from heart, liver and spleen of the dead birds in TSA plates. Characterization and identification of the isolate was done based on the morphology, cultural and biochemical characteristics. Further confirmation was done by conducting PM PCR (3.2).

3.10.3 Statistical Analysis

Data collected from various parameters were analysed as per the method of Snedecor and Cochran (1994) by using one way analysis of variance (ANOVA), followed by Duncan's multiple range test for grouping means having significance.

3.11 HISTOPATHOLOGICAL STUDIES.

3.11.1 Collection of Samples

Following first challenge test detailed postmortem examination of the birds died of pasteurellosis was conducted. Birds survived were also sacrificed and subjected to post mortem examination. Liver, spleen, caecal tonsils and bursa were collected from each bird for histopathological examination.

3.11.2 Preparation of Slides.

Tissues were fixed in 10 per cent buffered formalin. They were then processed and paraffin embedded as described by Sheehan and Hrapchak (1980). Sections were cut at four micron thickness and stained with routine Haematoxylin and Eosin stain (Bancroft and Cook, 1984). The sections were examined in detail under light microscope.

3.12 RECOMBINANT GHOST SYSTEM

3.12.1 Design of Primers

The complete nucleotide sequence of bacteriophage phiX174 (J02482) was downloaded from gene bank (http://www.ncbi.nlm.nih.gov/nuccore/J02482) in FASTA format. Based on gene map of phiX174 the location of *gene E* was identified. Based on these data, forward and reverse primes were designed using the software primer 3 for amplification of complete sequence of gene *E*.

3.12.2 Polymerase Chain Reaction for Amplification of *Gene E*

3.12.2.1 Materials

3.12.2.1.1 PCR Reaction Buffer (10x)

This included 500 mM KCl, 100 mM Tris-HCl pH 9.0 and 15 mM $MgCl_2$ procured from (M/s Sigma)

3.12.2.1.2 Taq DNA Polymerase (M/s Sigma)

Taq DNA polymerase enzyme with a concentration of 3 U/µl.

3.12.2.1.3 Magnesium Chloride

Magnesium chloride with strength of 25 mM obtained from M/s Sigma.

3. 12.2.1.4 Deoxy Ribonucleotide Triphosphate (M/s Sigma)

Deoxy ribonucleotide triphosphate (dNTP) mix

2.5 mM (10 mM each of dGTP, dCTP, dATP and dTTP in equal volume)

3. 12.2.1.5 Primers for Amplification of Gene E

The designed primers were custom synthesized by M/s Sigma.

3. 12.2.2 Method

3. 12.2.2.1 Preparation of Template DNA for PCR

PhiX174 genome (M/S Bangalore Genei Pvt. Ltd.), obtained as a solution of 0.5 µg / µl was diluted 100 times with Millipore water to contain 5 ng/µl and used as template DNA.

3. 12.2.2.2 Polymerase Chain Reaction for Amplification of Gene E

Polymerase Chain Reaction was conducted for the amplification of *Gene E.*

A 25 µl reaction mixture was prepared in 0.2 ml thin walled PCR tube (Axygen, India). The reaction mixture consisted of the following.

Template DNA	1 µl (5 ng/µl)
Primers	20 pmol of each primer
10 x PCR buffer	2.5 µl

Taq DNA polymerase	0.6 unit
dNTP mix	2.5 mM
Triple distilled water to	25 µl

The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

Initial denaturation		95°C for 3 min
30 cycles	denaturation	95°C for 30 sec
	annealing	55°C for 30 sec
	extension	72°C for 60 sec
Final extension		72°C for 7 min

The whole reaction was conducted under the heated lid. The product was analysed by submarine agarose gel electrophoresis as described in 3.2.3.2 using one per cent agarose gel.

Results

4. RESULTS

4.1 PURITY CHECKING OF THE ISOLATE

Purity checking of the isolate was done based on the assessment of morphology, cultural characteristics biochemical characterization and PM-PCR.

4.1.1 Cultural Characteristics and Morphology

The *P. multocida* serotype A1 isolate DP1 produced smooth, convex, translucent, and butyraceous colonies on blood agar. The size of the colonies on blood agar varied from one to three millimetres in diameter, after 24 h of incubation. Gram's staining revealed Gram negative cocco-bacillary organisms.

4.1.2 Biochemical Characterization

4.1.2.1 First Stage

The isolate grew aerobically and anaerobically and did not produce any growth on Mac Conkey's agar. The colonies on blood agar were non-haemolytic. It was catalase and oxidase positive and fermentative (Table 1).

4.1.2.2 Second Stage

The isolate was positive for indole and ornithine decarboxylase, negative for methyl red, Voges-Proskauer and urease reactions. It did not produced hydrogen sulphide (H_2S) in triple sugar iron agar medium, but reduced nitrate and was negative for citrate utilization.

Regarding the fermentation of the sugars, the isolate fermented glucose, sucrose, galactose, mannose, maltose, trehalose and sorbitol, but could not utilize lactose, arabinose, salicin and dulcitol (Table 2). Based on biotyping the isolate DP1 was characterized as *P. multocida* subsp. *multocida.*

Table 1. First Stage Biochemical Tests of DP 1

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

Table 2. Second stage biochemical tests of DP1

Test	Result
Indole production	+
Methyl red	–
Voges Proskauer	–
H_2S production	–
Citrate	–
Nitrate	+
Ornithine decarboxylase	+
Urease	–
Sugar fermentation	
Glucose	+
Sucrose	+
Galactose	+
Mannose	+
Lactose	–
Maltose	+
Arabinose	–
Salicin	–
Dulcitol	–
Trehalose	+
Sorbitol	+

4.2 *Pasteurella multocida* SPECIES SPECIFIC PCR (PM-PCR)

Agarose gel (1.5 per cent) electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (100 base pair DNA ladder) in 1 x TAE buffer. On viewing the gel under UV transilluminator a 460 bp amplicon was detected which was specific for *P. multocida* (Fig.1). In negative control no amplified product was detected. Thus the isolate DP1 was confirmed to be *P. multocida*.

4.3 PATHOGENICITY AND LETHAL DOSE 50 TESTING OF DP1 ISOLATE

4.3.1 Pathogenicity Testing in Mice

When 0.3 x 10^8 CFU of DP1 isolate was inoculated intra peritoneally into four Swiss albino mice, death occurred in all of them within a time period of eight to ten hours.

The control mice were alive even after an observation period of seven days. The gross lesions observed in the inoculated mice were petechial haemorrhages in the epicardium, general congestion of all the visceral organs, particularly of lung, liver and spleen. Fluid accumulation was also noticed in the peritoneal cavity of mice. 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, lungs, liver and spleen on bovine blood agar and TSA, following incubation at 37°C for 24 h. Re-isolated organisms were subjected to biochemical identification and PM-PCR and they showed characteristics similar to that of inoculated strain of organism as described in sections 4.1 and 4.2 respectively.

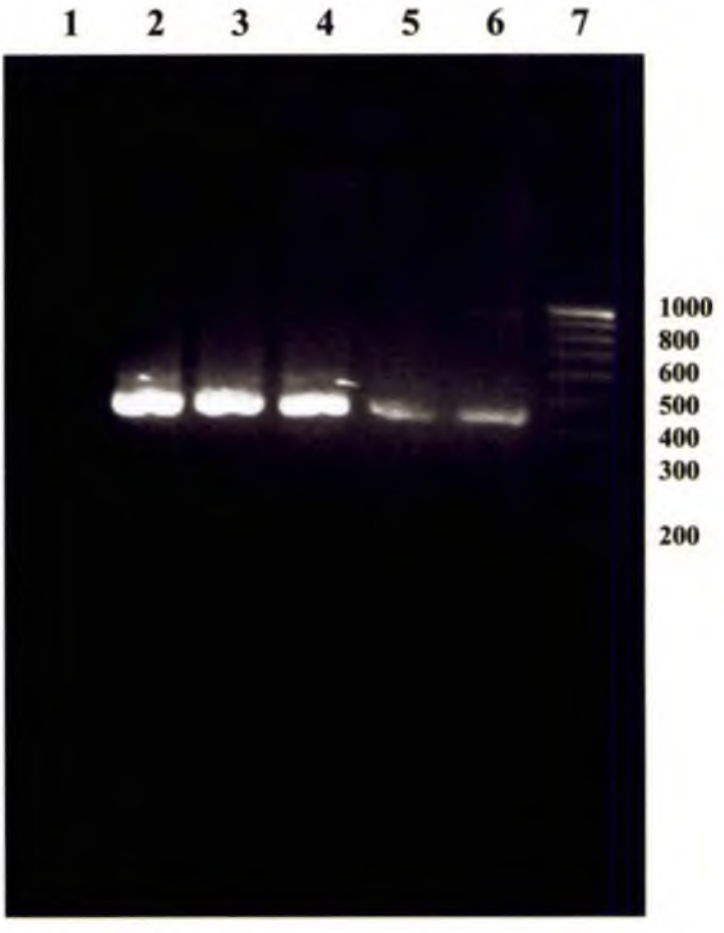

Fig.1 PM-PCR
Lane 1- negative control
Lane 2-4 - biofilm cell samples
Lane 5&6 - planktonic cell samples
Lane 7- 100 bp marker

4.3.2 Lethal Dose 50 (LD_{50}) of *P. multocida* in Ducklings/Adult Ducks

4.3.2 .1 Lethal Dose 50 of P. multocida in 11 Week Old Ducklings ($LD_{50(11w)}$)

Determination of $LD_{50(11w)}$ of *P. multocida* was done in 11 week old ducklings. The results of the experiment are furnished in Table 3.

Table 3. Determination of LD_{50} in 11 week old ducklings

Dilution	Organisms present in 0.1 millilitre of inoculum (CFU)	No: of birds inoculated	No: died	No: alive	Cumulative value		Ratio + ve	% + ve
					+ ve	–ve		
10^{0}	3×10^{6}	6	6	0	36	0	36/36	100
10^{-1}	3×10^{5}	6	6	0	30	0	30/30	100
10^{-2}	3×10^{4}	6	6	0	24	0	24/24	100
10^{-3}	3×10^{3}	6	6	0	18	0	18/18	100
10^{-4}	3×10^{2}	6	6	0	12	0	12/12	100
10^{-5}	3×10^{1}	6	6	0	6	0	6/6	100
10^{-6}	3×10^{0}	6	0	6	0	6	0/6	0

Calculation of proportionate distance

a=percentage of infectivity just above 50 percent ,

b=50 percent

c=percentage of infectivity just below 50 percent

Proportionate distance = (a-b) / (a-c)

= 100-50 / 100-0 = 50/100 = 0.5

Now added proportionate distance to dilution showing above 50 per cent infectivity i.e $10^{-(5+0.5)} = 10^{-5.5}$.

Therefore the 50 percent lethal dose end point was therefore at a dilution of $10^{-5.5}$ times the stock solution of organism. Stock solution of organism used was 3×10^6 organism / 0.1 millilitre.

$LD_{50\,(11\,w)} = 10^{-5.5} \times 3 \times 10^6 = 10^{0.5} \times 3 = 3.16 \times 3 = 9.5$

It was approximated to 10 organism. Thus $LD_{50\,(11\,w)} = 10$ Organism

4.3.2.2 Determination of Mean Death Time in 11 Week Old Ducklings.

Mean death time of 11 week old non inmmunised ducklings inoculated with decreasing doses of DP1 were recorded .The MDT varied from 23.75 h to 54.6 with decreasing dose of inoculum. The details are furnished in Table 4.

4.3.2.3 Lethal Dose 50 of P. multocida in 21 Week Old Ducks ($LD_{50(21w)}$)

Determination of $LD_{50(21w)}$ of DP1 was done in 21 week old ducks. The results of the experiment are furnished in Table 5. Dose of inoculum that produced 3 deaths among 6 birds was = 3×10^8 CFU of DP1. Therefore $LD_{50\,(21\,w)}$ was 3×10^8 CFU of DP1.

Table 4. Mean death time in 11 week old ducklings

Sl no	Group	Dose of inoculum (CFU/ bird)	Bird no:	Death time in h	Mean Death time in h
1	A	$3X10^6$	A1	10.5	23.75
			A2	19	
			A3	28	
			A4	19	
			A5	55.5	
			A6	10.5	
2	B	$3X10^5$	B1	16	26.75
			B2	40	
			B3	16	
			B4	28	
			B5	40	
			B6	20.5	
3	C	$3X10^4$	C1	19	29.5
			C2	19	
			C3	40	
			C4	37	
			C5	40	
			C6	22	
4	D	$3X10^3$	D1	19	42.42
			D2	78	
			D3	52	
			D4	22	
			D5	28	
			D6	55.5	
5	E	$3X10^2$	E1	66	49.8
			E2	28.5	
			E3	43	
			E4	40	
			E5	55.5	
			E6	66	
6	F	$3X10^1$	F1	78	54.6
			F2	55.5	
			F3	66	
			F4	28	
			F5	22	
			F6	78	

Table 5. Determination of LD_{50} in 21 Week Old Ducks

Dilution	Organisms present in 0.1 millilitre of inoculum	No: of birds inoculated	No: died	No: alive
10^{0}	3×10^{10}	6	5	1
10^{-1}	3×10^{9}	6	4	2
10^{-2}	3×10^{8}	6	3	3
10^{-3}	3×10^{7}	6	2	4
10^{-4}	3×10^{6}	6	1	5
10^{-5}	3×10^{5}	6	0	6

4.3.2.4 Determination of Mean Death Time in 21 Week Old Ducks.

Time required for death of 21 week old ducks when they were inoculated with increasing doses of DP1 were noted and furnished in Table 6.

4.3.2.5 Gross lesions observed in ducks.

The main lesions observed in post mortem examination of dead ducks during determination of LD_{50} were necrotic foci on liver (fig.2), haemorrhage in heart (fig.3), enlarged spleen with necrotic changes (fig.4) and haemorrhagic enteritis in intestine (fig.5).

Table 6. Mean death time in 21 Week Old Ducklings

Group	Dose of inoculum (CFU/ bird)	Bird number	Death time in h	Mean death time in h
A4	3×10^{10}	A41	43	35.4
		A42	17	
		A43	41	
		A44	36	
		A45	40	
B4	3×10^{9}	B41	41	38.38
		B42	34	
		B43	38.5	
		B44	40	
C4	3×10^{8}	C41	20.5	20.17
		C42	18	
		C43	22	
D4	3×10^{7}	D41	41	38.5
		D42	36	
E4	3×10^{6}	E41	17	17

Fig. 2. Necrotic foci on liver.

Fig.3. **Haemorrhage on heart .**

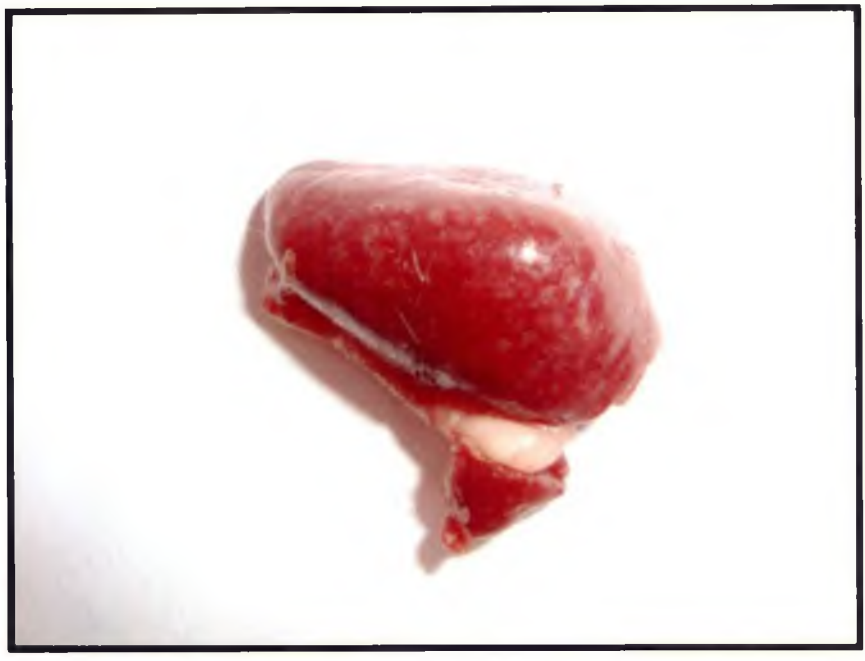

Fig.4. Enlarged spleen with necrotic changes

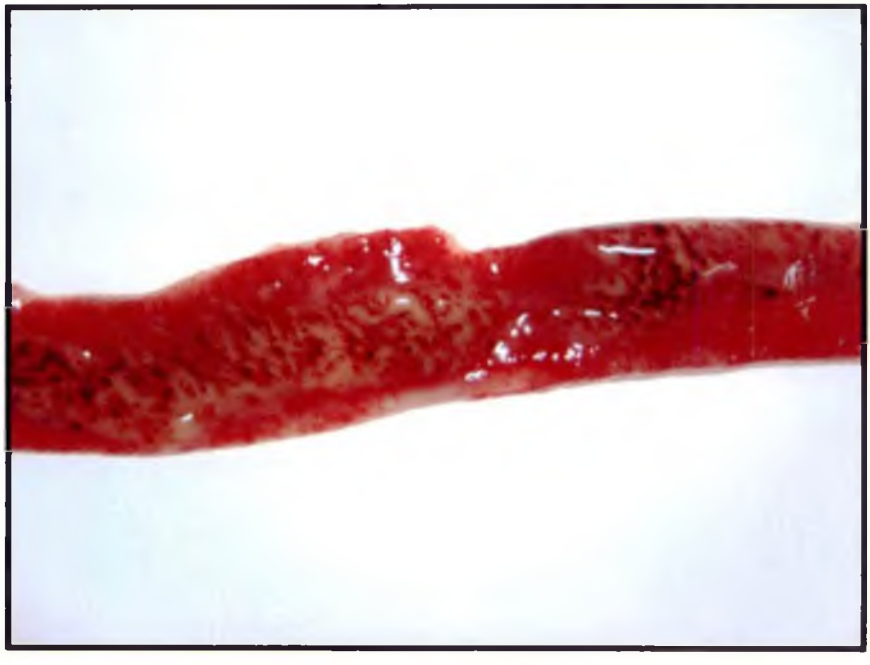

Fig.5. Hemorrhagic enteritis in intestine.

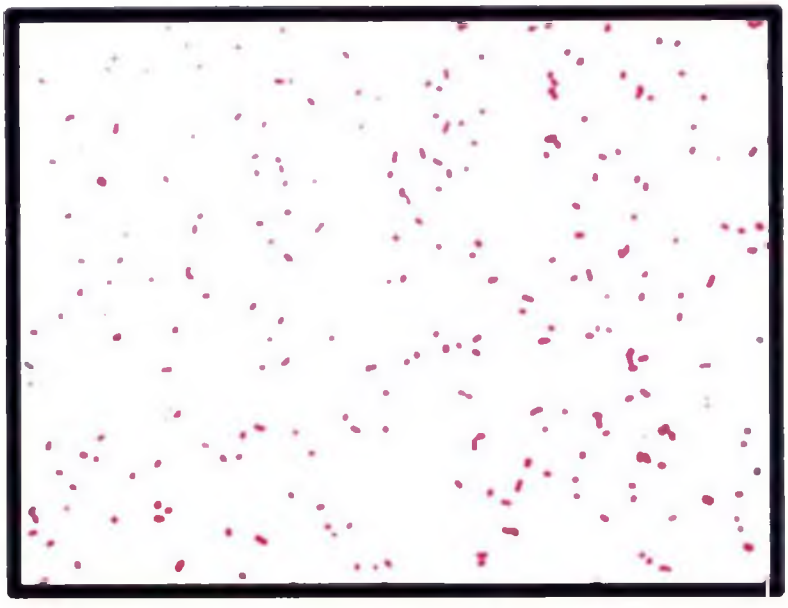

Fig.6. Grams staining of planktonic cells (1000x)

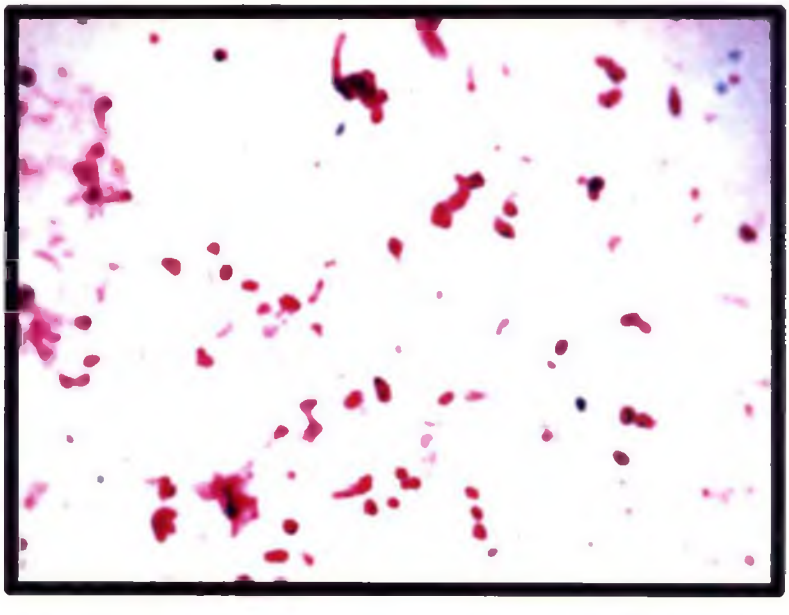

Fig.7. Grams staining of biofilm cells (1000x)

4.4 MORPHOLOGICAL STUDIES ON *P. multocida* BIOFILM AND PLANKTONIC CELLS

4.4.1 Light Microscopic Studies - *Gram's Stain*

The stained smears of planktonic cells appeared to be Gram negative coccobacillary organisms under oil immersion objective of the microscope (fig. 6).

The stained smears of biofilm cells of *P. multocida* appeared to be Gram negative, but pleomorphic. Bacterial cells were usually seen in aggregations although indivudal cells were also seen. They did not show a uniform morphology on microscopical examination. The different morphological forms observed were cocci, coccobacilli, rods, filamentous and pear shaped cells. Formation of hyphae like or stalk like extensions could be seen in certain cells. Cell to cell connections were seen as faintly stained networks. Cell aggregations and pleomorphism were the most distinguishing features observed in biofilm smears (fig.7).

4.4.2 Scanning Electron Microscopic Studies

4.4.2.1 Scanning Electron Micrograph at 1500x Magnification

Biofilm cells showed coral reef like structures and three dimensional formations, cells were arranged in an irregular fashion, and many micro colonies were seen embedded in a matrix material. In biofilm, the bacteria appeared to be more clumped rather than being a confluent monolayer of cells. The micro colonies were seen embedded in a common capsule. Filament formations were also seen under lower magnifications.

Planktonic cells appeared to be a confluent layer of cells like a cell sheet. The micro colony formation was absent and cells seemed to be arranged in an almost regular fashion.

4.4.2.2 Scanning Electron Micrograph at 2500x Magnification

At 2500 x magnification the observations are clearer although same as that at 1500 X (fig.8 and fig.9).

4.4.2.3 Scanning Electron Micrograph at 5000x and 8000x Magnification

Planktonic cells were almost uniform, oval or coccobacillary in shape. Filamentous formations were totally absent (fig.10).

Biofilm cells showed finger like filamentous formations, cells lacked uniformity in shape and size, pleomorphism of cells was more evident at this magnification (fig.11).

4.4.2.4 Scanning Electron Micrograph at 15000x Magnification

In the case of planktonic cells at 15000x, the tertiary structures were absent and cocco-bacillary cells were having a length of 0.4 μm and width of 0.2 μm (fig.12).

At very high magnification of 15000x the finger like projection formed by biofilm was very clearly visible. These finger like growth occurred in different directions, resulting in the formation of three dimensional tertiary structures of biofilm. The length of these structures varied while the diameter was about 0.5 μ m in most of the cases (fig.13).

4.5 EVALUATION OF PREPARED VACCINES

4.5.1 Preparation of Different Types of Vaccines

Firstly oil adjuvanted vaccines prepared consisted of 15 parts of antigen, nine parts of liquid paraffin and one part of lanolin. The aqueous phase was added drop wise to constantly stirred oil phase and homogenized the mixture at 18,500 rpm for 15 min. Drop test was employed to determine the emulsion type and then stored at 4°C. It failed to give an intact and immiscible emulsion with water. The vaccine

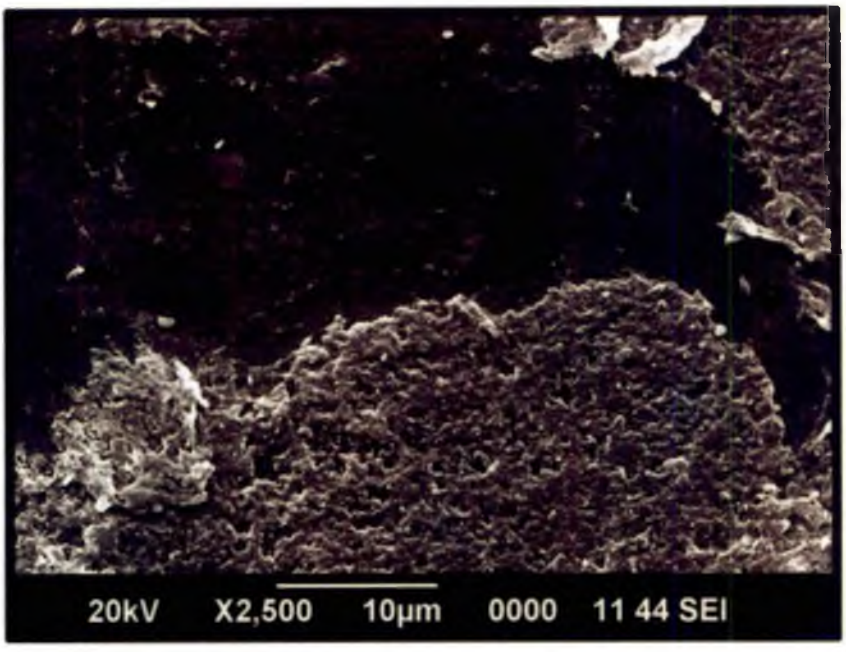

Fig.8. Scanning electron micrograph of *Pasteurella multocida* planktonic cells (2500 X)

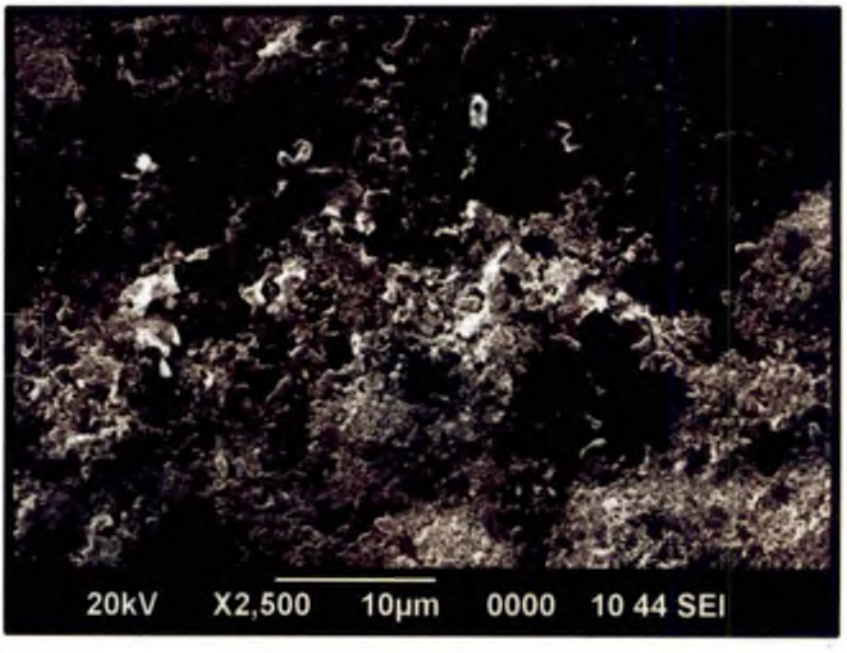

Fig.9. Scanning electron micrograph of *Pasteurella multocida* biofilm cells (2500 X)

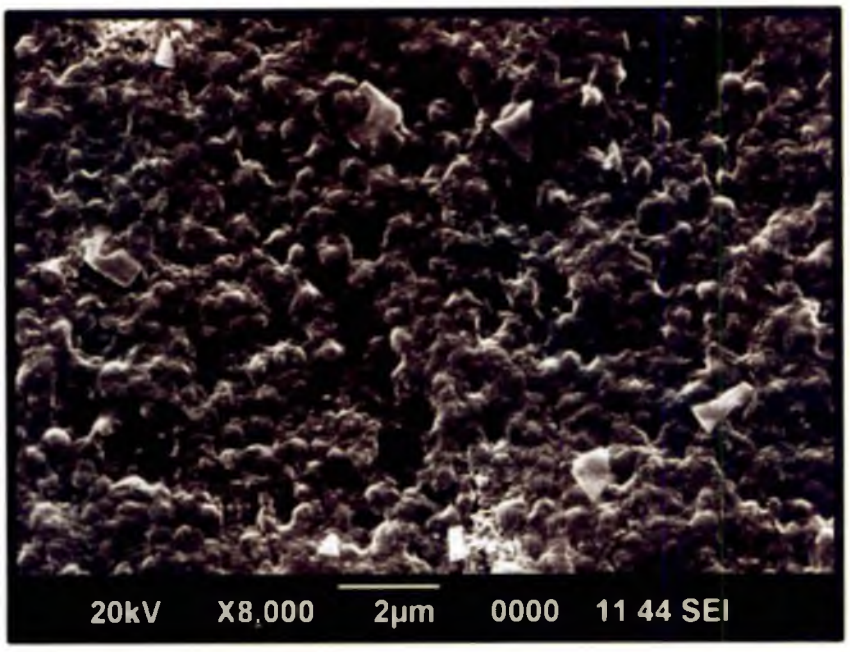

Fig.10. Scanning electron micrograph of *Pasteurella multocida* planktonic cells (8000 X)

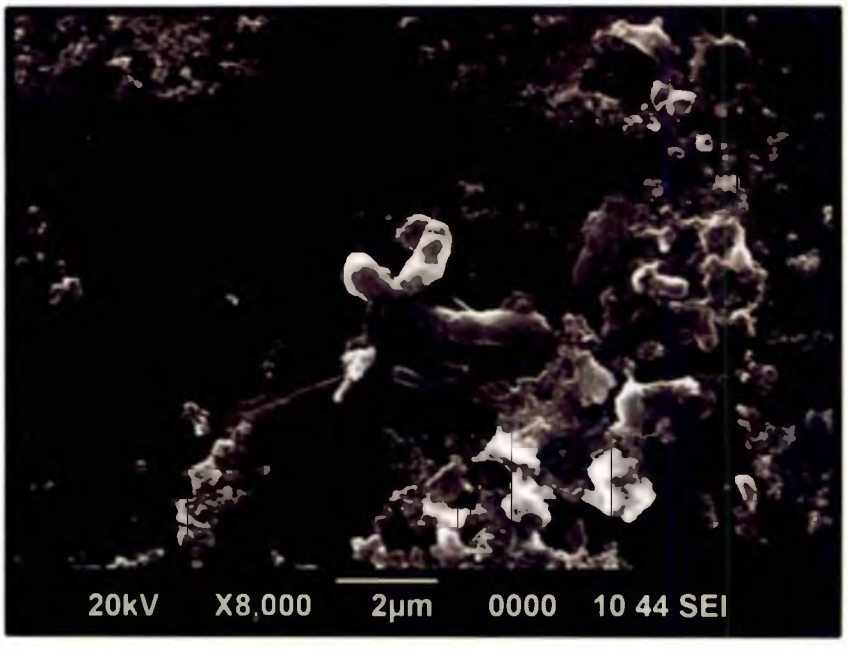

Fig.11. Scanning electron micrograph of *Pasteurella multocida* biofilm cells (8000 X)

Fig.12. Scanning electron micrograph of *Pasteurella multocida* planktonic cells (15000 X)

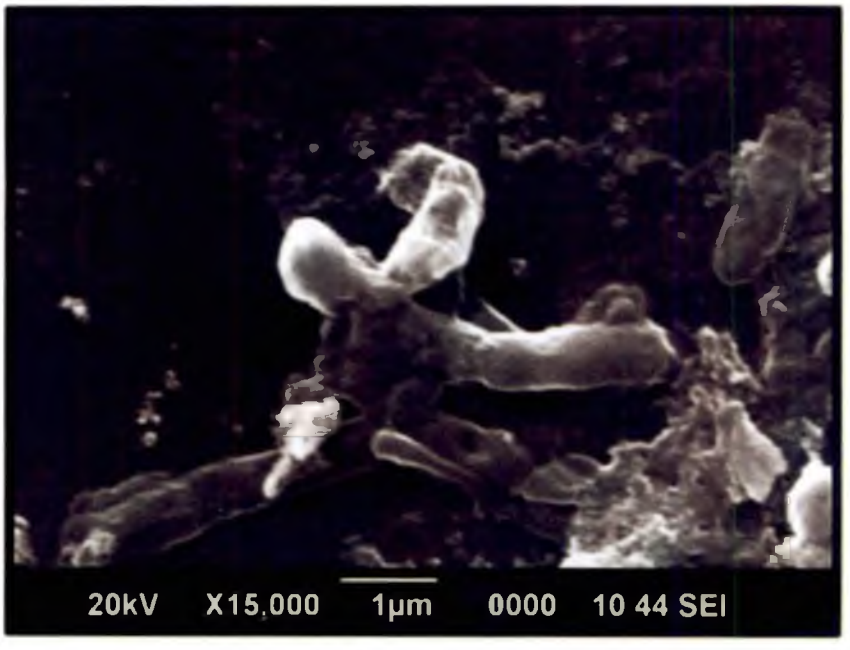

Fig.13. Scanning electron micrograph of *Pasteurella multocida* biofilm cells (15000 X)

immiscible emulsion with water. The vaccine spread on water surface on drop test. Also although homogeneity was observed at the time of preparation, it was lost after keeping for 48 hrs at 5 °C.

Hence, the homogenization of the mixture was tried again by altering the composition. The amount of liquid paraffin was reduced and that of lanolin increased by a level of 10 per cent sequentially. Each set of vaccines were subjected to drop test and keeping quality was evaluated for a period of 48 hrs at 5 °C. The optimum composition showing least viscosity and a positive drop test was found to be 12 parts of antigen, seven parts of liquid paraffin and one part of lanolin. When compared to vaccine prepared earlier, this was found to be homogenous even after keeping for a month at 5 ° C (fig.14). Hence, this modified composition was followed for preparation of oil adjuvanted vaccines throughout the study.

The OV, OBV, SV vaccines prepared were homogeneous suspensions which were easy for parenteral administration. But the SAV prepared failed to give a homogenous suspension. Hence, the SAV had to be mixed well just before administration.

4.5.2 Sterility Testing of the Vaccines

All the four vaccines prepared were found to be sterile as no growth was observed in blood agar, TSA, modified thioglycollate medium and 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 incubated at room temperature.

4.5.3 Safety Testing of the Vaccines

The safety of the vaccines was assessed by injecting 0.5 millilitre and one millilitre *i.e* double dose of vaccine intramuscularly to three ducklings each separately for the vaccines. All the four types of vaccines did not cause any untoward effects except for a transient lameness which subsided after one day post inoculation.

Fig.14. Improved (left) conventional (right)
(12:7:1) (15:9:1)

4.6 ASSESMENT OF IMMUNE RESPONSE TO VACCINATION BY INDIRECT HAEMAGGLUTINATION TEST

The serum was collected on day 0, 7, 14, 21, 35, 49,70, 77, 84 and 133 PV .The glutaraldehyde fixed-SRBC was prepared. Before fixation SRBC suspension appeared as bright red coloured solution while after fixation it became dark red (fig15 and fig 16). The humoral immune response to vaccination was assessed by indirect haemagglutination test (fig.17). The titre was expressed in $\log_2$. The entire data were tabulated and MIHAT was calculated and a graph was plotted using the software Microsoft excel 2007(fig.18). The tabulated data were analysed statistically using the software statistical package for social sciences (version 10).

4.6.1 Protein Concentration of Ultrasonicated *P. multocida* Antigen

Pasteurella multocida cell suspension was prepared to contain 3 X 10^{10} CFU / ml. The protein concentration of the ultra sonicated *P. multocida* antigen was estimated as per Lowry *et al.* (1951) and it was found to be 0.6 mg/ml.

4.6.2 Statistical Analysis

Analysis of variance (one way) of $\log_2$ IHA titres of sera collected on different days from the ducks was done. Based on MIHAT of individual sera collected at days 0, 7, 14, 21, 35, 49, 70, 77, 84, and 133 PPV ANOVA was done and results were shown in table 7.

4.6.2 .1 Day 0

There was no significant difference in mean titres among the groups.

Fig.15. Sheep RBC before glutaraldehyde treatment.

Fig.16. Sheep RBC after glutaraldehyde treatment.

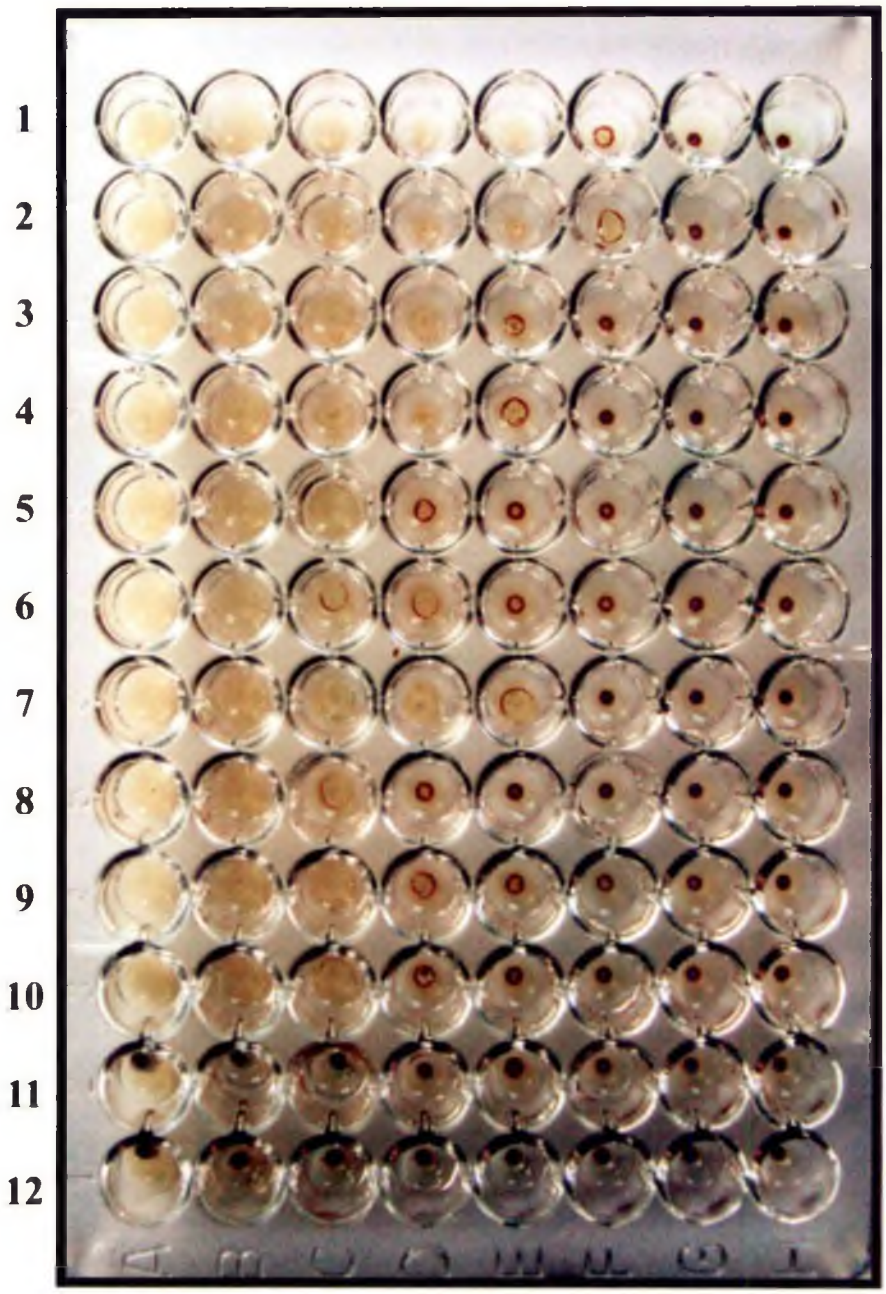

Fig.17. IHA test of different groups on day 21 PPV. Rows 1&2 -OV; Rows 3&4 - OBV; Rows 5&6 - SV; Rows 7&8 - SAV, Rows 9&10- Control, Rows 11&12- Negative test control

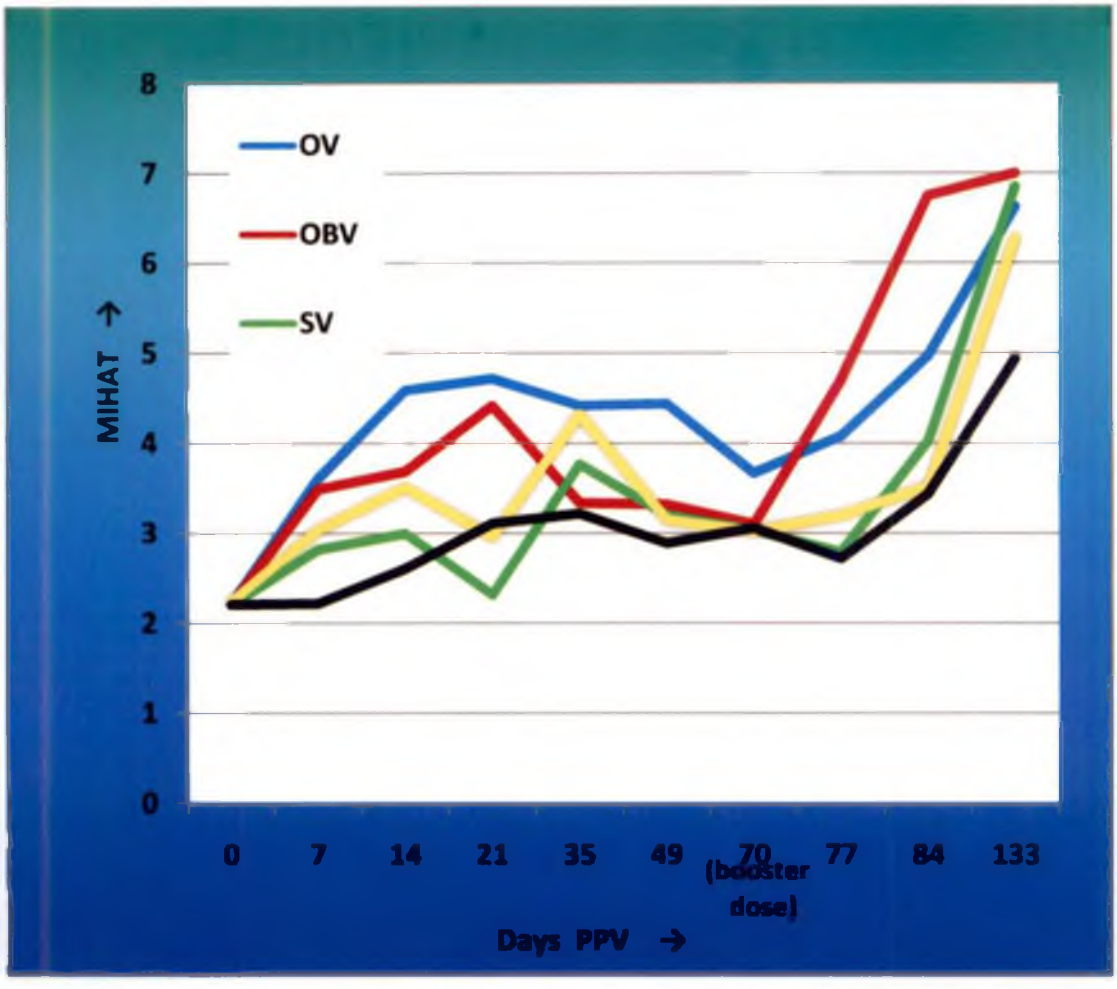

Fig.18. MIHAT of vaccine groups and control group on different days PPV

Table 7. Indirect Haemagglutination Test Titres of Serum Samples of Different Group of Birds on Different Days Post Vaccination

Day	OV (Mean±SE)	OBV (Mean±SE)	SV (Mean±SE)	SAV (Mean±SE)	Control (Mean±SE)
0*	2.21 ± 0.11	2.21 ± 0.10	2.21 ± 0.10	2.24 ± 0.11	2.21 ± 0.17
7	3.61^{a} ± 0.21	3.49^{a} ± 0.17	2.82^{b} ± 0.12	3.04^{ab} ± 0.11	2.22^{c} ± 0.17
14	4.59 ± 0.15^{a}	3.69^{b} ± 0.16	3.00^{c} ± 0.0777	3.51^{b} ± 0.12	2.61^{c} ± 0.16
21	4.72^{a} ± 0.12	4.42^{a} ± 0.11	2.32^{c} ± 0.0688	2.96^{b} ± 0.0992	3.11^{b} ± 0.0762
35	4.42^{a} ± 0.13	3.34^{b} ± 0.11	3.77^{c} ± 0.10	4.32^{a} ± 0.0978	3.22^{b} ± 0.10
49	4.44^{a} ± 0.12	3.32^{b} ± 0.0658	3.20^{bc} ± 0.0793	3.15^{bc} ± 0.10	2.89^{c} ± 0.11
70	3.67^{a} ± 0.0927	3.10^{b} ± 0.0926	3.03^{b} ± 0.0809	3.02^{b} ± 0.0949	3.06^{b} ± 0.0981
77	4.08^{b} ± 0.14	4.70^{a} ± 0.0974	2.79^{d} ± 0.0779	3.20^{c} ± 0.13	2.72^{d} ± 0.14
84	4.97^{b} ± 0.16	6.75^{a} ± 0.17	4.03^{c} ± 0.11	3.52^{d} ± 0.10	3.44^{d} ± 0.12
133	6.63^{ab} ± 0.18	7.00^{a} ± 0.20	6.85^{a} ± 0.20	6.27^{b} ± 0.0783	4.94^{c} ± 0.24

* - values are not significant ($p>0.05$)
- means bearing same superscripts in a row do not differ significantly ($p>0.05$)

4.6.2 .2 Day 7

There was no significant difference between OV, OBV, and SAV. Also there was no significant difference between SAV and SV. But control group differed significantly from all vaccinated groups.

4.6.2 .3 Day 14

Group OV differed significantly from all other groups. There was no significant difference between OBV and SAV. Also there was no significant difference between SV and control group.

4.6.2 .4 Day 21

There was no significant difference between OV and OBV. Also there was no significant difference between SAV and control group. But SAV differed significantly from other vaccinated groups *i.e* OV, OBV and SV.

4.6.2 .5 Day 35

There was no significant difference between OV and SAV. But SV differed significantly from all other groups. Also there was no significant difference between OBV and control group.

4.6.2 .6 Day 49

Group OV showed a higher mean $\log_2$ IHA titre of 4.44 which differed significantly from all other groups. There was no significant difference between OBV, SV and SAV. Also there was no significant difference between SV, SAV and control group.

4.6.2 .7 Day 70

Group OV showed a higher mean $\log_2$ IHA titre of 3.67 which differed significantly from all other groups. There was no significant difference between OBV, SV, SAV and control group.

4.6.2 .8 Day 77

The groups OBV, OV and SAV showed significant difference from each other as well as from SV and control groups. There was no significant difference between SV and control group.

4.6.2 .9 Day 84

The groups OBV, OV and SV showed significant difference from each other as well as from SAV and control groups. There was no significant difference between SAV and control group. On day 84 PPV all the vaccine groups and control group showed an increment in MIHAT from day 77.

4.6.2 .10 Day 133

There was no significant difference between OBV, SV and OV. Also there was no significant difference between OV and SAV. But control group differed significantly from all vaccinated groups.

4.6.2 .11 Comparison of vaccinated and control groups

The group OV showed a significant difference from control group on all the days post vaccination *i.e.* on days 7, 14, 21, 35, 49, 70, 77, 84 and 133. Next to OV it was the OBV, which showed a significant difference from control group on days 7, 14, 21, 49, 77, 84 and 133. Group SAV showed a significant difference from control group on days 7, 14, 35, 77, 133, while SV showed a significant difference from control group only on days 7, 21, 35, 84 and 133 PV.

4.7 HOMOLOGOUS CHALLENGE TEST

The efficiency of the prepared vaccines in offering protection from disease outbreak was assessed by conducting challenge test. Ten birds from each group were inoculated with 0.1 ml of inoculum containing 100 LD_{50} and observed for a period of 15 days.

4.7.1 First homologous challenge test

First homologous challenge experiment was conducted on day 49 PPV. The birds were challenged with 100 $LD_{50\ (11w)}$, *i.e.*, with 1000 organisms per bird. The results of challenge test were tabulated and given in table 8.The first challenge test revealed 80 per cent protection for OV group, 70 per cent protection for OBV group, 60 per cent protection for SAV group, 50 per cent protection for SV group. Birds survived in the control group were only 10 per cent. Time required for death was noted and furnished in table 9 and the mean death time was calculated. Mean death time in vaccinated birds varied from 36.25h-105h. For control group it was 32.61h.

4.7.2 Second homologous challenge experiment

Second homologous challenge experiment was conducted on day 84 PPV (day 14 PBV). The birds were challenged with 100 $LD_{50\ (11w)}$ (1000 CFU of DP1). No death was observed both in control and vaccinated groups for 15 days after challenge test.

4.7.3 Third homologous challenge experiments

Third homologous challenge experiment was conducted on 20 weeks old ducks. The birds were challenged with 200 $LD_{50\ (11w)}$ (2000 CFU of DP1) on day 98 PPV (day 28 PBV). No death was observed both in control and vaccinated groups for 15 days after challenge test.

Table.8 Homologous Challenge Experiments with 100 $LD_{50\,(11w)}$ (1000 CFU of DP1) Per Bird on Day 49 PV

Group	Vaccination status	No. of birds challenged	No. of birds survived	No. of birds died	Percentage protection
OV	Single vaccination	10	8	2	80
OBV	Single vaccination	10	7	3	70
SV	Single vaccination	10	5	5	50
SAV	Single vaccination	10	6	4	60
Control	Sham vaccination	10	1	9	10

Table 9. Mean Death Time on Day 49 Post Primary Vaccination.

Sl. no	Group	Bird no:	IHA titer	Death time (h)	Mean Death time (h)
1	OV	A41	5	44	54
		A42	4	64	
2	OBV	B41	3	23	40.66
		B42	3	25	
		B43	3	74	
3	SV	C41	3	20	105.8
		C42	4	20	
		C43	3	152	
		C44	3	152	
		C45	4	185	
4	SAV	D41	3	23	36.25
		D42	2	25	
		D43	2	25	
		D44	3	72	
5	CONTROL	E41	2	17.5	32.61
		E42	3	23	
		E43	2	25	
		E44	3	28	
		E45	3	35	
		E46	3	37.	
		E47	3	40	
		E48	3	43	
		E48	3	45	

4.7.4 Fourth homologous challenge experiment

Fourth homologous challenge experiment was conducted on day 133 PPV (day 63 PBV). The birds were challenged with 100 $LD_{50\ (21w)}$ (3×10^{10} CFU of DP1). The results of challenge test were tabulated and given in table 10. The fourth challenge test revealed 100 per cent protection for OV and OBV groups, 90 per cent protection for SAV group, 70 per cent protection for SV group while the protection obtained for control group was only 10 per cent .Time required for death was noted and the MDT was calculated. The MDT was lowest in SAV group (78 h) and highest in control group (102.33 h). The results are furnished in table 11.

4.8 HISTO PATHOLOGICAL STUDIES

The sections of organs obtained on postmortem examination following first challenge test on day 49 PPV were examined in detail under light microscope. The microscopic lesions observed in spleen, liver, caecal tonsil and bursa of Fabricius were noted both in live birds and in dead bird, tabulated and given in tables 12, 13, 14, and 15.

Table 10. Fourth Homologous Challenge Experiments with 100 $LD_{50\,(21w)}$ (3 $\times 10^{10}$ CFU of DP1) on day 133 PPV (day 63 PBV)

Group	Vaccination status	No. of days PBV	No. of birds challenged	No. of birds survived	Percentage protection
OV	Booster vaccination	63	10	10	100
OBV	Booster vaccination	63	10	10	100
SV	Booster e vaccination	63	10	7	70
SAV	Booster vaccination	63	10	9	90
control	Sham vaccination	63	10	1	10

Table 11. Mean Death Time on Day 133 Post Primary Vaccination.

Sl. no	Group	Bird no:	IHA titer	Death time (h)	Mean Death time (h)
1	SV	C41	8	76	89.33
		C42	6	100	
		C43	8	92	
2	SAV	D41	8	78	78
3	CONTROL	E41	6	100	102.33
		E42	4	78	
		E43	5	115	
		E44	7	115	
		E45	4	78.5	
		E46	4	102.5	
		E47	5	104	
		E48	4	104	
		E49	4	124	

Table 12. Histopathology of Spleen

Spleen		
Group	**Lesions in challenged sacrificed bird**	**Lesions in challenged dead bird**
OV	Normal spleen white pulp predominant	Splenic hemorrhage (fig.20) Lymphoid depletion Splenocyte necrosis
OBV	Normal spleen White pulp hyperplasia newly formed follicles seen as compact mass	Lymphoid depletion Vascular congestion White pulp
SV	Normal spleen White pulp predominant	Predominant splenic nodules Moderate periarteriolar lymphoid hyperplasia
SAV	Normal spleen White pulp predominant	White pulp predominant periarteriolar lymphoid hyperplasia Vascular congestion
Control	Periarteriolar lymphoid hyperplasia (fig.19)	Splenic nodules intact no change

Table 13. Histopathology of liver

Liver		
Group	**Lesions in sacrificed bird**	**Lesions in dead bird**
OV	Focal inflammatory cell infiltration Diffuse hepatic necrosis	Extensive fatty change Sinusoidal congestion Central venous congestion Kupffer cell reaction inflammatory cell infiltration
OBV	Inflammatory cell infiltration Diffuse hepatic necrosis Bile duct hyperplasia (fig.21) Vacuolation of liver	Sinusoidal congestion Central venous congested Focal hepatocyte degeneration
SV	Diffuse hepatic necrosis	Fatty change Diffuse hepatic necrosis Central venous congestion inflammatory cell infiltration (fig.22)
SAV	Diffuse necrosis Congestion	Fatty change Sinusoidal congestion Diffuse hepatocyte necrosis Focal inflammatory cell infiltration
Control	Central venous congestion Diffuse hepatic necrosis Multi focal inflammatory cell infiltration, Organism within vessel.	Sinusoidal congestion Diffuse hepatocyte necrosis Focal inflammatory cell infiltration

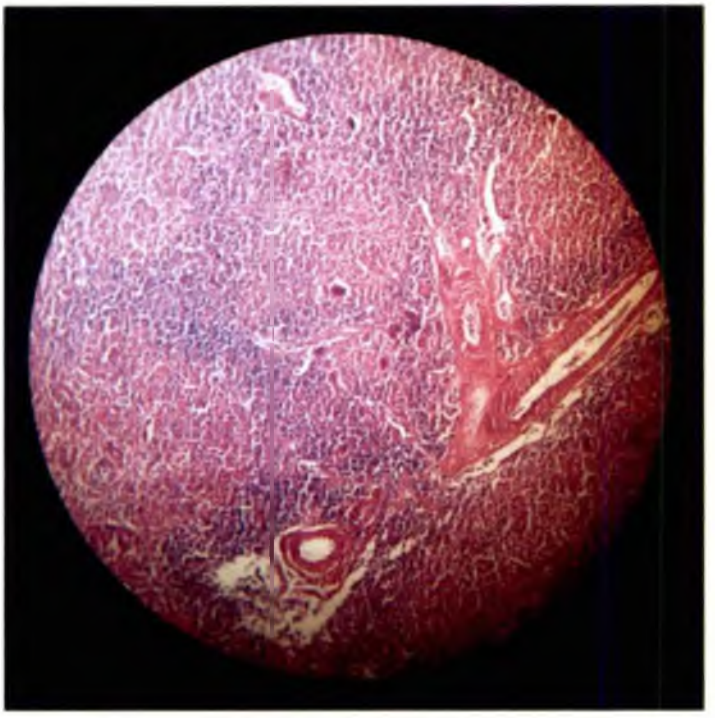

Fig.19. lymphoid hyperplasia in spleen (H&E X 100)

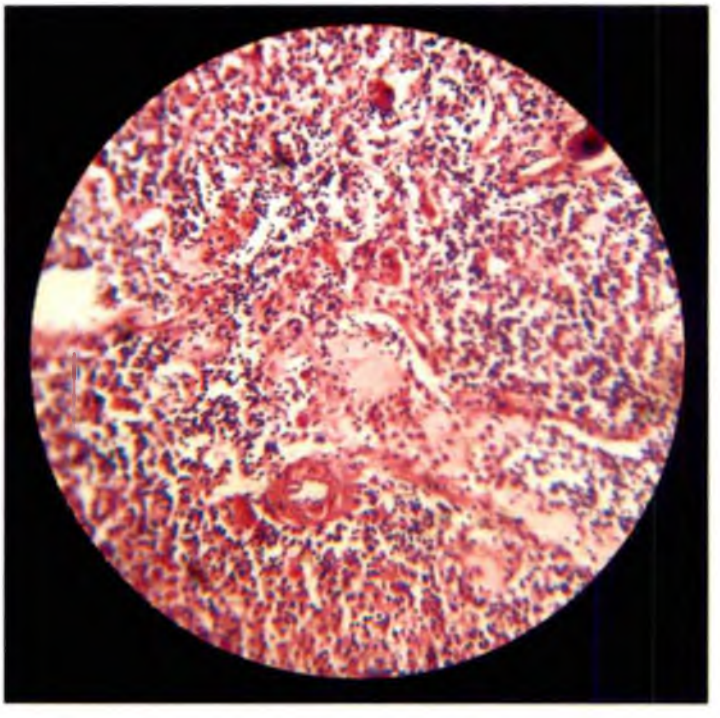

Fig.20. lymphoid depletion and hemorrhage in spleen (H&E X 100)

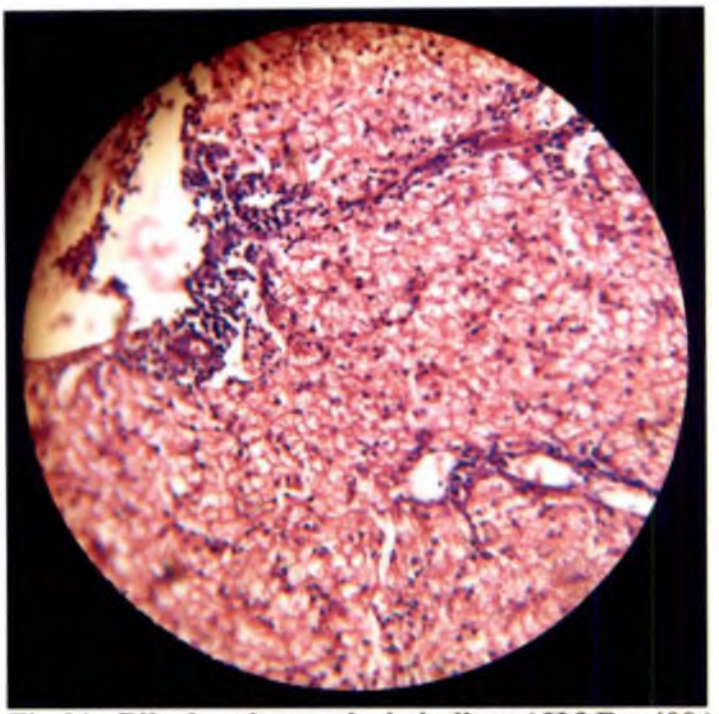

Fig.21. Bile duct hyperplasia in liver (H&E x 400)

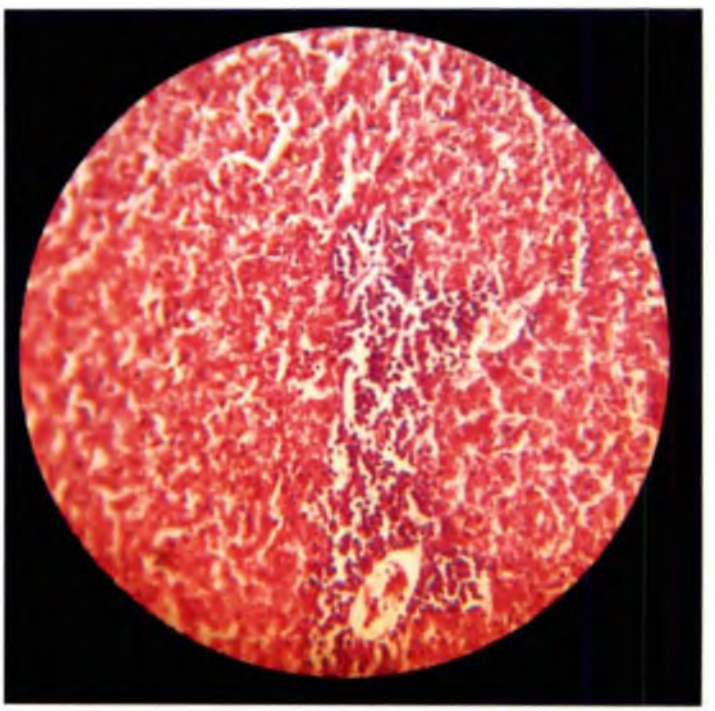

Fig.22. Inflammatory cell infiltration in liver (H&E x 400)

Table 14. Histopathology of Caecal tonsil

Caecal tonsil		
Group	**Lesions in sacrificed bird**	**Lesions in dead bird**
OV	Normal	Atrophy Glandular necrosis Lymphoid depletion(fig.24) Acute inflammation hemorrhage
OBV	Well developed follicles with compact aggregates of lymphocytes (fig.23)	Mild Lymphoid depletion
SV	Normal and intact	Lymphoid depletion
SAV	Normal and intact	Lymphoid depletion
Control	Depletion of lymphocyte and odema.	Lymphoid depletion, loose textured caecal tonsil

Table 15. Histopathology of Bursa of Fabricius

Bursa		
Group	**Lesions in sacrificed bird**	**Lesions in dead bird**
OV	Normal Well developed and compact follicle	Interstitial inflammatory cell infiltration Lymphoid necrosis Interstitial odema
OBV	Hyperplastic compact follicle packed with lymphocytes (fig.25)	Mild depletion of lymphocytes (fig.26)
SV	Lymphoid depletion in many follicles Necrotic changes	Lymphoid depletion in many follicles Follicles varied in size
SAV	Lymphoid depletion in many follicles necrotic changes	Atrophy of follicles
Control	Moderate Lymphoid depletion in few follicles	Lymphoid depletion in few follicles.

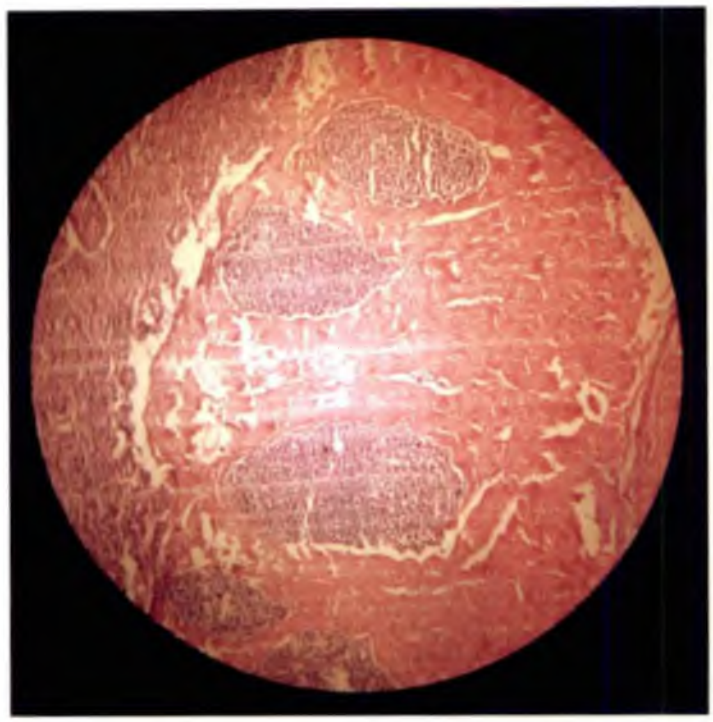

Fig.23. Well developed follicles in caecal tonsil (H &E x 40)

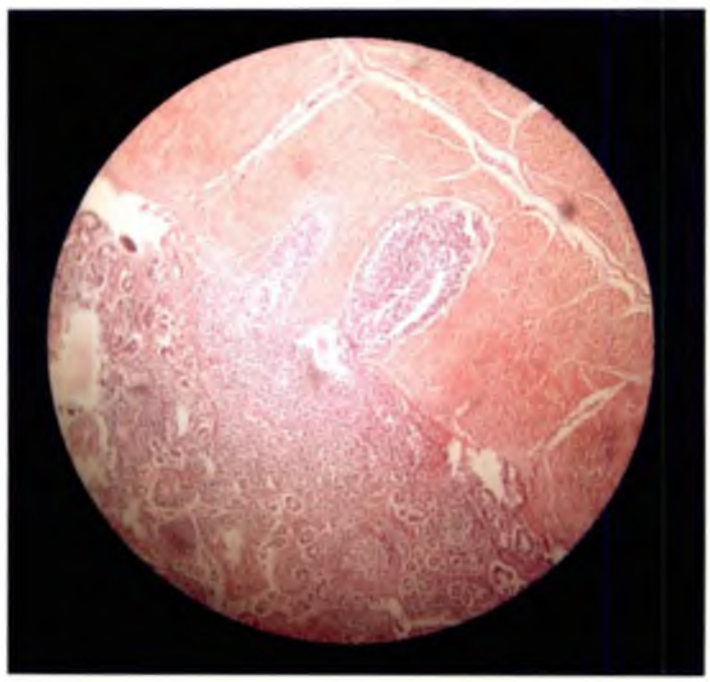

Fig.24. Lymphoid depletion in follicles caecal tonsil(H &E x 40)

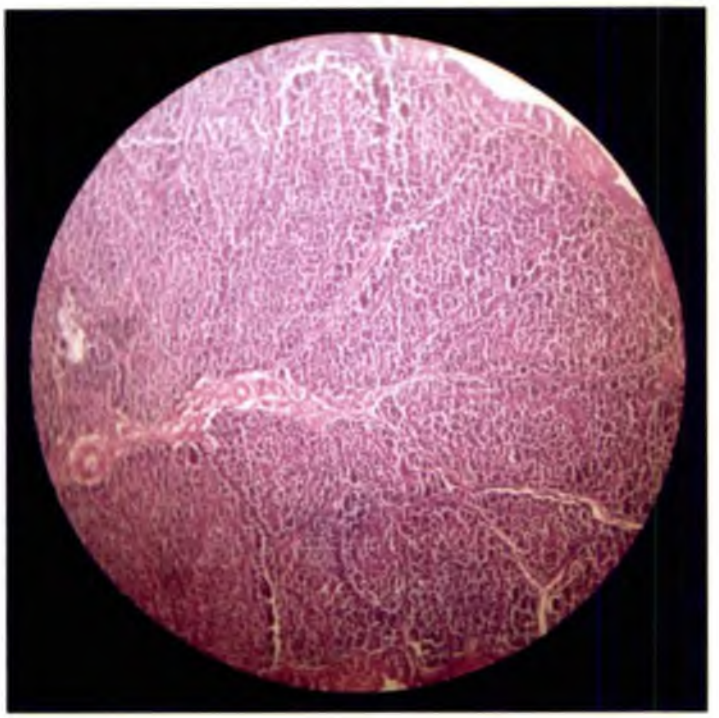

Fig.25. Lymphoiid hyperplasia in bursa (H&E x 100)

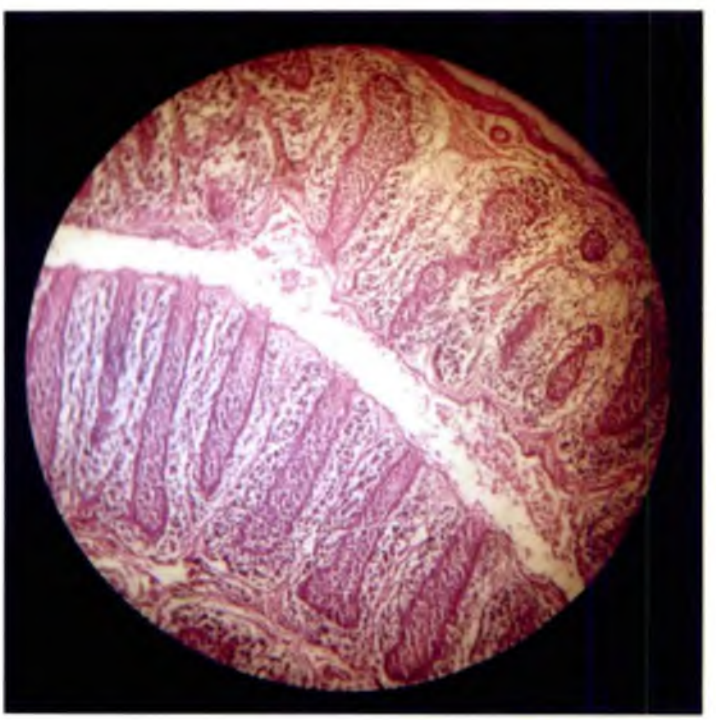

Fig.26. Lymphoid depletion in bursa (H&E x 100)

4.9 RECOMBINANT GHOST SYSTEM

4.9.1 Design of primers

The complete nucleotide sequence of bacteriophage phiX174 (J02482) was downloaded from gene bank (http://www.ncbi.nlm.nih.gov/nuccore/J02482) in FASTA format showed a sequence size of 5386 base pairs. Analysis of gene map showed that the gene *E* was located from 568 bp to 843 bp and gene *D* was located from 390 bp to 848 bp. A shine dalgarno sequence (GAGG) was seen from bp 555 to 558. Hence the primer was designed by including this shine dalgarno sequence using the software primer 3. A total of four primers were obtained as output out of which the best one was selected the details of which are given in table 16.

4.9.2 Polymerase Chain Reaction for amplification of *gene E.*

Agarose gel (one per cent) electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (100 base pair DNA ladder) in 1 x TAE buffer. On viewing the gel under UV transilluminator a 400 bp amplicon was detected which was specific for *gene E.* In negative control no amplified product was detected (fig.27).

Table 16. Primer 3 Output for Designing of Primers for Amplification of Gene *E.*

OLIGONUCLEOTIDE	start	length in bp	tm	GC%			3' seq
LEFT PRIMER (E1)	517	20	59.46	55.00	4.00	2.00	GGATTGCTACTGACCGCTCT
RIGHT PRIMER (E2)	916	20	59.90	55.00	4.00	2.00	GCCTTTAGTACCTCGCAACG
SEQUENCE SIZE: 5386							
INCLUDED REGION SIZE: 5386							
PRODUCT SIZE: 400, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00							
TARGETS (start, len): 555,289							

Discussion

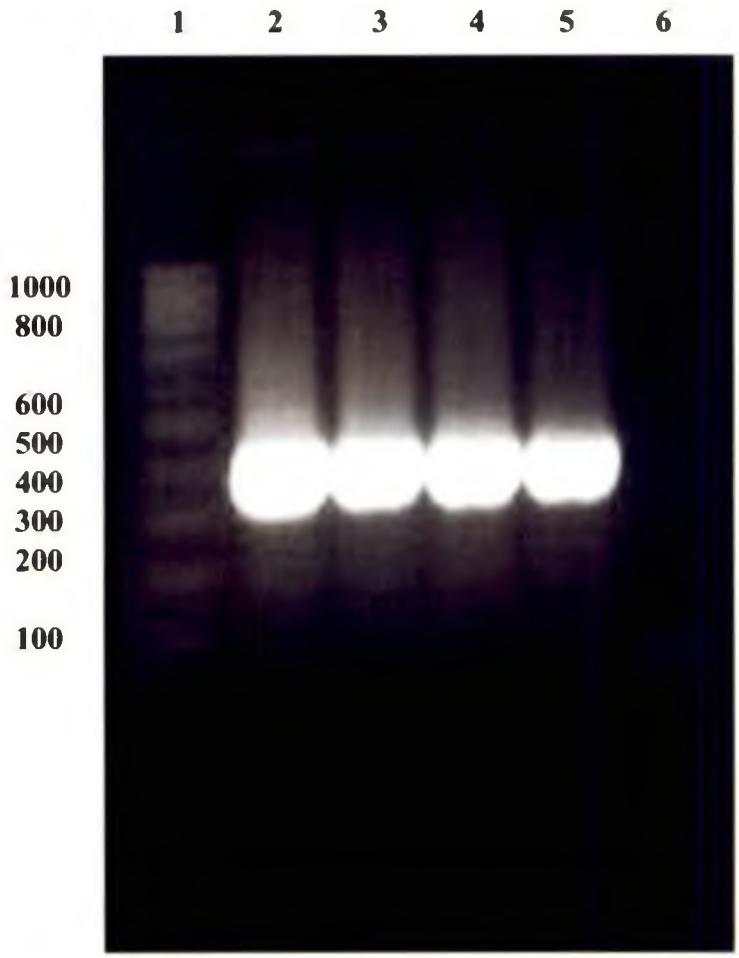

Fig. 27. Amplification of gene *E*
Lane 1- 100 bp marker
lane2-5 - samples
Lane 6 - negative control

5. DISCUSSION

Fowl cholera caused by *P. multocida* is a sporadic or enzootic bacterial disease among poultry population of the world (Rimler and Glisson, 1997). In both wild and domestic birds it is a septicaemic contagious disease with high morbidity and mortality (Glisson *et al.*, 2003) and thus has a major economic importance.

Empirically derived, live, attenuated vaccines often offer good protection against heterologous serotypes of *P. multocida,* but as the basis for attenuation is undefined, there is the risk of reversion of virulence (Adler *et al.*, 1999). Inactivated bacterin was found to be highly effective in controlling the outbreak of pasteurellosis among ducks (Murugkar and Ghosh, 1995).

In general the *P. multocida* vaccines currently used are bacterins, containing aluminium hydroxide or oil as adjuvant, prepared from multiple serotypes. Two doses of the killed vaccine are typically required. Live vaccines tend to impart greater protective immunity, but are used less frequently because of potential post-vaccination sequelae such as pneumonitis and arthritis. Also species wise identification of *P. multocida* is a frequently required step in vaccine production. The strain of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterised, of known serotype, pure, safe and immunogenic (OIE, 2004).

Pasteurella multocida formed biofilm at a high temperature of 42°C under nutrient restricted conditions and such a growth pattern resulted in production of novel immunogenic heat shock proteins and hence was recommended as candidate antigen for vaccines against fowl cholera (Hugar, 2004).

Hence, this study was aimed at the development of biofilm vaccines against duck pasteurellosis, using oil, saponin and aluminium hydroxide as adjuvants and experimental evaluation of the immunogenicity of these vaccines in ducks.

5.1 BIOCHEMICAL CHARACTERIZATION

Pasteurella multocida serotype A:1 isolate DP1 revived from lyophilized culture produced smooth, convex, translucent, and butyraceous colonies on blood agar after incubation at 37°C for 24 h. Gram's staining revealed Gram negative cocco-bacillary organism arranged singly or in pairs. This colony character is typical for the strain as described by Quinn *et al.* (2002).

The first stage and second stage biochemical identification tests and cultural characters were consistent with the description by Barrow and Feltham (1993) and fermentation patterns of dulcitol and sorbitol, were consistent with the description by Mutters *et al.* (1985).

Identity of the organism was further confirmed by PCR through specific amplification of an approximately 460 bp DNA fragment within the KMT1 gene, using species specific primers KMTISP6 and KMT1T7. The size of the amplified product obtained was in conformity with the observations of Townsend *et al.* (2001) and the negative controls did not give any amplified product. But they also mentioned that the same pair of primer, amplified template DNA from *P. canis* biovar 2 although it failed to amplify DNA from other members of *Pasteurellaceae* family, or unrelated bacteria. But *P. multocida* could be differentiated from *P. canis* based on biochemical tests (Barrow and Feltham, 1993). Thus, purity of the isolate used for vaccine production was established.

Although incorporation of presumptive identification using rapid technique like PCR helps in reducing time and cost for vaccine production, complete set of biochemical tests are also required to ensure identity of organism before commencement of production of a batch of vaccine.

5.2. PATHOGENICITY TESTING

5.2.1 Pathogenicity Testing

Virulence of the isolate was tested in Swiss albino mice. Collins (1973) reported that death of unvaccinated mice occurred 12 h after intravenous challenge of 10^4 viable *P. multocida* organisms due to the uncontrolled growth of the bacteria in all tissues. Hence he proposed mice as the animal of choice for pathogenicity testing of *P. multocida.*

Similar results were also reported by other workers. Murugkar and Ghosh (1995) tested the pathogenicity of *P. multocida* serotype A: 1 isolated from ducks in Tripura, in different hosts such as pigeon, duck, mice and rabbit by intraperitoneal route and found that the isolate killed all the hosts, though at varying time intervals. In their study the mice died within 12 h. Thus, isolates from ducks may be considered to be pathogenic to mice also. According to Basagoudanavar *et al.* (2006), LD_{50} of *P. multocida* was found to be 25 CFU in mice. Hence, mice may be considered to be a sensitive animal to *P. multocida.*

In the present study isolate DP1 killed the mice within eight hours, when inoculated intra peritoneally with 0.3 x 10^8 organisms. The identity of the organism re-isolated from mice was confirmed by biochemical tests and PM-PCR. Hence, mice could be used as a preferred laboratory animal for revival of freeze dried isolate DP1.

5.2.2 Post Mortem Lesions in Mice.

The gross lesions observed during post mortem examination of dead mice following intra peritoneal inoculation were epicardial petechiae, enlargement, congestion and pinpoint white, multiple, necrotic foci of the liver, congested spleen, haemorrhagic tracheitis and diffuse haemorrhages in internal organs like lungs and intestine. Similar findings had been reported by Rimler and Rhoades (1989).

The lesions indicated that death occurred due to acute septicaemia. The isolate DP 1 is a capsulated bacterium and its capsule had been demonstrated by Rajagopal (2007) by Maneval staining. According to Boyce and Adler (2000) capsulated *P. multocida* organisms were significantly resistant to phagocytosis and multiplied in the body following intra peritoneal challenge in mice. This was also evident from the high concentration of bipolar organisms in the visceral organs, following the examination of impression smears from dead mice.

5.2.3 Lethal dose 50 and Mean Death Time in Ducklings/Adult Ducks

5.2.3.1 Lethal dose 50 and Mean Death Time in 11 Week Old Ducklings

Fifty per cent lethal dose was described as a practical and reliable measurement of pathogenicity by Cruickshank *et.al.* (1975). Hence, LD_{50} testing was conducted to ensure the pathogenicity of the DP1 isolate for challenge studies.

The $LD_{50\ (11\ w)}$ of the DP1 isolate was 10 CFU / bird when tested in 11 week old ducklings. This result indicates that the isolate DP1 used for vaccine production was a highly virulent one and use of such types of isolates for vaccine trials was reported earlier (Ramanath,1994; Swamy,1994). Rajagopal (2007) determined the LD_{50} of DP1 as 23 viable cells. Ranjini (2007) and Indu (2008) estimated the same as 13 and 32 viable cells respectively when tested on duckling under similar experimental conditions. The $LD_{50\ (11w)}$ of birds observed in this study was lesser compared to studies conducted by others. This might be attributed to genetic differences among the different populations of ducks used by different workers.

The MDT of 11 week old ducklings when they were inoculated with increasing doses of isolate DP1 were recorded and furnished in Table 4. The table 4 showed that when 11 week old ducklings were challenged with a high dose of 3×10^6 CFU of *P. multocida* per bird the MDT was 23.75 h *i.e.* less than a day. Even though lot of work has been done on LD_{50} *P. multocida* serotype A:1, no data has

been available on the time required to produce death in the natural host of the isolate *i.e.* ducks. As pasteurellosis is an acute septicemic disease, the determination of MDT in its natural host helps to design control strategies during outbreaks.

Purushothaman *et al.* (2008) subjected *P. multocida* isolates obtained from wild geese in captivity to mouse bioassay and found them to be virulent and the MDT observed was between 12-18 h in mouse. Ramdani *et al.* (1990) observed that doses as low as 20 CFU of *P. multocida* M1404 when injected intra peritoneally into BALB/c mice, an overwhelming septicaemia was produced in mice in less than 30 h. They also opined that there was no evidence of inhibition of bacterial cell growth by natural host defense mechanisms of mice, even with this very small dose of bacterium. But in 11 week old kuttanad ducks the MDT was 54.67 h when the dose of inoculum was as low as 30 CFU. Thus, it might be inferred that compared to mice, ducks showed a small degree of natural resistance to challenge test even though that did not save the life of the bird. The inhibitory effect of avian serum to isolates of *P. multocida* had been demonstrated by various workers (Lee *et al.*, 1999; Diallo and Frost, 2000 and Muhairwa *et al.*, 2002).

In the present study MDT gradually increased as the dose of inoculum decreased.

5.2.3.2 Lethal dose 50 and Mean Death Time in 21 Week Old Ducks

In 21 week old ducks, the $LD_{50\ (21w)}$ of the isolate DP1 was estimated to be 3×10^8 CFU of *P. multocida*. The $LD_{50\ (11w)}$ of the isolate DP1 was 10 CFU / bird when tested in 11 week old ducklings. Hence, within a period of 10 weeks the LD_{50} increased by a factor of 3×10^7 times. This shows that the Kuttanad duck shows decrease in susceptibility to pasteurellosis with age. The findings were in contradiction with the opinion of others like Jordan and Pattison, (1996) and Petersen *et al.* (2001), who reported that adult birds seemed to be more susceptible than

younger stock. But the present finding was supported by an observation recorded by Ragagopal (2007), during his vaccine trials. When four Kuttanad ducks of 24 week age were inoculated subcutaneously with 1.5 X 10^7 CFU of isolate DP 1, for finding out LD_{50} no deaths were observed for a period of 10 days. In the present study death occurred only when 3 X 10^6 CFU were inoculated.

The resistance shown by avian serum to *P. multocida* has already been mentioned. Around 20 per cent of *P. multocida* strains obtained from fowl cholera outbreaks were found to be serum resistant in duck sera (Muhairwa *et al.*, 2002). From the table 5 it is evident that at doses up to 3 x 10^5 CFU and lower all the birds survived challenge with virulent DP1 isolate challenge at 21 W age. Hence serum resistance may be one of the possible reasons for resistance of 21 week aged Kuttanad ducks to isolate DP1. Devi *et al.* (2000) had already reported that pasteurellosis occurred more frequently during monsoon period and affected younger age groups more. This is in accordance with the observation made in the present study.

When 21 week old ducks were inoculated with decreasing doses of DP1 isolate, the MDT had not increased uniformly as in case of 11 week old ducklings. This again is supportive to the assumption that serum resistance developed with age and at the same time was a genetically coded factor and varied widely between individuals within a flock of birds. Also the sample size of the group used to determine MDT was less (six) to nullify this individual variation in serum resistance if present.

5.3 MORPHOLOGICAL STUDIES ON *P. multocida* BIOFILM AND PLANKTONIC CELLS

5.3.1 Light Microscopic Studies

On Gram's staining, planktonic cells appeared to be Gram negative coccobacillary organisms under oil immersion objective of the microscope. Biofilm cells were pleomorphic and showed cocci, coccobacilli, bacilli, filamentous and pear shaped cells (fig.7). Pleomorphism of cells was the most important morphological change during biofilm formation in this study. The loosely bound biofilm component contained Gram-negative filaments. Eighmy *et al.* (1983) carried light microscopic studies on biofilm formations and they observed similar type of Gram-negative filaments in biofilm formations.

A lot of genes were significantly up regulated in biofilm mode of growth and these included those encoding binding proteins, proteins involved in the synthesis of murein and glucosaminoglycan polysaccharide, intercellular adhesin and other enzymes involved in cell envelope synthesis and function (Genetik and Morgenstelle, 2005). This ultimately result in production of excess amount of exopolysaccharides and these polysaccharides interact with a wide range of other molecular species, including lectins, proteins and lipids, thereby forming various types of tertiary structures within a biofilm. In these tertiary structures, cells and cell products were also trapped (Sutherland, 2001). In brief, as opined by Costerton *et al.* (1995) during biofilm formation there would be formation of extra cellular matrix in which the bacterial cells were trapped and this was well demonstrated by Gram staining in which they appeared as faintly stained Gram negative networks or filaments. These faintly stained Gram negative networks were evidences for extracellular matrix of *Pasteurella* biofilm.

The resultant production of excess polysaccharides might also lead to deviation of shape of *P. multocida* from short rods to other forms, resulting in pleomorphism. Rimler and Rhoades (1989) commented that growth under unfavorable condition could induce pleomorphism in *P. multocida*. The conditions

provided for biofilm production in this study were quite unfavorable as nutritive conditions were oligotrophic and temperature was 42^0C and this resulted in pleomorphism. The reason for this irregularity in shape of cells might be attributed to irregularity in polysaccharide production which was well explained earlier.

5.3.2 Scanning Electron Microscopic Studies

Review of available literature indicated that prior published work related to these subjects was limited.

Scanning electron microscopy provides solid evidence for biofilm formation by *P. multocida* under oligotrophic conditions. Olson *et al.* (2002) commented that *P. multocida* required special growth conditions, such as the addition of fetal bovine serum, incubation under 10 per cent CO_2, and a longer culture time to form a biofilm on CBD. However, in the present study it was observed that *P. multocida* did not require special conditions or supplements and rather could form biofilm effectively under oligotrophic conditions in the presence of an attachment medium like chitin.

Scanning electron microscopic studies at lower magnifications were in concurrence with light microscopic studies while at higher magnifications extra morphological details about biofilm were revealed.

At 1500 x and 2500 x biofilm cells shows coral reef like structures with three dimensional formations. Such a description of biofilms was in concordance with the Dunne (2002), who reviewed that at a microscopic level biofilms might look like an underwater coral reef with pyramid or mushroom shaped projections extending away from the surface and channels and caverns running throughout.

The biofilm cells arranged in an irregular fashion and many micro colonies embedded in extra cellular matrix formed were the reasons for this coral reef like cell appearance. Planktonic cells appeared to be a confluent layer of cells. In this case

micro colony formation was absent and hence cells seemed to be arranged almost regularly.

In scanning electron micrograph of biofilm smear the bacteria appeared to be more clumped rather than being a confluent monolayer of cells and in planktonic cell smear the bacteria appeared to be a confluent monolayer of cells. Such an observation was contradictory to the only available scanning electro micrograph of *P. multocida* biofilm documented by Olson *et al.* (2002). They used CBD with TSB supplemented with serum as media and the *P. multocida* biofilm obtained appeared as a confluent layer of cells. Studies on *Serretia marcescens* biofilms by Rice *et al.*, (2005) indicated that the structure of biofilm depended on the nutrient availability and under high nutrient conditions a flat, undifferentiated biofilm was formed and under low nutrient conditions a classic differentiated biofilm was formed. Thus it might be deduced that the nutrient rich media used by Olson *et al.* (2002) might have resulted in flat undifferentiated biofilm which appeared as confluent layer of cells while the nutrient limited conditions used in the study resulted in production of well differentiated classic biofilm by *P. multocida* which appeared as coral reef like structures on SEM, as reviewed by Dunne (2002). This type of clumped nature of biofilm and micro colony formation was well described in other species (Eighmy *et al.*1983). This occurred due to extracellular matrix formation by the bacteria under nutrient limited conditions (Costerton *et al.*, 1995 ; Sutherland, 2001 and Olson *et al.* 2002). Thus in brief, just like *Serratia marcescens, P. multocida* can form well differentiated classic biofilm under nutrient limited conditions.

At 5000 x and 8000 x biofilm cells showed finger like filamentous formations, cells lacked uniformity in shape and size, and pleomorphism of cells was evident. Planktonic cells were almost uniform, oval or coccobacillary in shape and filamentous formations were totally absent. This type of pleomorphism among

biofilm cells was also observed in light microscopy after Gram's staining as described above.

At 15000 x the finger like outgrowth of biofilm cells in different directions resulting in formation of three dimensional tertiary structures was evident. The length of these structures varied while the diameter was about 0.5 µm in most of the cases. In case of planktonic cells at 15000 x the tertiary structures were absent and coccobacillary cells having a width of 0.2 µ m and length of 0.6 µm were detected. According to Rimler and Rhoades (1989) the *Pasteurella* cells usually measure 0.2-0.4 X 0.6-2.5 µm. Thus the size of planktonic cells observed in scanning electron microscopy lay within the range described by Rimler and Rhoades (1989).

In the present study the media used for biofilm production was 0.32 per cent TSB which was one tenth of the concentration of TSB used for production of planktonic cells. Nutrient limitation was also attributed to cause enhancement of capsule (Zaragoza and Casadevall, 2004). Rezende *et al.* (2005) were of the opinion that the capsular polysaccharides played an important role in the formation of biofilms. Also TSB was recommended as the medium of growth for inducing slime production in case of *Staphylococcus epidermidis* by Christensen *et al.* (1982) when other media like brain heart infusion broth were not able to support attachment of bacteria consistently.

Nalin *et al.* (1979) suggested chitin as a good substrate for adherence and multiplication of the bacteria. Colwell and Spira (1992) observed that in providing a surface of attachment for *Vibrio cholerae* biofilm, chitin was distinct from the others substrates like silicates and cellulose as many *Vibrio* species were able to use it as a sole carbon and nitrogen source.

Hence the 0.32 per cent TSB media supplemented with 0.5 per cent chitin used in this study for biofilm production seemed to be excellent medium which promoted biofilm formation by *P. multocida.*

5.4. PREPARATION OF VACCINES AND STERLITY AND SAFETY TESTING

As all the four vaccines prepared were found to be sterile and in safety testing, the vaccines did not elicit any untoward effects, except for a transient lameness which subsided after one day post inoculation. Hence it might be assumed that the vaccines prepared were safe and ready to use.

The newly composed oil adjuvanted vaccines consisting of 12 parts of antigen, seven parts of liquid paraffin and one part of lanolin was found to be homogenous even after keeping for a month at 5 $^{\circ}$ C when compared to older vaccines consisting of 15 parts of antigen, nine parts of liquid paraffin and one part of lanolin. Hence, this modification appeared to be better in terms of vaccine stability.

5.5 ASSESSMENT OF IMMUNE RESPONSE TO VACCINATION BY INDIRECT HAEMAGGLUTINATION TEST

5.5.1 Vaccination and Schedule of Serum Collection

Birds were vaccinated with 0.5 ml of each vaccine by intra muscular route. Leonchuk and Tsimokh (1977) reported that vaccination against *Pasteurella* by intra muscular route gave stronger and long lasting immunity than sub cutaneous route.

A total of 70 birds were maintained in each group and this helped to reduce the standard error due to difference in immune response among individual birds during statistical analysis. The serum was collected on days 0,7,14,21,35,49,70,77,84 and 133 PPV. The serum collected on day 0 helped to assess the basal level immune response. From day 7 to day 70 there were six collections which helped to evaluate the primary humoral immune response of the

bird to antigen given in different adjuvanted modes.

On 70 th day of primary vaccination a booster dose was given with the same antigen. Serum collected on day 77, 84 and 133 gave the trend of secondary humoral immune response. Primary immune response of ducks to *P. multocida* (DP 1) vaccine had been evaluated (Rajagopal, 2007; Ranjini, 2007; Indu 2008). Both primary and secondary immune response of chicken to *P. multocida* (CU strain) vaccine had also been evaluated (Selano *et al.*,1983) . But review of available literature indicated a lack of data on secondary humoral immune response of ducks against oil adjuvanted *P. multocida* bacterin. The data obtained in the present study would help to fill up this gap in scientific knowledge.

5.5.2 Assessment of Antibody Level before Vaccination

The group size was 70 and this helped to reduce error due to individual variation in IHA titre between birds. Although the individual birds varied in IHA titre, the maximum standard error observed was 0.21. Statistical analysis showed that the variation was not significant on day 0 among the different group of birds selected for the study.

In a serological study conducted among 400 waterfowls by Donahue and Olson (1969) although they are able to detect antibodies for *P. multocida,* no organisms were isolated from nasopharynx of any of them. The IHA titers of anti *pasteurella* antibodies in sera of one month old ducklings procured for experimental purpose were reported to be zero (Jayakumar, 1998). But when Akand *et al.* (2004) assessed the IHA titers of anti pasteurella antibodies in sera samples of healthy non vaccinated fayoumi birds, titres were found to be $\leq$ 4.0. Here in this study also the healthy birds procured showed an antibody titre against pasteurellosis.

When ducks were immunized with bacterin prepared from *P. multocida*, the mean titre varied within the range of 8 to 128 (Jayakumar, 1998). Hence it might be deduced that a titre less than 4 suggested that the birds did not receive an

immunostimulatory dose of *P. multocida* organisms, which naturally occured only as a sequelae to infection.

5.5.3. Primary Immune Response of Oil Adjuvanted Vaccine

On day seven OV group differed significantly from control group and SV, indicating that the vaccine evoked a humoral immune response on first week itself.

The mean IHA titre increased from day 0 (2.21), reached a maximum on day 21(4.72) and there after it started decreasing till day 70(3.67). Similar trend was observed by Selano *et al.* (1983) in chicken, although the titre values were lower. They vaccinated birds with oil adjuvanted *P. multocida* bacterin and a maximum titre of 3.8 was observed on day 21 PV which was lower than that observed in this study (4.72).

Homologous challenge test conducted on day 49 PPV, showed an 80 per cent protection among OV group, which was maximum among the four groups of vaccines. The OBV group showed 70 per cent protection, which was also higher that SV and SAV groups. In control group only 10 per cent birds survived. The MIHAT was also found to be highest for the OV group (4.44), followed by OBV (3.32) on day 49 PPV. So protection level was well correlated with mean IHA titre of OV and OBV group during first challenge test. Hence oil adjuvanted vaccines (OV and OBV) offered better protection compared to saponin and aluminium hydroxide adjuvanted vaccine groups (SV and SAV) following primary vaccination, up to seven weeks.

Emulsion adjuvants are depot adjuvants and can stimulate dendritic cells and macrophages which ultimately stimulate immune response (Tizard, 2004). They also induce actin polymerization and macro-pinocytosis, thereby enhancing antigen internalization by antigen presenting cells (Yang and Shen, 2007). The OV and OBV vaccines used in this study were oil in water emulsions and thus the immunostimulatory and inflammatory effects of the oil adjuvants might be the

reason for their better response, compared to saponin and aluminium hydroxide adjuvanted vaccines.

The fig.18 showed that the oil adjuvanted vaccines produced a stable prolonged response compared to saponin and aluminium hydroxide adjuvanted vaccines. The inflammation produced by emulsion adjuvants increases the persistence of antigen at the site of injection; thereby prolonging the immune response (Tizard, 2004). This type of enhanced and sustained antibody formation when the antigen was incorporated into a water-in-oil emulsion prepared with paraffin was also reported by Freund *et al.* (1948). This explains the reason for such a stable response.

The composition of the oil adjuvanted vaccines used in this study consisted of 12 parts of antigen, seven parts of liquid paraffin and one part of lanolin. When compared to vaccine prepared earlier, this was not only found to be homogenous as described earlier, but also more efficient in stimulating a humoral immune response following primary vaccination. Hence, this modification might be recommended for the preparation of oil adjuvanted vaccines.

5.5.4 *Primary Immune Response of Oil Adjuvanted Biofilm Vaccine*

The trend of mean IHA titres of group OBV showed that it increased from day 0 (2.21) and reached a maximum on day 21(4.42) and there after it started decreasing till day 70 (3.10). The antigen concentration used in all the vaccines was same in terms of CFU. Although the trend of primary humoral immune response of OBV group was almost same as group OV, the values were lower, indicating that OBV was less efficient in stimulating a humoral immune response compared to OV. The IHA titre of OBV was significantly lesser than that of OV on day 14, 35, 49 and 70 PPV. Homologous challenge test conducted at 49 days PV showed a 70 per cent protection among OBV group. Thus in this study it was observed that OBV gave 10 per cent lower protection than OV in homologous challenge and this lies in

correlation with the results of MIHAT which showed that *Pasteurella* biofilms were weak in inducing primary humoral immune response than planktonic cells.

Jensen *et al.* (1990) showed that biofilms induced an oxidative burst response by polymorphonuclear leukocytes, which was slow and was only 25 per cent of the response to planktonic bacteria. The findings of Yasuda *et al.* (1994) also showed that adherent bacteria had increased resistance to killing by phagocytes. As phagocytosis of antigen is an important step in antibody production, the resistance to phagocytosis may also contribute to decreased humoral immune response, hence leading to decreased protection.

In homologous challenge trials conducted by Rajagopal (2007), biofilm vaccine gave 10 per cent higher protection rates than ordinary bacterins, when challenged with 100 LD_{50} doses of *P. multocida.* This is against the results of present study which indicated that biofilm vaccine had 10 per cent lower protection with the same strain of organism. A probe into the methodology followed by Rajagopal (2007) for production of biofilm antigen helps to explain this variation. Rajagopal produced biofilm cells by growing in nutrient limited conditions *i.e.*, in 0.32 per cent TSB and the obtained bacterial cells were sub cultured in nutrient sufficient TSA (3.2 per cent) before use as vaccine antigen. This difference in methodology followed might be the reason for that variation.

Thus biofilms might be considered less immunogenic than planktonic *Pasteurella* cells in primary immune response against pasteurellosis.

Following primary vaccination, on days 7, 14 and 21 both the oil adjuvanted vaccines showed a higher MIHAT than saponin adjuvanted vaccines. Also oil adjuvanted vaccines showed a greater protection against challenge dose than saponin adjuvanted vaccines. Both of these observations showed that oil adjuvanation gave better protection than saponin and aluminium hydroxide adjuvanation. This better response of oil adjuvanted vaccines observed in this study was further supported by

studies conducted by Lalrinliana *et al.* (1988) on efficacy of different adjuvants employed in *P. multocida* vaccines. They found that highest antibody titre was observed with oil adjuvanted vaccines while the alum precipitated and aluminum hydroxide gel vaccines induced low titres.

5.5.5 Saponin Adjuvanted Vaccines

The MIHAT of SV group increased from day 0 (2.21) and on day 7 and 14 it was 2.82 and 3.00 respectively. On day 21 it decreased to 2.32, then increased on day 35 to 3.77, again stared decreasing on day 49 up to day 77. Even though booster vaccination was done on day 70 the MIHAT showed an increase only on day 84 PPV and the increment was maintained up to day 133 (6.85).

The SAV also showed a similar pattern. Mean IHA titre of group SAV increased from day 0 (2.24) to day 14 (3.51) and then on day 21 it decreased to 2.96, then increased on day 35 to 4.32, again decreased on day 49 and 70 PPV. On day 77 PPV, it again increased to 3.20 and the increment was maintained up to day 133 PPV (6.27). In case of SAV although the pattern of change in MIHAT was similar to that of group SV in primary immune response, in secondary immune response the pattern was similar to that of oil adjuvanted vaccines. Also on days 7, 14, 21, 35 and 77 PV SAV group showed a significantly higher MIHAT than SV group and this might be due to the capacity of aluminium hydroxide to potentiate immune response.

The use of quill-A (saponin) improved the immunogenicity of the aluminium adjuvant and thereby helped to avoid the use of oil components (Rurangirwa *et al.*, 1987). Hence saponin combined with aluminium hydroxide when used as adjuvant (SAV) gave better response than when saponin was used alone (SV). Similar results were also obtained by other workers. With saponin and aluminium hydroxide combined vaccine, antibodies peaked at a time, similar to that found when a vaccine containing single adjuvant was administered (Foggie *et al.*, 1971; Buonavoglia *et al.*,

1998). This was also supported by the homologous challenge test conducted on day 49 PV, in which the birds vaccinated with combined vaccine (SAV) secured 10 per cent better resistance to challenge. Thus it could be concluded that the presence of aluminium hydroxide potentiated the immunostimulating ability of saponin. The homologous challenge test also revealed that as in case of IHA titre the protection level of OBV and OV was more than SAV by 10 and 20 per cent respectively. Thus combined vaccine (SAV) although was better than single vaccine (SV), they cannot be used as a substitute to oil adjuvanted vaccines as reported by Rurangirwa *et al.* (1987).

5.5.6 Secondary Immune Response (Booster Vaccination)

Booster vaccination was given on day 70 PPV after blood collection for determination of IHA titre. Following booster vaccination, IHA titre of the different groups of birds was determined on day 77, 84 and 133 PPV (*i.e.* days, 7,14 and 63 PBV).

In case of OV on day 70, just before booster vaccination was given the MIHAT was 3.67 and after booster vaccination the MIHAT again started increasing and on day 133, it reached 6.63. As the MIHAT is the IHA titre expressed in $\log_2$, the increase in antibody titre was 2^3 times or eight fold. Wu *et al.* (1986) compared primary and secondary immune responses in chickens vaccinated with fowl cholera attenuated vaccine prepared from *P. multocida* strain 807 and established the importance of double vaccination in antibody raise. Onet *et al.* (1994) found that increment on second vaccination was at least 3 times. Hence booster vaccination definitely had added advantageous effect on protection and is a must, to prevent losses.

After booster vaccination the oil adjuvanted vaccine groups showed a higher response than saponin adjuvanted vaccines as in case primary vaccination. But among oil adjuvanated vaccines OBV showed a better MIHAT than OV on day 77,

84, and 133 PPV. The MIHAT of the OBV on day 133 (7.0) was the maximum MIHAT recorded in this study. In OBV the antigen used was biofilm cells instead of planktonic cells. As the secondary humoral immune response (following booster dose) was found to be highest for OBV on all the recorded days it could be assumed that *Pasteurella* biofilms, although weak in inducing a primary immune response has the potency to evoke a more powerful secondary response compared to planktonic cells.

An investigation on the effect of booster vaccination by Ling *et al.* (1998) revealed that serum antibody declined three days following booster vaccination. This decline would be probably because of immune elimination.

The surface-attached communities of bacterial cells in a bacterial biofilm are embedded in an extracellular matrix of biopolymeric substances (Costerton *et al.*, 1995).This extracellular slime the glycocalyx, of biofilm forms a physical barrier and resists penetration by larger molecules and provides a degree of protection (Leid *et.al.*, 2002). In OBV as the antigen used consisted of biofilm cells, they might be less available to the antibodies due to the physical barrier. Thus biofilm cells might be less neutralised by existing antibodies compared to planktonic cells. This might have resulted in higher MIHAT for OBV following booster vaccination.

In SV group the antigen was freely available as there was no depot forming agents. Also since the saponin has detergent effect it can easily penetrate the tissue and reach the vascular system, resulting in neutralisation of antigen by antibody produced during primary vaccination. This might be the reason for the decreased titre of SV group on day 77 PPV.

The SAV group produced a constant steady rise in immune response following booster vaccination. The MIHAT showed that this response was lower than that of oil adjuvanted vaccines and at the same time higher than that of SV group. When Aluminium hydroxide gel, was given as vaccine adjuvant, it elicited a

granulomatous inflammatory reaction, which persisted up to eight weeks or longer (Goto *et al.*, 1997). This would help in depot formation and persistence of antigen which in turn might result in an increasing MIHAT of SAV group following booster vaccination when compared to SV.

The booster response of oil adjuvanted vaccines also was significantly higher than that of saponin adjuvanted vaccines like SV and SAV in most of days PV. Also on all days following booster vaccination the oil adjuvanted vaccines showed significant difference from control group. Suli *et al.* (2004) already compared adjuvant activity of oil adjuvanted vaccines and aluminium hydroxide adjuvanted vaccines and found that the oil in water type lipoid adjuvant had 1.8-fold more immunogenic activity compared to aluminium hydroxide in eliciting antibody response to vaccine. Results of present study also support this observation.

As no observations were done after 133 days, no data were available regarding the time of decline of secondary humoral immune response.

5.5.7 Vaccination Schedule

Pasteurella multocida bacterin is usually administered as two doses at 2–4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course (OIE, 2004). In the present study immunization was done at six weeks age and a strong immunity was induced in oil adjuvanted vaccine groups which lasted for 10 weeks (70 days PPV). This response observed was in accordance with Layton (1984) who observed that when ducklings were immunized at six weeks of age, a strong immunity was induced which lasted for eight weeks. He also commented that at younger age they might be immunologically immature and unable to respond to antigenic stimuli, even though it was presented in an adjuvanted form. In the present study, 80 per cent protection was observed on day 49 PPV and hence the booster vaccination was given only at day 70 PPV. After booster vaccination 100 per cent

protection was observed in oil adjuvanted vaccine groups on challenge tests conducted on day 63 PBV.

Hence immunization at six weeks age, followed by booster vaccination at 16 weeks age seemed to be a better modification of existing schedule and might be recommended.

5.6 HISTOPATHOLOGICAL FINDINGS

5.6.1 Histopathology of Spleen

Lymphoid depletion was observed in both OV and OBV vaccine groups while splenic necrosis was observed in OV group that did not survive challenge test. These observations were in agreement with the necrosis of lymphoid follicles of spleen observed in both experimental fowl cholera (Hunter and Wobeser, 1980) and natural fowl cholera outbreaks in ducks (Fujihara *et al.* 1986). The spleen of all vaccinated birds that survived infection following challenge was found to be normal, with predominance of white pulp and absence of necrotic changes indicating that in them the vaccines were effective in preventing pasteurellosis.

Periarteriolar lymphoid hyperplasia was observed in survived control birds (non vaccinated birds) and in SV and SAV vaccine groups that did not survive challenge test. Other workers also reported lymphoid hyperplasia in pasteurellosis in different species. Morishita *et al.* (1996) observed splenic lymphoid hyperplasia and plasmacytosis in *P. multocida* infections in psittacines, Miguel *et al.*, (1998) reported the same lesion in sub acute to chronic fowl cholera in quails. Shilpa *et al.* (2005) observed lymphoid hyperplasia in experimental pasteurellosis in layers. The same observation in survived control birds (non vaccinated birds) and in SV and SAV vaccine groups that did not survive challenge test, point out that the lesion occurred due to *Pasteurella* infection in them. Also the MHIAT was low in them compared to other groups to offer protection against challenge dose. Thus lymphoid hyperplasia

observed in survived control birds (non vaccinated birds) and in SV and SAV vaccine groups that did not survive challenge test, indicated that persistence of *Pasteurella* organisms through mild infection might have occurred in them following experimental challenge. This in turn lies in concurrence with the observation made by Hunter and Wobeser (1980).

5.6.2 Histopathology of Liver

Diffuse hepatic necrosis was the main lesion observed in birds challenged with *P. multocida*. Inflammatory cell infiltration was also observed in most of the cases. These observations lie in alignment with the opinion of Rimler and Rhoades (1989) that in acute fowl cholera the commonly observed lesions were multiple small areas of coagulative necrosis and heterophil infiltration. Bile duct hyperplasia was observed in bird vaccinated with OBV that survived challenge. Hegde *et al.* (1996) also observed similar lesions in ducks in pasteurellosis in liver.

5.6.3 Histopathology of Caecal Tonsil

Lymphoid depletion was observed in all birds died during challenge test and in control bird that survived challenge test. Review of available literature shows that the data available on lesions of caecal tonsil during pasteurellosis are scanty. But similar lymphoid depletion had been reported in spleen during pasteurellosis in ducks. (Hunter and Wobeser, 1980; Nakamine *et al.*, 1992 and Fujihara *et al.* 1986). The present work indicated that during pasteurellosis lymphoid depletion occured in caecal tonsil also as in spleen. As the vaccinated birds that survived showed normal intact caecal tonsil, the course of disease and lesions might be less prominent in vaccinated birds during infection process.

5.6.4 Histopathology of Bursa

Bursa was found to be normal and well developed in OV and OBV group that survived challenge infection while in all other cases varying degree of lymphoid depletion were observed. As bursa was the organ of antibody production the well developed bursa indicated that the humoral immune response was well induced in OV and OBV birds that survived, compared to other groups. The MIHAT of OV and OBV groups observed in this study were significantly higher than other groups and this supported the above statement.

5.7 RECOMBINANT GHOST SYSTEM

Analysis of gene map showed that the gene *E* was located from 568 bp to 843 bp and gene *D* was located between bp 390 and 848, *i.e.* gene *E* was seen to be completely embedded in the reading frame of gene *D*. As primer was designed by including the shine dalgarno sequence it amplified the DNA of PhiX174 from 517 bp to 916 bp, thereby including certain regions of reading frame of gene *D*. Expression studies of genes *D* and *E* in *Escherichia coli* minicelis and lysis times obtained in the presence or absence of gene *D* translation showed that unlike other overlapping gene pairs, gene *E* expression was independent from the upstream translation of gene *D* (Blasi *et. al.*, 1990).

As the primers E1 and E2 amplified the gene E this pair of primers could be used for the production of amplified Gene *E* sequences for further studies.

Summary

6. SUMMARY

Poultry industry occupies a prominent position in agricultural sector all over the world. Among the domesticated avian species, ducks occupy the second position in India, with a population of about 24 million comprising 10 per cent of its poultry population. Pasteurellosis caused by *Pasteurella multocida* is a septicaemic disease with high morbidity and mortality thereby causing a serious threat to duck farming.

The present day vaccines give a protection only for a short duration of time and the immunity often wanes resulting in disease outbreaks. Thus booster vaccination becomes a requirement to produce a solid long lasting immunity. *Pasteurella multocida* biofilm was recommended as candidate antigen for biofilm vaccines against fowl cholera.

Hence this study was undertaken to develop biofilm vaccines against duck pasteurellosis using oil, saponin and aluminium hydroxide as adjuvants and to experimentally evaluate their immunogenicity in ducks.

A total of 440 unvaccinated one month old ducklings were procured and out of these 90 birds were utilised for determination of LD_{50} and rest 350 birds were used for vaccine trials.

Identity of *P. multocida* serotype A: 1 (DP1) used for study was confirmed by biochemical tests as and biotyping showed that the strain used was *P. multocida.* subsp. *multocida* . Further confirmation of the isolate was done by PM-PCR.

The pathogenicity of the isolate was established in Swiss albino mice before vaccine production. The $LD_{50\ (11\ w)}$ of the DP1 isolate was 10 CFU / bird and MDT was 23.75 h in 11 week old ducklings when a high dose of 3 X 10^{6} CFU of *P. multocida* was given per bird. At 11 Weeks age MDT gradually increased as the dose

of inoculum decreased. In 21 week old ducks, the $LD_{50\ (21w)}$ of the isolate DP1 was estimated to be 3×10^8 CFU of *P. multocida* and it indicated that the Kuttanad ducks had decreased susceptibility to pasteurellosis with age. The reason for this was attributed to the already reported serum resistance among birds which might have developed with age in kuttanad ducks.

On light microscopic studies, planktonic cells appeared to be Gram negative coccobacillary organisms under oil immersion objective of the microscope, while biofilm cells were Gram negative and pleomorphic with faintly stained networks due to formation of extra cellular matrix.

Electron microscopic studies revealed that *P. multocida* did not require special conditions or supplements and rather could form well differentiated classic biofilm effectively under nutrient limited conditions. Also the 0.32 per cent TSB media supplemented 0.5 per cent chitin seemed to be an excellent medium that support biofilm formation by *P. multocida*. The length of biofilm structures varied while the diameter was about 0.5 µm in most of the cases.

For vaccine trials, a total of 70 birds were maintained in each vaccine and control groups and this helped to reduce the standard error due to difference in immune response among individual birds during statistical analysis. The serum was collected on days 0, 7, 14, 21, 35, 49, 70, 77, 84 and 133 PPV.

In this study the healthy birds secured showed an antibody titre of around 2.2. As this titre was less than four, it suggested that the birds used did not receive an immunostimulatory dose of *P. multocida* organisms.

Four different vaccines *viz.* OV, OBV, SV and SAV were prepared and all of them were found to be sterile and safe. The oil adjuvanted vaccines (OV and OBV) offered better protection compared to saponin and aluminium hydroxide adjuvanted vaccine groups (SV and SAV) following primary vaccination, up to seven weeks.

The modified oil adjuvanted vaccines used in this study consisted of 12 parts of antigen, 7 parts of liquid paraffin and 1 part of lanolin. When compared to vaccine prepared earlier, this was not only found to be homogenous but also more efficient in stimulating a humoral immune response. Hence this modification may be recommended for the preparation of oil adjuvanted vaccines.

In this study it was observed that OBV gave 10 per cent lower protection than OV in homologous challenge and this lay in correlation with the results of MIHAT which showed that *Pasteurella* biofilms were weak in inducing primary humoral immune response than planktonic cells. Also oil adjuvanted vaccines showed a greater protection against challenge dose than saponin and aluminium hydroxide adjuvanted vaccines. Thus it was inferred that oil adjuvanation gave better protection than saponin and aluminium hydroxide adjuvanation.

The IHA titre and homologous challenge test conducted on day 49 PV indicated that SAV gave better protection than SV. Thus it could be concluded that the presence of aluminium hydroxide potentiated the immunostimulating ability of saponin. The homologous challenge test on day 49 PV also revealed that as in case of MIHAT the protection level of OBV and OV was more than SAV by 10 and 20 per cent respectively. The combined vaccine (SAV) although was better than single vaccine (SV) they cannot be used as a substitute to oil adjuvanted vaccines.

Booster vaccination was given on day 70 PPV and following booster vaccination, MIHAT of the different groups of birds was determined on day 77, 84 and 133 PPV (*i.e.* days 7, 14 and 63 PBV).

Following booster vaccination there was eight fold increase in MIHAT of oil adjuvanted vaccines. Fourth homologous challenge experiment conducted 63 days after booster revealed a better protection than that in first homologous challenge test conducted before booster vaccination. Hence booster vaccination definitely had

added advantageous effect on protection and is a must, to prevent losses. As the secondary humoral immune response (following booster dose) was found to be highest for OBV on all the recorded days it could be assumed that *Pasteurella* biofilms although weak in inducing a primary immune response had the potency to evoke a more powerful secondary response, compared to planktonic cells. As no observations were done after 133 days PV no data were available regarding the time of decline of secondary humoral immune response.

In the present study, immunization was done at six weeks age and a strong immunity was induced in oil adjuvanted vaccine groups, which lasted for 10 weeks (70 days PPV). As 80 per cent protection was observed on day 49 PPV the booster vaccination was given only at day 70 PPV. After booster vaccination 100 per cent protection was observed in oil adjuvanted vaccine groups even on challenge tests conducted on day 63 PBV. Hence immunization at six weeks age followed by booster vaccination at 16 weeks age seemed to be a better modification of existing schedule and may be recommended.

Following first challenge test postmortem examination of both sacrificed live birds and dead birds was conducted. Sections of spleen, liver, caecal tonsil and bursa of Fabricius were taken for histopathological studies and the microscopic lesions were noted.

Lymphoid hyperplasia observed in spleen in survived control birds (non vaccinated birds) and in SV and SAV vaccine groups that did not survive challenge test, indicated the persistence of *Pasteurella* organisms through mild infection in them following experimental challenge.

In this study following *Pasteurella* challenge, lymphoid depletion was observed in caecal tonsil also as in spleen. As the vaccinated birds that survived showed

normal intact caecal tonsil, the course of disease and lesions might be less prominent in vaccinated birds during infection process.

As bursa is the organ of antibody production the well developed bursa observed in OV and OBV birds that survived challenge test indicated that the humoral immune response was well induced in them, compared to other groups.

As an attempt for production of a recombinant bacterial ghost system, the complete nucleotide sequence of bacteriophage phiX174 was downloaded from gene bank and the location of *gene E* was identified. Based on these data, forward and reverse primers were designed using the software 'Primer 3' for amplification of complete sequence of gene *E*. The primers E1 and E2 amplified the gene E and hence, this pair of primers could be used for the production of amplified Gene *E* sequences for further studies.

In conclusion, 0.32 per cent TSB media supplemented 0.5 per cent chitin seemed to be an excellent medium that support classical biofilm formation by *P. multocida*. Booster vaccination definitely had added advantageous effect on protection. Immunization at 6 weeks of age with OV followed by booster vaccination at 16 weeks age with OBV seemed to be a better modification of existing schedule and may be recommended. The lesions were less prominent in vaccinated birds than control birds during infection process.

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DEVELOPMENT AND EVALUATION OF DIFFERENT VACCINES AGAINST DUCK PASTEURELLOSIS

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ABSTRACT

This study was undertaken to develop biofilm vaccines against duck pasteurellosis using oil, saponin and aluminium hydroxide as adjuvants and to experimentally evaluate their immunogenicity in ducks.

Identity of *P. multocida* serotype A: 1 (DP1) used for study was confirmed by biochemical tests and by PM-PCR and pathogenicity was established in Swiss albino mice before vaccine production.

The $LD_{50\ (11\ w)}$ of the DP1 isolate determined was 10 CFU / bird and MDT was 23.75 h in 11 week old ducklings when a high dose of 3 X 10^6 CFU of *P. multocida* per bird was given. At 11 Weeks age MDT gradually increased as the dose of inoculum decreased. In 21 week old ducks, the $LD_{50\ (21w)}$ of the isolate DP1 was estimated to be 3 $\times 10^8$ CFU of *P. multocida* and it showed that the Kuttanad duck had decreased susceptibility to pasteurellosis with age.

On light microscopic studies planktonic cells appeared to be Gram negative coccobacillary, while biofilm cells were Gram negative and pleomorphic. Electron microscopic studies revealed that *P. multocida* could form well differentiated classic biofilm and 0.32 per cent TSB media supplemented 0.5 per cent chitin seemed to be excellent medium for biofilm formation by *P. multocida*.

Four different vaccines *viz.* OV, OBV, SV and SAV were prepared and all of them were found to be sterile and safe. The oil adjuvanted vaccines (OV and OBV) offered better protection compared to saponin and aluminium hydroxide adjuvanted vaccine groups (SV and SAV) following primary vaccination, up to seven weeks. The modified oil adjuvanted vaccine prepared was not only found to be homogenous, but also more efficient in stimulating a humoral immune response and hence may be recommended.

The oil adjuvanation gave better protection than saponin and aluminium hydroxide adjuvanation. The SAV gave better protection than SV which might be due to the presence of aluminium hydroxide which potentiated the immunostimulating ability of saponin. The combined vaccine (SAV) although was found to be better than single vaccine (SV) they cannot be used as a substitute to oil adjuvanted vaccines. The booster vaccination was found to have added advantageous effect on protection and is a must, to prevent losses. *Pasteurella* biofilms although found to be weak in inducing a primary immune response had the potency to evoke a more powerful secondary response compared to planktonic cells. Vaccination done at six weeks age followed by booster vaccination at 16 weeks age seemed to be a better modification of existing schedule and may be recommended.

In histopathological studies, lymphoid hyperplasia was observed in spleen in survived control birds and in SV and SAV vaccine groups that did not survive challenge test, which indicated the persistence of *Pasteurella* organisms through mild infection in them following experimental challenge. Lymphoid depletion was observed in caecal tonsil in experimental pasteurellosis as in spleen. As the survived vaccinated birds following challenge test showed normal intact caecal tonsil, the course of disease and lesions might be less prominent in vaccinated birds during infection process. The well developed bursa observed in OV and OBV birds that survived challenge test indicated that the humoral immune response was well induced in them compared to other groups.

The designed primers E1 and E2 amplified the gene E and hence, this pair of primers could be used for the production of amplified Gene *E* sequences for further studies on recombinant ghost system.

In conclusion, 0.32 per cent TSB media supplemented 0.5 per cent chitin seemed to be an excellent medium that support classical biofilm formation by *P. multocida.* Booster vaccination definitely had added advantageous effect on protection. Immunization at 6 weeks of age with OV followed by booster vaccination at 16 weeks age with OBV seemed to be a better modification of existing schedule and may be recommended. In histopathological studies, the lesions were less prominent in vaccinated birds than control birds which indicated that the vaccines were effective.