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Immunogenicity and Protective Efficacy of Iron-inactivated *Pasteurella multocida* A:1 Vaccine against Fowl Cholera in Backyard Chicken

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ABSTRACT

Background: Fowl cholera is a highly fatal, contagious bacterial disease that incurs significant economic loss in commercial as well as back-yard poultry. Vaccination is the most effective way in controlling this disease. In this study, we prepared and evaluated the immunogenicity of iron- inactivated *Pasteurella multocida* A:1 vaccine and its protective efficacy against fowl cholera experimental infection in backyard chicken.

Methods: Scaled up *Pasteurella multocida* A:1 culture with 5 X 10^8 CFU/ml equivalent to 2.5 mg of antigen per dose was used for preparation of experimental vaccines. Formalin inactivated and mixed with APS adjuvant (FIA), formalin inactivated-Freund adjuvant (FIF), Iron inactivated and adjuvanted with iron (III), Iron inactivated from iron supplemented media and adjuvanted with iron (ISII) and commercial oil emulsion vaccine (CV) were used in the study. A total of 120 Vanaraja birds (n=20/group) of 2 weeks age were immunized with these vaccine and booster were given at 3rd and 6th week with respective vaccine. Specific antibody titers were assessed by iELISA in the serum at weekly intervals. The birds were challenged (n=6 /group) with 5x10^4 CFU/ml of virulent isolate by intraperitoneal route and morbidity, mortality percentage were observed.

Result: Protective antibody titers were induced by iron inactivated vaccine from 4th week of immunization and upon booster doses it induced significantly higher (P< 0.05) antibody response. The iron inactivated experimental vaccine gave equivalent protection as that of commercial vaccine upon challenge infection.

Key words: Fowl Cholera, Iron inactivation, Killed vaccine, *Pasteurella multocida*.

INTRODUCTION

Fowl cholera (FC), caused by avian *P. multocida,* is a highly fatal disease and has a major economic impact on commercial as well as rural poultry production (Rimler and Glisson 1997, Christensen and Bisgaard, 2000; Aye *et al.,* 2001; OIE, 2008). FC occurs sporadically or enzootically in most countries of the world (Heddleston, 1962). Vaccination is the effective way to prevent the infectious diseases. Commercially available vaccines against fowl cholera include inactivated vaccines and attenuated live vaccines. Adjuvants with vaccine antigen although facilitate to induce robust immune response, they are linked to drawbacks such as irritation and abscess at the site, costly etc. Live vaccines are in use in some countries including Australia; however, they pose a risk of reversion of virulence and may affect the laying rate. Hence, in the present study, we investigated a simple, inexpensive and safe method for preparing inactivated fowl cholera vaccine and evaluated its immunogenicity and protective efficacy in backyard chickens.

MATERIALS AND METHODS

Experimental birds

Day-old Vanaraja chicks were obtained from Hatchery, ICAR-Directorate of Poultry Research and were housed in battery brooders in the farm. Birds were fed with basal diet and water *ad libitum*. All experiments were carried out at Directorate of Poultry Research (ICAR-DPR), Hyderabad during 2017-18 with the approval of Institute Animal Ethics Committee (IAEC/DPR/18/5), by following all the guidelines of CPCSEA.

Bacterial culture and propagation

Pasteurella multocida A:1 isolated from fowl cholera outbreak and maintained at avian health lab, ICAR-Directorate of Poultry Research was used in the study. Brain heart infusion (BHI) agar (Himedia, India) was used for isolating single colonies and counting colony forming units

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(CFU). Purity of the culture was checked by colony morphology, Gram's stains and by PCR using specific primers. Live *Pasteurella* isolate was stored in BHI broth with 50% glycerol at -20°C for a period of not more than 4 months. For bulk propagation of *P. multocida,* BHI broth was used for culture and incubated for 24 hrs at 37°C. For iron-supplementation of BHI, stock solution of 1 M FeCl3 was added drop- by- drop to media up to a final concentration of 100 µM (Herath *et al*., 2010). The virulence of the culture was confirmed by inoculating in 12-week-old Vanaraja chicks. An inoculum of 0.5 ml of 1.9 x10^5 CFU/ml was given by intraperitoneal route and mean death time was observed (Reed and Muench, 1938).

Formalin-inactivated vaccine

Scaled up culture from seed culture of *P. multocida* isolate was prepared in BHI broth. Formalin inactivated vaccine was prepared as per the method described by Jones *et al*. (1988). Briefly, formaldehyde was added to bacterial culture harvested from bulk broth culture from 1 liter of 18h culture, washed and resuspended in 20 ml of normal saline up to the final concentration of 0.5%. After incubating for 24h at 37°C, the inactivated culture was washed and resuspended in normal saline. This was adjuvanted with 0.5% aluminum potassium sulfate (Merck) for the preparation of formalin inactivated with aluminum potassium sulfate vaccine (FIA). The formalin inactivated antigen was combined with Freund's complete and incomplete adjuvant (Sigma-Aldrich) and designated as formalin inactivated with Freund's adjuvant vaccine (FIF).

Iron-inactivated vaccine

BHI broth was enriched with 100 µM $FeCl_3$ was used to propagate the isolate. The bacterial culture harvested both from basal BHI and iron-enriched media were used for the preparation of iron-inactivated vaccines and designated as Iron inactivated (II) and Iron enriched iron inactivated (IEI) respectively. Iron inactivation of the culture was done as per the method described previously (Kumar and Singh, 2005; Herath *et al.,* 2010). Briefly, 1 M $FeCl_3$ was added drop by drop to the bacterial culture with intermittent shaking up to the final concentration of 100 mM. After incubating at 37°C for 4 hrs, the residual $FeCl_3$ Was removed by washing the bacterial pellet twice with normal saline.

Sterility and safety

The prepared vaccines were checked for sterility by inoculating on BHI plates and incubating for 24hrs at 37°C. The absence of bacterial growth on the plates indicated the sterility of prepared vaccines. Safety was assessed by inoculating the experimental vaccines at 0.5 ml dose through subcutaneous route and observed for survivability and adverse reactions (Nangia *et al*., 1966).

Immunization trial

A total of 120 two-weeks old Vanaraja chicks were randomly divided into six groups (n=20 each). Antigenic mass in each dose was adjusted 2.5 mg of wet weight of *P. Multocida* A:1 harvested from culture of 5 X 10^8 CFU/ml. At two weeks of age, the birds were immunized with formalin inactivated with freund's adjuvant vaccine (FIF), Formalin inactivated with aluminum potassium sulfate vaccine (FIA), Iron inactivated vaccine (II), Iron enriched iron inactivated vaccine (IEI) and Commercial oil emulsion vaccine (CV). One group was kept as unimmunized control. All vaccines were given by subcutaneous route with 0.5ml per dose. First and second booster were given at 3 and 6 weeks after first immunization with their respective vaccines by same route. After vaccination, birds were monitored for any post vaccine reactions either local or systemic *viz*., reactions at the site of injection, increase in body temperature *etc*. Sera from each group (n=6 at each interval) were collected at weekly interval from first immunization till 9 weeks. The sera were kept at -20°C until further use.

Humoral antibody response

Specific humoral antibody response to *P. multocida* was measured in serum samples collected at weekly interval from all groups by indirect ELISA. The antibody titers were quantified with IDEXX commercial kit (# 99-09251 FC antibody test kit, IDEXX, Westbrook, USA) by following the manufacturer's instructions. Briefly, the test was performed on 96-well ELISA plate precoated with *P. multocida* antigen, provided in the kit by manufacturer. Diluted (1:500) test sera were dispended (100 µl/well) in duplicates. Undiluted positive and negative controls (each 100 µl/well) provided along with the kit were also dispended on the coated wells. After incubating for 30 min at 25°C, the plates were washed with distilled water to remove any unbound material and followed by the addition of 100 µl conjugate. After 30 min at 25°C, the unbound conjugate was washed away and TMB substrate (100 µl) was added. The subsequent color developed was measured by spectrophotometer at 650nm and corresponding OD values were recorded. The respective antibody titers were calculated as given below:

$$\text{Titer} = \text{Antilog}\,[1.09\,(\log 10\ S/P)] + 3.36$$

Wherein S/P = [Mean OD of test sample-Mean OD of negative control] / [Mean OD of positive control- Mean OD of negative control]

The OD values were converted into titers using their software (xChekPlus, IDEXX). The titer greater than 396 is considered positive.

Protective efficacy

Six chicken from each group were challenged with 0.2 ml of 5 x 10^4 CFU /ml culture of virulent *P. multocida* A:1 isolate through intraperitoneal route at 3 weeks after second booster vaccine. The birds were observed at 6 hrs interval for morbidity. mortality pattern and gross lesions for up to 7 days post-challenge. Liver and spleen tissue from necropsied birds were collected and fixed in 10% neutral buffered formalin for histopathology. Sections were made and stained with hematoxylin and eosin (H & E).

Statistical analysis

The ELISA antibody titers were expressed as mean ± SEM. The titers of different groups were analyzed by one-way analysis of variance (ANOVA) to test for significance at overall effect followed by Duncan's post-hoc test to identify significant difference among groups. *P* values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Ferric chloride inactivates bacteria by precipitating proteins and by degrading the nucleic acids and lipids by free radical formation (Braun, 1997; Andrews *et al.,* 2003). However, this does not involve denaturation of antigenic epitopes as evidenced by the immune response induced by the iron inactivated in chicken in this study. Similar to earlier study, complete inactivation was attained by 100 mM final concentration of iron within 4 hrs. In contrast, for formalin inactivation by standard procedure takes 24 hrs. Innocuity or injectability of vaccine an important factor for vaccine-administers for large-scale, we observed that iron inactivated vaccine had water-like consistency and were comfortable for injections through s/c route. Moreover, iron inactivated vaccines did not cause local tissue reactions like swelling, muscle damage *etc*., that is normally found in oil adjuvanted vaccines and live a capsular *P. multocida* A:1 vaccine (Chung *et al.,* 2005).

All the experimental vaccines induced antigen specific serum antibody response during the immunization trial, however the efficiency varied. The protective antibody tires were induced at 7th week post-immunization, after two boosters by commercial oil adjuvant vaccine and experimental iron inactivated-iron enriched vaccine (IEI). The IEI vaccine induced significantly equivalent antibody response as that of commercial vaccine. The iron inactivated vaccine with iron supplementation in media also induced protective titers, however it was delayed. The unimmunized control birds remained negative for Pasteurella specific antibodies throughout the period (Fig 1). The delay in induction of immune response may be attributed to the difference in expression of antigenic peptides. This reiterates the hypothesis that iron supplementation induces the expression of immunogenic antigens in *Pasteurella*, which in turn helps in better immunogenicity. Formalin inactivated vaccines (both Freund's adjuvant and APS) induced protective titers. Among the two, Freund's adjuvant induced robust response. Similar trend of immune response was reported earlier also. Similar kind of low serum antibody response during initial days following primary vaccination and gradual increase in IgG titre over a 6-10 wk period after vaccination with oil adjuvanted vaccine was reported by earlier workers (Chandrasekaran *et al.,* 1994; Shah *et al.,* 2008).

Protective efficacy of the experimental vaccines was determined by challenge study by intraperitoneal inoculation of virulent *P. multocida* in immunized chickens. The experimental birds were challenged 3 weeks after second booster and observed for morbidity, mortality pattern, gross lesions and histopathological lesions. Iron inactivated vaccines gave equivalent protection to that of commercial vaccine. Formalin inactivated - APS adjuvant vaccine gave less (33%) protection compared to other groups (Table 1). The symptoms in challenged and succumbed birds included

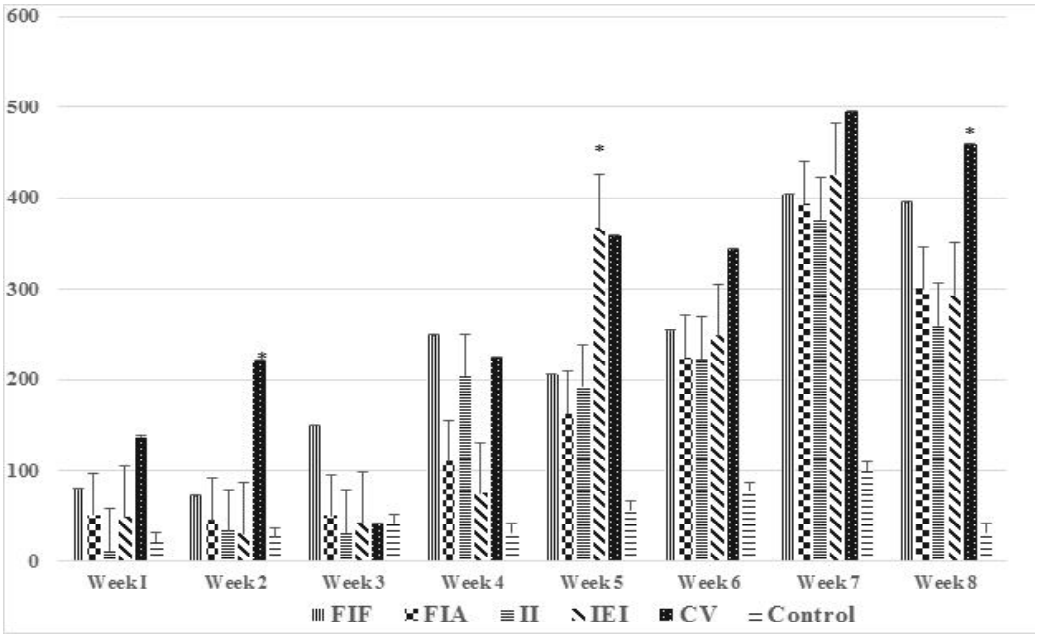


Fig 1: Antibody response to experimental vaccines assessed by iELISA.

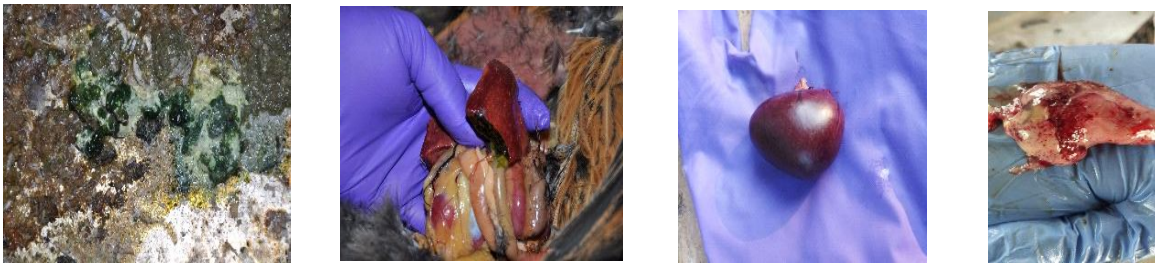
FIF: Formalin inactivated with Freund's adjuvant vaccine; FIA: Formalin inactivated with aluminum potassium sulfate; II: Iron inactivated; IEI: Iron enriched iron inactivated; CV: Commercial vaccine; Titers ≥ 396 are considered positive.

like that of acute form, *i.e.* fever, white diarrhoea, depression etc. The lesion in dead birds included necrotic foci on liver, spleen *etc*. (Fig 2). Gross lesions in the heart were congestion and petechial haemorrhages on the epicardium. The symptoms and lesions were similar to earlier findings by others (Rhodes and Rimler, 1989; Christensen and Bisgaard, 2000; Glisson *et al.,* 2003; Afifi *et al.,* 2007). Histopathological changes include micro abscess, necrosis, diffuse haemorrhages and congestion in liver and spleen (Fig 3). Overall, the mortality percentage in challenge study

Table 1: Mortality pattern in challenge study.

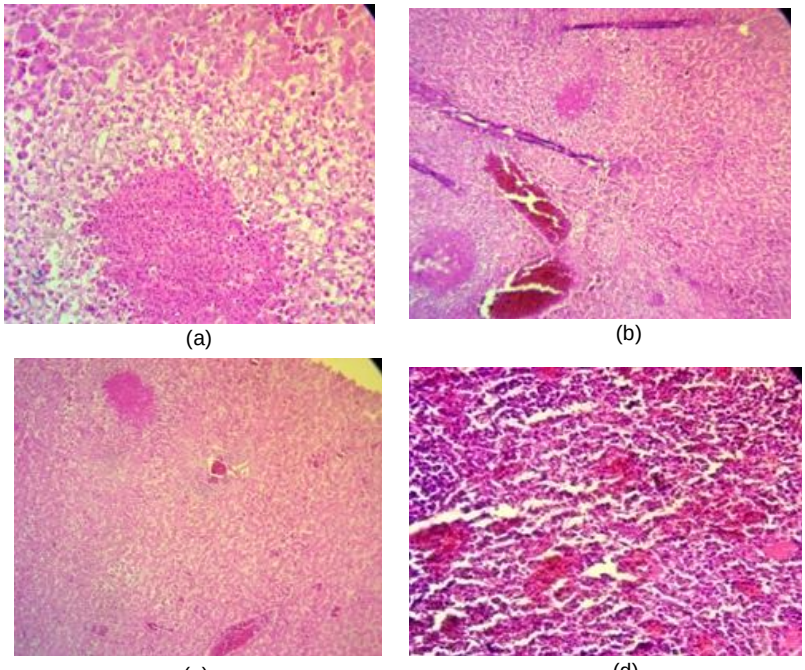
Group	No. of birds	Mortality (in hours)								% Mortality
		6	12	18	24	30	36	42	48	
FIF	6	-	-	-	2	1	-	-	-	50
FIA	6	-	-	-	1	1	-	-	-	33.3
II	6	-	-	-	1	2	-	-	-	50
IEI	6	-	-	-	2	-	1	-	-	50
CV	6		-	-	2	-	-	1	-	50
Unimmunized control	6	-	-	2	4	-	-	-	-	100

FIF: Formalin inactivated with freund's adjuvant vaccine; FIA: Formalin inactivated with aluminum potassium sulfate; II: Iron inactivated; IEI: Iron enriched iron inactivated; CV: Commercial vaccine.



(a) (b) (c) (d)

Fig 2: Clinical signs and gross lesions observed in challenge infected birds.
(a)Greenish white diarrahoea (b) Mottling and white necrotic foci on liver (c) White necrotic foci on spleen (d) Haemorrhages on epicardium of heart.



(a) (b) (c) (d)

Fig 3: Histopathological changes in the organs of challenge infected birds.
(a) Necrotic foci in liver (40x) (b) Microabscess, necrotic foci in liver (10x)
(c) Congestion of central hepatic vein and haemorrhages (10x) (d) Necrosis in spleen and lymphocytic infiltration (40x).

was higher than expected. The reason for this may be slightly higher microbial dose used for challenge, higher virulence of the field strain, age of the birds during challenge and also the route of injection selected for challenge study. Several authors earlier shown that pathogenicity of *P. multocida* A:1 varies with age of chicken and route of infection (Heddleston, 1962; Hungerford, 1968; Shivachandra *et al.* 2005). It has been earlier shown that highly virulent *P. multocida* serogroup A:1 cause mortality of chicken within 24 hrs even at low dose of inoculums (Wilkie *et al.,* 2000).

CONCLUSION

The present study, demonstrated a safe, easy and economical method of preparation of a killed vaccine for fowl cholera from *Pasteurella multocida* A:1 isolate that induced good antibody response in backyard chicken and equivalent protection as that of currently used oil adjuvant vaccine.

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Conflict of Interest

Authors declare that they have no conflict of interest.

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