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IMMUNE RESPONSES INDUCED IN CHICKENS BY CAPSULAR EXTRACT OF *P. multocida* ISOLATED FROM FARM RAT

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ABSTRACT

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An experiment was conducted to investigate the immune response induced in chickens by capsular extract of *Pasteurella multocida* isolated from rats wandering in and around the poultry farms. The rat isolate of *P. multocida* was isolated and identified by cultural, morphological, and biochemical characteristics, followed by capsular extract preparation and experimental vaccine development. The isolated *P. multocida* was found Gram-negative, non-motile, non-spore forming rod occurring singly or pairs and occasionally as chains or filaments in Gram's-staining method. The isolates consistently produced acid from dextrose, sucrose and mannitol but not fermented maltose or lactose. The Capsular antigen was extracted and confirmed by acriflavine test. Finally, experimental fowl cholera vaccine was prepared. Primary vaccination was performed at the dose rate of 5.6×10^7 CFU/ml through intramuscular and subcutaneous routes in birds of group A (10 birds) and group B (10 birds) and group C (10 birds) were control birds. Secondary vaccination was similarly performed after 15 days of primary vaccination in groups A and B. The levels of pre-vaccination and post-vaccination sera were determined by passive haemagglutination test. The passive haemagglutination antibody titre was recorded on 15 and 35 days of post vaccination in groups A and B. It was demonstrated that experimental capsular extract fowl cholera vaccine conferred 100% protection ($p < 0.01$) against challenge infection and found to be safe. It could be suggested that after thorough field trial, the experimentally prepared capsular extract FC vaccine using rat isolate of *P. multocida* may be used side by side with conventional FC vaccine.

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INTRODUCTION

Fowl cholera (FC), also known as avian cholera, avian pasteurellosis, avian hemorrhagic septicemia, is a contagious disease affecting domesticated and wild birds (Xiao *et al.*, 2016). Fowl cholera is caused by *Pasteurella (P.) multocida*, a Gram-negative, non-motile, non-spore forming rod, occurring singly or in pairs, and occasionally in chains or filaments (Ievy *et al.*, 2013). About 25% to 35% mortality in chickens of Bangladesh, is due to FC (Choudhury *et al.* 1985). Pathogenicity or virulence of *P. multocida* in relation to FC is complex and variable depending on the strain, host species and variation within the strain or host and conditions of contact between the two (Glisson *et al.*, 2008). Based on capsular antigen, *P. multocida* strains are categorized as serogroup A, B, C, D, and E, and based on lipopolysaccharide (LPS) antigens, further classified into 16 (1 to 16) serotypes (Kwaga *et al.*, 2013). In Asian countries, *P. multocida* type A:1, A:3, and type D are found to be responsible to cause FC, where the serotype A:1 causes 80% mortality in chickens (Ranjan *et al.*, 2011). The ability of *P. multocida* to invade and reproduce in the host is enhanced by the presence of capsule that surrounds the organism (Boyce *et al.*, 2000). Loss of ability of a virulent strain to produce capsule results in loss of virulence (Harper *et al.*, 2006). Snipes *et al.* (1987) also recorded that the capsular form of *P. multocida* were more virulent than that of non-capsulated forms because the capsulated form of bacteria might easily overcome the host's humoral defense.

Fowl cholera is considered as one of the major enzootic bacterial diseases of poultry, causes severe economic loss in Bangladesh (Biswas *et al.*, 2005). It occurs mainly in chickens, turkeys, ducks, geese, and quill (Parvin *et al.*, 2011), however, the causative agent, *P. multocida*, is also recovered from poultry farm rodents (Curtis *et al.*, 1980; Curtis, 1983; Snipes *et al.*, 1988; Snipes *et al.*, 1990). Curtis *et al.* (1980) isolated 41% (14 out of 34 rat-carcasses) *P. multocida* from 11 poultry farms. Besides, Snipes *et al.* (1988) reported 48 isolates of *P. multocida* of various somatic types from different species of mammals and bird in California, USA, and characterization of the isolates revealed that few were as same as the virulent *P. multocida* isolates of turkeys. Moreover, Curtis (1983) recorded the transmission of *P. multocida* infection from the brown rat (*Rattus norvegicus*) to domestic poultry.

Vaccination against FC is practiced as preventive measures in Bangladesh like other countries of the world to reduce the incidence of the disease (Kardos and Kiss, 2005). Two types of FC vaccines are available in Bangladesh, which are produced locally and reported to give better immunity (Rana *et al.*, 2010). Alum precipitated formalin killed FC vaccine is produced in the Livestock and Poultry Research and Production Centre (LPVRPC; formerly known as Poultry Biologics Unit) of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh, while the oil-adjuvant FC vaccine is produced in Livestock Research Institute (LRI), Mohakhali, Dhaka, under the Department of Livestock Services (DLS), Government of People's Republic of Bangladesh (Bag *et al.*, 2015). But, those which are imported or prepared from field cases of FC are much more expensive and there is a chance of being severe infection if sufficient measures are not taken (Akhtar *et al.*, 2016). It is scientifically established that immune responses are influenced by various factors, such as breed and rearing region (Rana *et al.*, 2010), age of the hosts (Dick and Avakian, 1991), and the isolate used as vaccine seed (Akhtar *et al.*, 2016). In this study, the vaccine was prepared with capsular extract of *P. multocida* which was isolated from the rats wandering in and around poultry farm as an alternative way to improve the immune status of chickens. The rat isolate of *P. multocida* can be considered as local isolates for vaccine seed development against FC in poultry (Snipes *et al.*, 1988). Therefore, the present study is focused on the immune responses induced in chickens by newly developed vaccine with capsular extract of *P. multocida* isolated from poultry farm rats through the different routes of vaccination to determine the efficacy of that vaccine against a challenge infection with virulent duck isolate of *P. multocida* PM-38, Serotype-1 (X-73).

MATERIALS AND METHODS

Design of the experiment

The entire experiment was conducted in the Bacteriology Laboratory of Department of Microbiology and Hygiene, Faculty of Veterinary Science, (BAU), Mymensingh. The research work was accomplished in three steps. Firstly, isolation and characterization of *P. multocida* isolated from the rats wandering in and around poultry farm; secondly, preparation of capsular extract to be used as FC vaccine and finally, vaccination and evaluation of the prepared capsular extract FC vaccine. A virulent isolate of *P. multocida* collected from the rats wandering in and around the BAU poultry farm was used for the preparation of capsular extract FC vaccine. The vaccine was prepared according to the procedures described by Choudhury *et al.* (1987).

A total of 30 Fayomi chickens of either sexes of 8 weeks age were selected for this study. The selected chickens were divided into three groups namely A, B and C, containing 10 chickens in each groups, A and B groups were used for trial vaccine while group C served as unvaccinated control. Pre-vaccination sera were collected to determine the pre-immunizing titre of the sera. Primary vaccination was given at the dose rate of 1 ml through intra-muscular (IM) and sub-cutaneous (SC) route in each chickens of group A and B, respectively. Booster dose was given with the similar dose and route to the chickens after 15 days of primary vaccination. 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 and 35 days post-vaccination. The degree of antibody level of Pre-vaccination and post-vaccination sera were determined by PHA test. Protection test was carried out in each vaccinated and control groups after 15 days of secondary vaccination.

Collection of samples

Rats were collected from wandering in and around the BAU poultry farm. Rat's feces / swab were collected aseptically in sterilized petri dishes and test tubes with the help of sterilized inoculating loop. Each sample was cultured on individual petri dishes containing respective media. After 24 hours incubation at 37°C, each plate were examined for identification of organisms and subcultured for pure culture and then stored at refrigerator for further study.

Culture of *P. multocida* organisms

P. multocida organisms were cultured according to the standard method described by Cowan (1985). The collected organisms were inoculated in Blood agar (BA), Nutrient agar (NA) and Nutrient broth (NB) enriched with yeast extract and beef extract for better growth. The inoculating media was incubated at 37°C in bacteriological incubator for characteristic colony formation. Subsequent subculture was done for getting pure culture.

Gram's staining and Carbohydrate fermentation reaction

The representative bacterial colonies were characterized morphologically using Gram's stain and biochemically using basic five sugars according to the method described by Merchant and Packer (1967).

Acriflavine test of capsular antigen

The test was carried out following the methods suggested by Furian *et al.* (2014) for identification of antigenic variants and colonial characteristics. A number of colonies were picked up from the growth on BA plates. Heavy suspensions of organisms were prepared on a clean grooved glass slides with one or two drops of 1:1000 acriflavine solution in distilled water. The reaction was read just after mixing. Isolates which indicated reactions like slimy precipitation, no precipitation or partial flocculation in acriflavine test were considered as positive.

Maintenance of stock culture of the organisms

Nutrient agar slants were used to maintain the stock culture. The *P. multocida* organisms were inoculated in slant by streaking and were incubated at 37°C for 24 hours. Finally, sterile mineral oil was overlaid and kept the slant at room temperature for future use.

Pathogenicity of the *P. multocida* isolate

In mice model: Pathogenicity test was done as described by Merchant and Packer (1967). Adult mice were used to observe the entero-pathogenicity of the selected isolated strains of *Pasteurella* spp. Adult mice were examined clinically and those found free from clinical symptoms were selected from the experiment. Mice were divided into 2 groups, test group (n=5) and control group (n=3). Then the strains of *Pasteurella* spp. were first grown on BA media. A small colony from BA media was added to the 5 ml to NB. The broth was incubated at 37°C for 24 hours. A dose of 0.5 ml of culture was injected *i/m* to test group and 0.5 ml of sterile NB was injected *i/m* to control group of mice and kept in separate cages. All the mice were reared in the laboratory animal shed and given diet, nourishment kept under observation for 24 hours and clinical signs and symptoms at every 6 hours interval were recorded.

In Fayoumi chickens: The fayoumi breed of poultry were divided into 2 groups (n=3) and control group (n=3). Each bird of group 1 was injected with 1 ml of bacterial suspension intramuscularly. The bird of group 2 served as control. All the birds were allowed to rear on same feed and environmental condition under observation for 24 hours and were observed for clinical signs and symptoms at every six hours interval.

Preparation of capsular antigen

The capsular antigen of *P. multocida* was prepared according to the method suggested by Siddique *et al.* (1997). The fresh subculture of *P. multocida* was diluted with PBS and heated at 56°C for 30 minutes in hot water bath to assist the removal of the capsular antigen. After heating, the suspension was centrifuged at 4500-6000 rpm for 20 minutes by using coarse stone bids. The supernatant was considered as a source of capsular antigen.

Preparation of capsular extract fowl cholera vaccine

This procedure was carried out according to the procedure described by Choudhury *et al.* (1987) with slight modification. In this study, laboratory mice were used for passages of isolates of *P. multocida*. For this, the isolates of *P. multocida* organisms were cultured in blood agar media and kept in bacteriological incubator at 37°C for 24 hours and examined the purity of culture and subsequently subcultured in the same media for 24 hours. The isolated colonies were then incubated in nutrient broth added with yeast extract 0.5 gm per liter (0.5gm/L) and beef extract 2 gm per liter (2gm/L) and incubated at 37°C for 24 hours for massive growth. Later on, formalin was added in the broth culture at the rate of 8 ml per liter (8ml/L), heated at 56°C for 30 minutes in hot water bath to assist the removal of the capsular antigen. After heating, the suspension was centrifuged at 4500-6000 rpm for 20 minutes by using coarse stone bids. The supernatant was considered as a source of capsular antigen after performing acriflavine test. Then, CFU estimation and sterility test were performed according to the method described by Michael *et al.* (1979) and Choudhury *et al.* (1987), respectively.

Vaccination of the chicken

This procedure was carried out according the procedure described by Choudhury *et al.* (1987). Fowl cholera vaccine was administered at the dose rate of 1 ml of 5.6×10^7 CFU through IM and SC route via thigh muscle in each selected group of A and B, respectively, at 9 weeks of age. Booster dose was given with the same dose and route after 14 days of primary vaccination in both groups.

Collection of serum from immunized birds

For collection of serum from the immunized birds the procedure described by Siddique *et al.* (1997) were followed. Five ml of blood was collected from the wing vein of all vaccinated chickens of each group without anticoagulant and was poured gently in the sterile glass test tubes at 63 days (as pre-vaccinated sera), after 2 weeks of primary vaccination (at 77 days) and after 3 weeks of secondary vaccination (at 98 days).

Protection test

A known highly virulent duck *P. multocida*, PM-38, Serotype-1 (X-73) which obtained from stock culture of the Department of Microbiology and Hygiene was inoculated in blood agar as subculture and incubated at 37°C for 24 hours. After three subsequent mice passage the subculture so prepared as broth culture to add 3ml PBS. Then CFU of each culture was determined by plate count method and was also tested for purity by Gram's staining method. For preparation of challenge dose, the procedure suggested by Choudhury *et al.* (1987) was followed. Both vaccinated and control group of birds were subjected to challenge, at 45 days of revaccination through IM inoculation following the procedure of Choudhury *et al.* (1987). The challenge inoculums contain 5.6×10^7 CFU. For the protection test, 10 birds from group A and B and 5 birds from group C were selected and challenged after 17 days of blood collection of secondary vaccination. Birds after challenge infection were observed daily up to one week for any clinical signs and symptoms of FC. The clinical findings of both the vaccinated and unvaccinated chickens were observed and recorded.

Passive haemagglutination (PHA) test

The test was used to determine titres of antibodies in birds having been inoculated with the antigen containing *P. multocida* as per the method by Tripathy *et al.* (1970), Choudhury *et al.* (1987) and Siddique *et al.* (1997) but with slight modification. According to Tripathy *et al.* (1970), the pH of PBS, concentration of tannic acid solution, strength of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, were 6.4, 1:25000, 0.15M and 0.15M respectively, but those values in the present study were 7.2, 1:20000, 0.20M and 0.20M, respectively.

Principle and method of the test

The sensitivity and specificity of PHA test procedure depends on the use of soluble antigen. In this case, capsular antigens (soluble antigen) of *P. multocida* are coupled to chemically modified erythrocytes (sheep erythrocyte) and then antigen coated erythrocytes readily react with specific antibodies and results in haemagglutination. This method was

carried out according to the method described by Tripathy *et al.* (1970). The end point was determined by observing the highest dilution at which cells are agglutinated. Agglutination was indicated by a flat deposition of a diffuse thin layer of clumping of RBC on the bottom of the wells. The results were recorded by deposition of a diffuse thin layer of clumping of RBC on the bottom of the wells which indicated HA positive and a compact buttoning with clear zone indicated HA negative. The reciprocal of the highest dilution of sensitized tanned SRBC was considered as titre of the serum.

Statistical analysis

The effect of vaccination on experimental birds in terms of PHA titre and protection capacity of vaccinated birds against challenge infection was subjected to analysis of Geometric mean with standard error, as per method of Zar (2014). The PHA titres were analyzed by t-test to determine the protective capacity of vaccinated birds against challenge exposure (Zar, 2014).

RESULTS AND DISCUSSION

Morphological, cultural and biochemical properties

For staining, smears were prepared from heart blood and liver impression of experimentally infected mice and chickens. Gram's stain revealed the presence of Gram negative, cocco-bacillary or rod shaped and generally occurred single or in paired and bi-polar organisms. Cowan (1985) and Cheesbrough (1985) also recorded similar staining characteristics of *P. multocida*. In blood agar and nutrient agar plate, the organisms produced small whitish colonies, which was opaque, circular and translucent in appearance. No hemolysis was observed in blood agar media. In nutrient broth, the *P. multocida* produced diffused turbidity. The selected *P. multocida* organism fermented dextrose, sucrose and mannitol but not maltose and lactose, and produced acid (indicated by the change of yellow colour of the medium) without gas, which was also observed by Cheesbrough (1985).

Results of pathogenicity test in mice

Experimental inoculation of isolated *P. multocida* in mice produced characteristic changes in different visceral organs. Following inoculation, mice died within 24 hours and there was a marked septicaemic lesions consisting of white necrotic foci and hemorrhages in lungs, trachea, liver and spleen. Glavits and Magyar (1990) found that mice and rabbits died of acute septicemia after intranasal infection with *P. multocida* isolated from acute case of FC. Mortality of mice indicated that the organisms were highly potent.

Result of acriflavine test

Slimy precipitation, no precipitation or partial flocculation was considered positive for capsule and complete flocculation were considered negative for capsule. Both the vaccine and challenge isolates were found positive for capsule in this study. Furian *et al.* (2014) also reported the same findings for acriflavine test.

Results of passive haemagglutination (PHA) test

Humoral immune response following vaccination of capsular extract FC vaccine was measured by PHA test. Choudhury *et al.* (1987) used the same method to measure the serum antibody titres following administration of FC vaccine. Prevacination PHA titres of sera samples of all vaccinates and control birds was found to be at a mean of $<4.0 \pm 0.00$ which was closely related with Parvin *et al.* (2011) and Bag *et al.* (2015). Individual serum sample of 10 chickens of experimental group A and B were tested by microplate PHA test. After 15 days of first vaccination, The PHA titre of group A (IM) and group B (SC) were 89.6 ± 33.05 and 51.2 ± 16.52 , respectively, after 15 days of primary vaccination, but at 35 days post vaccination (DPV) the antibody titres were 179.2 ± 66.09 and 99.2 ± 38.31 , respectively. The mean antibody titre of the survived chickens of group A and B were 268.8 ± 99.14 and 150.4 ± 54.83 , respectively, after 10 days of challenge experiment and result are presented in Table 1. The findings of this experiment were in agreement with the previously published reports (Bag *et al.*, 2015; Akhtar *et al.*, 2016). Moreover, Wubet *et al.* (2019) reported that the immunogenicity of the vaccine depended on the route of vaccination and IM route gave stronger and more lasting immunity than SC route.

Table 1. Mean PHA titres of sera of chickens vaccinated through different routes with capsular extract fowl cholera vaccine as determined by t-test

Groups	Route of vaccination	Secondary vaccination interval	Mean PHA±SE		
			At 0 day	At 15 DPV	At 35 DPV
A	IM	15	<4±0.00	89.6± 33.05 ^a	179.2± 66.09 ^a
B	SC	15	<4±0.00	51.2±16.52 ^b	99.2±38.31 ^b
C	Un-vaccinated	-	<4±0.00 ^c	<4±0.00 ^c	<4±0.00 ^c
Level of significance				**	**

** Significant at $p < 0.01$; PHA: Passive haemagglutination; DPV: Days post vaccination; IM: Intramuscular route; Mean: Geometric mean of 10 birds; SE: Standard error

Results of protection test

All the control and experimentally vaccinated chickens that were challenged with virulent vaccine strain of *P. multocida* at the dose rate of 5.6×10^7 CFU/ml at IM route and manifested the following clinical findings and postmortem lesions:

Clinical findings having post challenge

At 6 hours of post inoculation (PI), the non-vaccinated chickens, which served as control, did not show any clinical sign. The clinical signs first observed at 12 hours of PI were characterized by dullness and depression. There were dullness, depression and slight rise of body temperature (42.5°C) and increased respiratory rates (30-45/min) at 24 hours of PI. At 48 hours of PI, there were severe weakness, drowsiness, anorexia, rise of body temperature (43.6°C), increased respiratory rates (45-55/min), lameness, whitish (chalky) diarrhea with mucus were the important clinical manifestation. The clinical signs at 96 hours of PI were almost similar to that of 48 hours. At 96 hours the clinical signs were related with signs of chronic infection that was characterized by cyanosis, dark red colored wattles, comb and ear lobes. The other signs included anorexia, lameness, greenish diarrhea with mucus, subnormal temperature (41°C) and decreased respiratory rates (15-25/min). Death of one chicken observed first at 48 hours PI, two died at 72 hours and others died at 96 hours PI. The vaccinated chickens which were subjected to experimental challenge infection, did not exhibit any clinical signs and symptoms. Similar types of clinical signs were also recorded by Choudhury *et al.* (1987) and Akhtar *et al.* (2016).

Postmortem lesions

During postmortem examination of unvaccinated control chickens, septicaemic conditions, blood vascular congestion and hemorrhagic enteritis were the important lesions in control chickens. Hemorrhage in the lung, abdominal fat and intestinal mucosa was common. Liver was swollen and sometimes congested and multiple necrotic foci were observed throughout the dorsal surface. These findings were agreed with the reports of Panna *et al.* (2015). Heart was enlarged and edematous, and trachea and lungs were hemorrhagic (Zahoor and Siddique, 2006). Choudhury *et al.* (1985) also reported the similar types of postmortem lesions in birds that died after experimental challenge infection. After two weeks of challenge exposure, a few of the selected survivors of each challenged group were sacrificed and the organism was reisolated, as per recommendation of Choudhury *et al.* (1987).

Survivability at challenge experiment

Vaccinated chickens which were exposed to challenge infection survived. The survivability rate of vaccinated chickens of group A was 100% and that of group B was 80% (Table 2). But all chickens of control groups failed to survive when challenged with virulent isolate of *P. multocida*. Similar observation was recorded by Akhtar *et al.* (2016).

Table 2. The rate of survivability at challenge test

Groups and routes	Challenge DPB	Total chickens	No. of sick chickens	No. of chickens survived	No. of dead chickens	Survivability rate (%)
A (IM)	15	10	0	10	0	100%
B (SC)	15	10	2	8	2	80%
C (Control)	15	10	10	0	10	0

DPB: Days post boosting; IM: Intramuscular route; SC: Subcutaneous route; %= Percentage

CONCLUSION

To prevent and to reduce the incidence of FC, it may be suggested that capsular extract FC vaccine prepared with isolated rats *P. multocida* may be used side by side with conventional FC vaccine after thorough field trial.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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