Fowl cholera: Evaluation of a Trivalent *Pasteurella multocida* Vaccine Consisted of Serotypes 1, 3 and 4

Short Communication

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Summary

An inactivated trivalent fowl cholera vaccine consisted of serotypes 1, 3 and 4 *Pasteurella multocida* strains was prepared. The vaccine provided 70-100% protection against challenge with homologous starins. ELISA assay showed a considerable increase in antibody titer after towice vaccination of 8 weeks chickens. It was found that the trivalent vaccine can induce immunogenic response in vaccinated chickens.

Key words: *Pasteurella multocida*, fowl cholera, trivalent vaccine, ELISA

Introduction

Fowl cholera, caused by *P.multocida* can result in either an acute septicemia or chronic localized infections in domestic and wild birds (Sander *et al* 1998). Among the bacterial diseases of domestic birds, fowl cholera accounts for major economic losses to the industry through death, weight loss and condemnations. The disease has been recognized for over 200 years and has been the subject of many researches. Despite this attention fowl cholera still remains a problem in the modern poultry industry (Rimler & Roades 1989)

In Iran, since the first report of fowl cholera reported in 1971 (Bozorgmehrifard & Afghan) outbreaks of the disease have been reported by the Veterinary Organization in the northern part of Iran where fowl cholera is endemic (Kalaydari *et al* 2004,

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Tavasoli *et al* 1984). A monovalent killed aluminum hydroxide vaccine prepared from serotype A1 *P.multocida* (Sotoodehnia *et al* 1986) in Razi Vaccine & Serum Research Institute, is used for induction of immunity against avian cholera. It was demonstrated that the killed vaccine protected the chickens against homologous challenge (Sotoodehnia *et al* 1984) however; in spite of vaccination in the endemic area outbreak of fowl cholera has been reported (Kalaydari 1998). Bacterins have been widely used to prevent fowl cholera however these vaccines generally afforded homologous but not heterologous protection (Petersen *et al* 1991). Recently, presence of other serotypes of *P.multocida* was reported from Iran (Jabbari *et al* 2001). This investigation was undertaken to develop a trivalent vaccine containing serotypes 1, 3 and 4 of *P.multocida* strains and to evaluate its efficacy in chickens.

Materials and Methods

Chickens and laboratory animals. 8-week-old chickens were raised in an isolated facility. Feed and drinking water were available all the time. The mouse and rabbits for pathogenecity study were obtained from Laboratory Animals Productions Department, Razi Institute.

Vaccine preparation. The freeze dried stocks of serotype 1 (PMI030 strain), serotype 3 (PMI035 strain) and serotype 4 (PMI047 strain) of *P.multicided* isolated from field and natural outbreaks of fowl cholera in the northen provinces were used. Phenotypic and molecular characterization, and the minimum lethal dose (MLD) as a virulence indicator of the isolates were described previously (Jabbari *et al* 2002a, b, 2003). After reconstitution with 0.5ml triptose phosphate broth (TPB), each strain was streaked on sheep blood agar plate and incubated for 24h at 37°C. A single typical colony was cultured in 300ml TPB and incubated for 18h at 37°C with shaking. Cell suspensions was inactivated by adding 0.3% formaldehide and was left to stand for 24h. The cells were separated by centrifugation at 5000*g* for 30min and the pellet was resuspended in PBS (pH7.2). The optical density of each suspension was adjusted to 1 spectrophotometerically (Ultraspec 2000, Pharmacia) at 540nm. The aluminum hydroxide gel, as an adjuvant, was added to a mix equal proportion of each strain up to 1% final concentration. The vaccine was stored at 4°C. The purity and sterility tests were done according to OIE manual (2001).

Immunization and challenge procedure. 8-week-old SPF chickens were divided in two vaccinated and control groups. 1 ml dose of the prepared vaccine was intramascularly administrated and vaccination was repeated 3 weeks later. The chickens were challenged 2 weeks after booster injection. To challenge a typical colony of each strain was transferred to 10ml TPB and incubated for 6h at 37°C. The optical density of these cultures was then adjusted spectrophotometrically to 0.440 at 540nm. The concentration of these cultures was approximately 2×10^9CFU/ml. The cultures were diluted serially ten-fold in PBS and selected dilutions were used for challenging. For estimating the concentration of viable organisms, 0.5ml of selected suspensions were spread on BA plates. The colonies were counted after 24h incubation at 37°C. Blood samples were taken from the wing web brachial vein of all chickens before first vaccination, booster and before challenge exposure. Sera were collected and stored at –20°C until use.

Serological assay. An ELISA test was used to evaluate the immune response of vaccinated chickens by using two antigens, whole cell and sonicated antigens. Whole cell antigen was prepared according to the Kedrak *et al* (2000) method with some modifications. Briefly, the antigen was prepared using 18-h culture of agitated *P.multocida* in TPB medium at 37°C. The culture was inactivated with 0.3% formalin, centrifuged twice and washed with PBS, pH7.2. The bacterial pellet was suspended in carbonate buffer, pH9.6. The suspension was adjusted to optical density of 0.440 at 540nm spectrophotometrically. Antigens were stored at –20°C until use. In prelimnary examination optimum dilution of whole cell antigen was evaluated as 1:10. Sonicated antigen was prepared as described by Perelman *et al.* (1990). Coating and blocking of microplate (Polysorb, Nunc) and ELISA procedure were carried out according to Avakian and Pick (1985). Absorbancy of the plates were read at 405nm by an ELISA reader (MRXII-Dynex).

Results and Discussion

Pathogenecity of the P.multocida strains. Minimum Lethal Dose as a pathogenecity indicator of *P.multocida* isolates in mouse, rabbit and chicken is presented in table 1. It was found that *P.multocida* strain PMI030 (serotype 1) was highly virulent in all tested animals. Injection of *P.multocida* strains (with different MLD) induced lethality in rabbit. Strain PMI030 was found to be highly virulent for chicken. Injection of only 20CFU killed all birds in challenged group whereas, inoculation of 2×10^6CFU of PMI035 killed all inoculated birds. None of the birds injected by 2×10^8 CFU of PMI047 died, however all showed some signs of sickness such as anorexia, ruffled feather and diarrhea. *P.multocida* was isolated from kidney, spleen, heart and bone marrow of all sick and died birds.

Table 1. *Minimum lethal dose of P.multocida starins in mouse, rabbit and chicken*

***P.multocida* strain**	**Mouse**	**Rabbit**	**Chicken**
PMI030	20	2×10^3	0.2×10^2
PMI035	200	200	2×10^6
PMI047	2×10^4	2×10^6	Not done*

*This strain was low pathogen for chicken

Efficacy of the polyvalent vaccine. All chickens challenged with 0.75×10^2CFU of PM1030 and 2×10^9CFU of PM1047 were survived whereas 7 out of 10 challenged chickens with 2×10^7CFU of PM1035 were survived. Chickens immunized twice with trivalent vaccine were resistant (70-100%) to challenge with homologous serotypes. As serotype 4 (PMI047) *P.multocida* was a low virulent strain, inoccurance of sickness signs in challenged birds was considered as protective immunity. *P.multocida* could not be recovered from immunized chickens, which survived the challenge while it could be isolated from all dead or sick birds. The results of homologous and heterologous serotype challenge revealed that effective killed vaccine against fowl cholera should contain the important serotypes to induce broad-spectrum protection. It is well known that the most important serotypes of *P.multocida* which cause fowl cholera are serotypes 1, 3 and 4 (Glisson *et al* 2003). Results of measuring the antibody level by ELISA in immunized chickens detected with whole cell and sonicated antigens are shown in table 2. The trivalent fowl cholera vaccine gave significant protection against experimental challenge with each of three serotypes.

Table 2. *Mean antibody level (optical density) in chickens before vaccination, two and four weeks after vaccination detected by whole cell and sonicated antigens of P.multocida starins*

***P.multocida* strain**	**Whole cell antigen**					**Sonicated antigen**				
	Pre-vaccination	**2 weeks**	**P/N Ratio**	**4 weeks**	**P/N Ratio**	**Pre-vaccination**	**2 weeks**	**P/N Ratio**	**4 weeks**	**P/N Ratio**
PMI030	0.321 (0.041)	0.787 (0.123)	2.45	1.695 (0.149)	5.28	0.547 (0.069)	1.092 (0.227)	1.99	1.816 (0.136)	3.31
PMI035	0.291 (0.021)	0.638 (0.121)	2.19	1.371 (0.144)	4.71	0.415 (0.101)	0.754 (0.215)	1.81	1.329 (0.2050	3.2
PMI047	0.289 (0.045)	0.591 (0.101)	2.04	1.356 (0.136)	4.69	0.481 (0.071)	1.017 (0.127)	2.11	1.616 (0.169)	3.35

The potential use of ELISA test as a practical method for determination of immunological response of poultry to vaccination programs has been evaluated previously (Avakian *et al* 1989, Sender *et al* 1989). The method was introduced to detect antibody levels to *P.multocida* in turkeys (Marshall *et al*1980), chickens (Dick & Johnson 1984, Hofacre *et al* 1987) and geese (Kedrak *et al* 2000). In chickens and turkeys, the antibody titer was measured with ELISA highly correlated with protection against challenge with virulent organisms. Results of this study indicated that ELISA test could be valuable in the evaluation of the immune response of vaccinated chickens with killed vaccines after 8 weeks of age. All chickens showed a secondary response greater than that seen in chickens vaccinated once (Table 2). It seems that a significant immunolgical stimulus had been elicited by the second exposure. Such antibody response was well within the expected normal range and would provide protection against a cholera challenge. As antibody levels showed at virulent challenge exposure, it became evident that all birds with P/N ratio of 3.2 or higher survived. So far, whole cell and sonicated antigens of *P.multocida* have been generally used as coating antigen in ELISA test (Perelman *et al* 1990, Solano *et al* 1983).

In the present study, the whole cell was used as coating antigen resulted in high sensitivity. Howevere some nonspecific reactions happend when sonicated antigen was used. It seems that when bacterial cells were disrupted by ultrasonication, both internal and external antigenic substances can be solublized, which can be bind nonspecifically to antibodies in the serum.

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