

Original Article

Isolation of *Pasteurella multocida* from chickens, preparation of formalin killed fowl cholera vaccine, and determination of efficacy in experimental chickens

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ABSTRACT

Objectives: The objectives of this study were to isolate and identify *Pasteurella multocida* from fowl cholera (FC) suspected chicken, and to prepare and efficacy determination of formalin killed fowl cholera vaccine using the isolated *P. multocida* strain.

Materials and methods: A total of five suspected dead chickens were collected from Brothers Poultry Farm located at Gazipur district, Bangladesh. The samples were processed and the *P. multocida* was isolated through conventional bacteriological techniques, were finally confirmed by polymerase chain reaction using *P. multocida* specific primers targeting *cap* gene. The *P. multocida* isolate was used to develop a formalin killed fowl cholera vaccine. The efficacy of the newly prepared vaccine was determined in Starcross-579 chickens (n=30) aging 15 weeks either by injecting 1 mL (group-A; n=10) or 0.5 mL (group-B; n=10) vaccine containing approximately 3.2×10^8 CFU/mL *P. multocida* organism; 10 birds were kept as unvaccinated control. The sera from the vaccinated and control birds were collected and were subjected for antibody titre determination by enzyme-linked immunosorbent assay (ELISA). Finally the vaccinated birds were challenged using virulent strains of *P. multocida* to confer the protection against FC.

Results: *P. multocida* could be isolated from both the samples. The formalin killed vaccine prepared from the isolated bacteria was subjected for the determination of antibody titre in chicken, and found that the antibody titres in the birds of group A and group B were 4.513 and 4.07 respectively after primary vaccination, and 4.893 and 4.37 respectively after booster vaccination. Most of the vaccinated birds were found to be survived after challenging with virulent strain of *P. multocida*.

Conclusion: It is concluded that the causal agent of FC (*P. multocida*) was successfully isolated from FC affected dead chickens. The prepared formalin killed fowl cholera vaccine induces protective immune response and conferred protection against challenge infection caused by the virulent strain of *P. multocida*.

KEYWORDS

Efficacy, ELISA, Fowl cholera, Killed vaccine, *Pasteurella multocida*, Polymerase chain reaction

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INTRODUCTION

Pasteurella multocida subspecies *multocida* (*P. multocida*) is an important pathogen that causes fowl cholera (FC) in poultry and wild birds (Xiao et al., 2015). In poultry, *P. multocida* often associated with severe economic loss due to loss of cattle or poultry species (Biswas et al., 2005; Marza et al., 2015). The FC, a septicemic disease, is associated with high morbidity and mortality in poultry especially chicken and ducks. Signs and symptoms of FC in acute cases are often present for only few hours before death; the signs in chicken include fever, ruffled feathers, mucus discharge from mouth, nose and ears, and cyanosis of comb and wattles (Glisson et al., 2008).

Five capsular serotypes (A, B, D, E, and F) are usually found in *P. multocida*, and each is generally associated with a specific host, for example, Serotype A causes FC in avian species (Harper et al., 2006), and Serotype B causes hemorrhagic septicemia in cattle (Marza et al., 2015). Virulence of *P. multocida* varies depending on the strain involved and factors host species (Glisson et al. 2008). The FC is mostly prevalent in fall, winter and late summer (Heddleston and Rhoades, 1978). Laying flocks are mostly affected by FC because of their more susceptibility to the disease as compared to younger chickens (Choudhury et al., 1985; Wang et al., 2009). Choudhury et al. (1985) reported about 25-35% mortality of chickens due to FC in Bangladesh.

Accurate and early diagnoses are considered as the effective tools to frame the strategy for controlling of any infectious disease like FC. Conventional diagnostic system is not effective in all cases since it is time consuming and less sensitive as compared to molecular technique, for example, polymerase chain reaction (PCR). Vaccination is considered as one of the common preventive measures worldwide to reduce the prevalence and incidence of disease (Kardos and Kiss, 2005). Efficacy of a vaccine depends on many factors including the immunogenic characteristic of the vaccine strain. It is widely accepted that a local strain having immunogenic value should be selected as the ideal vaccine strain to prepare effective vaccine to control a particular disease like FC. The aim of the present study was to isolate *P. multocida* from suspected cases of FC from field, and confirm its identity by molecular technique. In addition, a formalin killed fowl cholera vaccine was prepared using the isolated strain to determine its efficacy.

MATERIALS AND METHODS

Sample collection, and identification of *Pasteurella*: Five dead chickens were collected from Brothers Poultry

Farm located at Konabari, Gazipur, Bangladesh. For the isolation of *P. multocida*, heart and liver samples were collected from the dead birds. Isolation and identification of *P. multocida* was done based on morphological study, staining properties, cultural and biochemical characteristic, as described by Cheesbrough (2006). All samples were collected as per standard sample collection procedure (Panna et al., 2015).

Polymerase Chain Reaction (PCR): Confirmation of the isolated organism as *P. multocida* was done based on PCR targeting capsular gene *cap* specific for *P. multocida* as described in OIE Manual (2008). Bacterial DNA was extracted using Wizard genomic DNA Purification Kit (Promega) according to the instruction of the manufacturer. Extraction of DNA and its quality was checked by running 5 µL suspension of the extracted DNA in a 1% (w/v) agarose-gel. The primers used in the PCR were PMcapEF (5'-TCC GCA GAA AAT TAT TGA CTC-3') and PMcapER (5'-GCT TGC TGC TTG ATT TTG TC-3') that amplified around 511-bp amplicon. All the PCR was done in a final 25 µL volume containing 12.5 µL PCR master mix, 1 µL of each primer (10 pmol), PCR grade water 8.5 µL and DNA template 2 µL. The thermal profile followed for PCR was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 90 sec, and a final extension at 72°C for 5 min. 5 µL PCR product was loaded into 1% agarose gel (w/v) along with 1 µL 6X loading dye for electrophoresis in 1X TBE buffer at 100 V for 30 min. A standard 100-bp DNA ladder was also loaded in the same gel to compare the size of the amplified PCR products. Prior to casting the gel, ethidium bromide (0.5 µg/mL) was added to the gel. The PCR products were visualized under UV light in an image documentation system (Bio Rad, USA).

Preparation of fowl cholera vaccine: A formalin killed fowl cholera vaccine was prepared from the isolate that was confirmed as *P. multocida* by PCR. For vaccine preparation, the isolate was grown in a 500 mL flask containing 230 mL nutrient broth at 37°C for 24 h in a bacteriological shaker. During this incubation, bacterial population was determined based on OD₅₄₀ (approximately 3.2x10⁸ CFU/mL). At this stage, 0.8% formalin was added into the broth culture and incubated at room temperature for 24 h to allow the killing process of the bacteria in a separated conical flask, 25% liquid paraffin and 2.5% Arlacil-80 was added along with a magnetic bar. The conical flask was then placed over the magnetic stirrer for 30 min for proper mixing. After that it was slowly mixed with the nutrient broth containing formalinized bacterial culture. Slowly 3.059 mL (1.3%)

Tween-80 was also added in the mixture and shaken well. Once all these ingredients mixed properly, the broth was dispensed in vials and stored at 4°C for future use. The sterility of the vaccine was checked according to the procedure described by Choudhury et al. (1985).

Ethical approval and vaccine efficacy test: A total of 30 Starcross-579 day old chicks were purchased and acclimatized with laboratory environment. Vaccine efficacy test was performed as per standard procedure without harming or giving stress to any birds. Pre-vaccinated sera were collected from all birds to perform ELISA test. The birds were divided into 3 groups namely Group A, B and C. Each group comprised of 10 birds. Group A and B were used for vaccine purpose, while group C was kept as unvaccinated control. Primary vaccination was done at the age of 15 weeks to all groups of birds (except control group) by injecting either 1 mL (Group-A) or 0.5 mL (Group-B) vaccine containing around 3.2×10^8 CFU/mL *P. multocida*. All the birds were vaccinated intramuscularly at thigh region except control bird. After 21 days of post-vaccination, sera were collected from the birds of group A and group B, and the sera were subjected for ELISA. Booster vaccination was done on day 21 after primary vaccination to all the birds of group A and B with same dose of vaccine. Sera were collected from group A and group B birds on day 35 after booster vaccination, and the sera were subjected for ELISA.

Challenge infection: Both the vaccinated and unvaccinated control chickens were challenged with virulent *P. multocida* isolate at 35 days after booster vaccination, as per the procedure of Choudhury et al. (1987). The challenge dose contained approximately 2.89×10^8 CFU/mL *P. multocida*.

Post-challenge observation of birds: Birds after challenge infection were observed frequently up to one week for any signs and symptoms of FC.

Post-challenge isolation of bacteria: After 15 days of challenge, liver and heart tissues from the alive and dead birds were collected for re-isolation of *P. multocida* through inoculation on to Blood agar media followed by identification by studying morphology, staining, cultural, biochemical test, and PCR.

Enzyme Linked Immunosorbent Assay (ELISA) Test: The ELISA was performed to determine the serum antibody titre of the experimental chickens as described by manufacturers using the IDEXX PM ELISA Kit (IDEXX Laboratories, Inc). This assay was designed to measure the relative level of antibody to *P. multocida* in

chicken serum. The test was performed on a 96-well ELISA plate coated with *P. multocida* antigen. Upon incubation of the test sample in the coated well, antibodies specific to *P. multocida* form a complex with the coated antigens. After washing away unbound material from the wells, a conjugate was added which binds to any attached chicken antibody in the wells. Unbound conjugate was washed away and enzyme substrate is added. Subsequent color development was directly related to the amount of antibody to *P. multocida* present in the test sample.

Statistical analysis: The effect of vaccination on the experimental chickens in terms of ELISA titre and protection capacity of vaccinated birds against challenge infection were statistically analysed based on Geometric mean and standard deviation according to the procedures described by Shil and Debnath (1985).

RESULTS AND DISCUSSION

The FC is a highly contagious bacterial disease of domestic and wild birds, and is considered as a major problem of poultry worldwide (Singh et al., 2014). Thus, the disease is often associated with severe economic loss in the poultry industries due to high mortality. For the prevention of FC, vaccination is one of the most important methods. In the past, several studies have been carried out in Bangladesh on the isolation and identification of *P. multocida* from field cases, vaccine development and its efficacy determination (Choudhury et al., 1985; Choudhury et al., 1987; Mondal et al., 1988; Khan et al., 1994; Ferdouset al., 2011). However, as far we know, none of these studies were carried out in detail at molecular level using PCR.

Out of five FC suspected dead birds examined from the Brothers Poultry Farm, two were found to be positive for *P. multocida*. Both the isolated organisms were found as Gram-negative. Morphologically, the bacteria were cocco-bacillary in shape, and the Leishman's staining revealed the presence of bipolar characteristics, as reported by Cheesbrough (2006). PCR is a rapid, robust and highly specific molecular technique for confirmatory detection of many species of bacteria including *P. multocida* (OIE, 2008; Panna et al., 2015). The technique has been successfully applied to detect *P. multocida* from avian and other species (Sellyeiet al., 2008; Mohamed et al., 2012). To confirm the isolate as *P. multocida* in this study, PCR was carried out successfully with primers specific for *P. multocida*, as recommended in OIE manual. PCR results revealed that both the isolates produced around 511-bp single amplicon specific for *P. multocida* capsular gene *cap*, as reported in the OIE manual (Figure

Table 1. Mean titre of OD₆₂₀ and antibody titre of sera of chickens as observed following booster vaccination with Fowl Cholera vaccine.

Group	Dose (mL)	Route	Days	Mean±SD of OD	S/P ratio	Antibody titre	P-value
A	1	IM	21	1.1321±0.12983	1.058	4.513	0.000**
B	0.5	IM	21	0.7283±0.13038	0.660	4.07	
A	1	IM	35	1.4863±0.15216	1.407	4.893	0.000**
B	0.5	IM	35	0.9978±0.05379	0.927	4.37	

**Significant at the level of 0.01; IM=intramuscular route

Table 2. The survivability rate of chicken at challenge infection after 21 days of booster vaccination.

Groups	Vaccination route	Birds (n)	Survived (n)	Died (n)	% survive	% died
A	IM	10	10	0	100%	0%
B	IM	10	8	2	80%	20%
C	Unvaccinated	10	2	8	20%	80%

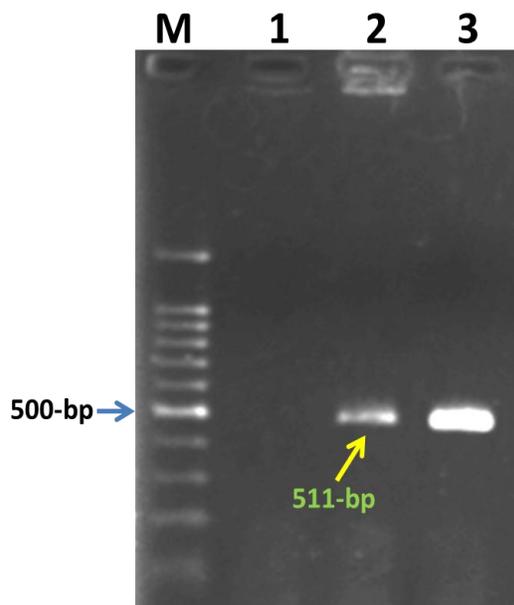


Figure 1. PCR amplification of capsular gene of *Pasteurella multocida*. Lane 1= Negative control, Lane 2= Isolates 1, Lane 2= Isolate 2, M=100-bp DNA marker

1). Recently, Panna et al., (2015) isolated *Pasteurella* spp. from chicken in Bangladesh, and confirmed the organism as *P. multocida* type A by PCR as used in this study.

ELISA has been used by many researchers to determine the titre of antibody against *P. multocida*, and thus to evaluate fowl cholera vaccine efficacy (Jabbri et al., 2005). In the present study, the efficacy of the prepared vaccine was successfully evaluated by ELISA. The titre of antibody was determined from this S/P ration. Results of antibody titre in various groups of chickens are presented in **Table 1**.

The ELISA test carried out here revealed that the S/P ratio of control group chickens (pre-vaccinated sera) as 0.0172 (antibody titre 3.37), however, following primary vaccination, the S/P ratio of group A and group B chickens raised to 1.058 (antibody titre 4.513), 0.660 (antibody titre 4.07) respectively at day 21 post-vaccination (**Table 1**). After the booster vaccination, the S/P ratio of group A and group B were found to be 1.407 (antibody titre 4.893) and 0.927 (antibody titre 4.37) respectively at day 35 post-boostering, indicating a significant increase in the antibody titre as compared to the titre of the primary vaccinated birds. In addition, from the results illustrated in the **Table 1**, it is clearly evident that administration of a dose of 1 mL of vaccine induced more immune response as compared to 0.5 mL vaccination.

The efficacy of the vaccine was further evaluated by challenging the vaccinated and control birds with the isolated *P. multocida*. The survivability rate of chicken at challenge infection after 21 days of booster vaccination is present in **Table 2**.

In post-challenge observations, control birds showed characteristic clinical signs of FC like depression, dullness, anorexia, laboured breathing, hyperthermia, greenish diarrhoea, lameness, and ultimately death. All birds of group A and 80% birds of group B were protected from FC, and did not show any clinical sign of FC. Thus, the challenge experiment demonstrated that the experimentally prepared fowl cholera vaccines conferred 100% protection against challenge infection in group A and 80% protection in group B. Besides, these findings suggested that the prepared vaccine was found to be highly effective against FC in chicken.

CONCLUSION

Two isolates of *P. multocida* were isolated from filed cases of FC of chicken. In addition to conventional methods such as staining, cultural and biochemical test, identification of the isolated organisms as *P. multocida* was confirmed by molecular approach *i.e.*, PCR using the primers specific for *P. multocida*. Finally, a formalin killed fowl cholera vaccine was prepared using the isolated bacteria. The experimentally prepared vaccine induces protective immunity in the vaccinated chicken as revealed by ELISA and challenge infection.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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