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EFFECT ON IMMUNE RESPONSE AGAINST *Pasteurella multocida* CAPSULAR TYPE A:1 AND A:4 ON SUPPLEMENTATION WITH PROTEIN PURIFIED DERIVATIVES OF *Mycobacterium bovis* IN CHICKEN

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KEYWORDS

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Tuberculo-protein

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ELISA

ABSTRACT

The adjuvant potential of tuberculo-protein of *Mycobacterium bovis* and Montanide ISA-206 adjuvants in different combination with *Pasteurella multocida* serotype A:1 and A:4 whole cell antigens have been evaluated in chicken model. Four different groups of chicken were immunized with different combination of adjuvants and humoral immune response was assessed by indirect ELISA. The immune response study revealed that chicken immunized with whole cell antigen in combination with Protein precipitated derivative (PPD) as well as montanide ISA-206 elicited a robust humoral immune response. To assess the protective ability two groups of chicken immunized with *P. multocida* A:1 were challenged with 1 MLD of *Pasteurella multocida* A:1; whereas two other groups of chicken immunized with *P. multocida* A:4 bacterin were challenged with 100 MLD of *P. multocida* A:4. Challenge studies indicated that both the groups of chicken immunized with *P. multocida* A:1 whole cell antigen adjuvanted with both Montanide ISA-206 and *Mycobacterium bovis* PPD as well as only Montanide ISA-206 conferred 100 % protection against *P. multocida* A:1. However two other groups immunized with *P. multocida* A:4 bacterins could not sustain 100 MLD dose of direct challenge and there were 100% death of all the birds in both the immunized group, but the group immunized with both Montanide and PPD showed delayed death.

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1 Introduction

Adjuvants play an important role in potentiating immune response and enhance protective ability of candidate vaccine antigen. Depending upon the nature of organism adjuvants are selected to get desired immune response. The humoral immune response plays a vital role in clearance from body against those pathogens which are extra cellular in nature and whose antigen presentation is through the MHC class II. With few exceptions, alum is most widely used adjuvant approved for human and animal use in the majority of countries worldwide. Although alum is able to induce a good antibody (Th2) response, it has little capacity to stimulate cellular (Th1) immune responses which are important for protection against many pathogens (Vogel & Powell, 1995). Freund et al., (1937) developed an emulsion of water and mineral oil containing killed *Mycobacteria*, thereby creating one of the most potent known adjuvants, Freund's complete adjuvant (FCA).

The adjuvant activity of bacteria derived adjuvants is mediated by N-acetyl muramyl-L-alanyl-D-isoglutamine, also called MDP. MDP activates many different cell types including macrophages, leucocytes, endothelial cells and fibroblasts inducing the secretion of various cytokines such as IL-1, B-cell growth factor and fibroblast activating factor (Petrovsky & Aguilar, 2004).

The oil in water emulsion without added *Mycobacteria* is known as Freund's incomplete adjuvant (FIA). Freund complete adjuvant (FCA) is considered better adjuvants in comparison to Freund incomplete adjuvant (FIA) which is due to killed *Mycobacterial* antigen. The use of Freund adjuvant is mainly restricted to hyper immune sera production as the animal may become sensitive to the tuberculo-protein. But this holds true only for those animals which are screened for tuberculosis by PPD. The large animals are tested for the tuberculosis by delayed type hypersensitivity therefore its use may have some limitations but the birds can be effectively immunized with formulations with tuberculo-protein if the formulation have positive effect on the immune response. Thus there is need to evaluate the potential of cellular components of *Mycobacteria* as an adjuvant with vaccine candidate in the species where it does not hinder testing procedure. The tuberculo-protein derivatives are better alternative in this direction. Further if such components are having adjuvant effect may be used effectively in vaccine formulation in those animals like birds where tuberculin testing is not done. Therefore potential of the purified protein derivative (PPD) of *M. bovis* along with Montanide formulation with *Pasteurella multocida* serotype A:1 and A:4 bacterin was explored in the birds following immunization. *Pasteurella multocida* type A:1 and A:4 was taken as target for the present study as it is the causative agent of

disease called fowl cholera which is an acute, fatal and septicemic disease of various domestic and wild bird species (Derieux, 1978; Glisson et al., 2013). In India it has been reported recurrently from the states of Assam (Lalrinthunga & Burah, 1993), Gujarat (Pande, 1993), Maharashtra (Kulkarni et al., 1990) and Orissa (Panda et al., 1951). Hence realizing the significance of this poultry pathogen and potential application of tuberculo-protein in immune response enhancement the present pilot study was undertaken to investigate the effect of PPD derived from *M. bovis* on immune response of whole cell antigen of *P. multocida* type A:1 and A:4 causing fowl cholera.

2 Materials and Methods

2.1 Preparation of Purified Protein derivatives

The Purified Protein derivative from indigenous strain of *Mycobacterium bovis* cultured in Dorset Hanley medium was used for the study was prepared as per the methods described in OIE (2009). The growth was steamed for 3 h at 100°C and subsequently clarified with 0.2 µm filter. The protein in culture filtrate was precipitated by adding 40% Trichloro acetic acid. After precipitation the centrifugation was done at 3,000 rpm for 30 minutes and supernatant was discarded. The precipitated protein was washed four times with 5% NaCl (pH 3.0) till pH of the protein was 2.7. The protein precipitate was then dissolved in alkaline solution (pH 6.6). The concentrate was then centrifuged to remove all insoluble impurities and then soluble proteins were diluted with glucose buffer solution (R-30). This was then tested for determination of protein concentration by Biuret method.

2.2 Biomass production of *Pasteurella multocida* type A:1 and A:4

2.2.1 *Pasteurella multocida*

Pasteurella multocida serotype A:1 and A:4 was procured from Division of Biological Standardization IVRI, Bareilly, was cultured on the 5 % sheep blood agar and characterized based on morphological, cultural characteristics on different media along with molecular characterization.

2.2.2 Production of bacterial harvest

Both the organisms were cultured in Casein Sucrose Yeast (CSY) broth for 18 h at 37°C. After examining each growth for purity and identity, 3ml of the same culture was used for sowing Roux flasks each containing 120-150 ml of CSY agar. The growth was harvested with the help of 10-20 ml of normal saline solution from each flask after incubation at 37°C for 24 h, purity and identity was rechecked. The pooled growth of both *P. multocida*

serotype A:1 and A:4 was then inactivated with 0.5% formalin and incubated for 24 h. Each pooled growth was stored for a week at 4°C. Before being manipulated for vaccine production the harvests were adjusted to Brown's opacity tube no. 7 and then retested for sterility and safety in the mice. The inactivated safe bacterial biomasses were subsequently used for immunization of PPD based adjuvant vaccine formulations for fowl cholera.

2.3 Preparation of vaccine formulations

Four different vaccine formulations using bacterial biomass from both (*P. multocida* A:1 & A:4) were prepared. First vaccine formulation was prepared using bacterial biomass from *P. multocida* A:1 adjuvanted with both Montanide as well as PPD where as second group of vaccine was prepared using only Montanide ISA-206 keeping the ratio of biomass and the Montanide (1:1) in water in oil formulation. Similarly third and fourth vaccine formulation was prepared using bacterial biomass from *P. multocida* A: 4. The emulsions were tested for viscosity and stability by drop test and were monitored for 9 months. PPD concentration in vaccine formulation was adjusted to 500 µg per dose. All the vaccine formulations were tested for sterility. These formulations so prepared were tested for immune response studies in birds.

2.4 Immunization trial

2.4.1 Grouping and rearing of birds

Fourteen healthy, 11 week old (both sexes) white leghorn birds were divided in five groups as follows:

Group 1: *P. multocida* A:1 adjuvanted with PPD as well as Montanide ISA 206 (n = 3, birds) Group 2: *P. multocida* A:1 adjuvanted with only Montanide ISA 206 (n = 3, birds) Group 3: *P. multocida* A:4 adjuvanted with PPD as well as Montanide ISA 206 (n = 3, birds) Group 4: *P. multocida* A:1 adjuvanted with only Montanide ISA 206 (n = 3, birds) and Group 5: Unvaccinated (n = 2, unvaccinated birds).

2.4.2 Vaccination, blood collection and testing

All the birds in different groups were vaccinated with one dose of 0.5 ml deep intramuscular route. Blood was collected at weekly interval from 0 day to 6 weeks. The serum was separated aseptically and stored at -20°C which was utilized for immune response studies.

2.5 Monitoring of immune response

Serum antibody titres of immunized birds were determined by indirect enzyme linked immunosorbent assay (ELISA) as per

Engvall & Pearlman, 1972 with some modifications. *P. multocida* A:1 whole cell antigen was used for coating the plate as antigen. Optimum antigen, antibody and conjugate dilution were determined by checker board titration. Optical density was read at 492 nm using an ELISA Reader plate (ASYS Hitech, GMBH Austria, Expert plus). The results were expressed in terms of Positive /negative ratio.

2.6 Determination of MLD of *Pasturella multocida* A:1 and A:4 and challenge studies

P. multocida serotype A:1 and A:4 were inoculated into two mice so that the culture may be brought to Phase 1. After death of birds the organisms were reisolated in pure form from the heart blood on fresh 5% blood agar. The isolated organism was cultivated in CSY broth at 37°C for 18 h. The dilutions of 18 h culture were prepared in fresh CSY broth was injected to birds. The dilution showing 100% death in the bird is taken as 1MLD. Challenge studies were done with 1MLD of A: 1 and 100 MLD of A: 4 of *P. multocida*.

2.7 Data analysis

All data were entered into Microsoft Office Excel sheet (Microsoft Corporation, 2013) and expressed as mean ± SEM data were analyzed with one way ANOVA with individual groups.

3 Results

3.1 Preparation of vaccine formulation

Four different adjuvanted vaccine formulations prepared by seppic oil (Montanide), PPD protein and NSS containing whole cell *P. multocida* adjusted to Browns opacity tube no.7 mixture in the ratio of 1:1 in a high speed homogenizer (14,000 rpm) for 1 h with 3 min interval of break after every 10 min. After about five times of the mixing of the emulsion - thin, creamy white emulsions of low viscosity was formed. After that, emulsion was mixed in high speed blender (usually 10 times total) to form viscous emulsions which had flow times of approximately 1-3 minutes per ml. These emulsions maintained their shape and remained largely submerged on addition to cold water. Moreover, these emulsions were highly stable, retaining their characteristic structure for more than 9 months when stored at 4°C.

3.2 Pathogenicity of the *P. multocida* strains

Injection of *P. multocida* type A:1 (with 1MLD) induced 100% protection in chicken immunized with *P. multocida* type A:1

bacterin formulation with or without PPD. A Challenge (100 MLD of *P. multocida* A:4) of immunized chicken with *P. multocida* type A:4 bacterin with or without PPD resulted into differential death pattern. The chicken immunized with type A:4 bacterin without PPD leads to death within 24 hours with apparently severe sickness. However, the *P. multocida* type A:4 bacterin with PPD showed mortality in the range of 36-48 hours.

3.3 Serum antibody titre in chicken immunized with adjuvanted *P. multocida* A1 bacterin

ELISA data showed differences in the onset, intensity and duration of the *P. multocida* type A:1 antibodies evoked by the different vaccine formulations from 7 day to 6 weeks. The serum antibody titers in both Montanide alone as well as Montanide & PPD adjuvanted group (Table no.1 & 2) showed classical pattern of immune response. Detectable antibody titer was found on 7th DPI which peaked on 42nd DPI in both the groups. Peak antibody titer in groups adjuvanted with both PPD & Montanide was (3.14±0.22) was higher than Montanide alone (2.62±0.53).

Table 1 Humoral immune response in chicken immunized with *Pasteurella multocida* type A:1 bacterin adjuvanted with montanide

A1 without PPD	0 day	7 day	14 day	21 day	42 day
1	1.08	1.08	1.79	1.79	1.68
2	1.07	1.09	1.16	2.06	3.52
3	1.08	1.12	1.39	1.79	2.69
Mean ± S.E	1.075±0.03	1.09±0.09	1.45±0.19	1.89±0.09	2.63±0.53
Control (Mean ± S.E)	1.10±0.02	1.05±.02	1.03±.09	1.12±.07	1.17475

Table 2 Humoral immune response in chicken immunized with *Pasteurella multocida* type A:1 bacterin adjuvanted with montanide and tuberculo-protein of *Mycobacterium bovis*

A1 with PPD	0 day	7 day	14 day	21 day	42 day
1	1.123	1.14	1.89	2.21	2.77
2	1.04	1.23	1.74	2.57	3.10
3	0.99	1.13	1.73	2.11	3.56
Mean ± S.E	1.05±.04	1.16±.03	1.79±.06	2.30±0.15	3.14±.23
Control (Mean ± S.E)	1.09±0.02	1.06±.02	1.01±.01	1.12±.07	1.18

3.4 Serum antibody titre in chicken immunized with adjuvanted *P. multocida* A:4 bacterin

The serum antibody titers as determined by Indirect ELISA in both Montanide alone as well as Montanide and PPD adjuvanted group (Table no.3 & 4) showed classical pattern of immune response. Detectable antibody titer was found on 7th DPI which peaked on 42nd DPI in both the groups. Peak antibody titer in groups adjuvanted with both PPD & Montanide was (2.66±0.36) was higher than Montanide alone (2.27±0.30).

Table 3 Humoral immune response in chicken immunized with *Pasteurella multocida* type A:4 adjuvanted with montanide

A4 without PPD	0 day	7 day	14 day	21 day	42 day
1	1.08	1.07	1.12	1.37	2.58
2	1.13	1.17	1.42	1.63	Died
3	1.09	1.13	1.24	1.35	1.97
Mean ± S.E	1.10±0.02	1.12±0.04	1.26±0.09	1.45±0.09	2.28±0.31
Control (Mean ± S.E)	1.01±0.02	1.06±.011	1.01±.01	1.12±.07	1.18

Table 4 Humoral immune response in chicken immunized with *Pasteurella multocida* type A:4 bacterin adjuvanted with montanide and tuberculo-protein of *Mycobacterium bovis*

A4 with PPD	0 day	7 day	14 day	21 day	42 day
1	1.17	1.16	1.34	1.63	2.56
2	1.08	1.17	1.33	1.83	3.08
3	1.18	1.17	2.01	1.98	2.36
Mean ± S.E	1.15±0.05	1.17±0.01	1.56±0.39	1.81±0.17	2.67±0.37
Control (Mean ± S.E)	1.01±0.02	1.06±.011	1.01±.01	1.12±.07	1.18

The immune response studies of both type A:1 and A:4 bacterin with tuberculo-protein vaccine showed that there was augmentation of immune response in both treated groups of birds in reference to control birds (Figure 1; Figure 2). The trends of immune response in both the treated groups were somewhat same. The significant variation in immune response was observed in between the both groups of tuberculo-protein *vis a vis* the formulations without tuberculo-protein.

4 Discussion

Fowl cholera is an acute, fatal and disease of economic importance in poultry production that may occur in different forms, such as peracute, acute, and chronic infections, often

Figure 1 Humoral Immune response against *Pasteurella multocida* type A:1 in birds vaccinated montanide adjuvanted bacterin of *Pasteurella multocida* type A:1 with and without tuberculo-protein of *Mycobacterium bovis* (Vaccine 1- A:1 with PPD, Vaccine 2- A:1 without PPD)

Figure 2 Humoral Immune response against *Pasteurella multocida* type A:4 in birds vaccinated montanide adjuvanted bacterin of *Pasteurella multocida* type A:4 with and without tuberculo-protein of *Mycobacterium bovis* (Vaccine 3- A:4 with PPD, Vaccine 4- A:4 without PPD)

causing high morbidity and mortality, thus resulting in large financial losses (Hirsh et al., 1990; Christensen & Bisgaard, 2000; OIE, 2015). The disease is caused by *Pasteurella multocida* serotype A:1, A:3, A:4 in birds and it has wide host range including domesticated chickens, turkeys, ducks, geese, quail, game birds reared in captivity, companion birds, birds in zoo and wild birds (Wang et al., 2009; OIE, 2015). Vaccines used against fowl cholera include inactivated bacterins or live attenuated bacteria. Bacterins are widely used, but must be injected and induce immunity only to homologous serotypes (Davis 1987; Rebers et al., 1988). In contrast, live vaccines can confer immunity against heterologous strains but may revert to virulence, possibly causing mortality in vaccinated flocks (Hofacre & Glisson 1986; Hopkin et al., 1988; Christiansen et al., 1989).

However, despite years of research and control efforts in medication and vaccination practices fowl cholera has remained prevalent.

The limitations in fowl cholera vaccine lies that the current vaccine formulations in the vaccinated stock gives 80% protection upon challenge with less than 1MLD. Thus there is need to search for such adjuvants which may help to provide 100% protection upon challenge with 1 MLD or more. In the present study though 100% protection has been observed in both the type A:1 vaccinated birds but the immune response was comparatively higher in vaccine formulation having both Montanide as well as PPD. Thus the present formulation added with purified protein antigen is likely to provide better protection even to higher challenge dose of bacteria. The similar observation in vaccine formulation derived from *P. multocida* type A:4 support above observation as there is prolong survival time Thus the present proposition not only a new dimension to the fowl cholera vaccine but can be effectively used in other vaccine formulations as well.

The current co-adjuvanted vaccine with seppic oil and PPD for type A:1 and A:4 study indicated that the PPD may be a future adjuvant for the vaccine. The possibilities of tuberculo-protein as future adjuvant have also been explored in past and showed that it may be a promising adjuvant. The potential of individual proteins like *M. tuberculosis* protein 50S ribosomal protein L7/L12 (rpL) from Rv0652, as an immunoadjuvant has been explored as and found to have the efficacy of a for DC-based tumor immunotherapy (Lee et al., 2014). The study conducted by the group indicated that Rv0652 induce DC maturation, and pro-inflammatory cytokine production (TNF-alpha, IL-1beta, and IL-6) that is partially modulated by both MyD88 and TRIF signaling pathways. In our study, immune responses elicited by adjuvanted vaccine formulations indicated that the PPD may be used as an adjuvant. Since the tuberculo-protein is an admixture of series of proteins derived from Mycobacterial spp, role of individual protein needs to be investigated along with various bacterial, viral immunogen in different species of animals and subsequent studies on the molecular mechanism in reference to different species of animals. The study conducted thus validate the earlier observation of adjuvant potential (Lee et al., 2014) but this needs to be further validated in terms of cytokine genes expression and TLR induction with crude tuberculo-protein which will help in better understanding about mechanism of protection conferred by these adjuvants, so that in future this may be used as adjuvant which may provide multi-factorial effect on the immunity.

Conclusion

The present study revealed that protein purified derivatives can be effectively used in the vaccine formulation to enhance the immune response against the immunogens. *Pasteurella multocida*

is an extracellular pathogen and purified protein derivatives have such a beneficial effect and it may likely to have sustainable effect on the intracellular organisms. Thus exhaustive studied be conducted in relation to various antigen encompassing bacterial, viral and protozoan pathogens. Simultaneously its role be further explored in immunotherapy of cancer as this may be the clean adjuvant. The findings clearly indicates that 100% protection can be provided in the oil based *Pasteurella multocida* vaccine formulation with co-adjuvantation with purified protein derivatives of *Mycobacterium bovis* and accordingly the vaccine validation protocols can then be further be modified.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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