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Original Article

Comparative Efficacy of a Conventional and a Newly Developed Fowl Cholera Vaccine in Commercial Layer Chickens

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The experiment was undertaken to find out the comparative efficacy of a conventional vaccine [prepared from an old isolate, PM-38 *P. multocida* serotype 1 (X-73) and a newly developed fowl cholera (FC) vaccine (prepared from a recent isolate, PNX-2, *P. multocida* serotype 1) in Fayoumi chickens, Morphology, staining, cultural and biochemical properties and pathogenecity of both the isolates of *P. multocida* were carried out by different bacteriological techniques. The PNX-2 bacterial inoculums were used as challenge dose for protection test. Both the vaccine dose and challenge dose contained approximately 5×10^7 CFU/ml. The immune responses were measured by passive hemagglutination (PHA) test. When conventional FC vaccine was administered intramuscularly, the serum mean PHA titers were recorded 44±5.85, 68±9.44 and 48±6.05 at 15 days post vaccination (DPV), 30 DPV and 45 DPV respectively. Whereas when the conventional FC vaccine was administered subcutaneously the serum mean PHA titers were determined as 48±6.05, 96±12.09, 80±10.47 at 15 DPV, 30 DPV and 45 DPV respectively. Therefore, the conventional FC vaccine that administered by subcutaneous route revealed higher immune response. On the other hand, the newly developed vaccine that was administered by subcutaneous route revealed higher immune response (the serum mean PHA titers were 60±4.00, 104±11.71, and 96±12.09 at 15 DPV, 30 DPV and 45 DPV respectively) compared to the newly developed FC vaccine (the serum mean PHA titers were 48±6.05, 80±10.47 and 52±5.86 at 15 DPV, 30 DPV and 45 DPV respectively) which was administered by intramuscular route. However, it was demonstrated that both the fowl cholera vaccines conferred 100% protection ($P<0.01$) against challenge infection at 30 days of secondary vaccination while unvaccinated control birds were found to be infected.

Key words: Fayoumi chickens, Fowl Cholera Vaccines, Passive hemagglutination (PHA) test, routes of vaccination

Introduction

Poultry sector in Bangladesh is facing various problems related to production and health management. Among the microbial diseases, fowl cholera is a major threat to poultry industry. It is a contagious bacterial disease caused by *Pasteurella multocida (P. multocida)* affecting domesticated chickens and other types of poultry, game birds rear in captivity, companion birds, birds in zoos and wild birds. Fowl cholera causes mortality of about 25% to 35% in chickens in Bangladesh[1]. According to central disease investigation laboratory (CDIL), Department of Livestock Services (DLS) Dhaka, Bangladesh, this disease is responsible for at least 25% of the total annual mortality of chickens[1].

P. multocida is a Gram-negative, non-motile, non-spore forming rod shaped organism occurring singly, in pairs and occasionally as chains or filaments. The organisms show bipolar character in Leishman's stain. The bacterium grows on blood agar, nutrient agar and nutrient broth and growth is enhanced with the addition of yeast extract and beef extract. *P. multocida* produce characteristics colonies with whitish opaque, circular and translucent appearance and no hemolysis on blood agar medium[2].

The disease is more prevalent in late summer, fall and winter[3]. It usually appears as septicemic form associated with high morbidity and mortality. Death loss from fowl cholera in chickens usually occurs in laying flocks, because the aged

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birds are more susceptible than younger chickens[1, 3]. *P. multocida* infection is transmitted primarily by excretion from mouth, nose and conjunctiva of infected birds that contaminate feed and water. The disease may also be transmitted through 'carrier birds' and insects vector. The acute form of disease is characterized by sudden death without premonitory signs and the clinical signs of fowl cholera are anorexia, fever, ruffled feathers, mucus discharge from mouth, rapid respiration and diarrhoea which is watery to yellowish initially and greenish with mucus in the late stage[4]. In chronic form of disease, clinical signs include depression, conjunctivitis, dyspnea, lameness and torticollis. Swelling of the wattle, sinuses, limb joints, foot-pads and sternal bursae may also be present[4].

As a means of control of this disease, prophylactic measures with strict biosecurity measures, sanitary conditions as well as vaccination are essential. Fowl cholera may be caused by any 01 of 16 Heddleston serotypes of *P. multocida*. The *P. multocida* vaccines in general use, are bacterins prepared from inactivated cells of serotypes selected on the basis of epidemiological information. In Bangladesh, fowl cholera vaccines are produced by Livestock Research Institute (LRI) under the Department of Livestock Services (DLS), at Mohakhali, Dhaka and Poultry biologics unit at Bangladesh Agricultural University (BAU), Mymensingh[5, 6].

A local strain of higher immunogenic value should be selected as a vaccine strain for preparation of bacterin with a view to controlling fowl cholera. Islam[6] stated that a vaccine of higher immunogenicity is required to prevent the loss of birds from fowl cholera. Therefore, the present research was conducted to develop a new fowl cholera vaccine with a recent isolate of *P. multocida* (PNX-2), to evaluate the efficacy of the newly developed vaccine with that of the conventional one, *P. multocida* (PM-38) serotype 1 (X-73) and also to evaluate the proper route(s) of vaccination.

Materials and methods

Vaccine and Challenge isolate

A field isolate of virulent *P. multocida* (PM-38) serotype 1 (X-73) was used for the preparation of conventional vaccine was obtained from the laboratory stock culture of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh and Phinix isolate (PNX-2) of virulent *P. multocida* from Phinix poultry Farm Ltd. Gazipur, was used in this study for the preparation and trial of vaccine. Vaccines were prepared from these two different isolate in the laboratory at the dose rate of $5x10^7$ cfu/ml. The same bacterial isolates were passaged at least thrice for the preparation of challenge dose as per the standard method.

Experimental chickens

A total of 40 day-old chicks of Fayoumi breed were collected from the central Govt. poultry farm of Department of livestock services (DLS) at Mirpur, Dhaka. These chicks were reared in the poultry shed of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. Then a total of 40 Fayoumi birds of either sexes at 14 weeks of age were divided into five groups namely A, B, C, D and E containing 8 birds in each group (n=8). Of these, A, B, C and D groups were used for trial of vaccines while group E served as unvaccinated control. Pre-vaccination sera were collected to determine the immune status of birds.

Vaccination Schedules

Fayoumi layer birds of 14 weeks aged were divided into five groups consisting of eight birds per group. Primary vaccination was given at the dose rate of 1 ml of $5x10^7$ CFU by conventional fowl cholera vaccine through intramuscular (IM) and subcutaneous (SC) route in either of the selected groups A and B respectively and the newly developed vaccine was administered through intramuscular (IM) and subcutaneous (SC) route in each selected groups C and D respectively. The same groups of birds were boostered with the same dose and route after 15 days of primary vaccination in groups A, B, C and D. The study was carried out in the animal shed of the Department of Microbiology and Hygiene with provision of a nutrient diet and ventilation following strict bio-security. Post-vaccination sera were collected at 15, 30 and 45 days post-vaccination (DPV). The antibody level of pre-vaccination and post vaccination sera were determined by passive haemagglutination (PHA) test. Protection test was carried out in each vaccinated and control groups after 30 days of secondary vaccination.

Determination of pathogenicity of the P. multocida isolates in mice

Pathogenicity of both the isolates in mice was determined following the procedure described by Tripathy *et al.*[8]. In brief, eight Swiss Albino mice were used and divided into two groups (4 mice in each group). Each mouse of group I was injected with 0.2 ml of bacterial suspension (2.6×10^8 cfu) with the help of a tuberculin syringe. The mice of group II served as control and fed on saline water. All the mice were reared in the laboratory animal shed for 24 hours and were recorded for clinical signs and symptoms at every six hours interval. The findings were observed as normal, sick or dead and any signs of sickness or death of the mice during the period was considered as their susceptibility to the bacteria or toxin present in the broth culture.

Preparation of fowl cholera vaccines from two different isolates and estimation of colony forming unit (cfu)

The vaccines were prepared according to the method described by Cowan[7] and the colony forming unit (cfu) was determined as per the method of Michael *et al.*[9] CFU was calculated according to ISO[10].

Sterility test of conventional and newly developed fowl cholera vaccines

The sterility test was carried out according to the procedure described by Choudhury *et al.*[11]. 1 ml of suspension from conventional FC vaccine and newly developed FC vaccine was inoculated separately in each of the 5 blood agar plates and kept overnight at 37^0C for bacteriological sterility. The plates showing no growth was indicated complete inactivation of *P. multocida* organisms and negative for other contaminating organisms.

Preparation of challenge dose for protection test

Both vaccinated and control groups of birds were subjected to challenge by intramuscular (IM) administration of a virulent Phoenix isolate of *P. multocida* (PNX-2), following the standard procedure[11]. From the stock culture, the organisms were inoculated in blood agar as subculture and incubated at

37°C for 24 hours. Then the culture was tested for purity of the organism by Gram's staining method. The challenge inoculum contains $5x10^7$ cfu/ml. For the protection test, 5 (five) birds of each group of vaccinated and control were randomly selected. The challenge infection was done in each group after 30 days of boostering and at the same time in unvaccinated control group.

Post-challenge observation of birds

Birds after challenge infection were observed frequently up to one week for any clinical signs and symptoms of fowl cholera. The clinical findings of both the vaccinated and unvaccinated chickens were observed and recorded every 6 hours interval.

Post-challenge isolation of P. multocida

The procedure suggested by Matsumoto and Helfer[13] was followed in this study. After 15 days of challenge exposure, a few of the selected survivors of each challenge group were sacrificed. Swab or tissue materials were taken from liver, lungs, spleen, kidney, bone marrow, brain and the inoculation sites in the thigh muscles from these chickens and were streaked on to blood agar plates. The plates were examined after 24 to 48 hours of incubation at 37°C for the growth of *P. multocida.* The positive cases were confirmed by the usual standard procedures described earlier.

Passive haemagglutination (PHA) test

The test was used to determine titers of antibodies in birds having been inoculated with the antigen containing *Pasteurella multocida* as per the method described by Chowdhury *et al.*[11], Carter[12], Mondal *et al.*[14], Sarker *et al.*[15], Siddique[16] and Tripathy *et al.*[17] but with slight modification. The modification for the tests was that in this test, capsular antigens (soluble antigen) of *P. multocida* are coupled to chemically modified erythrocytes (sheep erythrocytes) and then antigen-coated erythrocytes readily react with specific antibodies and results in haemagglutination.

Statistical analysis

The effects of vaccination on experimental birds in terms of PHA titer and protection capacity of vaccinated birds against challenge infection were subjected to analysis of geometric mean with standard error. The analysis was performed by using Statistical Package for Social Sciences (SPSS) program. The PHA titers were analyzed by F-test to determine the protective capacity of vaccinated birds against challenge exposure.

Results and Discussion

Both the organisms produced characteristic colonies and these included whitish, discrete, opaque, circular, convex and translucent in appearance. The both capsulated organisms produce larger colonies. No hemolysis was observed in blood agar media. The growth of *P. multocida* in nutrient broth was characterized by diffused turbidity. In some cases, pellicle was formed. The morphology of both vaccine strains of *P. multocida* were identified by using Gram's staining method. The organisms were coccobacillary or short rod shaped and generally occurred single or in pairs. Bi-polar characteristics of *P. multocida* were observed by Leishman's staning of the fresh culture of the organisms. The morphology, staining and cultural characteristics of both the isolates in different culture media as recorded were almost similar to those described by other studies[1, 18-19]. Experimental inoculation of two different isolated *P. multocida* in mice separately produced characteristic changes in different visceral organs. Following inoculation, mice died within 24 hours and there was a marked septicemic lesions consisting of white necrotic foci and hemorrhages in lungs, trachea, liver and spleen. Mortality of mice indicated that the organisms were highly potent. Heddleston and Watko[20] found that mice and rabbits died of acute septicemia after intranasal infection with *P. multocida* isolated from acute case of fowl cholera. *P. multocida* organisms from two different isolates fermented dextrose, sucrose and mannitol but not maltose and lactose separately. These fermented sugars produce acid without gas. Both the isolates were able to ferment dextrose, sucrose and mannitol completely and produced acid without gas but incomplete fermentation was recorded in case of maltose and lactose. These biochemical reactions were closely related with the findings of Calnek *et al.* [4] and Choudhury *et al.*[11].

Slimy precipitation, no precipitation or partial flocculation was considered positive for capsule and complete flocculation were considered negative for capsule. Both the isolates were considered positive for capsule. Both the vaccine organisms contained well developed capsules in fresh culture which were determined by acriflavine test according to the procedures suggested by Chowdhury *et al.*[1], Carter[12] and Cheesbrough[21]. Moreover, Chung *et al.*[22] and Boyce *et al.*[23] suggested that capsulated strains of *P. multocida* should be selected as vaccine strain. They also reported that capsular strain was more antigenic and produced better immune response in chickens against fowl cholera.

Table 1. *Mean PHA titers of sera of chickens vaccinated with conventional and a newly developed Fowl cholera Vaccine (FCV) through IM followed by IM as determined by F-test*

Groups	Route of vaccination	Secondary vaccination Interval	Mean PHA±SE of 0 day	Mean PHA±SE at 15 DPV	Mean PHA±SE at 30 DPV	Mean PHA±SE at 45 DPV
A (Conventional Vaccine)	IM followed by IM	15	<4±0.00	44±5.85b	68±9.44b	48±6.05b
C (Newly developed Vaccine)	IM followed by IM	15	<4±0.00	48±6.05a	80±10.47a	52±5.86a
E	Unvaccinated (Control)	-	<4±0.00	<4±0.00c	<4±0.00c	<4±0.00c
	Level of significance			**	**	**

PHA = Passive haemagglutination, DPV = Days post vaccination, SE = Standard error, IM= intramuscular,

***= Significant ($p<0.01$); Similar letters differed within column insignificant and dissimilar letters significant as per Duncan's Multiple Range Test (DMRT).*

Table 1 and 2 represents the mean PHA titers of sera of chickens vaccinated with two FC vaccines through IM and SC route respectively. The mean serum PHA antibody titers on 15,

30 and 45 DPV in group A (Table 1) were 44±5.85, 68±9.44 and 48±6.05, respectively, which were the lowest values among four experimental groups. On the other hand group D exhibited highest values which were 60±4.00, 104±11.71 and 96±12.09 (Table 2) for the aforementioned consecutive days post vaccination. While in group E served as control birds the PHA titer was <4.0±0.00 in all cases. The findings were closely related with the reports of Mondal *et al*[14] and Coates *et al*[24]. In this investigation it was found that chickens of group D which were vaccinated with newly developed fowl cholera vaccine through SC followed by SC route produced comparatively better immune response than group A, B and C.

All the control and experimentally vaccinated chickens that were challenged with virulent vaccine strain of Phoenix isolate of *P. multocida* (PNX-2) at the dose rate of 5×10^7 cfu/ml at IM route and the following clinical findings were observed. In post challenge observations, control birds showed characteristic clinical signs and symptoms of avian pasteurellosis like dullness, depression, anorexia, hyperthermia, laboured breathing followed by lameness, whitish (chalky) diarrhoea and ultimately death occurred. Vaccinated birds protected themselves and did not show clinical signs except dullness, depression and drowsiness. Sharma *et al.*[25] and Rhoades and Rimler[26] described that

Table 2. *Mean PHA titers of sera of chickens vaccinated with conventional and a newly developed Fowl cholera Vaccine (FCV) through SC followed by SC as determined by F-test*

Groups	Route of vaccination	Secondary vaccination Interval	Mean PHA±SE of 0 day	Mean PHA±SE at 15 DPV	Mean PHA±SE at 30 DPV	Mean PHA±SE at 45 DPV
B (Conventional Vaccine)	SC followed by SC	15	<4±0.00	48±6.05b	96±12.09b	80±10.47b
D (Newly developed Vaccine)	SC followed by SC	15	<4±0.00	60±4.00a	104±11.71a	96±12.09a
E	Unvaccinated (Control)	-	<4±0.00	<4±0.00c	<4±0.00c	<4±0.00c
	Level of significance			**	**	**

PHA = Passive haemagglutination, DPV = Days post vaccination, SE = Standard error, SC= Subcutaneous

**= *Significant (p<0.01); Similar letters differed within column insignificant and dissimilar letters significant as per Duncan's Multiple Range Test (DMRT).*

hyperthermia, dullness, incordination of movements, greenish yellow diarrhoea, laboured and painful breathing and unusual sitting posture were the most prominent clinical symptoms in case of experimentally produced avian pasteurellosis. On the other hand, Gordon and Jordan[27] observed that death of birds in good body condition was the only indication in some outbreaks of fowl cholera. All these findings were in agreement with the observation of the present study. Moreover, the authors also observed that marked depression, anorexia and mucus discharge from the orifices, cyanosis and fetid diarrhea were the most prominent clinical signs in acute cases of illness.

The vaccination dose was 1ml of 5×10^7 cfu for the production of better immune response which was closely related with Coates *et al.* [24] Khan *et al.* [28] and Scott *et al.* [29]. Booster dose was given with the similar dose and route at 15 days intervals in the group A, B, C and D respectively after primary vaccination. Choudhury *et al.*[1] and OIE[30] suggested that fowl cholera vaccine should be given through intramuscular (IM) or subcutaneous (SC) route.

Scott *et al.* [29] noted that at least 10^6 cfu of *P. multocida* P1 and 10^8 cfu of *P. multicoda* P3 were required to provide complete protection against challenge in all birds. Results of challenge exposure demonstrated that birds vaccinated with conventional and newly developed fowl cholera vaccines conferred 100% protection ($p<0.01$) against challenge infection with virulent phoenix isolate (PNX-2) of *P. multocida*, whereas none of the unvaccinated control birds survived. Similar observation was recorded by Mondal *et al.*[14], Coates[31] and Bhasin and Biberstein[32]. Coates[31] found 100 percent protection against challenge infection at 3 weeks of post vaccination following administration of fowl cholera vaccine at the dose of 1.2×10^7 cfu. Bhasin and Biberstein[32] found 60 percent survivability against challenge infection of an alum-precipitated fowl cholera vaccinated birds at 16 week post vaccination. However, Mondal *et al.*[14] found 100% protection against challenge infection of an alum-precipitated fowl cholera vaccinated birds at 5th week post vaccination.

All the vaccinated groups of birds of this experiment withstand the challenge infection. Thus survivability rate of vaccinated chickens was 100%. But all chickens of control group failed to survive when challenged with virulent Phoenix isolate of *P. multocida*. The stimulation of high degree of immunity induced by newly developed fowl cholera vaccine under investigation might be due to higher immunogenic characters of the recent isolate PNX-2 compared to PM-38 isolate of *P. multiocida*. Addition of alum as adjuvant might have further increased the immunogenicity of the vaccine. But, Bhasin and Biberstein[32] reported that aluminum hydroxide was more effective as adjuvant inducing immunity against fowl cholera.

Considering the prime objectives of this study it can be stated that both the fowl cholera vaccines are safe and effective for the vaccination of chickens against fowl cholera. However, newly developed fowl cholera vaccine produce better immune response than conventional FC vaccine and SC followed by SC route of vaccination found to be produced better immune response compared to IM followed by IM route of vaccination.

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