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Experimental iron-inactivated *Pasteurella multocida* A: 1 vaccine adjuvanted with bacterial DNA is safe and protects chickens from fowl cholera

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ABSTRACT

Fowl cholera is a serious problem in large and small scale poultry production. The present study describes the development and testing of an inactivated whole-cell, low-cost, safe, and effective vaccine for fowl cholera based on a previous work (*Vaccine* 23:5590–5598). *Pasteurella multocida* A: 1 grown in the presence of low $FeCl_3$ concentrations, inactivated with higher concentrations of $FeCl_3$, and adjuvanted with bacterial DNA from *P. multocida* B: 2 containing immunostimulatory CpG motifs protect chickens with a lethal *P. multocida* A: 1 challenge. Chickens were immunized with two whole-cell inactivated vaccine doses at 4 weeks apart and challenged 4 weeks after booster immunization. Experimental vaccines were pure, easy injectable, and caused very little distress in chickens due to their aqueous consistency. Vaccines and bacterial DNA (bDNA) posed no safety problems when chickens were injected subcutaneously (s.c.) with a single, double, and overdose of these preparations. Immunized chickens produced systemic IgY antibodies (Ab) responses and vaccine adjuvanted with bDNA protected 100% chickens from lethal intrapertoneal (i.p.) *P. multocida* A: 1 challenge. This work suggests that use of bDNA as an adjuvant can improve the cost-effectiveness of inactivated veterinary vaccines for their use in developing countries. Our future studies will focus on safety and potency evaluation of experimental and current vaccines using bDNA as an adjuvant.

1. Introduction

Pasteurella multocida infection in chickens causes highly fatal disease fowl cholera that has a great economical impact on commercial and back-yard poultry production [1,2]. Fowl cholera has global occurrence and is capable of infecting all avian species. Vaccination is definitely the most effective way to prevent this disease. For vaccination purposes, inactivated *P. multocida* is used with oil or aluminium adjuvants in most parts of the world except in North America where a live attenuated vaccine is in use [3]. Vaccine improvement and development is an integral part of disease prevention and eradication. Economic constraints of vaccine affordability in developing world could be overcome by developing inexpensive, safe, and better vaccines [4]. Vaccine cost-effectiveness is also a major criterion for effective disease control [5].

We have attempted to develop an inexpensive, safe, and better vaccine for fowl cholera based on a previous work [6] where authors developed a novel inactivated *Salmonella* vaccine formulation that afforded full protection against murine typhoid under experimental conditions. In present study we have attempted to develop an inactivated *P. multocida* A: 1 vaccine by using: (i) iron-sufficient brain-heart infusion (BHI) broth to grow bacteria with better antigenic complementation, (ii) mild inactivation agent like ferric chloride which it self is an adjuvant and preserve antigenicity, and (iii) CpG motif containing bDNA from *P. multocida* B: 2 as an adjuvant which enhances humoral and cellular immune responses of vaccines.

We report here the design, safety and efficacy of experimental vaccines in chickens. Vaccines adjuvanted with bDNA generated good systemic IgY Ab levels and were found to be a potent than formaldehyde-killed and alum adjuvanted vaccine in terms of protection from homologous lethal *P. multocida* A: 1 challenge.

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2. Methods

2.1. Chickens

Day-old chickens were obtained from Central Avian Research Institute, Izatnagar, India and housed in the animal sheds and Infectious disease challenge sheds of the Immunology Section, Division of Animal Biotechnology, Indian Veterinary Research Institute (IVRI), Izatnagar, India. Approval for the experimentation on the chickens was taken from Institutional Animal Ethical Committee and their guidelines were followed for maintenance, handling and care of chickens. Chickens were used for study of vaccine safety, humoral immune response development and vaccine efficacy studies. Blood was drawn from jugular vein in a volume not exceeding 1 ml/bleed/chick every week for serum collection.

2.2. Bacterial culture, maintenance, and virulence

P. multocida B: 2 strain P52 (vaccine strain) and *P. multocida* A: 1 poultry isolate 131 used in the study were obtained from Division of Bacteriology and Mycology, IVRI, Izatnagar, India. Blood agar (BA) was used for isolating single colonies and counting colony forming units (CFU) of the Pasteurellae. For broth cultures, Brain heart infusion broth (BHI) (HiMedia Labs, Mumbai, India; Cat #M210) was used for growth of *P. multocida* A: 1 and B: 2 for 24 h at 37 °C. This media was manufactured according to the provisions relevant to the Bovine Spongiform Encephalopathies (BSE)/Transmissible Spongiform Encephalopathies (TSE) safety of the products according to the certificate provided by the manufacturer. For iron-supplementation of BHI, stock solution of 1 M $FeCl_3$ was added drop-by-drop to media at a final concentration of 100 μM. Live Pasteurellae were stored in BHI-50% glycerol broth at −20 °C for a period of not more than 4 months. Virulence of 18 h grown *P. multocida* A: 1 culture in 6–8-week-old chickens was determined by calculating LD_{50} by the method of Reed and Muench [7].

2.3. Formalin-inactivated vaccine

Seed culture for vaccine production was produced by inoculating a single colony of *P. multocida* A: 1 in 5 ml BHI broth in a culture tube. This seed culture tube was incubated for 18 h at 37 °C as a static culture. Seed culture was then scaled up to 1 l fresh BHI broth at a ratio of 1:50. This scaled-up bacterial culture was grown as a static culture at 37 °C for 24 h in a 100 ml volume/1 l Roux culture bottle. The bacteria were spun down at 3500 rpm at 4 °C for 45 min and resuspended in 20 ml of normal saline solution (NSS). Formaldehyde inactivation of the bacteria was done by the method of Jones et al. [8] by adding formaldehyde drop-by-drop to bacterial culture up to the final concentration of 0.5% with intermittent shaking. Formaldehyde added bacteria were incubated at 37 °C for 24 h and residual formalin was removed by washing inactivated culture twice in NSS. Finally, this preparation was resuspended in NSS and stored at 4–8 °C. This preparation was designated as formalin-inactivated *P. multocida* A: 1 experimental vaccine (FIV). FIV was adjuvanted with 0.5% aluminum potassium sulfate (Merck India Ltd.) and this experimental vaccine was designated as FIV-Alum.

2.4. Iron-inactivated vaccine

Seed culture of *P. multocida* A: 1 was prepared essentially as described above in Section 2.3. BHI broth was supplemented with 100 μM iron as described in Section 2.2.

Bacterial growth and harvesting was done as mentioned in Section 2.3. Iron-inactivation of the bacteria was done by the method of Kumar and Singh [6] by adding 1 M $FeCl_3$ drop-by-drop into bacterial culture up to the final concentration of 100 mM with intermittent shaking. $FeCl_3$ added bacteria were incubated at 37 °C for 4 h with intermittent shaking and residual $FeCl_3$ was removed by washing the inactivated culture twice in NSS Finally, this preparation was resuspended in NSS and stored at 4–8 °C. This preparation was designated as iron-inactivated *P. multocida* A: 1 experimental vaccine (IIV). IIV was adjuvanted with *P. multocida* B: 2 genomic DNA (bDNA) and designated as IIV-bDNA.

2.5. Isolation, purification, and quantitation of bDNA

P. multocida B: 2 bDNA was isolated by a minor modification of method as mentioned by Wilson [9]. Our modification included bacterial cell wall digestion with lysozyme followed by RNA digestion by RNase. Protease treatment of bDNA was done to remove contaminating proteins. Repeated CTAB/NaCl extraction followed by phenol–chloroform extraction was done to remove bacterial polysaccharides. The purified bDNA was precipitated by two volumes of chilled ethanol, pelleted, air-dried, and resuspended in endotoxin free LAL reagent water (Pyroclear®, Associates of Cape Cod, USA). Sterility of the bDNA was checked as mentioned in Section 2.6. DNA quantification and purity determination was assessed by A_{260}/A_{280} ratio. Lipopolysaccharide (LPS) contamination of bDNA was assessed by limulus amebocyte lysate (LAL) assay. Known concentrations of control standard endotoxin from *E. coli O113:H10* (100 ng/ml) were two-fold diluted in LAL reagent water (LRW) ranging from 0.5 to 0.031 EU/ml that corresponded to 100–6.25 ng/ml of LPS. Test bDNA was appropriately diluted to 5 μg in 100 μl LRW (single adjuvant dose). Appropriate negative control (LRW) and positive control (Standard LPS) were used. Pyrotell LAL reagent of 0.125 EU/ml sensitivity and test samples were diluted 1:1 in pyrogen free glass tubes and kept at 37 °C in a water bath for 1 h. Positive results indicated by formation of purple gel-clot upon inverting the glass tubes. All reagents were from Associates of Cape Cod Inc., USA.

2.6. Sterility, safety, and innocuity of the experimental vaccines

Sterility of the experimental vaccine preparations was tested by inoculating 200 μl of vaccine on Sabouraud's dextrose agar (SDA), BA, and in 5 ml of BHI broth and then examining viable *P. multocida* A: 1, if any. Sterility of bDNA was also tested similarly. Safety trials of experimental vaccines were done by immunizing different groups of chickens as per recommendation of the World Organization for Animal Health [3,10]. Briefly, IIV-bDNA vaccine was administered with an antigenic mass of 25 mg equivalent to 5.2×10^8 CFU (10× overdose) along with 50 μg (10× overdose), 100 μg (20× overdose), 200 μg (40× overdose) of bDNA and 200 μg of bDNA alone. After injection, body temperatures of the chickens were recorded for a week. Same vaccines were also observed for the safety in a single normal dose (2.5 mg wet weight equivalent to 5.2×10^7 CFU) or repeated two doses. The ease with which the vaccines were injected and discomfort that chickens exhibited during and after immunization was used to measure innocuity/injectibility of vaccines.

2.7. Immunization of chickens with experimental vaccines

Five groups of 20 chickens of 1d old age were reared. The amount of antigenic mass in each dose of primary immunization with experimental vaccines was 2.5 mg of wet weight of *P. multocida* A: 1, which was equivalent to 5.2×10^7 CFU. Iron-inactivated vaccine (IIV) was adjuvanted with 5 μg of bDNA (IIV-5 μg bDNA) and 10 μg of bDNA (IIV-10 μg bDNA). FIV was adjuvanted with 0.5% alum (FIV-Alum). Control groups received 20 μg bDNA and NSS 200 μl NSS. All immunizations were done by s.c. route in a volume of 200 μl/dose. Secondary immunization of chickens was done after 4 weeks of pri-