Fowl cholera (FC) is a contagious disease affecting domesticated and wild birds. It usually appears as a septecemic disease associated with high morbidity and mortality, but chronic conditions often occur.

Several epornotics occurred in fowl in Europe during the second half of 18th century. Benjamin in 1851 gave a good description of the disease and demonstrated that it could spread by cohabitation. Pasteur (1880) isolated the organism and used it in his classic experiments in immunological studies.

Pathogenicity or virulence of *P. multocida* in relation to FC is complex and variable depending on the strain, host species and capsules that surrounds the organism, loss of ability of virulent strain to produce the capsule results in loss of virulence. Many isolates from cases of fowl cholera have large capsules but are of low virulence. Therefore virulence is apparently related to chemical substance like Lipopolysaccharides. In poults Lipopolysaccharide do not provoke a dermal Shawastzman reaction.

Pasteur used an avirulent culture and produced immunity that protected fowl against subsequent exposure, in field use. This was not practical because, uniform attention could not be obtained and heavy losses occurred sometimes in vaccinated flocks. Since Pasteur's classic
work numerous attempts have been made to produce efficient vaccines against FC but results have not been consistent.

Vaccine failure in field conditions in vaccinated flocks may be due to improperly prepared or administered vaccine, impaired immune response and difference in serotypes.

Antibacterial chemotherapy has been used extensively in the treatment of FC with varying success. It depends to a large extent on the promptness of treatment and drug used.

Vaccination has been considered as a tool in control of disease in areas where FC is prevalent. Two types of vaccines viz., bacterins and live vaccine have been used. Bacterins may not provide optimal protection against FC challenge. Three types of live vaccine are available for use in USA and the strains used are CU (Clemson University), M-9 a mutant of CU with low virulence and PM-1 a mutant of CU intermediate in virulence. Between CU and M-9, vaccination of chickens with these live vaccines induces protection against heterologus serotype challenge. The use of live FC vaccine stimulates an effective immune response but has the disadvantage of producing mortality in vaccinated birds.

While considering the most appropriate vaccination programme for FC, the following criteria should be considered: Prevalence of FC in the
area, most prevalent serotypes in area and duration of immunity afforded.

In the recent days to maneuver the disadvantage of conventional vaccines, biofilm vaccines have been considered as alternate methods of vaccine for better immune responses and long lasting immunity. Arun (2002), while working on antigenic analysis of biofilm and free cells of *P. multocida* A : 1 in comparison with field isolate opined that biofilm cell expressed more number of unique antigens when compared with field isolate. Hyperimmune sera produced against biofilm cell with *P. multocida* showed 1 : 16 titer in natural host.

Biofilm Cell

Biofilms are defined as an assemblage of microbial cells that are irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material allowing growth in sessile environment.

Biofilms are formed on wide variety of surfaces: Living tissues, Indwelling medical devices, Industrial or potable water piping and Natural aquatic systems.

In 1978, Costerton coined the term biofilm. In nature, biofilm constitute a protected growth modality that allows that bacteria to survive in hostile environment. They can colonize any humid surface like teeth, slippery river stones, infected tissue.

Biofilm contains pore through which nutrient circulate. In different zones of biofilm, the cells express different genes. The micro colony continues to grow in volume and bacteria in proximity to the surface have difficulties in gaining access to nutrients from the external environment. (Thien *et al.*, 2001). Only those, which are located in the upper layers of the colony, are able to continue multiplying and this situation creates a bacterial population with metabolic differences. Gradient of nutrients, waste products and signaling factors contribute to this heterogeneity. Biofilm is in some ways a misnomer, since biofilms are not continuous monolayer surface deposit, rather they are heterogeneous components such as water, polysaccharides and will contribute not only for the heterogeneity of the matrix but also for multicellular function. The matrix will change considerably as equilibrium between species is established and a balance between
competition and communalism is achieved within microbial community. Various structures are involved in biofilm formation, they are: Flagella, Fimbriae, OMP (outer membrane proteins), Curli (protenacious surface structure) and EPS (Expolysaccharide).

EPS production is known to be affected by: Nutrient status of the growth medium, Excess of carbon, Limitation of nitrogen, potassium or phosphate promotes EPS synthesis and Slow bacterial growth enhances production.

EPS contribute to antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm.

Biofilm formation is regulated by availability of surface, nutrients and environmental cues.

Biofilm is formed by Attachment, micro colony formation, development of 3-D community structure, maturation and detachment.

Designing of biofilm based vaccines

When a killed or attenuated vaccine is injected into an animal, an immune response occurs. However, this response may not afford protection because vaccine may not contain adequate immuno-dominant antigens.

In the production of conventional vaccines, laboratory often work with bacteria that no longer express such antigens since the capacity has been lost through successive replication invitro. Hence to mimic invivo conditions, in vitro bacteria have to be grown in liquid media by providing surface (bentonite clay), depleting nutrients and adding iron chelators and growing for longer periods. This leads to slow growth of the organism farming biofilms, which can express some novel proteins and ensure maximum EPS production.

The capsular EPS layer is thick, yet permeable to allow the passage of nutrients antibiotics or even large proteins. Antibodies are therefore able to penetrate without difficulty reaching and binding to the bacterial wall in their fab region, however due to thickness of bacterial EPS layer the bound antibodies are effectively masked or hidden and their FC region may be unable to establish contact with phagouste receptors.

In order to effectively phagocytose a bacterium surrounded by an EPS capsules the produced antibodies must be targeted to the EPS component rather than to the bacterial wall itself or alternatively vaccines can also be directed against bacterial adhesion factors such flagella, pili, which are important in attachment to host cell receptor prior to colonization, thereby avoiding the establishment of these micro colonies and obviating the relapsing of infections.

Keeping in view all these, the present study was undertaken with an objective to study the comparative kinetics of immune responses to biofilm vaccine against fowl
cholera, to assess the seroprevalence of Fowl Cholera and magnitude of cell mediated immune response. Prevalence of different disease conditions in Karnataka in poultry were studied based on the postmortem records from 1994-2004. Humoral and cell mediated immunity were studied in 4 groups of birds at 15 days interval for a period of 180 days. Challenge studies were conducted on these birds at the end of the study period. The results obtained are discussed as follows.

In the present study it can be observed from Table-1 that the order of prevalence of different diseases based on the percentage mortality was 14.45, 3.76, 1.65, 11.67 and 6.83 in pasteurellosis, hepatitis, arthritis, FLKS and miscellaneous, respectively. Whereas, Jha et al. (1996) observed the prevalence of fowl cholera as 2.5 per cent and Kulkarni et al., (1988) reported infection of pasteurellosis (prevalence) as 0.3 per cent. Amongst different diseases in the present study, coccidiosis showed highest prevalence (32.56 %) and arthritis was lowest (1.65 %).

Higher incidence of coccidiosis in the present study may be attributed to factors like managemental system viz., deep litter system, high moisture content in deep litter and resistance to coccidiostats used. Out of 40,7000 birds subjected to postmortem during 1994-2004. Mortality due to different disease in different districts of Karnataka, viz., Bangalore (21.37 %), Kolar (17.19 %), Shimoga (14.98 %), Bellary (12.28), Mysore (9.82 %), Chitradurga (8.84 %), Davanagere (5.65 %), Tumkur (4.91 %), Mandya (2.45 %) and Hassan (2.45 %). Highest overall mortality was observed in Bangalore and lowest was in Hassan and Mandya and lowest mortality in these district could be due to vast
irrigated land which might have driven farmer to take up agricultural activities than poultry rearing, non availability of lucrative market for their product.

Campi et al., (1990) monitored 45 premises in California for out breaks of fowl cholera and he opined that the flocks, which had outbreaks were located strategically closer to other livestock species. Carpenter (1996), showed that minimum of 7 days and maximum of 30 days is required for outbreak of fowl cholera in 720 flocks and he also recorded 53 outbreaks of disease.

However, present study is not in agreement of with Carpenter (1996) and Campi et al., (1990), where mortality was wide spread over the different parts of Karnataka and highest was in Bangalore probably due to commercialization of poultry industry leading to intensive poultry farming, high density of birds, and inadequate bio-security measures viz., labour movement (vehicles, men and material) poor management practices.

Prevalence of pasteurellosis (Fowl cholera) was 4.23 per cent in Bangalore, 2.95 per cent in Mysore, 2.21 per cent in Chitradurga, 1.67 per cent in Shimoga, 1.33 per cent in Kolar, 0.88 per cent in Tumkur, 0.74 per cent in Bellary, 0.25 per cent in Hassan, 0.20 per cent in Davanagere and 0 per cent in Mandya.

These results indicate that Fowl cholera infection is prevalent in different parts of Karnataka. Bangalore district showed highest prevalence of Fowl cholera and Mandya showed no Fowl cholera. Available literature does not throw much light on the prevalence of Fowl cholera. However, the variation in different parts of Karnataka can be attributed to wide host range (Kulkarni et al., 1990), inappropriate usage of antigens in the immunization programme. Lack of conscious effort in reporting the outbreak at field level and varied vaccination schedule (Arun, 2004). In the present study, Fowl cholera
was not recorded in Mandya district. This is probably due to the fact that this district has irrigated lands and people are concentrating mainly on agricultural activities, and the poultry farming is a low priority.

In the present study prevalence of fowl cholera in birds of variety was Cobb - 37.41 per cent, BV - 23.80 per cent, Giriraja - 21.59 per cent and Hubex - 17.17 per cent. There is paucity of information on specific mortality rate regarding variety of birds (Cobb, BV, Giriraja and Hubex). However, Saif et al., (1984) opined that in Turkeys, which are developed for increased egg production had higher mortality rate from fowl cholera suggesting genetic variation in resistance to these diseases. Miguel (1998) observed in a flock of 1300 breeder quails a moderate rate of mortality (13 %) which suggested that quails are susceptible to fowl cholera and are likely to develop sub acute to chronic fowl cholera whereas Sawada et al., (1999) reported 18 outbreaks of fowl cholera (acute) in myna birds, laying chickens and broiler chickens during 1976 and 1989, mortality in each outbreak ranged from 3-100 per cent. In the present study, there has been a wide variation in the prevalence of Fowl cholera amongst different varieties of birds. This may be due to improper usage of antigens/serotype in the immunization programmes, genetic variation in resistance to disease and development of chronic form of disease.

5.2 Evaluation of Immunity

Indirect haemagglutination, test was used to study the humoral immune responses in four groups of birds.
5.2.1 IHA Titers in Immunized Poultry

In the present study the results of Geometric mean titers (GMT) of Sera of Group-I (biofilm) were 7, 17, 42, 79, 79, 128, 194, 256, 338, 446, 549 and 426 at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 days. GMT was ranging from 7 to 549, and the lowest titer was observed on 15 days, post vaccination and highest titer was seen on 165 days post vaccination.

The GMT of sera Group-II (both) were 4, 6, 21, 42, 42, 79, 56, 52, 45, 30, 16 and 11 at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 days. GMT was ranging from 4 to 79, lowest was observed on 15 days post vaccination and highest was on 90 days post vaccination. The GMT of sera of commercial vaccines were 4, 7, 69, 34, 45, 52, 56, 52, 45, 32, 32 and 13 at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 days. GMT was ranging from 4 to 56, lowest was observed on 15 days PV & highest was on 105 days post vaccination. The results in the present study agreed well with Carter and Rappay (1963) who reported IHA titers of 20 to 320 against LPS antigen in sera of cattle vaccinated twice with formalized P. multocida vaccine and also with findings of Krishnamurthy (1981) who observed highest IHA at titers of 320 in sera of calves. However, Ramnatha, K.R. and Gopal, T. (1985) who recorded maximum IHA titers of 1280 and 10240 against crude LPS antigen respectively in sera of ducks vaccinated once and twice, collected at 58 days post vaccination from ducks.
The results of present study on GMT against Pasteurella with different vaccine indicate that the titer were higher in biofilm group at any interval compared to other groups. Thus, it can be hypothesized that biofilm vaccine evoked better immune response as compared to other groups. The quantitative GMT titers at different intervals using biofilm vaccines appear to be the first report in India.

In the present study, humoral antibody responses in Group-I rose from GMT 15 days, reached peak GMT of 549 in 165 days. In Group-II, peak titer was observed at 90 days (79) and in Group-III, peak was seen in 45 days (69). Whereas, Ramnatha et al. (1993) observed that IHA titers maintained without significant variation upto 94 days post vaccination.

Studies have indicated the utility of IHA as Penn and Nagy (1976) opined that the PHA titers should not be regarded as positive unless the immune sera had titers of at least four times that of control pre-immune serum, the present study is in agreement with Penn and Nagy (1976). Whereas, in pre-immune sera sample showed no PHA titers at all.

In the present study all three groups had titers above 20 from 45 days post vaccination and test results are in agreement with the findings of Carter and Rappay (1963) who reported that at least a titer of 1 : 20 should be regarded as positive reaction of moderate degree.
The present study indicates that biofilm vaccine afforded highest degree of immunity when compared with broth and commercial vaccines. Further, it is prudent to get the same result under field evaluation of biofilm vaccines.

5.2.2 Evaluation of Humoral Immunity against *Pasteurella multocida* Type-1 in Commercial farms

The results of PHA titers of different commercial layer farms were ranging from 1:2 to 1:28. This indicates that Fowl cholera is prevalent in commercial farms, based on sera titers. This corroborates with the observations of Carter and Raphy who had considered a titer of 1:20 as positive.

Based on the present study on humoral antibody response and epidemiological observation (based on postmortem data) it can be concluded that fowl cholera is prevalent in different parts of Karnataka.

5.3 Assessment of Cell-mediated Immunity by DNCB Test

The CMI response is the least understood component of immune response in fowl cholera. In recent years, it has drawn more attention, which has lead to evoke many methods to measure immune response as well as methods for diagnosis of diseases caused by certain intracellular organism such as brucella and mycobacteria. The term cell mediated immunity is applied to mean those biological and clinical events, which result from interaction between sensitized leucocyte and specific antigen.
There are many methods available to assess CMI viz., Leucocyte migration inhibition test, phagocytic index assessment and Dermal reaction test (using DNCB).

In the present study, Dermal test was selected because it is easy to perform, under field condition for rapid diagnosis of infection and the same can be used to take up corrective measure.

While assessing the cell mediated immunity in the present study, the birds, which had received the DNCB did not reveal any significant change in skin thickness, as compared to the control groups and it correlates well with Sharma (1976) who informed that CMI seems especially evident in virus infection and in lympho proliferative reaction. Liu Yongde et al. (1977) opined that in fowl cholera vaccine B-cell mediated immunity played a dominant role.

### 5.4 Challenge Studies

In the present study, after assessment of antibody titer for a period of six months, birds in different groups (Group-I, II, III) were subjected to challenge study with live *P. multocida* organism to assess the immunity level and protective titer afforded by biofilm, broth and commercial vaccine. For conducting challenge studies in birds LD 50 was calculated by utilizing mice.
The GMT in Group-1 (biofilm) at 180 days was 426, in Group-II it was 11 and in Group-III (Commercial vaccine) it was 13 and the mortality rate was nil in Group-I, 20 per cent in Group-II, 30 per cent in Group-III, and in control group it was 100 per cent. However, Ramnath (1985) observed survival rate of 20 per cent and 100 per cent respectively in ducks vaccinated once and twice with homologous *P. multocida* challenge of 1740 viable organisms present in 0.2 ml of 10.5 dilution. Present study is in agreement with the Ramnatha, K.R. and Gopal, T. (1985), especially with biofilm group whereas, other two groups did not correlate with Ramnatha, K.R. and Gopal, T. (1985).

Birds which received biofilm vaccine had 100 per cent protection, probably because of clear expression of more number of antigens (immunogens) as cited by Arun (2002) in his study that biofilm cell when subjected to SOS page and Western blot test revealed unique protein of 50-K-Da, 75-K-Da, which is of immuno protective value.

Further, it can be concluded that the present research work is carried out in experimental condition and its prudent to get the same results during vaccine trial (biofilm) in field conditions where lot of extraneous factor will be playing a major role in eliciting of immune response against FC biofilm vaccine.