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Isolation and Identification of *Pasteurella multocida* from Chicken for the Preparation of Oil Adjuvanted Vaccine

Samina Ievy, Mohammad Ferdousur Rahman Khan, Md. Ariful Islam and Md. Bahanur Rahman*

Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

*Corresponding author's e-mail: bahanurr@yahoo.com

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A B S T R A C T

The research work was performed for the isolation and identification of *Pasteurella multocida* from field cases, preparation of oil adjuvanted vaccine from isolated strain and determination of its efficacy. Samples were collected from suspected dead birds of three poultry farms of Bangladesh (*Code name: M and R)*. The *P. multocida* isolates were Gram negative, non-motile, non- spore forming rod occurring singly or pairs and occasionally as chains or filaments. Biochemically *P. multocida* ferment basic sugar and consistently produced acid except from maltose and lactose. After isolation formalin killed oil adjuvanted Fowl cholera vaccine was prepared in Laboratory of the Department of Microbiology and Hygiene, BAU and this experimental vaccine ($3.2x10^8$ CFU/ml) was administered in nine weeks old White Leg Horn chickens at the different dose rate through intramuscular (IM) route in each selected group A (1ml alum precipitated vaccine), B (0.5ml alum precipitated vaccine), C (1ml oil adjuvanted vaccine) and D (0.5ml oil adjuvanted vaccine). Pre-vaccinated sera were collected from all groups of birds. The mean of Passive Hemagglutination (PHA) titers of post-vaccination were 51±17.8, 76.8±17, 89.6±17, and 115±17.81 in group A, B, C and D respectively which consist of 5 birds in each. The vaccine produced better immune response when boostering with the similar dose and route at 15 days after primary vaccination. The mean PHA titers were higher at group D than other groups after boostering. Challenge infection was conducted on all the vaccinated and control group (n=5) of birds after 15 days of vaccination which protect 93.75% of birds and the PHA titers from different groups analyzed to determine the protective capacity of vaccinated chickens against challenge exposure. It was demonstrated that experimental oil adjuvanted fowl cholera vaccine with 0.5ml dose produce higher immune response against challenge infection and found to be safe.

Keyword: Isolation, *Pasteurella multocida*, killed vaccine, efficacy.

Introduction

Poultry farming is a growing business in Bangladesh at present time. Infectious disease outbreaks are considered as the most leading cause of economic loss and discouraging poultry rearing in Bangladesh (Das *et al*., 2005). Fowl cholera is a contagious bacterial disease of poultry caused by infection with *Pasteurella multocida* and identified as a major threat for poultry industry which hamper the profitable poultry production (OIE, 2008). Respiratory tract infection in poultry are majorly caused by *P. multocida* (Bisgaard *et al,* 2003) which also harbored in cloacal mucosa of asymptomatic birds and these strain remain as sources of outbreaks (Muhairwa *et al.,* 2000). 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 finally (Rhoades and Rimler, 1990). The chronic form of disease may be seen in chickens that survive after occurring the acute form of disease or it may result from infection with an organism of relatively low virulence (Gordon and Jordon, 1985). The clinical signs include depression, conjunctivitis, dyspnea, lameness and torticollis.

The swelling of the wattles, sinuses, limb-joints, footpads and sternal bursae may also present (Curtis, 1980). Many of the lesions are related to vascular disturbances, hyperemia in the vessels of the abdominal viscera, petechial and ecchymotic hemorrhages in sub-epicardial and sub-serosal locations. Increased amounts of peritoneal and pericardial fluids are seen frequently. The liver may be swollen and often develops multiple, small, necrotic foci. Fowl cholera occurs sporadically or enzootically in most countries of the world (Heddleston and Rhoades, 1978) including Bangladesh. Most reported outbreaks of fowl cholera are in chickens, turkeys, ducks, geese and also reported in quail. Turkeys are much more susceptible than chickens to infection with *P. multocida*, and the aged birds are more susceptible than younger (Wang *et al.,* 2009).

Vaccination is practiced as preventive measures in many countries of the world to reduce the incidence of the disease. Various scientists suggested that a local strain of higher immunogenic value should be selected as vaccine strain for preparation of bacterin with a view to control fowl cholera. In the present study, fowl cholera vaccine was prepared using capsular extract of *P. multocida* field isolates and was administered with a view to improve the immune status of chickens against fowl cholera.

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Materials and Methods

Study area and duration
The present study was conducted at the laboratory of Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh during the period of January 2012 to June 2012.

Collection of Samples and Isolation of *P. multocida*
Clinical specimens such as heart, liver and spleen were collected from chicken at different poultry farms of Mymensingh, suspected to be infected with fowl cholera. Collected samples were inoculated separately onto different bacteriological media under aseptic condition and incubated at 37 ^{0}C for 24 hours. The colonies on primary cultures were repeatedly sub-cultured in blood agar (BA) by streak plate method until the pure cultures with homogenous colonies were obtained. A total of 11 birds examined from two farms, 8 were found positive and subjected to further study and vaccine preparation.

Identification of Bacteria
In order to identify the bacteria from chicken cultural characteristics, morphological characteristics and biochemical characteristics were studied. For cultural characteristics or colonial morphology bacteria grown on the nutrient and blood agar media. Gram's staining method was performed to study the cellular morphology and staining characteristics of bacteria. Biochemical tests such as: sugar fermentation, coagulase, catalase, MR, VP, and indole tests were also performed according to the standard methods.

Preparation of oil adjuvanted killed vaccine
Isolated *P. multocida* was cultured in BA media and kept in bacteriological shaker incubator at 37^0 C for 24 hours. The purity of culture was examined and subsequently sub-cultured in the same media for 24 hours. The isolated colonies were then inoculated in 200 ml nutrient broth containing yeast extract and beef extract and kept in shaker incubator at 37^{0}C for 24 hours for massive growth. Bacterial count was determined and calculated as 3.2 x 10^8 CFU/ ml. Later on formalin was added in the broth culture and incubated at room temperature for 24 hours. Liquid paraffin and arlacil-80 was mixed slowly for 30 minutes with the help of magnetic stirrer. Then formalin killed culture was slowly added with it and tween – 80 was added slowly with this mixture at appropriate amount. These were mixed properly and finally it was dispensed in vials and stored at 4^{0}C as vaccine.

Vaccination of the Chicken
Prepared alum precipitated and oil adjuvant FC vaccines (3.2x10^8 CFU/ml) were administered at the dose rate of 1ml alum precipitated (AP), 0.5ml alum precipitated (AP) and 1ml oil adjuvant (OA), 0.5ml oil adjuvant (OA) at group of A,B,C and D respectively. The vaccines were administered intramuscularly at thigh region in each bird at 9 weeks of age except control bird. Booster dose was given to all the groups except control group with the same dose and route at 15 days after primary vaccination.

Collection of Chicken Serum
Blood was collected prior to and after 15 days of each vaccination using sterile syringe and needle. Syringes were then held in slanted position and blood was allowed to clot at room temperature for an hour. Blood clots were detached from the wall of the syringe by pressing the piston and were kept overnight in the refrigerator at 4^{0}C for separation of the serum. Then serum was carefully removed and centrifuged at 2000 rpm for 10 minutes for clarification and then stored at -20^{0}C in screw capped vials until used. The sera samples were heat inactivated at 56^{0}C for 30 minutes in water bath before PHA test.

PHA test
The procedure of the PHA test was followed according to the method descried by (Tripathy *et al.*, 1970).The reciprocal of the end point of highest dilution of test sera and sensitized tanned SRBC was considered as titre of the serum. 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 PHA positive, and a compact buttoning with clear zone indicated PHA negative.

Preparation of Challenge Dose
Both vaccinated and unvaccinated groups of chickens were challenged with virulent *P. multocida* isolate following the procedure of Choudhury *et al.* (1987). The challenge dose is 0.5 ml (3.2 x 10^8 CFU/ ml) was administered through intramuscular route after 15 days of booster vaccination.

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

Result

PHA antibody titres
The PHA antibody titers of the serum of chickens belonged to group A, B, C and D are presented in Table-1.The pre-vaccination mean PHA titer were <4±0.00 in sera of chickens of all groups. After 15 days of booster vaccination the mean PHA titres were 51±17.8, 76.8±17, 89.6±17, and 115±17.81 in group A, B, C and D respectively (Table-2). The mean PHA titres in chickens of unvaccinated control group E were <4±0.00.

Table 1. PHA titres of sera of group A, B, C and D chickens vaccinated with Alum Precipitated and Oil adjuvanted fowl cholera vaccine

Group	Age of chicken at primary vaccination	Age of chicken at booster vaccination	Dose and route of vaccination	Tag No.	Pre- vaccinated serum (PHA titre)	Post boostering serum (PHA titre)
A	9 weeks	11 weeks	1ml, I/M (APV)	01	<4	32
				02	<4	32
				03	<4	64
				04	<4	64
				05	<4	64
B	9 weeks	11 weeks	0.5ml, I/M (APV)	01	<4	128
				02	<4	64
				03	<4	64
				04	<4	64
				05	<4	64
C	9 weeks	11weeks	1ml, I/M (OAV)	01	<4	64
				02	<4	128
				03	<4	64
				04	<4	64
				05	<4	128
D	9 weeks	11 weeks	0.5ml, I/M (OAV)	01	<4	64
				02	<4	128
				03	<4	128
				04	<4	128
				05	<4	128

IM = Intramuscular route; APV=Alum Precipitated Vaccine; OAV=Oil adjuvant vaccine

Table 2. Mean PHA titres of sera of chickens vaccinated and revaccinated with fowl cholera vaccine through IM route as determined by *t*-test

Groups	Route of vaccination	Dose of Vaccination	PHA titer of control (Mean±SE)	PHA titer (Mean ±SE) at 15 DPV	P value
A	IM	APV, 1ml	<4±0.00	51±17.8	0.018
B	IM	APV, 0.5 ml	<4±0.00	76.8±17	
C	IM	OAV,1ml	<4±0.00	89.6±17	
D	IM	OAV,0.5ml	<4±0.00	115±17.81	

Significant at the 0.05 level
PHA=Passive Hemagglutination; DPV=Days post vaccination; IM = Intramuscular route; Mean= Geometric mean of 5 birds; SE = Standard error; APV=Alum Precipitated Vaccine; OAV=Oil adjuvant vaccine

Table 3. The survivability rate of chicken at challenge infection after 15 days of booster vaccination

Groups	Route of vaccination	Total birds	No. of birds survived	No. of birds died	Percentage of survivability	Average
A	IM	8	7	1	87.5%	93.75%
B	IM	8	7	1	87.5%	
C	IM	8	8	0	100.0%	
D	IM	8	8	0	100.0%	
E	Unvaccinated	8	1	7	12.50%	

Protection test
Challenge infection was conducted with all the groups of birds along with unvaccinated controls after 21 days of booster vaccination. Each bird was administered with 0.5 ml of vaccine (3.2×10^8 CFU/ml) through IM route. The experimental fowl cholera vaccine conferred 87.5%, 87.5%, 100%, 100% protection in group A, B, C, and D.

Discussion

Fowl cholera or avian pasteurellosis or avian hemorrhagic septicemia is a disease of domesticated and wild birds which is a devastating and killer disease. The disease causes severe economic loss and hampers to the development of poultry industry in the developing countries like our country where 25% to 35% mortality in chickens occurs due to fowl cholera (Choudhury *et al.,* 1985). For prevention of fowl cholera, vaccination is one of the most important methods. In this present study, *P. multocida* isolated from suspected dead chickens were used as antigen and formalin inactivated oil adjuvant fowl cholera vaccine was prepared using that isolates. Humoral immune response was measured by PHA test using formalin killed oil adjuvanted fowl cholera vaccine. Choudhury *et al.* (1985), Chang (1987), Mondal *et al.* (1988), Sarker *et al.* (1992) and Suman (2002) used the same method to measure the serum antibody titres following administration of fowl cholera vaccine in chicken.

The fowl cholera organism was characterized by using various microbiological techniques according to Cheesbrough (1985). The morphology, staining and cultural characteristics of the organism in different culture media were studied according to the procedure described by Choudhury *et al.,* (1985). Some strains of fowl cholera organism might not grow in media without blood or blood serum reported by Carter (1972). In the present investigation, the selected isolates were found to grow well in bovine blood agar media producing more or less characteristic colonies of *P. multocida* organism. Carter (1972), Heddleston and Rhoades, 1978, Kardos and Kiss, 2005 and Mbuthia *et al.,* 2008) used bovine blood agar for the culturing of *P. multocida.* All the isolated organisms in this study were Gram negative coccobacillary shape in Gram staining method and bipolar characteristics in leishman's staining method. Cowan (1985) and Cheesbrough (1985) also recorded similar staining characteristics of *P. multocida* isolate. In the present study, 8 isolates fermented dextrose, glucose, sucrose and mannitol completely and produced acid without gas but no fermentation was recorded in case of maltose and lactose which are also showed by Choudhury *et al.,* (1987), Calnek *et al.,*(1997), Shivachandra *et al.,*(2006) and Tabatabai, (2008).

Presence of well developed capsule in fresh culture of selected *P. multocida* was determined by acriflavine test according to the procedures suggested by Choudhury *et al.* (1985). Snipes *et al.,* (1987), and Khan *et al.,* (1997) observed that the virulence of the organisms were associated with the presence of capsule. Fowl cholera vaccine was administered at the different dose rate through IM route in each selected groups (A, B, C and D) in the laboratory. Booster dose was given with the similar dose and route 15 days after primary vaccination in groups A, B, C and D respectively. OIE Manual (2008) and Choudhury *et al.,* (1985) suggested that FC vaccine should be given through IM and SC routes.

PHA test was conducted to determine the humoral immune response of the serum of chickens having been inoculated at 9 weeks aged birds with the antigen containing *P. multocida* as per the method described by Chang (1987) but slight modification was done as suggested by Kuczkowski *et al.,* (2006) and Corney *et al.,* (2007). The pre-vaccination PHA titres of sera samples of all vaccinated and control birds was found with a mean of <4.00±0.00 that was closely related with Mondal *et al.* (1988). After 15 days of booster vaccination the mean PHA titres were 51±17.8, 76.8±17, 89.6±17, and 115±17.81 in group A, B, C and D respectively. The mean PHA titres in birds of unvaccinated control group F were always <4±0.00. Booster dose was given 15 days after primary vaccination in groups A, B, C and D respectively. In this present study, it was observed that group D produced comparatively better immune response than group A, B, and C.

In this study, results of challenge exposure demonstrated that the experimentally prepared fowl cholera vaccines conferred excellent protection against challenge infection following IM administration of fowl cholera vaccine at 21 days post boostering interval in control groups, out of eight birds, only one bird survived although exhibited clinical symptoms. Similar observation was also recorded by Coates (1972) and Mondal *et al.,* (1988). In post challenge observations, control birds showed characteristic clinical signs and symptoms of fowl cholera like dullness, depression, anorexia, hyperthermia, laboured breathing, lameness, greenish diarrhoea and ultimately death occurred. Vaccinated birds protected themselves and did not show clinical signs except dullness and depression which were similar to the findings of Sharma *et al.* (1974), Gordon and Jordan (1985), and Zahoor and Siddique (2006**)** described that hyperthermia, dullness, incoordination of movements, greenish yellow diarrhea, laboured and painful breathing and unusual sitting posture were the most prominent clinical symptoms in case of experimentally produced avian hemorrhagic septisemia. The authors observed that marked depression, anorexia, cyanosis and foetid diarrhea were the most prominent clinical signs in acute cases of illness.

Conclusion

The protective efficacy of fowl cholera vaccine was measured by determining the survivality rate of the birds of each vaccinated groups by challenge infection. The experimental fowl cholera vaccine conferred excellent protection of vaccinated birds in all groups while all the unvaccinated control birds were infected following challenge infection.

References

Bisgaard M, H Christensen, AM Bojese, and JP Christensen, 2003. Avian infections caused by species of *Pasteurellaceae.* Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University.

Calnek BW , HJ Barnes, CW Beard, LIL McDougald, and YM Saif, 1997. Diseases of poultry 10th edition. Iowa State University Press, Ames, Iowa. pp. 143-150.

Carter GR, 1972. Improved haemagglutination test for identifying type 'A' strain of *P. multocida*. *Applied Microbiol.* 24: 162-163.

Chang CF, 1987. In vitro assay for humoral and cell mediated immunity to pasteurellosis in ducks. *J. Chinese Soci. Vet. Sci.* 13: 67-74.

Choudhury KA, MM Amin, AJ Sarker, AH MR and AR Ahmed, 1987. Immunization of chickens against fowl cholera with oil-adjuvanted broth culture vaccine. *Bang. Vet. J.* 21: 63-73.

Choudhury KA, MM Amin, A Rahman, and MR Ali, 1985. Investigation of natural outbreak of fowl cholera. *Bang. Vet. J.* 19: 49-56.

Coates RS, JM Jensen, and ED Brown, 1977. The response of turkey to varying doses of live oral *P. multocida* vaccine. *Poult. Sci.* 56: 273-276.

Corney BC, IS Diallo, LL Wright, GR Hewitson, AJ Jong, PC Burrell, PF Duffy, CP Stephens, BJ Rodwell, DB Boyle, and UPJ Blacka, 2007. *P. multocida* detection by 5' Taq nuclease assay: A new tool for use in diagnosing fowl cholera. *J. Microbiol. Methods*. 25: 83-96.

Curtis PE, GE Ollerhead, and CE Ellis, 1980. Virulence and morphology of *P. multocida* of avian origin. *Vet. Rec.* 107: 105-108.

Das PM, DMM Rajib, M Noor, and MR Islam, 2005. Retrospective analysis on tile proportional incidence of poultry diseases in greater Mymensingh district of Bangladesh. In proceeding of 4th International Poultry show and seminar from February 28 to March 2, 2003 held in Bangladesh China Frienship Conference Centre, Agargaon. pp. 35-39.

Gordon RF, and FT Jordan, 1985. Poultry Diseases. 2nd edition, ELBS, Baillier Tindall, London, pp. 44-46.

Hasan N, 2009. Evaluation of vaccines in the control of avian pasteurellosis. MS thesis of Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh.

Heddleston KL, and KR Rhoades, 1978. Avian pasteurellosis in diseases of poultry. 7th edition. Iowa State University Press, Ames. Iowa, USA, pp: 181-199.

Kardos G, and I Kiss, 2005. Molecular epidemiology investigation of outbreaks of fowl cholera in geographically related poultry flocks. *J. Clin. Microbiol.* 43: 2959-2961.

Khan MANA, PM Das, KA Choudhury, and MR Islam, 1997. Pathology of experimentally induced fowl cholera in chickens. *Bang. Vet. J.* 1: 28-34.

Kuczkowski M, J Krol, A Wieliczko, A Gawel, J Schmidt, and M Mazurkiewicz, 2006. Prevalence of fowl cholera in poultry and characteristics of isolated *P. multocida* strains. *J. Microbiol*. 62: 481-600.

Mbuthia PG, LW Nyaga, PN Nyaga, LC Bebora, U Minga, J Kamundia, andJE Olsen, 2008. *Pasteurella multocida* in scavenging family chickens and ducks: carrier status, age susceptibility and transmission between species. *Avian Pathol.* 37: 51-57.

Mondal SK, KA Choudhury, MM Amin, MM Rahman, and AJ Sarker, 1988. Immune response in chickens induced by alum precipitated fowl cholera vaccine. Humoral immune response. *Bang. Vet. J.* 22: 7163-7169.

Muhairwa AP, JP Christensen, and M Bisgaard, 2000. Investigations on the carrier rate of *P. multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. *Avian Pathol.* 29:133-142.

OIE, 2008. Manual of standards for diagnostic test and vaccines.

Rhoades KR, and RB Rimler, 1990b. *P. multocida* colonization and invasion in experimentally exposed turkey poults. *Avian Dis*. 34: 381-383.

Sarker AJ, MNIIAmin, and WMA Hossain, 1992. Testing and quality control of poultry vaccines mid its monitoring in the field. *Bang. Agril. Univ. Res. Progress.* 6: 249-257.

Sharma RN, NP Bhalla, and JM Lall, 1974. Observation of experimental infection of *Pasteurella* septica (avian) in domestic fowl. Indian. *J. Ani. Heal.* 13:105-109.

Shivachandra SB, AA Kumar, R Gautam, S Joseph, MK Saxena, P Chaudhuri, and SK Srivastava, 2006. Identification of avian strains of *P. multocida* in India by conventional and PCR assays. *Vet. J.* 172: 561-564.

Snipes KP, GY Ghazikhanian, and DC Hirsh, 1987. Fate of *Pasteurella multocida* in the blood vascular system of turkey following intravenous inoculation: Comparison of an encapsulated virulent strain with its avirulent acapsular varient. *Avian Dis.* 31: 254-259.

Suman MSR, 2002. Study on the comparative efficacy of experimentally prepared formalin and heat killed fowl cholera vaccines. MS thesis of Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh.

Tabatabai LB, 2008. Identification of *P. multocida* CHAPS-soluble outer membrane proteins. *Avian Dis.* 52:147-149.

Wang C, Y Wu, X Xing, G Hu, J Dai, H He, 2009. An outbreak of avian cholera in wild waterfowl in Ordos wetland, Inner Mongolia, China. *J. Wildl. Dis.* 45: 1194-7.

Zahoor RMA, and M Siddique, 2006. Characteristics of *P. multocida* recovered from avian sources. *Pak. Vet. J.* 26: 41-43.