III. MATERIAL AND METHODS

Present research work envisaged (1) collection of data on prevalence of *Pasteurella multocida* type infection in poultry based on postmortem reports from the Department of Pathology, Veterinary College, Hebbal, (2) Preparation, standardization of biofilm antigen of *Pasteurella multocida* type A : 1 organisms and experimental evaluation of immunity engendered in layer birds of Giriraja breed, (3) Comparison of immunity conferred by broth vaccines, commercial vaccines with biofilm antigen vaccine and (4) An attempt was made to assess seroprevalence of *Pasteurella multocida* type A : 1 in various poultry farms using biofilm antigen.

To achieve the objectives of this study, postmortem (PM) data was collected to analyze prevalence of fowl cholera, antigen or vaccine was standardized, giriraja birds were procured, a meticulous experimentation was conducted to know the immunity level after administering three different vaccines and finally challenge studies were conducted.

3.1 Data on prevalence of *Pasteurella multocida* (Fowl Cholera) infection in poultry layer birds was collected from the department of Pathology, Veterinary College, Hebbal for the period between 1994-2004.

3.2 Preparation of Antigen
3.2.1 **Biofilm cell**

For production of biofilm cells, bentonite clay (0.3% W/V) was suspended in 0.32 per cent Tryptose Soya broth in conical flasks. Later inoculated with the standard inoculum and incubated at 37°C. The culture flasks with bentonite clay were agitated six times daily in a mechanical shaker at 50 rpm for one hour to keep the bentonite clay uniformly suspended in the media. The reagents required in the preparation of antigen, Alsever solution and nutrient broth were prepared as per the procedure described in Appendix-1.

3.2.2 **Biofilm cell antigen (BC antigen)**

The biofilm broth culture was sedimented and washed with PBS by centrifugation at 5000 rpm for 15 min. Bacterial suspension was treated with 0.5 per cent formalin and left at room temperature for 24 hours. Finally the cell concentration was adjusted to $10^9$ CFU.

3.2.3 **Determination of CFU**

Prior to inoculation, nutrient agar plates were dried at least for two hours at 37°C. Ten fold dilutions of the bacterial suspensions were directly obtained from broth culture in the flasks after vigourous vortexing. From each dilutions 0.02 ml was dropped onto the surface of the nutrient agar plate from a height of 2.5 cm so as to spread over an area of 1.5 to 2.0 cm diameter. Six dilutions were similarly inoculated on a plate and six such plates were used for each concentration. All the plates were then incubated at 37°C for 24 hours and the colony count was carried out. The average number of colonies for each
dilution per plate was multiplied by the dilution factor to obtain the viable count in the original suspension as described by Miles and Misra (1938) and expressed as CFU/ml.

3.3 Experimental evaluation of immunity engendered in layer birds with biofilm antigen of Pasteurella multocida type A:1

A total of 40 birds of Giriraja variety of five weeks old were procured from Department of Poultry Science, Veterinary College, UAS, Bangalore. Each bird was numbered using wing badge and they were randomly divided into four groups with 10 birds in each group weighing approximately 700-800 gms.

Birds were maintained in cage systems and were fed and watered routinely during the experiment.

Group-I, consisted of 10 birds and received biofilm antigen. Group-II, received conventional broth antigen. Group-III, received commercial antigen. Whereas, Group-IV served as control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of vaccine used</th>
<th>No. of birds</th>
<th>Age at first vaccine (wks)</th>
<th>Booster</th>
<th>Dose</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Biofilm</td>
<td>10</td>
<td>5</td>
<td>3 weeks</td>
<td>1 ml</td>
<td>i/m</td>
</tr>
<tr>
<td>II</td>
<td>Broth</td>
<td>10</td>
<td>5</td>
<td>3 weeks</td>
<td>1 ml</td>
<td>i/m</td>
</tr>
<tr>
<td>III</td>
<td>Commercial</td>
<td>10</td>
<td>5</td>
<td>3 weeks</td>
<td>1 ml</td>
<td>i/m</td>
</tr>
<tr>
<td>IV</td>
<td>Control</td>
<td>10</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>
Blood was collected from wing vein before and after vaccination and sera were stored at –20°C in aliquots after adding sodium azide (1 : 500 dilution).

Biofilm vaccine, broth vaccine and commercial vaccine were given to 10 birds respectively in each group by using intra muscular route. After vaccination, birds were monitored to assess any post vaccinal reaction either local or systemic viz., reaction at site of injection, increase in body temperature and alterations in feed intake and observations were recorded.

Collection of sera was done upto 6 months after vaccination in an interval of fifteen days. Sera from different poultry farms were collected to assess the seroprevalence of fowl cholera infection.

3.5 Assay of Humoral Immune response

The present research was undertaken to assess the immune response against fowl cholera biofilm vaccine in comparison with other broth vaccine and commercial vaccine.

3.5.1 Indirect haemaglutination test or passive haemagglutination test (PHA)

3.5.2 Extraction of crude capsular polysaccharide (CCPS)

The *P. multocida* Type A : 1 strain cultures grown for 18 hours in nutrient broth at 37°C were inoculated at the rate of 5 ml/Roux flask. The inoculated flasks were incubated at 37°C for 48 hours. *P. multocida*
cells were separated from the agar surface by agitating with sterile glass beads along with 20 ml of sterile NSS.

The culture suspension was thoroughly mixed and heated in water bath at 56°C for one hour. The cells were sedimented by centrifugation at 4000 rpm for 15 minutes. The cell free supernatant was collected and tested for the presence of carbohydrates by Molish’s test as per the method of Dua and Panduranga Rao (1978) and was used as a source of CCPS.

3.5.3 Preparation and stabilization of sheep erythrocyte suspension

Sheep blood was collected in an equal volume of fresh Alsever’s solution (Cruickshank, 1965). RBC in suspension was washed three times in normal saline solution. To a 10 per cent RBC suspension in NSS, glutaraldehyde solution was added drop wise so as to give final concentration of 1 per cent as described by Sawada et al. (1982). The mixture was kept at 4°C for 3 hours with frequent shaking to ensure glutaraldehyde fixation of erythrocyte. The erythrocytes were again washed six times with NSS to remove traces of glutaraldehyde. One per cent glutaraldehyde stabilized 10 per cent erythrocyte suspension was stored at 4°C and used as the source of sheep erythrocytes for sensitization with CCPS.

3.5.4 Sensitization of stabilized sheep RBC with CCPS

Ten ml of 10 per cent CCPS preparation was used to coat ten ml of 10 per cent unsensitized glutaraldehyde stabilized sheep erythrocytes
(GA-SRBC). The mix was agitated for thorough mixing and it was placed in water both at 37°C for one hour with intermittent shaking in order to expose the erythrocytes for antigen coating. After incubation the RBC suspension was centrifuged at 3000 rpm for 10 minutes and sediment containing sensitized erythrocytes was suspended in sterile normal saline to give 0.3 per cent final concentration and was used in PHA test.

3.5.5 PHA

Microtiter plates with round bottom wells (MT-80 Laxbro) were used to carryout the test. The PHA test was performed by mixing 0.05 ml vol of two-fold dilution of test sera in NSS and 0.05 ml of sensitized 0.3 per cent glutaraldehyde fixed sheep erythrocytes in HA plates. Serum dilution of 1 : 2 to 1 : 1280 was used in the test.

The control wells kept in the test were as follows:

1. 0.05 ml of sterile NSS + 0.05 ml of 0.3% sensitized RBC – Ag control.
2. 0.05 ml of test serum + 0.05 ml of unsensitized RBC – serum control

The plates were shaken for uniform mixing and incubated at room temperature for 2 hours and the first PHA reading was recorded. The plates were then placed in a refrigerator at 4°C overnight and second reading was made on the following day morning.

3.5.6 Recording of the results

A total of 520 sera samples collected from vaccinated and unvaccinated experimental chicken were screened and the PHA titers of sera were reciprocals of the highest dilution of serum sample showing complete agglutination.

3.6 Assay of CMI response

3.6.1 Dinitrochloro benzene test (DNCB)

DNCB test was carried out as per method described by Chauhan and Verma (1983). Dinitrochlorobenzene (DNCB) test was carried out in birds aged five
weeks. Ten birds from each group (I, II, III & IV) received a sensitizing dose of 0.1 ml of the one per cent DNCB intradermally at interdigital space between third and fourth digits after recording initial skin thickness. A challenge dose of 0.1 ml of one per cent DNCB was injected intradermally at the same sites seven days later. Skin thickness was measured using slide caliper at 24 hour, 48 hours post challenge.

3.7 Challenge studies

Challenge dose of *P. multocida* (10⁹ mg/ml) was prepared before inoculation as per the method of Raed and Munich (1938).

3.7.1 Calculation of L.D₅₀ for vaccination

LD₅₀ values of the isolates were estimated according to Raed and Munich (1938). A serial ten fold dilution of 18 hrs old nutrient both culture was made resulting in 10⁴ to 10⁹ dilutions and these were stored at 4°C until viable counts were made (24-48 hrs). Each of these dilutions was injected in 0.2 ml quantities S/C to six mice. The inoculated mice were observed for mortality upto seven days.
3.8 Seroprevalence studies

In order to assess the prevalence of fowl cholera under field conditions sera were collected in different poultry farm around Bangalore and Shimoga and assessed for antibody titer against fowl cholera using PHA tests.

3.9 The generated data were subjected to statistical analysis using graph pad prism software.