PRODUCTION AND EVALUATION OF VACCINES EMPLOYING *Pasteurella multocida* A:1 GROWN UNDER DIFFERENT GROWTH CONDITIONS

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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 **"PRODUCTION AND EVALUATION OF VACCINES EMPLOYING *Pasteurella multocida* A: 1 GROWN UNDER DIFFERENT GROWTH CONDITIONS"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"PRODUCTION AND EVALUATION OF VACCINES EMPLOYING *Pasteurella multocida* A: 1 GROWN UNDER DIFFERENT GROWTH CONDITIONS"** is a record of research work done independently by **Raja Gopal, R.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Introduction

INTRODUCTION

Ducks, the hardy anatids inhabit both fresh and sea water, exploring a variety of food sources such as grasses, aquatic flora and fauna and insects. They are farmed for their meat, eggs and feathers, besides being useful for aviculturists and for research. Duck farming is an effective tool for the socio-economic upliftment of rural masses as this imposes very less burden upon farmers and in turn provides excellent economy. Duck constitutes about 10 per cent of the total poultry population in India, occupying second place to chicken in the production of table eggs.

A long coastline and extensive water shed areas in several parts of our state offer excellent natural habitat for ducks. Kerala has a sizeable population of ducks, around 11.87 lakhs (Anon., 1996), mostly reared on a large scale in Alappuzha, Thrissur, Kottayam and Pathanamthitta districts.

Very few diseases actually plague the duck population, such as duck plague, duck hepatitis and duck pasteurellosis. Among the diseases, duck pasteurellosis caused by *Pasteurella multocida* (*P. multocida*), with its alarming mortality and morbidity have the potential to become a major bottle neck in the burgeoning of the industry. Adult ducks show some resistance to the disease but ducklings are very vulnerable and a protective umbrella at the early stages of their life is very essential. During the year 2001-2002 there were disease outbreaks in ducks in different parts of the State, which is still continuing. Systematic vaccination was not done for duck pasteurellosis in the State.

Ducks demand very less in terms of feed and maintenance costs and the only recurring expenditure troubling the farmers will be of that during an epidemic disease outbreak. So by curbing any such outbreaks, we could make duck rearing more profitable.

The rapid onset and spread of the disease makes medication practically impossible and ineffective. Rampant use of once effective antibiotics like sulpha drugs has led to the acquisition of drug resistance by the bacteria and the existence of chronic form of the disease adds to the problems of chemotherapy. Thus, curbing the disease by prophylactic immunization turns out to be the exclusive alternative to save the birds from this malady.

Several vaccines have been tried against pasteurellosis, like various antigenic extracts, whole cell inactivated bacterins, modified live organisms and subunit vaccines. Live vaccines are the most effective ones, with homologous and heterologous protection to their credit, but with possible reversion and dissemination of infection to vaccinates. Subunit vaccines are found to be effective, though not always. Subunit vaccines employing outer membrane proteins were proved to induce some amount of cross protection as well. With the advent of proteomics and genomics, it is becoming possible to fathom the complex mechanisms underlying the host-pathogen interaction and to bring into light better and more effective vaccine candidates. But in a developing country like India such ventures are still much farther to reach. We are still resorting to our good old inactivated bacterins, but unfortunately several researchers have questioned their efficacy. They did not provide a solid immunity which is lasting, necessitating a booster every time and also are ineffective against heterologous strains or else we have to use a blend of all the local strains as vaccine. It was found that the organisms grown under conditions mimicking *in vivo* confer better immunity than ordinary laboratory grown organisms and it was thus purported that *in vivo* derived cross protection factors might be responsible for conferring a broader immunity.

Biofilms are particularly pertinent in this context. Biofilms are structured community of bacterial cells enshrined in a protective covering of exopolysaccharide. They are increasingly similar to *in vivo* conditions and bacteria in biofilm mode undergo conspicuous changes in their genetic and phenotypic

expression, which also make them obviously different from their planktonic counter parts growing under conventional laboratory conditions. They express many novel proteins, especially outer membrane proteins and heat shock proteins, which may be having immunodominant epitopes. Moreover most of the organisms are shown to form biofilm *in vivo* during a disease process and the antibodies directed against the cell wall constituents of planktonic bacteria were utterly ineffective against the colonization of enmeshed bacteria. Biofilm bacteria are highly heterogeneous and along with its thick outer covering are unusually resistant to antibiotics and phagocytes. Thus to combat such a formidable defense strategy, antibodies should be specifically directed against the surface components of a biofilm itself. Studies with monoclonal antibodies against the bacterial adhesins is another prospect by which we can block the initial step of bacterial adherence and colonization. So biofilm which simulate *in vivo* condition with many novel epitopes can be considered as prospective vaccine candidate.

Bacterial capsule is endowed with multiple functions of *in vivo* adherence, resistance to opsonophagocytosis, complement mediated bactericidal activity and even in dissemination of infection from one host to another by preventing desiccation. The thickness of the capsule is critical in conferring these protective capabilities. *P. multocida* serotype A is heavily capsulated during *in vivo* growth and it adds to the virulence of the organism. Many researchers have insisted on the use of virulent form of bacteria for the preparation of inactivated vaccines, in order to derive better immunity. Though the immunogenic potential of capsular material when used alone is doubtful, capsular material along with other surface components may prove useful and so organisms with enhanced capsule form a valid vaccine candidate.

Hence the present study was conducted with the following objectives.

1. To determine the effect of supplementation of mammalian serum on the amount of capsule produced by *P. multocida* serotype A: 1.
2. To determine the potential of the organism to form biofilm under *in vitro* conditions.
3. Production of oil adjuvant vaccines employing *Pasteurella multocida* serotype A:1 grown under capsule enhancement conditions and biofilm mode.
4. Production of oil adjuvanated ordinary broth bacterin vaccine.
5. Comparing the immuno-potency of these vaccines by measuring the humoral immune response and the protection conferred by each vaccine during homologous challenge with virulent organisms.

Review of Literature

2. REVIEW OF LITERATURE

Pasteurella multocida are Gram negative coccoid or rod shaped bacteria causing several of economically significant diseases in numerous species of hosts *viz.*, cattle, buffaloe, sheep, goat, domestic fowl, turkey, horse and even camel. Of the diseases, the ones worth mentioning are fowl cholera, haemorrhagic septicaemia and porcine atrophic rhinitis.

2.1 NOMENCLATURE

Initially isolates of *Pasteurella* were named according to the animal from which they were isolated, *i.e.*, *P. avicida* or *P. aviseptica* from birds and *P. bovicida* or *P. boviseptica* from bovines. In 1929, it was suggested that all isolates be referred to as *P. septica* (Topley and Wilson, 1929). The name *P. multocida* proposed by Rosenbusch and Merchant (1939), has now been widely accepted and it is listed as the type species of the genus (Mannheim and Carter, 1984). The genus found its place in the family *Pasteurellaceae* under section five (facultatively anaerobic Gram negative bacilli) in the ninth edition of Bergey's Manual of Systematic Bacteriology (Mannheim, 1984).

Rimler and Rhoades (1989) considered *P. multocida* as the only name to represent this heterogeneous species.

The binomial nomenclature *Pasteurella multocida* has been universally accepted as the name of the etiological agent of avian pasteurellosis (Holmes, 1998).

2.2 HISTORY

Mailet (1836) first used the term "fowl cholera" to denote avian pasteurellosis. Toussaint (1879) isolated the bacterium and proved it as the sole causative agent of the disease. Pasteur (1880) isolated the organism and grew it in pure cultures in chicken broth.

Trevisan (1887) proposed the generic name *Pasteurella* in commemoration of the work of Pasteur on these bacteria.

Lignieres (1900) used the term "avian pasteurellosis" for the disease condition.

2.3 PREVALENCE

Fowl cholera (avian pasteurellosis) is a ubiquitously distributed avian disease to which all avian species are susceptible. A report concerning the international distribution of fowl cholera in 1986 indicated outbreaks in Africa, America, Asia, Europe and Oceania (FAO, 1987).

2.3.1 Prevalence in Other Countries

Carter (1955) was of the opinion that type A strains predominated as cause for fowl cholera, after performing serotyping of various strains with haemagglutination test employing type specific serum.

Curtis and Ollerhead (1978) reported seven separate outbreaks of pasteurellosis in broiler chicken in Britain with a mortality of one to three per cent. The acute form of the disease was characterized by sudden death and chronic form by lameness.

Prevalence study performed by Kawamoto *et al.* (1990) in rabbits and their environment revealed that *P. multocida* were isolated mostly from air, followed by surfaces of floor, bottles and cages.

Nakamine *et al.* (1992) reported the first outbreak of fowl cholera in Japan which occurred in a flock of Muscovy ducks wiping out 25 per cent of the birds in the farm.

Fowl cholera was first recognized in Indonesia in 1972 and there had been many subsequent outbreaks in ducks (Mariana and Hirst, 2000).

Prevalence of pasteurellosis in free ranging chicks and ducks had been reported from Tanzania (Muhairwa *et al.*, 2002).

A total of 21 *P. multocida* isolates were obtained from natural outbreaks of fowl cholera in layer flocks in and around Faisalabad and Rawalpindi. The serotype distribution of isolates showed that serotype 1 predominated (47.62 per cent) followed by serotype 3 (28.57 per cent), serotype 4 (14.29 per cent) and serotype 12 (9.52 per cent) (Arshad *et al.*, 2003).

2.3.2 Prevalence in India

In India, the incidence of fowl cholera in ducks has been reported as early as 1947 (Mulbagal *et al.*, 1972).

An outbreak of fowl cholera in six-day-old chicks in an organized poultry farm in Parbhani, Maharashtra, was reported by Kulkarni *et al.* (1988).

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

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

Out of the 71 samples collected from birds suspected for characteristic signs and gross lesions for pasteurellosis in Andhra Pradesh yielded *P. multocida* from a total of 57 specimens, in which 37 were from layer chicks, 19 from grower chicks and one from day old chick (Prasad *et al.*, 1997).

Investigation on 20 duck cholera outbreaks 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.

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

Bhattacharya (2005) reported 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 as early as six weeks of age, where as only 22.22 per cent of the isolates was from adult ducks.

2.4 BIOCHEMICAL CHARACTERIZATION

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

Heddleston (1976) assessed physiologic characters of 1268 cultures of *Pasteurella multocida* and concluded that all the cultures fermented glucose, fructose, sucrose and reduced nitrate. None of the cultures fermented inositol, inulin, rhamnose and salicin, hydrolyzed gelatin or produced urease and haemolysin.

No growth on Mac Conkey's agar, negative for haemolysis on blood agar and urease activity were also included as primary biochemical tests for identification of *P. multocida* by Buxton and Fraser (1977).

Fermentation patterns of dulcitol and sorbitol by *P. multocida* were of taxonomic significance (Mutters *et al.*, 1985). Based on these criteria the taxon *P. multocida* could be divided into three subspecies. The sorbitol and dulcitol positive variety became *Pasteurella multocida* subsp. *gallicida*; those strains negative for both became *Pasteurella multocida* subsp. *septica* and those positive for sorbitol but negative for dulcitol were *Pasteurella multocida* subsp. *multocida*.

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

Pasteurella haemolytica could be readily distinguished from *P. multocida* based on the inability of the former to produce indole, and their ability to ferment maltose, dextrin and after several days inositol (Adlam, 1989).

Bisgaard *et al.* (1991) described the taxonomic relationship of 131 strains of *P. multocida* by an extended phenotypic characterization. De-carboxylation of ornithine, production of acid from mannitol and indole production were used for the subspecies classification.

Mohan *et al.* (1994) were of the opinion that reactions of the organisms with dulcitol and sorbitol should be treated as variable characters of *P. multocida,* rather than to be used to split the taxon into different sub species.

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

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

Christensen *et al.* (1998) could biotype 45 isolates of *P. multocida* from two outbreaks of fowl cholera as *P. multocida* subsp. *multocida.*

Differentiation of *P. multocida* from *Riemerella anatipestifer* is based on indole production, glucose and sucrose utilization and orinithine decarboxylase reaction; while the former is positive for all the three reactions, the latter gives a negative reaction for all three tests (OIE, 2000).

2.5 VIRULENCE OF *P. MULTOCIDA* / PATHOGENICITY

Pasteurella multocida possess a multitude of specific virulence factors like hyaluronic acid capsule, lipopolysaccharides, adhesins, iron regulated outer membrane proteins (IROMPs) and nutrient acquisition genes. This makes the host-bacterium interaction very complex and it depends on the bacterial strain being studied, host model used and many other events occurring *in vivo*, most of which are yet to be elucidated.

Mice could be used as the animal of choice for testing pathogenicity of *P. multocida* and an overwhelming increase in the number of organisms in visceral organs was the cause of death of mice when experimentally inoculated (Collins, 1973).

Krekov (1976) found that as few as 10 organisms produced rapid death when given intravenously and intramuscularly, but infection was more difficult by other routes and five million organisms were required to infect via stomach.

Greater virulence was observed for strains representing serotypes A: 1, A: 3 and A: 4 which were more commonly isolated from fowl cholera, as reported by Rimler (1987).

Carpenter *et al.* (1989) found that the median times to death after inoculation using dilutions of 10^4-10^6 pasteurellae/ml in turkeys ranged from two days to eleven days.

In a virulence study Rhoades and Rimler (1989) demonstrated virulence of some capsular strains of *Pasteurella* which caused 73 per cent mortality in turkeys that received 900 organisms via intra-airsac route.

Morishita *et al.* (1990) associated the relative virulence of *P. multocida* strains to their ability to resist the bactericidal power of serum. Organism with a high

survival value were more virulent as confirmed by intravenous inoculation and mortality rates in turkeys.

Nakamine *et al.* (1992) found that the pathogenicity of the *P. multocida* serotype A isolated from Muscovy ducks was not very high when tested in 90-day-old chickens, although the natural disease among ducks was very severe.

Virulence study conducted by Rhoades *et al.* (1992) in two weeks old turkey poults revealed that intramuscular exposure of more than 20 organisms caused lethal infections in some poults.

According to Matsumoto and Strain (1993) *P. multocida* serotypes 3 and 4 were able to increase their pathogenicity by bird to bird transmission in a short period of time. The encapsulated original isolate revealed a mean infectious dose of more than $10^{8.2}$ CFU which after five passages produced 67 per cent mortality with a 10^2 CFU dose.

Exposing turkey poults to two different strains of *P. multocida*, Rhoades and Rimler (1993) established their difference in virulence. They differed in colonization ability as indicated by difference in isolation rates and ability to multiply *in vivo*, which was indicated by difference in mortality pattern and induction of septicaemia.

Murugkar and Ghosh (1995) tested the pathogenicity of *P. multocida* serotype A: 1 isolated from ducks in pigeon, duck, mice and rabbit by intraperitoneal inoculation. The pigeons and rabbit died in 48 h, ducks in 36 h and mice in 12 h post inoculation (PI).

Resorting different routes for the virulence study of raptorial *P. multocida* isolates in pekin ducks Pehlivanoglu *et al.* (1999) observed that intravenous route caused highest mortality while subcutaneous route caused the lowest mortality rates. They also stated that chronic lesions such as airsacculitis were observed in chicks inoculated with less virulent strains.

Wilkie *et al.* (2000) evaluated the virulence of five strains of *P. multocida* in chicken by intramuscular, intravenous, intratracheal and conjunctival routes. The relative virulence of each strain did not change when resorted to different routes.

Subcutaneous inoculation of 0.2 millilitre of 18 h broth culture of *P. multocida* to mice caused mortality in 24 h (Anupama *et al.*, 2003).

Capsulated type A strains of *P. multocida* caused 100 per cent mortality in chicken when inoculated intramuscularly and intravenously both at lower and higher doses, whereas low doses of non-capsulated variant strain caused no mortality (Borrathybay *et al.*, 2003).

Arshad *et al.* (2003) examined pathogenicity of *P. multocida* isolates in chicken by inoculating 10^6 organisms intramuscularly and observed 100 per cent mortality among serotype 1 and 3 strains, 80 per cent by serotype 4 and 60 per cent for serotype 12.

Kapoor *et al.* (2004) on assessing pathogenicity of *P. multocida* A: 1 in mice found that the isolates killed the mice in 24 h following subcutaneous injection of an 18 hour broth culture.

Pathogenicity testing of duck cholera isolates in pigeon by inoculating 0.5 ml of 8 hour old broth culture intraperitoneally caused death of the pigeon after 24 h (Bhattacharya, 2005).

Shivachandra *et al.* (2005) studied the pathogenicity of *P. multocida* A: 1 in 12 weeks old chicken and in embryonated eggs. An 18 hour broth culture was inoculated to chicken by parenteral routes and by yolk sac route to embryonated eggs. The minimum bacterial load required to cause mortality in chicken was more than $2.5\times10^{1.66}$ CFU/ml and mortality pattern showed dose dependence. Majority of chicken died between four to six days post inoculation. The minimum number of bacterium in the inoculum to cause mortality of embryonated eggs was more than 25 CFU/egg and mortality pattern showed dose dependence.

2.6 CAPSULE AS A VIRULENCE FACTOR

Bacterial capsule serves many functions which include determining the access of molecules and ions to bacterial cell envelope, promotion of *in vivo* adherence, enhancing *in vivo* survival by resisting opsonophagocytosis, complement mediated bactericidal activity, modulating the functions of B lymphocytes and can even act like cytokines (Cross, 1990). Capsule also is cardinal in dissemination of infection from one host to another by preventing desiccation and also takes part in formation of biofilm (Moxon and Kroll, 1990). Despite this capsule is not considered as the exclusive virulence factor, but constitutively with other virulence determinants it delivers much for bacteria to overwhelm the host.

Peterson *et al.* (1978) proposed that encapsulation of *S. aureus* strains interfered with phagocytosis by preventing effective opsonization and only in the presence of immune serum obtained one week after immunization with heat killed staphylococci, opsonization was found to be proper.

Using encapsulated strains Maheswaran and Thies (1979) evaluated the effect of encapsulation of *P. multocida* on phagocytosis by bovine neutrophils. The results showed that type A strains resisted both normal and hyper immune serum which was attributable to capsular hyaluronic acid, as hyaluronidase treatment made the bacteria susceptible to phagocytosis.

In vitro analysis of the effect of various fractions of *P. multocida* on bovine poly morpho nuclear (PMN) leukocyte functions indicated that hyaluronic acid of type A capsule was not responsible for inhibition of PMN function, instead the inhibitory factor was a heat stable, saline extractable capsular material which in turn could be associated with hyaluronic acid or cell surface (Ryu *et al.*, 1984).

Woolcock (1985) pointed out that combination of particular capsular antigen and the particular 'O' antigen restricted antibody independent classical complement

pathway activation by shielding deeper structures in the cell wall that were capable of activating the complement pathway.

Hansen and Hirsh (1989) concluded that the type A capsule of *P. multocida* shielded its outer membrane by preventing a stable interaction between the membrane attack complex and the outer membrane, thereby making it resistant to bactericidal action of turkey serum. Removal of capsule made the strain susceptible to complement activity.

Role of capsule in the pathogenesis of *P. multocida* was elucidated by Tsuji and Matsumoto (1989) by intravenous inoculation of an encapsulated form of the bacteria, enzymatically decapsulated form and a non-capsulated mutant into turkeys. They found that encapsulation was seemingly important for the survival of *P. multocida* after being entrapped in the liver and specific immunity appeared to be essential for the hepatic phagocytes for rendering the capsulated organisms inactive.

Chae *et al.* (1990) proved that capsule of *P. haemolytica* need to be considered as the principal virulence factor for they observed that decapsulated organisms were more susceptible to serum agglutination, complement mediated serum killing and phagocytosis by poly morpho nuclear leucocytes.

Studies by Harmon *et al.* (1991) linked encapsulation of *P. multocida* and its virulence as decapsulation made the strains susceptible to phagocytosis by avian heterophils and macrophages and they attributed this to inhibition of opsonization or physical interference of receptor ligand binding between phagocytes and opsonized bacteria.

Jacques *et al.* (1993) found it conflicting that the presence of capsule reduced the adhesion of *P. multocida* to porcine respiratory tract cells, suggesting possible masking of adhesins on bacterial cell surface by capsule, but capsule was proven to be an important virulence factor as encapsulated strains produced more severe lesions and were markedly more virulent by a magnitude of six to seven log units.

Hyaluronic acid capsule conferred resistance to phagocytosis and enhanced group A streptococcal virulence. The capsule mutant studied was highly susceptible to phagocytic killing during opsonophagocytic assays using 10 per cent serum and was moderately sensitive in whole blood (Moses *et al.*, 1997).

Boyce and Adler (2000) assessed the contribution of *P. multocida* capsule to its virulence. They observed that the acapsular mutant studied was significantly impaired in virulence as the 50 per cent infective dose (ID_{50}) on intraperitoneal injection to mice was 10^7 CFU compared to less than 10 CFU of the wild type ones. Acapsular bacteria were readily taken up and cleared by murine macrophages while the wild type was conspicuously resistant to phagocytosis.

Capsulated strains of *P. multocida* were more resilient to phagocytosis and killing by avian peritoneal macrophages (Poermadjaja and Frost, 2000).

Non capsulated *P. multocida* serotype B was found to stimulate protective immunity only at higher doses ($>10^5$) (Boyce and Adler, 2001). They suggested that this dose dependence was due to rapid clearance by host macrophages and that anti-capsule antibodies had only a minor role in conferring immunity against *P. multocida* serotype B. The protection against pasteurellosis could be provided even in the total absence of capsule/capsular polysaccharides suggesting that capsule was not an important protective antigen for *P. multocida* serotype B.

Capsule was shown to be a virulence factor for A serogroup of *P. multocida* in mice and chickens by Chung *et al.* (2001). The isogenic acapsular mutant used was avirulent in mice and chicken and the restoration of virulence on re-introduction of plasmid carrying the intact capsule gene corroborated capsule as an evident virulence factor. Based on serum sensitivity assays they proposed that the major function of capsule might be the protection from complement mediated bactericidal activity.

Among the *P. multocida* strains studied, the proportion of serum resistant strains was higher in outbreak strains than in strains from apparently healthy carriers and hyaluronidase treatment did not alter the serum activity as opined by Muhairwa *et al.* (2002). Further they added that the most severe lesions in experimental chicken were produced by serum resistant strains.

2.7 CAPSULAR STUDIES

2.7.1 Demonstration of Capsule

Acid dyes like acid fuchsin and congo red could be used for demonstration of bacterial capsule, with bacteria appearing in red and background taking blue colour leaving the capsule unstained (Maneval, 1941).

Jasmin (1945) suggested a modified method for demonstration of bacterial capsule using 0.5 to one per cent phenol and crystal violet which stained the organism dark blue to black, leaving the capsule as a distinct halo around the organism in a light blue or purple background.

The presence of capsular material of *P. haemolytica* was found to be age dependant and wide capsules were demonstrated from two to twelve hour broth cultures. Maneval stain correlated well with fluorescent antibody staining (FAT), as it clearly and consistently demonstrated the capsule (Corstvet *et al.*, 1982).

Tsuji and Matsumoto (1989) demonstrated the capsule of *P. multocida* organisms by Maneval staining of whole blood from infected birds.

Thies and Champlin (1989) found capsulated strains of *P. multocida* to be hydrophilic and repeated sub culture reduced encapsulation with a concomitant increase in hydrophobicity. They employed Maneval staining with some modifications for demonstration of the capsule.

By electron microscopical examination after polycationic ferritin labeling to confirm the presence or absence of capsular material of *P. multocida*, Jacques *et al.*

(1993) found a dense layer of ferritin granules of 40-60 nm in capsulated whereas the non-capsulated variants were totally devoid of capsular material.

By using Maneval stain, bacterial capsule of width greater than 150 nm could be demonstrated (Stoderegger and Herndl, 2001).

Watt *et al.* (2003) employed a modified acidic polysaccharide-specific congo red-acid fuchsin staining procedure for demonstration of capsule of *P. multocida* by light microscopy. The staining method had been proven to be as sensitive a method for visualization of capsular polysaccharide as electron microscopic assessments of ferritin labeled *P. multocida* cells.

The capsule thickness of *Mannheimia haemolytica* after Maneval staining and light microscopy was found to be 0.63 ± 0.09 μm (Reeks *et al.*, 2005).

2.7.2 Chemical characterization of crude capsular extract

Dubois *et al.* (1956) developed a simple method for colorimetric determination of simple sugars and polysaccharides with a free reducing group. Phenol sulphuric acid reaction gave a stable colour which was proportional to the sugar content.

Briefman and Yaw (1958) tentatively identified galactose and ribose as the major sugars present in the capsule of *P. multocida* serotype 1 and 3 variants, using ascending paper chromatography of acid hydrolyzed crude polysaccharides.

From various cultural and biochemical studies of *Pasteurella* strains of different hosts, colonial iridescence appeared to be closely linked with capsule for strains from pigs and cattle. The mucoid cultures contained more amount of hyaluronic acid than non-mucoid ones (Smith, 1958).

According to Prince (1969) the production of capsular β-antigen of *P. multocida* (corresponding to polysaccharide) was not affected by different media and also the bovine pleural exudates following infection contained more β antigen.

Chemical analysis of saline and potassium thiocyanate crude extracts had given a stronger positive Mollisch reaction for the latter, indicating its higher carbohydrate content. Proteins were present in both extracts and the saline extract was found to be toxic to mice on intraperitoneal injection (Mukkur and Nilakantan, 1972).

Revappa (1974) found that glucose and mannose was also present in the capsule of *P. multocida* in considerable amounts.

Purified crude capsular extracts of *P. multocida* obtained by saline extraction contained 1640 ± 300 µg of protein and 530 ± 240 µg of carbohydrate/ml (Kodama *et al.*1981).

Gentry *et al.* (1982) tried different methods for capsular extraction of *P. haemolytica* and determined the method of choice with minimal cell destruction to be that of incubating a phosphate buffered saline solution (PBSS) suspension of the organism at 41°C for one hour. Chemical characterization revealed that the amount of carbohydrate present was relatively constant (62-78 µg/ml) regardless of the extraction methods used. The protein content varied greatly (0.1-0.79 mg/ml) and the capsular extracts prepared at 56°C contained less amount of protein but viability was comparatively less.

Kodama *et al.* (1982) chemically analyzed the 2.5 per cent saline extracted fractions of *P. multocida* and revealed a protein content of 920 µg/ml and carbohydrate content of 340 µg/ml.

Syuto and Matsumoto (1982) purified the protective antigen of *P. multocida* after saline extraction by chromatographic methods, which revealed a carbohydrate to protein ratio of 1.5 and formed a single precipitin line with rabbit antiserum by gel diffusion and immunoelectrophoresis. They reported only minor shrinkage of the capsular structure and no lysis of the organisms. Sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed three major protein bands and one minor carbohydrate band.

Carter (1984) described that most virulent strains of *P. multocida* produced capsules of varying size which were of carbohydrate in nature and were lost after several subcultures.

Chemical characterization of the capsular extract of type A *P. multocida* grown in brain heart infusion broth supplemented with six per cent bovine serum and yeast extract obtained values of 0.26-0.38 mg/ml, 0.04-0.17 mg/ml, and 0.25 mg/ml for carbohydrate, hyaluronic acid and protein respectively (Ryu *et al.*, 1984).

Pandit and Smith (1993) demonstrated capsular hyaluronic acid of *P. multocida* type: A by sodium chloride gradient chromatography, turbidometric method employing acidified horse serum and cross streaking with hyaluronidase positive *Staphylococcus aureus*. Serotype A stock culture revealed a capsular hyaluronic acid concentration of 15.51 ± 4.46 µg/ml of crude capsular extract while it was 21.73 µg/ml for fresh strains.

Attached soil bacteria in sand columns had an average exopolysaccharide/protein ratio of 4.6 ± 0.9 and carbohydrates and extracellular polysaccharides both normalized to cell protein were greater than free cells (2.5 and five folds respectively). Carbohydrate assay was performed using phenol sulphuric acid method, after stripping the cell envelopes by sonication (Vandevivere and Kirchman, 1993).

Singh (1997) analyzed the crude capsular extract of *P. multocida* of duck origin. They found that the total carbohydrate content was 1250µg/ml and protein content was 3.8 mg/ml, by employing phenol sulphuric acid method and Bradford's method respectively.

Borrathybay *et al.* (2003) demonstrated a 39 kDa protein in the capsule of *P. multocida* type: A strains which was found to correlate with capsule thickness and

pathogenicity for chickens. Amounts of total proteins of crude capsular extract from capsulated strains were approximately twice from that of non-capsulated strains.

2.7.3 Capsule enhancement

Capsule helps the bacteria to evade many of the host defense mechanisms and the thickness of the capsule invariably influences these protective capabilities. The conditions employed for enhancement of capsule *in vitro* are reviewed below.

Vermeulen *et al.* (1988) observed that the more enriched the medium of growth, the more capsular polysaccharide *E. coli* would produce and log phase of growth consistently produced nearly five to ten times more capsule than cells harvested in stationary phase.

Gottschalk *et al.* (1993) recommended the use of three subcultures in non-supplemented broth rather than broth supplemented with serum for producing *Streptococcus suis* capsular antigens and also found that use of serum supplemented broth did not pose any advantage as it failed to induce higher antibody production in rabbits.

To maximize capsule production of *P. multocida* Boyce and Adler (2000) used dextrose starch agar containing six per cent avian serum.

For pneumococcal isolates, microaerophilic condition induced a significant increase in capsular polysaccharide expression when compared to atmospheric growth conditions, though maximal effect was observed under anaerobiasis (Weiser *et al.*, 2001)

Using sera of human and different animal origin, Zaragoza *et al.* (2003) studied the capsule induction in *Cryptococcus neoformans* (*C. neoformans*). Serum induced capsule growth at concentrations below five per cent was very small. No significant difference was found in capsule induction when sera from different species were used but rat and human sera proved less effective. Heat inactivation of

sera had no effect on capsule induction, suggesting no role of complement in induction.

Zaragoza and Casadevall (2004) using many different conditions for *in vitro* modulation of *C. neoformans* capsule observed that stimuli like alkaline conditions, increased serum concentration in the medium, dilution of the medium with basic buffers, increased carbon dioxide tension, nutrient limitation etc. enhanced the capsule size, while increased glucose and nutrients reduced capsule size.

2.8 *IN VIVO* ADHERENCE OF *PASTEURELLA MULTOCIDA*

In vivo adhesion forms the first and the primary step in pathogenesis of any bacterial infection and it is considered as an indicator of bacterial virulence. Several factors are involved in adhesion like capsule, fimbriae and outer membrane proteins. *Pasteurella multocida* is bestowed with several of these factors.

In vivo and *in vitro* studies conducted by Glorioso *et al.* (1982) on adhesion of *P. multocida* using experimental rabbits and HeLa cell lines respectively proposed that fimbriae observed in adhesive strains might be the mediator of adhesion and hyaluronic acid capsule masked these adhesins, thereby reducing the adhesion. Despite this all capsulated strains were found to be adhesive and serotype A strains were more adhesive.

Pruimboom *et al.* (1996) showed that hyaluronic acid was the capsular component that mediated adhesion of *P. multocida* to macrophages. It was noticed that capsulated *P. multocida* were adherent but not internalized. Contrastingly, capsule depolymerisation with hyaluronidase increased adhesion and subsequent internalization of the bacteria by recruited macrophages.

Al-Haddawi *et al.* (2000) observed that *P. multocida* type A: 3 strains attached more to lung tissue than trachea and aorta and opined that the capsular material of type A seemed to influence the adherence.

Pruimboom *et al.* (1999) performed adhesion and adhesion inhibition studies with *P. multocida* serotype A: 3 strains and turkey peripheral blood monocytes (TPBM). The findings suggested that the capsular hyaluronic acid of serogroup A strains of *P. multocida* recognized an isoform of CD_{44} expressed on blood monocytes. They hypothesized that recognition of CD_{44} in cell matrix interactions might be used by *P. multocida* to invade the host tissues.

Comparison of capsulated and non-capsulated strains of *P. multocida* adhesion onto chicken embryo fibroblast cells was done by Borrathybay *et al.* (2003).They found that adhesive property was enhanced almost three times by the presence of capsule. Depolymerisation of capsular hyaluronic acid enhanced adhesion while combined treatment with hyaluronidase and trypsin significantly reduced adhesion, stressing the role of the 39 kDa capsular protein in adhesion of *P. multocida.*

2.9 BIOFILM AND BIOFILM ASSAY

Bacterial biofilm is defined as "a structural community of bacterial cells enclosed in a self produced polymeric matrix and adherent to an inert or living surface" (Costerton *et al.*, 1999). The new definition of a biofilm is "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton, 2002).

The biofilms show much more resilience than the laboratory adapted strains which have lost their propensity to aggregate. Recently more and more studies are being done to unveil the complex mechanisms underlying the myriad ways of bacterial attachment by employing various *in vitro* models which are being reviewed here.

2.9.1 *In vitro* Biofilm Formation

Heukelekian and Heller (1940) found that when glass beads were present to augment the area of solid surface, *Escherichia coli* grew in either glucose or peptone solution as dilute as 0.5 milligram/litre. They also stressed the importance of aeration along with a surface for bacterial growth in dilute solutions.

Zobell and Grant (1943) proposed that when the concentration of food was very low, solid surfaces such as sand, glass beads or the walls of the culture receptacle which adsorbed food as well as bacteria would promote bacterial multiplication.

Incubation of *Vibrio cholerae* along with chitin particles resulted in adsorption of vibrios onto chitin, making it resistant to acid digestion which could be extrapolated to pathogenesis of vibrios, as adherence to ingested chitin of crustaceans might serve as a protection from gastric acid (Nalin *et al.*, 1979).

Krieg *et al.* (1986) observed that aeration of continuous culture of *Psuedomonas aeruginosa (P. aeruginosa)* at the rate of 0.5 ml/minute caused heavy polysaccharide production and selection of the mucoid phenotype occurred when a mixture of mucoid and non-mucoid revertants were used.

Pseudomonas aeruginosa (P. aeruginosa) formed biofilms on acrylic tiles when grown in a chemostat, with 2×10^8 cells attached per tile, by five days while the population of plankton reached 3×10^9 cells/ml on day one. Both biofilm and planktonic population remained constant throughout the study of seven days (Anwar *et al.*, 1989).

Ward *et al.* (1992) examined the growth of *P. aeruginosa* biofilm on peritoneal implants in rabbits and opined that pre-immunization with formalin killed whole cells of *P. aeruginosa* did not reduce the bacterial growth despite high levels of specific IgG indicating that preimmunization did not protect the host from colonization and chronic infections caused by the bacteria.

According to Vandevivere *et al.* (1993) soil bacteria formed very large clumps comprising 50 to 500 cells in sand columns, at an attached cell density of 2×10^8 cells/g of sand.

Ziebuhr *et al.* (1997) found that pathogenic strains of *Staphylococcus epidermidis* obtained from blood formed biofilms on plastic material, where as saprophytic strains isolated from skin did not.

Curli were morphological structures of major importance for inert surface colonization and biofilm formation in case of an *E. coli* K12 mutant strain (Vidal *et al.*, 1998).

Ammendolia *et al.* (1999) studied slime production by staphylococcal isolates in 96 well microtitre plates and observed a "carbohydrate effect", as addition of glucose at one per cent level increased slime production by *Staphylococcus aureus* strains, suggesting the inducible nature of its gene for slime production.

Ceri *et al.* (1999) described a new model *in vitro* system for biofilm studies - the Calgary Biofilm Device (CBD) which proved very amenable for antibiotic susceptibility testing of biofilms. The device consisted of a two part reaction vessel- the top component formed a lid with 96 pegs which fitted into the wells of a standard 96 well plate. Biofilms formed on the pegs were retrieved by vortexing.

Hydrophilic uncharged surfaces showed the greatest resistance to protein adsorption and bacterial cell attachment. A clear correlation between surface chemistry and biofilm formation was established (Cunliffe *et al.*, 1999).

Watnick and Kolter (2000) compared the complex highly differentiated multicultural community of biofilms to our own "city". They suggested that cell division might be infrequent in biofilms and the energy thus saved would be used for exopolysaccharide production rather than pro-creation.

Salmonella weltevreden (*S. weltevreden*) formed biofilm with a cell density of 3.4×10^7, 1.57×10^6 and 3.0×10^5 CFU/cm^2 on plastic, cement and stainless steel respectively, while another strain *Salmonella* FCM 40 biofilm on plastic, cement and stainless steel were of the order of 1.2×10^7, 4.96×10^6 and 2.23×10^5 CFU/cm^2 respectively, after ten days of incubation in 0.2 per cent TSB with a change of medium every alternate day (Joseph *et al.*, 2001).

Eriksson *et al.* (2002) studied a novel mode of bacterial growth on pyrene crystals in liquid medium by microscopy, isolation of pure cultures and analysis of cloned amplicons from the bacterial ribosomal operon. Single colonized pyrene crystal (approximate dimension of $1.5 \times 0.75 \times 0.35$ mm^3) yielded 10^{11} culturable heterotrophs and 10^5 biofilm propagules. Microbial growth on pyrene crystals was visible to the eye after three months of incubation of the primary enrichment culture at 22°C.

Neisseria subflava formed biofilm on polystyrene Petri plates as demonstrated by Kaplan and Fine (2002) and showed a novel dispersal phenotype of biofilm cells characterized by satellite colonies which grew firmly attached to the dish surface colonies in the area adjacent to each mature colony after 24 h.

Murphy and Kirkham (2002) demonstrated biofilm formation of *Haemophilus influenzae* strains isolated from otitis media and lower respiratory tract infections of humans by microtitre plate assay and using a pilus deficient mutant strain of *Haemophilus influenzae*, they proved the essentiality of pili in biofilm formation, as the mutant showed a three to four fold decrease in biofilm production than wild type organisms.

According to Borucki *et al.* (2003) biofilm formation by *Listeria monocytogenes* correlated with exopolysaccharide (EPS) production, high biofilm forming strains produced a dense, three dimensional structure, whereas the low biofilmer produced a thin patchy film.

The importance of temperature and relative humidity in biofilm formation by soil bacteria was shown by Else *et al.* (2003), using microcosm chambers and heavy metal coupons. They found that at 100 per cent relative humidity and 30°C the plate count values reached upto 4×10^4 CFU per coupon and the values decreased by 18 months of incubation.

Mycobacterium avium, an opportunistic pathogen in humans, formed biofilms on poly vinyl chloride (PVC) microtitre plates, which was found to be strain dependent. The highly virulent clinical isolates formed biofilms readily than the attenuated strains (Carter *et al.*, 2003).

In a study involving a multispecies biofilm formation under flowing and anaerobic conditions, Filoche *et al.* (2004) observed 65 ± 11.8 per cent coverage of the surface in 24 h and though sodium dodecyl suphate (SDS) treatment caused detachment of biofilm, re-establishment was apparent five hours after SDS application.

Jefferson (2004) discussed the reasons for biofilm formation by bacteria as defensive protection from external stressors, colonization to a nutrient rich and hospitable locale, benefits derived from a community life and as a default mode.

Vibrio vulnificus attachment to microtitre plate was enhanced with increased cell surface hydrophobicity, nutrient deprivation and by growth conditions like one per cent sodium chloride, pH 7 or 8 and 30 or 37°C (Joseph and Wright, 2004).

Parizzi *et al.* (2004) conducted adherence studies using *Listeria innocua* and *Staphylococcus aureus* on stainless steel, polypropylene and polycarbonate chips. The highest number of adhered cells for both the bacteria was between 10^5 and 10^6 CFU/cm^2, regardless of the surface studied. They also found plate count method to be more sensitive than epifluorescence microscopy for enumeration of attached cells during early log phase, while the latter proved good when attached cells per microscopic field ranged between 10 and 100.

Wakimoto *et al.* (2004) established biofilm formation by enteroaggregative *Escherichia coli* (EAEC) in polystyrene microtitre plate, when grown in high glucose Dulbecco's Modified Eagle's medium.

The *Staphylococcus epidermidis* clinical isolates attached to hydrophobic glass surfaces with a maximum of 7.4×10^6 cells/cm^2 whereas some strains revealed lower attachment potential with 3.2×10^5 cells/cm^2. It was also reported that substratum hydrophobicity influenced the initial attachment of the strains, but no relationship between bacterial surface hydrophobicity and extent of initial binding to substrates could be derived (Cerca *et al.*, 2005).

Transmission electron microscopic studies on *Salmonella gallinarum* revealed that bentonite clay provided in the growth medium acted as a good inert surface for bacterial attachment and biofilm formation. Bacteria were shown to express EPS and curli while repression of flagella and pili were noted. (Prakash *et al.*, 2005)

Rezende *et al.* (2005) observed better binding to glass and polystyrene surfaces by *S. enterica* serovar *Typhimurium* DT 104 which formed a rugose type colony. Rugose type cells formed biofilms that were 14 µm thick within 45 h.

Serratia marcescens (*S. marcescens*) formed classical biofilm and microcolony under reduced carbon and nitrogen conditions and quorum sensing played an important role in both formation and sloughing of the biofilm (Rice *et al.*, 2005).

Sriramulu *et al.* (2005) devised an artificial sputum medium to mimic growth of *P. aeruginosa* in the cystic fibrosis lung habitat and it was found that organisms grew in tight microcolonies attached to sputum components.

Studies conducted on biofilm formation by *Salmonella enterica* serovar *Enteritidis* PT4 on stainless steel at 20°C for 18 days with tryptone soya broth

(TSB) as growth medium indicated that biofilm developed better (10^7 CFU/cm^2) when the organism was grown in periodically renewed nutrient medium (Giaouris and Nychas, 2006).

2.9.2 Biofilm Formation by *Pasteurella multocida*

Only scanty literature was available to prove the biofilm formation by *P. multocida.* The available ones are quoted here.

Demonstration of biofilm formation by avian *P. multocida* on Calgary biofilm device (CBD) was done by Olson *et al.* (2002). For biofilm formation in TSB, supplementation with two per cent fetal bovine serum (FBS) and 10 per cent carbon dioxide tension was necessitated. By five hours the planktonic cells reached 5.5×10^9 CFU/ml and biofilm cells 1.5×10^5 CFU/peg.

Vadakel (2001) observed biofilm formation by *P. multocida* A: 1 in 0.32 per cent TSB supplemented with bentonite clay at 0.3 per cent level. The population of attached bacteria peaked on day three with a count of 1.5×10^{10} CFU/g of bentonite clay and persisted even after 50 days of incubation. The planktons declined rapidly after peaking on day one, proving the increased *in vitro* persistency of biofilm cells.

Pasteurella multocida formed biofilm under nutrient restricted conditions and produced altered outer membrane proteins, some of which were immunogenic heat shock proteins. *P. multocida* biofilm was suggested as a better candidate for vaccine against fowl pasteurellosis as it could be grown at high temperature (42°C), surface induced and nutrient restricted conditions, which mimicked the natural body environment of birds (Hugar, 2004).

2.9.3 Phenotypic Variation in Biofilm mode

Under biofilm mode of growth morphological variations can occur which may even be heritable to next generation, giving rise to different morphotypes and such a mutant may be a hyper biofilm former or the reverse. Perusal of the available

literature did not reveal any reference on the morphotypes of the *P. multocida* in biofilms.

On examination of human isolates of coagulase negative staphylococci (CNS) Barker *et al.* (1990) observed two phenotypes which produced slime differently under aerobic and anaerobic conditions. They opined that since oxygen was subject to concentration fluctuation in human hosts, these findings could have important implications regarding pathogenicity of the strains.

Rhoades and Rimler (1991) described the different colonial morphology of *P. multocida* isolated from birds with fowl cholera. The colonies obtained were iridescent, sectored with various intensities of iridescence or blue with little or no iridescence. Blue colonies of *Pasteurella* had undergone mutation while sub culturing and produced gray colonies, the cells of which were acapsular, avirulent and were arranged exclusively as chains of individual cells.

Biofilm forming strains of *Staphylococcus epidermidis* produced black colonies on congo red agar while saprophytic strains produced red colonies and these phenotypic variants were genetically similar to the attached strains (Ziebuhr *et al.*, 1997).

Pasteurella multocida grew fairly well on nutrient agar, typically forming circular colonies about 0.5-1 mm diameter after 24 h at 37°C. They were convex, amorphous, greyish yellow and translucent, with a smooth, glistening surface and an entire edge. The consistency was butyrous and easily emulsifiable (Holmes, 1998).

Escherichia coli and *Salmonella enterica* serovar *Typhimurium* produced a colony morphology variant called "rdar" (red dry and rough) phenotype which was characterized by over production of thin aggregative fimbriae called curli and the production of exopolysaccharide cellulose (Romling *et al.* 1998).

Fine *et al.* (1999) opined that fresh clinical isolates of *Actinomyces actinomycetemcomitans* produced rough colonies with characteristic central internal

star and this colony morphology after approximately 65 passages changed to smooth colonies lacking the internal star. The rough colonies formed tenaciously sticky growth on the sides or walls of culture vessels and exhibited long intertwining flexible fimbriae.

Phase variation studies suggested that opacity of *Vibrio parahaemolyticus* colonies correlated well with increased production of exopolysaccharide or capsular polysaccharides when compared to transluscent colony types (Enos-berlarge and Mc Carter, 2000).

Boles *et al.* (2004) reported considerable variation in colony morphology of *Pseudomonas aeruginosa* after two to seven days of growth as biofilms. Planktonic batch cultures grown under same conditions produced no variants. They also found that biofilm mode of growth induced multiple heritable genetic changes attributable to *rec* A gene and the diversity so produced provided an "insurance effect" for bacteria to tide over stressful conditions.

Kiristis *et al.* (2005) isolated small, rough, strongly cohesive colonial variants from *P. aeruginosa* biofilms which hyper-adhered to solid surfaces and shared traits with clinical isolates from cystic fibrosis. Transcriptional analysis of the "sticky" variant indicated an upregulation of type IV pili and exopolysaccharide loci, but this contributed only partially to colony morphology on solid media.

Owing to the intense expression of EPS and curli in the maturation phase of biofilm formation of *Salmonella gallinarum* on bentonite clay, the uranyl acetate stain was unable to penetrate and stain the bacteria. The cells were also observed to have lost their architecture by rounding from their initial rod shape (Prakash *et al.*, 2005).

Capsulated bacteria appearing in the form of chains during very late stationary phase of growth of *Salmonella enterica* serovar *Typhimurium* DT 104,

which comprised approximately five per cent of total population of cells, was reported by Rezende *et al.* (2005).

2.9.4 Varied Antigenic Expression- *In vivo* Derived Antigens

Bacteria during later stages of infection encounter host niches that require them to modify the expression of many genes and organisms grown under iron restriction and host derived ones show conspicuous variation in their antigenic profile, especially in case of outer membrane proteins. Now these putative host derived proteins are essentially considered as cross protective factors by many researchers.

Snipes *et al.* (1988) detected certain unique outer membrane proteins when *P. multocida* were grown in brain heart infusion broth (BHIB) supplemented with iron chelator 2, 2'-dipyridyl which were absent when the same strain was grown in BHIB alone and along with added iron. They suggested that one or more of these proteins might likely be responsible for the cross protection conferred when *in vivo* grown *P. multocida* was used for vaccination.

Truscott and Hirsh (1988) reported a 50 kDa protein localized in the outer membrane of an avian isolate of *P. multocida* that inhibited the *in vitro* phagocytosis of *Candida albicans* by mononuclear phagocytes. This could be extrapolated to pathogenesis of *P. multocida in vivo* as it allowed the multiplication of the organism to levels which could elicit an infection.

Staphylococcus epidermidis growing under *in vivo* conditions expressed many antigens distinct from those when grown *in vitro*, which possibly constituted adhesins or siderophores and that adherence might be a factor which determined the complete antigenicity of *in vivo* grown cells (Mc Dermid *et al.*, 1993).

Love and Hirsh (1994) were able to demonstrate that when grown at 42°C *P. multocida* A: 3 produced unique proteins particularly of 70 kDa and 40 kDa which

were not produced at 32°C. These proteins called as heat shock proteins induced specific antibody in turkeys exposed to *Pasteurella*, implying that some of these proteins were produced *in vivo* at higher body temperature of birds.

Studies using SDS-PAGE demonstrated that detergent insoluble fractions of *in vivo* grown *P. multocida* contained additional protein bands which were absent on *in vitro* growth and chicken antiserum directed against the *in vivo* insoluble fraction recognized the same antigens while two other antisera directed against the whole *in vitro* grown cells did not recognize the antigens on immunobloting (Wang and Glisson, 1994).

Variation in the expression of lipopolysaccharide (LPS) epitope and outer membrane proteins (OMP) under biofilm mode of growth was reported by Murphy and Kirkham (2002) for *Haemophilus influenzae*. The OMPs were conserved for biofilm and they were proved as potential vaccine candidates.

Probing into the physiology of biofilm cells of *Streptococcus mutans*, Welin *et al.* (2004) found that synthesis of almost 25 proteins was enhanced and that of eight proteins was reduced, along with the expression of a novel protein exclusive to the initial stages of biofilm formation.

Arun *et al.* (2005) observed many unique proteins of sizes 50, 46 and 14 kDa in nutrient deprived *P. multocida* and biofilm cells by SDS-PAGE analysis which made them profoundly different from their planktonic counterparts.

When the OMP profile of *P. multocida* grown *in vitro* was compared with that of bacteria recovered from the blood of infected chickens, it was found that only four proteins exhibited differential expression *in vivo* suggesting that most surface components involved in pathogenesis were either expressed constitutively or upregulated only during early stages of infection (Boyce and Adler, 2006).

Filia *et al.* (2006) did protein profiling of *P. multocida* grown under iron deplete and iron replete conditions and the comparison revealed two additional bands of 101 kDa and 104 kDa for iron deplete ones. On probing with homologous convalescent sera raised against *P. multocida* grown under iron deplete conditions, these bands were found to be immunodominant.

Gallaher *et al.* (2006) identified 265 proteins from extracellular matrix samples of *Haemophilus influenzae* biofilms which included a number of uncharacterized proteins as wells as proteins previously implicated in biofilm functions.

2.9.5 Exopolysaccharide (EPS) matrix

Costerton *et al.* (1978) pointed out the advantages of bacterial glycocalyx or the tangled mat of polysaccharide fibres on bacterial surface that they interact and attach to the specific host cell targets, enabling bacteria to resist removal by phagocytes and antibodies and could even function as a food reservoir for bacteria.

It was observed that 63 per cent of clinically implicated strains of *Staphylococcus epidermidis* grew as biofilms coating the polystyrene culture tube when propagated in TSB at 37°C and glucose was essential for biofilm formation (Christensen *et al.*, 1982).

Ziebuhr *et al.* (1997) detected the presence of "*ica*" (intercellular adhesion gene clusters) that mediated production of polysaccharide intercellular adhesin (PIA) in biofilm strains. This PIA was found to be synthesized by those bacterial cells that were organized in large clusters whereas bacteria that remained in suspension exhibited diminished adhesin production.

Brogden and Clarke (1997) reported extensive glycocalyx formation of *P. haemolytica* cells grown in bovine subcutaneous tissue chambers and when incubated *in vitro* in the presence of chamber fluid and heat inactivated ovine /bovine sera. They opined that glycocalyx formation in the presence of serum might be a

result of antibody attaching to capsular polysaccharide and the antibody would stabilize the structural integrity of capsular matrix.

Colanic acid, the exopolysaccharide of *Escherichia coli* biofilm was not required for the initial attachment of the bacteria to abiotic surfaces but was required for establishing the complex three dimensional structure of the biofilm (Danese *et al.*, 2000).

2.9.6 Resistance of biofilm cells

In biofilms, bacteria are subjected to ever changing surroundings and they assume themselves suitable changes to survive in those niches, giving rise to variation in genetic expression and multiple phenotypes. Bacterial plurality as a sequel of phenotypic heterogeneity and genetic flexibility equips bacteria to persist in the presence of the indefatigable host immune system and extraneous stressors.

Psuedomonas aeruginosa grown as biofilms were resistant to Tobramycin concentrations 2500 times greater than minimum inhibitory concentration (MIC) and 20 times greater than minimum bactericidal concentration (Nickel *et al.*, 1985).

Anwar *et al.* (1989) investigated the interaction of *P. aeruginosa* with antibiotics under conditions which mimicked *in vivo* iron limitation and their findings necessitated treatment of biofilm involving infections at the earliest, as older biofilms were practically impossible to get rid of.

Pseudomonas aeruginosa grown as biofilms induced an oxidative burst response by human polymorphonuclear leukocytes which was slow and only 25 per cent of the response to planktons, which could possibly play a role in persistence of biofilm infections (Jensen *et al.*, 1990).

Seven day old biofilms when exposed to antibiotics like Piperacillin and Tobramycin were very resistant to killing by the agents, conforming to the finding

that ageing biofilms made the persistence of *P. aeruginosa* possible in chronic as well as device related infections, despite antimicrobial therapy (Anwar *et al.*, 1992b).

Anwar *et al.* (1992a) observed that older biofilm cells of *S. aureus* exhibited extreme resistance to antibiotics Tobramycin and Cephalexin, which made them to persist inspite of the antibiotic exposure which was extended for an additional six days and even to re-grow on termination of the exposure.

Bacterial biofilms formed on the surface of cotton threads in an artificial medium *in vitro* resembled those grown in subcutaneous tissue pouches in rats in their surface structures and were equally resistant to phagocytic killing by polymorphonuclear leukocytes (Yasuda *et al.*, 1994).

Lewis (2001) reviewed that the increased resistance of biofilms to cidal action of antimicrobials was attributable to restricted penetration due to the diffusion barrier imparted by exopolysaccharide layer, decreased growth rate and expression of possible biofilm specific resistance genes.

Leid *et al.* (2002) demonstrated the penetration of phagocytes into the *Staphylococcus aureus* biofilm under conditions simulating *in vivo*. But the phagocytes were unable to penetrate deeper into the matrix and also to engulf the bacteria present in the core of biofilm, suggesting the presence of mechanisms that inhibited normal leukocyte function besides the physical barrier provided by EPS.

Planktonic and biofilm cells of *P. multocida* had similar antibiotic sensitivity profiles excepting Trimethoprim/Sulfadoxine, which explain why *Pasteurella* would show a positive response to most antibiotics, during infections (Olson *et al.*, 2002).

One to seven day old chemostat grown biofilm cells of *Streptococcus mutans (S. mutans*) were found to be highly resistant to acid killing at pH 3.5 for two hours, while the planktonic and dispersed biofilm cells were very sensitive (Mc Neill and Hamilton, 2003).

Teitzel and Parsek (2003) implicated heavy metal resistance of *Pseudomonas aeruginosa* biofilms to extracellular polymeric substance which enshrine and protect cells from heavy metals like copper, lead and zinc.

Biofilm mode of growth induced multiple heritable genetic changes attributable to *rec* A gene and the diversity so produced provided an "insurance effect" for bacteria to tide over stressful conditions (Boles *et al.*, 2004).

2.10 ENCAPSULATION AND BIOFILM FORMATION

No published report on the relationship between encapsulation and biofilm formation of *P. multocida* was available and so literature on other organisms are reviewed.

The presence of capsule in *Escherichia coli* blocked the function of a self recognizing protein antigen and physically interfered with biofilm formation (Schembri *et al.*, 2004).

Fimbriae could promote biofilm formation on abiotic surfaces and this property was blocked by capsular expression reducing surface adhesion by bacteria, suggesting physical interference of the function of type I fimbriae by capsule (Schembri *et al.*, 2005).

Martinez and Casadevall (2005) investigated the role of the capsule in cryptococcal biofilm formation and revealed that biofilm formation was dependant on the presence of polysaccharide capsule and correlated with the ability of capsular polysaccharide to bind to polystyrene solid support.

The findings by Rezende *et al.* (2005) asserted the role of capsular polysaccharides in biofilm formation by *Salmonella enterica* serovar *Typhimurium* DT 104 as they formed an integral part of EPS matrix of biofilm. They found the cells to be completely surrounded by refractile capsule when stained with India ink.

Davey and Duncan (2006) identified the gene responsible for polysaccharide synthesis in *Porphyromonas gingivalis* and studies using a mutant asserted that the production of capsule prevented attachment and the initiation of *in vitro* biofilm formation on polystyrene plates.

2.11 LETHAL DOSE 50

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

Mukkur (1977) determined the LD_{50} of *P. multocida* of bovine origin in mice as $5.0 \pm 2.1 \times 10^4$ CFU by intranasal inoculation and 5.0 ± 2.8 CFU by intraperitoneal inoculation.

Wijewardana *et al.* (1986) studied the *P. multocida* type B isolates from healthy bovines and found that the isolates were as virulent as those strains associated with outbreaks of haemorrhagic septicaemia, with an LD_{50} of one to six organisms in mice.

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

Ramanatha (1994) determined LD_{50} of *P. multocida* A: 1 in mice and obtained a mean value of 14.32 + 0.083 CFU.

Manoharan *et al.* (1997) performed LD_{50} testing of three strains of *P. multocida* in mice to be 3×10^4, 3×10^3 and 3×10^5 cells.

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

Pasteurella multocida A: 1 isolated from an outbreak in quails was used by Goto *et al.* (2001) to determine the LD_{50} both in quails and mice by intravenous inoculation, which was found to be 4.3×10^4 CFU and 3.9×10^2 CFU respectively.

Soodan *et al.* (2003) inferred that in hypocuprotic mice the LD_{50} of *P. multocida* (P_{52}) strain was 0.5 ml of 10^{-10} dilution of an 18 h broth culture while in control it was found to be 10^{-8} dilution.

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

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

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

During the acute course of pasteurellosis in poultry most of the post mortem lesions were associated with vascular disturbances which included sub epicardial and sub serosal haemorrhages mostly in lungs, abdominal fat and intestinal mucosa. Increased amounts of pericardial and peritoneal fluids were also reported to occur frequently. Liver of the affected birds revealed multiple small focal areas of coagulative necrosis (Rhoades and Rimler, 1991).

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

Necropsy findings in ducks that died after experimental infection with *P. multocida* included enlargement and greenish white discolouration of liver, enlarged

heart and epicardial hemorrhages, enlarged spleen, intestinal haemorrhages and airsacculitis (Pehlivanoglu *et al.*, 1999).

Arshad *et al.* (2003) described following lesions after experimental *Pasteurella* infection in chicken *viz.*, pinhead necrotic foci in liver, pericarditis and active inflammation at the injection sites. In survivors, airsacculitis, sternal bursitis, erosive lesions, petechiation and discolouration of muscle were observed.

Bhattacharya (2005) described necropsy lesions in natural duck cholera infection which included petechial haemorrhages on pericardium, enlarged liver with pinpoint necrotic foci on the surface, severe haemorrhages on the serosal surface of the proventriculus, intestine and inner surface of the abdominal wall.

Challenge infection of vaccinated and control birds revealed gross lesions like Petechiae on heart and thighs, congestion of lungs, liver, spleen and kidneys. Pericarditis and mottling of liver and spleen were also observed (Shilpa *et al.*, 2005).

2.13 VACCINE STUDIES

Several types of vaccines ranging from modified live vaccines to subunit vaccines had been tried for pasteurellosis. Several newer technologies and vaccine candidates like "bacterial ghosts" have been evolved which are being reviewed below.

Carter (1961) insisted the use of virulent capsulated organisms for the preparation of fowl cholera vaccine.

A relatively inexpensive method of evaluation of *P. multocida* vaccines was developed by Ose and Muenster (1968) which involved vaccination of mice and subsequent challenge with $\log_{10}$ dilutions of *P. multocida* and after seven days LD_{50} for each group compared with control. A minimum of two logs protection was required for an immunizing product.

Protection with adjuvant treated *Pasteurella* preparations was always superior to that seen with non-adjuvanated vaccines (Woolcock and Collins, 1976).

Stone *et al.* (1978) studied the influence of the composition of water in oil emulsions on their physical characteristics. In order to prepare vaccine, the 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. Oil emulsion vaccines against New Castle Disease thus prepared had low viscosity, were stable for more than 12 weeks at 37°C and induced a marked immune response in chicken.

Lalrinliana *et al.* (1988) conducted studies to compare efficacy of different adjuvants employed in *P. multocida* vaccines. The highest antibody titre was observed with oil adjuvant vaccines, followed by multiple emulsions. Alum precipitated and aluminum hydroxide gel vaccines induced low titres, whereas sodium alginate adjuvanated vaccine induced intermediate and quicker response.

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

2.13.1 Sub-cellular Components as Vaccine

According to Brown *et al.* (1970) a combination fraction of culture filtrate, cell wall and cytoplasm adjuvanated with Freund's incomplete adjuvant was better than commercial bacterin in turkeys, when challenged by exposure to an experimental epornitic of fowl cholera.

As per Srivastava *et al.* (1970) cell walls of *P. multocida* induced maximum protection (50 per cent) in mice when compared to other components like culture

filtrate and cytoplasmic components. There was lack of correlation between the response and the dose of the material used for immunization.

Ribosomal fraction from *P. multocida* was shown to have intense protective capability in mice and chicken when compared to lipopolysaccharide and other bacterial cell fractions (Baba, 1977).

Mice immunized with potassium thiocyanate extract of *P. haemolytica* serotype 1 were found to resist a challenge infection of *P. multocida* type A, demonstrating the cross protection (Mukkur, 1977).

Results of Rebers *et al.* (1980) indicated that capsular polysaccharide or lipopolysaccharide (LPS) of *P. multocida* were poorly immunogenic in mice and rabbits but induced protective antibodies in chickens, though purified LPS was more immunogenic.

Kodama *et al.* (1981) on immunization trails with crude capsular extract (CCE) showed that young adult turkeys were protected at the rate of 80 per cent or more by cell free CCA while purified antigen induced little protection and suggested that polysaccharide protein complex might be responsible for protection.

Following several treatments done on *P. multocida* cell lysates, Brogden and Rimler (1982) observed that cross protection was not induced by pepsin treated lysate, 50 per cent protection was induced by trypsin treated lysate and 100 per cent cross protection was induced in turkeys vaccinated with lysate heated at 56°C for one hour.

Immunization trials in turkeys revealed that the 50 per cent protective dose of the purified capsular antigen derived from *P. multocida* was between 10 and 50 μg of protein. When two doses were given at 14 days interval, the minimum protective dose was as little as 10 μg of protein (Syuto and Matsumoto, 1982).

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

Humoral protection was induced by ribosome-LPS vaccine against *P. multocida* in chicken as demonstrated by passive mouse protection test (PMT), but CMI was not detected by delayed type hypersensitivity skin test reaction (Rimler and Phillips, 1985).

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

Cell free culture filtrate (CCF) of *P. multocida* was found to be an effective immunogen for protecting turkeys, when administered via the lower respiratory tract (Ficken *et al.*, 1991).

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

Recombinant P six like protein (rP6lp) of *P. multocida* cloned in baculovirus expression system was used as vaccine via subcutaneous route in turkeys and was found that parenterally administered recombinant protein vaccine was incapable of protecting turkeys from avian cholera, as mortality observed was 100 per cent on contact challenging (Kasten *et al.*, 1997).

Sharma *et al.* (1999) compared saline extract and bacterin vaccine against *P. multocida* in mice and goats and saline extract vaccine was found to be superior in terms of protection (83 per cent vs. 66 per cent) and PHA titre.

Chawak *et al.* (2001) reported a protection rate of 66.6 per cent against heterologous challenge in birds vaccinated with OMPs of *P. multocida* grown in iron restricted medium and 33 per cent protection conferred by whole cells grown in iron restricted medium at nine weeks post vaccination. He proposed an additional immunogenic protein of 97.8 kDa expressed under iron restricted condition being responsible for the cross protection.

Kedrak and Borkowska-Opacka (2003) evaluated subunit vaccines comprising IROMPs (Iron Regulated Outer Membrane Proteins) of *P. multocida* serotype A: 1 in calves and found that antibodies against OMPs could be detected as early as seven days, which increased upto 14 days post vaccination.

Marchart *et al.* (2003) produced bacterial ghosts, which were empty cells devoid of cytoplasmic and genomic material from *P. multocida*, by the expression of phage phiX174 lysis gene E. These ghosts when used for immunization of rabbits and mice by subcutaneous route provided dose dependant protection (1.15×10^8 ghosts provided 100 per cent protection) against homologous challenge dose of upto 60 CFU (LD_{90}) and the results projected ghosts as new vaccine candidates.

Ofek *et al.* (2003) in their review discussed about the adhesin based vaccines and stated that whole attenuated or inactivated bacteria that carried adhesin had been employed for inhibiting bacterial adhesion and the anti-adhesin antibodies that were generated functioned in concert with antibodies against other virulence factors to protect the vaccinated host.

Tabatabai and Zehr (2004) identified the 39 kDa cross protective protein of *P. multocida* as *Pasteurella* lipoprotein B or PlpB, which constituted a family of virulence related proteins with an excellent candidature for vaccine experiments.

Wasnik *et al.* (2004) examined an OMP vaccine, whole cells grown with or without iron chelator in rabbits which revealed OMP to be more immunogenic than the other two vaccines while excellent cross protection was conferred by whole cell vaccine employing dipyridyl grown cells.

2.13.2 Live Vaccines

The study by Derieux (1983) to assess the response of broiler chicken to reducing numbers of live avirulent *P. multocida* by stick wing or subcutaneous route revealed the inability of reduced dosages such as 6.1×10^3 CFU/dose in conferring protection. This could be a reason for vaccination failures under field conditions, due to improper handling of vaccine. At higher dosages, protection was observed when challenged even after 80 weeks.

According to Solano *et al.* (1984) the most effective vaccination regimen against *P. multocida* in chicken was inoculation of a killed bacterin which was followed by wing web inoculation of a live Clemson University (CU) strain after five weeks.

Percy *et al.* (1985) tried Streptomycin dependant mutant of *P. multocida* serotype 12:A in rabbits and on intra nasal challenge with virulent homologous and heterologous strains, homologous challenged group showed more rapid nasal clearance, though elevated antibody response was noticed against both homo and heterologous challenge.

Studies done by Schlink and Olson (1986) on turkey breeder toms and hens suggested that the combination of vaccinations most effective in protecting breeders was vaccination in drinking water at seven and 11 week and inoculation into air spaces of the head at 15 weeks.

Schlink and Olson (1987) were successful in inducing high level of protective immunity by vaccination of turkeys with CU strain via alternative routes and stated that the parenteral route of vaccination that offered the most positive results for

immunizing turkeys was the wing web puncture. The wing web route and subcutaneous route showed dose dependency and high degree of immunity was noticed on twice vaccination.

Toth *et al.* (1987) suggested that activation of avian respiratory phagocytes by replicating avirulent *E. coli* could be used in the field at the appropriate time before an anticipated outbreak. They proposed extended use of vaccines to enhance the defense of avian respiratory tract by cellular immunity via nonspecific stimulation of the avian phagocytic system.

Studies by Toth *et al.* (1988) showed that intra tracheal administration of live CU strain increased the number of avian respiratory phagocytes (ARP) and such chickens did not show any sign of respiratory disease, which clearly indicated that activation of phagocytic cells might be a means of defending poultry against airsacculitis and pneumonia.

Confer *et al.* (1989) observed that calves vaccinated with bacterins or carbohydrate- protein subunit developed serum antibodies to capsular carbohydrates and these antibodies provided some opsonizing effect inducing some resistance to pneumonic pasteurellosis, but under conditions of intense pulmonary exposure the organisms might become resistant to opsonization by antibodies to capsular carbohydrates.

Comparing low virulent *P. multocida* mutants with CU strain as vaccine candidates in turkeys resorting to oculo-nasal-oral and drinking water routes of inoculation it was found that the former did not provide as much protection against virulent challenge as the CU strain did by drinking water route, but comparable protection was observed when given by oculo-nasal-oral route (Holfacre *et al.*, 1989).

Results of the study by Confer *et al.* (1996) indicated that vaccination of cattle with live *P. multocida* type A: 3 via aerosol or subcutaneous routes stimulated

antibody responses specific to several outer membrane proteins (OMP), which correlated with resistance to experimental challenge.

Hopkins and Olson (1997) compared an avirulent strain of *P. multocida* (PM-1) as live vaccine with the standard vaccine strain CU by drinking water route of administration. Vaccines containing higher concentration of organisms caused death of turkeys in both cases. Although the vaccine conferred high level of protection it was detrimental to body weight gain.

2.13.3 Inactivated Bacterin Vaccines

Carter (1950) compared the immunity produced in mice by broth bacterins and chicken embryo grown bacterin vaccine made from type I *P. multocida* only to show that the latter was superior.

Dougherty (1953) came to the conclusion that inoculation with chemically killed broth culture bacterins conferred poor protection to ducklings from lethal challenge as only 40 per cent of the ducklings were protected, while an egg embryo vaccine tried under similar conditions gave 83 per cent protection for a period of four weeks.

Iyer and Gopalakrishnan (1955) used mineral oil and lanolin to adjuvanate *P. multocida* bacterin and reported stronger and better immunizing values than alum precipitated vaccines. They also assessed the keeping quality of vaccine stored at 40°F to find that even 165 days storage did not deteriorate the vaccine.

After immunizing bull calves and rabbits with oil adjuvant vaccine Nangia *et al.* (1966) observed that single injection conferred immunity for a maximum of 850 days and vaccine was found immunogenic even after 814 days following storage at 45°F, but only for 20 days at 37°C or 42°C.

Bierer and Derieux (1972) opined that oil based bacterin alone was effective in preventing infection in turkeys but was not as good as avirulent live vaccine and the use of bacterin prior to the use of live vaccine proved more promising.

Heddleston and Rebers (1972) observed that cross protection was not induced in turkeys and mice by bacterins prepared from agar grown culture, but 90 per cent protection was conferred by *P. multocida* bacterins prepared 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 may be a deciding factor in the production of *P. multocida* immunogens.

Fowl cholera bacterins prepared from infected embryonated turkey eggs induced immunity in turkeys against heterologous strain when challenged intramuscularly /drinking water route. The cross immunity induced was host specific as the antigen responsible for cross protection was produced only when *P. multocida* was grown in turkey eggs (Heddleston and Rebers, 1973).

Mittal *et al.* (1977) employing a multiple emulsion vaccine with Tween 80 observed that it was equally immunogenic as that of oil emulsion vaccine in rabbits, as evidenced by direct challenge test and mouse protection test.

Dua and Pandurangarao (1978) observed that very high levels of antibodies were induced by oil adjuvanated bacterin when compared with the live vaccines in turkeys. The humoral immune response peaked two weeks post vaccination (PV) and persisted at appreciable levels upto eight weeks PV. The results also indicated that 100 per cent of the turkeys with a PHA titre of 64 or over were immune to challenge.

According to Harry and Deb (1979) formalinization was the most suitable method of inactivation for *P. anatipestifer* and prescribed the dose of the vaccine as 3×10^9 cells when administered intramuscularly.

Rimler *et al.* (1979) corroborated the finding by Heddleston and Rebers (1972) that the crude liver homogenates and *P. multocida* from heart blood of turkeys that died of fowl cholera were efficacious against both homologous and heterologous challenge while the *in vitro* grown bacterin vaccine induced only homologous protection. They also suggested that a host environment triggered change in phenotypic expression which could make the organism escape the immune mechanism of partially immunized host so that such hosts would succumb to challenge post vaccination.

Layton (1984) subcutaneously immunized two to three weeks aged ducklings with different dilutions of oil adjuvanated *P. multocida* bacterins and all were found to be effective in reducing the mortality caused by challenge. On heterologous challenging survival rates were lesser and lesion scores higher than homologously challenged groups. The immunized group challenged at eight to ten weeks of age showed significant protection but by 18 weeks low levels of immunity was demonstrated.

Layton and Sandhu (1984) prepared *P. anatipestifer* bacterins and two subcutaneous injections of the bacterin protected 70 to 85 per cent of ducklings against experimental challenge whereas it killed 90 to 100 per cent of unimmunized controls. The bacterin could be diluted five times without reduction in protection below 80 per cent, increasing the margin of error while administering the vaccine.

Confer *et al.* (1985) opined that antibody response to vaccination was not affected by pre-existing titres to *P. haemolytica* and live vaccines tried in calves induced consistent antibody titres, which the bacterins failed to do.

Among the variously inactivated vaccine preparations of *P. multocida*, formalin treated antigen was found to be the most immunogenic in mice which even conferred a low degree of cross protection against heterologous challenge (Kim *et al.*, 1986).

Pasteurella haemolytica bacterins adjuvanated with Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA) enhanced resistance to induced pneumonic pasteurellosis whereas alumininum hydroxide adjuvanated bacterins did not. It was observed that serum antibody to capsular polysaccharide (CPS) consistently correlated with protection against *P. haemolytica* challenge exposure whereas antibodies to somatic antigens did not (Confer *et al.*, 1987).

Avakian *et al.* (1989) tested a trivalent and quadrivalent bacterin vaccine of *P. multocida* in turkeys and antibody response was assessed by ELISA. Bacterin vaccines induced a protection of 86 per cent while the live vaccine (CU) compared induced 100 per cent protection. At 72 weeks of age the titres waned and challenge protection considerably diminished.

Vaccination studies was done by Dawkins *et al.* (1991) using *P. multocida* B: 2 strain oil adjuvant vaccine (OAV) and the vaccinal response showed that primary OAV induced a significant increase in protection to challenge with 80 per cent mice showing resistance to challenge with 10^6 viable bacteria and further 10 fold increase in protection was noticed on boostering. Oil adjuvant vaccine conferred 10 fold increased protection than broth bacterin vaccine. Protection lasted for atleast 20 weeks after primary vaccination.

Ireland *et al.* (1991) described a method to prepare the sonicated antigen from *P. multocida* which could be employed for the sensitization of sheep red blood cells while doing PHA for detection of antibodies against the organism.

Onet *et al.* (1994) vaccinated turkeys with different types of *P. multocida* vaccines and found that best protection was conferred by the inactivated 20 per cent oil adjuvanated vaccines.

Ramanatha (1994) assessed the efficacy of a formalin inactivated alum adsorbed (FAV) vaccine containing 50×10^{11} CFU/ml of *P. multocida* in ducks. Birds were vaccinated twice at 24 days interval and direct challenge was done with

homologous strain. The survival rates in twice challenged birds touched 100 per cent and the protection rates correlated well with IHA titres.

Swamy (1994) reported 100 percent survival in twice vaccinated ducks and only 57.1 per cent in once vaccinated ducks after challenging with 100 mouse LD_{50} dose of 1786 viable *P. multocida* organisms, stressing the necessity of a booster vaccination.

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 isolate was found to be highly effective in controlling the outbreak.

Subcutaneously administered ultra violet light killed *P. multocida* bacterin in goats induced protective immunity similar to that induced by virulent live *P. multocida* injected into the lung (Purdy *et al.*, 1997).

Confer *et al.* (1998) used a killed commercial vaccine against *P. haemolytica* in cattle to determine the duration of serum antibody responses after single vaccination and revaccination. Single vaccination stimulated antibodies from day seven to fourteen and then declined until revaccination. Revaccination on days 28 and 140 stimulated anamnestic response and the antibodies remained significantly increased for up to 84 days post re-vaccination.

Immuno potency testing of *P. multocida* A: 1 oil adjuvant vaccine in one month old ducklings gave 100, 100, 100 and 70 per cent homologous protection when challenged at 20, 40, 60 and 80 days PV respectively, with an average survivability percentage of 84.465 (Jayakumar, 1998).

Bacterin-toxoid killed vaccine against *P. haemolytica* proved to be more efficacious than the modified live vaccine employing a streptomycin dependent mutant (Mosier *et al.*, 1998). They reasoned it to be due to the inability of the vaccine strain to multiply *in vivo* and reach a substantial concentration of antigen.

Gupta *et al.* (1999) carried out studies using oil adjuvant whole cell (OAW) vaccine and oil adjuvant capsular (OAC) vaccine against *P. multocida* in chicks immunosuppressed biologically with duck anti-thymocyte globulins (DATG) or duck anti bursacyte globulins (DABG) and cyclophosphamide. The findings gave evidence of humoral immune response playing a larger role in protection, although both T and B cells were involved in host defense. Oil adjuvant capsular vaccine was found to be more immunogenic in chicks than OAW vaccine.

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 weeks aged chicken than the commercial vaccine tried and conferred cross protection, corroborating the notion that *P. multocida* when grown *in vivo* could express protective antigens common to several serotypes (Mariana and Hirst, 2000).

On intramuscular challenge testing using virulent *P. multocida* in chicken, Wilkie *et al.* (2000) found that the protection was lacking in intratracheal and conjunctival routes of inoculation. Conjunctival route proved to be the less preferred route for inoculation. Parenteral inoculation resulted in high degree of protective immunity, although the magnitude of serum antibody response measured by ELISA revealed poor correlation with virulent challenge.

Akand *et al.* (2004) reported 80 per cent protection in chickens vaccinated with washed cell fowl cholera vaccine containing 5.7×10^7 CFU/ml. The antibody titres obtained in the experiment indicated that intramuscular route of vaccination produced better results than subcutaneous route of vaccination.

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

Laboratory trial of a vaccine incorporated with local strains of *P. multocida* serotype A and D, using rabbits as experimental and target animals revealed that 100 per cent of vaccinated rabbits survived challenge, when the vaccine concentration was 4×10^9 cells/ml and the dose no less than one millilitre of the vaccine (Ruzauskas, 2005).

Shilpa *et al.* (2005) found that the formalin killed vaccine prepared from local isolate of *P. multocida* was better than OMP and commercial vaccine in layer chicken. Formalin killed vaccine conferred 100 per cent protection 28 days PV while 80 per cent and 70 per cent protection was reported for OMP and commercial vaccine groups respectively.

2.13.4 Biofilms as Vaccine Candidates

Shivaraj (1998) reported an exceptional 83.33 per cent protection for a biofilm based oral vaccine for *E. coli* biofilm while conventional vaccine could induce only 33.33 per cent protection

Employing immunohistochemistry and immunofluroscence Azad *et al.* (2000) demonstrated the uptake and processing of *Aeromonas hydrophila* vaccine antigens post vaccination with biofilm vaccine containing heat inactivated intact cells on chitin. The overall quantity of antigen available at different organs was greater with biofilm vaccine and the longer retention of the biofilm antigens was attributable to the larger size of biofilm flocs and protection offered by glycocalyx covering. They correlated the longer retention of antigens to enhanced serum agglutination titres and greater protection recorded post vaccination.

Most virulent clinical isolates and biofilm forming strains of coagulase negative staphylococci and *S. aureus* carry the *ica* gene locus expressing the polysaccharide antigen- PNAG (Poly-N-Acetyl Glucosamine) which forms an attractive vaccine candidate. Animal studies have shown that purified PNAG elicited protective immunity against both species of staphylococci, suggesting its potential as

a broadly protective vaccine for many clinically important strains (Maira-Litran *et al.*, 2004).

Martinez and Casadevall (2005) in an *in vitro* study observed that monoclonal antibodies (MAbs) to *C. neoformans* biofilm polysaccharide could inhibit biofilm formation, which in turn denoted the critical role of adaptive humoral immune responses in preventing microbial biofilms following an infection. They envisioned that the specific immunoglobulin will prevent capsular polysaccharide release and thus block the adhesion of yeast cells to surfaces.

2.14 ASSESSMENT OF IMMUNE RESPONSE

Carter (1955) described a haemagglutination test for identification of serotypes of *P. multocida*; the technique involved saline extraction of capsular antigen followed by adsorption of the extract onto human "O" erythrocytes. Appropriate dilutions of type specific serum produced agglutination of erythrocytes.

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

In a comparative study of five different serological tests for predicting the immune status of vaccinated and unvaccinated sheep, post challenge with virulent organisms, Dua and Pandurangarao (1978) revealed that PHA was the best for indicating immune status of unvaccinated sheep while agar agglutination test was found to be most reliable in predicting fate of vaccinated sheep.

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

Briggs and Skeeles (1984) found the advantage of ELISA over other serological tests for reporting exact antibody titres in that it was based on a colour change which was directly proportional to the amount of antibody present in serum samples. It was found that ELISA was highly sensitive and reproducible for use in detecting antibody response to *P. multocida* in chickens.

Solano *et al.* (1984) opined that enzyme linked immuno sorbent assay (ELISA) was almost twice as sensitive as that of IHA for measurement of antibody titres post vaccination with *P. multocida*, though the measured antibody titres by both the methods correlated well. Following primary vaccination with CU strain antibody titres peaked around 20 days PV.

Highest anti-*Pasteurella* antibody titre (1173 ± 43.5) in rabbits observed after vaccination with oil adjuvant vaccine was at 56 days post vaccination and the titre remained higher even upto 84 days (672 ± 66.4), as measured by tube agglutination test (Lalrinliana *et al.*, 1988).

Schlink and Olson (1989) noted a significant increase in titre one week after vaccination with CU and average titre remained higher than control until 13 and 15 weeks PV. The serum anti-*Pasteurella* antibody titres were the lowest before vaccination with the average geometric mean titre (GMT) being 1.58 and after one week post vaccination GMT peaked to 3.79.

Chae *et al.* (1990) observed no significant differences in opsonizing capacity of sera from vaccinated groups when encapsulated organisms were used while better opsonization was detected with decapsulated organism, emphasizing the disability of serum anti-capsular antibodies in exerting a complement mediated cidal effect.

A dot immunobinding assay (DIA) was found to be more specific than ELISA in detecting antibodies against *P. multocida* in turkey serum. Whole cell and sonicated antigen showed higher sensitivity while heat stable antigen and formalin extract were more specific (Choi *et al.*, 1990).

Patel and Jaiswal (1994) measured the hemolytic complement (HC) level of the pre and post challenge sera of chicks vaccinated with oil adjuvant vaccine against *P. multocida*. A rise in HC titre by 7th day, with maximum mean titre by 21st day of vaccination and sharp decrease by 2nd to 4th day post challenge which tended to increase by 6th to 10th day post challenge was observed.

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 while the titre of twice vaccinated ducks after 84 days ranged 160 to 640, the minimum protective titre assessed being 320.

The antibody titre following vaccination with a field isolate of *P. multocida* A: 1 was monitored by employing disc inhibition method by Murugkar and Ghosh (1995). It was observed that the zone of inhibition developed after 21 days PV and persisted for 120 days. Maximum zone of inhibition was 20 mm in diameter and minimum was five millimetre, considering a diameter of eight millimetre as positive.

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

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. Humoral antibodies were

detected as early as seven days post-immunization and persisted up to one year after immunization.

Jayakumar (1998) opined that the highest antibody titre of 128 was observed at day 20 post vaccination with oil adjuvant vaccines and there was decline in the antibody level to the lowest titre of eight which was recorded on 80^{th} day.

Sander *et al.* (1998) challenged vaccinated turkeys at 23 and 32 weeks of age with *P. multocida* and two doses of live PM-1 *P. multocida* vaccine protected broiler breeder hens. When antibody levels were measured using ELISA, a range of 1951-4346 with a geometric titre of 3000 were obtained.

Chawak *et al.* (2001) measured the humoral immune response post vaccination by employing IHA and ELISA while cellular immune response was studied using lymphocyte transformation assay. They observed good correlation between IHA and ELISA values which again correlated well with protection rates. They employed sonicated antigen at a dilution rate of 1: 4 for IHA test and cell mediated immune response showed to play little role in anti-*Pasteurella* immunity.

Passive haemagglutination antibody titres in ducks immunized with fowl cholera vaccines was found significantly increased post vaccination (124.6 ± 52.83) and post challenge (263.11 ± 99.8), in comparison to pre-vaccination (5.60 ± 1.96) values. Total leukocytic count (TLC) also significantly increased post vaccination and post challenge (Islam *et al.* 2004).

After vaccination of rabbits with OMP vaccine against *P. multocida* as per slide agglutination test (SAT) antibody titres in pooled sera increased from day 14 post vaccination (PV) reaching a peak at day 28 or 35 followed by a decline at day 50 and further at day 90 PV (Wasnik *et al.*, 2004).

Microtitre agglutination test (MTA) was carried out by Shilpa *et al.* (2005) to evaluate antibody titres on post-vaccination pooled serum. Formalin killed vaccine group showed an increase in titre from day zero till day 28 and highest titre observed

was 80. Outer membrane protein (OMP) and commercial vaccine group had same titres of 20 on 28 days PV. There was no strict correlation between levels of agglutinating antibodies and protection conferred.

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 BACTERIA

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

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

3.2 PATHOGENICITY TESTING OF DP1

3.2.1 Laboratory Animals

Swiss albino female mice, six to eight weeks of age (weighing approximately 20g) were procured from the Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy.

An 18 h broth culture of *P. multocida* containing approximately 3×10^8 organisms/ml was inoculated (0.1 millilitre) intraperitoneally and subcutaneously to four mice each. Four mice were kept as control which were mock inoculated with sterile PBS (pH 7.4). All the mice were observed for seven days post inoculation. Blood smears were prepared from the dead mice and stained with Leishman's stain. Re-isolation of *P. multocida* from heart blood, lung, liver and spleen of the dead

mice was carried out on blood agar, followed by incubation at 37°C under five per cent carbon dioxide tension.

3.3 CAPSULAR STUDIES

3.3.1 Capsule Enhancement Studies

3.3.1.1 Media Used

a) Dextrose starch agar (DSA)

b) DSA supplemented with 10 per cent foetal bovine serum (FBS) (Bioclot, FBS of South American origin, Imperial Biomedics)

c) DSA supplemented with 10 per cent FBS and 0.5 per cent yeast extract.

The organism was grown in different conditions to assess the amount the capsular material produced. It was grown in DSA, DSA supplemented with 10 per cent FBS and DSA supplemented with 10 per cent FBS and 0.5 per cent yeast extract under five per cent carbon dioxide tension. All the plates were incubated at 37°C for 18 h. The capsule enhancement was assessed by capsule demonstration using Maneval staining and by the chemical characterization of crude capsular extract.

3.3.2 Demonstration of Capsule

3.3.2.1 Aqueous Congo Red Solution (1 per cent)

Congo red	1 g
Distilled water	100 ml

Congo red was mixed with distilled water and filtered using Whatman number one filter paper.

3.3.2.2 Maneval Stain

Acid fuchsin (1 per cent aqueous solution)	1-2 ml
Ferric chloride(30 per cent aqueous solution)	4 ml

Glacial acetic acid (20 per cent aqueous solution)	8 – 10 ml
Phenol (5 per cent aqueous solution)	30 ml

The ingredients were mixed in fume hood and filtered using Whatman number one filter paper.

Capsular staining was done using the Maneval staining as described by Corstvet *et al.* (1982). One loopful each of 12 h old *P. multocida* culture from DSA and capsule enhancement media were suspended in 1.5 millilitre of PBS (pH 7.4) and one drop of the suspension was mixed with equal amount of one per cent aqueous congo red solution which then was spread into a thin smear on a clean glass slide. After air drying and without fixation the smear was counter stained with Maneval stain for two minutes. Then it was washed, air dried and observed under oil immersion objective of the microscope under reduced illumination.

Demonstration of capsule of biofilm cells of *P. multocida* was also tried using Maneval staining method. One loopful of three day old late logarithmic phase biofilm culture of *P. multocida* from 0.32 percent tryptone soya broth supplemented with 0.3 per cent bentonite clay was mixed with two drops of congo red solution and made into a thin smear. Maneval staining was done as quoted above.

3.3.3 Extraction of Crude Capsular Extract (CCE)

3.3.3.1 Phosphate Buffered Saline (PBS) [10 X Stock Solution]

Sodium chloride (NaCl)	80 g
Potassium chloride (KCl)	2 g
Disodium hydrogen phosphate (Na_2HPO_4. 12 H_2O)	11.32 g
Potassium dihydrogen phosphate (KH_2PO_4)	2 g

Distilled water	1000 ml

The pH was adjusted to 7.4 by 1 N NaOH and sterilized by autoclaving at 121° C for 15 min at 15 lbs pressure. The stock solution was diluted to 1 X before use with distilled water.

3.3.3.2 Sodium Chloride Solution (2.5 per cent)

Sodium chloride (NaCl)	25 g
Distilled water	1000 ml

3.3.3.3 Dialysis Membrane (Sigma Aldrich, Germany)

The dialysis membrane was pre-treated as per manufacturer's instructions.

3.3.3.4 Poly Vinyl Pyrollidone (SRL, Mumbai)

3.3.3.5 Thiomersal (BDH, England)

Extraction procedures were done as per the method employed by Syuto and Matsumoto (1982), with some modifications.

Pasteurella multocida grown under the three different conditions, *viz.*, on ordinary dextrose starch agar (DSA) at 37°C for 18 h, under capsule enhancement conditions and under biofilm mode in tryptone soya broth (0.32 per cent) supplemented with 0.3 per cent bentonite clay were used. The biofilm organisms were subcultured onto nutrient agar and subsequently harvested for extraction procedures. The organisms thus grown were harvested in PBS (pH 7.4) from respective conditions and washed thrice in the same buffer by centrifugation at 3000 x g for 15 min and finally resuspended in 2.5 per cent sodium chloride solution to yield 3×10^9 cells/ml by matching with Mc Farland's standard tube number ten. The suspension was then kept at 56° C for one hour in boiling water bath. It was then centrifuged at 17,000 × g for 20 min and the supernatant was collected and dialyzed for 48 h in 0.85 per cent sodium chloride solution containing 0.01 per cent

Thiomersal. The product was then concentrated using poly vinyl pyrollidone (PVP) and filtered through a 0.2 micron membrane filter; the filtrate was the crude capsular extract. The extraction from each growth condition was done in triplicate.

3.3.4 Chemical Characterization of CCE

3.3.4.1 Estimation of Total Carbohydrate Content of CCE

3.3.4.1a Crude Capsular Extract

Crude capsular extract was prepared from all the three conditions as described above.

3.3.4.1b Concentrated Sulphuric Acid (Merck, Mumbai)

3.3.4.1c Liquid Phenol (80 per cent) (Genei, Bangalore)

3.3.4.1d Standard Sugar Solutions

Aqueous solutions of glucose, galactose, mannose and ribose containing

50 to 500 µg/ml were used as standard solutions.

The total carbohydrate content of crude capsular extract was measured by phenol sulphuric acid method (Dubois *et al.*, 1956). As the important carbohydrates present in the capsule of *P. multocida* were glucose, galactose, mannose and ribose, the carbohydrate content was measured using those sugars as standards.

Two millilitres of sugar solution containing 50-500 µg/ml of the respective sugars were pipetted into test tubes and 0.05 millilitre of 80 per cent phenol was added to each tube separately. Then five millilitre of concentrated sulphuric acid was added rapidly and ensured uniform mixing. The tubes were allowed to stand for 10 min, followed by incubation at 30°C for 30 min. The colour absorbance was measured at 490 nm in UV-VIS spectrophotometer (Genesys 10 UV, Thermospectronic), using blanks prepared by substituting sugar solution with

distilled water. A standard curve was plotted and the estimation was done in triplicate.

3.4 BIOFILM ASSAY

3.4.1 Tryptone Soya Broth (TSB) (SRL, Mumbai)

3.4.2 Bentonite Clay Powder (Loba Chemie, Mumbai)

3.4.3 Nutrient Agar

3.4.4 PBS (pH 7.4) (3.3.3.1)

3.4.5 Method

Biofilm assay was performed as per the method described by Vadakel (2001).

3.4.5.1 Preparation of Inoculum for Biofilm Assay

For preparation of inoculum, four or five selected colonies of *P. multocida* from blood agar were suspended in TSB and incubated for 48 h at 37°C. The growth was harvested, washed thrice in PBS (pH 7.4) by centrifugation and resuspended in PBS (pH 7.4) to obtain the final inoculum having approximately 3×10^9 bacteria/ml as per Mc Farland's standard tube number ten. After confirming the sterility by Gram's staining, it was added to the biofilm culture media under sterile conditions.

3.4.5.2 Media and Conditions for Biofilm Assay

Tryptone soya broth at a concentration of 0.32 per cent was used as medium of growth in the present study. Bentonite clay powder at 0.3 per cent level was added as the inert substrate for providing surface of attachment or colonization to trigger biofilm formation.

Tryptone soya broth and bentonite clay were suspended in 100 ml of double distilled water in a 250 ml conical flask and sterilized by autoclaving at 121°C for 15 min. After checking the sterility of the broth by inoculating 0.1 millilitre of the broth to nutrient agar, the flasks were inoculated with *P. multocida* inoculum prepared as

above and incubated at 42°C for the required number of days. An initial attachment period of three to four hours was given and after that the culture flasks were agitated six times daily for duration of one hour each time, in an orbital shaker (Thermocon Instruments Private Limited, Bangalore) set at 50 rpm and 42°C to give proper aeration and uniform suspension of the substrate in the media. The flasks were incubated for one, three and six days separately.

3.4.5.3 Estimation of Biofilm and Planktonic Bacteria

3.4.5.3.1 Harvesting of Biofilm Cells

The biofilm cells were quantified by sedimenting the cells colonized on bentonite clay at 2000 rpm for 10 min. The supernatant was discarded and the pelleted bentonite clay carrying the biofilm was washed thrice with PBS (pH 7.4) to remove non-adherent bacteria, transferred to 10 ml of PBS (pH 7.4) and vortexed vigorously for 15 min to displace the biofilm cells from bentonite clay. Biofilm cells thus obtained in the supernatant were retrieved by another centrifugation at 2000 rpm for 10 min and the supernatant was used to perform the viable count by spread plate method.

3.4.5.3.2 Viable Count of Harvested Biofilm Cells

The viable count of the harvested biofilm cells was done by employing the spread plate method for counting bacterial cells. The nutrient agar plates were dried for two or three hours at 37°C prior to plate counting. Ten fold dilutions of the bacterial suspensions were made for biofilm cells in PBS (pH 7.4). From each dilution after proper mixing 0.1 millilitre was transferred to nutrient agar plate and the inoculum was spread uniformly with sterile "L" shaped glass rod. Duplicate plates were kept for each dilution. All the plates were incubated at 37°C for 48 h. After 48 h the colony count was carried out. The average number of colonies per

plate was multiplied by the dilution factor and the viable count was expressed as CFU/gram of bentonite clay.

3.4.5.3.3 Viable count of planktonic cells

One millilitre of the initial culture before harvesting was used to estimate the planktonic bacterial count. The procedure for plate count by spread plate method was the same as previously described and the viable count was expressed as CFU/ml of the culture.

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

3.5.1 Experimental Birds

For the study, unvaccinated one month old ducklings and six month old ducks were procured from a private breeder at Thrissur.

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

Pasteurella multocida A: 1 strain was passaged in mice to get a fully encapsulated virulent form. The virulent organisms isolated from mice were grown on DSA at 37°C for 24 h. The growth on DSA was harvested, washed thrice in PBS by centrifugation at 3000 × g for 15 min and resuspended in the same buffer to contain 3×10^9 cells/ml. Then serial ten fold dilutions were made upto 3×10^0 cells/ml. Plate count was done to assess the number of bacteria present in each dilution retrospectively.

Four week old ducklings were randomly assigned to nine groups of six ducklings each and the first eight groups were separately inoculated with the different dilutions of the bacteria with a dose of 0.1 millilitre per bird subcutaneously at wing web and the ninth group served as controls which were sham inoculated with 0.1 millilitre of sterile PBS (pH 7.4). Mortality was recorded one week 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. The method described by Reed and Muench (1938) was used for LD_{50} calculation.

The same procedure was followed for determination of LD_{50} in ducks, but the number of birds used for each dilution was only four and the dose given was 0.5 millilitre.

3.6 PREPARATION OF VACCINE

3.6.1 Organism and growth conditions used

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

1. Organisms grown in Tryptone soya broth (TSB) (3 per cent)
2. Organisms grown in TSB supplemented with 10 per cent foetal bovine serum (FBS) and 0.5 per cent yeast extract.
3. Organisms grown under biofilm mode in TSB (0.32 per cent) supplemented with 0.3 per cent Bentonite clay.

3.6.2 Formalin inactivated *P. multocida* bacterins

3.6.2.1 Formol saline (0.5 per cent)

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

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

3.6.2.2 Blood agar

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

Organisms grown in TSB (3 per cent) for 24 h and TSB (3 per cent) supplemented with 10 per cent FBS and 0.5 per cent yeast extract for 18 h at 37°C were checked for purity by Gram's staining. The culture was harvested by centrifugation at 3000 × g, washed thrice in the PBS (pH 7.4) and was then resuspended in 0.5 per cent formol saline to a concentration of 3×10^9 cells/ml. The culture thus prepared was allowed to stand at room temperature for 48 h with occasional shaking, for the formalin to act.

The bacterin for biofilm vaccine was prepared with a slight difference. The bacteria were first cultured under biofilm mode in 0.32 per cent TSB supplemented with 0.3 per cent bentonite clay and the biofilm obtained after three days of incubation at 42°C was poured onto nutrient agar plates at the rate of 0.1 millilitre per plate and incubated at 37°C for 24 h. The agar washed culture of biofilm cells in PBS (pH 7.4) was formalinized as previously described.

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

3.6.3 Liquid paraffin

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

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

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

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

3.7 STERILITY TESTING OF VACCINE

3.7.1 Blood Agar (3.6.2.2)

3.7.2 Tryptic Soy Agar

3.7.3 Modified Thioglycollate Medium

3.7.4 Sabouraud's Dextrose Agar

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

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

3.8 TOXICITY TESTING OF VACCINES

The toxicity of vaccines was assessed by injecting 0.5 millilitre and one millilitre of vaccine intramuscularly to three ducklings each separately for the

vaccines. The injected birds were observed for a period of seven days for any untoward reaction or clinical manifestations.

3.9 VACCINATION PROGRAMME

A total of 160 four week old ducklings were divided into four groups with 40 birds in each group and the first three groups were vaccinated as described below.

3.9.1 Group I

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

3.9.2 Group II

Group II was vaccinated intramuscularly at the thigh region with 0.5 millilitre oil adjuvanated bacterin vaccine prepared from *P. multocida* grown in TSB supplemented with 10 per cent FBS and 0.5 per cent yeast extract.

3.9.3 Group III

Group III was vaccinated intramuscularly at the thigh region with 0.5 millilitre oil adjuvanated bacterin vaccine prepared from *P. multocida* grown under biofilm mode in 0.32 per cent TSB supplemented with 0.3 per cent bentonite clay.

3.9.4 Group IV

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

3.10 COLLECTION OF SERUM SAMPLES PRE AND POST-VACCINATION

Blood was collected from all the 160 ducks grouped into four separate groups before the start of vaccination trial, at weekly intervals upto 28^{th} day post vaccination (PV) and on day 42 PV by cardiac puncture or by jugular venipuncture. The collected blood was allowed to clot and incubated at 37°C for 30 min. Serum was separated following overnight incubation at 4°C and stored at -20°C until use.

3.11 PASSIVE / INDIRECT HAEMAGGLUTINATION (PHA / IHA)

3.11.1 Sheep Blood For PHA

3.11.1.1 Alsever's Solution

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

Each ingredient in the above order was added to 500 ml of distilled water in graduated measuring cylinder and stirred until the chemicals dissolved completely. The volume was made up to one litre with distilled water and finally autoclaved at 121°C, 10 lbs pressure for 15 min. The solution was freshly prepared just before use.

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

3.11.2 Whole Cell Ultrasonicated Antigen

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

The organisms grown on brain heart infusion agar (BHIA) were harvested in PBS (pH 7.4) and washed twice by centrifugation at 8000 x g for 20 min at 4°C in the same buffer. The washed cells were resuspended in PBS to contain 3×10^9 cells/ml. The bacterial cell suspension held on an ice bath was then disrupted by

sonication at 250 V for a total of five minutes with 10 × 30 sec bursts in a sonicator (Branson Sonifier 450) fitted with a 12 mm diameter titanium probe, by placing the probe 1.0-1.5 centimetre deep into the suspension to avoid frothing. The sonication was interrupted for 30 sec between each burst for cooling. The suspension so prepared was centrifuged at 8000 x g for 30 min, the supernatant was collected and sterility was tested by plating 0.2 millilitre on DSA plates and observed for 48 h. The sterile antigen was stored at -20°C until further use.

3.11.3 Fixation of Sheep Red Blood Cells (SRBC)

3.11.3.1 Gluteraldehyde Solution (25 per cent) (Loba Chemie, Mumbai))

3.11.3.2 Gluteraldehyde Solution (1 per cent)

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

Sheep red blood cells collected in Alsever's solution was fixed using gluteraldehyde. The SRBC in Alsever's solution was washed by centrifugation (650 × g for 20 min) six times with 0.85 per cent saline. After the last wash, the packed cells were resuspended in PBS to yield a 10 per cent suspension (v/v) and chilled to 4°C in an ice bath. A 25 per cent solution of gluteraldehyde was diluted to one per cent (v/v) with PBS and chilled to 4°C. The 10 per cent washed SRBC suspension was mixed with an equal volume of one per cent gluteraldehyde solution and the mixture was incubated at 4°C for 30 min with gentle stirring. The gluteraldehyde solution was added only drop wise to the SRBC suspension in the ice bath. The mixture was then centrifuged at 650 × g for 10 min at 25°C. The pelleted, fixed cells were suspended in PBS, washed three times with PBS by centrifugation and re-suspended in PBS containing 0.1 per cent sodium azide to yield a 10 per cent suspension. The gluteraldehyde fixed-SRBC (GA-SRBC) was stored at 4°C.

3.11.4 Sensitization of GA-SRBC with whole cell ultrasonicated antigen

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

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

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

A 10 per cent suspension of GA-SRBC was mixed with an equal volume of a two fold diluted antigen. The mixture was incubated at 37°C for one hour with occasional shaking. The sensitized cells were washed three times in PBS containing 0.25 per cent bovine serum albumin (BSA) and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (v/v).

3.11.5 Indirect/Passive Haemagglutination (IHA/PHA) test

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

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

3.12 HOMOLOGOUS CHALLENGE OF VACCINATED BIRDS

Ten birds from each vaccinated group were subjected to homologous challenge by intramuscular route with 0.1 millilitre of inoculum containing 200 LD_{50} and 100 LD_{50} of fully encapsulated virulent form of *Pasteurella multocida* serotype A: 1 on 28^{th} and 42^{nd} day PV respectively. The challenged birds were observed for a period of two weeks post challenge for mortality/clinical signs. All the dead birds were examined for specific lesions of pasteurellosis and re-isolation of the organism on blood agar from heart blood, liver and spleen was attempted.

3.13 STATISTICAL ANALYSIS

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

Results

4. RESULTS

4.1 PURITY CHECKING OF THE ISOLATE

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

4.1.1 Cultural Characteristics and Morphology

The isolate produced typical colonies on blood agar which were smooth, convex, translucent, and butyraceous and one to three millimetres in diameter, after 18 to 24 h of incubation. Gram's staining revealed Gram negative coccobacillary organisms arranged singly or in pairs.

4.1.2 Biochemical Characterization

4.1.2.1 First Stage

The isolate grew aerobically and anaerobically, did not grow on Mac Conkey's agar, was non-haemolytic on blood agar and non motile. 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 did not produce hydrogen sulphide (H_2S), reduced nitrate and was negative for citrate utilization.

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

Table 1. First stage biochemical tests of DP 1

Tests	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

Tests	Result
Indole production	+
Methyl red	–
Voges proskauer	–
H2S production	–
Citrate	–
Nitrate	+
Ornithine decarboxylase	+
Urease	–
Sugar fermentation	
Glucose	+
Sucrose	+
Galactose	+
Mannose	+
Lactose	–
Maltose	+
Arabinose	–
Salicin	–
Dulcitol	–
Trehalose	+
Sorbitol	+

4.2 PATHOGENICITY TESTING IN MICE

Pasteurella multocida serotype A: 1 killed the mice inoculated with 0.3 x 10^8 organisms/0.1 ml intraperitoneally within eight hours and within 24 h when injected by subcutaneous route. The control mice were alive even after an observation period of seven days.

Similar lesions were observed in the inoculated mice irrespective of the route of inoculation. The gross lesions observed in the inoculated mice were petechial haemorrhages in the epicardium and general congestion of all the visceral organs, particularly of lung, liver and spleen. Fluid accumulation was also noticed in the peritoneal cavity of mice inoculated intraperitoneally. Blood smear and impression smears from spleen and liver following Leishman's staining revealed large number of bipolar stained organisms. Re-isolation of *P. multocida* in pure culture was done from the heart blood, lungs, liver and spleen on bovine blood agar at 37°C under five per cent carbon dioxide tension.

4.3 CAPSULAR STUDIES

The colonies produced by *P. multocida* grown in capsule enhancement media kept under five per cent carbon dioxide tension were confluent and highly mucoid. These colonies have shown the play of colours or iridescence when observed under oblique light.

4.3.1 Capsule Demonstration

Maneval staining was done for demonstration of capsule of DP1 grown under the three different conditions. The 18 h old culture of DP1 taken from DSA had shown capsule as a white unstained halo around the bacterial cell which had taken a red colour (Fig. 1). The background appeared as blue or red. The capsules of the organisms grown in capsule enhancement media (Fig. 2) were discernibly larger and denser when compared to the organisms grown in DSA alone. Maneval staining of

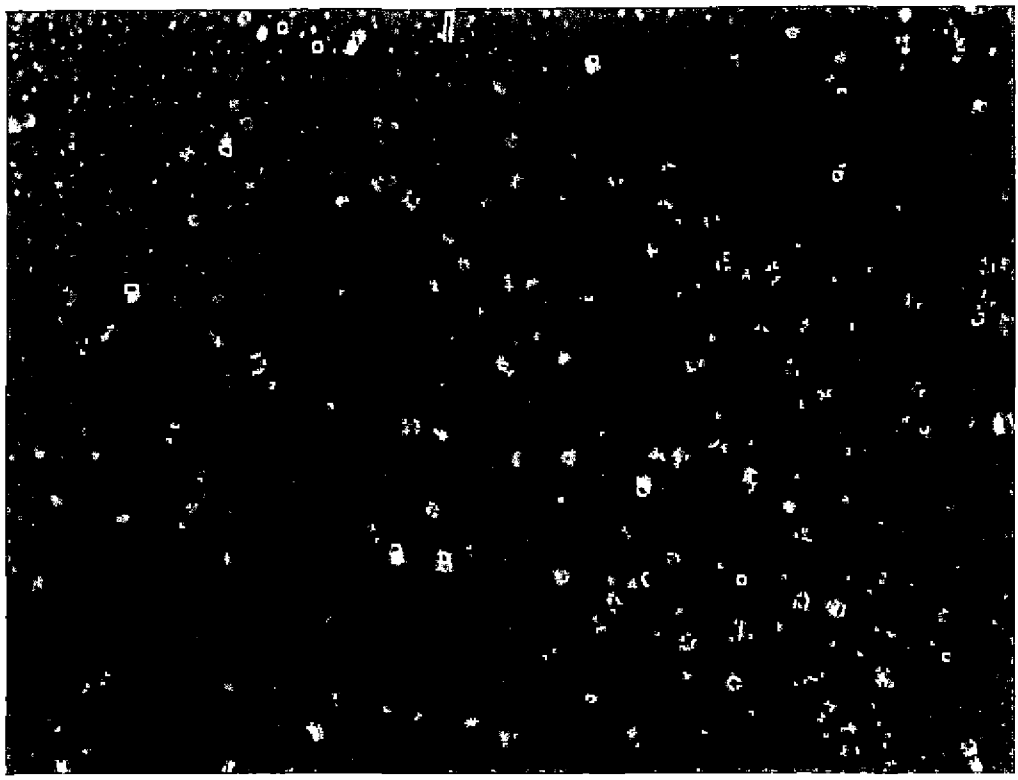


Fig. 1. DP 1 in Dextrose Starch Agar (Maneval stain, 1000 x)

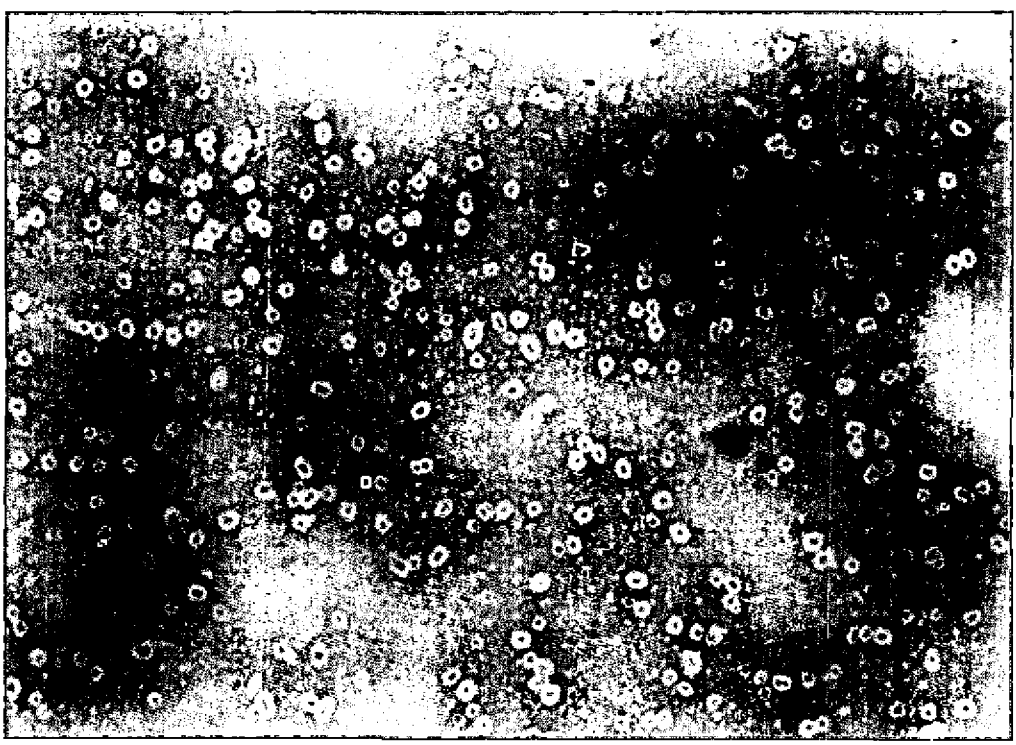


Fig. 2. DP 1 in Dextrose Starch Agar with FBS and Yeast Extract (Maneval stain, 1000 x)

late logarithmic phase of three day old biofilm culture revealed heavily capsulated cells of *P. multocida* attached as large aggregates (Fig. 3) and appearing as chains of coccobacillary cells (Fig. 4). The heavily capsulated aggregated cells even resisted the penetration of stain so that the bacteria remained unstained in their thick exopolysaccharide covering.

Maneval staining of the late logarithmic phase biofilm cultures also evidenced a change in morphology of the bacteria in that they formed a thick meshwork of aggregated and chain forming cells (Fig. 5).

4.3.2 Chemical Characterization of CCE

Capsular polysaccharides of the saline extract were estimated using phenol sulphuric acid method, employing different sugars such as glucose, galactose, mannose and ribose as standards. The values obtained for organism grown under each condition are presented in Table 3.

The capsular polysaccharides (when glucose was used as the standard) increased by approximately 1.6 times when the organism was grown in capsule enhancement media containing 10 per cent FBS and by about 2.06 times when grown in media supplemented with FBS and yeast extract. The capsular polysaccharide content of biofilm cells was still higher, approximately 3.25 times than that of planktonic bacteria grown in DSA alone. Carbohydrate content of the CCE of DP1 grown under different conditions is graphically represented in Fig. 6.

4.4 BIOFILM ASSAY

The results indicated that DP1 strain did form biofilm *in vitro* on bentonite clay under conditions of nutrient limitation.

4.4.1 Plate Count of Biofilm and Planktonic Cells

The results showed that the biofilm cells reached a peak on the third day of incubation with an average count of 1.54×10^6 CFU/g of bentonite clay while the

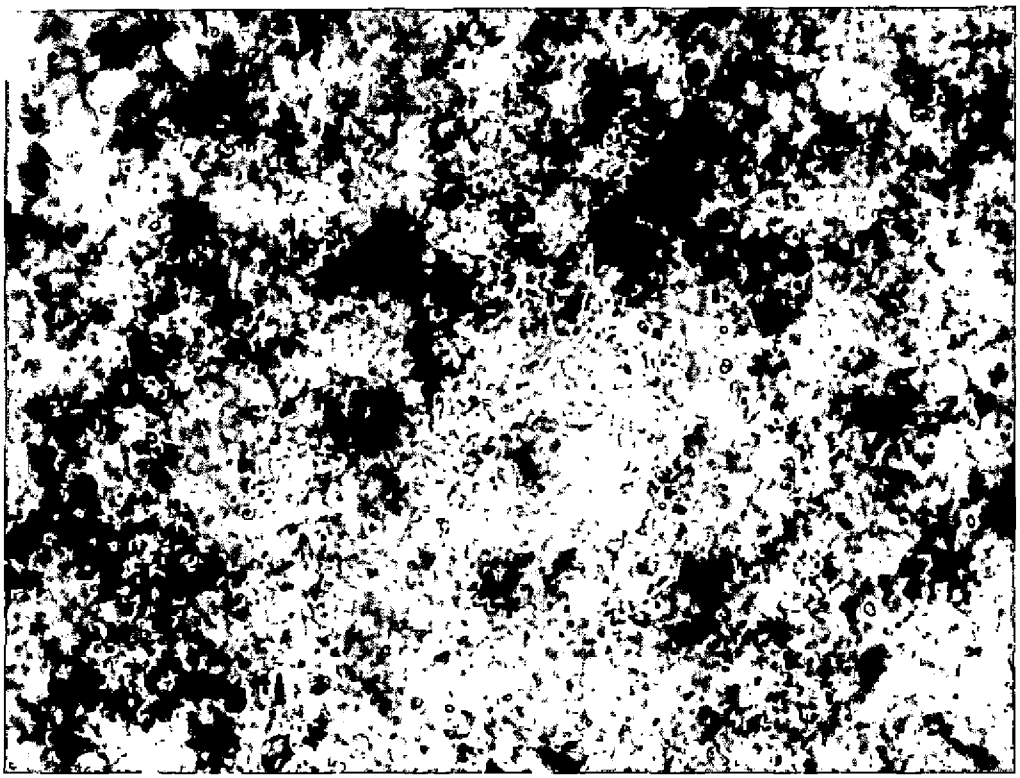


Fig. 3. Late stationary phase culture of biofilm cells in clusters (Maneval stain, 1000 x)

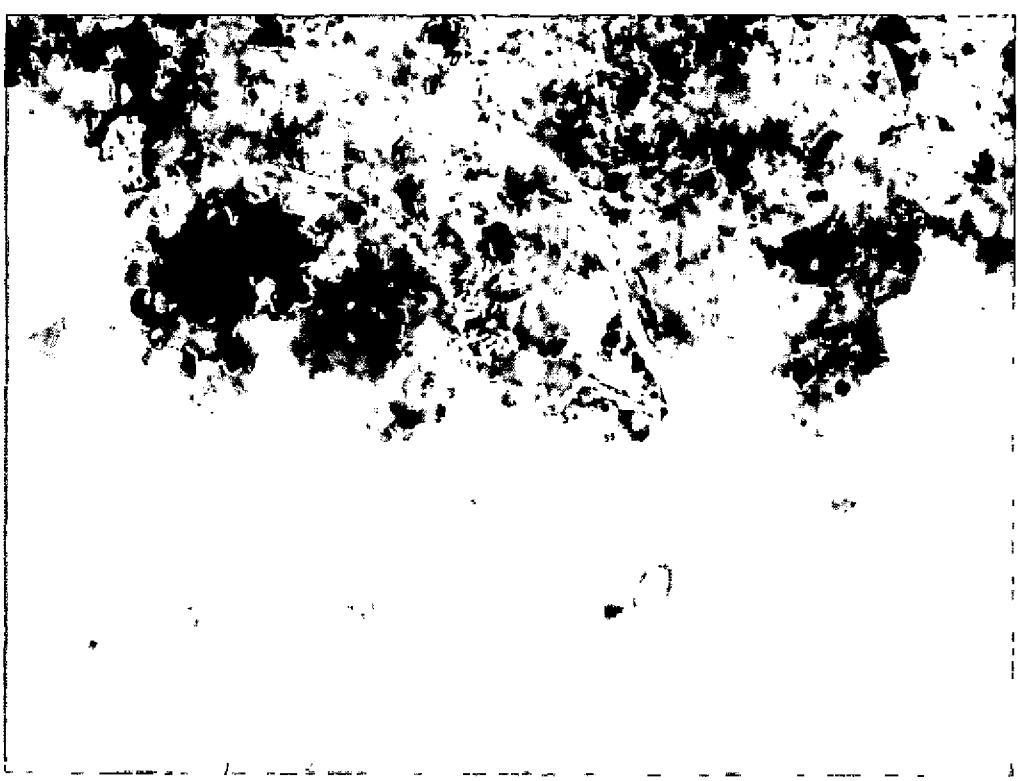


Fig. 4. Late stationary phase culture of biofilm - capsulated cells in chains (Maneval stain, 1000 x)

Fig. 1. DP 1 in Dextrose Starch Agar (Maneval stain, 1000 x)

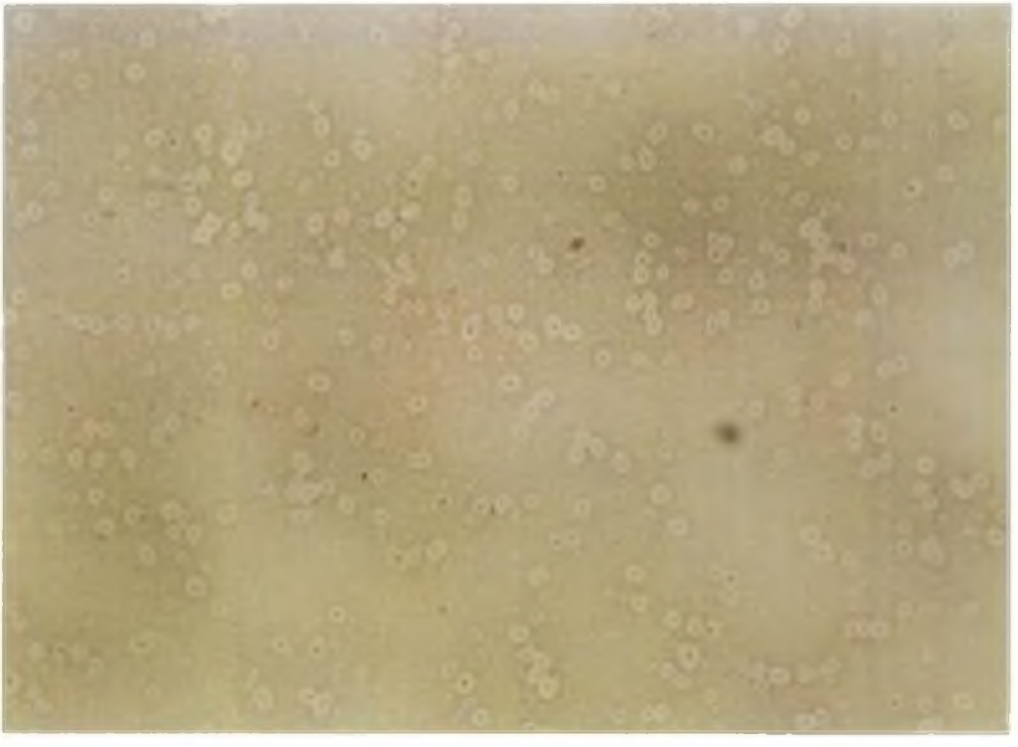


Fig. 2. DP 1 in Dextrose Starch Agar with FBS and Yeast Extract (Maneval stain, 1000 x)

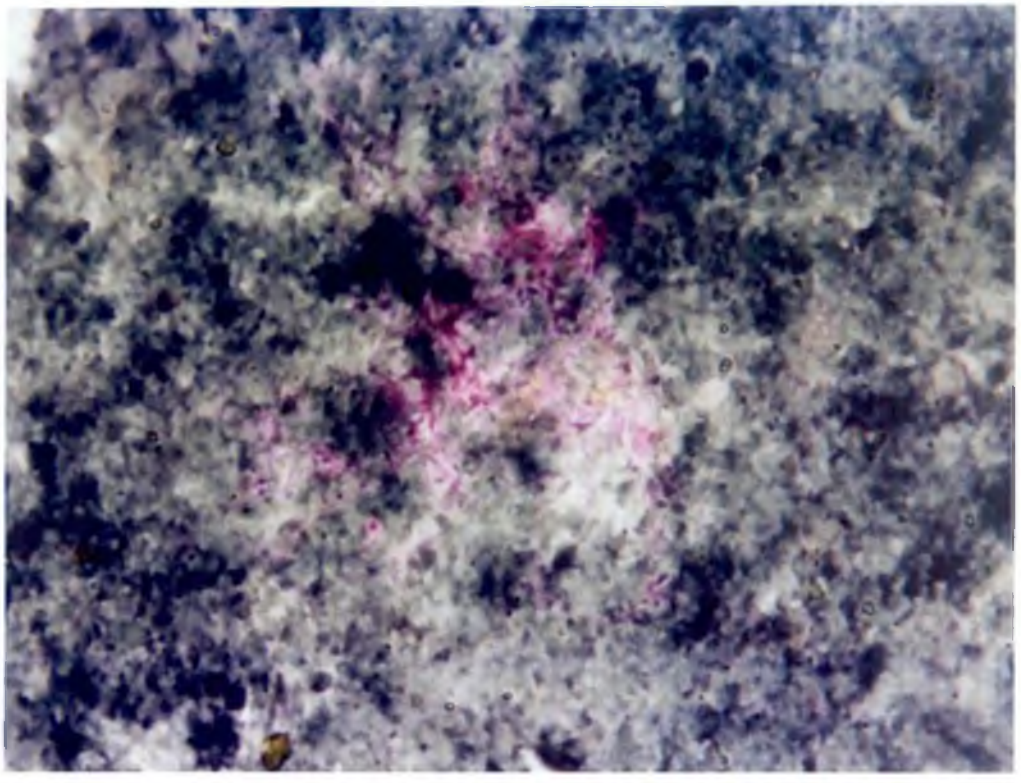
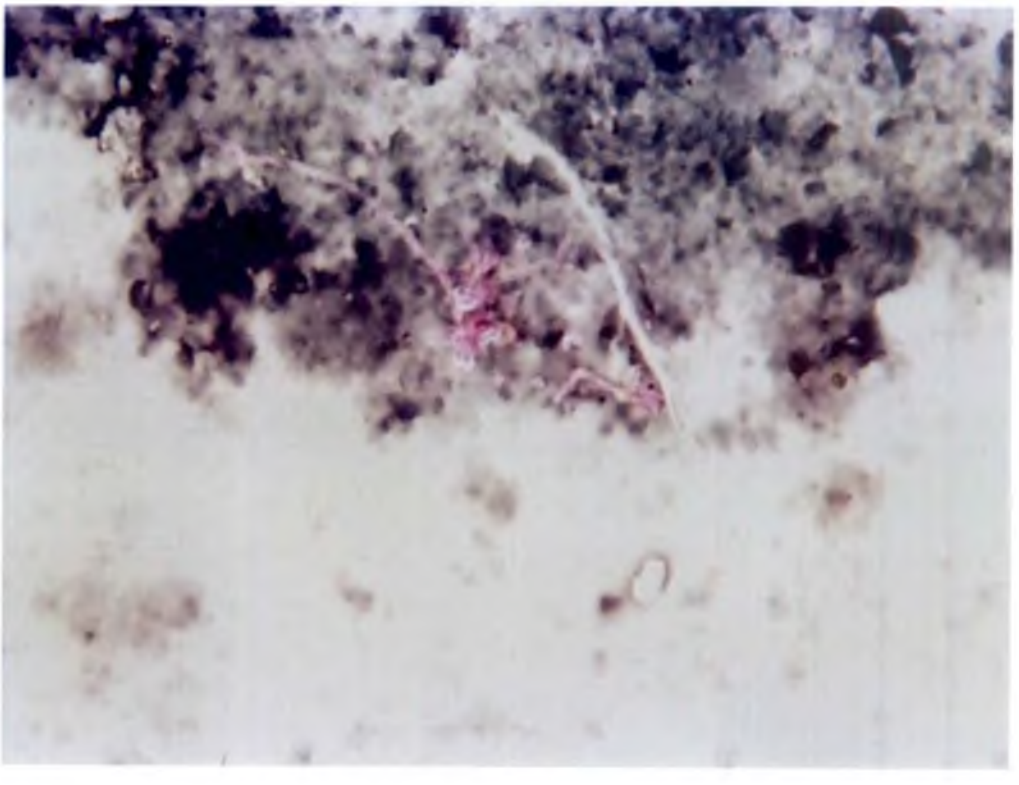


Fig. 3. Late stationary phase culture of biofilm cells in clusters (Maneval stain, 1000 x)



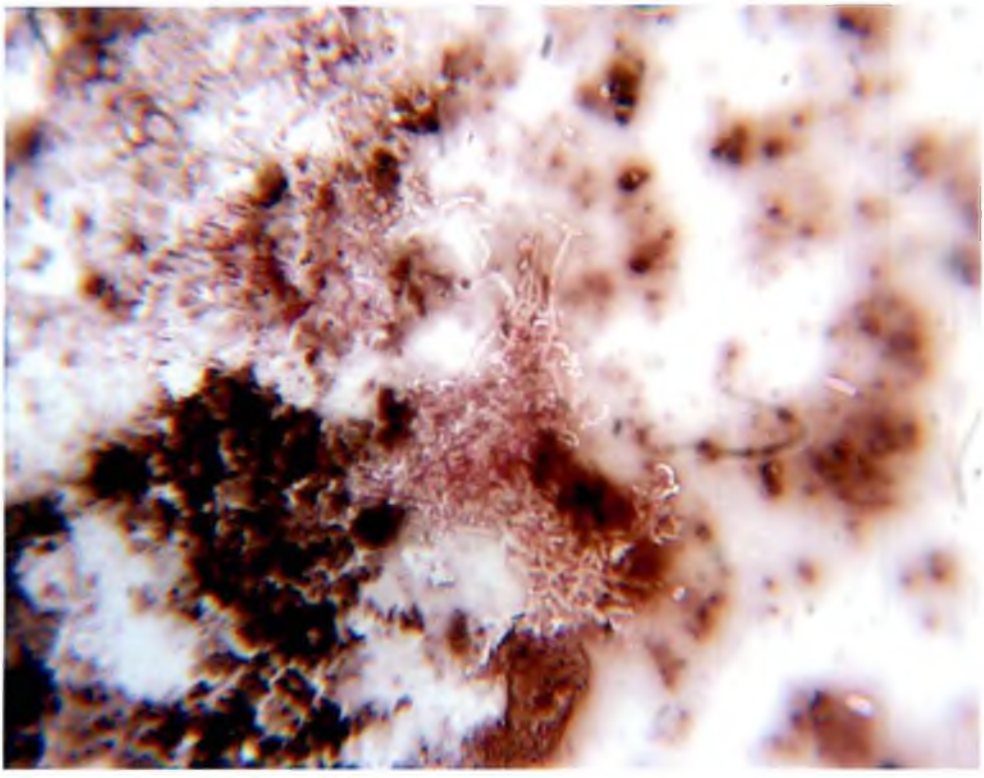


Fig. 5. Late stationary phase culture of biofilm - meshwork of attached cells (Maneval stain, 1000 x)

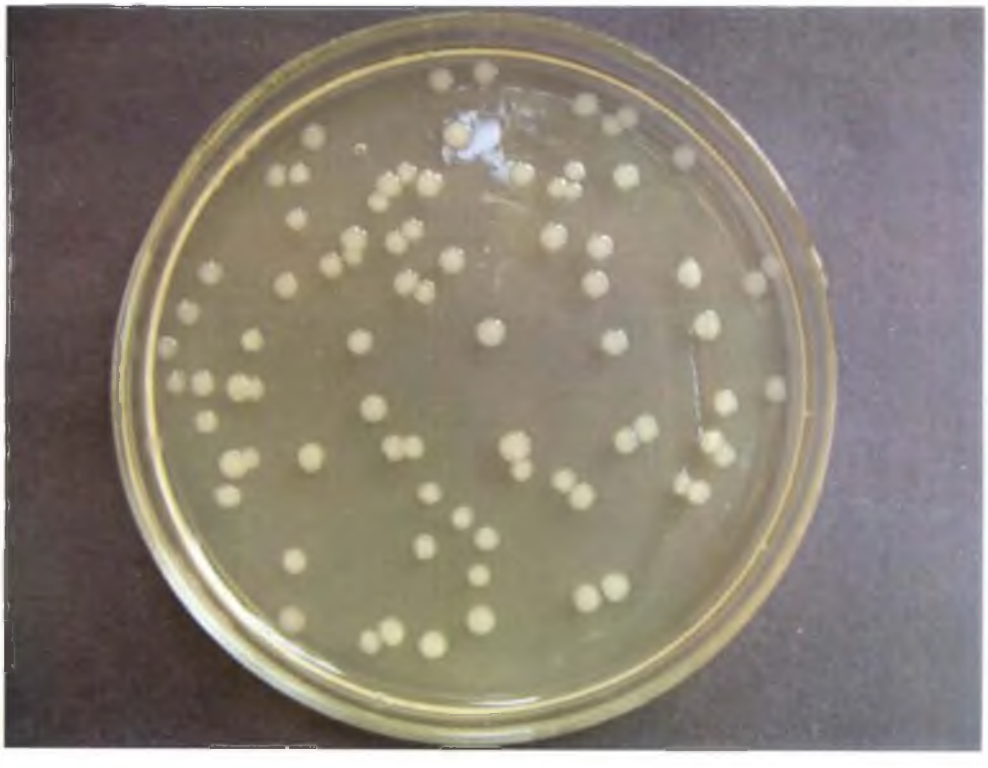


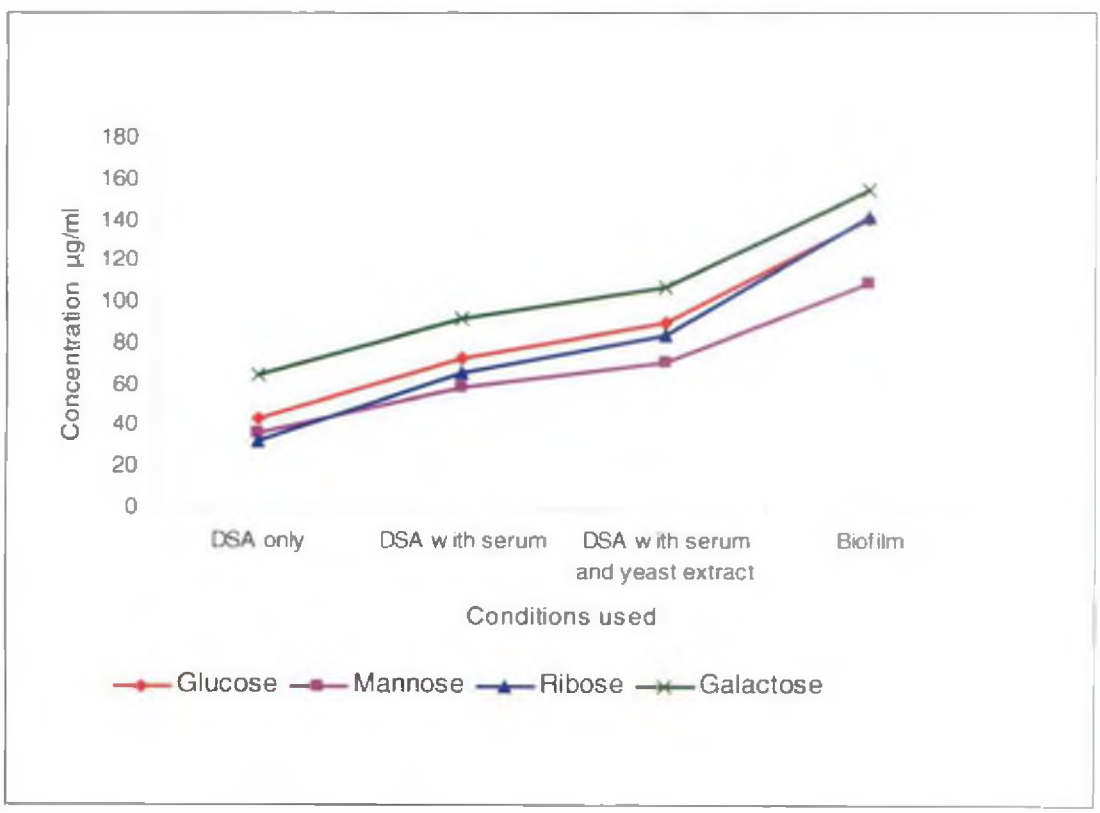
Fig. 7. Plate count of biofilm cells after 24 h

Table 3. Carbohydrate content of CCE of DP1

Conditions Employed	Concentration of polysaccharides in CCE (µg/ml)			
	Glucose	Mannose	Ribose	Galactose
DSA only	43.34	36.69	32.20	64.84
DSA with serum	73.16	58.76	65.64	92.43
DSA with serum and yeast extract	89.56	70.89	84.02	107.59
Biofilm	140.73	108.76	141.39	154.93

All the values obtained by finding the mean of triplicate experiments.

Fig. 6. Carbohydrate content of CCE of DP1 grown under different conditions (Estimated using different sugar standards)



planktonic cells were found to be maximum on day one post inoculation, with a peak count averaging about 8.10 $\times 10^8$ CFU/ml of the broth. The results of the plate count of biofilm and planktonic cells on days 1, 3 and 6 post inoculation are shown in Table 4. The colony of biofilm cells during plate count on nutrient agar after 24 h was shown in Fig. 7.

4.4.2 Colony Morphology of Biofilm and Planktonic Cells

Some of the biofilm colonies on nutrient agar during plate count consistently showed variation in colony morphology from that of planktonic *P. multocida*. The planktonic cells of *P. multocida* produced circular, convex, smooth and translucent colonies with an entire edge after 24 h at 37°C (Fig. 8). The biofilm cells on nutrient agar after 24 h at 37°C produced colonies characterized by radiating strands from centre to periphery (Fig. 9, 10). The colonies were smooth and transluscent, but the central portion of the colony showed opacity. Some of the colonies were having an undulating margin (Fig. 11) while most of the colonies revealed an entire edge. The colony morphotypes thus produced were lost on subsequent subcultures and there after were similar to planktonic colonies.

4.5 LETHAL DOSE 50

4.5.1 Lethal Dose 50 in Duckling

The LD_{50} of the isolate was 23 cells when tested in one month old ducklings. The dilution giving 50 per cent end point with 0.1 millilitre subcutaneous dose was $10^{-7.23}$. The results of the experiment are furnished in Table 5.

4.5.1.1 Gross Pathological Lesions in Experimentally Infected Ducklings

The gross lesions observed in experimentally infected ducklings were haemorrhages on epicardium, serous yellow fluid in pericardium (Fig. 12) pin point and diffuse patchy areas of necrosis on liver (Fig. 13 and 14), pin point and

Table 4. Plate count for biofilm and planktonic cells

Days of incubation	Plate count	
	Biofilm cells (CFU/g)	Planktonic cells (CFU/ml)
1	5.0×10^4	8.10×10^8
3	1.54×10^6	1.36×10^7
6	3.81×10^5	8.27×10^5

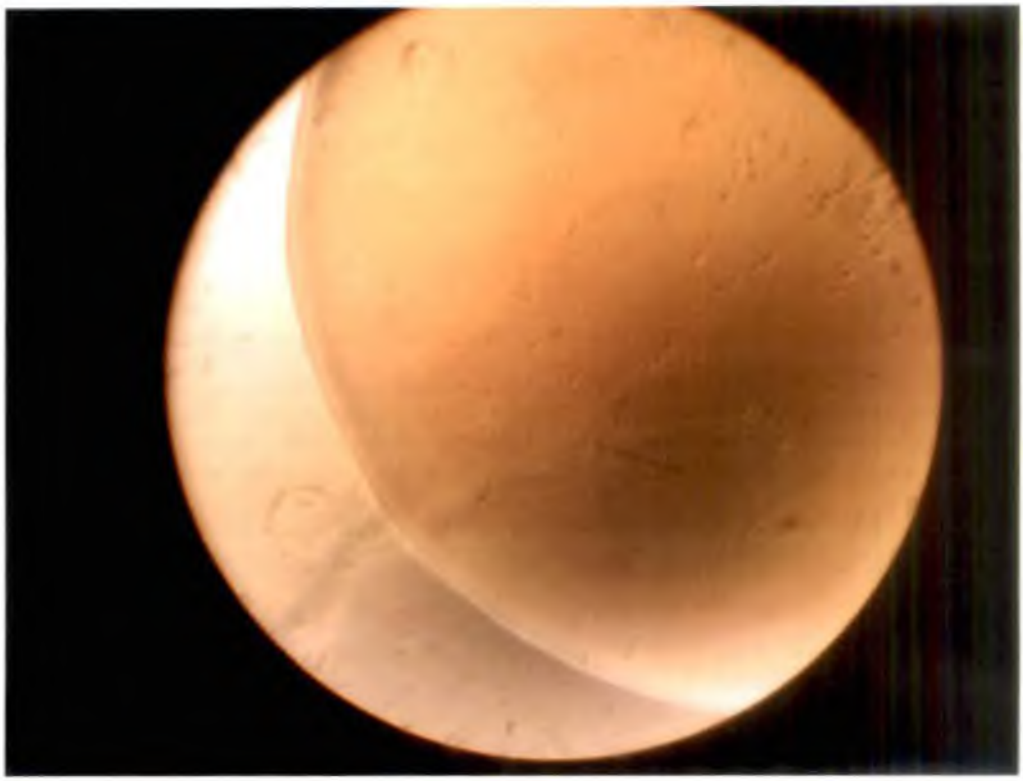


Fig. 8. 24 h old colony of planktonic cells (10 x)

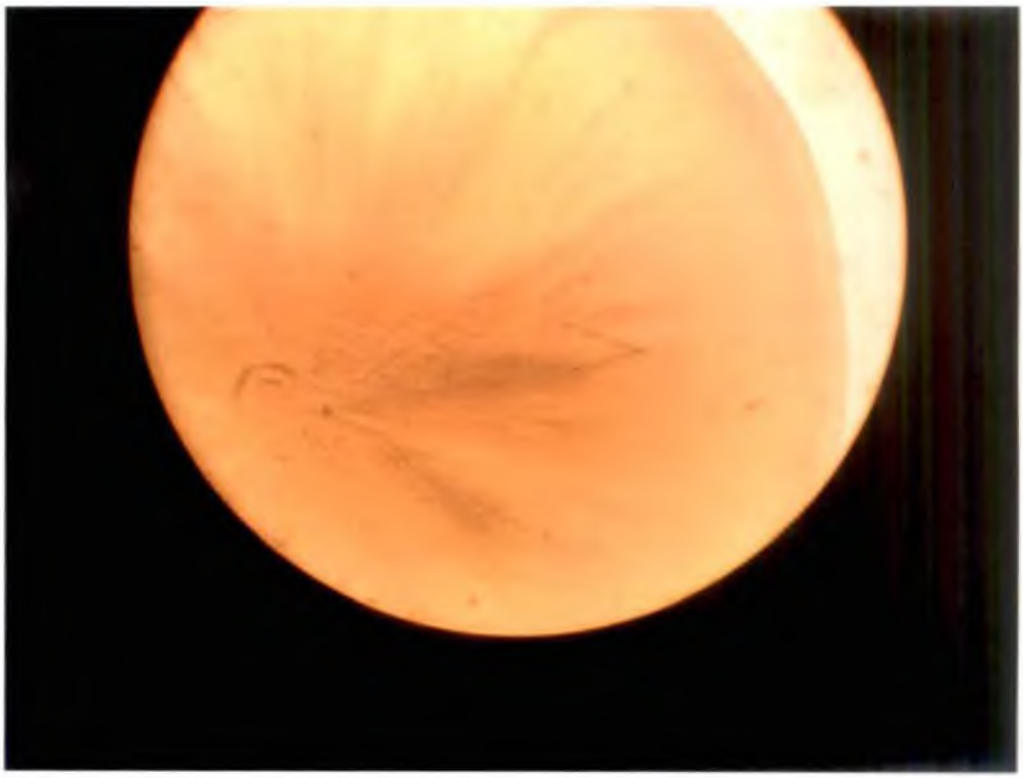


Fig. 9. 24 h old biofilm colony morphotype with radiating strands (10 x)

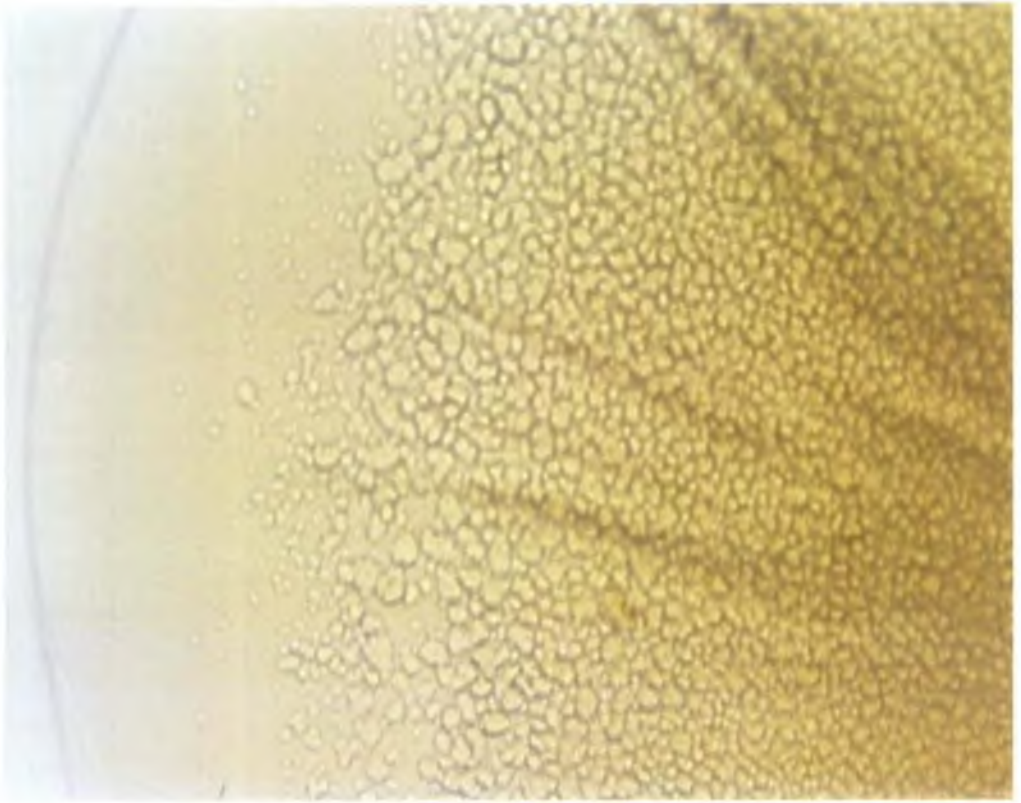


Fig. 10. 24 h old biofilm colony with radiating stands (40 x)

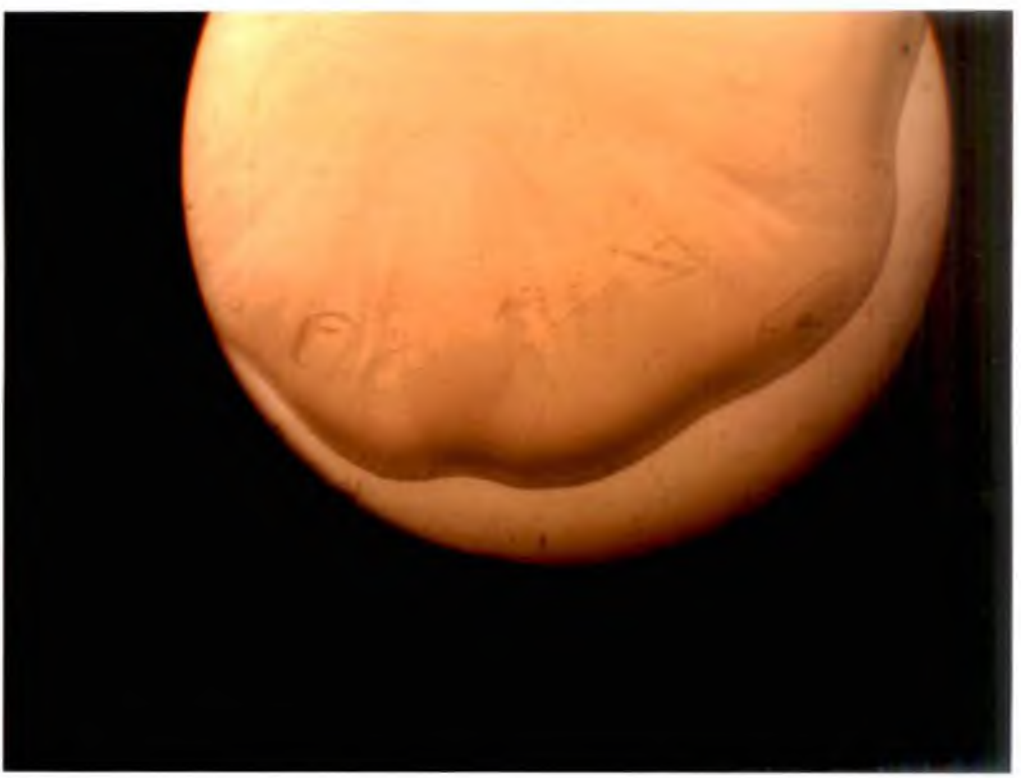


Fig. 11. 24 h old biofilm colony with radiating strands and wavy margin (10 x)

Table 5. LD_{50} in ducklings

Dilution	Organisms present in 0.1 millilitre of inoculum	No: of birds inoculated	No: died	No: alive	Cumulative value		Ratio + ve	% + ve
					+ ve	–ve		
10^{-1}	3×10^{7}	6	6	0	41	0	41/41	100
10^{-2}	3×10^{6}	6	6	0	35	0	35/35	100
10^{-3}	3×10^{5}	6	6	0	29	0	29/29	100
10^{-4}	3×10^{4}	6	6	0	23	0	23/23	100
10^{-5}	3×10^{3}	6	6	0	17	0	17/17	100
10^{-6}	3×10^{2}	6	6	0	11	0	11/11	100
10^{-7}	3×10^{1}	6	4	2	5	2	5/7	71.4
10^{-8}	3×10^{0}	6	1	5	1	7	1/8	12.5

$$\text{Proportionate distance} = \frac{71.4 - 50}{71.4 - 12.5} = \frac{21.4}{58.9} = 0.36$$

$$= 22.9 = 23 \text{ cells}$$

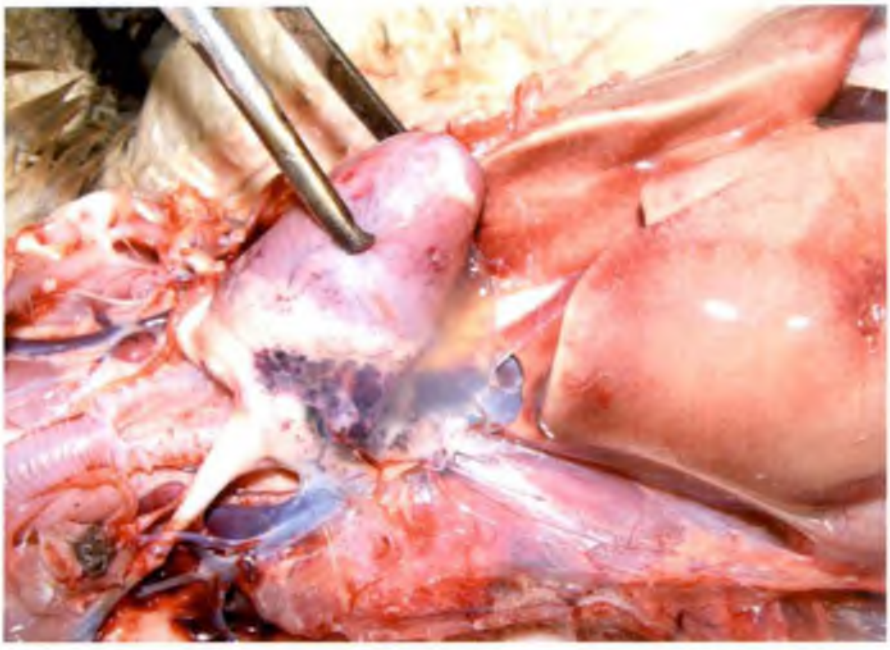


Fig. 12. Epicardial petechiae and hydropericardium in experimentally infected ducklings

Fig.13. Duckling - multiple pinpoint necrotic spots on liver.

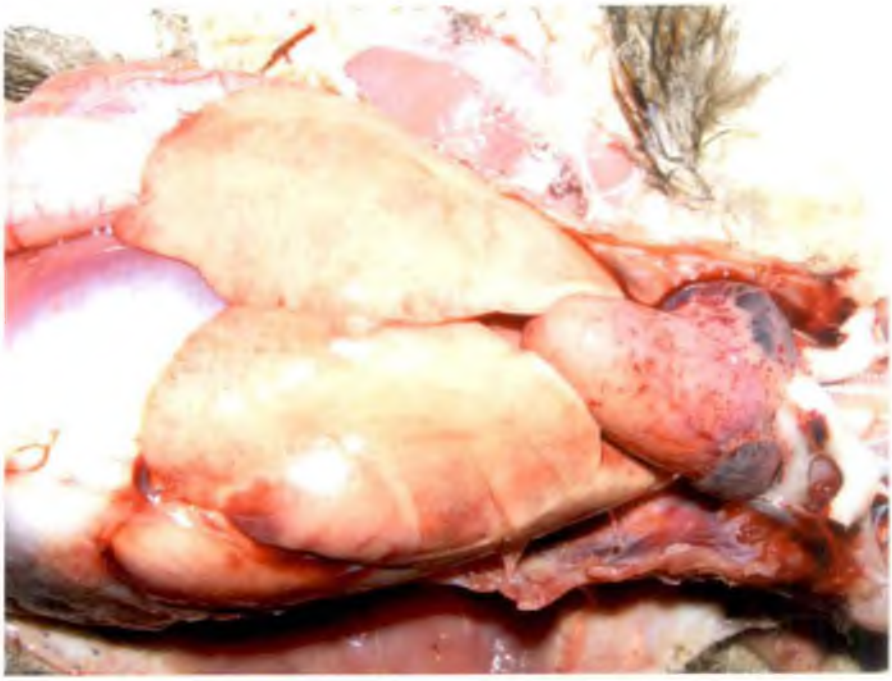


Fig.14. Duckling - petechiae on epicardium and extensive necrosis of liver

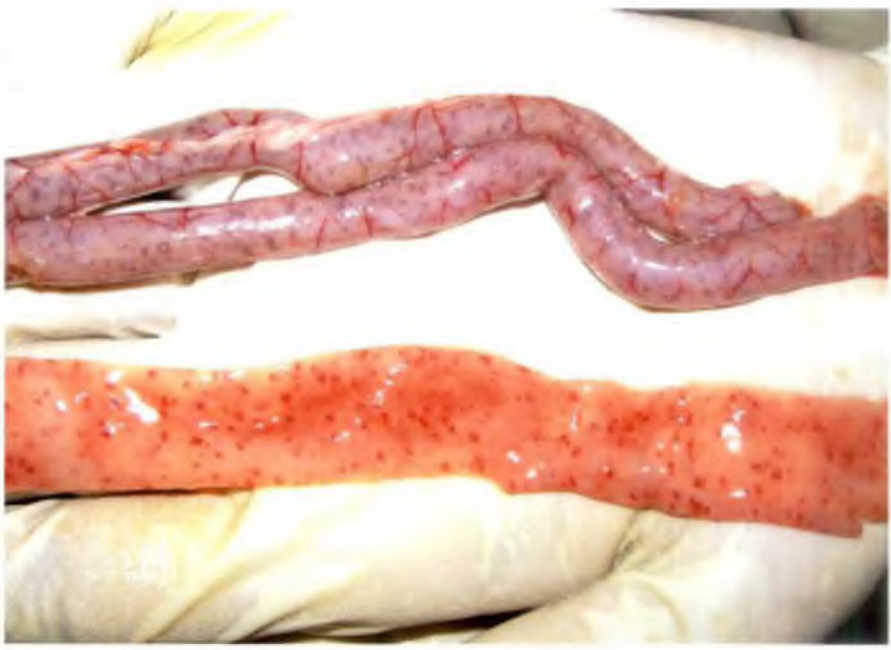


Fig.15. Multiple haemorrhagic spotsin the intestinal mucosa and serosa of experimentally infected ducklings

echymotic haemorrhages in intestinal serosa and mucosa (Fig. 15) and pulmonary oedema.

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

4.5.2 Lethal Dose 50 in Six Month Old Ducks

Initially when the LD_{50} testing was done in ducks with *P. multocida* whose virulence was enhanced by passage in mice, only one duck died which was given about 1.5×10^8 organisms/0.5 ml subcutaneously within the seven day observation period. The ducks did not succumb even when re-challenged with organisms whose virulence was further enhanced by two passages in ducks. Hence it was not possible to arrive at the median lethal dose. The results are presented in Table 6.

The dead duck revealed similar gross lesions as those observed in the case of ducklings. Additionally, extensive petechiation of the peritoneum was noticed (Fig. 16). Bipolar organisms were detected from blood smears and organ impression smears by Leishman's staining. The organism was isolated in pure culture on blood agar under five per cent carbon dioxide tension.

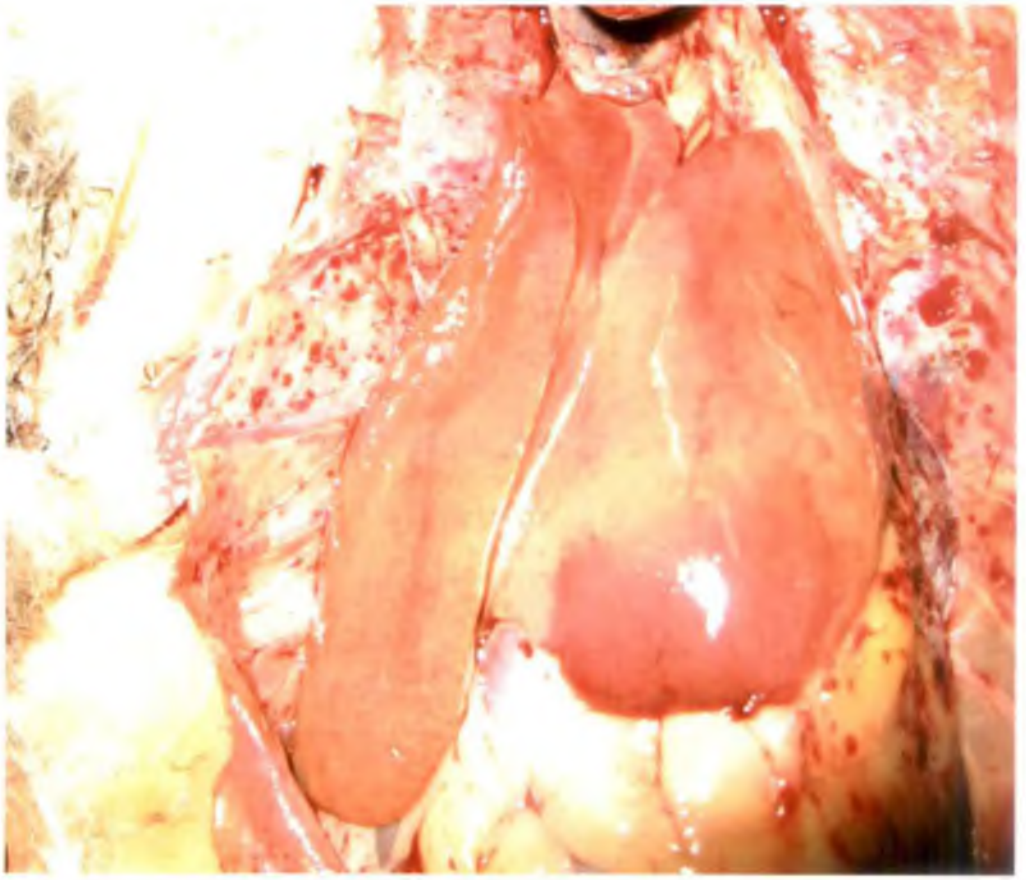
The live birds after 21 days were humanely sacrificed and performed post mortem examination, separately for each group. None of the birds revealed any of the gross lesions pertaining to avian pasteurellosis. Heart blood, liver and spleen cultured onto blood agar did not reveal any colonies.

PHA of the serum samples randomly collected from birds pre and post inoculation revealed slight antibody response (titre, 1 to 2 $\log_2$).

Table 6. LD50 in 6 month old ducks

Dilution	Organisms present in 0.5 millilitre of the dilutions	No: of birds inoculated	Dead/Live
10^{-1}	1.5×10^{8}	4	1/4
10^{-2}	1.5×10^{7}	4	0/4
10^{-3}	1.5×10^{6}	4	0/4
10^{-4}	1.5×10^{5}	4	0/4
10^{-5}	1.5×10^{4}	4	0/4
10^{-6}	1.5×10^{3}	4	0/4
10^{-7}	1.5×10^{2}	4	0/4
10^{-8}	1.5×10^{1}	4	0/4

Fig. 16 Necrosis of liver and extensive petechiation of peritoneum in experimentally infected ducks



4.6 PREPARATION OF DIFFERENT TYPES OF VACCINES

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

4.7 STERILITY TEST OF THE VACCINES

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

4.8 TOXICITY TEST OF THE VACCINES

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

4.9 POTENCY TESTING Of THE VACCINE

4.9.1 Passive / Indirect Haemagglutination (PHA / IHA)

The GA-SRBC could be stored at 4°C without haemolysis for the entire period of vaccine studies (two months) and the Sonicated antigen extracted from DP1 was found suitable for sensitization of fixed SRBC. The sensitized-GASRBC was intact without loosing its agglutinability for more than 3 weeks under refrigerated conditions.

4.9.1.1 PHA of Pre-Immunization Sera

Some of the pre-immunization sera randomly collected from the birds have shown titres ranging from 1 to 3 $\log_2$.

4.9.1.2 PHA of Vaccinated Birds

Antibodies were detected in the post vaccination sera as early as seven days in the first three vaccinated groups and the titre increased during subsequent days, though the pattern differed on different days. PHA showing the representative titres of each group on 42nd day PV were shown in Fig. 17.

4.4.1.2a Group I

Titres of most of the sera on day 7, 14, 21, 28 and 42 PV in ducks once vaccinated with oil adjuvanated ordinary bacterin ranged from 1 to 3 log_2 on day seven and 1 to 6 log_2 on days 14, 21, 28 and 42 PV.

4.4.1.2b Group II

The IHA titre of group II birds vaccinated with oil adjuvanated capsule enhanced bacterin on days 7, 14, 21, 28 and 42 ranged from 1 to 4 log_2, 1 to 6 log_2, 1 to 6 log_2, 1 to 7 log_2 and 1 to 6 log_2 respectively.

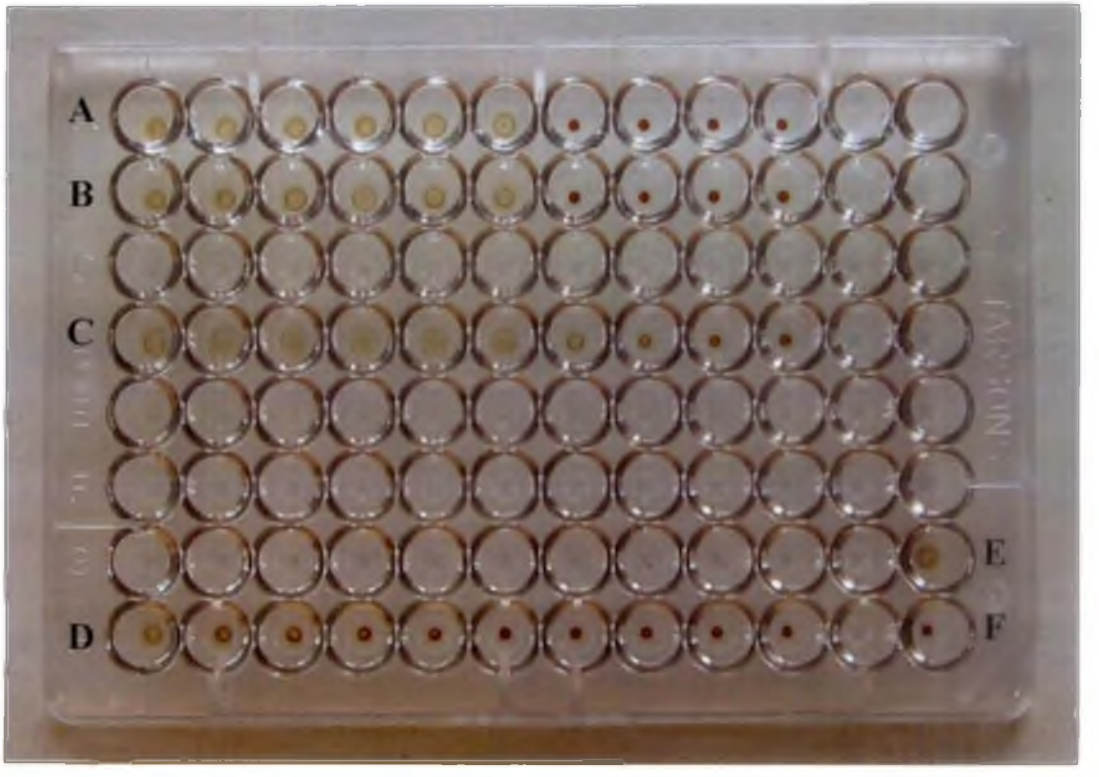
4.4.1.2c Group III

Titres of the sera on day 7, 14, 21, 28 and 42 day PV in ducks once vaccinated with oil adjuvanated biofilm vaccine ranged from 1 to 5 log_2, 1 to 8 log_2, 1 to 7 log_2, 1 to 6 log_2 and 1 to 6 log_2 respectively. The biofilm group had shown the highest titres at day 14 PV.

4.4.1.2d Group IV

The control group which was sham vaccinated with TSB did not reveal much antibody titres, with PHA values ranging from 1 to 3 log_2 irrespective of the days of collection.

Fig. 17. Passive haemagglutination test - day 42 PV



A : Group I
B : Group II
C : Group III
D : Group IV

E : Positive control
F : Negative control

4.9.1.2e Statistical Analysis

Analysis of variance (one way) of the logarithm of IHA tires of sera collected on different days from the ducks was done. The mean logarithmic (ML) IHA titres of individual sera collected at days 7, 14, 21, 28 and 42 PV are shown in Table 7.

There was no significant difference in mean titres among the groups during the 7th day. At day 14 PV biofilm group was significantly different from the rest of the two vaccine groups and the control. By day 21 and 28 all the vaccine groups were similar. At day 42, the biofilm vaccine group was found to maintain the titres while the titres of group I and group II decreased, making them significantly different. All the vaccine groups have shown significant difference from the control group at all the stages of the study. Group I and group II were having no significant difference in the mean titre during the entire study. The average PHA titres for the ducklings PV on different days are graphically represented in Fig. 18.

4.9.2 Homologous Challenge of Vaccinated Birds

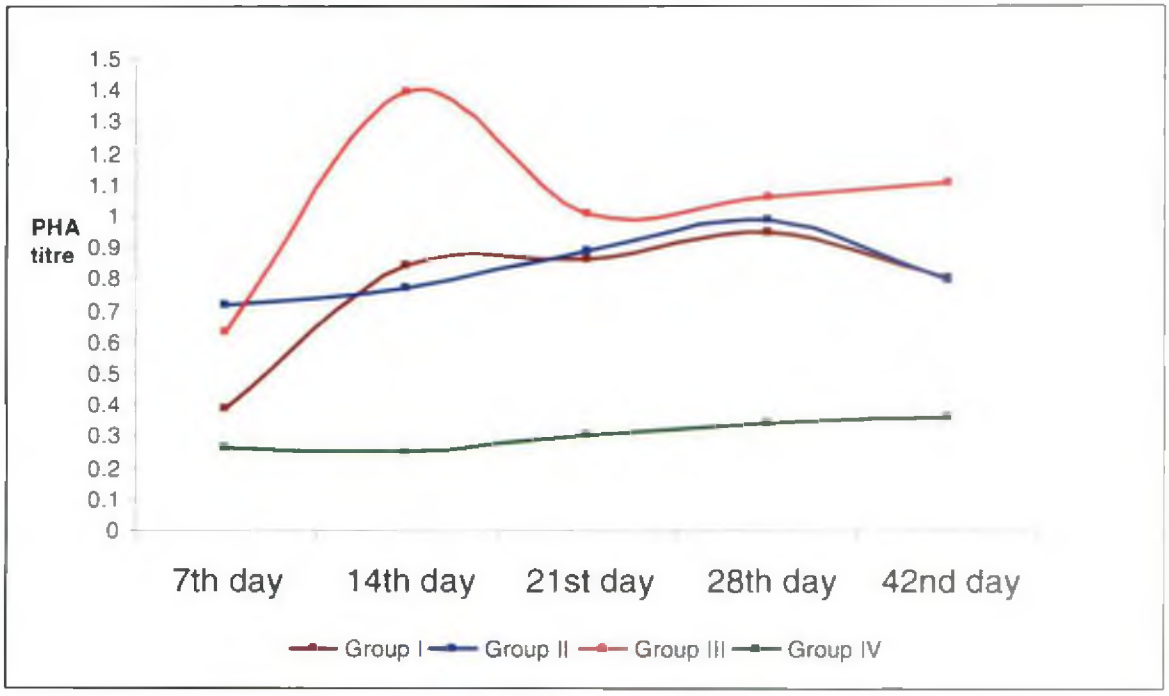
The vaccinated birds were challenged with virulent *P. multocida* A: 1 at a dose of 200 LD_{50} 28 days PV and with 100 LD_{50} 42 days PV. The results of challenge experiments are presented in Table 8 and 9.

The survivability of the birds of group I, II and III challenged with 200 LD_{50} dose on day 28 PV was 60 per cent, 60 per cent and 70 per cent respectively. The challenge dose killed 90 per cent of the control birds within 48 h.

The survivability of the birds of group I, II and III challenged with 100 LD_{50} dose on day 42 PV was 80 per cent, 80 per cent and 90 per cent respectively. The challenge dose killed 80 per cent of the control birds within 72h.

The control birds died within 48 to72 hours and revealed severe gross lesions characteristic of avian pasteurellosis. The blood smear and organ impression smears

Fig. 18 Comparative PHA titre of ducklings post vaccination



Group I – Ordinary Bacterin **Group II** – Capsule Enhanced Bacterin
Group III – Biofilm Vaccine **Group IV** – Control

Table 7. PHA titres of serum samples of vaccinated birds on different days PV

	Group I (Mean±SE)	Group II (Mean±SE)	Group III (Mean±SE)	Group IV (Mean±SE)
7th day*	0.386 ± 0.13	0.718 ±0.14	0.631 ± 0.18	0.266 ±0.1
14th day	0.84 ± 0.14^{b}	0.77 ± 0.16^{b}	1.397 ± 0.1^{a}	0.250 ±0.08^{c}
21st day	0.863 ± 0.06^{a}	0.889 ± 0.06^{a}	1.008 ± 0.08^{a}	0.302 ±0.05^{b}
28th day	0.946 ± 0.05^{a}	0.989 ± 0.06^{a}	1.057 ± 0.05^{a}	0.34 ±0.05^{b}
42nd day	0.801 ± 0.11^{b}	0.795 ± 0.09^{b}	1.108 ± 0.05^{a}	0.363 ±0.07^{c}

* - values are not significant ($p>0.05$)
- The values with same superscripts in a row are having no significant difference ($p>0.05$)

revealed high concentration of bipolar organisms. The culture of heart blood, liver and spleen on to bovine blood agar revealed typical colonies of *P. multocida.*

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

Table 8. Homologous challenge experiments - challenge with 200 LD_{50}

Group	Vaccination status	No. of days PV	No. of birds challenged	No. of birds survived	Percentage protection
I	Single vaccination	28	10	6	60
II	Single vaccination	28	10	6	60
III	Single vaccination	28	10	7	70
IV	Sham vaccination	28	10	1	10

Table 9. Homologous challenge experiments - challenge with 100 LD_{50}

Group	Vaccination status	No. of days PV	No. of birds challenged	No. of birds survived	Percentage protection
I	Single vaccination	42	10	8	80
II	Single vaccination	42	10	8	80
III	Single vaccination	42	10	9	90
IV	Sham vaccination	42	10	2	20

Discussion

5. DISCUSSION

Avian pasteurellosis is a septicaemic disease of poultry caused by avian strains of *P. multocida*. Among the birds, ducks are highly susceptible to pasteurellosis and it is a serious problem with high mortality and morbidity (Dougherty, 1953). The disease outbreaks occur more frequently during monsoon period and affect younger age groups more (Devi *et al.*, 2000). Because of its heavy toll, pasteurellosis is a major concern to the poultry industry.

The presently available live and killed vaccines are not capable of conferring a prolonged and total immunity against duck pasteurellosis. Several vaccine trials are going on with this aim and rapid strides have already been made on this aspect with the help of *in vivo* expression technology and protein mapping. Several newer vaccine candidates are to be identified, characterized and used as subunit vaccines. Even bacteria devoid of protoplasm-bacterial ghosts were reported to provide excellent protection (Marchart *et al.*, 2003).

Growth conditions could influence the antigenic profile and surface epitopes of an organism. Organisms grown in conditions simulating *in vivo* were proven to be superior in conferring immunity than the ordinary medium grown ones. Chicken embryo grown organisms have been successfully demonstrated to elicit better immune response (Dougherty, 1953; Heddleston and Rebers, 1973; Mariana and Hirst, 2000). The bacteria isolated from tissue fluid and blood were shown to provide broader immunity in terms of heterologous protection (Heddleston and Rebers, 1972) and this is hypothesized to be due to the expression of a putative cross protection factor which is having a dubious quality of getting expressed only *in vivo*. It is for sure that bacteria undergo some changes *in vivo* which we haven't yet understood completely, but these are of utmost importance for them to survive and also for us, to prevent them from overwhelming our body defense mechanisms.

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

The conditions employed 1. organisms grown in TSB (3 per cent) 2. organisms grown in TSB supplemented with 0.5 per cent yeast extract and 10 per cent FBS and 0.5 per cent yeast extract 3. organisms grown in TSB (0.32 per cent) supplemented with bentonite clay.

5.1 PURITY CHECKING AND CHARACTERIZATION

DP1 produced typical colonies of *P .multocida* on blood agar after incubation at 37°C for 24 h and were smooth, convex, translucent, and butyraceous. Gram staining revealed gram negative cocco-bacillary organism arranged singly or in pairs.

The biochemical reactions used for characterization of *P. multocida* (Barrow and Feltham, 1993) gave the expected results, confirming the identity of the organism. No growth on Mac Conkey's agar, negative for haemolysis on blood agar and urease activity were included as primary characters for *P. multocida* by Buxton and Fraser (1977). All these reactions were the same as above for DP1. It was catalase and oxidase positive and was fermentative. Heddleston (1976) reported that all the cultures of *P. multocida* he studied, fermented glucose and sucrose and also reduced nitrate, while none of the cultures fermented salicin or produced haemolysin. The reactions given by DP1 were in accordance with these findings.

Mutters *et al.* (1985) based on fermentation patterns of dulcitol and sorbitol categorized those positive for sorbitol but negative for dulcitol as *P. multocida* subsp. *multocida.* The reactions given by DP1 for these sugars were the same as described by them.

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). The isolate in this study gave a positive reaction for both indole and ornithine decarboxylase.

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

5.2 PATHOGENICITY TESTING IN MICE

When the pathogenicity of the confirmed *P. multocida* serotype A: 1 was done in mice, the mice inoculated with 0.3 x 10^8 organisms/0.1 ml intraperitoneally died within eight hours and within 24 h when injected by subcutaneous route.

Nakamine *et al.* (1992) found that the pathogenicity of *P. multocida* isolated from an outbreak in ducks was not very high when tested in chicken. So the selection of host on which the experiment is to be done becomes important. Contrastingly, Murugkar and Ghosh (1995) tested the pathogenicity 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 and the mice died within 12 h.

Collins (1976) proposed mice as animal of choice for pathogenicity testing of *P. multocida* and reasoned that the death of mice could be due to an overwhelming increase in the number of organisms in visceral organs. The gross lesions observed in the inoculated mice in this study were petechial haemorrhages in the epicardium and general congestion of all the visceral organs which was very much indicative of a severe septicaemic condition. This was also evident from the high concentration of bipolars in the visceral organs, following the examination of impression smears.

The virulence of the organism is dependent on the route of inoculation. Pehlivanoglu *et al.* (1999) observed that intravenous route of inoculation of *P. multocida* caused the highest mortality in ducks while the subcutaneous route caused

the lowest mortality. According to Collins (1976) infections in subcutaneously challenged mice developed more slowly than when the intravenous or intraperitoneal routes were used. Anupama *et al.* (2003) and Kapoor *et al.* (2004) reported that the *P. multocida* A: 1 isolate killed the mice in 24 h following subcutaneous injection. In the present study also similar results were arrived at. Though the subcutaneously challenged mice died only within 24 h and the infection was much slower than intraperitoneal route, the lesions developed were of similar intensity.

The DP1 isolate was able to kill the mice even by subcutaneous route, which is possible only if the isolate is considerably virulent.

5.3 CAPSULAR STUDIES

5.3.1 Capsule Enhancement

Most of the bacteria undergo extensive encapsulation *in vivo* and when the medium is supplemented with extra nutrients. Vermeulen *et al.* (1988) observed that the more enriched the medium of growth, the more capsular polysaccharide *E. coli* would produce. This nutrient mediated enrichment was vastly seen with the use of mammalian serum. Boyce and Adler (2000) used dextrose starch agar (DSA) containing six per cent avian serum in order to get *P. multocida* cells with maximum capsulation. Zaragoza *et al.* (2003) observed that serum induced capsule growth for *C. neoformans* at concentrations below five per cent was very small. In this study for capsule enhancement FBS was added at the level of 10 per cent to the medium.

In the present study also DSA supplemented with 10 per cent FBS was found to enhance the capsule production by *P. multocida.* The capsular polysaccharides (when glucose was used as the standard) increased by approximately 1.6 times, when the organism was grown in capsule enhancement media containing 10 per cent FBS. The enhancement was also studied with addition of 0.5 per cent yeast extract along

with 10 per cent FBS to the medium. This further increased the amount of capsule produced, by about 2.06 times the amount produced in ordinary media. Ryu *et al.* (1984) used brain heart infusion broth (BHIB) supplemented with yeast extract and 5 per cent serum for growing *P. multocida*. The effect of yeast extract on capsule enhancement is unknown. Increased carbon dioxide tension was also employed for these studies and this was found to have an effect on capsule production by *P. multocida*.

Microaerophilic condition induced a significant increase in capsular polysaccharide expression by pneumococcal isolates as per Weiser *et al.* (2001). Similar findings were also documented in case of *C. neoformans* by Zaragoza and Casadevall (2004). The results of the present study was also in accordance with these findings.

The effect of serum and carbon dioxide on capsule of *C. neoformans* was well documented by Zaragoza and Casadevall (2004). They opined that the organisms would encounter serum components in the course of infection; the phenomenon of serum-induced capsular growth may reflect the stimulus that induces the capsule during pathogenesis *in vivo*. Increased carbon dioxide also mimics an *in vivo* condition especially in lungs. This explanation becomes particularly significant when the organism is a respiratory pathogen like *P. multocida*.

Nutrient limitation was also attributed to cause enhancement of capsule (Zaragoza and Casadevall, 2004). When the capsular polysaccharide estimation was done for biofilm cells of *P. multocida* which were grown under nutrient limitation, the capsular polysaccharide content of biofilm cells was still higher, approximately 3.25 times than that of planktonic bacteria grown in DSA alone. This was again conceivable under the same explanation of *in vivo* stimulus since biofilm mode of growth is supposed to be increasingly similar to *in vivo* condition.

The colonies produced by *P. multocida* grown in capsule enhancement media kept under five per cent carbon dioxide tension were confluent and highly mucoid. These colonies have shown the play of colours or iridescence when observed under oblique light and this may be due to encapsulation. According to Smith (1958), the colonial iridescence was closely linked with capsule production of *P. multocida.*

5.3.1.1 Demonstration of Capsule

Capsule enhancement was assessed by demonstration of capsule by light microscopy.

A negative staining technique described by Maneval staining (1941) was employed for demonstration of capsule of DP1 grown under the capsule enhancement conditions and biofilm mode. Several workers had used this staining method for demonstration of capsule (Corstvet *et al.*, 1982; Tsuji and Matsumoto, 1988; Thies and Champlin, 1989; Stoderegger and Herndl, 2001; Watt *et al.*, 2003., Reeks *et al.*, 2004). This staining technique is a combined structural staining method, where the bacterial cell is stained with a basic dye and the background with an acidic dye, leaving the capsular envelope unstained. The stains used were congo red and acid fuchsin. The bacteria were stained red and background appeared in blue colour. This staining method was comparable to visualization of capsular polysaccharide by electron microscopic assessments of ferritin labeled *P. multocida* cells (Watt *et al.*, 2003) and to demonstration of capsule by fluorescent antibody technique (FAT) (Corstvet *et al.*, 1982).

The 18 h old culture of DP1 taken from DSA had shown capsule as a white unstained halo around the bacterial cell which had taken a red colour. The background appeared as blue or red. This may be due to the difference in the chemicals used for the preparation of the stain. It was observed that the capsule of the bacteria became more discernible when the density of the capsule increased. This was evidenced by the larger and clearer capsules of the organisms grown in capsule

enhancement media when compared to the organisms grown in DSA alone. Under capsule enhancement conditions all the bacterial cells revealed a clear capsule while some of them had extensive capsules. This kind of extensive capsulation was not seen in organisms grown under ordinary conditions.

Maneval staining of late logarithmic phase of three day old biofilm culture revealed heavily capsulated cells of *P. multocida* attached as large aggregates and appearing as chains of coccobacillary cells. Rezende *et al.* (2005) demonstrated similar capsulated cells forming chain during late logarithmic phase of *S. enterica* serovar *Typhimurium* DT 104 biofilm by India ink staining.

The heavily capsulated aggregated cells even resisted the penetration of stain so that the bacteria remained unstained in their thick exopolysaccharide covering. Maneval staining of the late logarithmic phase biofilm cultures also evidenced a change in morphology of the bacteria in that they formed a thick meshwork of aggregated and chain forming cells. This was in accordance with the findings of Prakash *et al.* (2005) who reported a refractoriness of *S. gallinarum* biofilm cells to uranyl acetate stain during electron microscopy and a change in architecture from rods to rounded forms.

There is contrasting reports on the presence of capsule and biofilm formation by different bacteria. Schembri *et al.* (2004) and Davey and Duncan (2006) proposed that the capsule physically interfered with biofilm formation while Martinez and Casadevall (2005) and Rezende *et al.* (2005) were of the opinion that the capsular polysaccharides played an important role in the formation of biofilms. No reports were available for *P. multocida*. However, in this study, the biofilm cells were extensively capsulated.

Corstvet *et al.* (1982) demonstrated the age dependency of capsular expression by broth grown *P. haemolytica*. Wide capsules were demonstrated on cells from 2 to 12 h cultures while on cells from 16 to 22 h cultures very little cell

associated capsular material were present. This age dependency was not experienced in this study, probably may be due to the use of solid medium instead of broth cultures.

5.3.1.2 Chemical Characterization of CCE

Carbohydrate estimation of the CCE of *P. multocida* cells extracted as per the method of Syuto and Matsumoto (1982) was done using phenol sulphuric acid method as described by Dubois *et al.* (1956).

Briefman and Yaw (1958) found that the major sugars present in the capsule of *P. multocida* were galactose and ribose. Revappa (1974) found that glucose and mannose were also present in the capsule of *P. multocida* in considerable amounts. So the estimation was done using these sugars as standards. The values obtained for DSA alone, DSA with serum, DSA supplemented with serum and yeast extract and biofilm cells were 43.34, 73.16, 89.56 and 140.73 µg/ml of CCE respectively, when glucose was used as the standard. The value of DSA alone was similar to Gentry *et al.* (1982) whose study revealed that the amount of carbohydrate present was 62 to78 µg/ml. Ryu *et al.* (1984) reported that the capsular extract of type A *P. multocida* contained 0.26-0.38 mg/ml of capsular polysaccharides. The values obtained by several workers are highly variable. This variation can be attributed to the strain used, conditions of extraction, extraction procedures employed and the method of estimation. In the present study, the values obtained are comparatively lower but capsule enhancement is very much obvious. The capsular polysaccharides increased by approximately 1.6 times when the organism was grown in capsule enhancement media containing 10 per cent FBS and by about 2.06 times when grown in media supplemented with FBS and yeast extract. The capsular polysaccharide content of biofilm cells was 3.25 times higher than that of planktonic bacteria grown in DSA alone.

5.4 BIOFILM ASSAY

Biofilm formation by bacteria is a well choreographed event and is dependent on many factors. Minor variations in the conditions employed can result in conspicuous difference in attachment process. Several substrates are used for triggering the bacteria to attach which may be hydrophobic or hydrophilic. Some of them are polystyrene or PVC plates (Wakimoto *et al.*, 2004; Parizzi *et al.*, 2004), flow cells (Filoche *et al.*, 2004), chitin flakes (Nalin *et al.*, 1979), stainless steel (Giaouris and Nychas, 2006) and glass surfaces (Rezende *et al.*, 2005).

In this study bentonite clay (0.3 per cent) was used as the substrate for bacterial attachment and TSB (0.32 per cent) as the medium of growth which was in accordance with Vadakel (2001).

Tryptone soya broth was recommended as the medium of growth for inducing slime production in case of *S. epidermidis* by Christensen *et al.* (1982) when other media like brain heart infusion broth were not able to support attachment of bacteria consistently. In this study, TSB was found to support the attachment of *P. multocida* onto bentonite clay. Bentonite clay, an inert hydrophilic material which forms a gel when mixed with water is cheap and easily available.

In this study, conditions of nutrient limitation and intermittent aeration was applied on biofilm cultures. Heukelekian and Heller (1940) stressed the importance of aeration along with a surface for bacterial growth in dilute solutions. Krieg *et al.* (1986) observed that aeration of continuous culture of *P. aeruginosa* caused heavy polysaccharide production and selection of the mucoid phenotype. So for uniform mixing of bentonite clay particles and for proper aeration, the biofilm cultures were shaken six times daily in an orbital shaker, each time shaking for a period of one hour.

The cultures were incubated at 42°C for a maximum of six days. Hugar (2004) observed biofilm formation of *P. multocida* at 42°C and nutrient limitation

with the expression of some heat shock proteins. To exploit this expression of novel proteins the biofilm cultures in the present study was incubated at 42°C. More over it simulated the natural body condition of birds.

In the present study the biofilm cells reached a peak on the third day of incubation with an average count of 1.54×10^6 CFU/g of bentonite clay while the planktonic cells were found to be maximum on day one post inoculation with a peak count averaging about 8.10×10^8 CFU/ml of the broth. The peaking on the third day of incubation was in accordance with the finding of Vadakel (2001). But the attachment capabilities of the bacteria was found to be slightly lower when compared to the biofilm count of 1.5×10^{10} CFU/g of bentonite clay as described by Vadakel (2001). Vadakel (2001) also reported a rapid decline of planktonic cells during the subsequent days of incubation which was not observed in this study. Similar finding was reported by Anwar *et al.* (1989) for *P. aeruginosa* that both biofilm and planktonic population remained constant throughout the study of seven days.

Vibrio vulnificus attachment to microtitre plate was enhanced with increased cell surface hydrophobicity (Joseph and Wright, 2004). Thies and Champlin (1989) found capsulated strains of *P. multocida* to be hydrophilic and repeated sub culture reduced encapsulation with a concomitant increase in hydrophobicity. Biofilm formation involves the production of exopolysaccharide. Borrucki *et al.* (2003) correlated biofilm formation by *Listeria monocytogenes* and its exopolysaccharide (EPS) production. Slime and capsule are very difficult to differentiate and so the heavy encapsulation of biofilm cells in this study can be extrapolated to the production of exopolysaccharide which is characteristic of biofilm formation. Circa *et al.* (2005) observed no relationship between bacterial surface hydrophobicity of *S. epidermidis* biofilm and extent of initial binding to substrates. It is again variable with bacterial species under study.

Circa *et al.* (2005) reported that substratum hydrophobicity influenced the initial attachment of *S. epidermidis*. Bentonite clay is hydrophilic but was found to trigger attachment of bacteria. Surface characteristics of the material are undefined. So further studies are required for elucidating the actual potential of bentonite clay as a substrate for bacterial attachment. According to Vandevivere *et al.* (1993) soil bacteria formed very large clumps comprising 50 to 500 cells in sand columns, at an attached cell density of 2×10^8 cells/g of sand. Similar clumps of bacterial cells were seen in this study also which was demonstrated using capsule staining.

Olson *et al.* (2002) opined that for biofilm formation of *P. multocida* in TSB, supplementation with two per cent fetal bovine serum (FBS) and 10 per cent carbon dioxide tension was necessitated. By five hours the planktonic cells reached 5.5×10^9 CFU/ml and biofilm cells 1.5×10^5 CFU/peg. The viable count they obtained was in accordance with the results of the present study except for the time of incubation. Further studies are required for quantification of biofilm formation of DP1 strain *in vitro*.

The results indicated that *P. multocida* was not a classical biofilm former but it could form biofilm *in vitro*.

5.4.1 Comparison of Colony Morphology of Biofilm and Planktonic Cells

Under biofilm mode of growth morphological variations can occur which may even be heritable to next generation, giving rise to different morphotypes. Such morphotypes were described for several bacterial species but no such literature is for *P. multocida*.

Pasteurella multocida on nutrient agar produced circular, convex, amorphous, greyish yellow and translucent colonies with a smooth, glistening surface and an entire edge (Holmes, 1998). Rhoades and Rimler (1991) described the different colonial morphology of *P. multocida* isolated from birds with fowl cholera.

The colonies obtained were iridescent, sectored with various intensities of iridescence or blue with little or no iridescence.

In this study, some of the biofilm colonies on nutrient agar consistently showed variation in colony morphology from that of planktonic *P. multocida.* The biofilm colonies were characterized by radiating strands from centre to periphery. It resembled the sectored colony type of *P. multocida.* The colonies were smooth and transluscent but the central portion of the colony showed opacity. Some of the colonies were having an undulating margin instead of the entire one of planktonic cells.

Rhoades and Rimler (1991) described a mutant colony type which produced gray colonies and were derived from the blue colonies of *P. multocida* while sub culturing. The cells of these colonies were acapsular, avirulent and were arranged exclusively as chains of individual cells. Biofilm cells also revealed chain formation though not in nutrient agar, but were capsulated and virulent.

Fine *et al.* (1999) described *Actinomyces actinomycetemcomitans* producing rough colonies with characteristic central internal star and this colony morphology after 65 passages changed to smooth colonies lacking the internal star indicating that it was a stable change. The colony morphotypes produced in this study were not stable and were lost on subsequent subcultures and there after were similar to planktonic colonies. So isolation and further characterization was not possible.

5.5 LETHAL DOSE 50

5.5.1 Lethal Dose 50 in Duckling

Virulence of the pathogen is measured in suitable experimental animals, the end result of which is death and this is an integral part of any vaccine study as it is required for computing the challenge dose during the potency testing of the vaccine. Fifty per cent lethal dose (LD_{50}) was described as a practical and reliable measurement of pathogenicity by Cruickshank *et al.* (1975).

Median lethal dose was determined in ducklings after the *in vivo* passaging in mice to increase the virulence of the organism. The LD_{50} of the isolate was 23 cells and the dilution giving 50 per cent end point was $10^{-7.23}$. This was exactly similar to the results obtained by Jayakumar (1998).

The LD_{50} of duck isolates of *P. multocida* was estimated in mice and it was found to be $10^{-7.83}$ which contained 14.96 viable cells per dose (Swamy, 1994). Ramanatha *et al.* (1995) determined LD_{50} of *P. multocida* A: 1 in mice and obtained a mean value of 14.32 + 0.083 CFU.

The dead birds revealed all the classical lesions of fowl cholera on post mortem examination and the blood smears and organ impression smears revealed bipolar organisms. The gross lesions observed were in agreement with Jayakumar (1998). Similar findings were described by Rhoades and Rimler (1991) and Shilpa *et al.* (2005). All the dead birds have shown acute signs of pasteurellosis and died within two to three days.

5.5.2 Lethal Dose 50 in Six Month Old Ducks

In the present study, it was unable to arrive at the median lethal dose in six month old ducks as only one duck died after 48 h, which was inoculated subcutaneously with 1.5×10^8 CFU/0.5 ml of inoculum. Initially the isolate was inoculated after an *in vivo* passaging in mice. The birds were observed for seven days for any mortality but none of the other birds died.

According to Matsumoto and Strain (1993) *P. multocida* serotypes 3 and 4 were able to increase their pathogenicity by bird to bird transmission in a short period of time. The encapsulated original isolate revealed a mean infectious dose of more than $10^{8.2}$ CFU which after five passages produced 67 per cent mortality with a 10^2 CFU dose.

Then the isolate was passaged in ducks twice and later re-challenged the different groups of birds with the same dose. The ducks did not succumb following

the second virulent challenge through out an observation period of 21 days. This may be due to the innate resistance of the breed rather than due to the pre-formed antibody response. Passive haemagglutination test of the post inoculation sera of the challenged and control birds were not having significant amount of antibodies with which they could resist the challenge. The inoculated *P. multocida* would have been cleared from the system as evidenced by the failure of re-isolation of the organisms from the organs of inoculated birds. The birds also had not shown any signs of chronic pasteurellosis.

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

5.6 PREPARATION OF DIFFERENT TYPES OF VACCINES

The effective control of fowl cholera in birds can be achieved only by developing a broadly effective vaccine against the local strains. Production of fowl cholera vaccine demands careful safety testing in the laboratory and potency testing.

Carter (1961) insisted the use of virulent capsulated organisms for the preparation of fowl cholera vaccine. Woolcock and Collins (1976) found that protection with adjuvant treated *Pasteurella* preparations was always superior to that seen with non-adjuvanated vaccines.

There are many adjuvants which can be incorporated into fowl cholera vaccines. Lalrinliana *et al.* (1988) conducted studies to compare efficacy of different adjuvants employed in *P. multocida* vaccines and the highest antibody titre was observed with oil adjuvant vaccines while the alum precipitated and aluminum hydroxide gel vaccines induced low titres. Iyer *et al.* (1955) used liquid paraffin (10 parts) and lanolin (one part) to adjuvanate 15 parts of *P. multocida* bacterin. A similar combination was used in the present study. Very few vaccines are available

against pasteurellosis in ducks in India and the avian vaccines usually necessitate booster as the duration of immunity conferred will be for a short period.

In the present study oil adjuvant inactivated vaccine was prepared from three different growth conditions. Tryptone soya broth (3 per cent) was used as the medium of growth for ordinary bacterin, TSB (3 per cent) with 10 per cent FBS and 0.5 per cent yeast extract. Since capsule production was reported to be age dependent, an early logarithmic culture before 12 h of incubation was used for capsule enhanced bacterin. The cultures were continuously shaken at 50 rpm for aeration. TSB supported the growth of the organisms and aeration notably increased the yield of the cells. Yeast extract was also having an effect in increasing the yield of the bacterial cells.

For biofilm vaccine preparation, the bacteria were first grown in biofilm mode and then they were once subcultured on to nutrient agar and those cells were harvested for vaccine preparation. This method was followed because it was not possible to produce sufficient amount of intact biofilm cells per dose of vaccine (3×10^9 cells/ml), since the bacterial count in the biofilm was at the level of 3×10^6 cells/ml.

According to Harry and Deb (1979) formalinization was the most suitable method of inactivation of *P. anatipestifer* for vaccine preparation. The bacterin prepared in this study was successfully inactivated with 0.5 per cent formol saline after an incubation period of 72 h at room temperature. Even the biofilm cells which were supposed to be more resistant to chemical agents were effectively inactivated by formalin. A formalin treated antigen of *P. multocida* was found to be the most immunogenic in mice, out of the variously treated antigens and even conferred a low degree of cross protection against heterologous challenge (Kim *et al.*, 1986).

The vaccine was prepared as per the method of Stone *et al.* (1978). The composition of vaccine was fixed according to Jayakumar (1998). The mixture was

tested for the emulsion type using drop test. The vaccine so prepared by homogenization at 18,000 rpm for 30 sec was not thick and was easier to administer. The oil phase of vaccine constituted by lanolin and liquid paraffin is cheaper and easily available.

Since *P. multocida* is very pathogenic to ducklings, toxicity testing of the prepared vaccine is very important. Toxicity studies carried out by injecting exact and double doses caused no ill effects both locally and systemically.

5.7 POTENCY TESTING

Harry and Deb (1979) prescribed the dose of the vaccine as 3×10^9 cells for *P. anatipestifer,* when administered intramuscularly. So this concentration of vaccine was used for the potency testing in ducklings in the present study.

The potency of oil adjuvant whole cell vaccines was doubted by many scientists. Dougherty (1953) came to the conclusion that inoculation with chemically killed broth culture bacterins conferred poor protection to ducklings from lethal challenge. Bierer and Derieux (1972) opined that oil based bacterin alone was effective in preventing infection in turkeys but was not as good as avirulent live vaccine and the use of bacterin prior to the use of live vaccine proved more promising.

Conversely, many reports suggest that oil adjuvanated bacterins elicit good immune response. Dua and Pandurangarao (1978) observed that very high levels of antibodies were induced by oil adjuvanated bacterin when compared with the live vaccines in turkeys. Onet *et al.* (1994) vaccinated turkeys with different types of *P. multocida* vaccines and found that best protection was conferred by the inactivated 20 per cent oil adjuvanated vaccines. Shilpa *et al.* (2005) found that the formalin killed vaccine prepared from local isolate of *P. multocida* was better than OMP and commercial vaccine in layer chicken.

Glisson *et al.* (1990) evaluated different injection sites in turkeys for *P. multocida* bacterin and there were no differences in the immune response elicited irrespective of the injection sites. Akand *et al.* (2004) indicated that intramuscular route of vaccination produced better results than subcutaneous route of vaccination. Since the intramuscular route of administration was easier and much faster to perform, vaccinations were done by that route.

In the present study, the three types of vaccines were injected to one month old ducklings by intra muscular route and blood samples were collected at weekly intervals upto day 28 PV and on day 42 to perform PHA test.

5.7.1 Passive Haemagglutination Test

Individual blood samples from the birds of all the four groups were collected on different days and assessed the antibody titre by PHA. The test was done as per Sawada *et al.* (1982) using GA-SRBC. The GA-SRBC was found to be stable during the entire period of study (2 months) when kept under refrigerated conditions which was in accordance with Sawada *et al.* (1982), Ramanatha (1994) and Jayakumar (1998). Also in the present study the sensitized GA-SRBC maintained its agglutinability for about a month. Sawada *et al.* (1982) stated that sensitized GA-SRBC can be stored for atleast one week. This provided uniformity to the study as GA-SRBC of the same lot can be used for the entire study.

The IHA titres obtained for biofilm vaccine group on day 14 was very much higher than the other two groups. The antibody titre was observed from day 7 onwards for all the groups. The antibody titre of group I and group II increased during the 21 and 28 days PV. The highest titre obtained was 256 during the day 14 PV for biofilm group. The antibody titre of biofilm group slightly reduced after day 14 PV but was able to maintain a reasonable titre till day 42 PV. The titre of other two groups decreased from 28 to 42 days PV. The values obtained for Jayakumar (1998) was higher but the pattern was almost similar. Ramanatha (1994) got similar

mean logarithmic titres as that of this study and opined that the antibody titre was considerably increased by giving a booster dose of vaccine at 24 days interval. Akand *et al.* (2004) obtained a similar pattern of antibody response for washed cell fowl cholera vaccine in chicken but they got a prolonged response upto day 42. In this study only the biofilm group was able to maintain its titre upto 42nd day. Similar results as that of biofilm group in this study was obtained for Confer *et al.* (1998) when they used a killed commercial vaccine against *P. haemolytica* in cattle to determine the duration of serum antibody responses after single vaccination and revaccination. Single vaccination stimulated antibodies from day seven to fourteen and then declined until revaccination. Revaccination on days 28 and 140 stimulated anamnestic response and the antibodies remained significantly increased for up to 84 days post re-vaccination. So it was evident that a booster dose with the biofilm vaccine on 21 days PV would have elicited a much higher immune response. Kedrak and Borkowska-Opacka (2003) evaluated subunit vaccines comprising IROMPs (Iron Regulated Outer Membrane Proteins) of *P. multocida* serotype A: 1 in calves and found that antibodies against OMPs could be detected as early as seven days, which increased upto 14 days PV.

In this study the capsule enhanced vaccine could not elicit a better response although it managed to match the ordinary bacterin. Crude Capsular material was shown to elicit better immune response as in the studies of Gupta *et al.* (1999), Kodama *et al.* (1981) , Sharma *et al.* (1999). Oil adjuvant capsular vaccine was found to be more immunogenic in chicks than oil adjuvant whole cell vaccine by Gupta *et al.* (1999). Kodama *et al.* (1981) showed that young adult turkeys were protected at the rate of 80 per cent or more by cell free CCA while purified antigen induced little protection and suggested that polysaccharide protein complex might be responsible for protection. Sharma *et al.* (1999) compared saline extract and bacterin vaccine against *P. multocida* in mice and goats and saline extract vaccine was found to be superior in terms of protection and PHA titre. Crude capsular extract contains

proteins in addition to capsular polysaccharides. From this it is evident that capsular polysaccharides are not so immunogenic but constitutively along with proteins and lipopolysaccharide it elicits better response. So there is not much advantage in increasing the capsular content of the organism and subsequently using them as vaccine. By capsule enhancement probably only the content of capsular polysaccharides is increasing and not the proteins. Measurement of protein content of the CCE obtained from organisms under capsule enhancement would have given a clearer picture, but it was unable to do in this study.

In the present study, analysis of variance (one way) of the logarithm of IHA titres of sera collected on different days from the ducks was done. There was no significant difference in mean titres among the groups during the 7^{th} day. At day 14 PV biofilm group was significantly different from the rest of the two vaccine groups and the control. By day 21 and 28 all the vaccine groups were similar. At day 42, the biofilm vaccine group was found to maintain the titres while the titres of group I and group II, decreased making them significantly different. All the vaccine groups have shown significant difference from the control group at all the stages of the study. Group I and group II were having no significant difference in the mean titre during the entire study.

5.7.2 Homologous Challenging

The challenge results have shown that the biofilm group has given 10 per cent more protection than the other groups when challenged with 100 and 200 LD_{50} doses.

Swamy (1994) reported 100 percent survival in twice vaccinated ducks and only 57.1 per cent in once vaccinated ducks after challenging with 100 mouse LD_{50} dose of 1786 viable *P. multocida* organisms, stressing the necessity of a booster vaccination. In this study also the ducks once vaccinated with ordinary and capsule enhanced bacterin have shown only 60 per cent protection while the biofilm vaccine

group showed 70 per cent. Jayakumar obtained 100 per cent protection in once vaccinated birds during 20, 40 and 60th post vaccination while by about 80 days only 70 per cent protection. Akand *et al.* (2004) reported 80 per cent protection in chickens vaccinated with washed cell fowl cholera vaccine containing 5.7×10^7 CFU/ml. In the present study, similar results were obtained for ordinary and capsule enhanced bacterin group when challenged with 100 LD_{50} dose on day 42. Biofilm group when challenged with 100 LD_{50} dose on day 42 have given a protection rate of 90 per cent.

Islam *et al.* (2004) vaccinated ducks with alum precipitated formalin killed fowl cholera vaccine and observed that the immunized ducks with PHA titre less than 1: 64 died on virulent challenge exposure. In the present study, correlation between PHA titres and protection rate was not assessed, but biofilm group which showed more titre exhibited better protection rates. Also more number of birds survived when the challenge dose was only 100 LD_{50}. So there may be a positive correlation between antibody titre and protection.

From all these results it can be concluded the biofilm vaccine was the best among the three vaccines tried. Biofilms exhibit many novel proteins (Murphy and Kirkham, 2002; Mcdermid *et al.*, 1993 and Welin *et al.* 2004) and other immunodominant epitopes and presentation and familiarization of such epitopes to the host body may be one reason for the better immune response. Biofilms are increasingly similar to *in vivo* growth and it is an established fact that in vivo grown organisms exhibit the cross protective factor. This fact is corroborated by evidence of better immune response elicited by *P. multocida* grown under *in vivo* conditions like embryonated eggs, host blood and tissue fluids (Dougherty, 1953; Heddleston and Rebers, 1972. and Heddleston and Rebers, 1973).

Ward *et al.* (1992) examined the growth of *P. aeruginosa* biofilm on peritoneal implants in rabbits and opined that pre-immunization with formalin

killed whole cells of *P. aeruginosa* did not reduce the bacterial growth despite high levels of specific IgG indicating that preimmunization did not protect the host from colonization and chronic infections caused by the bacteria. This signifies the importance of familiarizing the host body with antigens specific to biofilm which may not be present in planktonic bacteria.

Martinez and Casadevall (2005) observed that monoclonal antibodies (MAbs) to *C. neoformans* biofilm polysaccharide could inhibit biofilm formation, which in turn denoted the critical role of adaptive humoral immune responses in preventing microbial biofilms following an infection. They envisioned that the specific immunoglobulin will prevent capsular polysaccharide release and thus block the adhesion of yeast cells to surfaces. So by using biofilms with such exopolysaccharide expressed as vaccine, we will be able to block the initial step of any infection- the adherence and subsequent colonization.

Shivaraj (1998) reported an exceptional 83.33 per cent protection for a biofilm based oral vaccine for *E. coli* biofilm while conventional vaccine could induce only 33.33 per cent protection.

Azad *et al.* (2000) demonstrated the uptake and processing of *Aeromonas hydrophila* vaccine antigens post vaccination with biofilm vaccine. The overall quantity of antigen available at different organs was greater with biofilm vaccine and the longer retention of the biofilm antigens was attributable to the larger size of biofilm flocs and protection offered by glycocalyx covering. They correlated the longer retention of antigens to enhanced serum agglutination titres and greater protection recorded post vaccination. Glycocalyx of biofilm could serve as a microcapsule and helps to deliver the immunogen at immune responsive sites and persist for long time *in vivo*. In the present study also the immune response was more prolonged for biofilm vaccine and more protection conferred which is attributable to the longer retention of antigens inside the body of the host.

The present study is only a preliminary investigation which provided some outlooks on the biofilm formation of *P. multocida* and the utility of biofilm as vaccine. The results of the study prove promising and warrant elaborate investigation. Outer membrane subunit vaccines were found to be effective by various researchers and it would be more effective if the outer membrane proteins of biofilm cells are used. In the present study, production of sufficient quantity of biofilm for vaccine preparation was found to be difficult. So newer techniques are to be devised for large scale production of biofilm cells. In the present study, the birds were examined only for 42 days and we did not know for how long the immune response will last by single vaccination. Booster doses were not given in the study which also can be done in future studies. One real advantage of the biofilm vaccine will be its ability to induce cross protection which is also not assessed here. Extrapolation of the laboratory study to field and better results from the field trial will actually decide the prospects of biofilm vaccine.

Summary

SUMMARY

The present day whole cell vaccines against pasteurellosis in ducks often produce poor immunogenicity and shorter durations of immunity. Also, continuous medication with prophylactic levels of antibiotic is not cost effective. Hence, a research work was undertaken to prepare three different types of vaccines using *P. multocida* grown under different conditions and compare their efficacy in ducklings.

The purity checking of the *Pasteurella multocida* A: 1 strain (DP1) isolated from Niranam Duck farm (Pathanamthitta district), serotyped at IVRI, Izatnagar and maintained in the Department of Veterinary Microbiology, COVAS, was done as per standard procedures. The isolate gave all the specific reactions of *P. multocida*.

Pathogenicity testing of the isolate was done in six to eight weeks old mice by inoculation via two different routes *viz.*, intraperitoneally and subcutaneously. The isolate killed the mice inoculated with 0.3×10^8 organisms per 0.1 ml intraperitoneally within eight hours and within 24 h when injected by subcutaneous route while the control mice stayed alive throughout the observation period of seven days. The gross lesions observed were the same for both the routes, excepting fluid accumulation in the peritoneum which was noticed only in the case of intraperitoneal inoculation. The organism could be re-isolated from the dead mice.

The organism was grown in different media to assess the amount of capsular material produced. The media employed were DSA, DSA supplemented with 10 per cent FBS and DSA supplemented with 10 per cent FBS and 0.5 per cent yeast extract. The capsule enhancement media were kept under five per cent carbon dioxide tension. All the plates were incubated at 37°C for 18 h. The capsule enhancement was measured by capsule demonstration using Maneval staining and by characterization of crude capsular extract. The capsular extraction was done by hot

saline extraction using 2.5 per cent sodium chloride solution. The capsules of the organisms grown in capsule enhancement media when demonstrated using Maneval staining were discernibly larger and denser, when compared to the organisms grown in DSA alone. Capsular polysaccharides of the saline extract were estimated using phenol sulphuric acid method, employing different sugars such as glucose, galactose, mannose and ribose as standards. The carbohydrate content of the CCE from organisms grown in DSA alone, DSA plus serum and DSA plus serum and yeast extract were 43.34, 73.16, 89.56 µg/ml of CCE respectively (when glucose was used as standard). The capsular polysaccharides increased by approximately 1.6 times when the organism was grown in capsule enhancement media containing 10 per cent FBS and by about 2.06 times when grown in media supplemented with FBS and yeast extract. The enhancement was similarly seen when the other standard sugars were used for estimation.

The *in vitro* biofilm formation of the organism was assessed by growing it under nutrient restricted conditions. The organism was grown in TSB (0.32 per cent) supplemented with 0.3 per cent bentonite clay. The culture after inoculation with an inoculum containing 3×10^9 bacteria/ml was incubated at 42°C for one, three and six days separately. The flasks were agitated six times daily for a duration of one hour each time, in an orbital shaker set at 50 rpm. The quantification of biofilm and planktonic cells was done by performing plate count by spread plate method. The results showed that the biofilm cells reached a peak on the third day of incubation with an average count of 1.54×10^6 CFU/g of bentonite clay while the planktonic cells were found to be maximum on day one post inoculation, with a peak count averaging about 8.10×10^8 CFU/ml of the broth.

Maneval staining of late logarithmic phase of three day old biofilm culture was done and it revealed heavily capsulated cells of *P. multocida* attached as large aggregates and appearing as chains of coccobacillary cells. Also they appeared as a meshwork of aggregated and chain forming cells. The capsular polysaccharide

estimation of biofilm cells was also done employing the same method as above and obtained a value of 140.73 µg/ml of CCE which was 3.25 times than that of planktonic bacteria grown in DSA alone.

The biofilm cells on nutrient agar after 24 h at 37°C produced some colony morphotypes characterized by radiating strands from centre to periphery. Some of the colonies were having an undulating margin different from that of the entire margin of planktonic bacteria.

Median lethal dose (LD_{50}) of *P. multocida* was determined in one month old ducklings and also in six month old ducks.

Four week old ducklings were randomly assigned to nine groups of six ducklings each and the first eight groups were separately inoculated with the different dilutions of the fully encapsulated virulent bacteria with a dose of 0.1 millilitre per bird subcutaneously at wing web and the ninth group served as control which was sham inoculated with 0.1 millilitre of sterile PBS. The LD_{50} of the isolate was found to be 23 cells and the dilution giving 50 per cent end point was $10^{-7.23}$. All the dead birds revealed classical gross lesions of avian pasteurellosis. Blood smear and organ impression smears revealed bipolar organisms and re-isolation was successful on blood agar from all the birds.

The same procedure was followed for determination of LD_{50} in ducks, but the number of birds used for each dilution was only four. In the present study, it was unable to arrive at the median lethal dose in six month old ducks as only one duck died after 48 h, which was inoculated subcutaneously with 1.5×10^8 CFU/0.5 ml of inoculum. The dead duck revealed the same gross lesions as that of ducklings. Haemorrhages on the peritoneum was observed as an additional finding.

Oil adjuvant inactivated bacterin vaccines were prepared from DP1 grown under three different conditions *viz.*, 1. organisms grown in TSB (3 per cent) 2. organisms grown in TSB supplemented with 10 per cent FBS and 0.5 per cent yeast

extract 3. organisms grown under biofilm mode in TSB (0.32 per cent) supplemented with 0.3 per cent Bentonite clay.

The innocuity of all the bacterins was tested on blood agar at 37°C for 72 h under five per cent carbon dioxide tension. Vaccine emulsions were prepared by combining aqueous and oil phase vaccine components. The aqueous phase of emulsion consisted of 15 parts of formalin inactivated bacterin and oil phase was formed of nine parts of sterile light liquid paraffin and one part of sterile lanolin. The mixture was homogenized at 18,500 rpm for 30 sec.

All the vaccines prepared were homogeneous suspensions which were easy for parenteral administration. The sterility of the prepared vaccines was tested individually in blood agar and tryptic soy agar (TSA) for aerobic bacteria, modified thioglycollate medium for anaerobic bacteria and Sabouraud's dextrose agar (SDA) was used for detecting any fungal contaminant. No growth was observed on any of the media.

The toxicity of vaccines was assessed by injecting exact and double doses of vaccine intramuscularly to three ducklings each, separately, for the vaccines. The birds did not show any untoward effects systemically and locally. Only a transient lameness was noticed, which subsided within one day PV.

A total of 160 four week old ducklings were divided into four groups with 40 birds in each group and the first three groups were vaccinated with ordinary bacterin, capsule enhanced bacterin and biofilm vaccine respectively. The fourth group served as sham vaccinated control. The birds were vaccinated with 0.5 millilitre of vaccine intramuscularly. Blood was collected from all the ducks pre-vaccination, at weekly intervals upto 28^{th} day post PV and on day 42 PV by cardiac puncture or by jugular venipuncture. Passive haemagglutination using GA-SRBC sensitized with sonicated antigen of DP1 was used to measure the humoral immune response. The IHA titres obtained for biofilm vaccine group on day 14 was very much higher than the other

two groups. The antibody titre was observed from day seven onwards for all the groups. The antibody titre of group I and group II increased during the 21st and 28th days PV. The highest titre obtained was log 8 during the day 14 PV for biofilm group.

Analysis of variance (one way) of the logarithm of IHA tires of sera collected on different days from the ducks was done to compare the efficacy of different vaccines. There was no significant difference in mean titres among the groups during the 7th day. At day 14 PV biofilm group was significantly different from the rest of the two vaccine groups and the control. By day 21 and 28 all the vaccine groups were similar. At day 42, the biofilm vaccine group was found to maintain the titres while the titres of group I and group II decreased. All the vaccine groups have shown significant difference from the control group at all the stages of the study. Group I and group II were having no significant difference in their mean titres during the entire study.

Ten birds from each vaccinated group were subjected to homologous challenge with 0.1 millilitre of inoculum containing 200 LD50 and 100 LD_{50} of fully encapsulated virulent form of *Pasteurella multocida* serotype A: 1 on 28th and 42nd day PV respectively and observed for a period of two weeks post challenge for mortality/clinical signs. Group I, group II and group III had given 60, 60 and 80 per cent protection respectively when challenged with 100 LD_{50} dose. The challenge results on 42nd day was 80, 80 and 90 for group I, group II and group III respectively.

Biofilm vaccine was proved to be the best among the three vaccines tried. The capsule enhanced vaccine did not provide any additional advantage over the ordinary bacterin vaccine.

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PRODUCTION AND EVALUATION OF VACCINES EMPLOYING *Pasteurella multocida* A:1 GROWN UNDER DIFFERENT GROWTH CONDITIONS

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Abstract

A research work was undertaken to prepare effective vaccines against *P. multocida* grown under different conditions and their immunopotency assessed in one month old ducklings.

The purity of the *Pasteurella multocida* A: 1 strain (DP1) was confirmed as per standard procedures. Pathogenicity of the isolate was assessed in six to eight weeks old mice. The isolate killed the intraperitoneally inoculated mice within eight hours and within 24 h when injected by subcutaneous route.

The organism was grown in different media to assess the amount of capsular material produced. The media employed were DSA, DSA supplemented with 10 per cent FBS and DSA supplemented with 10 per cent FBS and 0.5 per cent yeast extract. The capsule enhancement was measured by capsule demonstration using Maneval staining and by characterization of crude capsular extract. The capsules of the organisms grown in capsule enhancement media when demonstrated using Maneval staining were discernibly larger and denser, when compared to the organisms grown in DSA alone. The capsular polysaccharides increased by approximately 1.6 times when the organism was grown in capsule enhancement media containing 10 per cent FBS and by about 2.06 times when grown in media supplemented with FBS and yeast extract.

The potential of the organism to form *in vitro* biofilm was assessed by growing the organism in nutrient restricted conditions. The organism was grown in 0.32 per cent TSB supplemented with an inert substrate called bentonite clay, for the bacteria to attach and colonize. For quantification of biofilm, plate count by spread plate method was employed and the results showed that the biofilm cells reached a peak on the third day of incubation with an average count of 1.54×10^6 CFU/g of bentonite clay while the planktonic cells were found to be maximum on day one post inoculation, with a peak count averaging about 8.10×10^8 CFU/ml of the broth.

Maneval staining of late logarithmic phase of three day old biofilm culture revealed heavily capsulated cells of *P. multocida* attached as large aggregates and appearing as chains of coccobacillary cells. Also they appeared as a meshwork of aggregated and chain forming cells. The capsular polysaccharide estimation of biofilm cells revealed a 3.25 times increase over the planktonic bacteria grown in DSA alone.

The biofilm cells on nutrient agar after 24 h at 37°C produced some colony morphotypes characterized by radiating strands from centre to periphery and wavy margin.

Median lethal dose (LD_{50}) of *P. multocida* when determined in one month old ducklings was 23 cells. In the present study, it was unable to arrive at the median lethal dose in six month old ducks as only one duck died after 48 h of virulent challenge.

Oil adjuvant formalin inactivated bacterin vaccines were prepared from DP1 grown in TSB, capsule enhancement medium and under biofilm mode and performed the sterility, safety and potency tests of the vaccine employing standard procedures. A total of 160 four week old ducklings were divided into four groups with 40 birds in each group and the first three groups were vaccinated with ordinary bacterin, capsule enhanced bacterin and biofilm vaccine respectively. The fourth group served as control. The birds were vaccinated with 0.5 millilitre of vaccine intramuscularly in the thigh region. Blood was collected from all the ducks pre-vaccination, at weekly intervals upto 28^{th} day post PV and on day 42 PV by cardiac puncture or by jugular venipuncture. Passive haemagglutination using GA-SRBC sensitized with sonicated antigen of DP1 was used to measure the humoral immune response. The IHA titres obtained for biofilm vaccine group on day 14 was very much higher than the other two groups. The antibody titre was observed from day seven onwards for all the groups. All the vaccine groups have shown significant

difference from the control group at all the stages of the study. Groups I and II were having no significant difference in their mean titres during the entire study. On homologous challenging, biofilm vaccine gave higher protection rates of 70 and 90 per cent than the 60 and 80 per cent protection rates of ordinary and capsule enhanced bacterins, when challenged with 200 and 100 LD_{50} doses respectively.

Biofilm vaccine was proved to be the best among the three vaccines tried. The capsule enhanced vaccine did not provide any additional advantage over the ordinary bacterin vaccine. Elaborate field trials are to be done before advocating the vaccine for commercial use.