ISOLATION AND IDENTIFICATION OF *PASTEURELLA MULTOCIDA* FROM POULTRY FOR PREPARATION OF VACCINE AND DETERMINATION OF ITS EFFICACY

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ABSTRACT

The present research work was performed for the isolation and identification of *P. multocida* from field cases, preparation of its vaccine and determination of its efficacy. For this purpose, samples were collected from suspected dead birds of Phenix Hatchery Ltd, Gazipur. The isolates consistently produced acid from dextrose, sucrose and mannitol but not fermented maltose or lactose. Capsular antigen was extracted from the organisms and fowl cholera (FC) vaccine was prepared. The experimentally prepared FC vaccine was administered in 9 weeks aged Sonali chickens at the dose rate of 0.5 ml of 2.93×10^8 CFU through subcutaneous (SC) route in each selected groups (A, B, C, D and E) in the laboratory. Booster dose was given with the similar dose and route at 15^{th}, 21^{st}, 28^{th}, 35^{th}, 42^{nd} days after primary vaccination in groups A, B, C, D and E respectively and group F was kept as unvaccinated/control. Prevaccination sera were collected from all the groups of birds. The mean Passive haemagglutination (PHA) titers of post-vaccination were 96±12.09, 88±11.71, 88±11.71, 80±10.47 and 80±10.47 in group A, B, C D and E respectively. The mean PHA titer in birds of unvaccinated control group F were <4±0.00. The vaccine produced better immune response when booster was given at 15^{th} days after primary vaccination compared to 21^{st}, 28^{th}, 35^{th}, 42^{nd} days after primary vaccination. Challenge infection was conducted on all the vaccinated and control group of birds at 15 days of post vaccination. The PHA titer obtained from different group of birds was analyzed by *t*-test to determine the protective capacity of vaccinated chickens against challenge exposure. It was demonstrated that experimental fowl cholera vaccine using capsular extract conferred 100% protection against challenge infection and found to be safe.

Keywords: *Pasteurella multocida*, PHA titer, Vaccine, Immunogenicity, Chicken.

INTRODUCTION

Pasteurella multocida (*P. multocida*) is a Gram-negative encapsulated bacterium that is the causative agent of a range of animal pasteurellosis diseases, including fowl cholera in poultry and wild birds, hemorrhagic septicemia in cattle and buffalo, atrophic rhinitis in swine, and

snuffles in rabbits (Harper, M *et al*, 2006). Fowl cholera (FC), caused by *P. multocida* can result in either an acute septicemia or chronic localized infections in domestic and wild birds (Sander *et al.*, 1998). FC is also known as avian cholera, avian pasteurellosis, and avian hemorrhagic septicemia. It is a contagious bacterial disease of domesticated and wild avian species which hamper the profitable poultry production (OIE, 2008).

P. multocida can be harbored in the respiratory tract or cloacal mucosa of asymptomatic birds and these strains can become sources of outbreaks (Muhairwa *et al.*, 2000). Wild birds and rats have been suggested as other possible sources of infection (Botzler, 1991) as well as contaminated water or aerosols (Simensen *et al.*, 1980). The acute form of disease is characterized by sudden death without premonitory signs, and the clinical signs of fowl cholera were anorexia, fever, depression, ruffled feathers, mucoid discharge from the mouth, rapid respiration and diarrhea which was watery to yellowish initially and greenish with mucus finally (Rhoades and Rimler, 1990a). The chronic form of disease may be seen in chickens that survive the more acute disease or it may result from infection with an organism of relatively low virulence (Gordon and Jordon, 1985). The disease is more prevalent in late summer, fall and winter (Heddleston and Watko, 1964). Death from fowl cholera in chickens usually occurs in laying flocks, because the aged birds are more susceptible than younger chickens (Heddleston and Watko, 1964; Choudhury *et al*, 1985).

In Bangladesh there are several constraints for the expansion of Poultry industries. Of those, infectious diseases are considered as the most leading causes of economic loss and discouraging poultry rearing in this country (Das *et al.*, 2005). Among the bacterial diseases, fowl cholera is a major threat to the poultry industry. About 25% to 35% mortality in chickens of Bangladesh is due to fowl cholera (Choudhury *et al.* 1985).

Preventive measures in Bangladesh like other countries of the world to reduce the incidence of the disease (Michael *et al*., 1979) 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.

The objective of this study was to isolate and identify *P. multocida* from naturally infected chicken and determination of efficacy of experimentally prepared fowl cholera vaccine using *P. multocida* chicken isolates.

MATERIALS AND METHODS

Isolation and Identification of the Organism

The present study was conducted at the laboratory of Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. Clinical specimens such as Heart, liver, spleen and ovarian follicle were collected from chicken at Phenix Hatchery Ltd suspected to be infected with fowl cholera. For isolation and identification of *P. multocida*, Nutrient broth (NB), Nutrient agar (NA), EMB agar, MacConkey agar and Blood agar (BA) media were used. Yeast extract and beef extract were also used with NB. Morphological characteristic of *P. multocida* colonies were studied by using Gram's stain and Leishman's stain. Gram's staining was performed according to the method described by Merchant and Packer (1967). The carbohydrate fermentation test was performed by inoculating a loopful of NB culture of the organisms into the tubes containing different sugar media (five basic sugars such as dextrose, sucrose, lactose, maltose and mannitol) and incubated at 37^0C for 24 hours. The changing of color from used to yellow indicated the acid production and accumulation of

gas bubbles in inverted Durham's tube indicated gas production. Methyl red (MR) test, Voges-Proskauer (VP) test, Indole test were performed.

Preparation of Fowl Cholera Vaccine

Fowl cholera vaccine was prepared in the laboratory at the dose rate of 0.5 ml of 2.93×10^8 CFU (colony forming unit) and kept in the laboratory. For this, *P. multocida* isolated from suspected chicken at Phenix Hatchery Ltd. were cultured in BA media and kept in bacteriological incubator at 37ºC for 24 hours. The purity of culture was examined and subsequently subculture in the same media for 24 hours. The isolated colonies were then inoculated in 100 ml NB containing yeast extract and beef extract and incubated at 37ºC for 24 hours for massive growth. Later on formalin was added in the broth culture and after 24 hours alum was also added. Finally, it was dispensed in vials and stored at room temperature for future use. Thus the vaccine is formalin inactivated alum-precipitated type. The vaccine was prepared according to the method described by OIE Manual (1992) and Supar *et al.* (2002). After vaccine preparation sterility test was performed according to Chowdhury *et al* (1985).

Vaccination of the Chickens

FC vaccine was administered at the dose rate of 0.5 ml of 2.93×10^8 CFU through subcutaneous route at the neck region in each bird of either sex at 9 weeks of old except control bird. Then the vaccinated birds were grouped into A, B, C, D and E. Booster dose was given to the A, B, C, D and E with the same dose and route respectively after 15^{th}, 21^{st}, 28^{th}, 35^{th} and 42^{nd} days after primary vaccination. OIE Manual (1992) and Choudhury *et al.*, (1985) suggested that FC vaccine should be given through IM and SC route.

Collection and Inactivation of Chicken Serum

Blood was collected prior to vaccination, 15^{th} days of booster vaccination and 15^{th} days of challenge infection using sterile syringe and needle. Derieux (1978) and WU *et al.*, (2007) suggested that two doses were required with an interval of two to four weeks and with inactivated vaccines protective level of immunity could not be produced until approximately two weeks after the second dose of a primary vaccination. 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 4^0C for separation of the serum. Then serum was carefully removed and centrifuged at 2000 rpm for 10 minutes for clarification and then stored at -20^0C in screw capped vials until used. The serum samples were inactivated at 56^0C for 30 minutes in water bath before PHA test.

Challenge Exposure to Experimental Chicken

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 containing 2.93 x 10^8 colony forming unit (CFU)/0.5ml was administered through intramuscular route after 15 days of booster vaccination.

Passive Haemagglutination (PHA) Test

The test was used to determine the antibody titers in chickens against *P. multocida* after vaccination. The test was performed according to the methods described by Tripathy *et al.* (1970a), Chowdhury *et al.* (1987), Mondal *et al.* (1988), Sarker *et al.* (1992) and Siddque (1997) with slight modifications (Nime *et al* 2016).

Statistical analysis

The effect of vaccination on experimental chickens in terms of PHA titre and protection capacity of vaccinated birds against challenge infection were analyzed by Geometric mean with standard error. The analysis was performed according to the procedures described by Shil and Debnath (1985). The PHA titres were analyzed by *t*-test to determine the protective capacity of vaccinated birds against challenge exposure.

RESULTS AND DISCUSSION

Isolation and Identification of *P. multocida* (PM)

The presumptive *P. multocida* produced small, whitish, discrete, opaque, circular and translucent colonies on NA media and BA media. No hemolysis was noticed on BA media. Culture on MacConkey agar plates yielded no colonies. Culture of *P. multocida* on EMB agar yielded small, circular, smooth, convex, translucent, glistening colonies which had a tendancy to coagulase. Metallic sheen was absent. Gram negative coccobacillary shape in Gram staining method and bipolar characteristics in Leishman's staining method were shown by the organism. Cowan (1985) and Cheesbrough (1985) also recorded similar staining characteristics of *P. multocida.* The selected organism *P. multocida* fermented dextrose, glucose, sucrose and mannitol completely and produced acid without gas but no fermentation was recorded in case of maltose and lactose. These Biochemical reactions were closely correlated with Choudhury *et al.,* (1987) and Calnek *et al.,* (1997). The presence of well developed capsule in fresh culture of selected *P. multocida* was determined by acriflavine test according to the procedures suggested by Cheesbrough (1985) and Choudhury *et al.,* (1985).

Result of Sterility Test

One milliliter of FC vaccine was inoculated in separate BA plates and kept overnight at 37ºC in bacteriological incubator. The plate showing no growth indicated complete inactivation of *P. multocida* organisms and negative for any other contaminating organisms.

PHA Antibody Titer

The PHA antibody titres of the serum of chickens belonged to group A, B, C, D and E 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 96.00±12.09, 88.00±11.71, 88.00±11.71, 80.00±10.47 and 80.00±10.47 in group A, B, C D and E respectively (Table 2). The mean PHA titres in chickens of unvaccinated control group F were <4±0.00. In this present study, it was observed that group A produced comparatively better immune response than group B, C, D & E and group B and C produced comparatively better immune response than group D & E (table 2).

Table 1: Antibody titres of group A, B, C, D and E by PHA after boostering

	Tag no.	Groups							Tag no.	Groups				
		A	B	C	D	E	F			A	B	C	D	E
Prevaccin-ation Antibody Titres	1	≤4	≤4	≤4	≤4	≤4	≤4	Antibody Titres after 15 days of boostering	1	64	128	64	64	64
	2	≤4	≤4	≤4	≤4	≤4	≤4		2	128	64	128	128	64
	3	≤4	≤4	≤4	≤4	≤4	≤4		3	128	64	64	64	128
	4	≤4	≤4	≤4	≤4	≤4	≤4		4	64	64	64	64	64
	5	≤4	≤4	≤4	≤4	≤4	≤4		5	64	64	128	64	64
	6	≤4	≤4	≤4	≤4	≤4	≤4		6	64	128	64	64	64
	7	≤4	≤4	≤4	≤4	≤4	≤4		7	128	128	128	128	64
	8	≤4	≤4	≤4	≤4	≤4	≤4		8	128	64	64	64	128

Table 2: Mean PHA titers with standard error of sera of chickens vaccinated with experimentally prepared FC vaccine.

Groups	Route of vaccination	Secondary vaccination interval	PHA titer of unvaccinated control (Mean±SE)	PHA titer (Mean ±SE) at 15 DPV	P value
A	SC	15	<4±0.00	96±12.09	0.620
B	SC	21	<4±0.00	88±11.71	
C	SC	28	<4±0.00	88±11.71	
D	SC	35	<4±0.00	80±10.47	
E	SC	42	<4±0.00	80±10.47	

Level of significance: NS (P>0.05)

Result of Challenge Infection

Challenge infection was conducted with all the groups of birds along with unvaccinated controls after 15 days of booster vaccination. Each bird was administered with 0.5 ml of 2.93×10^{8} CFU through IM route. The experimental fowl cholera vaccine conferred 100% protection of birds while all the unvaccinated control birds succumbed to such infection. The rate of survivality at challenge infection performed after 15 days of booster infection are presented in Table 3.

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

Group	Route of vaccination	Total birds	No. of birds survive	No. of birds died	Percentage of survivality	Percentage of died
A	SC	8	8	0	100%	0%
B	SC	8	8	0	100%	0%
C	SC	8	8	0	100%	0%
D	SC	8	8	0	100%	0%
E	SC	8	8	0	100%	0%
F	Unvaccinated	8	0	8	0%	100%

CONCLUSION

The experiment was undertaken to identify and characterize *P. multocida* from poultry, preparation of vaccine and determination of its efficacy. To identify and characterize the organism different bacteriological techniques were carried out. The degrees of immunity produced in each vaccinated group of birds were measured by determining their serum antibody level using PHA test. The protective efficacy of fowl cholera vaccine was also measured by determining the survivality rate of the birds of each vaccinated groups by challenge experiment. The study had proved that the experimentally prepared fowl cholera vaccine was safe, effective and conferred 100% protection of vaccinated birds for the vaccination of chicken against fowl cholera. The vaccine produced better immune response when booster dose was given at 15^{th} days compared to 21^{st}, 28^{th}, 35^{th} and 42^{nd} days after primary vaccination.

REFERENCES

Botzler, R. G. (1991) Epizootiology of avian cholera in wildfowl. *Journal of Wildlife Diseases,* 27, 367-395.

Calnek, B.W.; Barnes, H.J.; Beard, C.W.; McDougald, L.I.L. and Saif, Y.M. (1997). Diseases of poultry 10th edition. Iowa State University Press, Ames, Iowa. pp. 143-150.

Cheesbrough, M. (1985). Medical laboratory manual for tropical countries. Volume II pp. 87-90.

Choudhury, K. A.; Amin M. M.; Sarker, A. J.; AH, M. R. and Ahmed, A. R. (1987). Immunization of chickens against fowl cholera with oil-adjuvanted broth culture vaccine. *Bangladesh Veterinary Journal,* 21, 63-73.

Choudhury, K.A.; Amin, M.M.; Rahman, A. and Ali, M.R. (1985). Investigation of natural outbreak of fowl cholera. *Bangladesh Veterinary Journal*, 19, 49-56.

Cowan, S.T. (1985). Cowan and Steel's Manual for Identification of Bacteria. 2nd edition. Cambridge University Press, Cambridge, London, pp. 122-125.

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

Derieux, W.T. (1978). Response of young chickens and turkeys to virulent and avirulent Pasteurella multocida administered by various routes. Avian Disease, 22, 131-139.

Gordon, R.F. and Jordan, F.T. (1985). Poultry Diseases. 2nd edition, ELBS, Baillier Tindall, London, pp. 44-46.

Harper, M.; Boyce, J.D.; Adler, B. (2006).Pasteurella multocida pathogenesis: 125 years after pasteur. FEMS Microbiol. Lett. 265, 1–10.

Heddleston, K. L. and Watko, L. P. (1964). Fowl cholera: Susceptibility of various animals and their potentials as disseminators of the disease. Vet. Bull. 34, 247-251.

Merchant, I. A. and Packer, R. A. (1967). Veterinary Bacteriology and Virology. 1st edition. Iowa State University Press, Ames. Iowa, USA. pp. 339.

Michael, A.; Geier, E.; Konshtok, R.; Hertman, I. and Markenson, J. (1979). Attenuated live fowl cholera vaccine. I. II. Laboratory and field vaccination trials in turkeys and chickens. *Avian Disease,* 23, 878-885.

Mondal, S.K.; Choudhury, K.A.; Amin, M.M.; Rahman, M.M. and Sarker, A.J. (1988). Immune response in chickens induced by alum precipitated fowl cholera vaccine. Humoral immune response. *Bangledesh Veterinary Journal,* 22, 7163-7169.

Muhairwa, A. P., J. P. 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 Pathology,* 29,133-142.

Nime, J; Hasan, M; Sattar, M.A; Nuri, M.D; Rahman, M.B; Khatun, A. (2016). Preparation of Fowl Typhoid Vaccine from Field Isolates and Determination of Efficacy. Article no. BMRJ.24994, 14(1): 1-7, ISSN: 2231-0886, NLM ID: 101608140

OIE, (1992). "Manual of Standards for Diagnostic Test and Vaccines. Second Edition."

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

Rhoades, K.R. and Rimler, RJB. (1990a). Virulence and toxicology of capsular serogroups D *P. multocida* strains isolated from avian hosts. *Avian Disease,* 34, 384-388.

Sander, J.E., Resurreccion, R.S., Waltman, W.D. and McMurray, B.L. (1998). Pasteurella challenge and ELISA serology evaluation of broiler breeders vaccinated with live cholera vaccine. Avian Diseases, 42,190-193.

Sarker, A.J.; Amin, M.N.1.1. and Hossain, W.M.A. (1992). Testing and quality control of poultry vaccines mid its monitoring in the field. *Bangladesh Agril. Univ. Res. Progress.* 6: 249-257.

Shil, R.N. and Debnath, S.C. (1985). An Introduction to the Theory of Statistics. First ed. City Press. Mymensingh, Bangladesh. PP: 32-35.

Siddique, A. B. (1997). Studies on the physico-chemical, antigenic and immunogenic properties of pigeon pox virus isolates of Bangladesh. Ph.D. thesis of Microbiology and Hygiene, Vet. Science. Bangladesh Agricultural University. Mymensingh.

Simensen, E., and L. D. Olson. (1980). Aerosol transmission of *P. multocida* in turkeys. *Avian Disease*, 24,1007-1010.

Supar; Setiadi, Y.; Djaenuri; Kurniasih, N.; Poerwadikarta, B. and Sjafei (2002). The development of fowl cholera vaccine: II. Pathogenicity and vaccine protection of P. multocida local isolates in experimental ducks. J. Tmu Ternak dan Veteriner, 6, 120-125.

Tripathy, D.N.; Hanson, L.E. and Myers, W.L. (1970a). Passive haemagglutination test with fowl pox virus. *Avian Disease*, 14, 29-38.

Wu, J.R.; Shieh, H.K.; Shien, J.H.; Gong, S.R. and Chang, P.C. (2007). Protective immunity conferred by recombinant P. multocida lipoprotein E (PlpE). Vaccine, 25, 4140-4148.